Hochschule für Angewandte Wissenschaften Hamburg

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Development of a real time PCR-based method for the detection of the two spotted cricket *Gryllus bimaculatus* in food

Bachelorarbeit

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[redacted]

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List of abbreviations

16S: 16S ribosomal RNA

18S: 18S ribosomal RNA

28S: 28S ribosomal RNA

bp: base pair

BHQ-1: Black Hole Quencher 1

BOLD: Barcode Of Life Databank

COI: Cytochrome c oxidase subunit I

C_q: Quantification cycle

CTAB: Cetyltrimethylammonium bromide

Cytb: Cytochrome b

DBPCFC: Double-Blind Placebo Controlled Oral Food Challenge

DIAAS: Dispensable Amino Acid Score

dNTPs: Deoxynucleotide triphosphate

dsDNA: double stranded DNA

EC: European Comission

EDTA: Ethylenediaminetetraacetic acid

EFSA: European Food Safety Association

FAO: Food and Agriculture Organization of the United Nations

FAM: Carboxyfluorescein

GHG: Greenhouse gas

IgE: Immunoglobulin E

IPIFF: International Platform of Insects for Food and Feed

LOD: Limit of detection

MM: Master mix

NCBI: National Center of Biotechnology Information

ND1: NADH-ubiquinone oxidoreductase chain 1

NGS: Next generation sequencing

NRL-AP: National Reference Laboratory for Animal protein in Feed

NTC: No template control

PCR: Polymerase Chain Reaction

PDCAAS: Protein Digestibility Corrected Amino Acid Score

PVPP: Polyvinylpolypyrrolidone

qPCR: quantitative PCR (Real time PCR)

RT: Room temperature

TAE: Tris-acetate-EDTA

TBE: Tris-borate-EDTA

Abstract

During the past decades insects have attracted much attention as an alternative food source. High nutritional value and a small carbon food print, as well as the ability of easily being farmed in a high scale potentially make them one of the solutions to the challenges posed by a rapidly growing population. Even in western societies, where "entomophagy", the eating of insects, does not have much tradition and is mostly refused, more and more insect derived foods are entering the market. Such as for other novel foods, edible insects come with risks for the consumer, due to the potential presence of chemical and biological hazards and the possibility of allergic reactions. The two spotted cricket *Gryllus bimaculatus* is a species not permitted in food or feedstuff for livestock, but it is anyway farmed as food for pets and as fish bait. Its presence on the market could derive into a misuse of this insect and of its products thereof, potentially leading to a food fraud. Furthermore, contaminations between allowed and prohibited species could occur in farms where different kinds of crickets are kept. Hence, a reliable method for the detection of the species not yet allowed is needed to ensure consumers safety, which was the aim of this bachelor thesis. For the development of the system a real time PCR-based approach was chosen. After *in-silico* analyses for the detection of the most suitable target genes, several sets of primers were ordered and tested using endpoint PCR followed by gel electrophoresis. Afterwards, the primers were tested in a real time PCR assay employing SYBR® green, and for the best performing set a TaqMan™ probe was developed. Several DNA samples isolated from different species were tested to ensure specificity, among which insects, crustaceans, mollusks, fishes, land animals and plants. Additionally, sensitivity and efficiency of the method were determined, and the limit of detection was derived. Finally, the practical applicability was verified on a small selection of foods. The system generally produced satisfying results, but further experiments must be made to complete the validation.

1 Introduction

The practice of eating insects is known as "entomophagy" and around 2000 species are recorded to be part of the human diet (Jongema 2017). Entomophagy has a long history and is recorded in Mexican codices, Egyptian papyrus, and Chinese annals (Costa-Neto and Dunkel 2016), but it is still quite unusual in other parts of the world like the EU (figure 1), where insects in meals are often triggering the feeling of disgust (van Huis et al. 2013) and are associated mostly with negative aspects, such as dirt, diseases, and fear of contamination (Deroy, Reade, and Spence 2015).

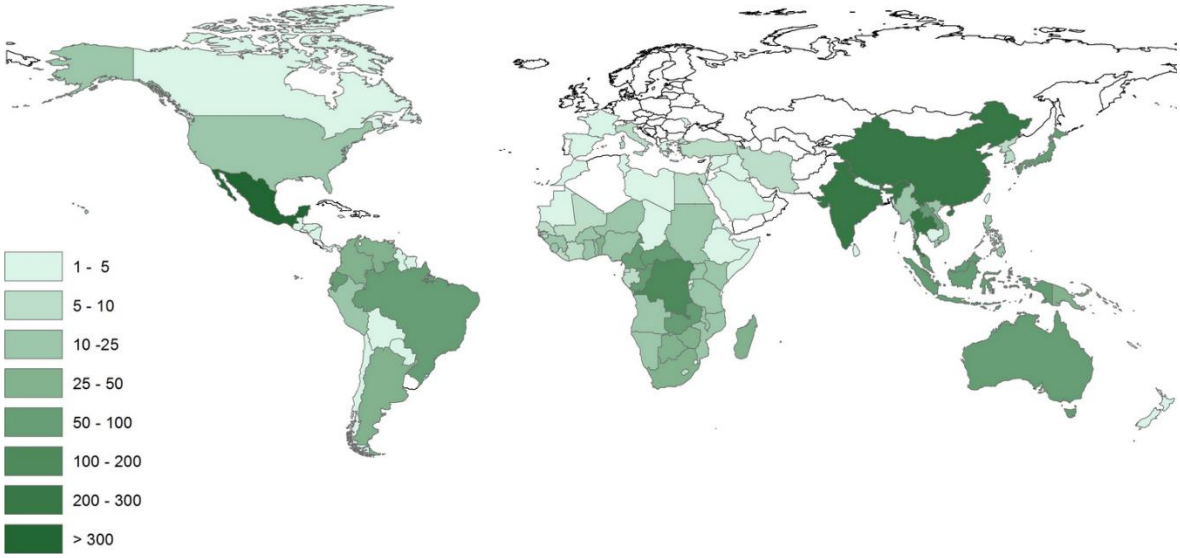

Figure 1 Numbers of recorded edible insect species in different countries
Source: (Jongema 2017)

However, the world population is increasing and is likely to reach the 9 billion-mark in 2050 (United Nations 2019). Supplying this large number of people with food poses a great challenge and, as an alternative protein source, insects are getting more and more attention, especially since the Food and Agriculture Organization of the United Nations (FAO) proposed them as one of the solutions to this problem (van Huis et al. 2013). Today, several products like burger patties, pasta, protein bars and candies with different proportions of insects can be found in grocery stores and especially on the online market. But the consumption of insects also presents some risks, due to the potential presence of chemical or biological hazards, or of allergenic proteins (EFSA 2015), which can especially be a danger to people already sensitized to other arthropods, like dust mites or crustaceans (Broekman et al. 2016; Kamemura et al. 2019; Verhoeckx et al. 2014).

Orthopteran species like crickets, grasshoppers and locusts are ranging among the most consumed insects in the world, surpassed only by the coleopterans, lepidopterans and hymenopterans (Jongema 2017). The *Gryllus bimaculatus* is a species belonging to the *Gryllidae* family, which is part of the *Orthoptera* order. The species is distributed especially throughout Africa and as far as eastern Asia (Bellmann et al. 2019), where it is a commonly consumed species usually collected

from the wild, but also farmed for profit (van Huis et al. 2013). In Europe instead, it is widespread from the Iberian Peninsula through Italy to Greece, lives in the plains and mostly near the coast (Bellmann et al. 2019). Here this insect is often farmed as feed for terrarium pets or as fish bait (van Huis et al. 2013). However, it is not allowed as food or feed for livestock, which creates a precarious market situation that makes the possibility of accidental contamination or even fraud realistic. Therefore, it is necessary to have a reliable method for its detection in food and feeds.

2 Theoretical background

2.1 Insects as food

2.1.1 Opportunities

Polluted and overfished oceans result in a continuing depletion of the world's fish stock (Global Ocean Comission 2015). Doubling the food production in the past decades led to intensification of agriculture, which degraded and exhausted soil and land (Gomiero 2016). Despite the continuous increase in food production, an increasingly large segment of the population, especially in low and middle-income countries, is undernourished due to protein and energy deficiency in their diet, resulting in 462 million adults being underweight and 149 million or 45 million children, respectively, being stunted (too short for age) or wasted (too thin for height). Conversely, in other parts of the world 1.90 billion adults and 38.9 million children are overweight or obese (WHO 2021). Between 2010 and 2050 the demand of animal protein like milk and meat is expected to increase by 53 % or 72 % respectively (FAO 2011), which will put even more pressure on oceans and soils if no alternative protein sources will be efficiently used. Furthermore, according to the United Nations World Water Development Report, almost half of the world's population (47 %) suffers freshwater scarcity for at least one month of the year, and by 2050 this number is expected to increase to 57 % (United Nations 2018).

The protein content of insects, assessed between 20 and 76 %, depending on the type and on the developing stage of the insect (Kouřimská and Adámková 2016), is high, although the nitrogen in the chitin armor of the insects can lead to an overestimation when this is measured using the Kjeldahl-method (Jonas-Levi and Martinez 2017). The quality of these proteins is generally high, with suitable amounts of essential amino acids, which however differ by species and rearing conditions (Stull 2021). Proteins can be easily digested, as reported in the case of the Eri silkworm (*Samia ricinii*) and the large black chafer (*Holotrichia parallela*), where a protein digestibility corrected amino acid score (PDCAAS) of 0.86 and 0.89 respectively has been determined (Longvah, Mangthya, and Ramulu 2011; Yang et al. 2014), which is a better score than the one achieved by most plants, such as oats (0.57) and peas (0.67), and almost at the same level as beef (0.92) (van Vliet, Burd, and van Loon 2015). Even though the PDCAAS is no longer the standard for protein

quality evaluation and is nowadays replaced by the digestible indispensable amino acid score (DIAAS) (FAO 2013), the values are basically comparable. The fat content of insects can vary broadly between 2 and 50 % of the dry mass, but up to 70 % of the fatty acids in insects are polyunsaturated. Furthermore, a large variety of minerals (K, Na, Ca, Cu, Fe, Zn, Mn and P) as well as all vitamins can be present in reasonable amounts (Kouřimská and Adámková 2016). The bioavailability of iron from insects is comparable to that of beef or, in case of the buffalo worm *Alphitobius diaperinus*, even higher (Latunde-Dada, Yang, and Vera Aviles 2016).

High meat consumption, particularly red meat and highly processed meat is connected to an increased risk of total mortality, cardiovascular diseases, diabetes mellitus type 2 and colorectal cancer (Richi et al. 2015). Although the nutrient composition of insects is highly diverse and it cannot be claimed that their consumption is generally heathier than the consumption of meat, in 2016 a study by Payne and collaborators indicated that some species of cricket, palm weevil and mealworm have proven better health scores than beef or chicken, while none of the insects included in the study had worse health scores than meat. Hence, one conclusion of the study is that entomophagy, as an alternative to meat consumption, could exacerbate diet-related public health problems related to overnutrition (Payne et al. 2016).

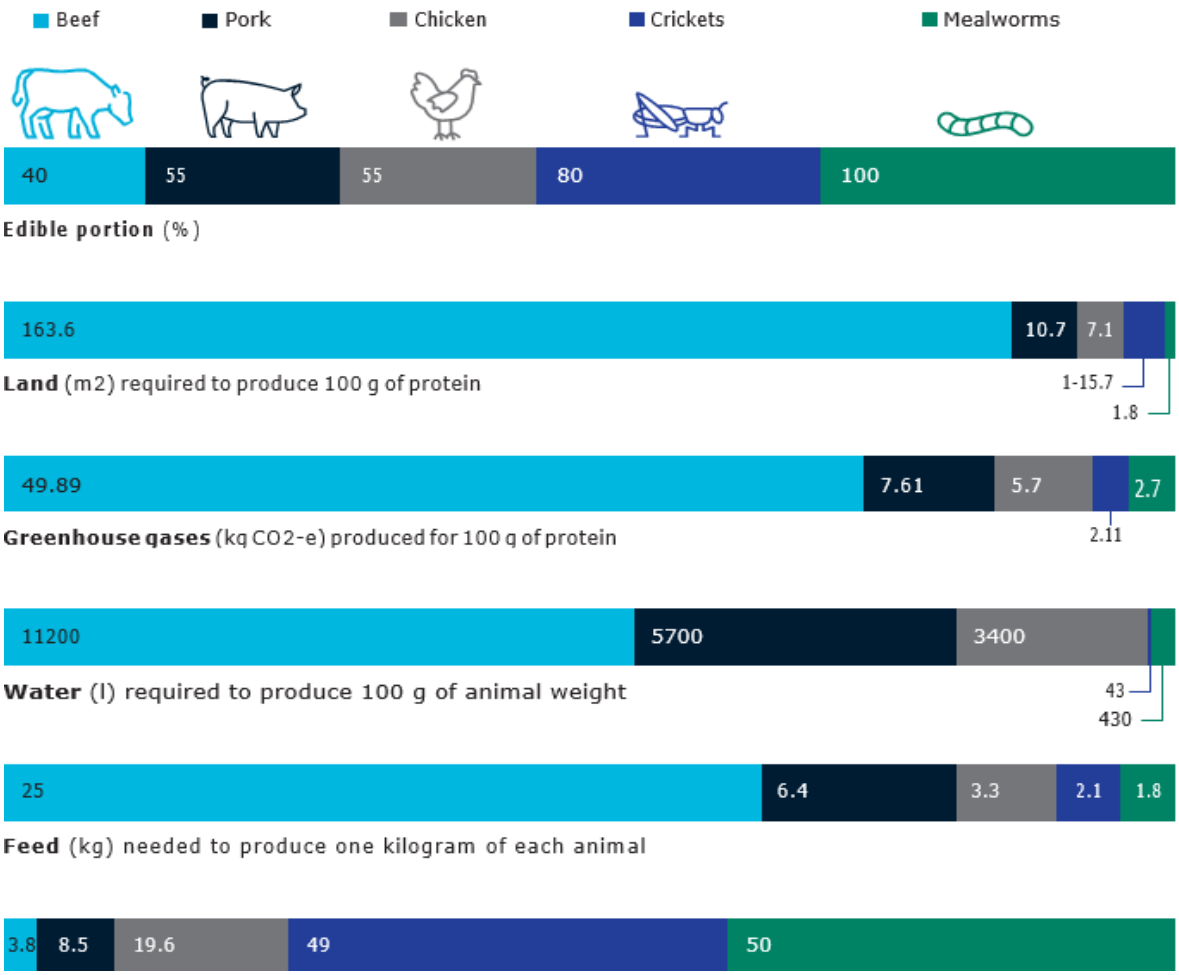

Figure 2 Environmental footprint of crickets and mealworm compared to conventional farm animals
Source: (Ponce-Reyes and Lessard 2021)

With emissions at 7.1 gigatons CO_2-equivalent each year, commonly farmed livestock is a significant contributor to climate change, representing 14.5 % of human induced greenhouse gas (GHG) emissions mainly produced during the making of beef (41 % of the sector's emission) and cattle milk (20 %), while pigs and poultry, for meat and egg production, contribute for 9 % and 8 % respectively (Gerber et al. 2013). As shown in figure 2, most of the insect body is edible, differently from other livestock, where only 40-55 % can be consumed (Alexander et al. 2016; Halloran et al. 2016). Furthermore, the figure shows that insects outperform other livestock in terms of GHG emissions (Nadeau et al. 2015; Poore and Nemecek 2018), water requirement (Halloran et al. 2017; Miglietta et al. 2015; Poore and Nemecek 2018), feed requirement (Alexander et al. 2016; Bawa et al. 2021) and land use (Nadeau et al. 2015; Oonincx and de Boer 2012; Poore and Nemecek 2018; Suckling et al. 2020). They could even be farmed vertically in skyscrapers, hence in densely populated areas (Dossey, Tatum, and McGill 2016). The protein-feed conversion efficiency of 2 g of feed per g of protein gain is much better than that of cattle, pig and chicken (Alexander et al. 2016; Halloran et al. 2016). One reason is that insects are poikilotherm (cold blooded), which also means that they demand constant warm temperatures (Dossey et al. 2016). This implies that farming insects in cold countries, at least in the case of the mealworms *Tenebrio molitor* and *Zophobas morio*, requires more energy than farming chickens or dairy cows, but about the same as farming pigs and still less than farming beef cattle (figure 3) (Oonincx and de Boer 2012; de Vries and de Boer 2010). Other farming methods are investigated, for example using the metabolic heat of larger larvae to warm the smaller ones, which would reduce the need of energy significantly (Dossey et al. 2016). However, current insect farming is in devolopment and undergoes quite a transition. In the past, insects were mainly farmed for fish bait and pet food, and despite the fact that industrial insect production is increasing, farms are still small and lack technology and innovation, making them not much different from household processes, only on a larger scale (Dossey et al. 2016). Thus, more research and development could make the farming of insects even more efficient. Another advantage over the farming of larger animals is the rapid growth of insects due to the short life span and high fecundity. House crickets (*Acheta domesticus*) for example, can lay 1200-1500 eggs in a period of 3-4 weeks (Dossey et al. 2016).

