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Detection of *Bombyx mori* as a Protein Source in Feedingstuffs by Real-Time PCR with a Single-Copy Gene Target

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Abstract: The silkworm, *Bombyx mori*, is reared on a large scale, mainly for silk production. The waste from this silk production, like pupae, is underused. As an edible insect, *B. mori* is a good source of protein in human food and animal feed. In recent years, European legislation on the use of insects has evolved and a multitude of European companies have initiated the rearing of insects specifically for food and feed applications. Regarding animal feed, Commission Regulations (EU) 2021/1372 and 2021/1925 authorize eight insect species, including silkworm, as processed animal proteins for use in fish, pig, and poultry feed. The incorporation of edible insects into the human diet falls within Regulation (EU) No. 2015/2283 concerning novel foods. Implementation of authentication methods is imperative to ensure the conformity of the products. In the present study, we propose a specific real-time PCR method for the detection of silkworm (*B. mori*). The developed PCR test amplifies a 98 bp fragment of the cadherin gene. This gene is present in a single-copy per haploid genome, as demonstrated by experimental evidence. The qualitative method was successfully evaluated on the performance criteria of specificity, sensitivity, efficiency, robustness, and transferability. The applicability of the test was assessed on samples of *B. mori* from industry. Light microscopy and DNA metabarcoding approaches were used as a complement to genomic analysis as a means of providing authentication of the samples.

Keywords: insect; *Bombyx mori*; silkworm; lepidoptera; detection; real-time PCR; cadherin; feed

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1. Introduction

Bombyx mori, silkworm, is an insect primarily used for silk production. Ninety percent of the world's silk production comes from *B. mori* [1]. Sericulture generates various natural products with diverse applications. The cocoons are mainly exploited for silk production. The pupae are used for oil extraction, biogas and biodiesel production, mushroom cultivation, and chitosan production [2–4]. Silkworm frass is a combination of partially digested plant material and fecal matter with eventually dead larvae. It can be used as fertilizer [3].

Silkworm pupae are rich in proteins, calcium, vitamins, minerals, and lipids (essential fatty acids) [3–5]. Therefore, silkworm pupae and pupal waste (after oil or chitosan extraction) are good substitutes for fish meal or plant proteins in feed [2]. Their use as foodstuff for fish, poultry, and pigs has contributed to improved growth, increased resistance to pathogens, and reduced feed costs [5–7].

Comparing the protein content across different insect species is not straightforward. Indeed, the protein content is calculated based on the nitrogen content multiplied by a nitrogen-to-protein conversion factor, which varies between studies [8]. In terms of protein quality, a study published by Brogan et al. [9] showed that although the protein content is lower for *B. mori* (>50 g of crude protein/100 g of sample) than that of *Acheta domesticus* and *Locusta migratoria* adult powders (>70 g of crude protein/100 g of sample), the protein quality of *B. mori* pupae powder is higher based on a better ratio of essential amino acids to total amino acids content.

Another advantage of using *B. mori* pupae lies in the valorization of an abundantly available by-product of silk production, which is not the case for other species that are specifically reared for the purpose of making protein powders. Moreover, sericulture causes minimum environmental impact because a lot of silk producers cultivate mulberry trees to harvest their leaves, which serve as food for *B. mori*. Mulberry cultivation allows producers to reduce the carbon footprint of the sericulture [10].

In Europe, processed animal proteins of *B. mori* were authorized for use in fish, pig, and poultry feed in 2021. This species received approval because it only consumes leaves from mulberry trees (*Morus alba* and *Morus nigra*), ensuring no risk of contamination with feed products of animal-origin, which are currently banned for insect feeding [11,12].

However, the use of silkworm in feed was permitted later than the other seven insect species (*Hermetia illucens*, *Tenebrio molitor*, *A. domesticus*, *Alphitobius diaperinus*, *Gryllodes sigillatus*, *Gryllus assimilis*, and *Musca domestica*), which have been authorized in aquafeed since 2017 [13]. To avoid losses in cocoon harvesting during rearing, antibiotics are employed to prevent bacterial infections. The most used antibiotic is chloramphenicol [14,15], a broad-spectrum antibiotic. In Europe, chloramphenicol is prohibited in food-producing animals since it constitutes a hazard to human health at any concentration level [16].

In terms of human nutrition, *B. mori* is an insect consumed as a food in Asia for its nutritional benefits [17]. In 2004, the Chinese Ministry of Health recognized the silkworm chrysalis as a common food, and in 2016, a region of South China promulgated a local food safety standard for frozen fresh silkworm pupae as safe to eat [18]. In Europe, the consumption of insects falls within Regulation (EU) No. 2015/2283 on novel foods [19]. This implies that food containing insects or insect-derived products must undergo authorization after the European Food Safety Authority (EFSA) conducts risk assessments to ensure their safety [20]. In 2015, a list of insect species potentially usable as food was published by the EFSA [21]. *B. mori* was included in this list. However, at present, only certain products from four insect species have received approval to be placed on the food market: frozen, dried, and powder forms of *T. molitor* larvae [22,23] of *L. migratoria* [24], *A. domesticus* [25], and *A. diaperinus* larvae [26] as well as partially defatted powder of *A. domesticus* [27]. Since *B. mori* has traditionally been used for human consumption in some countries [21], it could also be employed in Europe once the EFSA has endorsed it as a safe novel food. Silkworms are good sources of high-quality proteins and lipids that can enhance the nutritional properties of high-energy biscuits, for example [28]. Additionally, some of these components may have potentially beneficial effects on chronic diseases [17]. However, as with all edible insects, attention must be paid to the risk of allergies. Indeed, a review published by de Gier and Verhoeckx [29] indicates that allergy to silkworms has been one of the most frequently reported, despite the fact that it is not the most commonly consumed insect. Correct labelling of products containing insects is therefore essential for the consumer.

Testing is necessary for both the producer's self-monitoring and for control laboratories that monitor the insects market. Indeed, it was demonstrated in a previous article on the *A. diaperinus* species that labeling errors were present [30]. Detection of insects can be achieved using real-time PCR, which remains the reference technique for DNA detection in food or feed products [31–35]. Therefore, real-time PCR assays have been published for insect detection. To facilitate DNA detection in processed food and feed where DNA may be degraded, tests target a fragment of mitochondrial DNA. Indeed, a mitochondrion possesses multiple copies of its genome, while a single cell can contain several

mitochondria [36]. Such tests for specific detection in feed and food have been published for *H. illucens* [37,38], *Oxya chinensis* (Thunberg) [39], and *G. sigillatus* [40]. Specific PCR tests to detect *B. mori* based on a portion of mitochondrial DNA were proposed by Kim et al. [41] and by Zarske et al. [15]. However, for quantitation purposes, this multicopy characteristic is a disadvantage since the copy number per cell will be variable depending on the considered tissue [37]. This is why a single-copy gene is used in other publications such as the real-time PCR tests for *T. molitor* [42] and *A. diaperinus* [30] detection. It should be noted that a specific PCR test to detect *B. mori* based on a single-copy gene has already been published by Zarske et al. [15].

In this study, we chose the real-time PCR method for specific detection of *B. mori* by focusing on a different chromosomal single-copy target per haploid genome, the *cadherin* gene, which was characterized for *A. diaperinus* by Hua et al. [43] and has already been used to develop real-time PCR tests for the detection of *T. molitor* [42] as well as *A. diaperinus* [30]. Considering that these last two tests share the same thermal program, the proposed test was developed in such a way that it can be used under the same amplification conditions. As such, it is possible to combine them on the same PCR plate, thereby facilitating the work for control laboratories. All the performance criteria recommended for the evaluation of a qualitative PCR test were performed under recommendations [44,45]. According to current analytical requirements in the EU [46], real-time PCR is combined with light microscopy (LM) for the detection of processed animal proteins in the context of the feed ban. Microscopic analyses were carried out to visually complement the detection of insects. A DNA metabarcoding approach was also used as a complement to PCR analysis as a means of providing authentication of the samples.

2. Materials and Methods

2.1. Samples

2.1.1. Insect Samples

Insects were either collected in their natural environment by trained entomologists, purchased from specialized companies, or provided by the Functional and Evolutionary Entomology Lab of Gembloux Agro-Bio Tech (ULiège, Gembloux, Belgium). Insects were selected to cover several taxonomic groups or to include close relatives with practical relevance to the species considered. All insects were either dead upon arrival at the laboratory or were killed by freezing at −80 °C, a method considered to be the best one by entomologists [47]. DNA extraction was primarily performed on single individuals, except for smaller insects, which required several individuals to yield sufficient material.

2.1.2. Pure *B. mori* Samples and Mixes Containing 0.1% of *B. mori*

Samples of chrysalis and caterpillars after cocoon formation of *B. mori* were obtained through the International Producers of Insects for Food and Feed (IPIFF, Brussels, Belgium) but the origin of the samples must remain anonymous. Two processed animal proteins (PAPs) of *B. mori* were bought online from a company specializing in ornamental fish feed.

