



Multiplex real-time PCR for the detection of insect DNA and determination of contents of *Tenebrio molitor*, *Locusta migratoria* and *Achaeta domestica* in food

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Abstract

In Europe, edible insects are not a part of a common diet. But according to the Food and Agriculture Organization of the United Nations (FAO), animal protein from insects could be an ecological, economic and healthy addition to human diets. In the EU there are currently no regulations on insects as food for human consumption. The production and sales of products containing insects are not prohibited. Several companies are producing and marketing food products containing insects already. In Switzerland, the recently revised food law allows and regulates insect production and products for human consumption. Like other ingredients, such food additives, insects have to be labeled correctly. To enforce such labeling, food control laboratories need analytical tools to detect insect ingredients and determine their species and quantity. We, therefore, developed a multiplex real-time PCR method detecting insect DNA generally and the three approved species specifically. The performance of this multiplex real-time PCR method was assessed during validation including data from other laboratories. These results indicate that the method is fit for purpose.

Keywords Multiplex real-time quantitative PCR · Insect · *Tenebrio molitor* · *Locusta migratoria* · *Achaeta domestica*

Introduction

Eating insects (entomophagy) is not new. Traditions reach back to the very beginning of human food culture and are practiced by a majority of cultures (FAO). In contrast, in the so called developed countries, entomophagy is replaced by meat consumption. According to FAO (<http://www.fao.org/ag/againfo/themes/en/Environment.html>) this has a severe ecological impact, as meat consumption is high and leads to negative ecological impact as meat production is very inefficient considering the loss of calories during growth of the animals. Insects may be a more

efficient way to produce animal protein (<http://www.fao.org/edible-insects/en/>). Insects often are a byproduct of e.g., silk production. Insects can be fed by poor quality organic material like all kinds of organic waste. However, insect production for human or animal consumption must fulfill all legal requirements like hygiene and chemical residues like pesticides and antibiotics and must not transmit diseases. According to Swiss food law, insect components must be listed correctly on the package of the food products. An upcoming problem may be the already established production of insects for pet food. Adulteration of high-quality insect products for human consumption with lower quality pet food insects may take place. In addition, the use of not recognizable insects, such as insect flour, may be more acceptable to consumers than entire insects. In any case, food control laboratory must be capable to detect insects in food products. However, to a certain degree and as inevitable ingredient, insect material is expected to be found in many food products already. It may be a result of contamination during harvest, storage and production process as insects are omnipresent. Therefore, quantitative analysis methods are desirable to fix a threshold value to

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distinguish usual contamination from intentional insect ingredients.

According to the Swiss law, only *Tenebrio molitor* (mealworm), *Locusta migratoria* (migratory locust) and *Achaeta domestica* (house cricket) are approved as Novel Food ingredients.

Already previously, molecular biological methods served to define systematic relationship of insects [1, 2]. Sets of primers were developed specific for the sequencing of unknown insects [3, 4] leading to rearrangements in insect systematics [5]. Certain regions turned out to be more suitable for this goal and were used as so called barcoding regions [6–8]. For quantitative gene expression studies, reference genes expressed constantly within insect cells were assigned. In this context, more quantitative aspects were investigated and appropriate tools were developed [9–14]. Most recent attempts use deep sequencing methods [15] expanding the set of known sequences on e.g., Genbank substantially. However, all this work used single organisms and not mixed food products with e.g., a high background of vertebrate and plant DNA. For this purpose, to our knowledge, no PCR-system was developed yet.

Therefore, we had to develop new appropriate PCR systems. We decided to develop a multiplex real-time PCR system to detect and determine all three allowed insects at once in an economic way. In addition, food have to be screened for major insect ingredients at all. An additional unspecific real-time PCR system based on SybrGreen for the determination of as many insect species as possible, without cross reaction to vertebrates and plants, was developed and integrated in the final tetraplex real-time PCR system. The usual parameters like sensitivity and specificity were validated. Reference material was produced to enable calibration for quantitative analysis, showing that this system may be suitable to screen food products for insect ingredients in routine analysis. The involvement of other laboratories showed that the system is transferrable. The system revealed to be robust and fit for purpose.

Materials and methods

Samples

Insects were purchased from different food and pet-food internet shops as whole insects. To verify the exact species the DNA from all these samples was extracted and sequenced using primers for the Cytochrome b [16] and Cytochrome oxidase region [17]. The sequences were compared to the Databank of the National Center for Biotechnology Information using the BLASTn tool.

DNA extraction

DNA extraction from all sample matrices was performed using the column based Wizard Plus Miniprep DNA purification system (Promega, Madison, USA). 200 mg of ground sample material was extracted and the DNA was eluted into 50 µl elution buffer according to the supplier's manual. The concentration was determined by spectrophotometry (denatured single strand DNA, 320/260 nm) and adjusted by dilution to 20 ng/µl using PCR-grade water.

