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Detection of *Hermetia illucens* by real-time PCR

A. Marien[1*], F. Debode[1], C. Aerts[1], C. Ancion[1], F. Francis[2] and G. Berben[1]

[1]Walloon Agricultural Research Center (CRA-W), Unit Traceability and Authentication, chaussée de Namur 24, 5030 Gembloux, Belgium; [2]Gembloux Agro-Bio Tech – University of Liège, Functional and Evolutionary Entomology, Passage des Déportés 2, 5030 Gembloux, Belgium; a.marien@cra.wallonie.be

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RESEARCH ARTICLE

Abstract

Insects are rich in proteins and could be an alternative source of macronutrients to feed animals and humans. Over the past few years, numerous companies have started producing insects for feed purposes. In Europe, the processed animal proteins obtained from seven insect species have been authorised for aquaculture by Commission Regulation (EU) 2017/893 since 1 July 2017. Methods of authentication are required to check the conformity of the products. In this study, we propose a real-time PCR method for the specific detection of the black soldier fly (*Hermetia illucens* L.), one of the most widely used insects for feed production. The developed PCR assays amplify a 67 bp fragment based on the mitochondrial *COX3* gene coding for subunit 3 of the cytochrome c oxidase. The qualitative method was tested according to several performance criteria. The specificity was tested against 51 insect species. The specificity was also checked against plant species and other animal species such as crustaceans, mammals and birds. The sensitivity, efficiency and robustness of the PCR test were successfully tested. The applicability of the test was proven through the analysis of real-life processed samples (industrial meals) of *H. illucens*.

Keywords: insect, *Hermetia illucens*, black soldier fly, detection, real-time PCR

1. Introduction

Farmed production of fish and shellfish nearly tripled in volume between 1995 and 2007 leading to an increase in demand for fish feed by the aquaculture sector (Naylor *et al.*, 2009). Fishmeal has been the usual source of animal protein to feed farmed fish for several decades. As fishmeal is becoming a finite resource, the aquaculture industry is searching for new alternative sources of high quality proteins (Riddick, 2014). Insect meal from Diptera could be one of the possible alternatives (Barroso *et al.*, 2014). The high protein and fat content of dried black soldier fly prepupae reinforces the high potential of fly meal as animal feed (Diener *et al.*, 2009). The processed animal proteins from seven insects were authorised in aquafeed in Europe by Commission Regulation (EU) 2017/893 on 1 July 2017 (EC, 2017). Insect-rearing for commercial purposes exists both inside and outside Europe. The most promising insect species for industrial production of feed are black soldier fly (*Hermetia illucens)*, the common house fly (*Musca domestica*), the yellow mealworm (*Tenebrio molitor*), the lesser mealworm (*Alphitobius diaperinus*), and several cricket species (*Acheta domesticus*, *Gryllodes sigillatus*, *Gryllus assimilis*) (EC, 2017; Van Huis, 2012).

The identification of insect species in feed products is therefore an emerging application. DNA-based methods have generally been used for taxonomic classifications (Cameron, 2014; Cook *et al.*, 2002; Hebert *et al.*, 2003; Mandal *et al.*, 2014), forensic entomology (Dawnay *et al.*, 2007; Malewski *et al.*, 2010; Wells and Škaro, 2014), and predation (Hoogendoorn and Heimpel, 2001; Pons, 2006; Pons *et al.*, 2006; Sheppard *et al.*, 2005). Recently, methods by real-time PCR were proposed for the detection of *T. molitor* in food and feed (Debode *et al.*, 2017a). Presently, real-time PCR remains the reference technique for the detection of DNA in food or feed products. For a detection in processed products, the amplicon size must be small (Debode *et al.*, 2007, 2017b). This study proposes a real-time PCR method for the specific detection of the black soldier fly (*H. illucens*).

2. Materials and methods

Samples

Insects were collected in the environment or provided by the Functional and Evolutionary Entomology unit of Gembloux Agro-Bio Tech (ULiège, Gembloux, Belgium). Insects were selected in order to cover several taxonomic groups.

