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Research

A Set of Multiplex Polymerase Chain Reactions for Genomic Detection of Nine Edible Insect Species in Foods

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Abstract

On 1 January 2018, a new regulation on 'Novel Food' has come into application in the EU. Insects and insect-based products are therefore included among the categories of food which constitute novel foods. Insects are nutrient-rich, produce fewer greenhouse gases and ammonia than conventional livestock, and have high feed conversion efficiency. Insects may be an alternative food source in the near future, but consideration of insects as a food requires scrutiny due to the risk of allergens. The aim of the present study was to develop a set of multiplex polymerase chain reaction (PCR) to detect nine edible insect species directly in foods. Four sets of mPCRs were designed to detect *Locusta migratoria migratorioides* (Reiche & Fairmaire, 1849) (Orthoptera: Acrididae), *Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae) (mPCR-I), *Acheta domesticus* (Linnaeus, 1758) (Orthoptera: Gryllidae), *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae (mPCR-II), *Alphitobius diaperinus* (Panzer, 1797) (Coleoptera: Tenebrionidae), *Schistocerca gregaria* (Forskål, 1775) (Orthoptera: Acrididae), *Zophobas atratus* (Fabricius, 1775) (Coleoptera: Tenebrionidae) (mPCR-III), *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae), and *Gryllodes sigillatus* (Walker, 1869) (Orthoptera: Gryllidae) (mPCR-IV). Results demonstrate that the panel of mPCRs allowed a rapid genetic identification of the insect species and has proved to be a sensible and highly discriminatory method. The assay is a potential tool in issues related to the labeling of products and food safety, in case of allergic consumers.

Key words: edible insect, detection, multiplex PCR, food product

Trends toward 2050 predict a steady population increase to 9 billion people, forcing an increased food/feed output from available agro-ecosystems resulting in an even greater pressure on the environment (FAO 2009). For this reason, the need for alternative protein sources is urgent. Promoting edible insects may mitigate the livestock crises. About 1,900 species of insects are eaten worldwide, mainly in developing countries (Van Huis 2013). In particular, insects are a widespread human food source in many parts of the world predominantly in Asia, Africa, and Latin America (Klunder et al. 2012). Insects are nutrient-rich and in some cases have a high protein and fat content when compared to other animal foods such as pork, beef, and poultry. They produce less greenhouse gases and ammonia than conventional livestock and use significantly less water. They have high feed conversion efficiency (Van Huis et al. 2013, Palmer 2016).

On 1 January 2018, the new Regulation (EU) No 2015/2283 of the European Parliament and of the Council on 'Novel Food' (European Union 2015), repealing Regulation (EC) No 258/97 (European Commission 1997), has come into application in the EU. The insect species reported to have the greatest potential for use as food in the EU include *Tenebrio molitor*, *Acheta domesticus*, *Gryllodes sigillatus*, and *Bombyx mori* (EFSA Scientific Committee 2015). As from last January 2018, edible insects can be available in EU markets and restaurants. Currently, insects represent a niche food market in the EU, with several Member States reporting occasional human consumption. Particularly, a selection of insects, which are offered for human consumption in some European countries is shown in Table 1. For example, whole mealworms (*T. molitor*), crickets (*A.* domesticus), and grasshoppers (*Schistocerca gregaria*) are on sale in the United Kingdom. In the Netherlands and Belgium, insect burgers and nuggets, as well as vegetable spreads made with mealworms, are available in some supermarkets. In particular, in Belgium, a former list of insects species accepted as food if produced in EU, if the whole insects are used and if requirements for food safety have been respected, drawn up by the Federal Agency for the Safety of the Food Chain (FASFC) has been recently amended; only three insects species have been left from the former list, encompassing, originally, all the species included in this study.

Insects may be an alternative food source in the near future, but consideration of insects as a food requires scrutiny due to risk of allergens. Several allergens, such as tropomyosin, arginine kinase, and chitin, cannot be detected directly by application of polymerase chain reaction (PCR) methods, but detection of insect species will indicate the presence of potentially allergenic compounds. Besides, authenticity testing of the insect species is important for identification of fraud and adulteration in industrial insect products. Thus, specific species detection enables to verify the product composition as given on labels.