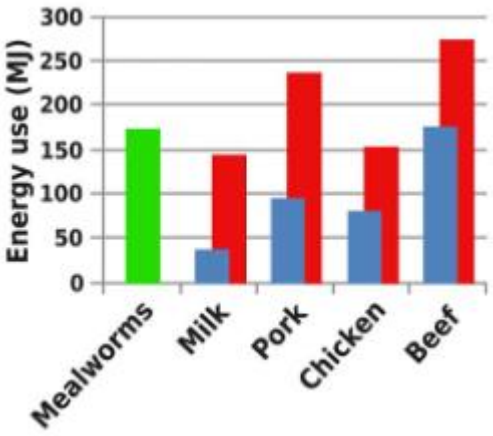

Figure 3 Energy use for the production of mealworms versus conventional farm animals. Blue= minimum, red= maximum
Source: (Oonincx and de Boer 2012)

2.1.2 Risks

In contrast to the variety of opportunities offered by entomophagy, there are several risks that should not be overlooked. A scientific opinion published by the European Food Safety Authority (EFSA) in 2015 assessed that the presence of biological, chemical and environmental contaminants in foods derived from farmed insects depends on the production methods, the substrate used for feeding, the stage of harvest, the species chosen and the methods used for further processing the insects, and that all these factors can potentially lead to a risk for the consumer (EFSA 2015).

Insects can have high microbial loads with total aerobic counts that might reach up to $8.9x10^7$ cfu/g in raw crickets (Megido et al. 2017), and they are generally eaten without removing the gut, where 10^6 to 10^{12} bacteria per ml of gut content were found in different species (Cazemier et al. 1997). Still, the risks posed by the intrinsic microbial flora of the insects is relatively low. They are genetically very different from higher vertebrates, and their microbiota lacks cross reactivity between mammals and insects (Ferri et al. 2019). Important foodborne bacteria have not (for example *Listeria monocytogenes*) or only occasionally (*Salmonella* spp. and *Escherichia coli*) been detected (Dries et al. 2018; Grabowski and Klein 2016; Megido et al. 2017; Osimani et al. 2018). On the other hand, among farmed insects the external microbial flora, namely the microbiota coming from substrate, feedstuff and litter, can be a hazard for consumers. When industrially farmed insects enter the same food production processes as other food commodities, they are confronted with the same bacteria, including pathogens like *Cronobacter* spp. or *Pseudomonas* spp. (Ferri et al. 2019). An indicator of inadequate hygiene during processing can be the presence of *Staphylococcus aureus* (GHAFIR et al. 2008; Jacxsens et al. 2009). Boiling raw insects for at least 10 minutes, or alternatively acidifying them at pH 4.5, will reduce the microbial load to an acceptable value.

Regarding fungi, the situation is similar to that of bacteria. Many fungi can be found on the surfaces and in the guts of insects, some of which can be entomopathogenic, but because of their species-specificity their safety record for vertebrates is very good (EFSA 2015). In cricket farms, however, the FAO reported that the presence of fungi increases, probably because of humidity and overcrowding (van Huis et al. 2013). Some of these fungal species can produce toxins dangerous for human health. These mycotoxins can be specifically dangerous due to their heat-resistance (Magan and Olsen 2004).

Entomopathogenic viruses can lead to mortality and colony collapses among insects (Belluco et al. 2013), but as for bacteria and fungi, their species-specificity makes them generally not dangerous to humans or other vertebrates (Fernandez-Cassi et al. 2019). However, insects can passively or mechanically carry viruses pathogenic to humans and farm animals (Wanaratana et al. 2013). In its scientific opinion about the safety of insects, the EFSA panel mentions that viruses typically

adapted to vertebrates are able to survive in the substrates used for insect farming, thus posing a potential risk to humans. The Authors also mention that insects could be mechanical vectors for prions (EFSA 2015), which are not able to replicate in insects as they do in mammals and hence cannot be biologically carried or amplified by them (Ferri et al. 2019).

In terms of chemical toxins, particularly the possibility of insects to accumulate or bio-conjugate environmental substances like heavy metals, pesticides, dioxins and mycotoxins is problematic. The accumulation of cadmium for example was investigated in several cricket species (Bednarska et al. 2015; Devkota and Schmidt 2000; Vijver et al. 2003; Zhang et al. 2009). Their presence depends on the species and the growth state, with larvae showing higher concentrations than adults. Insect species with short live span, such as crickets, are less likely to accumulate high amounts of toxic substances. Some insects also produce chemical toxins and venoms or accumulate toxic substances "purposely" as a defense mechanism (Fernandez-Cassi et al. 2019). Like for traditional farmed livestock, an extended use of antibiotics in insect farming could lead to antibiotic-resistant bacteria populations, which would be a risk for humans as well (Ferri et al. 2019).

In general, it is advisable to heat insects before eating them to inactivate microorganisms and thermolabile toxins. Ignoring this can have severe consequences. In Western Africa for example, a beri-beri like vitamin deficiency occurred because large quantities of a thiaminase containing caterpillars (*Anaphe venata)* were eaten raw, against traditional rules (Adamolekun and Ibikunle 1994).

The presence of biological and chemical contaminations can be avoided by following the guidelines suggested by the International Platform of Insects for Food and Feed (IPIFF) on good hygiene practices for producers of insects as food and feed (IPIFF, 2022). Conversely, when insects are collected from the wild, unpredictable amounts of environmental contaminants, such as heavy metals and pesticides, can accumulate. This practice also has a negative impact on the biodiversity (van Huis et al. 2013).

In addition to the microbiological and chemical hazards, the EFSA opinion also mentioned the allergic potential of insects as a risk of entomophagy.

2.2 Food allergies

Allergies are pathogenic hypersensitivities to otherwise harmless substances foreign to the body. Food allergies are immunologically mediated, individually occurring, abnormal reactions to food, caused by allergens, which are proteins from animals or plants. They are increasing in prevalence in western industrialized countries (Kasper 2020).

Food allergies predominately fall under the immunoglobulin E (IgE)-mediated type I allergies. After a first contact with the allergen, B-lymphocytes are transformed into plasma cells, which form IgE-antibodies against the allergen. These antibodies bind to mast cells, which primary does not lead to an activation of the mast cells. That only happens after another contact with the allergen. The antibodies capture the allergen and the activated mast cells degranulate, resulting in the release of inflammatory mediators such as histamine, serotonin, prostaglandins, and leukotrienes, which determine the clinical picture of the allergic reaction (Valenta et al. 2015), as shown in figure 4. The symptoms occur 10-30 minutes after the exposure and can be hives, diarrhea, abdominal pain, and bronchial asthma, or, in the worst-case, an anaphylactic shock that can lead to death (Kasper 2020).

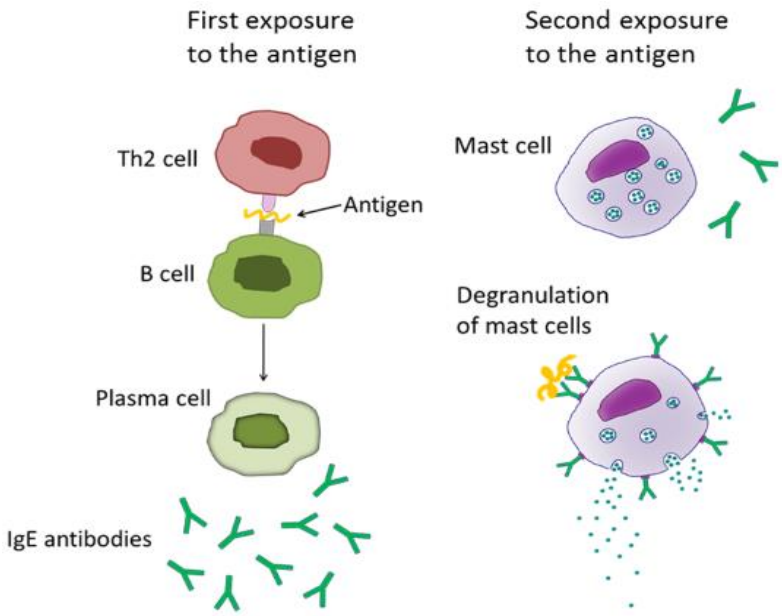

Figure 4 IgE mediated allergy
Source: (Linnéa Barman, 2015)

The gold standard for the diagnosis of a food allergy is a double-blind placebo controlled oral food challenge (DBPCFC). The patient ingests increasing doses of the concerned food while allergy related medications are discontinued. The method requires an experienced clinician who must be able to recognize and treat symptoms of anaphylaxis immediately. Much less stressful options for the patient are skin tests or serum specific IgE testing. But the results can neither rule out a food allergy nor differentiate between sensitization versus a true IgE-mediated allergy and have to be interpreted cautiously based on the clinical history (Anvari et al. 2019).

Cross reactivities occur when the immune system reacts to an allergen that has a similar structure to the allergen to which it is already sensitized (Bonds et al., 2008). In the case of insects, this means that people who are already allergic to crustaceans or house dust mites have a high chance to show

allergic symptoms when they eat insects (Kamemura et al. 2019; Verhoeckx et al. 2014). In a study by Broekman and colleagues (2016) 13 out of 15 patients allergic to shrimp displayed an allergic reaction following the consumption of mealworm in a DBPCFC.

2.2 Legal background

In the EU, edible insects are considered novel foods under the current EU Regulation 2015/2283 (EU 2015), as they were not traditionally eaten before 15th of May 1997, when the previous regulation No 258/97 came into force (EU 1997). The new regulation applied from 1st of January 2018. However, at that time some products were already on the market without being formally authorized by the EU. The reason was that the previous Novel Food Regulation did not mention insects specifically, and generally referred to ingredients "consisting of or isolated from plants and […] animals". After the company Entoma SAS brought the case against two French ministries in front of

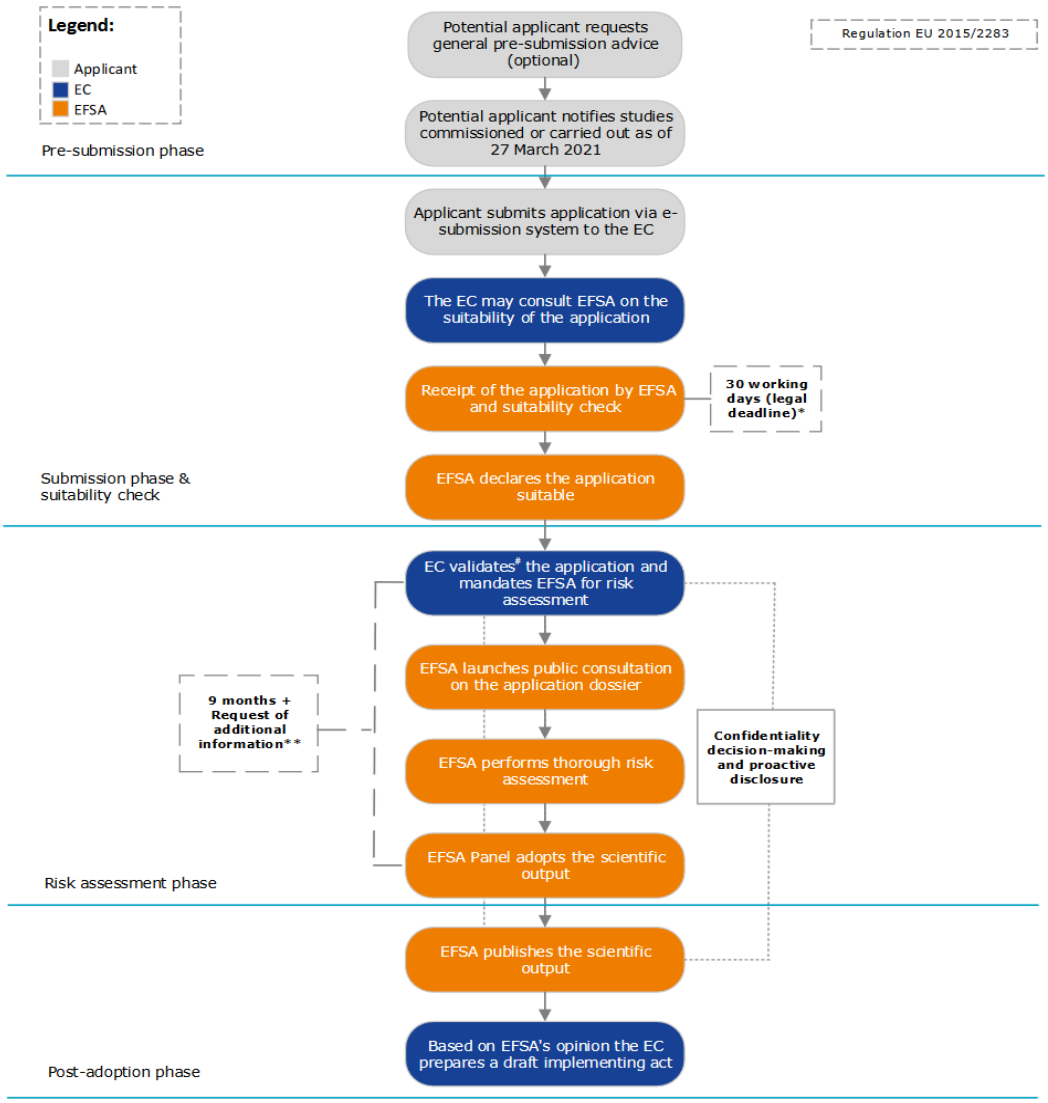

Figure 5 Application procedure for novel foods
Source: (EFSA 2021)

the Supreme Administrative Court of France, the European Court of Justice ruled that whole insects did not fall under the previous Novel Food Regulation (European Court of Justice (Third Chamber) 2020). Therefore, all insect-based foods placed on the market before 2018 were temporarily allowed, provided that a formal application as novel food was submitted to the EU. Thus, a very heterogenous situation arose, where the different EU member states had different interpretations of the law (Lähteenmäki-Uutela, Marimuthu, and Meijer 2021).

Today, there are two possible ways to place a novel food on the EU market. One way is to submit an application demonstrating the safety of the product to the European Commission (EC). The decision of the EC is based on the opinion of the EFSA, which evaluates data to assess the risk (figure 5). At the time this work was written, four applications for insects as novel foods was accepted by the EC: the dried larvae of *T. molitor* mealworm (Regulation 2021/882) (EU 2021a), the frozen, dried and powder forms of the locust *Locusta migratoria* (Regulation 2021/1975) (EU 2021b), the frozen, dried and powder forms of yellow mealworm (*T. molitor* larva) (Regulation 2022/169) (EU 2022a) and the frozen, dried and powder forms of the cricket *Acheta domesticus* (Regulation

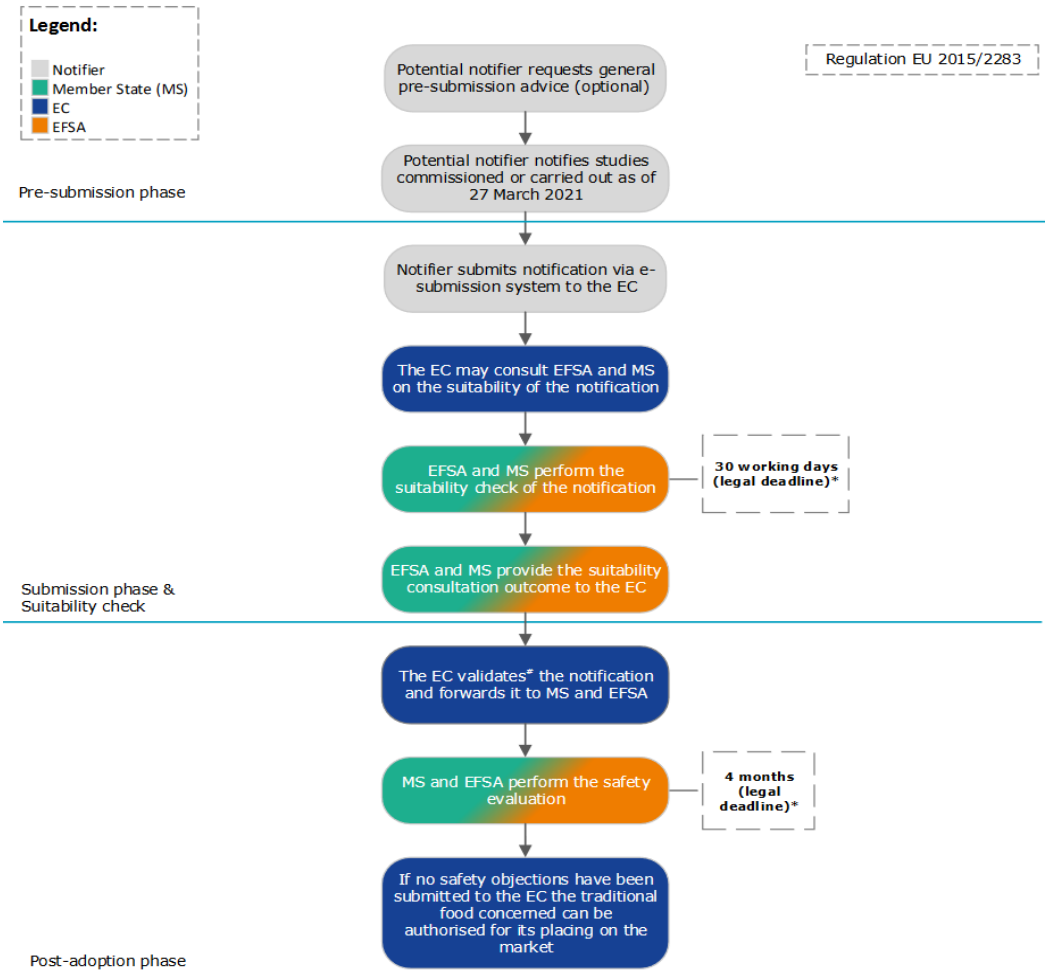

Figure 6 Notification procedure for traditional foods
Source: (EFSA 2021)

2022/188) (EU 2022b). Moreover, two scientific opinions for insects not yet authorized by the EC have been published by EFSA, one on the safety of the frozen lesser mealworm *Alphitobius diaperinus* (EFSA 2022a) and one of the partially defatted house cricket *Acheta domesticus* powder (EFSA 2022b). The following insect-based products are still under risk assessment:

- meal, refined fat and dried defatted powder of black soldier fly (*Hermetia illucens*)
- honeybee (*Apis mellifera*) male pupae
- protein powders of the lesser mealworm (*Alphitobius diaperinus*)
- UV-treated powder and protein-rich flour of yellow mealworm (*Tenebrio molitor*) larvae

Another application for the banded cricket *Gryllodes sigillatus* has been recently withdrawn by the applicant (EFSA, n.d.). Other insects may continue to be distributed until the EC makes its decision because they were legally marketed before 1st of January 2018, when the adopted version of the Novel Food Regulation entered into force, and an application or notification was submitted by January 2019 (Lähteenmäki-Uutela et al. 2021).

