



RESEARCH ARTICLE

Detection of *Acheta domesticus* by real-time PCR in food and feed

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Abstract

Edible insects are rich in protein and can serve as a significant source of vitamins and minerals. The house cricket (*Acheta domesticus*) stands out as one of the most nutritious edible insects. In various parts of the world, crickets are consumed roasted, baked, fried, boiled and in the form of cricket flour, a powder of dried and ground crickets. In Europe, processed animal proteins derived from eight insect species (including *A. domesticus*) have received authorization for use in fish, pig and poultry feed. Therefore, the development of a method for detecting house crickets is essential to verify product compliance and provide accurate labelling information to the end user. In this study, we have established a real-time PCR assay for the specific detection of house crickets. This method is based on the amplification of a mitochondrial fragment that codes for cytochrome b. We assessed its specificity by testing it against 39 other insect species, 7 plant species and 31 other animal species. Furthermore, we successfully evaluated the amplification efficiency, sensitivity, robustness, applicability on commercial samples and transferability to a second laboratory.

Keywords

Acheta domesticus – detection – real-time PCR – food – feed

1 Introduction

With projections indicating that the global population might reach 9.8 billion people in 2050 (United Nations, 2022), the demand for food is set to rise substantially. Anticipated figures predict a 72% increase for demand of animal proteins over the next 35 years (Wu *et al.*, 2014). At present, the primary source of protein is derived from animal farming. This surge in meat

demand will inevitably lead to elevated land and water usage, along with increased greenhouse gas emissions, consequently contributing to environmental strain (von Hackewitz, 2018).

Numerous researchers, food companies and consumers are shifting their focus towards innovative and sustainable food options for the future (Kim *et al.*, 2018). Among the promising protein sources for both human and animal nutrition, insects stand out.

Insect consumption is a global practice, with thousands of insect species being consumed by humans, as well-documented in various impactful publications (Dreon and Paoletti, 2009; Rumpold and Schlüter, 2013a, Anankware *et al.*, 2015). Insects are consumed in approximately 200 countries across the world, though primarily outside Europe (Belluco *et al.*, 2015). The literature records over 1,900 species of edible insects, with the majority found in tropical regions. Commonly consumed insect groups encompass beetles, caterpillars, bees, wasps, ants, grasshoppers, locusts, crickets, cicadas, leaf and planthoppers, scale insects and true bugs, termites, dragonflies and flies (Van Huis *et al.*, 2013). Due to insects' ectothermic nature, they exhibit high feed conversion efficiency. For instance, crickets require 12 times less feed than cattle, and only half as much feed as pigs and broiler chickens to produce an equivalent amount of protein (Van Huis *et al.*, 2013).

Commercial insect farming is a global phenomenon encompassing the rearing of insects for the production of animal feed. While a growing number of companies are emerging in Europe, markets have been constrained by prevailing legislation that initially prohibited the utilization of insects for livestock feed (Debode *et al.*, 2017a). However, the legislative landscape in Europe has since evolved regarding processed animal proteins (PAP). In 2017, the PAP obtained from seven insect species, namely *Acheta domesticus*, *Alphitobius diaperinus*, *Gryllosides sigillatus*, *Gryllus assimilis*, *Hermetia illucens*, *Musca domestica* and *Tenebrio molitor*, received authorization for use in aquafeed (EU regulation 2017/893). In 2021, this authorisation was extended to encompass pig and poultry feed (EU regulation 2021/1372). The same year *Bombyx mori* was added as the eighth species on the authorised list (EU regulation 2021/1925).

For human consumption in Europe, insects are regulated under (EU) Regulation 2017/2470, which establishes a list of authorized novel foods and outlines the conditions under which each novel food can be used, following the guidelines of (EU) Regulation 2015/2283 on novel foods. A novel food is defined as food that had not been significantly consumed by humans in the EU before May 15, 1997. Regarding *A. domesticus*, a first authorization was granted in 2022 for the introduction of frozen, dried, and powder forms of *A. domesticus* onto the market (as detailed in the (EU) Regulation 2022/188, which amends (EU) Regulation 2017/2470). A second authorization was given in 2023 for the sale of a defatted powder derived from *A. domesticus* (as specified in

(EU) Regulation 2023/58, which amends (EU) Regulation 2017/2470).

The house cricket, *A. domesticus*, belongs to the Orthoptera order. Boasting a protein content of 66.6% of its dry weight (von Hackewitz, 2018), the house cricket ranks among the highest-protein edible insects, significantly surpassing conventional protein sources (Fernandez-Cassi *et al.*, 2019). The house cricket also contains 29-31% of polyunsaturated fatty acids. Additionally, due to its composition, crickets can be considered as a valuable source of vitamins (Rumpold and Schlüter, 2013b). House crickets contain allergenic proteins similar to those found in other arthropods (e.g. prawns, crabs, lobsters), including tropomyosin, arginine kinase, and glyceraldehyde 3-phosphate dehydrogenase. Moreover, house crickets also contain specific allergens such as hexamerin. To mitigate health risks for allergen-susceptible consumers, proper labelling of crickets and cricket-derived products is essential (Fernandez-Cassi *et al.*, 2019).

With the growing interest in edible insects within the food and feed industries, the need for reliable methods to authenticate insect species has become paramount. Real time PCR methods have already been proposed for *H. illucens* (Marien *et al.*, 2018, Zagon *et al.*, 2018), *T. molitor* (Debode *et al.*, 2017a), *A. diaperinus* (Marien *et al.*, 2022), *B. mori* (Kim *et al.*, 2018) and *Oxya chinensis* (Kim *et al.*, 2019). A real-time PCR protocol for detecting the house cricket (*A. domesticus*) was developed by Garino *et al.* (2021), utilizing a real-time PCR method based on COI detection and tested specifically against 22 insect species. In pursuit of multiple insect species detection authorized for feed use, both PCR-based tests (Tramuta *et al.*, 2018) and real-time PCR tests (Kim *et al.*, 2019; Köppel *et al.*, 2019) have been proposed.