Primers and probes

Unspecific insect system: The primers are located in the cytochrome oxidase gene, subunit 1. Its origin is mitochondrial. This system was designed to fit as many insect families as possible without fitting to sequences of e.g., vertebrates. The design leads to extensive aligning of sequences and extensive practical testing. The given reference sequence of drosophila melanogaster fits with one mismatch (A instead of T).

Tenebrio molitor: The primers and probes are located in the NADH dehydrogenase subunit 4L. Its origin is mitochondrial.

Achaeta domestica: The primers and probes are located in the cytochrome oxidase gene, subunit 1. Its origin is mitochondrial.

Locusta migratoria: The primers and probes are located in the cytochrome oxidase gene, subunit 1. Its origin is mitochondrial.

Primer and probe sequences are listed in the “[Appendix](#)” Table 1.

Real-time PCR procedure

5 µl DNA extract was added to 20 µl of reaction mix containing Sensifast™ Probe No-Rox kit (BL-BIO-86020 2000, Bioline GmbH, Germany), primers and probes listed in “[Appendix](#)” Table 1. The final concentrations of primers and probes are also listed “[Appendix](#)” Table 1. PCR was performed on a Rotor-Gene Q real-time PCR system (Qiagen, Germany) according to the following cycling protocol: Initial step of 5 min at 95 °C; followed by 35 cycles of 5 s. at 95 °C and 15 s. at 55 °C. In addition, a melting curve was determined using the following parameters: starting at 65 °C for 90 s. temperature increment 1 °C, 5 s each step until 95 °C was reached. To control the thermocycler and for calculations of the cycle threshold (*C_t*) values, the Rotor-Gene Q Series Software 2.0.2 was used. For the calculation of the percent values, DNA dilution rows were applied.

Production of spiked cookies

The Insects were dried and milled to a fine powder. The composition is listed in “Appendix” Table 2. By adding of the water, a dough was made and knead to homogeneity. The dough was baked for 25 min at 180 °C. After that the cookies were milled to a homogenous powder and stored at −20 °C. From this material, the DNA was extracted like described above.

Production of asymmetric spiked hamburgers

The insects were dried and milled to a fine powder. The composition is listed in “Appendix” Table 3. This powder was added to cryo-milled beef. After mixing a hamburger was formed and backed in rapeseed oil until brown and crispy. After that, the hamburger was frozen and milled to a homogenous powder and stored at −20 °C. From this material the DNA was extracted like described above.

Sequencing of amplicons of the unspecific system

In cases where no specific system shows an amplification but the unspecific systems indicate insect DNA, the amplicon of this unknown species can be sequenced directly using the primer Ins3.2F. In cases where only one species leads to this amplicon a BLAST-search may reveal the corresponding insect species.

Results

Specificity

Close relatives of *Tenebrio molitor*, *Achaeta domestica* and *Locusta migratoria*

The most important issue was to check the primers for cross reactivity to close relative insects. A major problem was the availability of insects. In relation to the large number of different insects only a minor part could be tested for detection or cross reactions. “Appendix” Table 4 presents these results. No unexpected cross reactions were observed.

The unspecific PCR-system amplified all insect template DNA except DNA from *Blattella germanica*.

The sequence of the cytochrome oxidase subunit I (KT268256) was aligned with every primer sequence of the unspecific system. The primers Ins3.2F and Ins3.3R position (grey highlighted) showed similar sequences at position 170 and 450. The similarities were biased by mismatches (capital letters):

161 tgccaatt **T**taattggAggattggaaattga ttagtacc
ttaatatata ggtgctcctg atatagcatt tctcgtata aataatataa

gtttttgact ttacctcca tctctatctc ttctattagc tagaagccta gtcgaaa-
gag gagctgggac tggttgaacc gtataccac ccttagctag aggaattgct
catgctggag cttcagtaga tttagctatt ttctattac atcttgcagg ggtatcat
caattTtaggTgcCGtaaattttattTcAaca attattaaca.

Both primers have two mismatches or more. It is, therefore, highly probable that no amplification can take place, confirming the experimental data.