The second way to introduce an insect as food on the EU market is to file a notification for a traditional food according to article 14 of the Regulations 2015/2283, in case a distributer opts to place an insect on the market that has been traditionally eaten in a third country outside of the EU and has a history of safe use. This notification is then forwarded to the member state concerned and to EFSA for safety evaluation. If there are any safety concerns, the notified food will not be authorized (figure 6), and a new application may be submitted following the procedure of novel foods. If the notification gets accepted, it is only applicable on the traditional way of processing and serving that food. In the case of modifications, a new novel food application must be made.

It is still possible to order many kinds of edible insects from countries outside the EU on the internet, thus circumventing EU regulations.

As for the protection of the allergic consumers, so far insects do not fall under the EU Regulation No 1169/2011 (EU, 2011) on food labelling, which, among other things, includes the list of allergens to be mandatorily reported on the label. However, the recently issued regulations on *Tenebrio molitor* (Reg. 2021/882 and 2022/169), *Locusta migratoria* (Reg. 2022/1975) and *Acheta domesticus* (Reg. 2022/188) report additional specific labelling requirements addressed to consumers allergic to crustaceans.

2.3 Food fraud

In the annual report of 2020, the Agri-Food Fraud Network of the European Commission describes food frauds as "activities characterized by their intentional nature, including the aim to make an economic gain, in violation of legal rules at the expense of the immediate or the final customer" (The Agri Food Fraud Network, 2020). A food fraud can be identified with the help of four criteria, as shown in figure 7.

Figure 7 Food fraud criteria
Source: (The Agri Food Fraud Network 2020)

If a case matches all four criteria, a suspicion of fraud could be considered. The referred "EU rules" are codified in the EU agri-food chain legislation (Article 1(2) of Regulation (EU) No 2017/625). However, it is challenging to recognize fraudulent activity due to the many different forms of food fraud (figure 8) but also because it is hard to distinguish deliberate from accidental or unintentional acts (EC n.d.-a).

Figure 8 Different types of food fraud
Source: (EC n.d.-a)

There are different techniques being used for the detection of food frauds. In a study conducted by a Korean team led by Hong, it was found that the largest proportion of food frauds (20.6 %) were detected using mass spectrometry (MS)-based methods (according to the number of uses in literature between 2005 and 2015), followed by polymerase chain reaction (PCR)- (18.5 %) and liquid chromatography (LC)-based methods (11.6 %) (Hong et al. 2017). An overview of which technique is used for which kind of products can be obtained from figure 9.

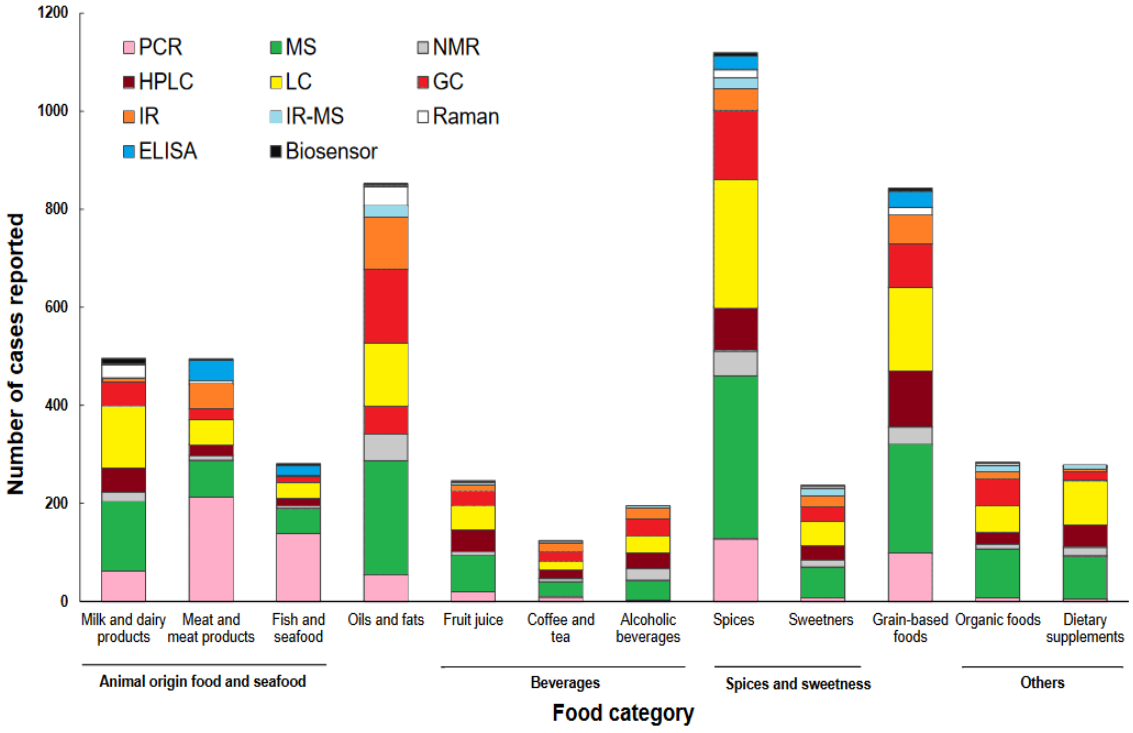

Figure 9 Major technologies for the detection of food fraud, as reported in the literature from 2005-2015. HPLC= high-performance liquid chromatography, IR= infrared, ELISA= enzyme-linked immunosorbent assay, Isotoperation mass spectrometry, NMR= nuclear magnetic resonance, GC= gas chromatography
Source: Based on (Hong et al. 2017)

Compared to mass spectrometric methods, PCR-based methods have the advantage of being less expensive regarding acquisition- and operating-costs and require less educated operators (Baltes and Kroh 2004).

A prominent case of food fraud in which a PCR method was employed was the "horsemeat scandal" in 2013, in which packaged beef products in several EU countries were found to contain undeclared horsemeat (EC n.d.-b).

2.4 DNA-extraction

2.4.1 CTAB-extraction

The first step to extract DNA is the lysis of the sample, which is performed by a combination of mechanical, thermal and chemical disruption. The extraction buffer, containing surfactants and chaotropic agents, is added at 65 °C. The buffer contains Ethylenediaminetetraacetic acid (EDTA), which inhibits the activity of DNases, and Cetyltrimethylammonium bromide (CTAB). In high ionic strength this cationic detergent builds complexes with proteins and most acidic polysaccharides (which are present in plants but also in the chitin armor of insects). Proteinase K is another important component in the process of extraction. In contrast to other proteinases, it is still active at 65 °C. Proteinase K degrades all the proteins, while RNase acts on the single stranded RNA. Polyvinylpolypyrrolidone (PVPP), a water-insoluble polymer, can be added to bind polyphenols and anthocyans co-eluting with DNA, which might hamper the polymerase during Polymerase Chain Reaction (PCR). The whole lysis procedure is carried out while vortexing the extraction tube several times, in order to facilitate the breakdown of the cells and the release of the cytosol (Reinard 2010).

After the separation from insoluble cell debris by a centrifugation step, chloroform is added to separate the lipids in the organic phase from the aqueous phase, which contains the nucleic acids. Following centrifugation the denatured proteins sink in the aqueous phase on the interphase with the organic phase, while the DNA remains dissolved (figure 10) (Reinard 2010).

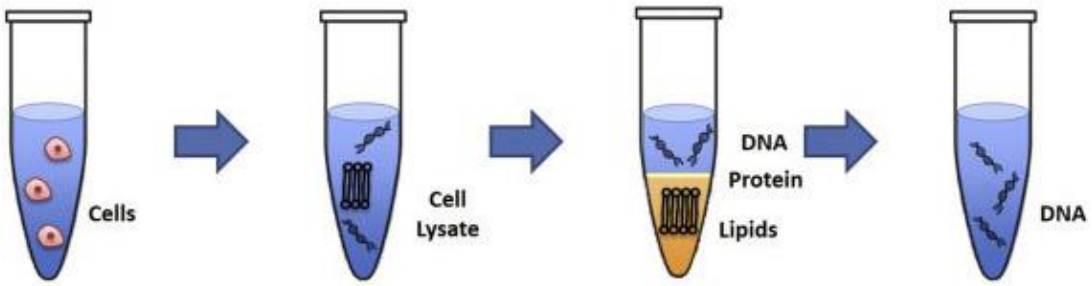

Figure 10 Major steps of DNA isolation, using chloroform
Source: (McKiernan and Danielson 2017)

The DNA precipitates with ethanol or isopropanol, which is also a step of purification since many unwanted substances stay in the aqueous phase. This is followed by a washing step with 70 % ethanol, after which the pellet is dried, so all ethanol is removed, and the precipitated DNA can be eluted again (Reinard 2010).

2.4.2 Extraction kits

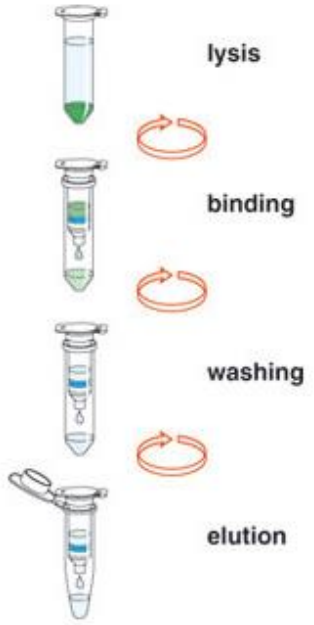

Figure 11 Procedure of NucleoSpin™ food kit
Source: (Bioké 2019)

There is a series of commercial extraction kits on the market for all kinds of different tasks, such as the purification of DNA from blood, food, plants and many more. These kits provide all the necessary components that are needed for the DNA-isolation. They have similar principles than other extraction-methods. The most significant difference is the way how DNA is separated from the rest. In most cases they make use of the ability of silica surfaces to bind nucleic acids. The silica appears in the form of columns, membranes, or silica-coated magnetic beads. High concentrations of chaotropic salts cause the binding of DNA to the silica surface by removing the hydration shell of the biomolecules. The DNA bound to the silica can be washed with ethanol and solutions with high salt concentration to remove proteins and short nucleic acids up to 70 base pair (bp). Eventually the DNA gets eluted from the silica with pure water or elution buffer (figure 11) (Reinard 2010).

2.5 Polymerase Chain Reaction

The PCR is a method developed in 1984 by Kary Mullis to amplify certain DNA section. It runs in special machines called thermocyclers in repeating cycles, which mainly consists of three steps that repeat themselves:

Denaturation:

Initially, double stranded DNA (dsDNA) is denatured at 95 °C, causing the two strands to separate. A complete denaturation of the DNA is crucial for the further success of the method. If the DNA does not denature completely, the annealing efficiency of the primers is reduced (Müller & Prange, 2016a).

Annealing:

Specific oligonucleotides (primers) anneal to their complementary regions on the two ends of the section that needs to be amplified. This happens by lowering the temperature to a certain degree at which this specific annealing can take place. If the temperature is too high the primers will not anneal. If it is too low the primers will also anneal in other parts of the DNA that are not 100% complementary, thus generating unspecific products (Müller and D. R. Prange 2016a).

Extension:

Afterwards, the enzyme polymerase elongates the primers complementary to the DNA strand, using free Deoxynucleotide Triphosphates (dNTPs). Most of the DNA polymerases, including the human ones, work naturally at physiological temperatures (37°C in our case), and get degraded at the high temperatures used by the thermocycler. To prevent this, most PCRs employ the polymerase of a thermostable bacteria strain called *Thermus aquaticus*, known as *Taq*-polymerase, which works well around 72 °C and can resist 95°C without being inactivated (Müller and D. R. Prange 2016a). Heating again to 95°C starts the next cycle. This procedure will be continued until enough DNA is reproduced. This is usually the case after 25-30 cycles in endpoint- or up to 45 cycles in a real time-PCR (qPCR) (Müller & Prange, 2016a). The process is visualized in figure 12. Another important component of the PCR is Magnesium Chloride. It raises the melting temperature of the

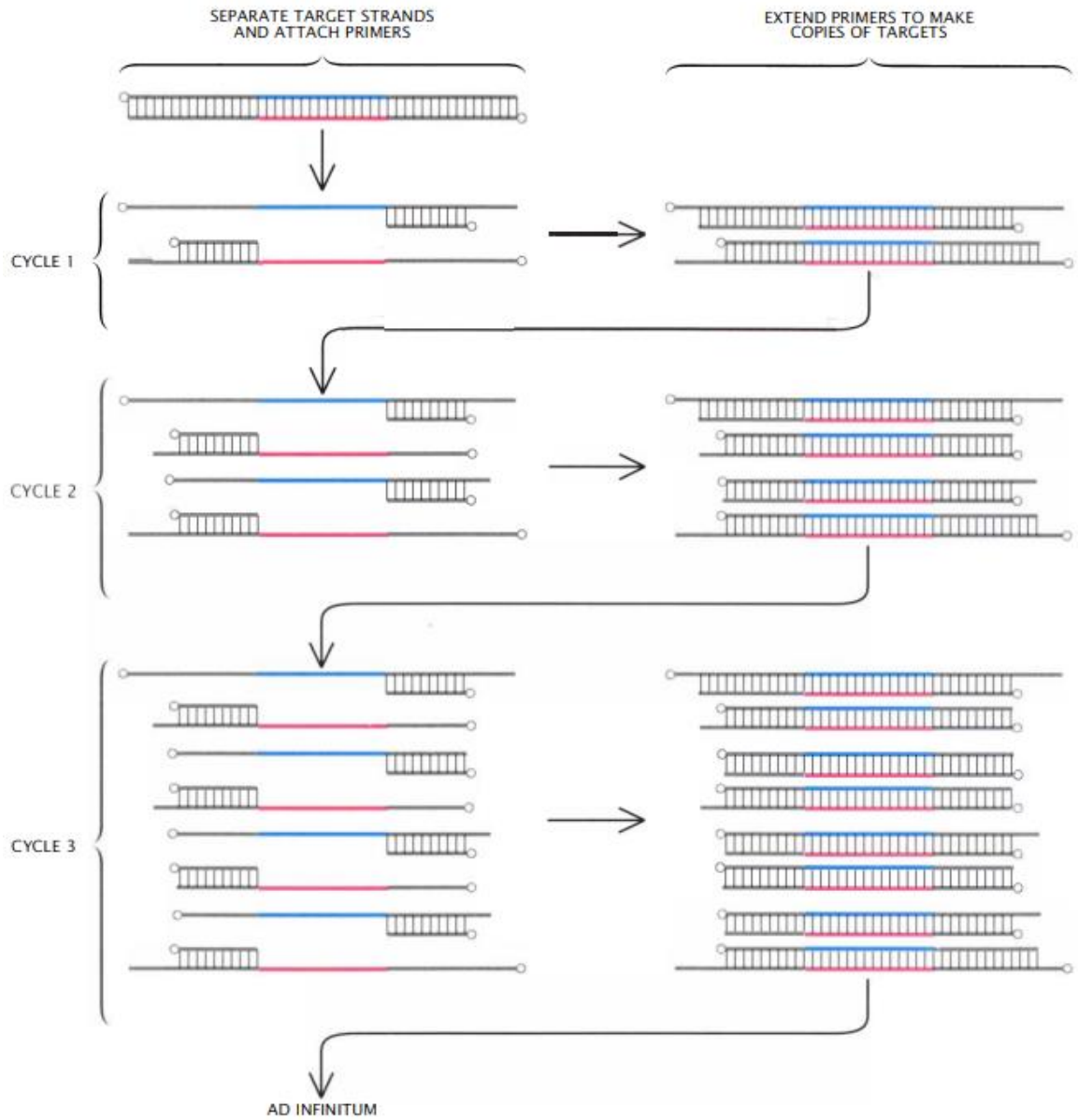

Figure 12 PCR-process
Source: (Mullis 1990)

dsDNA, and it builds complexes with the nucleotides which can be recognized by the polymerase (Müller & Prange, 2016a).

If the primer design is incorrect, it can lead to self-hybridization, which causes unwanted secondary structures (figure 13):

- Hairpin: caused by intra-primer homology, three or more bases are complementary to the other bases within the same primer.
- Self-dimerization: caused by inter-primer homology in same-sense primers, when forward or reverse primer have complementary sequences.
- Hetero-dimerization: caused by inter-primer homology in anti-sense primers, when forward and reverse primers are partially complementary (Eva Mészáros 2022).