Approaches based on DNA metabarcoding coupled to high throughput sequencing are now starting (Hilinger *et al.*, 2023). Additionally, proteomic methods employing mass spectrometry are also in development but accurate identification will only be possible after a sequencing effort due to the evident scarcity of proteomic data for insect species (Francis *et al.*, 2020).

In this study, we present a robust real-time PCR for the detection of house crickets (*A. domesticus*). This assay relies on the amplification of a mitochondrial fragment coding for cytochrome b. The method has been developed to be used under conditions compatible with other PCR targets (Debode *et al.*, 2017a; Zagon *et al.*, 2018; Garino *et al.*, 2021; Marien *et al.*, 2022) allowing for multiple PCR tests to be conducted on the same plate. The method was thoroughly evaluated to ensure it met

TABLE 1 Primers and probe used for the *Acheta domesticus* detection and primers for the *Gryllobates sigillatus* detection (in EvaGreen format) by real-time PCR

Target	Name	Sequences 5'-3'	Amplicon size
<i>Acheta domesticus</i>	AchetaD_cytB_F1	ATAGTAGGTATTCTAATCTTATTCCTA	149 bp
	AchetaD_cytB_R1	CATTGTACTAGATCAGTTCCTAGATA	
	AchetaD_cytB_P1	FAM- AATAGCTGCCGCTTTCATAGGTTAC – TAMRA	
<i>Gryllobates sigillatus</i>	GSIFw	GATCAAACAATCCCCTAGGTGTC	149 bp
	GS1re	CTGGGTCTCCAAGTATATAAGGATTAG	

various performance criteria, such as specificity, efficiency of amplification, sensitivity, applicability, robustness and transferability.

2 Materials and methods

Samples

The insects were collected from the environment by trained entomologists, purchased from specialized companies or provided by the Functional and Evolutionary Entomology unit of Gembloux Agro-Bio Tech (ULiège, Gembloux, Belgium). The selection of insects aimed to encompass various taxonomic groups. All insects were dead at the arrival at the laboratory, either dried or frozen. DNA extraction was mostly performed on a single whole individual, except for smaller insects for which several individuals were required. All samples used in the specificity testing are detailed in Table 3 later in this article.

Real-life processed samples consist of industrial meals sourced from different companies based in the European Union and acquired through the International Producers of Insects for Food and Feed (IPIFF). As stipulated in the material transfer agreement (MTA), the identity of these companies cannot be disclosed in the article. Others industrial meals and food samples labelled to contain *A. domesticus* or crickets were purchased online. The industrial samples employed in this study are itemized in Table 4 later in this article. Real-life samples were ground in an Ultra-Centrifugal Mill – ZM 200 (Retsch, Haan, Germany) using sieves with a mesh size of 0.5 mm. The centrifugal grinding results in homogenisation of the sample.

DNA extraction

Genomic DNA was extracted and purified from all samples using the CTAB-based method outlined in Annex A.3.1 of the international standard ISO 21571:2005 (ISO 2005). The quality and quantity of DNA extracted

from samples were assessed spectrophotometrically using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA), measuring absorbance at 260 nm (A260) and 280 nm (A280). Other species were tested with targets developed or evaluated within the framework of the European Union Reference Laboratory for Animal Proteins in feedstuff activities (EURL-AP, 2017; Marien *et al.*, 2019) or with the 18S target (Garikipati *et al.*, 2006, Debode *et al.*, 2017a). Ten ng of DNA were used in the PCRs.

Regarding the industrial meals of *A. domesticus* classified as processed animal proteins (PAP), and the food samples, DNA extraction was done following the method recommended by EURL-AP. This method is an adaptation of the protocol from the Wizard Magnetic DNA Purification System for Food kit (Promega, Madison, USA) and is outlined in the EURL-AP Standard Operating Procedure (EURL-AP, 2014).

Primers and probe for the real-time PCR

The primers and probes used for the real-time PCR were synthesized by Eurogentec (Seraing, Belgium). The sequences of the primers and probe designed for the detection of *A. domesticus* are provided in Table 1. The probe 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.

During applicability testing, the PCR test for *G. sigillatus* detection described by Daniso *et al.* (2020) was used for the sample where *A. domesticus* was not detected. A slight modification was made to the reverse primer for which one base was missing (the A at position 17 in the primer) compared to the sequence KR903358.1 referenced in the article and available on the NCBI website. The sequences of the primers are included in the Table 1.

Real-time PCR

Real-time PCR was conducted with a total reaction volume of 25 µl on a QuantStudio™ 5 Real-time PCR System

(Thermo Fisher Scientific, Foster City, CA, USA) using the Universal Master Mix from Diagenode (Seraing, Liège, Belgium). The reaction mixture consisted of 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 (10 ng). The reaction mixtures were dispensed into 96-well reaction plates (Thermo Fisher) designed for the specific thermocyclers. The wells were covered with adhesive film and centrifuged (2 min at 500 rpm) to eliminate any air bubbles in the well bottoms. The thermal cycling program was as follows: 2 minutes at 50 °C; 10 minutes at 95 °C; 50 cycles of 15 seconds at 95 °C; and 1 minute at 60 °C.

Specificity testing

The specificity of the targeted sequence was first investigated *in silico* using the BLAST tool of NCBI (National Centre for Biotechnology Information, U.S. National Library of Medicine, USA).

Specificity was experimentally tested on DNA from 40 insect species (including *A. domesticus*) spanning various taxonomic orders including 7 Orthoptera other than *A. domesticus*, 6 Diptera, 16 Coleoptera, 2 Hemiptera, 3 Hymenoptera, 3 Lepidoptera, 1 Blattodea and 1 Dermaptera.