Real-life processed samples (industrial meals from different EU-based companies) of *H. illucens* were obtained through the International Producers of Insects for Food and Feed (IPIFF). For each *H. illucens* industrial meal, a mix containing 0.1% (in mass fraction) of *H. illucens* in a commercial fish feed (composition: fishmeal, fish oil, wheat gluten, protein concentrate extracted from pea, maize starch, yeast, lecithin, vitamins and minerals) was prepared.

DNA extraction

Genomic DNA was extracted and purified from all samples following the CTAB-based method described in Annex A.3.1 of the international standard ISO 21571:2005 (ISO, 2005). The quality and quantity of DNA extracted from samples were estimated spectrophotometrically using a Nanodrop ND-1000 spectrophotometer at 260 nm (A260) and 280 nm (A280) absorbance (Nanodrop Technologies, Wilmington, NC, USA). DNA purity was determined using the A260/A280 ratio. The amplifiability of the DNA extract was checked by real-time PCR with the 18S target for insects (Debode *et al.*, 2017a) and rbcL (Debode *et al.*, 2012) for plants. Other species were tested with targets developed or evaluated within the framework of the European Union Reference Laboratory for Animal Proteins in feedingstuff activities (EURL-AP, 2013, 2014a) or with 18S target (Debode *et al.*, in press; Garikipati *et al.*, 2006). Ten ng of DNA were used in the PCR reactions.

The industrial meals and the mixes at 0.1% of *H. illucens* were extracted following the method recommended by EURL-AP and based on the adaptation of the protocol of the Wizard Magnetic DNA Purification System for Food kit (Promega, Madison, WI, USA). This method is described in the EURL-AP Standard Operating Procedure (EURL-AP, 2014b).

Primers and probe for the real-time PCR

Eurogentec (Seraing, Belgium) synthesised the primers and probes. The primers and probe sequences developed for the detection of *H. illucens* are presented in Table 1. The probe for this latter method was labelled with the reporter dye FAM™ (6-carboxyfluorescein) at the 5' end and the quencher dye TAMRA™ (tetramethyl-6-carboxyrhodamine) at the 3' end.

Real-time PCR method

Real-time PCR (total reaction volume of 25 µl) was performed on an ABI7500 Fast (Thermo Fisher, Foster City, CA, USA) using the Universal Master Mix of Diagenode (Seraing). The reaction mixture included 12.5 µl of Master Mix, 1.7 µl of each primer (5 µM), 1.5 µl of probe (9 µM), 2.6 µl of bidistilled water, and 5 µl of DNA. Reaction mixtures were distributed on 96-well reaction plates (Thermo Fisher) developed for the specific thermocyclers. Wells were covered with adhesive film and centrifuged (2 min at 500 rpm) to eliminate any air bubbles in the well bottoms. The thermal programme was as follows: 2 min at 50 °C; 10 min at 95 °C; 50 cycles of 15 s at 95 °C; and 1 min at 50 °C.

Specificity of the PCR method

The specificity of the method was checked on 51 insect species of different taxonomic orders including nine Diptera other than *H. illucens*, eighteen Coleoptera, seven Orthoptera, five Hemiptera, six Hymenoptera, three Lepidoptera, one Nevroptera, one Dermaptera and one Blattodea. The specificity tests were performed on five crustaceans (also members, as insects, of the Arthropoda phylum), one mollusc and nine other animal species (six mammals, two birds, one fish). As insect meals, the meals produced from these animal species are considered by the legislation as processed animal protein (PAP), and could be found in PAP used to feed animals. The possibility of a cross-reaction with human DNA was envisaged too. Seven plant species were also tested and correspond for the most part to plant species frequently used in feed (Table 2). Ten ng of DNA were used in the PCR reactions. Each DNA extract was tested at least in duplicate.

Table 1. Primers and probe used for the *Hermetia illucens* detection by real-time PCR.

