Set-up and validation of end-point polymerase chain reaction for the detection of edible insect DNA – *Acheta domesticus* and *Tenebrio molitor* – in food products

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Abstract

Insects offer an alternative protein supply for both animal and human consumption. Recently, companies in Europe have begun insect production for use in food and animal feed. Following Regulation (EU) No. 2015/2283, edible insects are classified under the novel food category and can be marketed and consumed within the European Union. As a result, methods of authentication are necessary to verify the compliance of these insect-based products. In this study, two end-point polymerase chain reaction assays for the detection of the 16S rRNA gene of *Acheta domesticus* and *Tenebrio molitor* were fine-tuned and validated. The primer specificity was tested on insect samples, other animals, and plants, and the gene was amplified only in target samples. The limit of detection was evaluated using different food matrices contaminated with decreasing concentrations of insect powder and was below 0.05% (w/w).

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Introduction

Trends estimate that by 2050, the world population will grow to approximately 9 billion people, and, as a result, the environmental impact of the food production chain will also increase (FAO, 2009; Smetana *et al*., 2023). Insects have gained increasing public attention in Europe as a potential solution to today's food and nutrition challenges, particularly considering the rising demand for animal protein, offering a food source that is both health-conscious and environmentally sustainable (Orsi *et al*., 2019; Gałęcki *et al*., 2023). Indeed, insect farming produces limited greenhouse gas emissions compared to traditionally farmed animals and allows for lower use of land and water (Oonincx and Boer, 2012; Nadeau *et al*., 2015). Additionally, insects require less feed and can be raised on organic waste while providing nutritional values comparable to those of other commonly consumed animals and plant-based products (FAO, 2013; EFSA Scientific Committee, 2015).

According to Regulation (EU) No. 2015/2283, insect-based food products have been classified as novel foods, that is, food which had not been significantly consumed by humans in the European Union before 15 May 1997 (European Parliament and Council of the European Union, 2015). Currently, four insect species are listed in the Regulation (EU) No. 2015/2283: *Locusta migratoria*, *Tenebrio molitor* (yellow mealworm), *Acheta domesticus* (house cricket), and *Alphitobius diaperinus* (lesser mealworm) (Conway *et al*., 2024; Turck *et al*., 2024). In particular, the authorized novel foods, as listed in Regulation (EU) 2017/2470 (in Table 1), in accordance with Regulation (EU) 2015/2283, are frozen, dried, and powder of *L. migratoria*, *A. domesticus*, and *A. diaperinus* and *T. molitor* larvae; and partially defatted powder of *A. domesticus* (European Commission, 2017).

Food authenticity is crucial for preventing food fraud, maintaining ingredient quality and safety, and minimizing cross-contamination (Hillinger *et al*., 2023). High-value or premium products are

particularly vulnerable to adulteration with lower-quality or inexpensive substitutes, requiring analytical verification for effective monitoring (Haiminen *et al*., 2019). DNA barcoding techniques have been established for species classification based on the cytochrome oxidase I (*COI*) gene; however, these methods typically require DNA fragments ranging from 600 to 800 bp. However, DNA in processed food and feed can be highly degraded, requiring shorter target sequences. In these cases, polymerase chain reaction (PCR) methods are currently recognized as valuable for detecting and identifying animal and plant species in food products due to their high sensitivity and specificity, as well as their rapid execution and cost-effectiveness (Debode *et al*., 2017; Böhme *et al*., 2019). The aim of this study was to set up and validate end-point PCR methods for the detection of the 16S rRNA gene of *A. domesticus*, *T. molitor* and *L. migratoria* in human food products. A method for *A. diaperinus* was not tested, since at the moment this insect and products using this species are not available on the Italian market.

Materials and Methods

Samples collection

T. molitor dried larvae, dried *A. domesticus*, and live *L. migratoria* were purchased from online retailers. Other adult insects to test primers specificity (n=31) were either purchased alive from online retailers (*e.g.*, bait or collectors' shops) or collected in the natural environment. Sampling was focused on testing the primers on the highest possible number of species and orders among those available. Moreover, attention was given to those species that most commonly are found in anthropic environments and, therefore, more likely to appear as accidental contaminants. Other animal and plant samples were bought from supermarkets.