The specificity was also evaluated on 2 arachnids (1 scorpion and 1 spider), 8 crustaceans, 1 mollusk, 9 mammals, 7 birds and 4 fishes. Seven plant species corresponding to the plant species frequently used in feed and food were also tested. Each DNA extract was tested at least in duplicate. Ten ng of DNA were placed in the tubes for specificity testing.

Cloning of the target and copy number determination of the plasmid DNA

In the laboratory where the method was developed (CRA-W), the amplified 149-bp fragment from *A. domesticus* was ligated into the 3.9-kb pCR^{2.1}-TOPO plasmid vector (Invitrogen, Merelbeke, Belgium) following the TOPO[®] TA Cloning[®] kit instructions (Invitrogen). The presence of the fragment and its amplicon size were confirmed through gel electrophoresis, as well as by real-time PCR. Plasmid DNA was isolated from bacterial cultures using the Genopure Plasmid Maxi Kit (Roche Diagnostics GmbH, Mannheim, Germany). The plasmid DNA was then linearised with the *Bam*HI restriction enzyme (New England Biolabs, Ipswich, MA, USA) and purified using GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA). The quantity of plasmid DNA was measured using a Nanodrop ND-1000 spectrophotometer at 260 nm. The subsequent

copy number of plasmid was calculated as described by Debode *et al.* (2010).

To evaluate the sensitivity, efficiency, and robustness of the PCR test, diluted plasmid DNA samples were prepared. These dilutions were realised in water until an estimated copy number of 10,000 copies/5 µl was reached. For further dilutions below this estimated copy number, a solution containing 12 ng/µl of maize DNA was used as background DNA. Low binding tubes were selected to minimize DNA losses caused by binding to the tube walls.

In the second laboratory (FRIP) where the transferability was tested, new plasmids were prepared. The amplified 149 bp fragment was inserted into the pGEM[®]-T Easy Vector (Promega, Madison, WI, USA) following the prescriptions of the kit. Plasmids containing the inserted fragment were extracted using the PureYield[™] Plasmid Miniprep System (Promega) and subsequently linearized using the *Bam*HI restriction enzyme (New England Biolabs). The linearized plasmid was further purified using the PureYield[™] Plasmid Miniprep System (Promega).

Determination of the linearity and efficiency

The PCR assay's efficiency was determined using a dilution series of plasmid DNA containing 5,000, 1000, 500, 100 and 50 copies of the target. Each dilution was analysed in triplicate across 5 separate runs. The efficiency range was required to be between 90 and 110% as outlined by (Broeders *et al.*, 2014).

Determination of the sensitivity

Target sensitivity assessed according to the principles outlined in the former AFNOR XP V03-020-2 standard (AFNOR 2003). This standard no longer exists, but the principles detailed in it are still valid (Marien *et al.*, 2018). The absolute limit of detection (LOD) was determined for the PCR assay (primers + probe + amplification program) on dilutions of plasmid DNA.

Subsequent dilutions were prepared to contain 100, 50, 20, 10, 5, 2, 1 and 0.1 copies of the target. In accordance with the AFNOR XP V03-020-2 (LOD₆) standard, six PCRs were conducted for each dilution. The method's LOD₆ was defined as the lowest copy number for which all six PCRs produced positive signals, but only if the final dilution containing 0.1 copy per reaction yielded a maximum of one positive PCR signal out of the six replicates. If more than one positive signal was observed for the 0.1 copy dilution, the DNA quantities had to be revised. This LOD₆ approach has since evolved into LOD₁₀ (with ten repetitions instead of six).

TABLE 2 Experimental conditions tested to evaluate the robustness of the developed *A. domesticus* PCR protocol

PCR machine	QS5 (Life Technologies) or LightCycler 480 (Roche Diagnostics)				
PCR reagent kit	ABI Taqman 2× Universal PCR Master mix (Applied Biosystems™) or Universal Mastermix (Diagenode)				
Annealing temperature	59 °C or 61 °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)

in the recommendations for verification of analytical methods for GMO testing when implementing interlaboratory validated methods (ENGL, 2017). The copy number corresponding to LOD₁₀ was tested 60 times on the same plate to determine 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 number for LOD₁₀ and LOD_{95%} is 20 copies.

Digital PCR

The copy number of the plasmid DNA dilution, at approximately 200 copies/µl, was estimated by digital PCR using the Biomark™ HD system (Fluidigm Corporation, South San Francisco, CA, USA) with the 12.765 Digital Array™. These digital arrays comprise 12 panels (12 wells thus 12 samples). Each reaction loaded into a panel is partitioned into 765 individual PCR of 6 nl each. The reaction mixture included 4 µl of Universal Master Mix with passive reference (Diagenode, Seraing, Belgium), 0.15 µl of each primer (18.1 µM), 0.15 µl of probe (28.8 µM), 0.4 µl GE sample loading reagent (Fluidigm Corporation) and 3.15 µl of plasmid DNA. Eight µl of reaction mix were dispensed into each sample inlet and approximately 4.6 µl of this reaction mix was distributed throughout the partitions within each panel using an automated NanoFlex IFC Controller (Fluidigm Corporation) (Corbisier *et al.*, 2010). Two arrays were analysed with for each, six and five replicates. The thermal program was as follows: 10 min activation step at 95 °C, 50 cycles of 15 s at 95 °C for denaturation and 60 s at 60 °C for annealing and extension.

The number of target molecules per panel was determined using the BioMark HD Digital PCR software.