In addition, the specificity using DNA from the following non-insect animal species was tested:

Pig (*Sus scrofa*), cow (*Bos taurus*), chicken (*Gallus gallus*), sheep (*Ovis aries*), goat (*Capra aegagrus*), horse (*Equus caballus*), buffalo (*Bubalis bubalus*), duck (*Anatidae*), goose (*Anser domesticus*), turkey (*Meleagris gallopavo*), trout (*Salmo trutta*), Cod (*Gadus morhua*), Thuna (*Thunnus albacares*), Swordfish (*Xiphias gladius*), Dolphin (*Tursiops truncatus*) [shark (*Carcharodon carcharias*), Oyster (*Ostrea edulis*), Venus clam (*Veneridae striatula*), Scallop (*Pecten maximus*), common mussel (*Mytilus edulis*), Squid (*Teuthida*), crawfish (*Astacoidae*), common shrimp (*Dendrobranchiata*), Shrimp sea tiger (*Penaidae*), Black Tiger shrimp (*Penaeus monodon*), Norway lobster (*Nephrops norvegicus*), Brown crab (*Cancer pagurus*), Giant freshwater prawn (*Macrobrachium rosenbergii*), Deep water prawn (*Pandalus borealis*), Pacific White Shrimp (*Litopenaeus vannamei*), Red swamp crayfish (*Procambarus clarkii*)

Only for crawfish and common shrimp a slight cross reactivity was observed. 2 ng of purified crawfish DNA resulted in a positive signal of <0.1% in the unspecific insect system. As the signal was below 0.1% compared to purified insect DNA and as the use of crawfish DNA as contaminant is rare, we consider this cross reactivity as irrelevant, but must be kept in mind in the case of potential foods such as insect enhanced fishcakes.

The following plant species were tested: almond (*Prunus dulcis*), hazelnut (*Corylus avellana*), walnut (*Juglans regia*), apricot (*Prunus armeniaca*), coconut (*Cocos nucifera*), wheat (*Triticum spp*), rice (*Oryza spp*), potato (*Solanum tuberosum*), paprika (*Capsicum annuum*), tomato (*Solanum lycopersicum*), onion (*Allium cepa*), carrot (*Daucus carota sativus*), parsley (*Petroselinum crispum*), nutmeg (*Myristica fragrans*), cinnamon (*Cinamomum verum*), lentil (*Lens culinaris*), peas (*Pisum sativum*), mung beans (*Vigna radiata*), chickpeas (*Cicer arietinum*), lupine (*Lupinus*), peanut (*Arachis hypogaea*), beans (*Phaseolus vulgaris*), garlic (*Allium sativum*), celery (*Apium graveolens*), pepper (*Piper nigrum*), aniseed anise (*Illicium anisatum*), potato (*Solanum tuberosum*), soybean (*Glycine max*), rice (*Oryza sativa*), maize (*Zea mays*), kiwi (*Actinidia chinensis*), papaya (*Carica papaya*), fig (*Ficus*), plum (*Prunus domestica*), peach, khaki (*Diospyros kaki*) cherry (*Prunus padus*), mango (*Mangifera odorata*), passion fruit (*Passiflora edulis*), avocado (*Persea Americana*), blueberry (*Vaccinium*

myrtilus), raspberry (*Rubus occidentalis*), strawberry (*Fragaria*), mulberry (*Morus nigra*), aronia (*Aronia*), cranberry (*Vaccinium macrocarpon*), cowberry (*Vaccinium vitis-idaea*), grape (*Vitis*), squash (*Curcubita*), eggplant (*Solanum melongena*), ginger (*Zingiber officinale*), curcuma (*Curcuma longa*), cucumber (*Cucumis sativus*), watermelon (*Citrullus lanatus*), Pomgranade (*Punica granatum*), date (*Ziziphus jujube*), olive (*Olea europea*), fennel (*Foeniculum vulgare*), Saskatoon serviceberry (*Amelanchier alnifolia*).

No unspecific signal was observed.

Sensitivity, precision and uncertainty.

The accuracy, the mean deviation to the true value was calculated and the absolute values were averaged over all dilutions. This was done by three laboratories. The averaged data are compiled in “Appendix” Table 5. All samples were detectable and quantifiable within the assigned dilution down to a proportion of 0.1% of insect content w/w. The measurement uncertainty ranged between 7.8% (unspecific insect system) and 29.3% (*Achaeta domestica* system) which corresponds to experienced RSD in the field of allergen determination in food using real-time PCR [16]. A maximal measurement uncertainty of 35% was calculated for a single determination (not extended).

Results of spiked cookies and burgers

The DNA of the spiked cookies and burgers were isolated and analyzed in eight runs by three persons over a time period of 2 months. The results from the spiked burgers were taken to assess the three specific systems. For the unspecific system, only the spiked cookies could be used as with asymmetric spiking like done with the burgers the results of the unspecific system can't be used as the sum is unknown. These results were taken from the analysis using the spiked cookies. From the raw data the RSD and the accuracy (relative deviation to the spiked value) was calculated and geometrically summarized to estimate the measurement uncertainty (“Appendix” Table 6).

Analysis of products from the market

To assess a possible background of insect DNA in usual products we isolated the DNA from the Products listed in “Appendix” Table 7 and applied AllInsect for analysis. We expected a certain background signal as a result of small contaminations in the supply chain and during the processing of food. Noodles, bars and composed food products like ready to eat menus. In addition, 44 rice-and rice products and 60 dried herbs were analyzed. As contamination can occur with any insect, we focused specially on the unspecific insect-system detecting (Fig. 1).