Few PCR protocols have been proposed to identify insects (Tayutivutikul et al. 2003, Veer et al. 2013) and in particular in foods or feeds (Debode et al. 2017). No PCR protocols, which could detect simultaneously edible insect species are yet available to the best of our knowledge. Multiplex PCR is a necessary and promising approach to produce results in a more economic and faster way. In the present study, a method is described to detect and to differentiate nine different edible insect species, by using a set of multiplex PCRs (mPCRs), directly in food products for human consumption.

Materials and Methods

Positive Controls

As positive controls for mPCRs, genomic DNA extracted by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) from the insects *A. domesticus*, *Alphitobius diaperinus*, *B. mori*, *Galleria mellonella*, *Gr. sigillatus*, *Locusta migratoria migratorioides*, *S. gregaria*, *T. molitor*, and *Zophobas atratus* were used. Insects were obtained from a commercial supplier.

Design and Synthesis of Oligonucleotides

Different pairs of primers specific for each of the nine insect's target genes were designed on the basis of alignment of published gene sequences obtained from GenBank (Table 2). Only reverse primer of *L. migratoria migratorioides* was selected from the literature (Veer et al. 2013). The specificity of methods was checked in silico on 16S and COI genes of 75 insect species including different taxonomic groups: 19 Lepidoptera, 13 Hymenoptera, 13 Orthoptera, 9 Coleoptera, 9 Diptera, 6 Hemiptera, and 6 Isoptera (Table 3) (Williams et al. 2016). Primers specificity was also tested in silico using 16S and COI sequences of nontarget animal and plant species: crustacean (*Homarus gammarus, Paralithodes camtschatieus, Gammarus* sp., *Paleomon serratus*), mollusca (*Teuthida sp.*), mammals (*Homo sapiens*, *Bos taurus*), and plants (*Glycine max*, *Zea mays*, *Brassica rapa*, *Triticum aestivum*, *Oryza sativa*, *Solanum lycopersicum*). Sequences were then aligned using multiple-alignment software provided in the BioEdit package, version 7.2.6.1 (Hall 1999).

Multiplex PCRs Protocol

Initially, individual pair of primers were tested by PCR, using DNA extracted from each positive control. Then, four sets of mPCRs were designed to detect *L. migratoria migratorioides*, *T. molitor* (mPCR-I),

Table 1. Insects for human consumption in European countries

Insect species	Order	Family	Belgium	Netherlands	Switzerland	France	United Kingdom
Acheta domesticus	Orthoptera	Gryllidae	X		X	X	X
L. migratoria migratorioides	Orthoptera	Acrididae	X	X	X		X
Tenebrio molitor	Coleoptera	Tenebrionidae	X	X	X	X	X
Alphitobius diaperinus	Coleoptera	Tenebrionidae	X	X			

Table 2. Primers used in the multiplex PCRs for amplification of edible insect species

Insect species	Target gene	Primer sequence (5′-3′)	Annealing Temperature (°C)	Amplicon size (bp)
Multiplex PCRI				
Locusta migratoria migratorioides	16S rRNA	CAAAGGTAGCATAATCATTAGT CTCCGGTTTGAACTCAGATC	55°C	370–420
Tenebrio molitor	16S rRNA	CAAAGGTAGCATAATCATTAGT AGTTAAATAAATTTTCTAACCG	55°C	240
Multiplex PCRII				
Acheta domesticus	16S rRNA	CAAAGGTAGCATAATCATTAGT TCATTCCATAATACAGGATCA	55°C	290
Bombyx mori	16S rRNA	CAAAGGTAGCATAATCATTAGT CGTCTTTTTAAATAATTTTAACT	55°C	134
Multiplex PCRIII				
Alphitobius diaperinus	COI	AGTAGGAATAGACGTAGATACTCG TAGCAAATACGGCTCCTATTGAT	55°C	284
Schistocerca gregaria	16S rRNA	CAAAGGTAGCATAATCATTAGT TCATTAAATATAGAAAGACAAACA	55°C	198
Zophobas atratus	COI	GGGCATCAGTCGATCTCGCA CGATCAAAAGTTATTCCTTGTGG	55°C	120
Multiplex PCRIV				
Gryllodes sigillatus	16S rRNA	CAAAGGTAGCATAATCATTAGT TATTTAAGTCATAATACTATCCT	50°C	195
Galleria mellonella	COI	TATTAATATAAAATTAAATGGTT GAACAGGCAATGAAAGAAG	50°C	100