With these four pure *B. mori* samples (P1 to P4, Table 1), mixes containing 0.1% (in mass fraction) of *B. mori* in blank feed (feed free from *B. mori*) were prepared (M1 to M12). For blank feed, three types of commercial feed (BF1 to BF3) were used: a fish feed, a piglet feed, and a poultry feed. Their compositions are described in Table 2.

Table 1. List of collected pure samples of *B. mori*.

No Sample	Composition	Brands
P1	Chrysalis of *B. mori*	Confidential
P2	Caterpillars after cocoon formation	Confidential
P3	Silkworm meal	Vivani baits (Venlo, the Netherlands)
P4	Silkworm protein meal	Feed stimulants (Zoetermeer, the Netherlands)

Table 2. Composition of three commercial feeds used to prepare mixes containing 0.1% (in mass fraction) of *B. mori*.

No Sample	Types of Commercial Feed	Composition
BF1	Fish feed	Fishmeal, fish oil, wheat gluten, protein concentrate extracted from pea, maize starch, yeast, lecithin, vitamins, minerals
BF2	Piglet feed	Wheat, maize, soybean oilcake (genetically modified), barley, wheat gluten, wheat bran, palm kernel flakes, sunflower seed oilcake, pork fat, rapeseed oilcake, peas, calcium carbonate
BF3	Poultry feed	Maize, wheat, soybean oilcake (genetically modified), wheat by-product, calcium carbonate, soybean oil, premix, sodium chloride, lysine, methionine, essential oils

2.1.3. Compound Feed Containing *B. mori*

Eight industrial compound feed samples containing *B. mori* were purchased online. The compositions of compound feed (CF1 to CF8) are mentioned in Table 3.

Table 3. Composition of eight industrial compound feed samples labelled or promoted to contain *B. mori* purchased online.

No Sample	Kind of Samples	Brands and Product Names	Composition	Claims
CF1	Supplementary feed for fish	Feed stimulants Insect cream, boilies 12 mm (Zoetermeer, the Netherlands)	Animal meals, vegetable meals, enhancers, amino acids, preserver, flavor, sweetener, vitamins, minerals.	"crafted with defatted black soldier fly meal and silkworm meal" in the information mentioned on the site
CF2	Supplementary feed for fish	Feed stimulants Insect cream, boilie mix (Zoetermeer, the Netherlands)	Animal meals, vegetable meals, enhancers, amino acids, preserver, flavor, sweetener, vitamins, minerals.	"crafted with several insect meals such as black soldier fly meal, silkworm and insect powder" in the information mentioned on the site
CF3	Complementary feed for Koi	Hikari® Silkworm selects™ (Hyogo, Japan)	Insects, cereals, by-products of plant origin, algae, mineral substances.	"silkworm" in the name of product
CF4	Supplementary feed for carp	Vivani Baits *Bombyx mori* boilies (Venlo, the Netherlands)	Maize, wheat, silkworm meal, soy flour, chicken protein powder, egg powder, mulberry Florentine aroma, salt.	"*Bombyx mori*" in the name of product
CF5	Feed for ornamental fish	JBL Propond® silkworms (Neuhofen, Germany)	Salmon meal (23%), silkworms (14%), wheat germs, maize meal, krill meal (9%), gammares, soja meal, rice meal, wheat gluten, spirulina (4%), yeast extract, seaweed meal (1%), minerals, herb flour.	"silkworm" in the name of product
CF6	Feed for ornamental fish	Tropical® Insect menu flakes (Chorzów, Poland)	Insects (*Hermetia illucens* larvae meal 15%, silkworm pupae meal 15%, mealworms meal 15%), cereals, vegetable protein extracts, algae, oils, fats, minerals.	Presence of silkworm is explicitly mentioned in the composition
CF7	Pet food for dogs and cats	Truffe délice Mulberry *Bombyx* & carrots treats (Poisat, France)	Carrot, mulberry bombyx, potato flour, flaxseed oil.	"mulberry bombyx" in the name of product
CF8	Pet food for dogs	Antos® Insecta nibbles silkworm with carrot (Zaltbommel, the Netherlands)	Carrot, silkworm, potato flour, flaxseed oil.	"silkworm" in the name of product

For three samples (CF1 to CF3), the composition is vague, with reference to "animal meals" or "insects", but the presence of *B. mori* is stated on the sales site or in the product name.

2.1.4. Egg Samples

Five types of *B. mori* eggs (E1 to E5) were purchased from a specialized company www.bombyxstore.fr (accessed on 1 March 2022). According to the supplier's information, each type has mutations that produce different cocoons and caterpillars. When the eggs hatched, the caterpillars (second instar) were killed by freezing at −80 °C.

2.2. DNA Extraction

Genomic DNA extracts from pure species samples that were used for specificity testing were obtained via two extraction methods, depending on the matrix type. Blood samples were extracted with a "Qiagen Genomic DNA tip 20/G" kit (Qiagen GmbH, Hilden, Germany) from a test portion of 500 µL while meat and plant samples (test portion of 200 mg) were submitted to the CTAB method in accordance with the protocol described in Annex A.3.1, of the ISO 21571:2005 international standard [48]. The CTAB method was also used to extract DNA from insects. 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. DNA purity was determined using the A260/A280 ratio. The amplifiability of the DNA extract was successfully checked by real-time PCR with the 18S target for insects [42], rbcL [49] for plants, and a generic fish target [50] for fish. Other species were tested with targets developed or evaluated within the European Union Reference Laboratory for Animal Proteins in Feedingstuff [33,51–53] or with the 18S target [54,55]. Ten ng of DNA were used in these PCR reactions.

DNA of caterpillars (issued from eggs claimed to bear some mutations, see sample paragraph) was also extracted following the CTAB-based method; the quality and quantity of extracted DNA were estimated spectrophotometrically using a Nanodrop ND-1000 spectrophotometer at 260 nm (A260) and 280 nm (A280) absorbance. DNA purity was determined using the A260/A280 ratio.

The pure industrial samples (P1 to P4), compound feed (CF1 to CF8), and the feed mixes containing 0.1% of *B. mori* (M1 to M12) 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 [56]. The quantities tested for this purpose are also in line with the EURL-AP SOP.

Compound feed, CF6 to CF8, were also extracted following the CTAB-based method described in Annex A.3.1 of the international standard ISO 21571:2005 [48] for metabarcoding analysis.

2.3. Primers and Probe for the Real-Time PCR

Eurogentec (Seraing, Belgium) synthesized the oligonucleotides. The primers and probe sequences developed to detect *B. mori* are presented in Table 4. 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. Table 4 also lists the primer sets targeting portions of the cytochrome c oxidase subunit I (COI) gene used for Sanger sequencing analysis as well as primers for metabarcoding for high-throughput sequencing (HTS).

Table 4. Primers and probes used within this study.

Assay	Target	Name	Sequences 5′-3′	Amplicon Size (bp)
qPCR	*B. mori* cadherin gene	Bombyx-Cad-F	TTTCAGACACCGACCATGACA	98
		Bombyx-Cad-R	CCAAAATGATGCCGAAGTACTG	
		Bombyx-Cad-P	FAM–AGCTCTGGAGCATTGTCGTTCACATCAA–TAMRA	
Sanger sequencing (Specimen identification)	*B. mori* COI gene	COI_F6	CAATTTATCGCTTATTATTCAGCC	826
		COI_R6	CCTCTTTCTTGTGAAATAATATGAG	
High-throughput sequencing (Metabarcoding)	Arthropod COI gene	BF3	CCHGAYATRGCHTTYCCHCG	458
		BR2	TCDGGRTGNCCRAARAAYCA	

2.4. Real-Time PCR Method

Real-time PCR (total reaction volume of 25 µL) was performed on thermocycler CFX96 Deep Well Real-time PCR Detection Systems (Bio-rad, Hercules, CA, USA) using the Uni-

versal Mastermix provided by Diagenode (Seraing, Belgium). Following an optimization of oligonucleotide concentrations, the following conditions yielded the best results: the reaction mixture included 12.5 µL of Master Mix, 1.7 µL of each primer (2.5 µM), 1.5 µL of probe (5 µM), 2.6 µL of bidistilled water, and 5 µL of DNA. Reaction mixtures were distributed on Hard-Shell® 96-Well PCR Plates (Bio-rad) developed for the specific thermocyclers. One positive control consisting of *B. mori* DNA from sample P2 and two no template controls (negative PCR control) per PCR plate were analyzed. Wells were covered with an adhesive film and centrifuged (2 min at 500 rpm) to eliminate any air bubble in the well bottoms. The thermal program was applied as follows: 2 min at 50 °C; 10 min at 95 °C; 50 cycles of 15 s at 95 °C, and 1 min at 60 °C. The PCR results were analyzed with CFX Manager™ Software (version 3.1) in Quantification Cq Results with the parameters set to 1000 for threshold and automatic baseline.

The eight industrial compound feed samples (CF1-CF8) were analyzed with real-time PCR tests for *T. molitor* [42], *H. illucens* [37], *A. diaperinus* [30], *A. domesticus* [57], and *G. sigillatus* (test from Daniso et al. [40] with a slight modification in reverse primer described by Jilkova et al. [57]) detection. Table 5 shows the primers and probes used.