Seven products showed a signal in the unspecific insect system. Nine products containing mainly rice and wheat

and/or maize showed a positive result for *Tenebrio molitor*. One basmati rice and one pasta product showed positive signals for *Achaeta domestica*. All signals were far below 0.1% comparing to the spiked cookies which were taken as calibrators. In a further investigation, we analyzed 37 samples of rice, 22 sausages and 20 herbs using AllInsect (not shown). None of the samples exhibited signals for insects. We judge therefore the natural contamination by insects as a not significant problem and can be ignored.

Sequencing of amplicons of the unspecific system

The following species were determined by sequencing using the Ins3.2F primer: *Schistocerca gregaria* (Accession-No. KY980902.1), *Tenebrio molitor* (Accession-No. KF418153.1) and *Locusta migratoria* (Accession-No. JN858191.1). *Gryllus bimaculatus* (Accession-No. KJ850234.1), *Galleria mellonella* (Accession-No. KT82456.1), *Bombyx mori* (Accession-No. AH010009.2), *Drosophila melanogaster* (Accession-No. KY310613.1), *Hermetia illucens* (Accession-No. KC192965.1), *Harmonia axyridis* (Accession-No. LC318295.1), *Apis mellifera* (Accession-No. KY271930.1). The following species could be determined only to the species level *Zophobas Drosophila Gryllus*, *Blaps*. *Blattella germanica* could not be sequenced. In this case an earlier published system revealed the correct sequencing information [16].

Conclusion

The multiplex real-time PCR system AllInsect exhibited amplification efficiency, correlation, precision and accuracy showing its functionality. Possible matrix effects could be diminished by the dilution of the sample template DNA. The high copy numbers of the target amplicons still lead to a LOD of 0.1% (w/w). Calibration was possible using spiked cookies or hamburgers. Applying AllInsect and the described calibration, several product categories were assessed for insect DNA. Only traces were found, far below 0.1% showing that contamination by insects is not a general problem in these food products.

An unresolved issue is data on the specificity to other insect species, as the insects are a very inhomogeneous and a very big group of animals. Especially the general insect system detects several insect species. But which ones remain unknown. Only a few insect species are bred industrially. Material of entomological collections is usually not available. This has to be ameliorated in future development. Limiting is still the availability of defined insect material.

The positive real-time signal in the no template control can be distinguished by melting curve from amplicons from insect DNA. It showed no significant cross reaction with