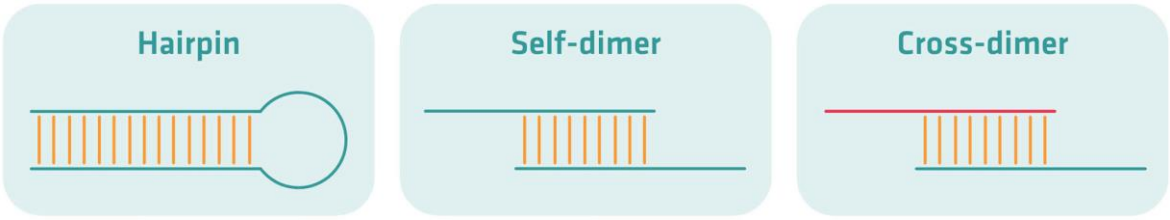

Figure 13 Types of secondary structures
Source: (Eva Mészáros 2022)

2.5.1 Endpoint PCR and agarose gel electrophoresis

In an endpoint PCR, the DNA is amplified in a thermocycler over a certain number of cycles and the resulting products can be further analyzed. The term endpoint comes from the fact that the reaction is only measured at the end, when all the amplicon has formed. The agarose gel electrophoresis is often used to verify the success of an endpoint PCR. The gels are prepared with 1-3% agarose, a sugar coming from red seaweed, and a saline buffer, usually Tris Acetate EDTA (TAE)- or Tris Borate EDTA (TBE). Amplicons are loaded in the resulting polymerized gel immersed in saline buffer inside an electrophoretic cell. The cell is then connected to a voltage source, and the DNA-molecules, that have a negative electric charge because of the phosphate backbone, start to move in the direction of the cathode. Since DNA molecules do not have distinct tertiary structure and since their weight is proportional to their length, their traveled distance on the gel correlates with their size. To estimate the size of the bands, a standard mixture of differently sized known DNA fragments, called ladder, is also loaded on the gel (Löffler 2008; Müller and D.-R. Prange 2016).

2.5.2 Real time PCR

In qPCR assay the amplification of the DNA can be monitored directly while the process is taking place (in real time). The technique enables the operator to estimate the starting amount of the template DNA in the sample. The reason is that the number of cycles required for amplification to become linear (figure 14) is inversely proportional to the initial amount of template. During the

ground phase the fluorescence emission does not rise above the background. Fluorescence starts slowly to increase significantly over the background during the exponential phase, which is followed by the log-linear phase, where the optimal amplification period is reached and after every cycle the PCR product is doubled under ideal conditions. This continues until the plateau stage is reached where the reaction components become limited (Wong and Medrano 2005).

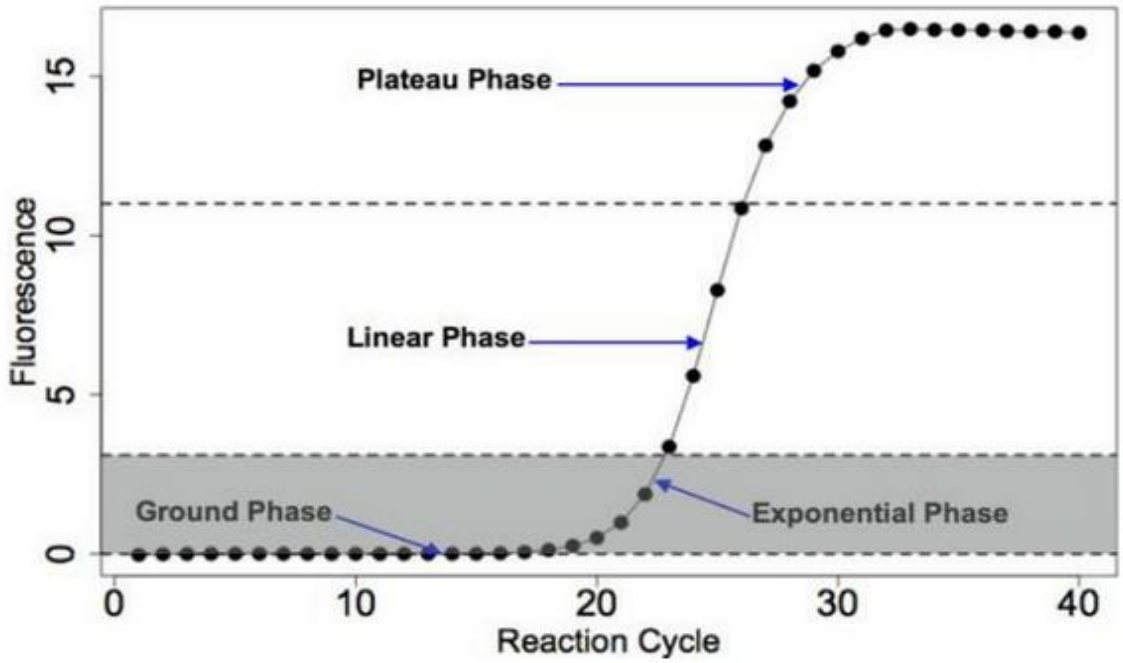

Figure 14 Phases of real time PCR
Source: (Page and Stromberg 2011)

Usually, either a fluorogenic probe or an intercalating dye are needed (Freeman, Walker, and Vrana 1999). An example for an intercalating dye is the SYBR® Green. It has no fluorescence when free in solution but becomes brightly fluorescent when it binds to DNA (Kubista et al. 2006). In a Taq-Man™ assay, another oligonucleotide called probe is employed. It has a fluorophore at the 5'-end (reporter) and a quencher-molecule at the 3'-end. As long as the probe is intact, the quencher molecule is close enough to the reporter that it suppresses its fluorescence. The probe fits complementary to the target-DNA, and it anneals in the space between the forward and the reverse primers. When the reaction of amplification starts, the polymerase elongates the forward primer until finally the enzyme hits the fluorophore at the 5'-end of the probe, disassembling it. This moves the fluorophore away from the quencher, causing it to emit light, which is measured by a detector. With increasing copy-numbers of the amplicon, the fluorescence increases as well due to the rising amount of free fluorophore (figure 15) (Müller and D. R. Prange 2016b).

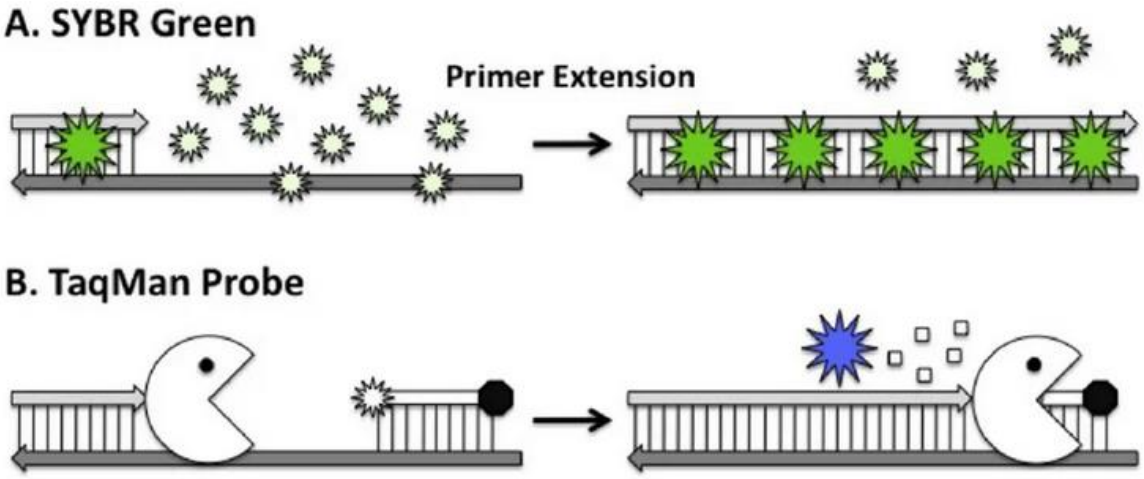

Figure 15 Functionality of SYBR® Green and TaqMan™-probe
Source: (Eva Mészáros 2022)

3 Materials and Methods

A list of all employed chemicals, samples, devices, instruments, and software can be found in the appendix.

3.1 *In silico* preliminary work

To find an appropriate gene on which to write the primers, the National Center of Biotechnology Information (NCBI) database and the Barcode Of Life Databank (BOLD) was used. Potential genes are those that contain sequences unique for the species *Gryllus bimaculatus*.

3.1.1 Alignment

Table 1 Degenerated bases – nomenclature Source: (Cornish-Bowden 1985)

A or G	R
C or T	Y
G or C	S
A or T	W
G or T	K
A or C	M
C or G or T	B
A or G or T	D
A or C or T	H
A or C or G	V
Any base	N
. or -	Gap

To find differences or/and homology in the base sequences, the alignment-tool "Multalin" was used. This tool allows aligning DNA-sequences to find matching or diverging regions. The sequences of interest were entered in the program in FASTA format. The program now aligns one sequence below the other, and for the highest corresponding sequences, a consensus sequence is displayed. To find conserved regions, all available entries of the target gene from the *Gryllus bimaculatus* were entered in the software. Afterwards all bp, in which the information was not consistent, were substituted by degenerated bases, as it is shown in table 1. After that procedure, one combined sequence for the *Gryllus bimaculatus* gene was present.

To detect differences to other taxonomically close species, the nucleotide sequence of the same genes of other species of the family *Gryllidae* were aligned with the combined sequence of the *Gryllus bimaculatus*. The primers were to be written in the regions with multiple differences in the bp-sequence.

3.1.2 Primer design

To design primers and probes successfully, there is a couple of requirements to follow that are shown in table 2.

The software Primer3 has been used to write the probes and the primers. The target sequence and all the parameters were entered in the software. Since this software always proposes the best performing primers, which not necessarily are good in terms of specificity, primers and probes were also written without the use of any software. To write the primers in exactly the targeted region, the parameters of table 2 often had to be bent.

Primers that have not been written using Primer3 software were checked with the eurofins Oligo Analyse Tool for their compatibility in terms of melting temperature or the tendency of forming hairpins or primer-dimer.

Table 2 Requirements for primer- and probe-design (Baltes et al., 2004; McLennan et al., 2013; Müller & Prange, 2016b)

	Primer	Probe
Length	18-26 Nucleotides	20-30 (max. 35) Nucleotides
Location		5'-end close to the 3'-end of the primer
Guanine-cytosine-content	40-60% One or two guanine- or cytosine molecules at the 3'-end	40-60% No guanine at 5'-end
Melting temperature	55-70°C, forward and reverse primer in 3°C-range	5-10°C beyond the primer-T_m
Other	• Avoid more than 4 of the same bases in a row • No complementary between primer or between primer and probe • Avoid secondary structures like hairpin or primer-dimer	

The company TIB Molbiol synthesized the primers and probes. They arrived lyophilized and after the addition of 101.6 µL water or buffer respectively, a concentration of 100 µmol/l was achieved. Aliquots of this source were adjusted to a concentration of 10 µmol/l and stored at -20 °C for further usage. The probes quencher is the Black Hole Quencher-1 (BHQ-1) and the reporter is Carboxyfluorescein (FAM).

3.2 DNA-Extraction

The DNA of whole insects and plants was extracted, using the CTAB-Method, while for foods the NucleoSpin™ Food kit (Macherey Nagel™) has been used. All samples were extracted in triplicates. A variety of insects and insect-containing foods and additionally a couple of plants were extracted. Other DNA samples that were used in this work have already been extracted by different operators.

3.2.1 CTAB-Method

The following steps were carried out:

- 200 mg of the sample were homogenized with a mortar and added to a 2 ml reaction vessel.
- 1000 µl CTAB-extraction buffer was added and, for very colored samples, tip of a spatula of PVPP was added.
- The mixture was vortexed and 20 µl RNAse [40 mg/ml] was added to remove ribonucleic acid (RNA).
- 10 minutes of incubation at 65 °C in the thermomixer

- 10 µl of proteinase K [20 mg/ml] were added, and the mixture was vortexed.
- Another incubation over 30 min at 65 °C in the thermomixer.
- Centrifugation at 14500 g over 10 min
- The supernatant was transferred in a new 2 ml reaction vessel. Twice the sample volume of chloroform/isoamyl (24:1) alcohol was added and mixed well.
- After another 5 minutes of centrifugation at 14500 g the upper, aqueous phase was transferred in a new 2 ml reaction vessel and the volume was measured precisely.
- Two times that volumes of pure ethanol and 1/10th of the volume of Sodium acetate [3M] were added
- Incubation for 60 minutes at room temperature.
- Centrifugation at 14500 g for 15 minutes
- The supernatant was discarded, and the remaining pellet was dried completely.
- The pellet was dissolved in 350 µl of NaCl [1.2 M] and 350 µl chloroform/isoamyl alcohol (24:1) was added.
- After mixing and another 10 minutes centrifugation at 14500 g, the upper aqueous phase was transferred in a new 1.5 ml reaction vessel and 0.8 times the volume of isopropanol, cold from the freezer, was added.
- Everything was mixed by hand and left at -20 °C until the next day.
- Another centrifugation at 14500 g over 20 minutes at 4° C.
- The supernatant was discarded and 500 µl of ethanol [70%] was added and the vessel was shaken until the pellet came off the bottom.
- After a final 3 minutes in the centrifuge at 14500 g the supernatant was carefully decanted, and the pellet was dried completely until all the ethanol was gone
- The pellet was resuspended in 100 µl elution buffer
- The DNA was stored at -20 °C.

3.2.2 NucleoSpin™ Food Mini kit (Macherey-Nagel™)

The following steps were carried out:

- 200 mg material were homogenized and added to a 2 ml reaction vessel.
- 550 µL buffer CF (preheated to 65 °C) were added. Mixed 15 s, added 10 µL proteinase K and mixed again.
- 30 min incubation at 65 °C in the thermomixer.
- 10 µL RNase A [20 mg/ ml] was added.
- 30 min incubation at RT.
- The clear supernatant was transferred into a 2mL reaction tube. 1 vol buffer C4 and 1 vol ethanol were added, mixture was vortexed for 30 s.

- 700 µL of the mixture were pipetted onto a NucleoSpin™ food column placed in a collection tube and centrifuged for 1 min at 11000 g.
- Flow-through was discarded and the step was repeated with the rest of the sample
- 1st wash: 400 µL CQW were added to the column and centrifuged for 1 min at 11000 g.
- 2nd wash: 700 µL buffer C5 were pipetted onto the column and centrifuged for 1 min at 11000 g.
- 3rd wash: Another 200 µL buffer C5 were pipetted onto the column and centrifuged for 2 min to remove C5 buffer completely.
- The column was placed in a 1.5 mL reaction vessel and 50 µL elution buffer CE (preheated to 70 °C) were pipetted onto the column, incubated for 5 min at room temperature (RT) and centrifuged for 1 min at 11000 g to. This step was done twice.
- DNA was stored at -20 °C.

3.3 Quantification

One microliter of the DNA sample was put on the lens of the Nanodrop, and the flap was closed. The measurement was made at an absorbance between 200 and 400 nm. The concentration of nucleic acid is shown by the measured value at the absorption of 260 nm. Indications of purity were provided by the ratios of absorbance at 260/280 and 260/230 nm. Since not only double, but also single-stranded nucleic acids like RNA absorb at 260 nm, the Nanodrop often provides an overestimation. So, to determine the concentration of dsDNA, a fluorometric approach was followed, using the Qubit which provides generally lower estimates of concentration compared to the Nanodrop. Two microliters of the sample and 198 µL of the Qubit buffer from the 1x dsDNA HS-kit (containing the fluorescent dye) were added to a Qubit assay tube. The tube was placed in the holder and the concentration was taken from the display.

3.4 PCR

Two different types of PCR assay were used: Endpoint-PCR and qPCR. The qPCR was used with SYBR® Green and with TaqMan™ probe. In all assays a sample volume of 20 µL was employed, which included 2 µL of the DNA sample that was before diluted down to 10 ng/µL. This way, a total DNA quantity of 20 ng was used. The content of the remaining 18 µL varied, depending on the type of PCR. The preparation of any kind of PCR was done under a chemical hood. In every experiment a positive and no template control (NTC) was used, represented by *Gryllus bimaculatus*-DNA or PCR-pure grade water, respectively. All samples were tested in triplicates for the qPCR and in duplicates for the endpoint PCR.

3.4.1 Endpoint PCR and gel electrophoresis

DNA, master mix (MM) and both primers were put in 200 µL PCR-tubes which than were placed in a thermocycler for 45 cycles. Variable temperatures were employed. Concentration and quantity of each component is shown in table 3.

Table 3 Concentrations and quantities of endpoint PCR components

Component	Storage concentration	Final concentration	Volume per sample [µL]
Water			6.8
Takyon™ Low ROX MM	2X	1X	10
Primer F [µM]	10	0.3	0.6
Primer R [µM]	10	0.3	0.6
DNA [10 ng/µL]			2.00
Total			20.00

After the run was over, 3 µL of 5x gel loading dye were added to each PCR tube. For the following electrophoreses an agarose gel (2 %) was prepared. Three grams of agarose were put in 150 ml 0.5x TBE buffer and heated in a microwave until it completely dissolved. After cooling down to about 70 °C, 5 µL of DNA intercalating agent Ready Red™ were added in the flask. The gel was filled into the gel caster where it polymerized after 25 minutes. It was then transferred to the electrophoresis flow-cell and the samples were loaded in the wells. In one well a 100 kb ladder was loaded, consisting of 3 µL ladder with 2 µL 5x gel loading dye. After 45 minutes at 150 V the gel was put in the imaging device, where a photo was taken.