The genomic DNA from all samples was extracted using the commercial DNeasy® mericon food kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

End-point polymerase chain reaction assays

Three pairs of primers (Table 1), previously designed by Tramuta *et al.* (2018), were selected for the species-specific amplification of the mitochondrial 16S ribosomal RNA gene (16S rRNA) of *A. domesticus*, *T. molitor*, and *L. migratoria* using end-point PCR. Simplex PCR reactions were performed with a total reaction volume of 25 µL, consisting of GoTaq® G2 Hot Start Master Mix (1X) (Promega, Madison, Wisconsin, USA), 0.2 µM of the primer pair, 2.5 µL of extracted DNA and distilled water. The PCRs were loaded in the ProFlex PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with the following thermal profile: initial denaturation at 95°C for 2 minutes, 35 cycles of denaturation of 30 seconds at 94°C, annealing at 55°C for 90 seconds, extensions at 72°C for 90 seconds, and a final extension of 10 minutes at 72°C. The PCR products were visualized with capillary electrophoresis on a QIAxcel Advanced System (Qiagen, Hilden, Germany) using the QIAxcel® DNA Screening Kit (v2.0) with QX Alignment Marker 15 bp/1 kb and QX DNA Size Marker 50-800 bp v2.0 (5 ng/µL).

Primer specificity

The specificity of the primers was evaluated using 34 insect samples collected from the natural environment and belonging to different taxonomic orders. The species of the tested samples was confirmed by sequencing the *COI* gene using the previously designed primer set jgLCO1490 and jgHCO2198 (~700 bp) (Geller *et al*., 2013) on an Applied Biosystems SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The resulting sequences were submitted to the BLAST tool (https://www.ncbi.nlm.nih.gov/) for species identification. Additionally, the specificity was also verified against four other animal phyla, in particular two crustacean samples, two mollusks, two fish, and two mammal species, and against three plant species.

Polymerase chain reaction limit of detection in insect-based food

The sensitivity of the method was tested on four food matrices purchased at the supermarket, namely white flour, dry biscuits, cereal bars, and plant-based burgers, corresponding to categories of food currently available in Europe, with the addition of insect powder. The matrices were homogenized and supplemented with decreasing concentrations of dried and ground *A. domesticus* and *T. molitor*, purchased from an online retailer and previously tested with the two PCRs. In baked goods formulations, insect powder is used on average to replace 5-25% of conventional flours (Yazici and Ozer, 2021). Therefore, to determine sensitivity, decreasing concentrations from 2% down to 0.00001% were arranged, extracted using the DNeasy® mericon food kit (Qiagen, Hilden, Germany), and tested with the two PCR methods. For each dilution, the four food matrices were prepared and analyzed in 10 replicates. The food samples were also extracted and tested without any addition of insect powder to verify that they were not contaminated with *A. domesticus* and *T. molitor*.

Results

Primer specificity

In total, the primers were tested on insects belonging to 9 different taxonomic groups (12 Coleoptera, 7 Diptera, 4 Lepidoptera, 3 Hymenoptera, 3 Rhynchota, 2 Orthoptera, 1 Blattoidea, 1 Homoptera, and 1 Thysanura), 4 other animal phyla, and 3 plant samples. Table 2 shows the taxonomic assignment of the

Table 1. Primers detailed by Tramuta et al. used for the amplification of the 16S rRNA of *A. domesticus, T. molitor,* and *L. migratoria.* Reproduced from: Tramuta *et al.* (2018).