Table 5. Primers and probes from literature used to authenticate insects present in eight industrial compound feed samples labelled or promoted to contain *B. mori*.

Target	Name	Sequences 5′-3′	Publication
Tenebrio molitor	Cadherin-2F	AATAGACGAAGACAACCAGCTTGA	[42]
	Cadherin-2R	TCTCTATCGGCATCACTATATGTTAGATT	
	Cadherin-2P	FAM–CCGGACGACACCCTCAACGGA–TAMRA	
Tenebrio molitor	TM-WING-F	CAGGGTTGAACGGGTTCAGT	
	TM-WING-R	ATACTATTTCGGGCAACAGCATC	
	TM-WING-P	FAM–AAGCCGTACTTGTGTTACGGCGGTTCAC–TAMRA	
Hermetia illucens	HI-mito-2F	ACCATTCTTCAAGCCTATGA	[37]
	HI-mito-2R	TTGAGCCGTAGACTGCG	
	HI-mito-P	FAM–TGAAGCCCCTTTTACTATTGCTG–TAMRA	
Alphitobius diaperinus	Alphi-Dia-Cad-F	CCAAGTGACTCTCATCATTCAGGAT	[30]
	Alphi-Dia-Cad-R	CTGAAACCGTAATGTCTAGTTCACCTA	
	Alphi-Dia-Cad-P	FAM–CCATTGCAGATCCAAGTCCCCGAAA–TAMRA	
Acheta domesticus	AchetaD_cytB_F1	ATAGTAGGTATTCTAATCTTATTCCTA	[57]
	AchetaD_cytB_R1	CATTGTACTAGATCAGTTCCTAGATA	
	AchetaD_cytB_P1	FAM–AATAGCTGCCGCTTTCATAGGTTAC–TAMRA	
Gryllodes sigillatus	GS1Fw	GATCAAACAATCCCCTAGGTGTC	[40] with a modification as described in [57]
	GS1re	CTGGGTCTCCAAGTATATAAGGATTAG	

The published PCR conditions were applied. Real-time PCR was performed on thermocycler LightCycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) using the Brilliant II QPCR Low ROX Master Mix (Agilent Technologies, Santa Clara, CA, USA). The PCR results obtained on a LightCycler 480 thermocycler (Roche Diagnostics Ltd.) were analyzed with LightCycler® 480 Software (version 1.5.1.62) with analysis mode in Abs. quant/second derivative max and high confidence.

2.5. Specificity of the PCR Method

The specificity of the method was verified on 43 insect species of different taxonomic orders, including six Lepidoptera taxa other than *B. mori*, thirteen Coleoptera, seven

Diptera, eight Orthoptera, four Hemiptera, two Hymenoptera, and two Blattodea. The specificity tests were performed on two arachnids and six crustaceans, which, like insects, belong to the Arthropoda phylum, as well as one mollusk, one polychaeta, and thirty-three vertebrates (twelve terrestrial mammals, six sea mammals, eight birds, seven fish). The possibility of a cross-reaction with human DNA was also considered. Seven plant species frequently used in feed and food were included in the experimental set-up (Table 6). Ten ng of DNA were used in the PCR reactions. Each DNA extract was tested in duplicate.

Table 6. Specificity of *B. mori* PCR test on animal and plant species (n = 2). For positive samples, mean Cq values (m) and standard deviations (σ) are given in brackets (real-time PCR settings on CFX96: threshold 1000, automatic baseline). Origin of samples is specified with "a" for insects collected by trained entomologists, "b" for insects purchased from specialized companies, "c" for the insects provided by the Functional and Evolutionary Entomology lab of Gembloux Agro-Bio Tech (ULiège, Gembloux, Belgium), and "d" for the EURL-AP sample bank.

Taxonomic Classification		Scientific Name (Species or Species Group)	Common Name	Origin	Results
Insects	Lepidoptera	*Bombyx mori* L.	Silkworm	b	+ (m = 27.72, σ = 0.03)
		Samia ricini (Jones)	Eri silkmoth	b	-
		Rothschildia lebeau (Guérin-Meneville)	Rothschild silkmoth	b	-
		Galleria mellonella L.	Greater wax moth	a	-
		Omphisa Fuscidentalis (Hampson)	Bamboo worm	b	-
		Cadra cautella (Walker)	Almond moth	a	-
		Ephestia kuehniella (Zeller)	Mill moth	a	-
	Coleoptera	*Alphitobius diaperinus* (Panzer)	Lesser mealworm	a	-
		Tenebrio molitor L.	Mealworm	b	-
		Zophobas morio F.	Superworm	b	-
		Staphylinidae (Latreille)	Rove beetles	a	-
		Trypodendron domesticum L.	European hardwood ambrosia beetle	a	-
		Coccinella septempunctata L.	The seven spot ladybird	a	-
		Melolontha melolontha L.	Cockchafer	c	-
		Cassida viridis L.	Green tortoise beetle	c	-
		Protaetia cuprea F.	Copper chafer	a	-
		Lucanus cervus L.	Stag beetle	a *	-
		Rhynchophorus ferrugineus (Olivier)	Red palm weevil	b	-
		Cybister limbatus F.	Diving beetle	b	-
		Cetonia aurata L.	Rose chafer	b	-

Table 6. *Cont.*

Taxonomic Classification	Scientific Name (Species or Species Group)	Common Name	Origin	Results
Diptera	*Hermetia illucens* L.	Black soldier fly	b	-
	Tabanus sudeticus (Zeller)	Dark giant horsefly	a	-
	Episyrphus balteatus (De Geer)	Marmalade hoverfly	a	-
	Musca domestica L.	House fly	a	-
	Drosophila melanogaster (Meigen)	Common fruit fly	b	-
	Lucilia sericata (Meigen)	Common green fly	b	-
	Pollenia angustigena (Wainwright)	Blow fly	a	-
Orthoptera	*Locusta migratoria* L.	Migratory locust	c	-
	Acheta domesticus L.	House cricket	b	-
	Gryllus bimaculatus (De Geer).	Mediterranean field cricket	b	-
	Gryllus assimilis F.	Jamaican field cricket	b	-
	Gryllodes sigillatus (Walker)	Tropical house cricket	b	-
	Gryllus campestris L.	European field cricket	a	-
	Patanga succincta (Johannson)	Bombay locust	b	-
	Brachytrupes portentosus (Lichtenstein)	Giant cricket	b	-
Hemiptera	*Palomena prasina* L.	Green shield bug	a	-
	Pyrrhocoris apterus L.	Firebug	a	-
	Belostomatidae sp. (Leach)	Giant water bug	b	-
	Cicadidae sp. (Latreille)	Cicada	b	-
Hymenoptera	*Bombus terrestris* L.	Buff-tailed bumblebee	a	-
	Oecophylla smaragdina F.	Weaver ant	b	-
Blattodea	*Blatta orientalis* L.	Oriental cockroach	c	-
	Blaptica dubia (Serville)	Dubia cockroach	b	-
Arachnida	*Heterometrus longimanus* (Herbst)	Black scorpion	d	-
	Haplopelma albostriatum (Simon)	Tarantulas	d	-
Polychaeta	*Lumbricus terrestris* L.	Earthworm	d	-
Crustacean	*Euphausia superba* (Dana)	Antartic krill	d	-
	Penaeus vannamei (Boone)	Whiteleg shrimp	d	-
	Crangon crangon L.	Common shrimp	d	-
	Nephrops norvegicus L.	Langoustine	d	-
	Homarus gammarus L.	European lobster	d	-
	Paralithodes camtschatieus (Tilesius)	Red king crab	d	-
Mollusca	Teuthida sp. (Naef)	Squid	d	-

Table 6. *Cont.*

Taxonomic Classification	Scientific Name (Species or Species Group)	Common Name	Origin	Results
Terrestrial mammals	*Bos taurus* L.	Beef	d	-
	Sus scrofa domesticus (Erxleben).	Pork	d	-
	Sus scrofa scrofa L.	Wild boar	d	-
	Ovis aries L.	Sheep	d	-
	Capra hircus L.	Goat	d	-
	Equus caballus L.	Horse	d	-
	Equus asinus L.	Donkey	d	-
	Lepus europaeus (Pallas)	Hare	d	-
	Capreolus capreolus L.	Roe deer	d	-
	Cervus elaphus L.	Stag	d	-
	Rattus rattus L.	Rat	d	-
	Homo sapiens L.	Human	d	-
Sea mammals	*Stenella coeruleoalba* (Meyen)	Striped dolphin	d	-
	Tursiops truncatus (Montagu)	Bottlenose dolphin	d	-
	Grampus griseus (G. Cuvier)	Risso's dolphin	d	-
	Ziphius cavirostris (G. Cuvier)	Cuvier's beaked whale	d	-
	Phocoena phocoena L.	Harbor porpoise	d	-
	Phocidae (Gray)	Seals	d	-
Fish	*Salmo salar* L.	Salmon	d	-
	Gadus morhua L.	Atlantic cod	d	-
	Scomber scombrus L.	Atlantic mackerel	d	-
	Clupea harengus L.	Atlantic herring	d	-
	Mallotus villosus (Müller)	Capelin	d	-
	Sprattus sprattus L.	Sprat	d	-
	Engraulis encrasicolus L.	European anchovy	d	-
Birds	*Gallus gallus* L.	Chicken	d	-
	Meleagris gallopavo L.	Turkey	d	-
	Numida meleagris L.	Guinea fowl	d	-
	Cairina moschata L.	Muscovy duck	d	-
	Anser sp. L.	Goose	d	-
	Coturnix japonica (Temminck and Schlegel)	Quail	d	-
	Struthio camelus L.	Ostrich	d	-
	Turdus merula L.	Blackbird	d	-