Target	Name	Sequences 5'-3'	Amplicon size (bp)
H. illucens	HI-mito-2F	ACCATTCTTCAAGCCTATGA	67
	HI-mito-2R	TTGAGCCGTAGACTGCG	
	HI-mito-P	FAM-TGAAGCCCCTTTTACTATTGCTG-TAMRA	

Table 2. Specificity of *Hermetia illucens* PCR test on animal and plant species (n≥2). For positive samples mean quantification cycle (Cq) values (m) and standard deviations (σ) are given in brackets.[1]

	Taxonomic classification	Latin name or family	Common name	Results
Insects	Diptera	*Hermetia illucens* L.	black soldier fly	+ (m=13.02, σ=0.20)
		Stomoxys calcitrans L.	stable fly	–
		Tabanus sp.	horse fly	–
		Bibio marci L.	St. Mark's fly	–
		Calliphora vicina R.-D.	blow fly	+ (m=36.06, σ=0.48)
		Sarcophaga carnaria L.	common fresh fly	–
		Bombylius major L.	large bee-fly	–
		Chironomus plumosus L.	buzzer midge	–
		Syrphidae	hover fly	–
		Musca domestica L.	house fly	–
	Orthoptera	*Locusta migratoria* L.	migratory locust	–
		Acheta domesticus L.	house cricket	–
		Gryllus bimaculatus De G.	Mediterranean field cricket	–
		Gryllus assimilis F.	Jamaican field cricket	–
		Gryllus sp.	cricket	–
		Locusta sp.	locust	–
		Acheta sp.	cricket	–
	Coleoptera	*Pachnoda* sp.	dola's worm	–
		Tenebrio molitor L.	mealworm	–
		Zophobas morio F.	superworm	–
		Alphitobius diaperinus P.	lesser mealworm	–
		Carabus sp.	beetle	–
		Staphylinidae	rove beetles	–
		Curculionidae/Scolytidae	true weevils	–
		Coccinellidae	ladybird	–
		Scarabidae	scarab beetles	–
		Oxythyrea funesta P.	white-spotted rose beetle	–
		Melolontha melolontha L.	cockchafer	–
		Leptinotarsa decemlineata S.	colorado potato beetle	–
		Cassida viridis L.	green tortoise beetle	+ (m=37.66, σ=0.39)
		Cicindela campestris L.	green tiger beetle	+ (m=40.64, σ=0.55)
		Nicrophorus humator G.	black sexton beetle	–
		Nicrophorus vespillo L.	common burying beetle	–
		Cetonia aurata L.	rose chafer	–
	Hemiptera	Aphididae	aphid	–
		Anthocoridae	bugs	–
		Palomena prasina L.	green shield bug	–
		Pyrrhocorus apterus L.	firebug	–
		Psyllus sp.	jumping plant louse	–
	Hymenoptera	*Apis* sp.	bee	–
		Bombus terrestris L.	buff-tailed bumblebee	–
		Bombus campestris P.	field cuckoo bumblebee	–
		Componotus sp.	carpenter ant	–
		Vespa sp.	hornet	–
		Vespula sp.	wasp	–
	Lepidoptera	*Biston betularia* L.	peppered moth	–
		Tineola sp.	moth	–
		Bombyx mori L.	silkworm	–
	Neuroptera	*Chrysoperla carnea* S.	green lacewing	–
	Blattodea	*Blatta orientalis* L.	oriental cockroach	–
	Dermaptera	*Forficula auricularia* L.	common earwig	–

Table 2. Continued.

Taxonomic classification	Latin name or family	Common name	Results
Crustacean	*Euphausia superba* D.	Antartic krill	–
	Penaeus vannamei B.	whiteleg shrimp	–
	Nephrops norvegicus L.	langoustine	–
	Homarus gammarus L.	European lobster	–
	Paralithodes camtschatieus T.	red king crab	–
Mollusca	Teuthida sp.	squid	–
Mammals	*Bos taurus* L.	beef	–
	Sus scrofa L.	pork	–
	Ovis aries L.	sheep	–
	Equus caballus L.	horse	–
	Equus asinus L.	donkey	–
	Homo sapiens L.	human	–
Fish	*Salmo salar* L.	salmon	–
Birds	*Gallus gallus* L.	chicken	–
	Meleagris gallopavo L.	turkey	–
Plants	*Glycine max* M.	soybean	–
	Zea mays L.	maize	–
	Brassica rapa L.	rapeseed	–
	Triticum aestivum L.	wheat	–
	Oryza sativa L.	rice	–
	Solanum lycopersicum L.	tomato	–
	Beta vulgaris L.	sugar beet	–

[1] + = positive signal; – = negative signal; Cq>36 = indicative value corresponding to a late signal.