Target	Primer sequence (5'-3')	
Acheta domesticus	Forward	CAAAGGTAGCATAATCATTAGT
	Reverse	TCATTCCATAATACAGGATCA
Tenebrio molitor	Forward	CAAAGGTAGCATAATCATTAGT
	Reverse	AGTTAAATAAATTTTCTAACCG
Locusta migratoria	Forward	CAAAGGTAGCATAATCATTAGT
	Reverse	CTCCGGTTTGAACTCAGATC

sequenced samples and the associated percentage of identity. The two end-point PCRs enabled the specific amplification of the 16S gene only in *A. domesticus* and *T. molitor* samples (Table 2). The *L. migratoria* primers exhibited insufficient specificity, producing a positive amplification across all tested insects, crustaceans, and mammal samples, and in two plant species. For this reason, the validation for this species was interrupted in the early stages of the study.

Polymerase chain reaction limit of detection in insect-based foods

The PCR methods detected target species DNA for both *A. domesticus* and *T. molitor*, with sensitivity ranging from 0.05% to 0.0001% (w/w) in the various food matrices analyzed. In particular, the limit of detection (LOD) for *A. domesticus* in white flour, cereal bars, and plant-based burgers was 0.01%, while in dry biscuits it was 0.001% (Table 3). Instead, regarding the PCR for *T. molitor*, the LOD was 0.05% for dry biscuits, 0.001% for cereal bars, and 0.0001% for white flour and plant-based burgers (Table 3).

Discussion

Around 2000 insect species are consumed globally (Kim *et al*.,

Table 2. Results of the taxonomic classification, along with the associated percentage of identity, obtained through COI gene sequencing, and the 16S polymerase chain reaction results for *Acheta domesticus, Tenebrio molitor,* and *Locusta migratoria* on insects, other animals, and plant species.

Taxonomic classification		Species identification	% identity	*A. domesticus*	*T. molitor*	*L. migratoria*
Insecta	Coleoptera	*Tenebrio molitor*	99.56	-	+	+
		Pentodon bidens	98.18	-	-	+
		Harpalus dimidiatus	99.85	-	-	+
		Gonioctera fornicata	100	-	-	+
		Hister quadrimaculatus	99.23	-	-	+
		Oxythyrea funesta	100	-	-	+
		Protetia morio	92.85	-	-	+
		Calathus fuscipes	98.76	-	-	+
		Cidnopus pilosus	97.12	-	-	+
		Cetonia aurata	99.39	-	-	+
		Isomira testacea	99.69	-	-	+
		Harmonia axyridis	99.69	-	-	+
	Diptera	*Calliphora vomitoria*	99.70	-	-	+
		Epistrophe nitidicollis	100	-	-	+
		Voria ruralis	99.37	-	-	+
		Fannia canicularis	99.83	-	-	na
		Bibio hortulanus	98.33	-	-	+
		Musca domestica	99.09	-	-	+
		Calliphora vicina	99.71	-	-	+
	Lepidoptera	*Galleria mellonella*	99.85	-	-	+
		Plodia interpunctella	99.56	-	-	+
		Cadra cautella	97.68	-	-	+
		Papillio machaon	99.69	-	-	+
	Hymenoptera	*Vespula germanica*	99.70	-	-	+
		Polistes dominula	99.39	-	-	+
		Apis mellifera	99.40	-	-	na
	Rhynchota	*Halyomorpha halys*	99.54	-	-	+
		Pyrrhocoris apterus	98.27	-	-	+
		Lygaeus equestris	99.54	-	-	+
	Orthoptera	*Acheta domesticus*	99.85	+	-	+
		Locusta migratoria	100	-	-	+
	Blattodea	*Blatta orientalis*	99.41	-	-	na
	Homoptera	*Haematoloma dorsatum*	93.04	-	-	+
	Thysanura	*Ctenolepisma calvum*	99.24	-	-	+
Crustacea		*Litopenaeus vannamei*	100	-	-	+
		Pleoticus muelleri	100	-	-	+
		Armadillidium vulgare	99.70	-	-	+
Mollusca		*Ruditapes phillippinarum*	100	-	-	-
		Mitilus edulis	99.20	-	-	-
Teleostei		*Salmo salar*	97.20	-	-	-
		Oncorincus mykiss	100	-	-	-
Mammalia		*Bos taurus*	100	-	-	+
		Sus scrofa	100	-	-	+
Plant		*Oryza sativa*	100	-	-	+
		Origanum onites	99.74	-	-	+
		Allium sativum	99.68	-	-	-

na, not available.