Table 6. *Cont.*

Taxonomic Classification	Scientific Name (Species or Species Group)	Common Name	Origin	Results
Plants	*Glycine max* (Merr)	Soybean	d	-
	Zea mays L.	Maize	d	-
	Brassica napus L.	Rapeseed	d	-
	Triticum aestivum L.	Wheat	d	-
	Oryza sativa L.	Rice	d	-
	Solanum lycopersicum L.	Tomato	d	-
	Beta vulgaris L.	Sugar beet	d	-

Note: + = Positive signal, - = No signal. * The *Lucanus cervus* (protected species) was not collected in the environment but obtained from an old insect box from a private collection.

Specificity tests were also performed on five types of *B. mori* eggs, which, according to supplier information, would have mutations, thereby producing different cocoons and caterpillars (Table 7). Ten ng of DNA were used in the PCR for four types while a slightly lower amount of DNA was used for the last type. Each DNA extract was tested in duplicate.

2.6. Copy Number Determination of *B. mori* Genomic DNA and Dilutions

The target copy numbers were estimated based on C-value data from the animal genome size database (www.genomesize.com (accessed on 2 February 2018)) at the University of Guelph (Ontario, Canada). Given that two close C-values are referenced for *B. mori* species, the mean value was considered in the calculation of the number of copies.

The quantity of genomic DNA corresponding to 20,000 target copies was estimated at 10.5 ng for *B. mori* based on data from the animal genome size database. The sensitivity, efficiency, and robustness of the PCR test were determined on diluted genomic DNA. These dilutions were performed in water until an estimated copy number of 10,000 copies/5 µL was reached. Higher dilutions of the target DNA were prepared in a solution containing 50 ng/µL of salmon sperm DNA as background DNA. Low-binding tubes were chosen to minimize DNA losses.

Table 7. Mean Cq (n = 2) obtained with the *Bombyx mori* PCR test on larvae from five kinds of *B. mori* eggs, which, according to supplier information, would have mutations, thereby producing different cocoons and caterpillars (real-time PCR settings on CFX96: threshold 1000, automatic baseline).

No Sample	Identification of Samples	Mean Cq Obtained with *B. mori* PCR Test
E1	Kind 1 of *B. mori* eggs	26.78
E2	Kind 2 of *B. mori* eggs	26.63
E3	Kind 3 of *B. mori* eggs	26.45
E4	Kind 4 of *B. mori* eggs	27.10
E5	Kind 5 of *B. mori* eggs	28.23

2.7. Limit of Detection (LOD)

Target sensitivity was evaluated following the recommendations of the former AFNOR XP V03-020-2 standard [58]. This standard no longer exists, but the principles detailed in it remain valid. The absolute limit of detection (LOD) was determined for the PCR assay (primers + probe + amplification program) on dilutions of genomic 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, but only if the highest dilution supposed to contain the 0.1 copy per reaction generated a maximum of one positive PCR signal on six replicates. If more than one positive signal was observed for the 0.1 copy, the DNA

quantities had to be revised. The copy number corresponding to LOD_6 was then tested 60 times on the same plate (determination of $LOD_{95\%}$). The $LOD_{95\%}$ is validated if at least 59 positive signals are recorded out of the 60 replicates. The highest acceptable copy number for both LOD_6 and $LOD_{95\%}$ is 20 copies.

2.8. Efficiency

The efficiency of the PCR assay was calculated with a dilution series of genomic DNA at target levels of 5000, 2500, 1000, 500, and 100 copies. Each dilution was analyzed in six replicates and on four runs. Efficiency had to be between 90% to 110% [44,45].

2.9. Digital PCR

The number of copies of the nuclear DNA dilutions at approximately 500 copies/5 µL (5 µL being the volume of the DNA extracts added in the real-time PCR mix) were checked by digital PCR. The assay was performed on the Biomark™ HD system (Fluidigm Corporation, South San Francisco, CA, USA) using the 12.765 Digital Array™. These digital arrays comprise 12 panels (12 wells, thus 12 samples), each of which is partitioned into 765 individual PCR of 6 nL. 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), and 3.15 µL of DNA dilution. Eight µL of reaction mix was 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) [59]. Two arrays were analyzed with eleven replicates of DNA dilution and one no-template control per array. 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 Fluidigm Digital PCR Analysis software (version 4.0.1) with analysis settings set to 0.4 for quality threshold, baseline correction in linear, and Ct threshold method in Auto (Global).

Since transferability was conducted after the internal performance evaluation, a new stock solution of genomic DNA for the various dilutions was prepared. The number of copies of this new solution was verified using digital PCR. Digital PCR was performed on the Biomark™ HD system (Fluidigm Corporation) using the qdPCR 37K™ IFC. These digital arrays comprise 48 panels (48 wells, thus 48 samples), each of which is partitioned into 770 individual PCR of 0.85 nL. The reaction mixture included 2 µL of Universal Master Mix with passive reference (Diagenode, Seraing, Belgium), 0.1 µL of each primer (13.6 µM), 0.2 µL of probe (10.8 µM), 0.2 µL GE sample loading reagent (Fluidigm Corporation), 0.2 µL of water, and 1.2 µL of DNA dilution. Four µL of reaction mix was dispensed into each sample inlet and approximately 0.65 µL of this reaction mix was distributed throughout the partitions within each panel using an automated NanoFlex IFC Controller (Fluidigm Corporation). A total of 36 replicates, run on two plates (15 replicates on the first plate and 21 replicates on the second plate), were analyzed. Three no template controls per array were also analysed. The thermal programme 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 Fluidigm Digital PCR Analysis software (version 4.0.1) with analysis settings set to 0.4 for quality threshold, baseline correction in linear, and Ct threshold method in Auto (Global).

2.10. Robustness of the PCR Method

The method's robustness was tested by introducing some slight deviations to the standard experimental conditions [45,60]. Parameters considered were as usual [37,44,45,61]: the annealing temperature (60 °C +/− 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 involved a final reac-

tion volume of 25 µL +/− 1 µL. Six replicates of the genomic DNA at 20 copies/5 µL were tested under the conditions described in Table 8. Robustness was performed on two real-time PCR platforms: thermocycler Bio-Rad CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) with Universal Mastermix by Diagenode (Seraing, Belgium) and thermocycler QuantStudio™ 5 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) with ABI TaqMan 2x Universal PCR Mastermix No AmpErase UNG (Applied Biosystems, Thermo Fisher Scientific, Warrington, United Kingdom).

Table 8. Experimental conditions tested to evaluate the robustness of the described *B. mori* PCR test. The variations tested are given for each of the parameters given in bold in the first column.

PCR machine	Bio-Rad CFX96™ Real-Time System (Bio-Rad) or QuantStudio 5 (Applied Biosystems)				
PCR reagent kit	Universal Mastermix (Diagenode s.a.) or Master Mix ABI No AmpErase™ (Applied Biosystems)				
Annealing temperature	59 or 61 °C				
Concentration of each primer (final concentration in the PCR)	Minus 30% (0.119 µM)	Standard (0.170 µM)	Standard (0.170 µM)	Standard (0.170 µM)	Standard (0.170 µM)
Probe concentration (final concentration in the PCR)	Standard (0.300 µM)	Minus 30% (0.210 µM)	Standard (0.300 µM)	Standard (0.300 µM)	Standard (0.300 µM)
PCR volume	Standard (20 µl mix + 5 µl DNA)	Standard (20 µl mix + 5 µl DNA)	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 acceptance criterion is that all deviations to the standard protocol had to give a positive result at a level of 20 copies of the target in the reaction [44,45].

2.11. Applicability of the PCR Method

The applicability of the PCR method was verified on different types of samples, including four samples of *B. mori* pure species, which included two samples from the industry produced in the EU and two PAPs from a specialised company producing ornamental fish feed (P1 to P4). Three commercial feeds were mixed with the previous pure samples to contain 0.1% in mass fraction of each *B. mori* pure species sample, leading to 12 samples in total (M1 to M12). For each sample, two DNA extracts were tested by PCR at two dilutions.

2.12. Practical Interest

The practical interest of the method was tested on eight commercially purchased compound feed samples labelled or promoted to contain silkworm (CF1 to CF8). For each sample, two DNA extracts were tested by PCR at two dilutions.