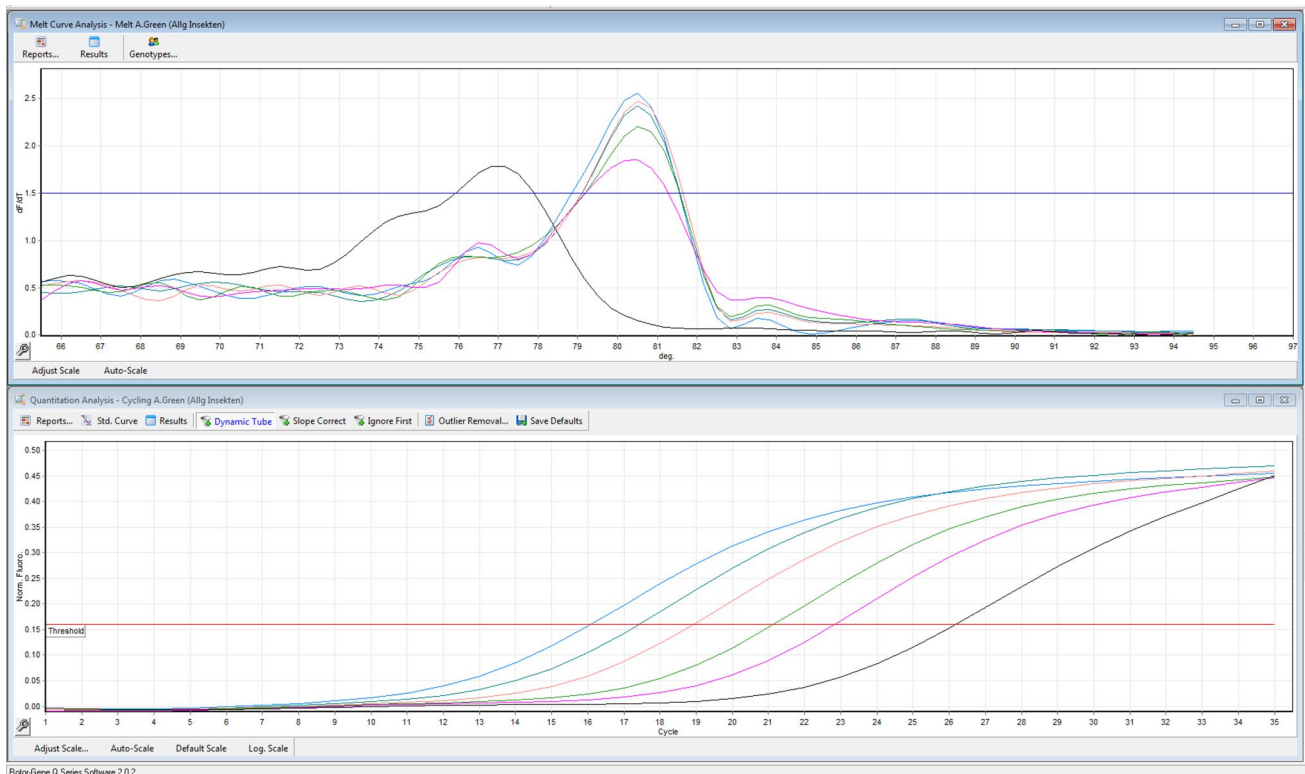


Fig. 1 The unspecific system uses SybrGreen for visualization of any double strand DNA. This enables the detection of a wide range of insect DNA. However, the drawback is that the NTC shows up at about 24 cycles. To assay true positive from arbitrary signals a melting curve can be measured after the cycling. The melting point of the unspecific insect-system show only one amplicon (one melting point)

the templates used in this work, especially all tested animal DNA. This feature is sensitive to any change of the primer sequences presented here. It was one of the difficulties to develop such primers. Systems published in the past, in contrary, often amplify mammalian DNA too. Another issue in the past was the cross reaction to crustacean, mollusk and mussels. No tested crustacean, mollusk and mussels gave an amplification curve for this system.

After all, the tetraplex real-time PCR system AllInsect is fit for the analysis of various food products potentially containing insect residues or ingredients, but quantitative results must still be interpreted with caution.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or mammalian subjects.

using insect DNA as a template which can be distinguished from the negative template control (NTC, left black). In addition, the ct distance to the 0.1% calibration sample still is three cycles and also clearly distinguishable from the NTC. We finally concluded that the NTC can be clearly assigned by using these two criteria

Appendix: Illustrations and Tables 1, 2, 3, 4, 5, 6 and 7

Tetraplex real-time PCR system AllInsect

The primer Ins3.2F can also be used for sequencing of amplicons of the unspecific system

The primers Ins3.2F/R, Ins3.1R and Ins3.3R were designed on a fusion sequence of KX771130.1 KY883616.1 KX053394.1 and KX431795.1 to cover a broad range of insects (mismatches in capital letters):