Table 3. Insects included for the detection of primers specificity

Coleoptera		Diptera		Hemiptera		Hymenoptera		Lepidoptera		Orthoptera	
Family	Genus	Family	Genus	Family	Genus	Family	Genus	Family	Genus	Family	Genus
Cerambycidae	*Callipogon*	Caenidae	*Povilla*	Coreidae	*Pachilis*	Apidae	*Apis*	Cossidae	*Chilecomadia*	Acrididae	*Melanoplus*
Curculionidae	*Rhynchophorus*	Calliphoridae	*Lucilia*	Membracidae	*Hoplophorion*	Formicidae	*Atta*		*Comadia*		*Oxya*
	Scyphophorus	Chaoboridae	*Chaoborus*		*Umbonia*		*Limetopum*	Erebidae	*Ascalapha*	Blattellidae	*Blatella*
Passalidae	*Oileus*	Drosophilidae	*Drosophila*	Pentatomidae	*Edessa*		*Oiecophylla*	Noctuidae	*Agrotis*	Blattidae	*Blatta*
	Passalus	Muscidae	*Musca*		*Euchistus*	Trigonalydae	*Parachartegus*		*Helicoverpa*	Gryllidae	*Brachytrupes*
Scarabaeidae	*Cetonia*	Sirfidae	*Copestylum*				*Trigon*		*Pseudaletia*		*Gryllus*
	Pachnoda	Stratiomyidae	*Hermetia*			Vespidae	*Brachygastra*		*Spodoptera*	Gryllotalpidae	*Gryllotalpa*
							Brachygastra	Notodontidae	*Anaphe*	Pyrgomorphidae	*Sphenarium*
							Polistes	Pieridae	*Catasticta*		*Zonocerus*
							Polybia	Saturniidae	*Arsenura*	Romaleidae	*Taeniopoda*
							Vespa		*Hyalophora*		
							Vespula		*Hylesia*		
									Imbrasia		
									Usta		
								Sphingidae	*Manduca*		

A. domesticus, *B. mori* (mPCR-II), *Al. diaperinus*, *S. gregaria*, *Z. atratus* (mPCR-III), *G. mellonella*, and *Gr. sigillatus* (mPCR-IV).

The set of mPCRs were performed to amplify the mitochondrial 16S ribosomal RNA gene (16S rRNA) and the mitochondrial Cytochrome Oxidase I (COI) gene. PCR reactions were carried out using a total reaction volume of 25 µl containing 20 pmol of each primer, 1X Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 2.5 µl of genomic DNA and H_2O. All the PCR reactions were carried out on GenAmp 2720 thermal cycler (Applied Biosystems, Foster City, CA) under the following cycle conditions: 95°C for 15 min of initial denaturation followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C (mPCRs I, II, III) for 90 s or 50°C (mPCR IV) for 90 s, extension at 72°C for 90 s and amplification ended with a 10-min final extension step followed by a 4°C hold. The PCR products were electrophoresed on 2.5% (w/v) agarose gel (Sigma-Aldrich, Poole, Dorset, United Kingdom). Products were visualized using an ultraviolet transilluminator (Gel-Doc, Bio-Rad, Richmond, CA) and molecular weight sizes were determined by comparison with a 100–base pair (bp) DNA ladder plus (Bio-Rad, Richmond, CA).

Multiplex PCRs Limit of Detection

Whole-genome DNA from insect was used to determine the limit of detection (LOD) of the mPCRs. A serial dilution series of the DNA solution containing a mixture of DNA was produced: 10, 5, 2.5, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 ng/µl. To produce the dilution series, ultrapure water was used. The quality and quantity of DNA extracted from insects were estimated spectrophotometrically using a Nanodrop ND-1000 spectrophotometer (Life Technologies, Grand Island, NY) at 260 nm (A260) and 280 nm (A280) absorbance.