2.13. Transferability of the PCR Method

The efficiency and LOD (LOD_6 and $LOD_{95\%}$) of the PCR assay were tested on genomic DNA in the laboratory of Eurofins Biologie Moléculaire France with similar conditions to those of the developer's laboratory (CRA-W, Gembloux, Belgium). The genomic DNA dilutions, provided by CRA-W, were prepared from a solution quantified to contain 6351 copies per 5 µL using digital PCR (Table 9).

Real-time PCR were performed on thermocycler CFX96 Deep Well Real-time PCR Detection Systems (Bio-rad, Hercules, CA, USA) using the Applied Biosystems™ TaqMan™ Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Warrington, UK). Reaction mixtures were distributed on Hard-Shell® 96-Well PCR Plates (Bio-rad) developed for the CFX96 thermocyclers. Wells were covered with adhesive film and the plates were centrifuged to eliminate any air bubbles in the well bottoms. The PCR results

were analyzed with CFX Manager™ Software (version 3.1) in Quantification Cq Results, with the parameters set to 100 for threshold and automatic baseline.

2.14. Specimen Identification

To confirm specimen identity, portions of the cytochrome c oxidase subunit I gene were amplified and subjected to Sanger sequencing. To do so, the primer set COI_F6/COI_R6, specifically designed to amplify the DNA of *Bombyx* specimens, was used (Table 4). These primers were used on DNA extracted from *B. mori* surface-decontaminated legs, from caterpillars of *B. mori* specimens supposedly displaying different mutations in their genomes, as well as from Eri silkworm (*Samia ricini* (Jones)) and Rothschild silkmoth (*Rothschildia lebeau* (Guérin-Meneville)) specimens. The PCR amplifications were performed using 5 µL of 5X GoTaq® Flexi Buffer (Promega, Leiden, The Netherlands), 2.5 µL of 2 mM dNTP mix (Thermo Scientific, Waltham, MA, USA), 1.5 µL of 25 mM $MgCl_2$ (Promega), 1 µL of 10 µM forward and reverse primers (Eurofins Genomics, Ebersberg, Germany), 0.15 µL of GoTaq® G2 Flexi DNA Polymerase (Promega), 1 µL of DNA, and nuclease-free water (Qiagen GmbH, Hilden, Germany) resulting in a final volume of 25 µL. The thermal cycling conditions were set as follows: initial denaturation at 95 °C for 3 min followed by 30 cycles at 95 °C for 30 s, 47 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified fragments were sent to Eurofins Genomics (Ebersberg, Germany) for Sanger sequencing. The obtained sequences were deposited in GenBank under accession numbers PP922534 to PP922541.

Table 9. Copy numbers obtained on a dilution of genomic DNA prepared to be the basis for dilutions used for transferability. Measurements made in digital PCR on Biomark™ HD system. Thirty-six replicates were analyzed on qdPCR 37K™ IFC plate (n = 36).

Plate	Copy Number of Target/5 µL	Copy Number Mean of Target/5 µL ± SD (σ)	Coefficient of Variation
1	7487	6351 ± 422.51	6.65%
	6672		
	6748		
	6188		
	6825		
	6315		
	6239		
	6417		
	6213		
	6239		
	5857		
	6315		
	5857		
	6519		
	6188		
2	6595		
	7334		
	5806		
	5908		
	6570		
	6137		
	6494		

Table 9. *Cont.*

Plate	Copy Number of Target/5 µL	Copy Number Mean of Target/5 µL ± SD (σ)	Coefficient of Variation
	6417		
	5806		
	6595		
	5857		
	5704		
	5933		
	6494		
	5959		
	6952		
	6901		
	6112		
	6035		
	6188		
	6774		

2.15. High-Throughput Sequencing

Several commercial products were analyzed for their insect composition using a DNA metabarcoding approach. Briefly, DNA was extracted using the CTAB protocol. The primer set selected to perform the metabarcoding analysis amplified the cytochrome c oxidase subunit I (COI) region and was constituted of BF3 [62] and BR2 [63] primers (Table 4), given their excellent performances [62]. They were appended with Illumina universal adapters. Amplifications were carried out using 12.5 µL of 5X GoTaq® Flexi Buffer (Promega), 3 µL of 2 mM dNTP mix (Thermo Scientific, Waltham, MA, USA), 1.8 µL of 25 mM $MgCl_2$ (Promega), 3 µL of 10 mM BSA (Roth, Karlsruhe, Germany), 1.5 µL of 10 µM forward and reverse primers (Eurofins Genomics, Constance, Germany), 0.2 µL of 5 U/µL GoTaq® G2 Flexi DNA Polymerase (Promega), 1 µL of DNA, and 9 µL of nuclease-free water (Qiagen). The thermal cycling conditions were set as follows: initial denaturation at 95 °C for 3 min followed by 35 cycles at 95 °C for 30 s, 46 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 5 min. Triplicate PCR products were pooled during purification using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). The amplicon quality was checked by running 5 µL of PCR products on a 1% agarose gel, and the DNA concentration was measured using a Qubit 4 fluorometer (ThermoFisher, Waltham, MA, USA). These amplicons were then sent to Eurofins Genomics for HTS on an Illumina MiSeq device with 2 × 300 sequencing chemistry. The HTS data generated in this study are available in the NCBI sequence read archive under the BioProject number PRJNA1141787. Raw sequencing data were imported into QIIME2 [64] for bioinformatics processing. Demultiplexed paired-end reads were denoised with DADA2 [65] to generate amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs with the blastn standalone tool (v2.15.0) [66] from BLAST command line applications, using a custom insect COI database developed and curated using the DB4Q2 pipeline [67].

2.16. Microscopic Observations

Microscopic observations were carried out on the eight industrial feed samples labelled as containing *B. mori* (CF1 to CF8). The samples were prepared in accordance with Annex VI of Commission Regulation (EC) No 152/2009 [46] and by applying the Standard Operating Procedures (SOP) established by EURL-AP. Permanent slides from the flotate fraction were prepared according to the method suggested by Veys and Baeten [68] from the double sedimentation protocol. No staining reagent was used.

Observations were conducted on a Carl Zeiss Axio Imager A1 (Zeiss, Oberkochen, Germany) under conventional transmitted bright field (BF) and performed at several magnifications. Micrographs of the different insect particles observed were taken with a Carl Zeiss AxioCam MRc (Zeiss, Germany) coupled with a 0.63 port.

3. Results

The cadherin gene of *B. mori* was used to select a piece of DNA that is specific to the considered insect species. Appropriate primers and probes were designed to amplify a 98 bp fragment of the cadherin gene (Table 4). The latter is considered as a single-copy gene in several insect species such as Lepidopteran *Ostrinia nubilalis* (Hübner) [69], Coleoptera *Diabrotica virgifera virgifera* (Le Conte) [70], and *A. diaperinus* [30], which is an advantage for quantitation purposes.

Specificity was first investigated in silico using the Blast tool of the National Center for Biotechnology Information (NCBI) database. The different blasts and the alignments of DNA sequences performed with available sequences indicated that the PCR test should be specific to the target species *B. mori*. Specificity was also experimentally tested on DNA from *B. mori* of various origins, as well as on forty-two other non-target insect species, including six Lepidoptera. Positive results were obtained only with all samples of *B. mori*. No signal was obtained with the 42 other insect species.

For Lepidoptera, it was important to check if other species also bred for their silk production were not detected by this assay. Eri silkworm (*S. ricini* or *Philosamia ricini*) and Rothschild silkmoth (*R. lebeau*) belong to the order Lepidoptera and are also producers of silk [71,72]. Silk fibers from the Eri silkworm are produced commercially [71,73]. In India, the Eri silkworm is the second most used species for silk production, accounting for 16% of the total [72]. The test with DNA from *S. ricini* and *R. lebeau* species showed no amplification signal. It was also important to prove that the PCR test did not respond positively with DNA from food moths. The PCR test does not demonstrate any specificity with *Ephestia kuehniella* and *Cadra cautella* (Lepidoptera), which are invasive moth species that proliferate in feed intended for insect farms. The DNA of *Bombyx mandarina*, the wild species of *B. mori*, could not be tested but it is unlikely that this species will be found in feed or food since it is not used for silk production and is not commercialized.

No signal was obtained with the 43 other animal species (arachnids, crustaceans, mollusks, and vertebrates) and the seven plant species tested (Table 6).

A sequencing of the 5′ region of the COI gene was performed on different samples to confirm their identity. DNA from *S. ricini* and *R. lebeau* specimens, used for specificity tests, were sequenced and compared to that of *B. mori* (Figure 1). This sequencing allowed confirmation of the identity of these specimens.

DNA of caterpillars from five types of *B. mori* eggs, reported to have mutations according to supplier information, was sequenced with COI primers specifically designed to amplify the DNA of *Bombyx* specimens. DNA extraction was performed on caterpillars immediately after hatching to avoid any DNA contamination from food sources. Fragments sequenced showed very few divergences, only two nucleotide positions showing variations (Figure 2). For all the five specimens, a BLASTn analysis showed 100% identity with different vouchers of *B. mori*.