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ataccaatc ataattggTggatttgaaattga ctagtac gattaatatt
aggagctccc gatagatcat tctcgcact aaataatata ccattttgac ttctc-
ccacg atctttgtct atcttaatta taggtcttta taaagaagga gcacga
acaggatgaacTgtttatccacc tctttccc tcgaataaat aatatgagat
tttgattatt gattccatca ttatttttat taattttaag atctataatt aatattggtg
taggnactgg gtgaactgtt tatcctcctt taccattaaa tataagtcac agag-
gaatat cagttgattt agctattttt tctttacata ttgctggagt atcct
caattataggagcaataaattttattactaca att

```

Table 1 Tetraplex real-time PCR system AllInsect for the simultaneous determination of three specific insects and an insect system detecting a broad range of insects

Insects unspecific system, <i>cytochrome oxidase I</i>				
The system uses SybrGreen for monitoring the signal				
Primer/probe	Final conc. μ M	Sequence	Amplicon	Source/GenBank acc.no./labelling
Ins3.2F	0.3	ATA ATT GGT GGA TTT GGA AAT TGA	173 bp	This work KX771130.1 KY883616.1 1 Mismatch
Ins3.1R	0.3	GGT GGA TAA ACT GTT CAT CCT GT	173 bp	This work Consens sequence KX053394.1 KX431795.1
Ins3.2R	0.2	GGT GGA TAA ACa GTT CAT CCT GT		
Ins3.3R	0.2	CTG TAG TAA TAA AAT TTA TTG CTC CTA TAA TTG	280 bp	
Alph F	0.4	CAGGAATTAAAATTTTCAGATGA	178 bp	This work KR052883.1
Alph R	0.4	GGC AACTACAT AGTAAGTATC		

Table 2 Specificity tests of close relatives to *Tenebrio molitor*, *Achaeta domestica* and *Locusta migratoria*

Testet insects	Insect unspecific	<i>Acheta domestica</i>	<i>Tenebrio molitor</i>	<i>Locusta migratoria</i>
<i>Gryllus bimaculatus</i> 1%	pos	neg	neg	neg
<i>Locusta migratoria</i> 1%	pos	neg	neg	pos
<i>Acheta domestica</i> 1%	pos	pos	neg	neg
<i>Tenebrio molitor</i> 1%	pos	neg	pos	neg
<i>Schistocerca gregaria</i> 1%	pos	neg	neg	neg
<i>Alphitobius diaperinus</i> 100%	pos	neg	neg	neg
<i>Zophobas morio (atratus)</i> 1%	pos	neg	neg	neg
<i>Galleria mellonella</i> 1%	pos	neg	neg	neg
<i>Bombyx mori</i> 100%	pos	neg	neg	neg
<i>Blattella germanica</i> 1%	neg	neg	neg	neg
<i>Drosophila melanogaster</i> 1%	pos	neg	neg	neg
<i>Drosophila prolongata</i> 1%	pos	neg	neg	neg
<i>Hermetia illucens</i> 1%	pos	neg	neg	neg
<i>Gryllus assimilis</i> 1%	pos	neg	neg	neg
<i>Gryllus bimaculatus</i> 1%	pos	neg	neg	neg
<i>Harmonia axyridis</i> 1%	pos	neg	neg	neg
<i>Apiformes</i> 1%	pos	neg	neg	neg
<i>Bombus</i> 1%	pos	neg	neg	neg
No template control	Ct > 28	neg	neg	neg

Bold indicates primer sites

For the species-specific systems, no cross reactivity was observed. The unspecific system showed positive signals for all tested insect DNA except for *Blattella germanica*

The primers Alph F/R for *Alphitobius diaperinus* were added to cover this species as it is used to produce insect proteins.

Location in the genome of KR052883 cytochrome oxidase I, position 178–356:

gttccta **caggaattaaaatttcagatga** ttatgaactc ttcattggcac
tcaactaaat tatagaccct cctctctgtg agcttagga ttgtattcc tattca-
cagt aggaggatta accggagtag tattagcaaa ctcatcaatt gatattatat
tacat **gatacttactatgtagttgcc** cacttccattatgt

Table 3 Performance of the AllInsect-system

AllInsect level 1%	Insect unspecific	<i>Acaeta domestica</i>	<i>Tenebrio molitor</i>	<i>Locusta migratoria</i>
Limit of detection % (w/w)	0.1	0.1	0.1	0.1
Amplification efficiency	1.2	1.1	1.1	1.4
Correlation R ²	1.0	1.0	1.0	1.0
Precision (RSD) $\pm$ %	10.9	11.3	8.5	9.1
accuracy $\pm$ %	25.0	12.4	35.6	21.3
Measurement uncertainty $\pm$ %	25.6	11.4	31.3	21.3

Serial dilutions of insect-DNA were used as calibrators to assess the amplification efficiencies, correlation, precision and accuracy. The precision was calculated by averaging the individual precisions (relative standard deviations) of all dilutions. The accuracy was calculated by averaging the absolute values of the individual dilutions. The measurement uncertainty listed represent the measurement uncertainty at the limit of detection. The correlations and amplification efficiencies were taken from the Rotorgene software algorithm directly and were averaged as well. The values shown represent mean values from three Laboratories. The Limit of detection (LOD) was estimated at the lowest dilution of the template DNA where all experiments resulted in an amplification signal (0.1% equal to 0.1 ng/assay DNA, photospectrometrically determined)

Table 5 Recipe of burgers to be used as calibration material