Multiplex PCRs Specificity

PCR products were examined for correct size by agarose gel (2.5%) (Sigma-Aldrich) electrophoresis in 1X TAE buffer. Successively, amplicons were purified using a commercial purification kit (ChargeSwitch PCR Clean-Up Kit, Invitrogen, Grand Island, NY) and sequenced (ABI Prism 310 Genetic Analyser, Applied Biosystems, Foster City, CA) to confirm the identity of the insect species and of the PCR amplified products. The chromatograms of the nucleotide sequences obtained were submitted for BLAST analysis.

Multiplex PCRs in Insect-Based Foods

The developed set of mPCRs was applied on 42 food samples. In particular, two cookies containing 25% and 50% (w/w) of each insect species (n = 36 spiked samples), three cricket nut fudge with dark chocolate and three cricket nut fudge with milk chocolate (Eat Grub) (n = 6 commercial products) were tested. Genomic DNA was extracted and purified from foods by DNeasy mericon Food (Qiagen, Hilden, Germany). The quality and quantity of DNA extracted from samples were estimated spectrophotometrically using a Nanodrop ND-1000 spectrophotometer (Life Technologies) at 260 nm (A260) and 280 nm (A280) absorbance. PCR products were examined for correct size by agarose gel (2.5%) (Sigma-Aldrich) electrophoresis in 1X TAE buffer.

Results

Multiplex PCRs

Primers were designed to produce amplicons of different sizes that could be combined in one reaction and be distinguished from each other. To develop the set of mPCRs, we tested the progressive incorporation of primers corresponding to the different insect genes and several combinations of melting temperatures and primer

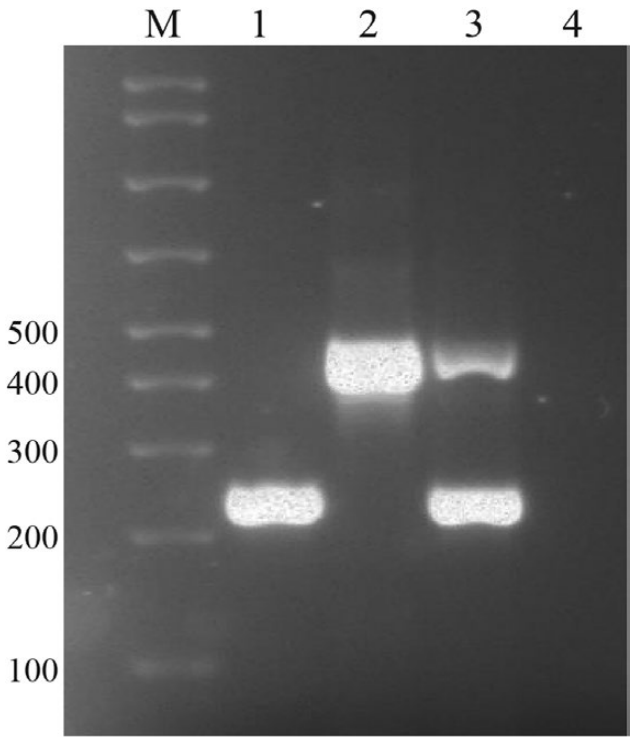

Fig. 1. Amplification products obtained by the set of multiplex polymerase chain reaction I (mPCRI) assays. Lane M: 100–base pair DNA ladder plus; lane 1: PCR positive-samples for *Tenebrio molitor*; lane 2: PCR positive-samples for *Locusta migratoria migratorioides*; lane 3: PCR positive-samples for *L. migratoria migratorioides* and *Tenebrio molitor*; lane 4: negative sample for mPCRI.