DNA from these five types of *B. mori* eggs was tested with the *cadherin* PCR test to ensure that even if mutations were observed at the level of the COI, it does not affect the *cadherin* target. All five types of eggs were successfully detected.

The amplification efficiency, LOD, and robustness were evaluated on genomic DNA. To check that the number of copies in the dilutions used to assess the performance criteria were correct, the dilution estimated at 500 copies/5 µL was checked by digital PCR on the Biomark™ HD system. The average obtained over the 22 measurements by digital PCR was 574 copies/5 µL with a coefficient of variation at 8.22% (Table 10). This mean measured copy number was close to the initial value (based on the genome size of *B. mori* and considering the *cadherin* gene as a single-copy gene). The obtained results confirm

that the *cadherin* gene is a single-copy gene per haploid genome, as mentioned in other studies [30,69,70].

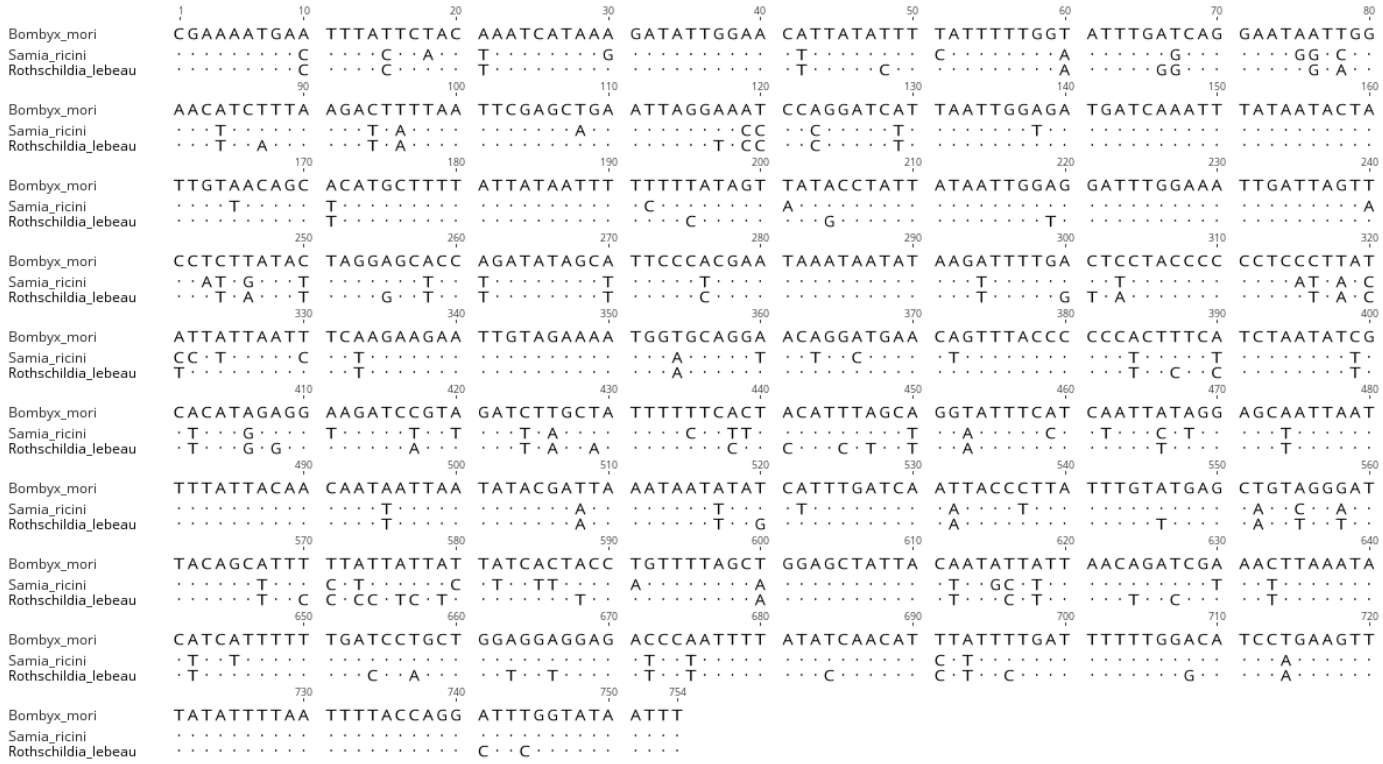

Figure 1. Alignment of COI DNA fragments sequenced from specimens of silkworm (*B. mori*), Eri silkworm (*S. ricini*), and Rothschild silkmoth (*R. lebeau*).

Figure 2. Alignment of DNA fragments sequenced from five *B. mori* caterpillars to confirm their identity. Primers specifically designed to amplify the DNA of *Bombyx* specimens were used to analyze the nucleotide composition of the cytochrome c oxidase subunit I (COI) coding gene.

PCR efficiency was evaluated to be at 97.8%. This was calculated considering the mean Cq (quantification cycle) values obtained at the different copy numbers tested (from

5000 to 10), and no outliers were encountered (Table 11). When calculated per plate, the efficiency was always higher than 90% and therefore met the acceptance criterion proposed by Broeders et al. [44].

Table 10. Copy numbers obtained on a dilution of genomic DNA at approximately 500 copies/5 µL by digital PCR on Biomark™ HD system. Twenty-two replicates were analyzed on 12.765 Digital Array™ (n = 22).

Array	Copy Number of Target/5 µL	Copy Number Mean of Target/5 µL ± SD (σ)	Coefficient of Variation
1	578	574 ± 47.12	8.22%
	650		
	642		
	443		
	553		
	528		
	617		
	573		
	551		
	501		
	523		
2	540		
	606		
	620		
	600		
	598		
	606		
	598		
	589		
	562		
	551		
	589		

Concerning the sensitivity testing, LOD_6 was estimated at 5 copies following the AFNOR XP V03-020-2 standard approach [58] and at 5 copies for $LOD_{95\%}$ with 59/60 positive signals. Therefore, the PCR test easily reaches the recommended performance criterion (≤20 copies).

The robustness of the PCR method was also evaluated with success. All tested deviations (Table 8) to the standard protocol delivered positive results at the level of 20 copies in the PCR.

Positive signals were present on industrial samples of *B. mori*, showing the applicability of the PCR test on real-life samples (Table 12, samples P1 to P4). Applicability was also tested on three commercial feeds (for fish, piglet, and poultry) adulterated with 0.1% of different samples of *B. mori* (Table 12, samples M1 to M12). These commercial feeds were tested as free of *B. mori* and the amplifiability of their DNA extracts was checked with a 18S rDNA target [54,55]. The twelve mixes of feed containing 0.1% of different *B. mori* samples were tested and gave positive results with the *cadherin* PCR test. Table 12 shows the mean Cq value achieved with undiluted DNA extracts.

The practical interest of the method was finally tested on eight samples of commercial compound feed labelled or promoted to contain *B. mori*. For all samples, undiluted DNA extracts and tenfold diluted DNA extracts were analyzed. The PCR results shown in Table 13 were obtained on undiluted extracts, except for three samples (CF3, CF5, and CF6)

containing PCR inhibitors. For these extracts, further dilutions (20, 40, and 80-fold dilution) of DNA extracts allowed the removal of inhibition and enabled the detection of the species present in the samples. The Cq values indicated for these three samples correspond to the Cq obtained when the inhibition is completely removed for the PCR test concerned. Given that different reagents were used for the *B. mori* PCR test compared to the other PCR tests, it is not unusual to have different dilution factors required to remove the inhibition entirely. Indeed, the Brilliant II QPCR Low ROX Master Mix seems to be less sensitive to the presence of inhibitors.

Table 11. Cq values obtained on dilutions of genomic material used for efficiency calculation and for $LOD_{95\%}$ (real-time PCR settings on CFX96: threshold 1000, automatic baseline). For efficiency, each concentration was analyzed in six replicates and on four PCR plates (n = 24). For $LOD_{95\%}$, the concentration at five copies was analyzed in sixty replicates on one PCR plate with fifty-nine positive results (n = 60).

Copy Number of Target	Cq (Mean Value) ± SD (σ) and (n)
5000	27.62 ± 0.21 (24)
2500	28.58 ± 0.08 (24)
1000	29.99 ± 0.13 (24)
500	31.01 ± 0.17 (24)
100	33.33 ± 0.19 (24)
5	38.47 ± 0.79 (60)

Table 12. Mean Cq obtained with the *Bombyx mori* PCR test on samples from *B. mori* and on mixes containing 0.1% in mass fraction of *B. mori* in three commercial feeds. Results on undiluted extracts (real-time PCR settings on CFX96: threshold 1000, automatic baseline).