Robustness of the PCR method

The robustness of the method was assessed by introducing slight deviations to the standard experimental conditions (CCMAS, 2010; Broeders *et al.*, 2014; Debode *et al.*, 2017b), including adjustments to the annealing temperature (60 ± 1 °C), primer concentrations (standard or reduced by 30%), probe concentration (standard or reduced by 30%) and the volume of the real-time PCR mastermix (standard or ± 1 µl) resulting a final reaction volume of 25 ± 1 µl. Six replicates of the target at 20 copies/5 µl (plasmid DNA) were tested with the conditions outlined in Table 2. The robustness evaluation was performed on two real-time PCR platforms with appropriate Mastermixes: the QuantStudio™ 5 Real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with the Taqman 2× Universal PCR Mastermix (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA, USA) and the LightCycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) with the Universal Mastermix of Diagenode. The acceptance criterion was that all deviations from the standard protocol must yield a positive result at a level of 20 copies of the target (Broeders *et al.*, 2014).

Transferability of the method

The efficiency of amplification and the LOD₁₀ were tested on plasmid DNA (target cloned in the pGEM®-T Easy Vector) in a second laboratory (Food Research Institute Prague, Czech Republic).

Real-time PCR tests were performed on a StepOne™ Real-Time PCR System (Thermo Fisher Scientific) with software version 2.3. The SensiFAST™ Probe No-ROX Kit (Meridian Bioscience, Cincinnati, OH, USA) was used for the PCRs. To determine the efficiency of the PCR assay, a dilution series of plasmid DNA containing

5,000, 1000, 500, 100 and 50 copies of the target was prepared. Each dilution was analysed in triplicates.

The limit of detection (LOD) was established for the PCR assay (primers + probe + amplification program) on dilutions of plasmid DNA. The subsequent dilutions were set to contain 100, 50, 20, 10, 5, 2, 1 and 0.1 copies of the target.

3 Results and discussion

Specificity results

The *A. domesticus* specific primers and probe were designed on basis of the mitochondrial cytochrome b gene, yielding a PCR product with a size of 149 bp. The short size of the amplicon gives the possibility of detection and identification of house cricket DNA in processed food and feed products where the risk of DNA degradation due to processing factors such as high temperature, mechanical damage and exposure to chemicals, nucleases, or low pH exists (Ali *et al.*, 2015; Debode *et al.*, 2017c; Zdenkova *et al.*, 2018). The advantage of mitochondrial DNA is that thousands of copies can be present in cells (Rooney *et al.*, 2015). Therefore, focusing on mitochondrial DNA ensures good sensitivity of detection methods, even in processed products. However, its abundance can vary significantly per cell and depending on the tissues considered. This makes that genomic sequences, present at single or low copy numbers, are generally preferred for quantitation purposes. The low number of sequences for *A. domesticus* in the database makes it difficult to propose a test based on a single copy sequence.

The *A. domesticus* species-specific primers and probe were initially tested *in silico* with the primer-BLAST tool of NCBI (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). On the basis of the sequences available in the NCBI database, this test was considered as specific to the mitochondrial cytochrome b gene of the house cricket. Sequences alignment of the targeted cytochrome b region for five insect species belonging to the Gryllidae family is presented in Figure 1.

Experimental specificity testing was conducted on DNA from *A. domesticus* but also on 39 other insect species spanning various orders: Orthoptera (7 species), Diptera (6 species), Coleoptera (16 species), Hemiptera (2 species), Hymenoptera (3 species), Lepidoptera (3 species), Blattodea (1 species) and Dermaptera (1 species). Among the eight insect species from the Orthoptera order, five belong to the Gryllidae family (*Gryllus bimaculatus*, *Brachytrupes portentosus*, *G. assimilis*, *G.*

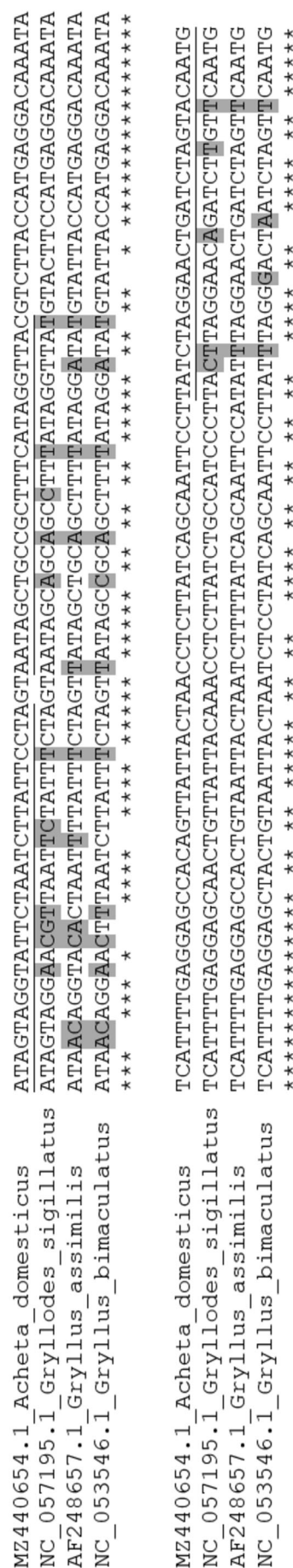


FIGURE 1 Alignment of nucleotide sequences of the part of cytochrome b gene from 4 species of the Gryllidae family. The location of the primers and probe specific to *Acheta domesticus* is underlined. The differences between sequences are highlighted in grey and stars indicate identical nucleotides for all sequences.

sigillatus and *A. domesticus*). Three of them (*G. assimilis*, *G. sigillatus* and *A. domesticus*) are authorised in fish, pig and poultry feed and can be produced on large scale. Late signals were observed for five insect species, two of which are also from the Gryllidae family (Orthoptera). The mean Cq value obtained for *G. sigillatus* exceeds 37 cycles and, for *G. assimilis*, it exceeds 40 cycles. For the three other species (*Curculionidae/Scolytidae* (Coleoptera), *Blatta orientalis* L. (Blattodea) and *Forficula auricularia* L. (Dermaptera)), the mean Cq values obtained exceeded 40 cycles. The few observed unspecificities are not problematic as the amplification signals are late with concentrated DNAs. Moreover, the two Gryllidae species that exhibit non-specificity are also authorized for use in feed. All other insect species gave negative signals.