Cloning into pCR2.1 and dilutions

The 67 bp amplified fragment from *H. illucens* was ligated into the 3.9 kb pCR®2.1-TOPO plasmid vector (Invitrogen, Merelbeke, Belgium) following the TOPO® TA Cloning® kit instructions (Invitrogen). Plasmid DNA was isolated from bacterial cultures using the Genopure Plasmid Maxi Kit (Roche Diagnostics GmbH, Mannheim, Germany). The plasmid DNA was linearized with the *Hin*dIII restriction enzyme (Promega) and then purified using phenol-chloroform-isoamyl alcohol.

The quantity and quality of plasmid DNA were measured using a Nanodrop ND-1000 spectrophotometer at 260 nm (A260) and 280 nm (A280) absorbance. DNA purity was determined using A260/A280 ratio. The quantity of plasmid DNA was converted to copy number as follows: firstly, taking into consideration that 1 unit of absorbance at 260 nm corresponds to a concentration of 50 µg/ml; secondly, that the molar weight of one plasmid molecule is derived from the combination of its size in base pairs and the mean molar weight of one base pair set at 635 Da (Sambrook *et al.*, 1989); and thirdly, by knowing the number of moles available, it is possible to deduce the copy number of plasmids by means of the Avogadro number (Debode, 2017; Debode *et al.*, 2010).

The sensitivity, efficiency and robustness of the PCR test were determined on diluted plasmid DNA. These dilutions were realised in water until an estimated copy number of 10,000 copies/5 µl was reached. Higher dilutions were done in a solution containing 5 ng/µl of organic maize DNA as background DNA. Low binding tubes were chosen to minimise DNA losses due to tube wall binding.

Determination of the limit of detection

Target sensitivity was evaluated following the recommendations of the former AFNOR XP V03-020-2 standard (AFNOR, 2008). This standard no longer exists, but the principles detailed in it are still valid. The absolute limit of detection (LOD) was determined for the PCR assay (primers + probe + amplification programme) on dilutions of plasmid material.

The subsequent dilutions had to contain 50, 20, 10, 5, 2, 1 and 0.1 copies of the target. Six PCRs had to be achieved for each dilution. The method's LOD_6 was the smallest copy number for which the six PCRs were positive, and only

if the final dilution supposed to contain the 0.1 copy per reaction generated a maximum of one positive PCR signal on the six replications. If more than one positive signal was observed for the 0.1 copy then, the DNA quantities had to be revised. The copy number corresponding to LOD_6 is then tested 60 times on the same plate (determination of the $LOD_{95\%}$). The $LOD_{95\%}$ is validated if at least 95% of positive signals are recorded out of the 60 replicates. The highest acceptable copy numbers for LOD_6 and $LOD_{95\%}$ are 20 copies.

Determination of the efficiency

The efficiency of the PCR assay was calculated with a dilution series of plasmid material at target levels of 5,000, 2,500, 1000, 500 and 100 copies. Each dilution was analysed in 6 replicates and on 4 runs. The efficiency has to be between 80 and 120% (Broeders *et al.*, 2014).

Robustness of the PCR method

The robustness of the method was tested by introducing some slight deviations to the standard experimental conditions (Broeders *et al.*, 2014; CCMAS, 2010; Debode *et al.*, 2017c) such as the annealing temperature (50±1 °C), the primer concentrations (standard or reduced by 30%), the probe concentration (standard or reduced by 30%) and the real-time PCR master mix volume (standard or ±1 µl) which involves a final reaction volume of 25±1 µl. Six replicates of the plasmid borne target at 20 copies/5 µl were tested in the conditions described in Table 3. The robustness was performed on two real-time PCR platforms: thermocycler ABI7500 Fast (Thermo Fisher) with Universal Mastermix by Diagenode (Seraing) and thermocycler Lightcycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) with ABI Taqman 2x Universal PCR Mastermix (Thermo Fisher). The acceptance criterion is that all deviations to the standard protocol must give a positive result at a level of 20 copies of the target (Broeders *et al.*, 2014).