No Sample	Identification of Samples		Mean Cq obtained with *Bombyx mori* PCR Test
P1	Chrysalis of *B. mori*	Extract 1	19.98
		Extract 2	19.95
P2	Caterpillars of *B. mori* after cocoon formation	Extract 1	21.85
		Extract 2	22.52
P3	Silkworm meal	Extract 1	23.76
		Extract 2	23.35
P4	Silkworm protein meal	Extract 1	24.34
		Extract 2	24.03
M1	Fish feed (BF1) containing 0.1% of *B. mori* from P1	Extract 1	33.00
		Extract 2	32.54
M2	Piglet feed (BF2) containing 0.1% of *B. mori* from P1	Extract 1	30.67
		Extract 2	29.35
M3	Poultry feed (BF3) containing 0.1% of *B. mori* from P1	Extract 1	32.21
		Extract 2	30.62
M4	Fish feed (BF1) containing 0.1% of *B. mori* from P2	Extract 1	34.41
		Extract 2	33.17
M5	Piglet feed (BF2) containing 0.1% of *B. mori* from P2	Extract 1	33.90
		Extract 2	33.96
M6	Poultry feed (BF3) containing 0.1% of *B. mori* from P2	Extract 1	35.28
		Extract 2	34.18

Table 12. *Cont.*

No Sample	Identification of Samples		Mean Cq obtained with *Bombyx mori* PCR Test
M7	Fish feed (BF1) containing 0.1% of *B. mori* from P3	Extract 1	34.45
		Extract 2	37.19
M8	Piglet feed (BF2) containing 0.1% of *B. mori* from P3	Extract 1	33.63
		Extract 2	32.18
M9	Poultry feed (BF3) containing 0.1% of *B. mori* from P3	Extract 1	34.34
		Extract 2	34.97
M10	Fish feed (BF1) containing 0.1% of *B. mori* from P4	Extract 1	35.24
		Extract 2	35.34
M11	Piglet feed (BF2) containing 0.1% of *B. mori* from P4	Extract 1	33.71
		Extract 2	33.70
M12	Poultry feed (BF3) containing 0.1% of *B. mori* from P4	Extract 1	36.71
		Extract 2	35.27

Table 13. Results obtained by real-time PCR tests, light microscopy (LM), and high-throughput sequencing (HTS) with eight industrial compound feed samples labelled or promoted to contain *B. mori*. PCR results on undiluted extracts, except when marked with a letter referring to the dilution used. For positive results, mean Cq values are presented for *B. mori* PCR test (n = 2) and Cq values for other PCR tests (as routine analysis) are given in brackets. Expected PCR positive results are marked with a grey background. (Real-time PCR settings on CFX96 set to threshold 1000 and automatic baseline; on LightCycler 480 set to second derivative max and high confidence).

Samples Identification		Results with PCR Tests for the Detection of							LM Results	HTS Results
		B. mori	*T. molitor* (Cadherin)	*T. molitor* (TM-Wing)	*H. illucens*	*A. diaperinus*	*A. domesticus*	*G. sigillatus*		
CF1	Extract 1	+ (29.99)	-	-	+ (18.70)	-	-	-	*B. mori* + *H. illucens* +	NA
	Extract 2	+ (29.65)	-	-	+ (18.14)	-	-	-		
CF2	Extract 1	+ (30.64)	-	-	+ (21.74)	-	-	-	*B. mori* + *H. illucens* +	NA
	Extract 2	+ (31.74)	-	-	+ (22.40)	-	-	-		
CF3	Extract 1	+ (29.65 [a])	+ (38.39)	+ (41.33)	-	-	-	-	*B. mori* + *T. molitor* −	NA
	Extract 2	+ (27.52 [a])	+ (38.05)	+ (42.14)	-	-	-	-		
CF4	Extract 1	+ (29.76)	-	-	+ (29.10)	-	-	-	*B. mori* + *H. illucens* +	NA
	Extract 2	+ (29.09)	-	-	+ (30.18)	-	-	-		
CF5	Extract 1	+ (33.25 [d])	-	-	+ (38.35 [b])	-	-	-	*B. mori* ~ *H. illucens* ~	NA
	Extract 2	+ (33.22 [d])	-	-	+ (37.55 [b])	-	-	-		
CF 6	Extract 1	+ (33.23 [d])	-	-	+ (25.45 [c])	-	-	-	*B. mori* + *H. illucens* +	*B. mori* *H. illucens*
	Extract 2	+ (33.93 [d])	-	-	+ (25.12 [c])	-	-	-		
CF7	Extract 1	-	+ (32.73)	+ (33.76)	+ (23.32)	-	-	-	*T. molitor* + *H. illucens* +	*T. molitor* *H. illucens*
	Extract 2	-	+ (32.77)	+ (33.36)	+ (23.40)	-	-	-		
CF8	Extract 1	-	+ (29.88)	+ (31.35)	+ (35.92)	-	-	-	*T. molitor* + *H. illucens* −	*T. molitor*
	Extract 2	-	+ (29.83)	+ (31.41)	+ (35.58)	-	-	-		

[a] 10-fold dilution, [b] 20-fold dilution, [c] 40-fold dilution, [d] 80-fold dilution, NA = Not analyzed, + = positive, − = negative, ~ = below the limit of decision established for the method.

The results for *B. mori* PCR obtained on the eight compound feed samples show that despite what is claimed by the supplier, samples CF7 and CF8 do not contain *B. mori* (Table 13). To ensure that this negative result is not due to the presence of inhibitors, the dilutions, as used for the samples showing inhibition, were analyzed.

In light of these results, PCR authentication methods already published for other insect species (Table 5) were employed to identify the insect species present in the feed. Furthermore, all samples underwent LM analysis.

By comparison with observed particles of *B. mori*, *H. illucens*, and *T. molitor* in pure meals, the species identified by LM in the eight commercial feeds were *B. mori*, *H. illucens*, and *T. molitor*. The distinction made by LM between these different insect species was based on various morphological criteria, mainly concerning their cuticles. In the case of *B. mori*, the cuticle appears yellowish and is alveolate (Figure 3G,H) [74]. For *H. illucens*, the cuticle is often brown or very pale brown with circular or mosaic adornments [75] (Figure 3C,D). Finally, cuticle fragments of *T. molitor* are light yellow or orange, foveolate and puncticulate [74], corresponding to vestiges of bristle insertion (Figure 3E,F). For all the species described, bristles (sensillae and setae) have also been observed (Figure 3A,B) but are not sufficient on their own to determine the species.

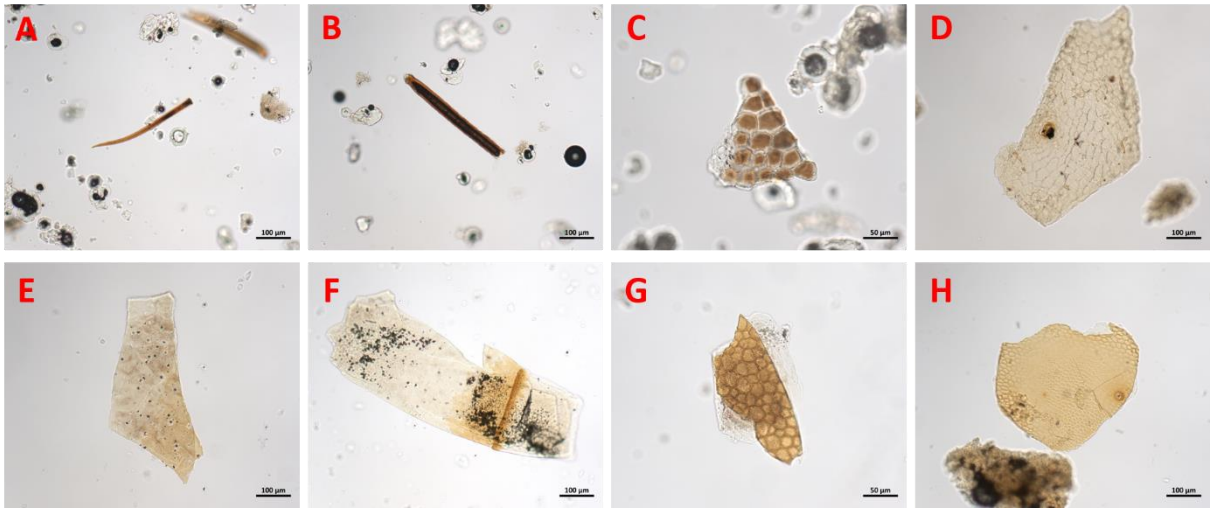

Figure 3. Micrographs of different insect particles observed in the eight industrial feed samples in bright field. (**A**,**B**) Example of sensilla and setae particles observed in CF1, CF2, CF4, CF5, CF6, and CF7. (**C**,**D**) Example of cuticle particles consistent with the presence of *H. illucens* observed in CF1, CF2, CF4, CF5, CF6, and CF7. (**E**,**F**) Example of cuticle particles consistent with the presence of *T. molitor* observed in CF7 and CF8. (**G**,**H**) Example of a cuticle particle consistent with the presence of *B. mori* observed in CF1, CF2, CF3, CF4, CF5, and CF6.

In addition, HTS was performed on the PCR products of two samples that tested negative with the *B. mori* PCR assay (CF7 and CF8) and the sample CF6, labelled to contain a mixture of three different insect species at a concentration of 15% (Figure 4).