No signals were obtained with the 31 other animal species (vertebrates, mollusc and crustaceans) and the seven plant species tested (Table 3).

Applicability

Different types of samples were analysed: three industrial meals of *A. domesticus* intended for animal feed, eight food samples labelled to contain *A. domesticus* (from the European Union) and six food samples labelled only with the mention “cricket” without specifying the species (three from European Union and three from countries outside the European Union). Two DNA extracts from each sample were analysed by PCR in duplicate. The PCR assay successfully detected *A. domesticus* in all samples where the presence of *A. domesticus* was specified. In five out of six samples where the cricket species was not declared/specified, *A. domesticus* was detected; while in the last one, *A. domesticus* was not detected. For this last sample, an analysis using the PCR test for the detection of *G. sigillatus* published by Daniso *et al.* (2020) confirmed that the cricket used was *G. sigillatus*. The composition of samples and obtained results are presented in Table 4.

Please note that the production process for the commercial samples in Table 4 is unknown. Therefore, it is not possible to establish a relationship between the Cq values obtained and the treatments undergone. We can only observe that, with the exception of the first industrial meal listed in the table, signals obtained are earlier for the pure meals. It is also important to mention that for the applicability testing, DNA quantities were not normalized to 10 ng because in products containing multiple ingredients, a significant portion of the DNA may originate from non-insect ingredients. Conse-

quently, for the commercial samples, 5 µl of extracted DNA was used for PCR amplifications.

Linearity and efficiency

The cytochrome b gene, located in mitochondrial DNA, can be considered as a multicopy target. In order to have a better control of the copy number, the sensitivity, efficiency and robustness were conducted on plasmid DNA (target cloned in the pCR2.1 plasmid). To evaluate the real-time PCR system's efficiency and linearity, five standard curves were generated, each containing five calibration points (5,000, 1000, 500, 100 and 50 copies). Two independent dilutions were prepared for each calibration point, and each dilution was tested in triplicate.

The acceptance criterion were satisfied with an average slope value of the standard curves within the range of $-3.60 \leq \text{slope} \leq -3.10$, corresponding to an amplification efficiency of 90-110%, and a correlation coefficient (R^2) > 0.98, in accordance with Hougs *et al.* (2017). The slope and correlation coefficient (R^2) of the standard curve were -3.58 and 0.99, respectively, resulting in a PCR efficiency of 91.6%.

Sensitivity

The sensitivity of the developed real-time PCR assay was measured using plasmid DNA dilutions (target cloned in PCR2.1 plasmid) containing different copy numbers, including 100, 50, 20, 10, 5, 2, 1, 0.1 and 0 copies in a single reaction. Each level was tested in 10 replicates. The LOD₁₀, which represents the level at which all 10 replicates are positive, was determined to be 5 copies. This result meets the acceptance criterion, as the LOD₁₀ must be ≤20 copies. However, at the level of 0.1 copies, 2 out of 10 positive results were observed while a maximum of 1 out of 10 positive was expected. A replication of the experiment with new dilutions yielded the same results, which can be explained considering the Poisson distribution (Tellinghuisen and Spiess, 2015).

To ensure that the number of copies in the dilutions was not underestimated, digital PCR was performed on the BioMark HD (Fluidigm Corporation) for a more accurate estimation of the present copy numbers. The level of 1000 copies (in 5 µl) was estimated at 1005 copies by digital PCR so we considered that the copy number in the different dilutions was not underestimated.

The LOD_{95%} was tested with 60 replicates at the determined LOD₁₀ level of 5 copies, resulting in 59 out of 60 positive results. This fulfils the acceptance criteria. At the level of 10 copies, all 60 out of 60 replicates yielded positive answers.

TABLE 3 Evaluation of the specificity of the *Acheta domesticus* PCR test. For positive samples, the mean quantification cycle values (m) and its standard deviations (σ) are given in brackets. The origin of the insect samples is specified with "a" for insects collected by trained entomologists, "b" for insects purchased from specialised companies and "c" for the insects provided by the Functional and Evolutionary Entomology lab of Gembloux Agro-Bio Tech (ULiège, Gembloux, Belgium)

Taxonomic classification	Latin name or family	Common name	Origin	Mean Cq obtained on undiluted extract and standard deviation σ
Insects	Orthoptera	<i>Acheta domesticus</i> L.	b	+ (m = 21.88, σ = 0.01)
		<i>Locusta migratoria</i> L.	c	–
		<i>Gryllus bimaculatus</i> (De Geer)	b	–
		<i>Pantaga succincta</i> (Johannson)	b	–
		<i>Schistocerca</i> sp. (Forsskål)	b	–
		<i>Brachytrupes portentosus</i> (Lichtenstein)	b	–
		<i>Gryllus assimilis</i> F.	b	+ (m = 40.33, σ = 0.01)
		<i>Gryllodes sigillatus</i> (Walker)	b	+ (m = 37.67, σ = 0.45)
		<i>Hermetia illucens</i> L.	b	–
		<i>Bibio marci</i> L.	c	–
		<i>Sarcophaga carnaria</i> L.	c	–
		<i>Musca domestica</i> L.	a	–
		<i>Episyrphus balteatus</i> (De Geer)	a	–
		<i>Tabanus</i> sp. L.	a	–
	Diptera	<i>Tenebrio molitor</i> L.	b	–
		<i>Pachnoda</i> sp. (Burmeister)	b	–
		<i>Zophobas morio</i> F.	b	–
		<i>Melolontha melolontha</i> L.	c	–
		<i>Leptinotarsa decemlineata</i> (Say)	c	–
		<i>Cassida viridis</i> L.	c	–
		<i>Nicrophorus humator</i> (Gleditsch)	c	–
		<i>Nicrophorus vespillo</i> L.	c	–
		<i>Phyllophaga</i> (Harris)	b	–
		Coccinellidae sp. (Latreille)	a	–
		Staphylinidae (Latreille)	a	–
		Curculionidae/Scolytidae (Latreille)	a	+ (m = 41.07, σ = 0.20)
	Coleoptera	House cricket	b	
		Migratory locust	c	
		Mediterranean field cricket	b	
		Bombay locust	b	
		Bird grasshoppers	b	
		Giant cricket	b	
		Jamaican field cricket	b	
		Banded cricket	b	
		Black soldier fly	b	
		St. Mark's fly	c	
		Common fresh fly	c	
		House fly	a	
		Marmalade hoverfly	a	
		Horse fly	a	
		Mealworm	b	
		Dola's worm	b	
		Superworm	b	
		Cockchafer	c	
		Colorado potato beetle	c	
		Green tortoise beetle	c	
		Black sexton beetle	c	
		Common burying beetle	c	
		May Beetle/June bug or July beetle	b	
		Ladybird	a	
		Rove beetles	a	
		True weevils	a	