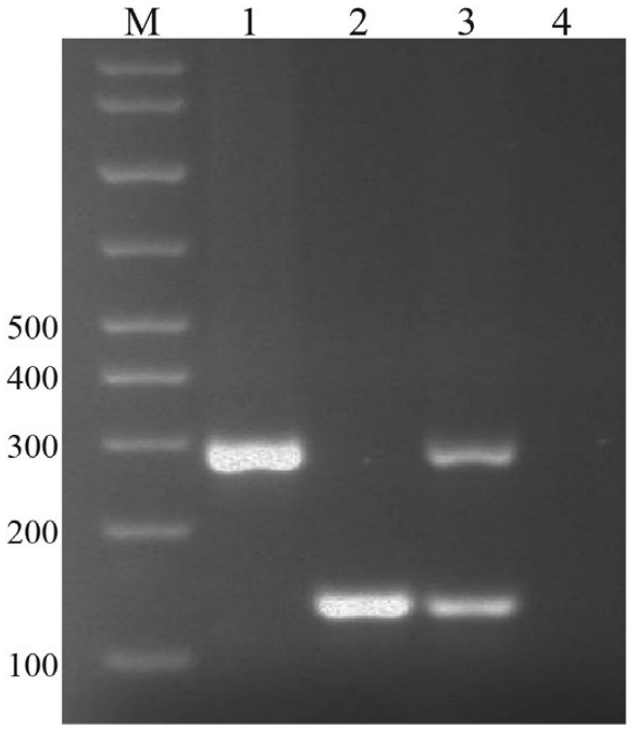

Fig. 2. Amplification products obtained by the set of multiplex polymerase chain reaction II (mPCRII) assays. Lane M: 100–base pair DNA ladder plus; lane 1: PCR positive-samples for *Acheta domesticus*; lane 2: PCR positive-samples for *Bombyx mori*; lane 3: PCR positive-samples for *A. domesticus, B. mori*; lane 4: negative sample for mPCRII.

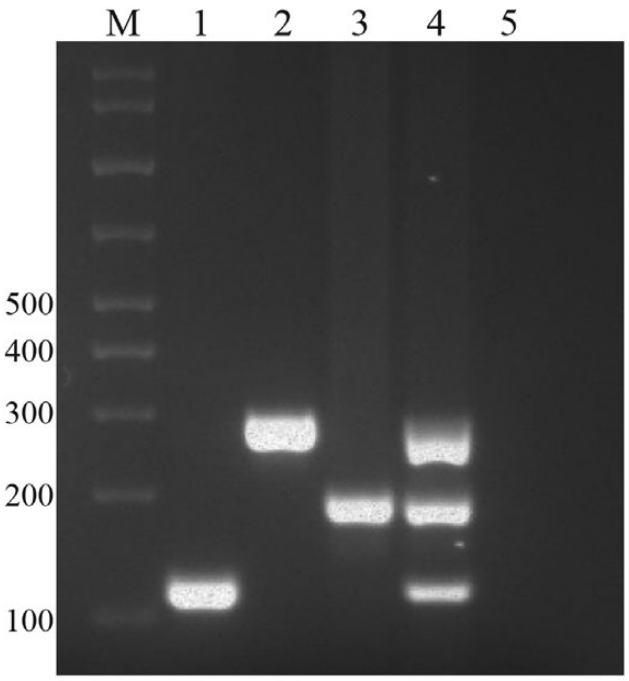

Fig. 3. Amplification products obtained by the set of multiplex polymerase chain reaction III (mPCRIII) assays. Lane M: 100–base pair DNA ladder plus; lane 1: PCR positive-samples for *Zophobas atratus*; lane 2: PCR positive-samples for *Alphitobius diaperinus* lane 3: PCR positive-samples for *Schistocerca gregaria*; lane 4: PCR positive-samples for *Z. atratus*, *S. gregaria*, *Al. diaperinus;* lane 5: negative sample for mPCRIII.