Applicability of the PCR method

The applicability of the PCR method was checked on four real-life processed samples (industrial meals) of *H. illucens* produced in the EU. The DNA extract of each meal was tested by PCR in triplicate.

The sensitivity of the PCR assay was also evaluated on a fish feed containing 0.1% in mass fraction of *H. illucens* industrial meal. Two DNA extracts with 2 dilutions were tested by PCR in triplicate.

3. Results and discussion

Presently, only a few DNA sequences are available for *H. illucens* in the National Center for Biotechnology Information database and the DNA Data Bank of Japan. Therefore, several problems were met to select regions of interest: firstly, even for mitochondrial DNA, which is generally better referenced for animal species, only partial sequences were available prior to June 2017; secondly, when alignments were possible, they showed numerous similarities with other insect species and sometimes even with unexpected species (e.g. for some regions, there were greater similarities with crustacean species than with insect species); and thirdly, some regions of interest were AT-rich, preventing their use in primers and probes if the recommended GC content or length has to be met (Debode *et al.*, 2017b; Rodriguez *et al.*, 2015). Therefore, the *COX3* gene coding for subunit 3 of cytochrome c oxydase (mitochondrial DNA) was used to select the primers and probe. Working on mitochondrial DNA can be an advantage for detection as a mitochondrion contains several copies of its genome and several mitochondria can be present in a single cell (Cavelier *et al.*, 2000). However, this multicopy characteristic is a disadvantage for quantitation purposes as the copy number per cell will be variable depending on the considered tissue.

Table 3. Experimental conditions tested to evaluate the robustness of the described *Hermetia illucens* PCR test.

PCR machine	ABI7500 fast (Life Technologies) and Lightcycler 480 (Roche Diagnostics Ltd)				
PCR reagent kit	Universal Mastermix (Diagenode s.a.) and ABI Taqman 2x Universal PCR Master mix (Life technologies)				
Annealing temperature	49 and 51 °C				
Primer concentration	Minus 30%	Standard	Standard	Standard	Standard
Probe concentration	Standard	Minus 30%	Standard	Standard	Standard
PCR volume	Standard	Standard	Standard (20 µl mix + 5 µl DNA)	Standard + 1 µl Mastermix (21 µl mix + 5 µl DNA)	Standard – 1 µl Mastermix (19 µl mix + 5 µl DNA)

The specificity was tested on DNA from *H. illucens* but also on 50 other non-target insect species, including nine Diptera. Positive results were obtained with *H. illucens*. On the 50 other insect species, late signals were observed with *Calliphora vicina* (Diptera), *Cassida viridis* and *Cicindela campestris* (Coleoptera). These aspecificities should not cause problems because the amplification signals are late (see Cq values in Table 2). Moreover they were not reproducible during a second analysis. No signal was obtained with the 15 other animal species (vertebrates, mollusc and crustaceans) and the seven plant species tested. The results are presented in Table 2. As it was hard to obtain individuals of the Stratiomyidae family identified with certainty, the black soldier fly was the only representative of Stratiomydae family. However, on the eight other Diptera tested, six species belong to the Brachycera suborder like *H. illucens*. In the current EU legislation, only two Diptera species are authorised in fish feed and can be produced at large scale. The specificity tests also consider this second authorised Diptera, *M. domestica*, which in practice could be mixed with *H. illucens* in meals.