TABLE 3 (Continued)

Taxonomic classification	Latin name or family	Common name	Origin	Mean Cq obtained on undiluted extract and standard deviation σ
Coleoptera	<i>Rhynchophorus ferrugineus</i> (Olivier)	Red palm weevil	b	–
	<i>Cybister limbatus</i> F.	Diving beetle	b	–
	<i>Lucanus cervus</i> L.*	Stag beetle	a	–
	<i>Alphitobius diaperinus</i> (Panzer)	Lesser mealworm	a	–
Hemiptera	Cicadidae sp. (Latreille)	Cicada	b	–
Hymenoptera	Belostomatidae sp. (Leach)	Giant water bug	b	–
	<i>Oecophylla smaragdina</i> F.	Weaver ant	b	–
	<i>Bombus terrestris</i> L.	Buff-tailed bumblebee	a	–
	<i>Vespula</i> sp. (Thomson)	Wasp	a	–
Lepidoptera	<i>Bombyx mori</i> L.	Silkworm	b	–
	<i>Omphisa fuscidentalis</i> (Hampson)	Bamboo worm	b	–
	<i>Galleria mellonella</i> L.	Greater wax moth	a	–
	<i>Blatta orientalis</i> L.	Oriental cockroach	c	+
Blattodea	<i>Forficula auricularia</i> L.	Common earwing	c	(m = 40.07, σ = 0.60)
Dermaptera	<i>Haplophelma albostriatum</i> (Simon)	Tarantulas	–	+
	<i>Heterometrus longimanus</i> (Herbst)	Black scorpion	–	(m = 42.62, σ = 0.41)
	<i>Daphnia</i> sp. (Müller)	Plankton for fish	–	–
		Daphnies	–	–
Cladocera	<i>Cyclops bicuspidatus</i> (Claus)	Cyclops	–	–
	<i>Euphasia superba</i> (Dana)	Antartic krill	–	–
	<i>Crangon crangon</i> L.	Common shrimp	–	–
	<i>Nephrops norvegicus</i> L.	Langoustine	–	–
Cyclopoida	<i>Homarus gammarus</i> L.	European lobster	–	–
	<i>Penaeus vannamei</i> (Boone)	Whiteleg shrimp	–	–
	<i>Teuthida</i> sp. (Naef)	Squid	–	–
			–	–
Euphausiacea				
Decapoda				
Mollusca				
Decapodiforme				

TABLE 3 (Continued)

Taxonomic classification		Latin name or family	Common name	Origin	Mean Cq obtained on undiluted extract and standard deviation σ
Mammals	Artiodactyla	<i>Bos taurus</i> L.	Beef	–	–
		<i>Ovis aries</i> L.	Sheep	–	–
		<i>Sus scrofa domestica</i> (Erxleben)	Pork	–	–
		<i>Cervus elaphus</i> L.	Stag	–	–
		<i>Sus scrofa scrofa</i> L.	Wild boar	–	–
		<i>Capra hircus</i> L.	Goat	–	–
		<i>Equus caballus</i> L.	Horse	–	–
		<i>Rattus rattus</i> L.	Black rat	–	–
		<i>Homo sapiens</i> L.	Human	–	–
		<i>Gadus morhua</i> L.	Atlantic cod	–	–
Fish	Scombriformes	<i>Melanogrammus aeglefinus</i> L.	Haddock	–	–
		<i>Scomber scombrus</i> L.	Atlantic mackerel	–	–
		<i>Engraulis encrasicolus</i> L.	European anchovy	–	–
Birds	Galliformes	<i>Gallus gallus</i> L.	Chicken	–	–
		<i>Meleagris gallopavo</i> L.	Turkey	–	–
		<i>Phasianus colchicus</i> L.	Common pheasant	–	–
		<i>Coturnix japonica</i> (Temminck. & Schlegel)	Japanese quail	–	–
		<i>Cairina moschata</i> L.	Muscovy duck	–	–
		<i>Anser Sp.</i> L.	Goose	–	–
		<i>Columba livia</i> (Gmelin).	Common pigeon	–	–
		<i>Glycine max</i> (Merr)	Soybean	–	–
		<i>Zea mays</i> L.	Maize	–	–
		<i>Triticum aestivum</i> L.	Common wheat	–	–
Plants	Caryophyllales	<i>Oryza sativa</i> L.	Asian rice	–	–
		<i>Brassica napus</i> L.	Rapeseed	–	–
		<i>Solanum lycopersicum</i> L.	Tomato	–	–
		<i>Beta vulgaris</i> L.	Sugar beet	–	–

* The *Lucanus cervus* (protected species) was not collected in the environment but obtained from an old insect box coming from a private collection.