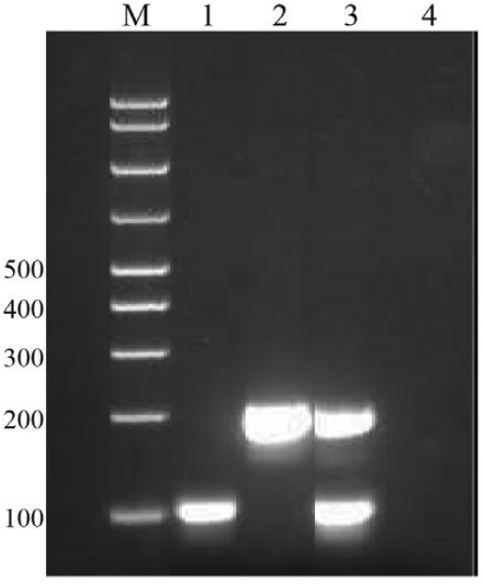

Fig. 4. Amplification products obtained by the set of multiplex polymerase chain reaction IV (mPCRIV) assays. Lane M: 100–base pair DNA ladder plus; lane 1: PCR positive-samples for *Galleria mellonella*; lane 2: PCR positive-samples for *Gryllodes sigillatus*; lane 3: PCR positive-samples for *G. mellonella, Gr. sigillatus*; lane 4: negative sample for mPCRIV.

concentrations. PCR products of the expected sizes (Table 2) were obtained for each insect species. Two bands were obtained for mPCRI (*L. migratoria migratorioides*, *T. molitor*), mPCRII (*A. domesticus*, *B. mori*) and mPCRIV (*G. mellonella*, *Gr. sigillatus*) and three bands were obtained for mPCRIII (*Al. diaperinus*, *S. gregaria*, *Z. atratus*) (Figs. 1–4).

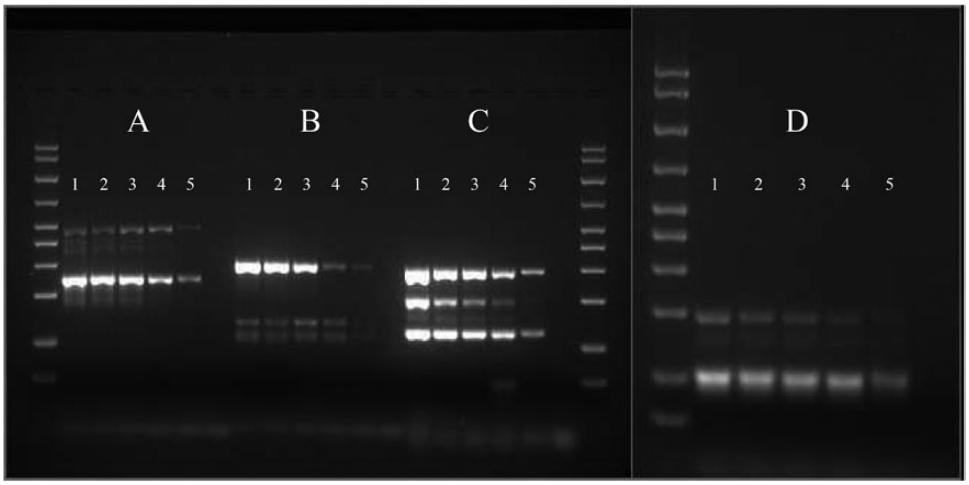

Fig. 5. LOD of the mPCRs, obtained from serial dilutions of DNA extracted from insects. A: mPCRI, B: mPCRII, C: mPCRIII, D: mPCRIV. Lane 1: 0.5 ng/µl; lane 2: 0.1 ng/µl; lane 3: 0.05 ng/µl; lane 4: 0.01 ng/µl; lane 5: 0.005 ng/µl.

The sensitivity and specificity of the reactions were assayed with the insects. Analytical sensitivity/LOD of the mPCRs, obtained from serial dilutions of DNA extracted from insects, was determined to be as low as ~0.01 ng/µl (0.025 ng/tube) (Fig. 5). No cross-reactions occurred and the primers were determined to be specific. All mPCRs correctly detected insect DNA species. Sequencing of PCR products showed a similarity of 98–100% with the sequences of *A. domesticus* (KR903672), *Al. diaperinus* (KR052902), *B. mori* (AF027951), *G. mellonella* (KT782456), *Gr. sigillatus* (AF514593), *L. migratoria migratorioides* (AF514505), *S. gregaria* (KY980799), *T. molitor* (AJ438153), and *Z. atratus* (KR916703), retrieved from the GenBank database.