3.4.2 Real time PCR

For the qPCR, the Maxima SYBR® Green/ROX qPCR Master Mix from Thermo Scientific was employed when no fluorescent probe was present. In case the TaqMan™ technology was used, a probe was added as an additional component and like in the endpoint PCR, the Takyon™ MM was utilized. For the qPCR with TaqMan™ technology, initially a primer- and probe-concentration of 0.1 µM was utilized which later was reduced to the final concentration of 0.05 µM. Concentration and quantities can be taken from table 4 and table 5.

Table 4 Concentrations and quantities of SYBR® Green-qPCR components

Component	Storage concentration	Final concentration	Volume per Sample [µL]
Water			7.6
SYBR® Green MM	2X	1X	10
Primer F [µM]	10	0.1	0.2
Primer R [µM]	10	0.1	0.2
DNA [10 ng/µL]			2
Total			20

Table 5 Concentrations and quantities of TaqMan™-qPCR components

Component	Storage concentration	Final concentration	Volume per sample TaqMan™ [µL]
Water			7.7
Takyon™ Low ROX MM	2X	1X	10
Primer F [µM]	10	0.05	0.1
Primer R [µM]	10	0.05	0.1
Probe [µM]	10	0.05	0.1
DNA [10 ng/µL]			2
Total			20

The components were added to a 96 well qPCR-plate which was sealed afterwards, followed by a centrifugation step of 1 minute at 1000 rpm. Finally, the plates were loaded in the instrument to start the run. The annealing temperature was set to 60 °C.

3.4.3 Determination of efficiency and LOD

3.4.3.1 Efficiency

The systems efficiency, meaning the likelihood to which a molecule of DNA is duplicated after one cycle (Lalam 2006), was evaluated by performing a qPCR with seven serial dilutions, going from 0.2 ng down to 2 x 10^{-7} ng in tenfold increments. To calculate the efficiency, the average Ct of the first four or five dilution steps were plotted. The resulting linear equations slope was inserted into the efficiency calculation:

$E = (10^{-1/m} - 1)x100$ with: E = Efficiency

m = slope

The result of this formula gives the efficiency in percent. It should be between 90 % and 100 % with a linearity (R^2) of at least 0.98 (Broeders et al. 2014).

3.4.3.2 LOD

To find the limit of detection (LOD), seven different DNA quantities were tested, starting at 5 x 10^{-4} ng, and going down in twofold increments to around 3.1 x 10^{-5} ng. Each dilution step was made in 12 replicates. The LOD is achieved when the system is working at 95 % confidence (Forootan et al. 2017), which means that all 12 replicates must be positive.

To express the result in genome copy numbers, the number of bp in the genome of the *Gryllus bimaculatus* was retrieved from Genbank and multiplied to the average weight of one base pair to obtain the weight of the whole genome.

In order to find the practical LOD, meaning the lowest level of target detectable target matrix in an actual food sample, a model food was created by mixing breadcrumbs to decreasing amounts of *Gryllus bimaculatus* flour. Starting from a 1% mixture, four more levels were created, containing 100 ppm, 1 ppm, 100 ppb and 10 ppb of *Gryllus bimaculatus* flour. Each prepared sample was

mixed in a rotating mixer (Turbula T2 F, W.A. Bachofen GmbH, Germany) for one hour, to ensure homogenization. The DNA was extracted, and a qPCR was performed to see until which concentration the system is capable to detect the target.

4 Results

4.1 *In silico* analyses

The following genes have been explored:

- 16S ribosomal RNA (16S)
- 18S ribosomal RNA (18S)
- 28S ribosomal RNA (28S)
- Cytochrome c oxidase subunit I (COI)
- Cytochrome b (Cytb)
- NADH-ubiquinone oxidoreductase chain 1 (ND1)
- NADH-ubiquinone oxidoreductase chain 4 (ND4)
- NADH-ubiquinone oxidoreductase chain 5 (ND5)

Of these genes, only the ND5 and the Cytb showed enough promising interspecies variability for the primer design. Eventually five primer-systems were written, four on the ND5 and one on the Cytb (Table 6).

Table 6 List of ordered primer-pairs

System	**Primer and probe (if ordered)**		**Amplicon length**	**Gene**	**Source**
BimND5-1	BimND5-F1	TTACACTGTTTGGGTGATGAAAG	150 bp	ND5	manually designed
	BimND5-R1	CGCCAAAATCCTCATATAAAAC			
BimND5-2	BimND5-F2	GTCTAATCGTATTGGTGATGTG	100 bp	ND5	Primer3
	BimND5-R2	CAAATCTCCAAAATTGTTTACCC			
	BimND5-P	TGCTTGAATAATARGTTATGGTAGCTG			
BimND5-3	BimND5-F3	GGATCAATATGGTTTATACCC	105 bp	ND5	manually designed
	BimND5-R3	TCTCTTCAACCATAATCTACC			
BimND5-4	BimND5-F4	CTGTTTGGGTGATGAAAGTTATGG	100 bp	ND5	Primer3
	BimND5-R4	ACCACCAATAAAACTAACAACCAAC			
BimCytB	BimCytb-F	GACAGGCATCTTCTTAGCCATG	122 bp	Cytb	manually designed
	BimCytb-R	CTCCATTAGCATGTATTGTTCGTAG			

The differences at bp level can be observed in figure 16. For the Cytb-system, more entries belonging to species of the *Gryllidae* family were found, so the list of compared species differs from the one of the ND5-gene.

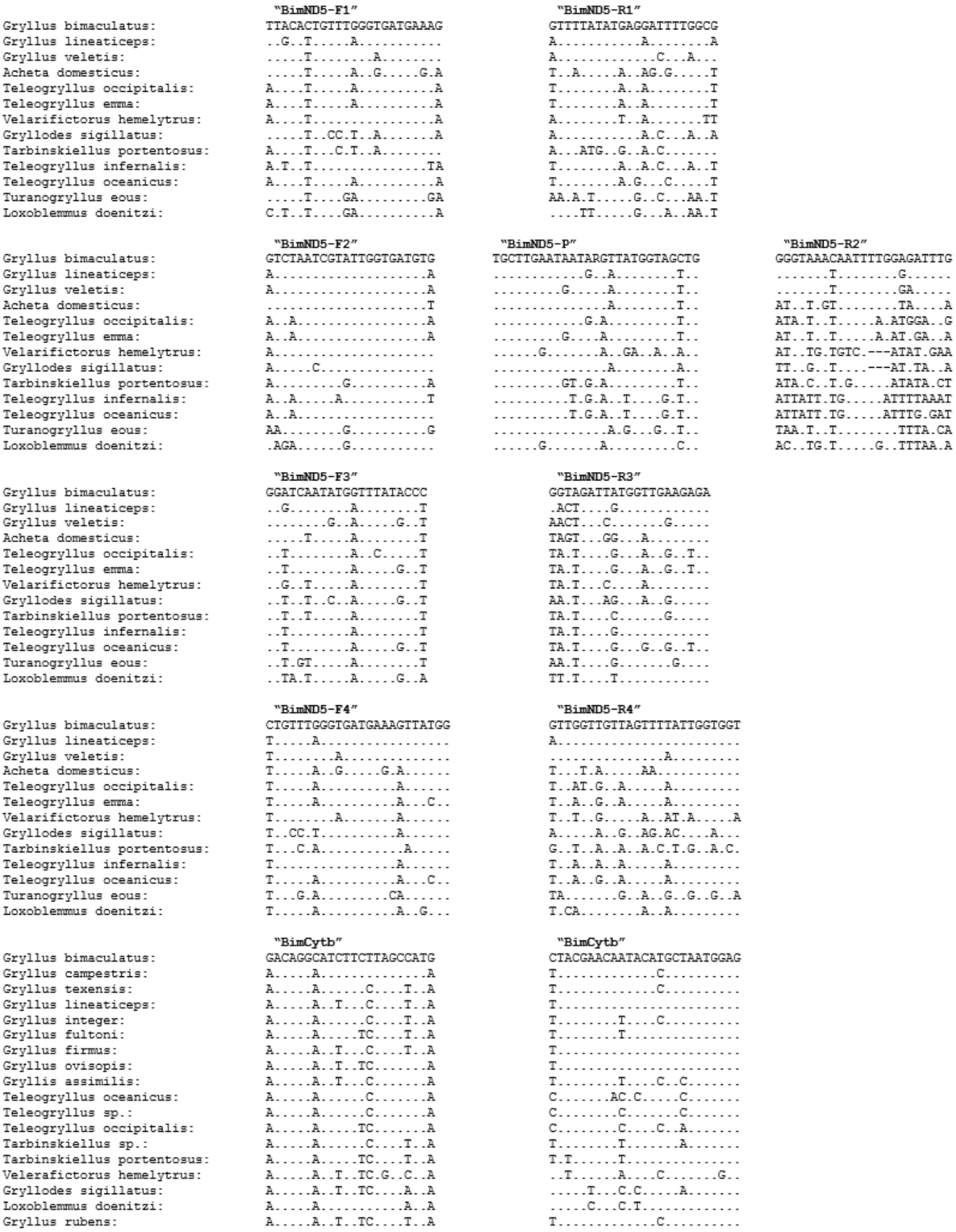

Figure 16 Alignment of all systems. Differences highlighted, dots= agreements, hyphen= gaps

4.2 Endpoint-PCR

Before any DNA was used to work with the developed systems, the extracted DNA was tested with universal primers, to check it for general ability to amplify. This was mainly done by other operators, so the results are not shown in this work.

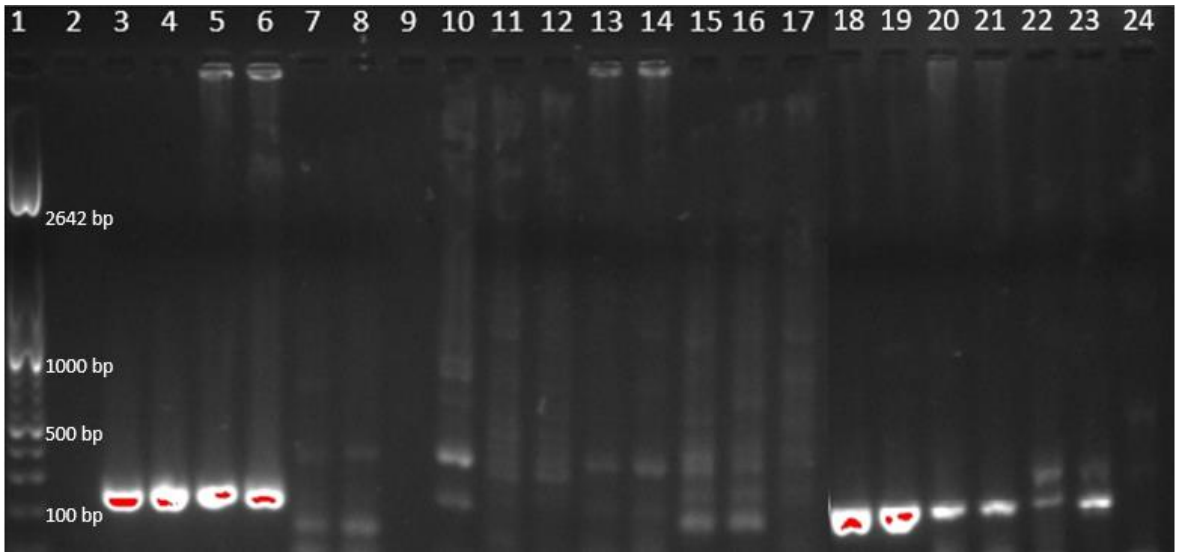

Figure 17 First endpoint experiment. Well assignment: 1: Ladder. Cytb: 3-4: Gryllus bimaculatus; 5-6: Gryllus assimilis; 7-8: Tenebrio molitor; 10: NTC. ND5-1: 11-12: G. bimaculatus; 13-14: Acheta domesticus; 15-16: Tenebrio molitor; 17: NTC. BimND5-4: 18-19: Gryllus bimaculatus;

All primers were tested initially on the target species *Gryllus bimaculatus* and on the cricket *Acheta domesticus,* as well as on another insect taxonomically more distant, *Tenebrio molitor*. The Cytb-system seemed not to be able to distinguish between the two crickets *Gryllus bimaculatus* and *Acheta domesticus*. For the BimND5-4-system, *Acheta domesticus* as well as *Tenebrio molitor* were positive. The BimND5-1 on the other hand, did not amplify anything (figure 17). These three systems were dismissed. The other two systems delivered more promising results and did only amplify the target species (Figure 18). In further experiments the attention was therefore focused on the two systems BimND5-2 and BimND5-3.

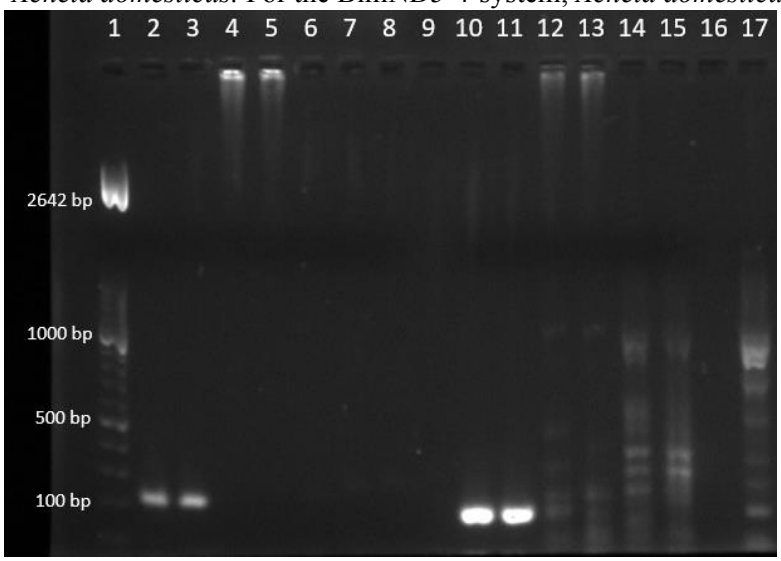

Figure 18 Second endpoint experiment: Well assignment: 1: Ladder. BimND5-3: 2-3: Gryllus bimaculatus, 4-5: Acheta domesticus, 6-7: Tenebrio molitor, 8: NTC. BimND5-2: 10-11: Gryllus bimaculatus, 12-13: Acheta domesticus, 14-15: Tenebrio molitor, 17: NTC.

In the next experiment, the two remaining systems were tested on all the orthopteran species available at the National Reference Laboratory for Animal protein in Feed (NRL-AP). The two species *Tarbinskiellus portentosus* and *Gryllotalpa pluvialis* displayed bands in both systems. For the ND5-2 also some minor bands were visible regarding the species: *Oxya yezoensis*, *Acheta domesticus*, *Gryllodes sigillatus*, *Teleogryllus derelictus* and *Gryllus assimilis* (Figure 19). In a further experiment, the systems were tested again with an increased annealing temperature of 60 °C instead of 55 °C, in the attempt of increasing the specificity as well. This specifically helped to limit the amplification in the *Gryllus assimilis* species.