TABLE 4 Cq mean values obtained with the *Acheta domesticus* PCR test on industrial meals and food samples labelled to contain *A. domesticus* or crickets (n = 2). The standard deviations (σ) are given in brackets

Description and composition of samples containing <i>A. domesticus</i> or crickets	Feed or food destination	Produced in the EU or outside the EU	Extract number	Cq mean and standard deviations σ
Industrial meal of <i>Acheta domesticus</i> for feed	Feed	EU	1	23.26 ($\sigma = 0.10$)
			2	22.30 ($\sigma = 0.11$)
Industrial meal of <i>Acheta domesticus</i> for feed	Feed	EU	1	17.95 ($\sigma = 0.04$)
			2	18.01 ($\sigma = 0.09$)
Industrial meal of <i>Acheta domesticus</i> for feed	Feed	EU	1	18.06 ($\sigma = 0.10$)
			2	17.70 ($\sigma = 0.25$)
Meal: 100% cricket powder <i>Acheta domesticus</i> .	Food	EU	1	16.18 ($\sigma = 0.05$)
			2	15.92 ($\sigma = 0.07$)
Meal: 100% house cricket <i>Acheta domesticus</i> .	Food	EU	1	17.90 ($\sigma = 0.14$)
			2	17.88 ($\sigma = 0.04$)
Meal: 100% domestic crickets <i>Acheta domesticus</i>	Food	EU	1	18.34 ($\sigma = 0.00$)
			2	18.38 ($\sigma = 0.14$)
Breakfast product: oat flakes, maple syrup, hazelnuts, agave syrup, sunflower oil, cricket powder <i>Acheta domesticus</i> (5%), pumpkin seeds, quinoa, sunflower seeds, flax seeds, sesame seeds, cinnamon, nutmeg, salt.	Food	EU	1	24.25 ($\sigma = 0.28$)
			2	25.61 ($\sigma = 0.12$)
Breakfast product: sprouted, oat flakes, cricket flour (<i>Acheta domesticus</i>) (10%), dried sprouted red quinoa flakes, panela, rice powder (rice syrup powder, rice starch, rice flour), dried apples, apple powder, sea salt, natural flavouring, ground cinnamon.	Food	EU	1	22.11 ($\sigma = 0.06$)
			2	22.30 ($\sigma = 0.10$)
Snack product: hazelnuts, agave syrup, pumpkin seeds, oat flakes, quinoa, rice syrup, sesame seeds, buckwheat, cricket powder <i>Acheta domesticus</i> (5%), hemp seeds, flax seeds.	Food	EU	1	19.98 ($\sigma = 0.06$)
			2	21.01 ($\sigma = 0.04$)
Snack product: pea flour, cricket flour (<i>Acheta domesticus</i>) (10%), garlic, sunflower oil, herbs (wild garlic, paprika, satureja, basil, pepper, oregano, thyme), potato starch, yeast extract, salt.	Food	EU	1	25.04 ($\sigma = 0.02$)
			2	24.95 ($\sigma = 0.07$)
Pasta: Red lentil flour, cricket flour (<i>Acheta domesticus</i>) (10%).	Food	EU	1	22.82 ($\sigma = 0.03$)
			2	22.78 ($\sigma = 0.04$)
Meal: cricket powder	Food	EU	1	15.06 ($\sigma = 0.14$)
			2	15.12 ($\sigma = 0.05$)
Snack product: wheat flour, water, sunflower oil, potato flakes, dried glucose syrup, cricket powder, salt.	Food	EU	1	27.96 ($\sigma = 0.30$)
			2	26.45 ($\sigma = 0.16$)

TABLE 4 (Continued)

Description and composition of samples containing <i>A. domesticus</i> or crickets	Feed or food destination	Produced in the EU or outside the EU	Extract number	Cq mean and standard deviations σ
Snack product: pea powder, cricket powder (10%), poppy powder, lentil flour, pumpkin seed powder, rapeseed oil, spices, yeast, starch, sea salt.	Food	EU	1	24.81 ($\sigma = 0.07$)
			2	25.46 ($\sigma = 0.05$)
Meal: 100% cricket powder	Food	Outside the EU	1	18.53 ($\sigma = 0.07$)
			2	18.38 ($\sigma = 0.07$)
Meal: 100% cricket powder	Food	Outside the EU	1	18.11 ($\sigma = 0.05$)
			2	17.98 ($\sigma = 0.03$)
Meal: cricket powder	Food	Outside the EU	1	NR*,**
			2	NR*,**

* Non reproducible signal, only 1 late signal on 2 repetitions.

** Identified as *G. sigillatus* with the PCR methods proposed by Daniso *et al.* (2020) slightly modified.

Robustness

The robustness testing procedure followed the orthogonal setup proposed by Broeders *et al.* (2014). Robustness testing aims to determine whether a method remains relatively unaffected by deviations from the standard protocol. Various deviations were introduced, including using different types of Master Mixes (Diagenode vs Life Technologies), altering concentrations of Mix components (30% less primers, 30% less probe, less/changed Master Mix volumes by adding or subtracting 1 μ l), employing different instruments (QuantStudio™ 5 and LightCycler 480) and adjusting annealing temperatures (deviating by more or less than 1 °C from standard conditions). The modifications tested are outlined in Table 2.

Examining the histograms (Figure 2), the largest differences occur when the annealing and elongation temperature increases to 61 °C while using Taqman 2× Universal PCR Mastermix from Thermo Fisher on the Quantstudio™ 5 thermocycler. However, when using the LightCycler 480 with Universal Mastermix from Diagenode, the differences between annealing temperatures of 59 °C and 61 °C are less pronounced. Regardless of the Master Mix used, reducing the concentration of primers appears to have the most substantial impact on the observed Cq values.

The robustness assessment was carried out at a level of 20 copies of the target and all deviations to the standard experimental protocol delivered a positive result at this very low level. The method remained unaffected and the acceptance criteria were met.