Recipe of reference burger	10%	3.2%	1%	0.32%	0.1%
Minced meat	10.5 g	13.6 g	14.55 g	14.86 g	14.95 g
<i>Tenebrio molitor</i> dried	1.5 g	0.47 g	0.15 g	0.047 g	0.01 g
<i>Locusta migratoria</i>	0.015 g	0.047 g	0.15 g	0.47 g	1.5 g
<i>Achaeta domestica</i>	0.15 g	0.47 g	1.5 g	0.015 g	0.047 g

The insects were dried and milled to a fine powder. By adding of water a mixture with minced beef and knead to homogeneity. The burgers were baked for 10 min without oil. After that the burger was milled with liquid nitrogen for homogenization

Table 4 Recipe of cookies to be used as calibration material

Recipe of reference cookies	10%	3.2%	1%	0.32%	0.1%
Wheat	10.5 g	13.6 g	14.55	14.86	14.95
<i>Tenebrio molitor</i> dried	1.5 g	0.47 g	0.15	0.047	0.015
<i>Locusta migratoria</i>	1.5 g	0.47 g	0.15	0.047	0.015
<i>Achaeta domestica</i>	1.5 g	0.47 g	0.15	0.047	0.015
Water	9 ml	9 ml	9 ml	10 ml	12 ml

The insects were dried and milled to a fine powder. By adding of water a dough was made and knead to homogeneity. The dough was baked for 25 min at 180 °C. After that the cookie was milled for further homogenization

Table 6 Results of the calibration cookies (for insect unspecific) and burger for the specific systems

AllInsect using reference cookies	Insect unspecific	<i>Acaeta domestica</i>	<i>Tenebrio molitor</i>	<i>Locusta migratoria</i>
Precision $\pm$ % (RSD)	8	29	11	13
Accuracy $\pm$ %	13	19	22	22
Measurement uncertainty $\pm$ %	15	35	25	26

The DNA was isolated and used as template in the AllInsect PCR. Here the amplification characteristics are compiled. The template DNA was always diluted 1:100 without spectrophotometrically determination of the DNA concentration after DNA isolation

Achaeta domestica

Primer/probe	Final conc. μ M	Sequence	Amplicon	Source/Gen-Bank acc.no./labelling
Ache4.1F	0.8	GGT TAT ACC AAT TAT AAT TGG TGG	125 bp	This work
Ache4.1R	0.8	GGG TTA GTG AGG GTG GTA AAA GTC		
Ache2Joe	0.08	GGT GCA CCC GAT ATA GCC TTT CCT CG		Joe/BHQ1

Location in the genome of KR919588 Cytochrome oxidase I cytochrome oxidase I, position 159–284

121 atcgtaactgcacatgcatttgcataatttttttcatt
gggtataccaattataattgggtgg attcggaaattgattagtagccccaat-
atta ggtgcacccgatagccttctcga aataaacaatataagatttt
gactttaccaccctcactaacc ttttataaccagaaga 301

Table 7 Results of a market survey

Products declared insect ingredients	Insect unspecific	<i>Acheta domesticus</i>	<i>Tenebrio molitor</i>	<i>Locusta migratoria</i>
Halva, speciality of sesam/honey	neg	neg	neg	neg
Potatoesoup	neg	neg	neg	neg
Mohini apero snack	neg	neg	neg	neg
St John's bread	neg	neg	neg	neg
Bulgur	neg	neg	neg	neg
Almonds crushed	neg	neg	neg	neg
Desert indian wheat	neg	neg	neg	neg
Grapeseedwheat	neg	neg	neg	neg
Quinoa	neg	neg	neg	neg
Maniokbread	neg	neg	neg	neg
Potatoeflakes	neg	neg	neg	neg
Soy hached	neg	neg	neg	neg
Chestnutwheat	neg	neg	neg	neg
Cocoswheat	neg	neg	neg	neg
Tortilla chips	neg	neg	neg	neg
Maniokwheat	neg	neg	neg	neg
Wheat snack	neg	neg	neg	neg
Cracker with thyme	neg	neg	neg	neg
Potato chips	neg	neg	neg	neg
Fries with ketchup	neg	neg	neg	neg
Bacon snack	neg	neg	neg	neg
Mexican salsa spirals	neg	neg	neg	neg
Nacho cheese	neg	neg	neg	neg
Multigrain snack paprika	neg	neg	neg	neg
Snacks sour cream and onion	neg	neg	neg	neg
Flips	neg	neg	neg	neg
Mixed meat with noodles	neg	neg	neg	neg
Nasi goreng with chicken	(positive)	neg	neg	neg
Mah mee, chef select	(positive)	neg	neg	neg
Chicken sweet and sour, chef select	(positive)	neg	neg	neg
Spaghetti bolognese	neg	neg	neg	neg
Hachis parmentier	neg	neg	neg	neg
Poulet madras	neg	neg	neg	neg
Chili con carne	neg	neg	neg	neg
Ravioli napoli	neg	neg	neg	neg
Classic lasagne bolognese	(positive)	neg	neg	neg
Riz Casimir (mix rice, sauce and chicken)	(positive)	neg	neg	neg
Minced meat with pasta	(positive)	neg	neg	neg
Chicken satay with vegetables	(positive)	neg	neg	neg
Hached meat with pasta	neg	neg	neg	neg
Maize wheat	neg	neg	(positive)	neg
Pasta	neg	(positive)	(positive)	neg
37 rice and rice products	neg	neg	neg	neg
7 rice and rice products	neg	neg	(positive)	neg
60 dried herbs and spycies mix	neg	neg	neg	neg

Bold indicates primer sites

We analysed few products from the market for insect residues by AllInsect. Seven samples contained traces of unknown insect DNA. Eight other samples traces of DNA of *Acheta domesticus* and *Tenebrio molitor* were detectable but not quantifiable