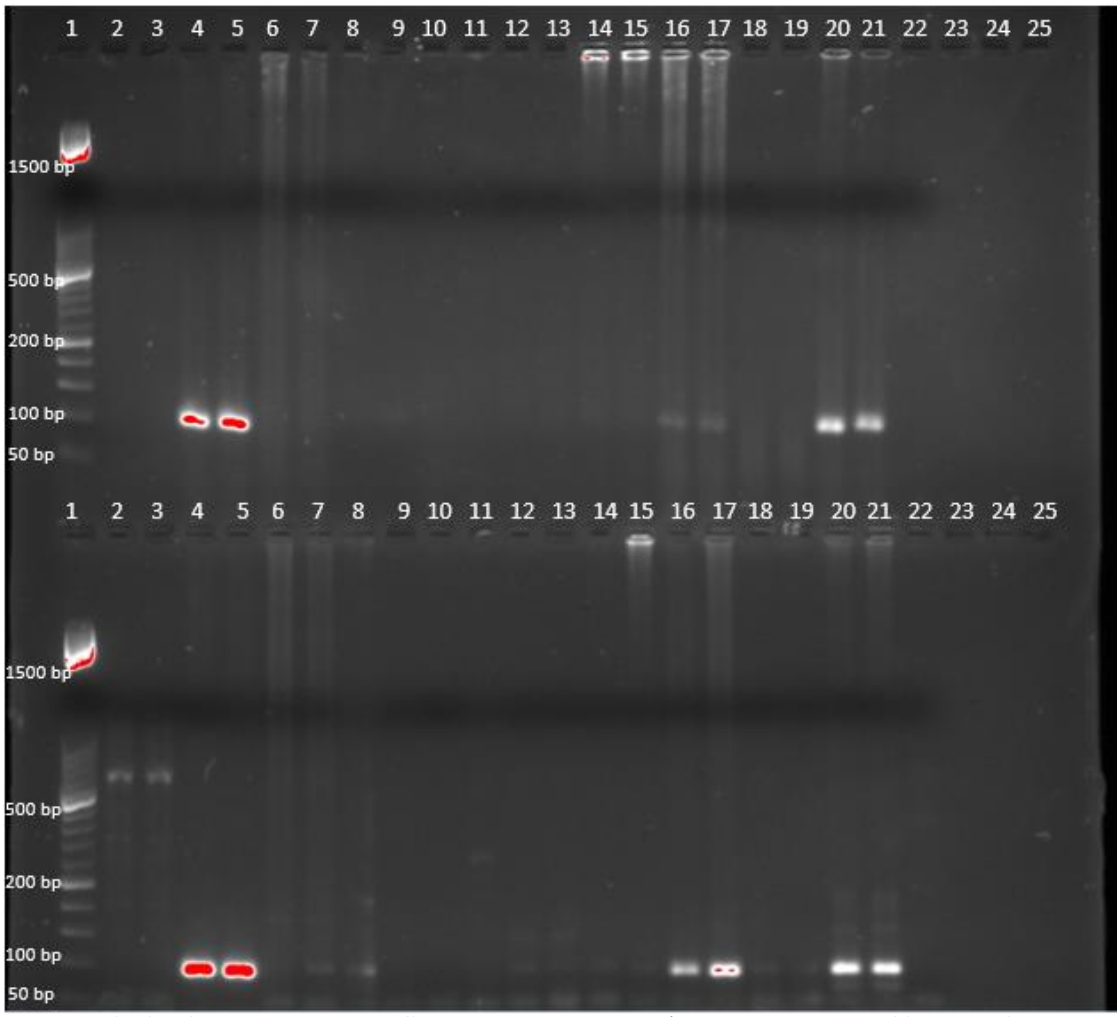

Figure 19 Third endpoint experiment. Well assignment:. Top: ND5_3|Bottom: ND5_2. 1: Ladder, 2-3: Schistocerca gregaria; 4-5: Gryllus bimaculatus; 6-7: Gryllus assimilis, 8-9: Teleogryllus derelictus, 10-11: Locusta migratoria, 12-13: Gryllodes sigillatus, 14-15: Acheta domesticus, 16-17: Tabinskiellus portentosus: 18-19 Oxya yezoensis; 20-21: Gryllotalpa pluvialis; 22: NTC

4.3 Real time PCR

4.3.1 SYBR® Green approach

The first PCR experiment was executed with SYBR® Green. Some *orthopteran* species were tested with both primer pairs. The amplification plot of the ND5-2-system can be seen in figure 20. For a better overview just one of the triplicates is shown, representative for all three.

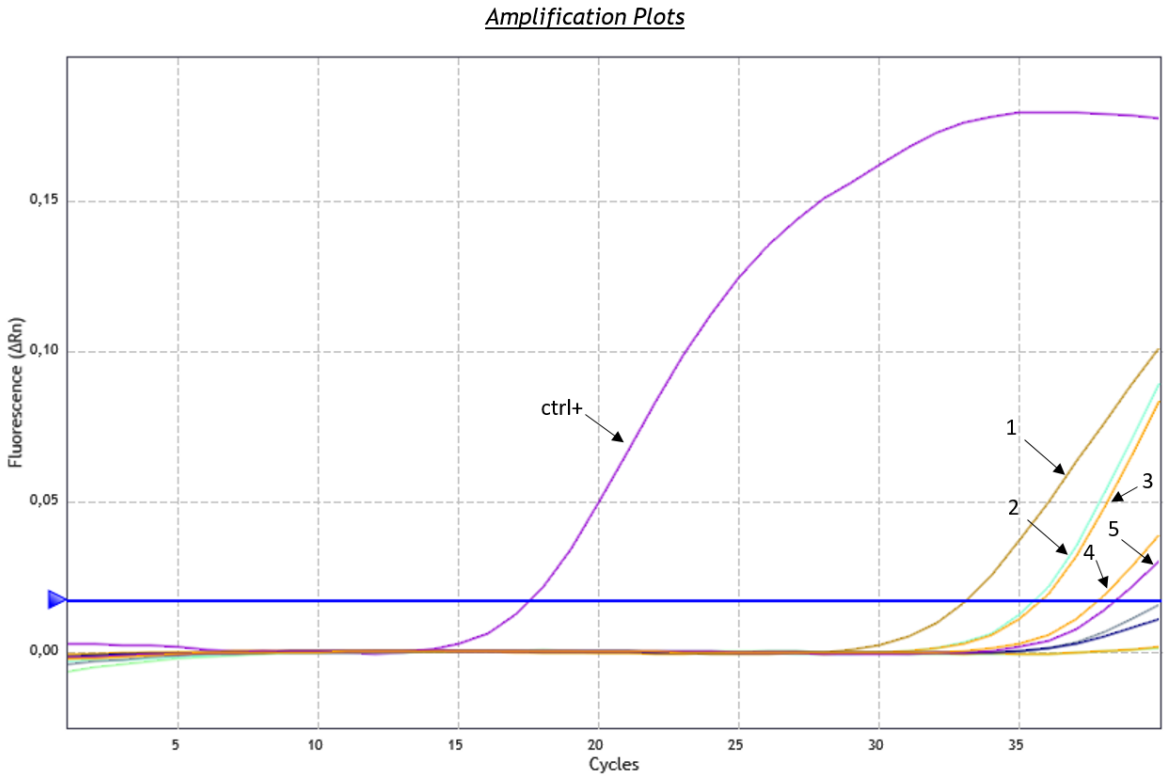

Figure 20 Amplification plot of the SYBR® Green experiment with the ND5-2-system. 1: Tarbinskiellus portentosus, 2: Teleogryllus derelictus, 3: Gryllodes sigillatus, 4: Schistocerca gregaria, 5: Meconema meridionale, ctrl+: Gryllus bimaculatus

Since the ND5-3 system did not show any signals at all, the experiment was repeated, using an increased primer concentration of 300 nM. Afterwards a signal was observed. However, with an average quantification cycle (C_q) of 33.02 for the *Gryllus bimaculatus*, the performance of the system was considered insufficient, and due to its poor sensitivity the system was dismissed as well.

4.3.2 TaqMan™ approach

To increase the specificity even further, the probe for the ND5-2 system was designed. We tested again the species previously used in the SYBR® Green assay and we included some more insects. Since some of the tested samples had late C_q-values past 35, we decided to set a threshold. So, samples with C_q-values higher than 35 were considered negative.

Nevertheless, few species kept on displaying an early amplification sign before the cut-off of 35 (figure 21).

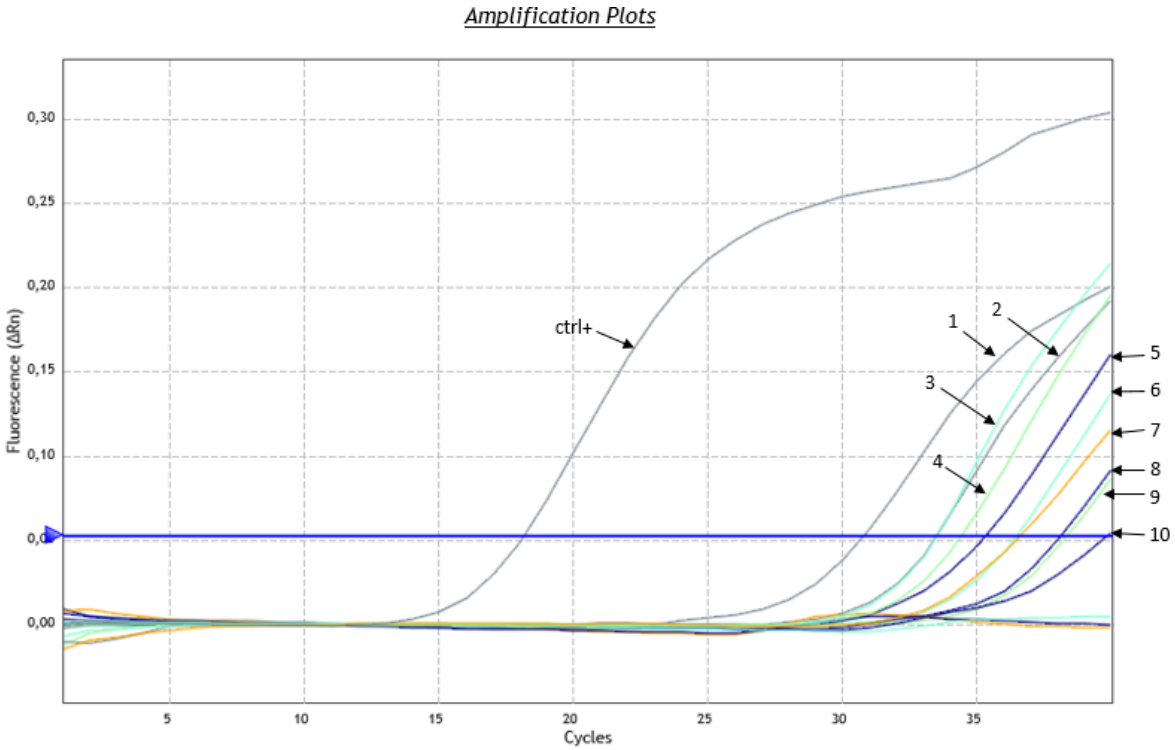

Figure 21 Amplification plot of the first experiment with probe. 1: Oecophylla smaragdina, 2: Tarbinskiellus portentosus, 3: Teleogryllus derelictus, 4: Gryllodes sigillatus, 5: Oxya yezoensis, 6: Acheta domesticus, 7: Gryllus assimilis, 8: Tenebrio molitor, 9: Hermetia illuciens, 10: Tenebrio molitor, ctrl+: Gryllus bimaculatus

4.3.3 Efficiency

Until this point, we were working with a primer-concentration of 100 nM. In the attempt of decreasing the number of amplified non-targeted species, we investigated a lower primer concentration. Therefore, the efficiency of the system was evaluated at decreasing primer concentrations of 100, 75 and 50 nM. The system showed an optimal efficiency of 94,49 % also at 50 nM primer concentration, until the 0.2 pg-point (figure 22).

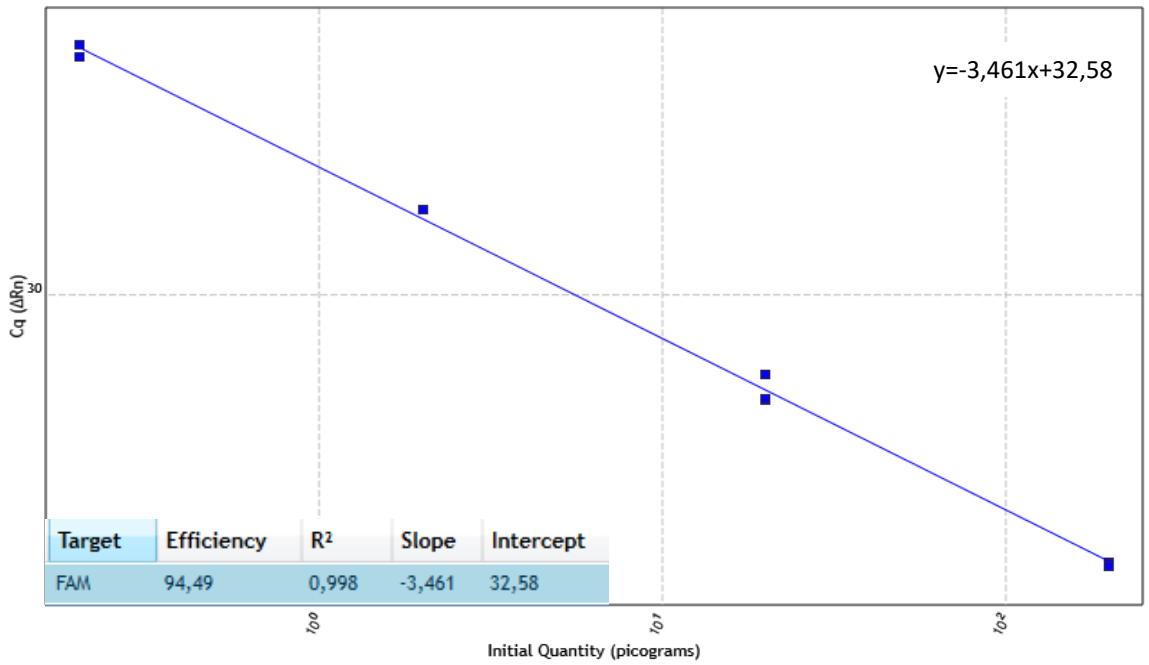

Figure 22 Efficiency for the 50 nM primer concentration

4.3.4 Specificity

Table 7 Positive not targeted species

Samples	**C_q**
Omphisa fuscidentalis	33.41
Tarbinskiellus portentosus	32.57
Lethocerus indicus	31.39
Oecophylla smaragdina	30.45
Gryllotalpa pluvialis	28.09

In further experiments, the ND5-2 system was tested on all insect-samples present in the NRL-AP. Furthermore, a variety of crustaceans, mollusks, fishes, land animals and plants that are relevant in the food industry were tested as well. Eventually, all non-insect samples resulted negative, however we still had five "false" positive results on insect samples that were not the target (Table 7).

4.3.5 Limit of detection

At the 1.25 x10^{-4} ng (125 fg) dilution all 12 replicates were still positive. Since the size of the genome of *Gryllus bimaculatus* is known, it is possible to express the LOD not only in terms of DNA concentration but also in terms of genome copy number.

Number of bp in the genome: 1.658*10^{9} (Genbank, ID: 99306)

Average weight of one bp: 1.1*10^{-21} g

The genome weight is therefore 1.82 pg, in which case 125 fg correspond to approximately 0.07 target copies.

The practical LOD was achieved at 1 ppm (Figure 23), since not all the DNA replicates obtained from the two lower levels showed amplification before the cut-off.

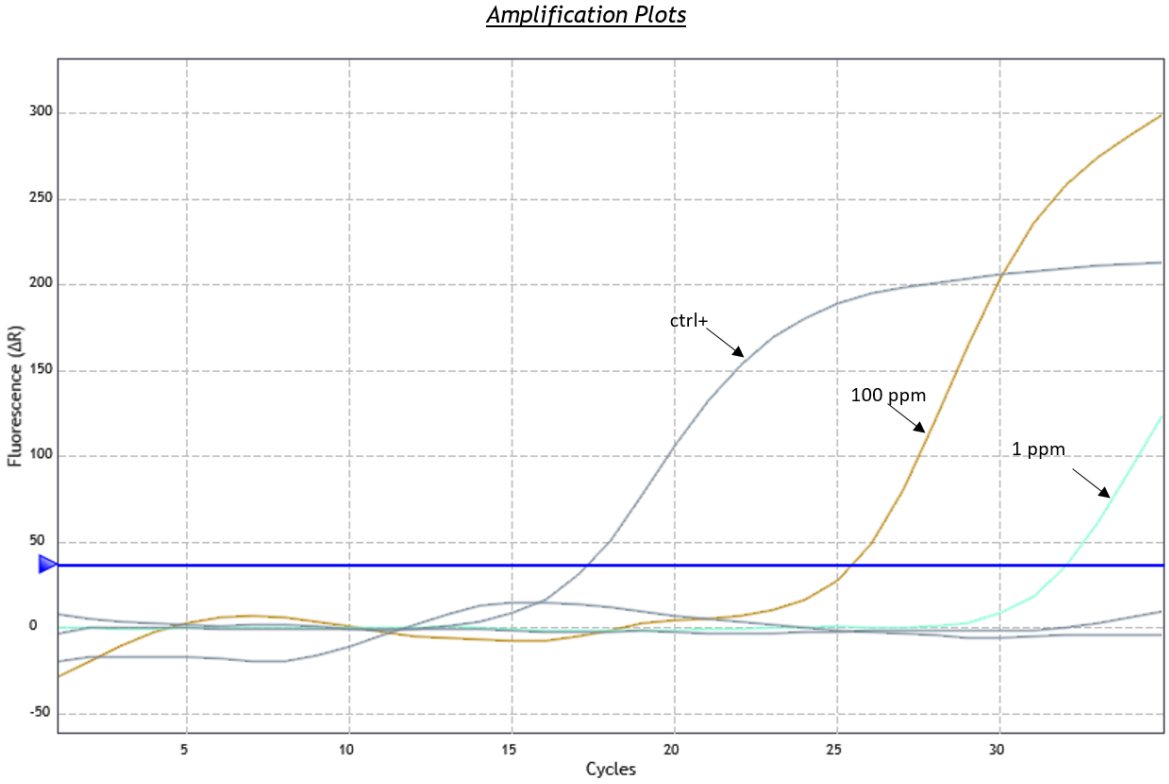

Figure 23 Amplification plot for the technical LOD.

4.3.6 Applicability to foods

To test the system's applicability on real commercial foods, a variety of foods containing different insects were tested. Since there are no foods on the marked that contain the *Gryllus bimaculatus*, it was only possible to test samples that were expected to be negative, which was for all tested samples the case (see appendix).