Multiplex PCRs in Insect-Based Foods

Once the PCR conditions were established using insects, mPCRs were used in insect-based foods. Genomic DNA extracted by DNeasy mericon Food (Qiagen) has allowed to obtain high amounts of insect DNA from complex food matrices as chocolates and cookies. All mPCRs correctly detected insect DNA contained within the 42 foods analyzed both at 25 and 50% (w/w).

Discussion

The use of insects as a source of food has important environmental and economic benefits. The insect species reported to have the greatest potential for use as food in the EU include mealworms (*T. molitor*), crickets (*A. domesticus* and *Gr. sigillatus*) and silkworms (*B. mori*). Different studies showed that insects are considered food with satisfactorily energy and protein content, good amino acid and fatty acid profiles and high contents of a variety of micronutrients, e.g., minerals (K, Na, Ca, Cu, Fe, Zn, Mn, and P) and vitamins (B, A, E, K, and C) (Rumpold and Schlüter 2013, Van Huis 2013). In contrast, there are few informations about some safety issues, in particular regarding insect protein allergenicity. The structural similarities of insect proteins to known allergens in more widely consumed arthropods present a risk of allergic reactions due to cross-reactivity in individuals with preexisting allergies (Palmer 2016). The tropomyosin, the chitin and the arginine kinase in insects are similar to the ones in crustaceans (Palmer 2016). As there is no specific treatment for food allergies, strict avoidance of food allergens is the only way to prevent serious health consequences. For consumer protection, several countries, including member states of the EU, require the labeling of ingredients that can trigger allergic or intolerance reactions. An effective control of fraud in insect products and violations of labeling requirements for insect products is important for economic, safety and health reasons.

Molecular methods based on mitochondrial DNA sequences allow for specific species identification of animal tissues in food and animal feeds (Bottero et al. 2003, Kusama et al. 2004). In particular, PCR targets short segments of DNA of a targeted gene from an allergenic source to amplify and identify. Currently, there is no PCR panel for the simultaneous detection of edible insect in foods. The set of mPCR assays described in the present study would be a time- and cost-effective way of simultaneously detecting edible insect species in foods. Results demonstrate that the set of mPCRs allowed a rapid genetic identification of the insect species and has proved to be a sensible, simple and discriminatory method. In particular, we have developed a panel of mPCRs that is able to identify and discriminate nine edible insect species with the aim to reduce the number of tests needed to identify insect species offered for human consumption in European countries including Italy. Whereas, different molecular studies showed that 16S rRNA gene of mitochondrial DNA and mitochondrial Cytochrome Oxidase I gene are powerful markers for species identification and classification (Cook et al. 2002, Porter et al. 2014), we have designed six sets of novel primers within 16S rRNA gene to detect *A. domesticus, B. mori, Gr. sigillatus, L. migratoria migratorioides*, *S. gregaria, T. molitor*, *and* three sets of primers within COI gene to detect *Al. diaperinus, G. mellonella*, and *Z. atratus*. Moreover, all primers were developed to create short PCR products, allowing the use of the assay also in foods subjected to heat-temperature processing (Rodríguez et al. 2004, Martin et al. 2008). Thus, the set of multiplex PCRs described herein could be used to detect minimal amounts of insect DNA in raw and heat-treated food products. Given that a large number of insect species are found in nature, the risk of false positive results as a consequence of cross reaction with non-target sequences cannot be excluded: in particular, similarities of 16S rRNA sequences of some insects considered in this study (e.g., *B. mori, L. migratoria migratorioides,* and *A. domesticus*) and other species (e.g., *Spodoptera, Agrotis,* and *Blatella*) could be observed. In addition, 16S rRNA and COI sequences deposited on Genbank can hardly be considered comprehensive of insect species that can be found in nature.

In conclusion, the assay here developed can be considered as a screening for issues related to the labeling of products containing insects and a potential tool in response to the need for ensuring avoidance from exposure to allergens for allergic consumers.

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