Table 13 shows several discrepancies between announced composition in terms of the insect species and analytical results. For two samples, CF1 and CF2, the composition remains vague with the mention of "animal meals". Information available on the sales site provides details about the species present. Silkworms and black soldier flies were used to formulate these feeds. Both species were detected by real-time PCR and LM (Figure 3). The composition of sample CF3 is also unclear as it only states "insects", but the presence of *B. mori* is indicated by the term "silkworm" in the product name. The presence of *B. mori* was confirmed by both PCR and LM. Although *T. molitor* was detected by PCR, it showed very late signals, indicating a very low quantity, which explains why it was not detected by LM. For sample CF4, the composition claimed to include only *B. mori*. However, analysis by PCR and LM also detected *H. illucens*. Concerning sample CF5, the composition listed only *B. mori* but PCR results revealed the presence of both *B. mori* and *H. illucens* DNA. While LM detected particles of both *B. mori* and *H. illucens*, their quantities were too small to be considered positive. On the other hand, the compositions announced for samples CF6, CF7, and CF8 are incorrect and mislead the user. CF6 is labelled to contain a rate of 15% of *B. mori*, *H. illucens*, and *T. molitor* but the latter species was not detected by real-time PCR, HTS, and LM. Only *B. mori* and *H. illucens* species were found in sample CF6. Using the metabarcoding approach (HTS), 2% of the reads were assigned to two Diptera species

(*Chrysomya megacephala* and *Exorista sorbillans*), which may reflect trace contamination. The results obtained with these three techniques show that neither sample CF7 nor CF8 contain *B. mori*. All three techniques reveal the presence of *T. molitor* in both samples and *H. illucens* in sample CF7. In the HTS assay, a very low proportion of reads (0.2%) were assigned to *Sitophilus oryzae*. Belonging to the Coleoptera order, this could indicate minor contamination along with Coleoptera listed on the product label (*T. molitor*). PCR analysis of sample CF8 also gave late signals in the *H. illucens* PCR test. Given that the *H. illucens* PCR test targets a multi-copy DNA fragment, the quantity of *H. illucens* present is low, which explains the non-detection in LM. In HTS, this low quantity in *H. illucens* may be masked by the higher *T. molitor* content. Samples CF7 and CF8 are very similar and have the same description of composition but they are from different brands.

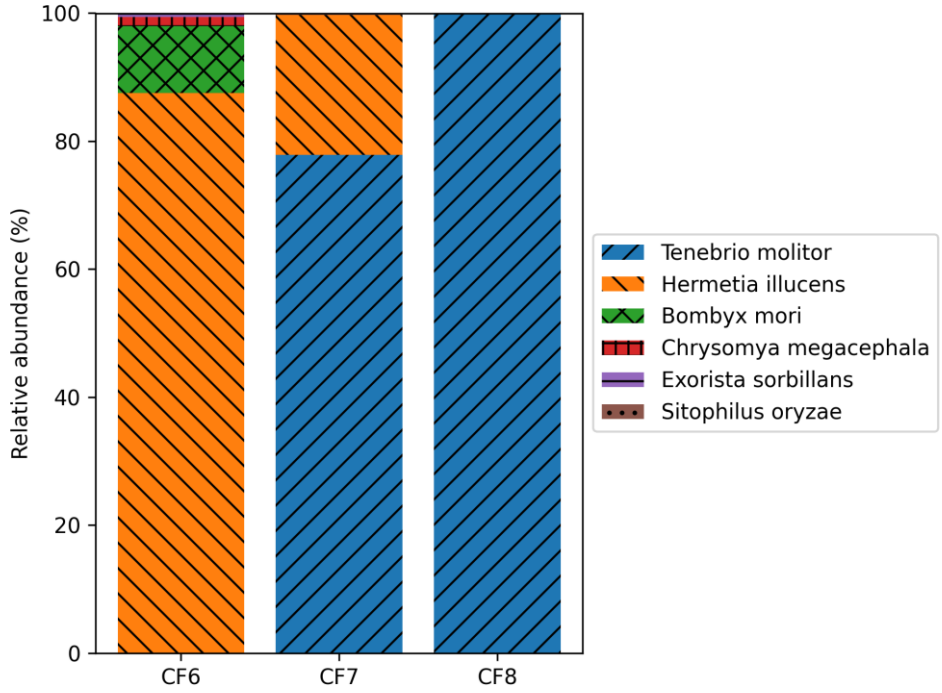

Figure 4. Taxonomic composition of samples CF6, CF7, and CF8 inferred from sequence abundance with a DNA metabarcoding analysis (the amounts of *Exorista sorbillans* and *Sitophilus oryzae* are too low to be visible on the graph).

Concerning transferability, the PCR efficiency was evaluated at 93.1% on genomic DNA in a second laboratory and therefore met the acceptance criterion proposed by Broeders et al. [44]. Table 14 indicated the mean Cq values obtained with the different copy numbers tested (from 5000 to 10), and no outliers were encountered.

Table 14. Data of the transferability study: Cq values obtained on dilutions of genomic material used for efficiency calculation and for $LOD_{95\%}$. For efficiency, each concentration was analyzed in six replicates and on four PCR plates (n = 24). For $LOD_{95\%}$, the concentration at 10 copies was analyzed in 60 replicates on 1 PCR plate (n = 60).

Copy Number of Target	Cq (Mean Value) ± SD (σ) and (n)
5000	27.52 ± 0.12 (24)
2500	28.76 ± 0.18 (24)
1000	30.12 ± 0.18 (24)
500	31.27 ± 0.26 (24)
100	33.49 ± 0.22 (24)
10	37.48 ± 0.90 (60)

Efficiency was higher than 90% for all plates when taken separately. LOD_6 was estimated at 5 copies following the former AFNOR XP V03-020-2 standard approach [58] and at 10 copies for $LOD_{95\%}$ with 60/60 positive signals. The mean Cq value at 10 copies is 37.48 cycles. The PCR test easily reached the recommended performance criterion ($\leq$20 copies). The transferability of the method was therefore evidenced.

4. Discussion

The study describes a specific, sensitive, and robust test to detect *B. mori*. With the recent authorisation in EU legislation to use eight insect species, including silkworm, in aquafeed and pig and poultry feed, the interest in this type of PCR test is increasing.

In silico and experimental specificity have been demonstrated. The applicability of the PCR method was also successfully tested on pure samples of *B. mori* from industrial production (P1 to P4) and feed containing a low level of *B. mori* (M1 to M12). In terms of performance, the validated PCR test is very similar to the already existing SP1 test of Zarske et al. [15], but the test described here seems slightly more sensitive when considering the obtained LOD, while the efficiency is also somewhat better (this statement is based on the results of the developers of the test as well as those of the transfer laboratory). Robustness was not considered by Zarske et al. [15].

The tests carried out on commercial samples (CF1 to CF8) clearly illustrate the importance of authentication tests. Indeed, most products do not correspond to the announced composition at the insect species level, with three mislabelled samples found out of the eight analyzed samples. This was revealed using three different detection techniques: real-time PCR, HTS (metabarcoding), and LM. LM is one of the official techniques for detecting PAPs. Therefore, it seemed important to use it on the different samples from this study to highlight its advantages and disadvantages. Although insect particles could easily be detected and identified in the commercial samples, as described in the article by Veys and Baeten [68], light microscopy can only distinguish insects at the level of their order. *B. mori* is a species belonging to the Lepidoptera order, so it is possible, when using LM, to distinguish it from Coleoptera and Diptera, which were also present in the commercial samples analyzed. However, it is clearly not possible to differentiate between several Lepidoptera species using this technique, and so, if several *Bombyx* species are present in the samples analyzed, it would not be possible to differentiate between them. In this study, the insect particles observed in the commercial samples were compared with particles from pure insect meal samples. This made it possible to identify the particles observed, but it is evident that it is not possible to be sure of this identification, which is why it is essential to combine this technique with genomic methods.

Finally, the real-time PCR test developed is suitable for quantification given that it is only present once per haploid genome. The challenge for quantification remains the need to have a single-copy general insect target, which, to our knowledge, has not yet been identified.

5. Conclusions

The developed PCR assay for the detection of *B. mori*, targeting a 98 bp fragment located on the *cadherin* gene, is fit for purpose. Indeed, the acceptance criteria were reached for efficiency, sensitivity (LOD_6 and $LOD_{95\%}$), and robustness. The specificity produces good results since only *B. mori* was detected. However, interference with *B. mandarina* cannot be excluded as it could not be tested but this has no major practical implications. The PCR method was successfully applied to pure industrial samples and allows the detection of 0.1% (in mass fraction) of *B. mori*. Issues with labelling accuracy of commercial products were identified in relation to the verified composition. Finally, the transferability of the method was also demonstrated by testing the efficiency and LOD in a second laboratory.

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analyzed the data and interpreted the results. A.M. wrote the manuscript with the help of B.D., A.A. and F.D. G.B. and P.V. provided valuable comments to improve the quality of the manuscript. All authors have read and agreed to the published version of the manuscript.

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