Since the *COX3* gene is a multicopy target, sensitivity, efficiency and robustness were performed on the target in their plasmid DNA in order to have better control of the copy number. These three parameters reached the recommended acceptance criteria. Indeed, for the sensitivity, the LOD_6 was estimated at 5 copies following the AFNOR XP V03-020-2 standard approach (AFNOR, 2008) and for the $LOD_{95\%}$, tested at the 5 copies level, 60/60 positive signals were obtained with a mean Cq value of 36.82. Therefore, the PCR test easily reaches the recommended performance criteria (≤20 copies). The PCR efficiency was evaluated at 92.7%. The Table 4 presents the mean Cq values obtained with different copy numbers tested (from 5,000 to 5). When calculated per plate, the efficiency was always higher than 90% and therefore met the acceptance criterion set by Broeders *et al.* (2014). The robustness of the PCR method was also positively evaluated. All tested deviations to the standard protocol delivered positive results at a target copy number of 20 in the PCR.

Positive signals were also obtained on industrial samples (pure meals of *H. illucens*) showing the applicability of the PCR test on real-life samples (Table 5). The four mixes of

Table 4. Quantification cycle (Cq) values obtained on dilutions of plasmid material used for efficiency calculation and for $LOD_{95\%}$. For efficiency, each concentration was analysed in 6 replicates and on 4 PCR plates (n=24). For $LOD_{95\%}$, the concentration at 5 copies was analysed in 60 replicates on 1 PCR plate (n=60).

Copy number of target	Cq (mean value)	Standard deviation and (n)
5,000	26.75	0.21 (24)
2,500	27.78	0.18 (24)
1000	29.07	0.17 (24)
500	30.15	0.14 (24)
100	32.72	0.27 (24)
5	36.82	0.72 (60)

Table 5. Quantification cycle (Cq) mean values obtained with the *Hermetia illucens* PCR test on processed samples from *H. illucens* and on mixes containing 0.1% in mass fraction of *H. illucens* in a commercial fish feed (n=3).

Identification of samples			Cq	
			Dilution 1×	Dilution 10×
Pure industrial meals of *H. illucens* produced in the EU	n°1	Extract 1	15.32	18.83
		Extract 2	15.33	18.71
	n°2	Extract 1	13.73	17.14
		Extract 2	13.40	17.12
	n°3	Extract 1	13.97	17.18
		Extract 2	14.04	17.56
	n°4	Extract 1	13.16	16.26
		Extract 2	14.08	16.61
Fish feed containing 0.1% of *H. illucens* from the industrial meal n°1		Extract 1	26.90	29.05
		Extract 2	26.99	28.46
Fish feed containing 0.1% of *H. illucens* from the industrial meal n°2		Extract 1	26.51	27.98
		Extract 2	27.05	28.20
Fish feed containing 0.1% of *H. illucens* from the industrial meal n°3		Extract 1	28.23	28.51
		Extract 2	27.22	28.50
Fish feed containing 0.1% of *H. illucens* from the industrial meal n°4		Extract 1	27.33	27.99
		Extract 2	27.54	28.09

fish feed containing 0.1% of different processed *H. illucens* meals were also tested. All the PCR tests gave positive results (Table 5). The tenfold dilutions provide evidence of a slight inhibitory effect of feed on the amplification of the *H. illucens* target. On the one hand, the complete fish feed used was checked to see if it was free of the *H. illucens* target before its spiking with black soldier fly meal. On the other hand, the 18S target (Debode *et al.*, in press; Garikipati *et al.*, 2006) provided evidence of the amplifiability of DNA from DNA extracts of this feed.

4. Conclusions

The PCR method for the detection of *H. illucens* based on the *COX3* gene coding for subunit 3 of the cytochrome c oxidase is fit for purpose. This qualitative PCR test is based on mitochondrial DNA, consequently, it is a multicopy target which allows detection at low level even on processed samples. The specificity gives good results with respect to the insect, animal and plant species tested. Only three insect species showed aspecificities. However, the signals with these non-target species are late and not reproducible. The risk of interference is extremely reduced when considering the aim for which the test is designed and the earliest signal is in fact close to the LOD. The acceptance criteria were reached for sensitivity (LOD_6 and $LOD_{95\%}$), efficiency and robustness. The PCR method is also applicable to real-life samples from industry even when used at 0.1% in mass fraction in fish feed.

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Competing interests

The authors declare that they have no competing interests.

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