5 Discussion

The choice of the target DNA region for the design of the primers and probe depends on several factors, the most important of which is the availability of known sequences on publicly available databases. If the target region is located within a single-copy gene on the genome, the parallel "one amplicon = one genome copy" is easier, and it is possible to express the quantitative results obtained via the real time PCR assay as genome copy number without knowing the size of the genome for that species. Conversely, when the target region is located on a multi-copy gene, the number of amplicons is not equal to the number of genome copies, therefore it is better to express the quantitative results in terms of DNA concentration. Then, if the size of the genome for that species is known, it is possible to express the concentration into genome copies. The advantage of using a multi-copy region, is that the sensitivity of the assay increases. Our plan was to design primers not only on mitochondrial, multi copy genes like ND5 and Cytb, but also on at least one genomic single copy gene. However, due to the lack of genomic sequences in the available databases and the limited interspecies differences on the few genomic single copy genes that were found, it was not possible to do so. In order to be as much specific as possible, for each of the selected mitochondrial genes we tried to retrieve the corresponding sequences from all the taxonomically closest species. It was often hard to find a region with sufficient point mutations among the species analyzed. To maximize our chances to find a good candidate system, we explored different primer pairs, even those where only very few inter-species differences were highlighted, in the hope to reach necessary specificity after the addition of the probe. Furthermore, for all the *Gryllus bimaculatus* genes that were selected, only one or maximum two entries could be obtained. Thus, hardly any statement could be made about the intra species variability of these sequences. The developed probe and primers have very few bp of difference with the species *Gryllus lineaticeps* and *Gryllus veletis*, and there were no biological samples from these species we could test experimentally. In our specificity assay, we could only include the species *Gryllus assimilis*, belonging to the same genus as *Gryllus bimaculatus*, but a cross reactivity of our developed system with other *Gryllus* species cannot be ruled out based on the *in-silico* analysis.

Two different extraction methods were utilized. Due to the high yield of polysaccharides, insects and plants have been extracted using a CTAB-protocol. Problematic samples were especially those coming from the Thai company "Thailand unique". As a ready to eat product these insects were dried and salted, a procedure that can damage the DNA. This often resulted in a low yield (less than 10 ng/ µl) and a low 260/280 ratio (less than 1.80). The other whole insect samples, ordered at "Six feet to eat" in Germany, were only dried but not salted, and showed, apart from *Hermetia illucens*, better results regarding yield and 260/280 ratio. The food kit delivered mainly satisfying results regarding the DNA quality and yield, even though the latter was generally lower than for most of the

CTAB-extractions. Somewhat tricky were the “Crispies” from the company Entomos, which provided low yield and low DNA-quality, as well as the Crupuk chips of the same company, which had a low yield. The samples were highly processed and, compared to the other extracted foods, very hygroscopic, which may have led to an insufficient buffer addition. Additionally, the breadcrumbs and all admixtures that were prepared with them, had partly low DNA-yields. However, all samples have proven their general ability to be amplified with universal primers.

Both primer- and probe concentration and the optimal annealing temperature were not chosen based on their performances on the positive *Gryllus bimaculatus* DNA control, but rather to obtain the lowest number of false positive, in order to achieve the highest species-specificity. Already during the preliminary tests in endpoint-PCR approach a temperature of 60 °C showed better results in terms of specificity compared to 55 °C, at which the non-target species *Gryllus assimilis* displayed amplification. Reducing the primer and probe concentrations in the real time PCR from 100 nM to 50 nM helped to exclude the late amplification of the species *Rhynchophorus ferrugineus*, *Gryllodes sigillatus* and *Teleogryllus derelictus.* This may have also caused a shift in the C_q of *Gryllus bimaculatus*, but it also reduces the cost of the system because less reagents are needed. Five species have indicated a potential cross reactivity with the system. Of these species, the DNAs extracted from *Gryllotalpa pluvialis* and *Lethocerus indicus* showed a contamination with other undefined members of the *Gryllidae* family, while the *Oecophylla smaragdina* showed minor contaminations with *Gryllus bimaculatus* in a next generation sequencing (NGS)-test (not part of this work). This is a possible explanation for the “false positive” results. Regarding the *Tarbinskiellus portentosus* and the *Omphisa fuscidentalis* samples*,* the reason of cross reactivity is unclear. However, only the *Tarbinskiellus portentosus* is a cricket belonging to the same subfamily of *Gryllus bimaculatus* (*Gryllinae*), but the *in-silico* alignment shows many bp different from the target species in the primers annealing sites, which can be seen in figure 16 on page 28. The *Tarbinskiellus portentosus*, as well as the *Gryllotalpa pluvialis,* did also amplify with the ND5-3-system, which later was dismissed for its poor sensitivity. The other species are taxonomically even further distant, so also in these cases a contamination in the sample used is more likely than an actual cross reactivity due to genetic identity or proximity. We tested the applicability of the system to real food by mixing flour of the *Gryllus bimaculatus* with breadcrumbs, and the results were satisfactory, indicating a sensitivity down to 1 ppm of the target in food. However, no thermally processed samples were designed and tested, which should be done in the further validation of the system.

With 94,6 %, the efficiency of the system is a bit lower than the one obtained in other studies about the detection of insects in food or feeds (Garino et al. 2022; Zagon et al. 2018) where the mitochondrial gene COI gene was selected as target. Still, it easily exceeds the minimum of 90 % to count as valid and, with an R^2 of 0.998, the linearity was sufficient (Broeders et al. 2014). Comparing the sensitivity, with all 12 replicates being positive at 125 fg the system appears to be less sensitive

than the one specific for *Alphitobius diaperinus* (circa 30 fg, Garino et al., 2022), but when the LOD is expressed as genome copy numbers the results are approximately the same (circa 0.1 copies). The practical LOD at 1 ppm is comparable to that of other qPCR-systems (Garino et al. 2022).

Searching the literature, no other real time PCR-based assay for the detection of *Gryllus bimaculatus* was found. But Korean scientists have developed an ultra-fast PCR assay for the detection of six species of edible insects and one of them is the *Gryllus bimaculatus* (Kim et al. 2019). In their system, the primers were written on the COI gene, which was also considered in this work, but no sufficient regions with enough inter-species variability was identified for the primer design. In fact, regarding the specificity, the team of Kim and collaborators did only test nine other species, while 38 insect species were included in this work. Furthermore, unlike in this work, neither crustaceans, a subphylum of arthropods genetically related to the *Insecta* class, nor any other animals or plants were tested.

6 Outlook

Regarding nutritional and environmental aspects, insects deliver a row of arguments to be increasingly considered as an alternative protein source, and they appear to be an urgently needed puzzle piece in the challenge of feeding the worlds growing population. The development of socially accepted recipes and clever marketing strategies will likely manage to overcome Western people's dislike for edible insects. Entomophagy could play a significant role in inducing a much-needed change of the meat-oriented diet currently followed in many developed countries. But despite the opportunities offered by entomophagy, its possible risks should not be overlooked. Due to their allergenic potential, insects will likely be labeled as allergens in the future. It is likely that more species of edible insects will become part of the European food market, and the *Gryllus bimaculatus* could be one of them since it is already being farmed as pet food in Europe and as food for humans in Asia.

To detect deliberate or accidental contaminations with prohibited species, the development of reliable detection methods is mandatory to ensure the safety of consumers.

Even if this work was focused on the development of a method to detect *Gryllus bimaculatus* in food, it is conceivable to apply it as well on feeds. Especially the possibility to distinguish between *Gryllus bimaculatus* and the other cricket *Gryllus assimilis*, which is allowed in feeds for aquaculture, pigs and poultry, makes it particularly interesting. However, the applicability to real and model feeds needs to be tested experimentally.

If the system will be completely validated and scientifically published in the future, it will be used in combination with other species-specific real time PCR based systems directed against crickets and grasshoppers playing a role in the food- or feed market. However, for a complete validation of the system, its intra- and inter-laboratories robustness must still be determined.

7 Literature

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Appendix

List of employed software and databases

Name	Version	Link	Publisher
BLAST	-	https://blast.ncbi.nlm.nih.gov/Blast.cgi	National Center for Biotechnology Information (NCBI
Nucleotide Database	-	https://www.ncbi.nlm.nih/	National Center for Biotechnology Information (NCBI)
Primer3web	4.1.0	https://primer3.ut.ee/	Untergasser, A. *et al.*
Multalin	-	https://multalin.toulouse.inra.fr/multalin/	Florence Corpet, France
Oligo Analysis Tool	-	https://eurofinsgenomics.eu/en/	eurofins Genomics

Chemicals, instruments and devices

DNA-Extraction with CTAB-protocol

Chemicals	Manufacturer
Chloroform/isoamyl alcohol (24:1)	Merck, Germany
CTAB	Merck, Germany
EDTA (pH 8.0)	Roth Werke, Germany
Elution buffer	Macherey-Nagel, Germany
Ethanol	Merck, Germany
Isopropanol	AppliChem, Germany
Proteinase K solution (20 mg/ml)	Macherey-Nagel, Germany
PVPP	Sigma-Aldrich, Canada
RNAse (40 mg/ml)	Macherey-Nagel, Germany
Sodium acetate 3M	Merck, Germany
Sodium chloride	Merck, Germany
Tris-HCL (pH 8.0)	Roth Werke, Germany
Instruments & devices	**Manufacturer**
Analyze scale (BP 210)	Sartorius Mechatronik, Germany
Centrifuge 5425 R	Eppendorf, Germany
Concentrator plus	Eppendorf, Germany
Mortar	Haldenwanger, Germany
Pipettes (1000 µL/100 µl/20 µl)	Eppendorf, Germany
ThermoMixer C	Eppendorf, Germany
Vortex-Genie 2	Scientific Industries, USA

DNA-Extraction with Food Kit

Chemicals	Manufacturer
Lysis buffer CF Buffer C4 Collection tubes (2 mL) Elution buffer CE NucleoSpin™ Food Columns Proteinase buffer PB Wash buffer C5 Wash buffer CQW	Macherey-Nagel, Germany

Mortar	Haldenwanger, Germany
Water bath OLS 200	Grant instruments, UK
Instruments & devices	**Manufacturer**
Analyze scale (BP 210)	Sartorius Mechatronik, Germany
Centrifuge 5425 R	Eppendorf, Germany
Pipettes (1000 µl/200 µl/100 µl/20 µl)	Eppendorf, Germany
ThermoMixer C	Eppendorf, Germany
Vortex-Genie 2	Scientific Industries, USA

Determination of DNA quality and quantity

Chemicals	**Manufacturer**
Qubit buffer (1x dsDNA HS Assay Kit)	Thermo Fisher Scientific Inc., USA
Elution buffer	Macherey-Nagel, Germany
Instruments & devices	**Manufacturer**
Fluorometer "Qubit"	Sartorius AG Mechatronik, Germany
Nanophotometer "Nanodrop"	Implen, Germany
Pipettes (200 µl/2,5 µl)	Eppendorf, Germany
Qubit assay tubes	Thermo Fisher Scientific Inc., USA

Endpoint DNA

Chemicals	**Manufacturer**
Takyon™ Low Rox Probe MM	Eurogentec, Germany
Primer (forward and reverse)	TIB Molbiol, Berlin
Water, nuclease- free (PCR water)	Thermo Fisher Scientific Inc.
Instruments & devices	**Manufacturer**
Colored PCR tubes	Eppendorf, Germany
Mini centrifuge	NeoLab, Germany
Pipettes (1000 µl/100 µl/10 µl)	Eppendorf, Germany
Vortex V-1 plus	BioSan, Latvia

Gel-electrophoresis

Chemicals	**Manufacturer**
Agarose powder	Eurogentec, Germany
Gel pilot loading dye, 5x	Qiagen, Netherlands
DNA molecular weight marker 100 bp ladder	Roche diagnostics, Germany
DNA molecular weight marker 50 bp ladder	Roche diagnostics, Germany
ROTI®-Gelstain RED	Roth Werke GmbH, Germany
Tris-Borat-EDTA buffer	Thermo Fisher Scientific Inc., USA
Instruments & devices	**Manufacturer**
Electrophoresis comb	Roth, Germany
Electrophoresis gel chamber	Roth, Germany
Electrophoresis power supply	Consort, Belgium
Gel caster	Roth, Germany
Imaging device Gel Doc XR	Bio-Rad, USA
Microwave	Bosch, Germany
Pipettes (20 µl/10 µl)	Eppendorf, Germany

Real time PCR

Chemicals	Manufacturer
Maxima SYBR® Green/ ROX qPCR MM (2x)	Thermo Fisher Scientific Inc., USA
Primer (reverse and forward)	TIB Molbiol, Germany
Probe	TIB Molbiol, Germany
qPCR probe dilution buffer pH 8.0	eurofins Genomics, Luxemburg
Takyon™ Low Rox Probe MM	Eurogentec, Germany
Water, nuclease- free (PCR water)	Thermo Fisher Scientific Inc.
Instruments & devices	**Manufacturer**
96-well plate	Biozym Scientific, Germany
Biozym adhesive clear PCR seal	Biozym Scientific, Germany
Centrifuge for 96 well plates	Thermo Fisher Scientific, USA
PCR instrument Aria DX	Aligent technologies, USA
Pipettes (1000 µl/100 µl/10 µl)	Eppendorf, Germany
Vortex V-1 plus	BioSan, Latvia

Production of model foods

Instruments & devices	
Analytic scale ABT 100-5NM	Kern & Sohn, Germany
Sample beakers	Boettger, Germany
Rotating mixer Turbula T2 F	W.A. Bachofen, Germany
Other materials	**Manufacturer**
Gloves	Kimtech, USA
Pipette tips (1000 µl/200 µl/100 µl/20 µl/10 µl)	Eppendorf, Germany
Racks	Eppendorf, Germany
Reaction vessels (0,5 ml/1,5 ml/2 ml/5 ml)	Eppendorf, Germany
Spatulas	Bochem Instrumente, Germany

Results of DNA-extraction with CTAB-protocol and corresponding PCR results

Insects

Species	English name	Sample origin	DNA-concentration	Date of extraction	260/280 ratio	Negative with BimND5-2	Date of qPCR
Acheta domesticus	House cricket	Six Feet To Eat, Germany	88.4 ng/µl 242 ng/µl 540 ng/µl	04.11.21	1.97 1.95 1.97	Yes	28.03.22
Alphitobius diaperinus	Lesser mealworm	Snack-Insects, Germany	134 ng/µl 104 ng/µl 151 ng/µl	10.11.21	1.97 1.96 1.96	Yes	28.03.22
Gryllodes sigillatus	Banded cricket	Six Feet To Eat, Germany	365 ng/µl 540 ng/µl 750 ng/µl	10.11.21	1.93 1.96 1.97	Yes	06.05.22

Grylls bimaculatus	Two-spotted cricket	Six Feet To Eat, Germany	85.7 ng/µl 650 ng/µl 760 ng/µl	02.03.22	1.98 1.97 1.99	No (15,20)	21.04.22
Hermetia illucens	Black soldier fly	Six Feet To Eat, Germany	13,2 ng/µl 15,7 ng/µl 16,3 ng/µl	10.11.21	1.65 1.76 1.72	Yes	28.03.22
Locusta migratoria	Migratory locust	Six Feet To Eat, Germany	568 ng/µl 538 ng/µl 588 ng/µl	04.11.21	1.86 1.91 1.86	Yes	28.03.22
Omphisa fuscidentalis	Bamboo worm	Thailand unique, Thailand	14.5 ng/µl 16.8 ng/µl 9.32 ng/µl	02.11.21	1.57 1.45 1.23	No (33,21)	24.05.22
Oxya yezoensis	Grasshopper	Thailand unique, Thailand	4.83 ng/µl 10.2 ng/µl 14.8 ng/µl	02.11.21	1.32 1.66 1.10	Yes	24.05.22
Rhynchophorus ferrugineus	Sago palm weevil	Thailand unique, Thailand	13.4 ng/µl 2.92 ng/µl 9.34 ng/µl	02.11.21	1.91 1.85 1.87	Yes	24.05.22
Tenebrio molitor	Mealworm	Six Feet To Eat, Germany	92.6 ng/µl 268 ng/µl 222 ng/µl	04.11.21	1.97 1.96 1.96	Yes	28.03.22
Termitidae	Higher termites	Thailand unique, Thailand	2.00 ng/µl 2.80 ng/µl 8.70 ng/µl	16.11.21	1.61 1.84 1.87	Yes	19.05.22

Plants

Species	**English name**	**Sample origin**	**DNA-concentration**	**Date of extraction**	**260/280 ratio**	**Negative with BimND5-2**	**Date of qPCR**
Foeniculum vulgare	Fennel	Edeka, Germany	104 ng/µl 218 ng/µl 216 ng/µl	10.05.22	1.97 1.95 1.97	Yes	16.05.22
Zea mays	Corn	Edeka, Germany	116 ng/µl 110 ng/µl 162 ng/µl	10.05.22	1.97 1.96 1.96	Yes	16.05.22
Solanum lycopersicum	Tomato	Edeka, Germany	53.0 ng/µl 38.2 ng/µl	10.05.22	1.93 1.96	Yes	06.05.22

Results of DNA-extraction with extraction kit and corresponding PCR results

Commercial Foods

Label	**Claimed insect content**	**Manufacturer**	**DNA-concentration**	**Date of extraction**	**260/280 ratio**	**Negative with BimND5-2**	**Date of qPCR**
Acheta Knusper Müsli	30% *Acheta domesticus*	Entomos, Switzerland	43.4 ng/µl 47.6 ng/µl 48.2 ng/µl	26.10.21	1.94 1.92 1.92	Yes	31.05.22