Tenebrio molitor

Primer/ probe	Final conc. μ M	Sequence	Amplicon	Source/Gen- Bank acc.no./ labelling
Tene2F	0.6	CCA TGA GTA CGA ATA AGA GAA ACC	156 bp	This work
Tene2R	0.6	GCT TGA ATT TGT TGT TTT ATC TG		
TeneRox	0.08	AAT AGA TAG ACC AAG AAC GCC TTC ACA		Rox/BHQ2

Location in the genome of KP994554 NADH dehydrogenase subunit 4L, position 9477–9533

9361 ttgaaataa tcgttt ccatgagtacgaataagagaaacc aa
aatagatagaccaagaacgccttcaca aactctaaaag ttaaaaaat tata-
gaaaaa aaataatctc ttcccaaata agataaataa ataaaaagaa cataataaa
cagataaaacaacaaattcaagc cttaaaaag 9541

Locusta migratoria

Primer/ probe	Final conc. μ M	Sequence	Amplicon	Source/GenBank acc.no./labelling
Locu4.2F	0.6	CTG TAT TTT ATA TTC GGG GCA TG	120 bp	This work
Locu4.4R	0.6	GAT TAC ATT ATA TAC TTG ATC ATC G		
Locu3Cy5	0.08	TTG TTC CTG GTT GAC CTA ATT CAG CTC GAA		Cy5/BHQ2

Location in the genome of KM362655 cytochrome oxidase I, position 5-124

1 taca ctgtattttatattcggggcatg agctggaatagtaggaacat-
caataagaataattattcgagct gaattaggtcaaccaggaacaa taattaa
cgaatgatcaagatatataatgtaac attacagcacacgcat 141

References

- Kambhampati S, Smith PT (1995) PCR primers for the amplification of four insect mitochondrial gene fragments. *Insect Mol Biol* 4(4):233–236
- Ji Y-J, Zhang D-X, He L-J (2003) Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates. *Mol Ecol Notes* 3:581–585
- Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Vogler AP (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Syst Biol* 55(4):595–609
- Cognato AI (2006) Standard percent DNA sequence difference for insects does not predict species boundaries. *J Econ Entomol* 99(4):1037–1045
- Caterino MS, Cho S, Sperling FAH (2000) The current state of insect molecular systematics: a thriving tower of babel. *Annu Rev Entomol* 45:1–54
- Hebert PD, Cywinska A, Ball SL (2003) Biological identifications through DNA barcodes. *Proc R Soc Lond B Biol Sci*, 270(1512), 313–321
- Hebert PD, Gregory TR (2005) The promise of DNA barcoding for taxonomy. *Syst Biol* 54(5):852–859
- Park DS, Suh SJ, Hebert PDN, Oh HW, Hong KJ (2011) DNA barcodes for two scale insect families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae). *Bull Entomol Res* 101:429–434
- Van Hiel MB, Van Wielendaele P, Temmerman L, Van Soest S, Vuerinckx K, Huybrechts R, Simonet G (2009) Identification and validation of housekeeping genes in brains of the desert locust *Schistocerca gregaria* under different developmental conditions. *BMC Mol Biol* 10(1):56
- Xue JL, Salem TZ, Turney CM, Cheng XW (2010) Strategy of the use of 28S rRNA as a housekeeping gene in real-time quantitative PCR analysis of gene transcription in insect cells infected by viruses. *J Virol Methods* 163(2):210–215
- Majerowicz D, Alves-Bezerra M, Logullo R, Fonseca-de-Souza AL, Meyer-Fernandes JR, Braz GRC, Gondim KC (2011) Looking for reference genes for real-time quantitative PCR experiments in *Rhodnius prolixus* (Hemiptera: Reduviidae). *Insect Mol Biol* 20(6):713–722
- Teng X, Zhang Z, He G, Yang L, Li F (2012) Validation of reference genes for quantitative expression analysis by real-time RT-PCR in four lepidopteran insects. *J Insect Sci* 12(60):1–17
- Reim T, Thamm M, Rolke D, Blenau W, Scheiner R (2013) Suitability of three common reference genes for quantitative real-time PCR in honey bees. *Apidologie* 44(3):342–350
- Yang C, Pan H, Liu Y, Zhou X (2014) Selection of reference genes for expression analysis using quantitative real-time PCR in the pea aphid, *Acyrtosiphon pisum* (Harris)(Hemiptera, Aphididae). *PloS One*, 9(11), e110454
- Zhou X, Li Y, Liu S, Yang Q, Su X, Zhou L, Huang Q (2013) Ultra-deep sequencing enables high-fidelity recovery of biodiversity for bulk arthropod samples without PCR amplification. *Gigascience* 2(1):4
- Morf NV, Wood KL, Köppel R, Felderer N, Daniels M, Tenger B, Kratzer A (2013) A multiplex PCR method to identify bushmeat species in wildlife; forensics *Forensic Sci Int Genet Suppl Ser*, 4, 1, e202–e203
- Herbert PD, Cywinska A, Ball SL, de Waard JR (2003) Biological identifications through DNA barcodes *Proc Biol Sci* 270 (1512) 313–321

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