QS5 / LC480 qPCR instruments used. Annealing temperature = 59 °C/61 °C; St = Standard conditions; St + 1 = increase of 1 μ l of Master Mix (total volume 26 μ l); St – 1 = decrease of 1 μ l of Master Mix (total volume 24 μ l); St – 30%FR = decrease of 30% of the concentration of the Forward and Reverse primers; St – 30%P = decrease of 30% of the concentration of the probe.

Transferability

Transferability testing was conducted in a second laboratory, the FRIP laboratory (Prague, CZ), which is distinct from the laboratory where the method was originally developed (CRA-W, Gembloux, BE). The tests were performed using the StepOne™ real time PCR machines with the SensiFAST™ Probe No-ROX MasterMix. Results showed similar performances with a PCR efficiency of 99.0%, a slope of –3.35 and a R² of 0.99. The limit of detection (LOD₁₀) was estimated at 5 copies of the target, which aligns with the LOD obtained at the CRA-W laboratory. The values are consistent with the recommendations outlined in this study. Moreover, the utilization of different real-time PCR machines and MasterMix further emphasizes the robustness of the method.

4 Conclusion

Given the growing interest in incorporating edible insects in food and feed products, the need for effective identification methods has become necessary. In this study, a real-time PCR method with TaqMan™ probe was developed for the detection of the house cricket in

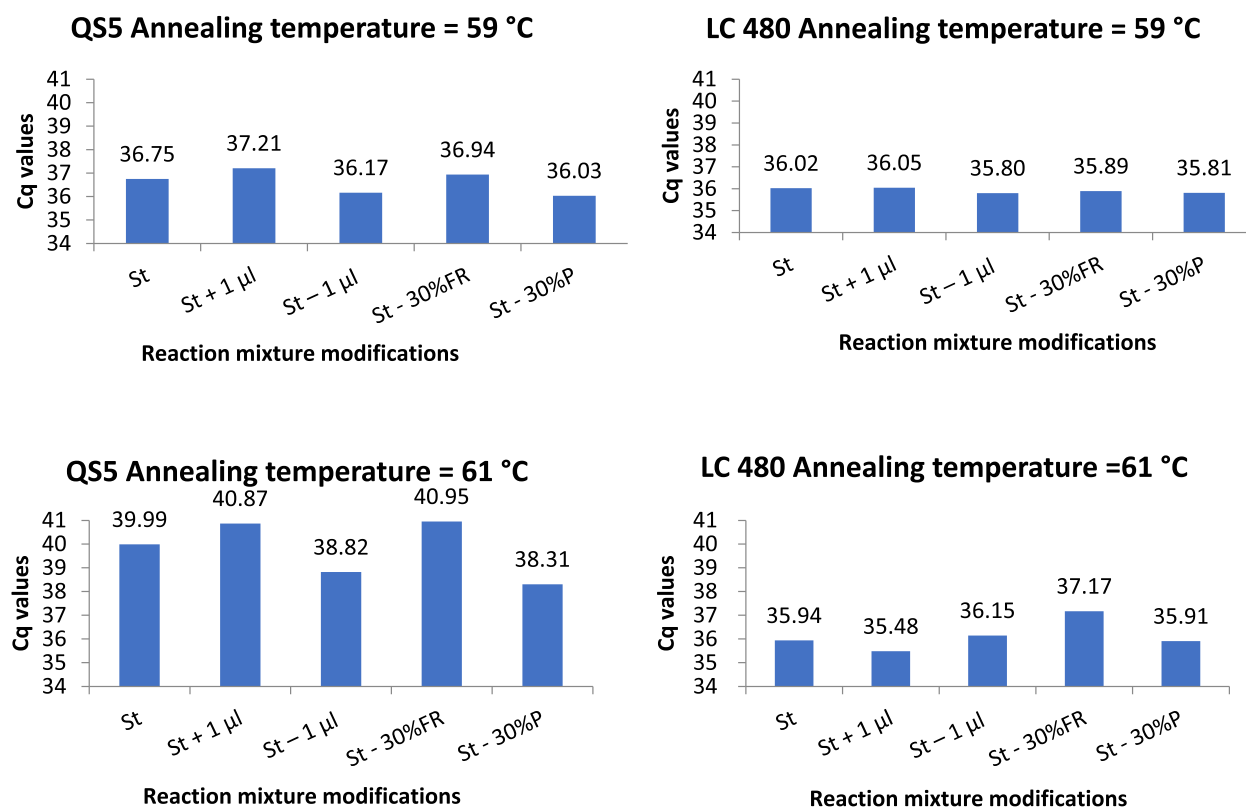


FIGURE 2 Robustness testing. Mean Cq observed for each experimental condition during robustness testing.

food and feed. The method utilizes the mitochondrial cytochrome b gene as its target, a multicopy region. It is advantageous for detection, enabling the identification of even trace amounts of *A. domesticus* in a sample. The specificity testing yielded favourable outcomes, demonstrating the method's accuracy in distinguishing between insect, animal, and plant species. Only five insect species exhibited unexpected late positive signals. The proposed PCR test was found to be well-suited for its intended purpose, meeting criteria related to amplification efficiency, specificity, sensitivity, robustness and transferability. The applicability of the method has been demonstrated on a wide variety of samples. This study provides an additional tool for the detection of *A. domesticus*.

The melting temperature of the primers and probes allows for this *A. domesticus* method to be employed on the same PCR plate as tests for other insect species that require a hybridization temperature of 60 °C, such as *Tenebrio molitor* (Debode *et al.*, 2017a), *Hermetia illucens* (Zagon *et al.*, 2018), *Oxya chinensis* (Kim *et al.*, 2019b) and *Alphitobius diaperinus* (Garino *et al.*, 2021; Marien *et al.*, 2022). This compatibility arises because all these tests share the same thermal program.

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