Acheta pasta	17 % *Acheta domesticus*	Entomos, Switzerland	220 ng/µl 299 ng/µl 252 ng/µl	22.10.21	1.91 1.91 1.91	Yes	31.05.22
Crispies	47 % *Tenebrio molitor*	Entomos, Switzerland	2.40 ng/µl 3.36 ng/µl 3.82 ng/µl	26.10.21	1.54 1.50 1.61	Yes	31.05.22
Crupuk Chips	18% *Tenebrio molitor*	Entomos, Switzerland	5.59 ng/µl 7.33 ng/µl 6.35 ng/µl	28.10.21	1.98 1.99 1.95	Yes	31.05.22
Feel Good Bar	12% *Alphitobius diaperinus*	Isaac Nutrition, Germany	46.2 ng/µl 60.8 ng/µl 5.58 ng/µl	27.09.21	1.79 1.75 1.88	Yes	31.05.22
Proteinpulver	15% *Alphitobius diaperinus*	Isaac Nutrition, Germany	56.2 ng/µl 63.2 ng/µl 59.2 ng/µl	28.09.22	1.80 1.83 1.84	Yes	31.05.22
Tenebrio Knusper Müsli	50% *Tenebrio molitor*	Entomos, Switzerland	7.50 ng/µl 10.7 ng/µl 14.2 ng/µl	26.10.21	1.90 1.93 1.95	Yes	31.05.22
Tortillas nature	38 % *Tenebrio molitor*	Entomos, Switzerland	57.6 ng/µl 49.6 ng/µl 51.2 ng/µl	22.10.21	1.93 1.93 1.94	Yes	31.05.22
Paniermehl	None (Blank control for model foods)	Thüringer Mühlenwerke, Germany	7.13 ng/µl 8.97 ng/µl 11.7 ng/µl 12.6 ng/µl 5.70 ng/µl 11.5 ng/µl	09.05.22	1.94 1.89 1.91 1.89 1.88 1.92	Yes	16.05.22

Model Foods

Description	**Insect content**	**Manufacturer**	**DNA-concentration**	**Date of extraction**	**260/280 ratio**	**Negative with BimND5-2**	**Date of qPCR**
G. bimaculatus – breadcrumbs mix	1 % *G. bimaculatus*	In house	9.30 7.40 6.45	18.05.22	2.01 1.90 1.96	No (15,94)	19.05.22
G. bimaculatus – breadcrumbs mix	100 ppm *G. bimaculatus*	In house	3.70 3.80 9.88	18.05.22	2.08 2.03 1.95	No (26,49)	01.06.22
G. bimaculatus – breadcrumbs mix	1 ppm *G. bimaculatus*	In house	10.0 11.2 11.5	18.05.22	1.93 1.94 1.93	No (30,62)	01.06.22
G. bimaculatus – breadcrumbs mix	100 ppb *G. bimaculatus*	In house	10.6 12.0 12.6	18.05.22	1.93 1.94 1.96	Yes	01.06.22
G. bimaculatus – breadcrumbs mix	10 ppb *G. bimaculatus*	In house	5.45 10.8 4.06	18.05.22	2.00 1.94 2.20	Yes	01.06.22

Employed DNA samples, extracted by other operators

Insects

Species	English name	Negative with ND5-2	Date of qPCR
Blaptica dubia	Dubia roach	Yes	28.03.22
Bombyx mori	Silkworm	Yes	28.03.22
Calliphorid vomitoria	Blue bottle fly	Yes	31.03.22
Callosobruchus maculatus	Cowpea weevil	Yes	31.03.22
Cetonia aurata	Rose chafer	Yes	31.03.22
Chilecomadia moorei	Chilean moth	Yes	28.03.22
Drosophila melanogaster	Fruit fly	Yes	31.03.22
Drosophila subquinaria	unknown	Yes	31.03.22
Galleria mellonella	Greater wax moth	Yes	31.03.22
Gryllotalpa pluvialis	Mole crickets	No (28.09)	06.05.22
Gryllus assimilis	Jamaican field cricket	Yes	28.03.22
Gryllus campestris	European field cricket	Yes	16.05.22
Lethocerus indicus	Giant water bug	No (31.39)	24.05.22
Lucilia caesar	Blow fly	Yes	31.03.22
Lucilia sericata	Common green bottle fly	Yes	31.03.22
Meconema merdionale	Southern oak bush cricket	Yes	28.03.22
Musca domestica	Housefly	Yes	28.03.22
Nauphoeta cinerea	Cinereous cockroach	Yes	31.03.22
Oecophylla smaragdina	Queen Weaver Ants	No (30.34)	24.05.22
Pachnoda marginata	Sun beetle	Yes	31.03.22
Phoetalia pallida	Pallid cockroach	Yes	28.03.22
Polyrhachis	Black ant	Yes	31.03.22
Schistocerca gregaria	Desert locust	Yes	28.03.22
Stegobium paniceum	Drugstore beetle	Yes	31.03.22
Tanyptera atrata	Large crane fly	Yes	31.03.22
Tarbinskiellus portentosus	Large brown cricket	No (32.57)	31.05.22
Teleogryllus Derelictus	unknown	Yes	06.05.22
Zophobas morio	Dark beetle	Yes	31.03.22
Crusaceans			
Cancer pagurus	Brown crab	Yes	01.04.22
Cherax quadricarinatus	Australian red claw crayfish	Yes	01.04.22
Crangon crangon	Common shrimp	Yes	01.04.22
Eriocheir sinensis	Chinese mitten crab	Yes	01.04.22
Euphausia superba	Antarctic krill	Yes	01.04.22
Homarus americanus	American lobster	Yes	01.04.22
Macrobachium rosenbergii	Giant river prawn	Yes	01.04.22
Pandalus borealis	Northern prawn	Yes	01.04.22
Paralithodes camtschaticus	Red king crab	Yes	01.04.22
Penaeus monodon	Giant tiger prawn	Yes	01.04.22
Pleoticus muelleri	Argentine red shrimp	Yes	01.04.22
Portunus pelagicus	Flower crab	Yes	01.04.22
Varuna litterata	River swimming crab	Yes	01.04.22
Xiphopenaeus kroyeri	Atlantic seabob	Yes	01.04.22

Mullusks

Achatina fulica	Giant African land snail	Yes	01.04.22
Amphioctopus aegina	Marbled octopus	Yes	01.04.22
Buccinum spp.	Whelk	Yes	01.04.22
Cerastoderma edule	Common cockle	Yes	01.04.22
Crassostrea gigas	Pacific oyster	Yes	01.04.22
Glycymeris spp.	Unknown	Yes	01.04.22
Helix lucorum	Unknown	Yes	01.04.22
Littorina sp.	Unknown	Yes	01.04.22
Loligo reynaudii	Cape Hope squid	Yes	01.04.22
Mytilus edulis	Blue mussel	Yes	06.05.22
Placopecten magellanicus	Atlantic deep-sea scallop	Yes	01.04.22
Ruditapes philippinarum	Manila clam	Yes	01.04.22
Sepia officinalis	Common cuttlefish	Yes	01.04.22
Spisula solidissima	Bar clam	Yes	01.04.22

Fishes

Anguilla anguilla	European eel	Yes	05.05.22
Merluccius merluccius	European hake	Yes	05.05.22
Oncorhynchus mykiss	Rainbow trout	Yes	05.05.22
Oreochromis niloticus	Nile tilapia	Yes	05.05.22
Pleuronectes platessa	European plaice	Yes	05.05.22
Salmo salar	Atlantic salmon	Yes	05.05.22
Sardina pilchardus	European pilchard	Yes	05.05.22
Scomber scombrus	Atlantic mackerel	Yes	05.05.22
Sebastes marinus	Rose fish	Yes	05.05.22
Spondyliosoma cantharus	Black seabream	Yes	05.05.22
Theragra chalcogramma	Alaska pollock	Yes	05.05.22
Thunnus albacares	Tuna	Yes	05.05.22

Plants

Allium sativum	Garlic	Yes	16.05.22
Anacardium occidentale	Cashew	Yes	21.04.22
Apium graveolens	Celery	Yes	21.04.22
Arachis hypogaea	Peanut	Yes	19.05.22
Arthrospira	Spirulina	Yes	21.04.22
Avena sativa	Oat	Yes	21.04.22
Bertholletia excelsa	Brazil nut	Yes	21.04.22
Brassica rapa ssp	Canola	Yes	21.04.22
Cannabis sativa	Hempseed	Yes	21.04.22
Capsicum annuum	Bell pepper	Yes	21.04.22
Carum carvi	Caraway	Yes	21.04.22
Carya illinoinensis	Pecan	Yes	21.04.22
Coriandrum sativum	Coriander	Yes	21.04.22
Daucus carota	Carrot	Yes	16.05.22
Fagopyrum esculentum	Buckwheat	Yes	21.04.22
Glycine max	Soy	Yes	21.04.22
Horeum vulgare	Barley	Yes	21.04.22
Juglans regia	Walnut	Yes	06.05.22
Lupinus perennis	Lupine	Yes	21.04.22
Macadamia integrifolia	Macadamia	Yes	21.04.22
Malus domestica	Apple	Yes	16.05.22

Musa acuminata	Banana	Yes	16.05.22
Myristica fragrans	Nutmeg	Yes	21.04.22
Ocimum basilicum	Basil	Yes	16.05.22
Origanum vulgare	Oregano	Yes	21.04.22
Oryza sativa	Rice	Yes	21.04.22
Pimpinella anisum	Anise	Yes	21.04.22
Piper nigrum	Black pepper	Yes	21.04.22
Pistacia vera	Pistachio	Yes	19.05.22
Pisum sativum	Pea	Yes	21.04.22
Prunus amygdalus	Almond	Yes	21.04.22
Salvia rosmarinus	Rosemary	Yes	16.05.22
Secale cereale	Rye	Yes	21.04.22
Sinapis alba	Mustard	Yes	21.04.22
Syzygium aromaticum	Cloves	Yes	21.04.22
Thymus vulgaris	Thyme	Yes	16.05.22
Triticum aestivum	Wheat	Yes	06.05.22
Triticum spelta	Spelt	Yes	21.04.22
Vaccinium	Blueberry	Yes	21.04.22
Zingiber officinale	Ginger	Yes	21.04.22

Land animals

Anatidae sp.	Duck	Yes	05.05.22
Ovis aries	Lamb	Yes	05.05.22
Capra hircus	Goat	Yes	05.05.22
Bos taurus	Cattle	Yes	05.05.22
Anser anser	Goose	Yes	05.05.22
Gallus domesticus	Chicken	Yes	05.05.22
Sus domesticus	Pig	Yes	05.05.22
Meleagris	Turkey	Yes	05.05.22
Cervidae sp.	Deer	Yes	05.05.22
Leporidae sp.	Rabbit	Yes	05.05.22

Commercial foods

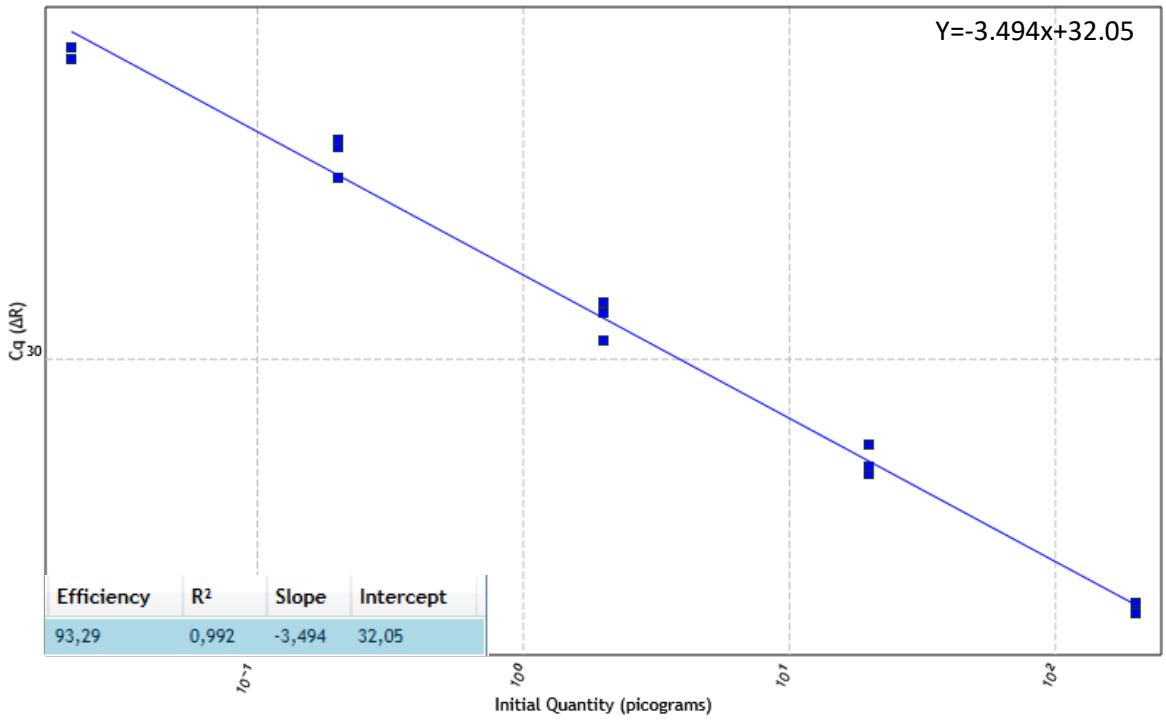
Label	**Insect content**	**Manufacturer**	**Negative with ND5-2**	**Date of qPCR**
Energieriegel Zartbitterschokolade & Orange	20 % *Acheta domesticus*	SENS Foods	Yes	25.05.22
Insektensnack, Salzige Schokolade	6 % "Cricket flour"	INSTINCT	Yes	25.05.22
Insect-pasta	10 % *Alphitobius diaperinus*	PlumentoFoods	Yes	31.05.22
Grannola Himbeer und Kürbiskerne	5% Alphitobius diaperinus	Jiminis	Yes	31.05.22
Cricket burger	10% *Acheta domesticus*	imago	Yes	31.05.22
Dinkelbrot mix	10% *Acheta domesticus*	imago	Yes	31.05.22

Results of the SYBR® Green-experiment

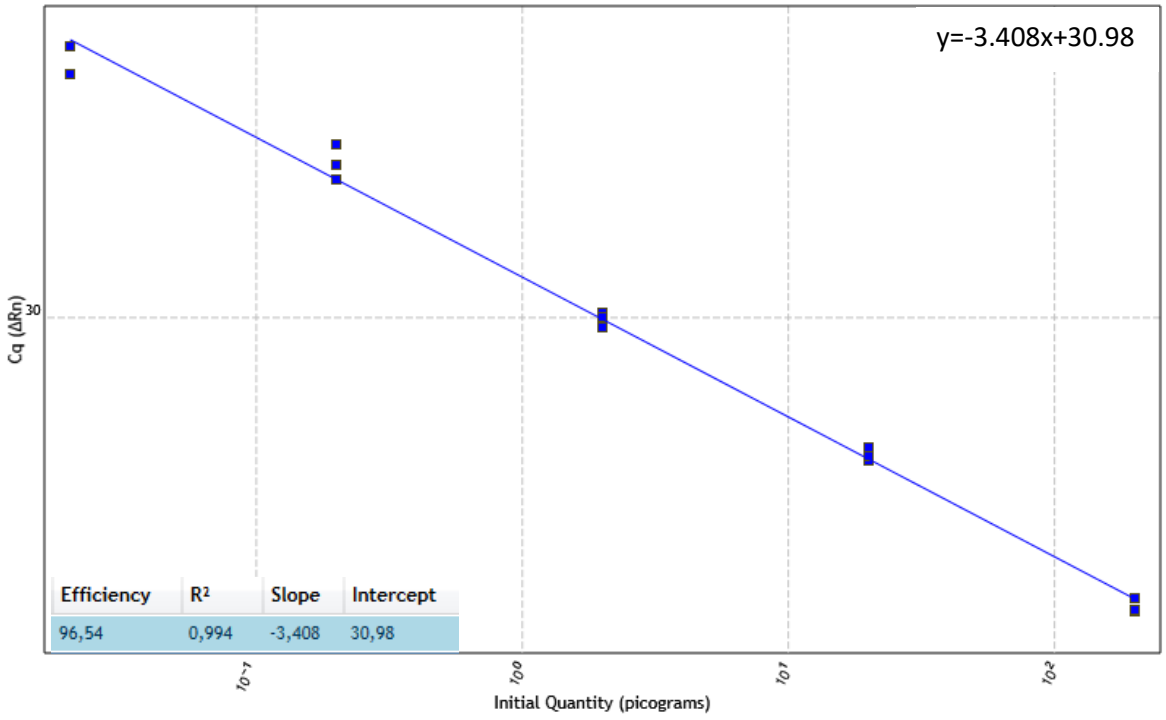
Species	**C_q with BimND5-2 (17.03.22)**	**C_q with BimND5-3 (21.03.22)**
Acheta domesticus	No	No
Gryllus assimlis	No	No
Gryllus bimaculatus	17.76	33.01
Gryllus sigillatus	35.81	No
Locusta migratoria	No	No
Meconema meridionale	38.67	No
Oxya yezoensis	No	No
Schistocerca gragaria	No	No
Tarbinskiellus portentosus	33,08	No
Teleogryllus derelictus	35.83	No

Efficiency results (03.05.22)

75 nM:



100 nM:



Eidesstattliche Erklärung

Ich versichere, dass ich die vorliegende Arbeit ohne fremde Hilfe selbstständig verfasst und nur die angegebenen Hilfsmittel benutzt habe. Wörtlich oder dem Sinn nach aus anderen Werken entnommene Stellen sind unter Angabe der Quelle kenntlich gemacht.

Hamburg, 28.09.2022 [redacted]