Table 3. Detection results of Acheta domesticus and Tenebrio molitor 16S gene using end-point polymerase chain reactions in the different tested food matrices.

% contamination (w/w)	White flour		Dry biscuits		Cereal bars		Plant-based burgers	
	Ad	Tm	Ad	Tm	Ad	Tm	Ad	Tm
0.1	+	+	+	+	+	+	+	+
0.05	+	+	+	+	+	+	+	+
0.01	+	+	+	-	+	+	+	+
0.001	-	+	+	/	-	+	-	+
0.0001	/	+	-	/	/	-	/	+
0.00005	/	-	/	/	/	/	/	-

Ad, *Acheta domesticus*; Tm, *Tenebrio molitor.*

2019). Even though only 34 insect species were included in the study, our findings demonstrated that these methodologies allowed a rapid, specific, and sensitive detection of the presence of two edible insect species currently authorized for sale in Europe. With the recent approval in EU legislation to include different insect species, such as *A. domesticus* and *T. molitor*, the relevance of these tests is becoming increasingly significant. The methods are suitable for processed products and can be used to verify the presence of specific species in a food ingredient list. The development of methods that enable the detection of insects in food products, thereby also allowing the control of potential fraud, is essential for consumer protection from both ethical and health perspectives. In this context, the assessment of declared ingredients assumes greater significance, as products supplemented with insect powder currently available on the market exhibit a significantly higher cost compared to traditional food products.

The short amplicons (290 bp for *A. domesticus* and 240 bp for *T. molitor*) are advantageous because they allow for the analysis of food products in which DNA is frequently degraded into small fragments due to the effects of high temperatures and pressure during the manufacturing process (Natonek-Wiśniewska *et al*., 2022). An alternative and highly effective method for detecting insect DNA in food samples is real-time PCR, which ensures accurate species authentication due to its high specificity. Its exceptional sensitivity allows the detection of minimal amounts of target DNA, while its ability to perform both detection and quantification in a single step eliminates post-PCR procedures, significantly reducing labour time (Velasco *et al*., 2020). However, PCR-based methods (*e.g.*, PCR end-point and real-time PCR) allow the simultaneous detection of a limited number of insect species (Hillinger *et al*., 2023). Next-generation sequencing technologies based on a target sequence enable the parallel analysis of all DNA molecules within a sample, including those found in trace amounts. This approach represents one of the most promising analytical methods for verifying the authenticity of complex food authenticity (Giusti *et al*., 2024). As a result, this technique allows the identification of all insect species present in a sample, including those not declared on the label.

Regarding *L. migratoria*, the primer set used in this study cross-reacted with all the insect samples analysed, as well as with some samples from other animals and plants. The result of the primer analysis using the Primer-BLAST tool confirmed that they are able to align with no mismatches and produce a fragment of the expected size (~460 bp) for various insect genera (*e.g.*, *Xestia* spp., *Thraulus* spp., *Habrophlebia* spp., *Pantomorus* spp., *etc*.). When specifically queried for *A. domesticus* and *T. molitor*, the reverse primer has only two mismatches, thus allowing for positive amplification. Further studies to identify specific primers will be required, possibly involving the development of real-time PCRs. In addition to allowing the quantification of different species in foods, these may also provide increased sensitivity due to the use of probes.

Conclusions

The assays developed in this study can be considered as screening methods for managing labelling issues in products containing insects for animal and human consumption.

In the future, we aim to investigate alternative protocols for the detection of *L. migratoria,* also evaluating the performance of real-time PCRs to increase specificity and extend and validate new methods for the detection of DNA from other insect species of interest (*e.g.*, *A. diaperinus*).

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