DEVELOPMENT OF A NEXT GENERATION SEQUENCING (NGS)-BASED METHOD FOR THE IDENTIFICATION OF INSECT SPECIES IN FEEDS

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INTRODUCTION

The recent EU Regulation 2021/1372 allows a shortlist of seven insect species (Hermetia illucens, Musca domestica, Tenebrio molitor, Alphitobius diaperinus, Acheta domesticus, Gryllodes sigillatus and Gryllus assimilis) to be included in the formulation of feeds for aquaculture, poultry and pigs. The introduction of this new Regulation raises the issue of switching from a classical detection method based on microscopy to a more sophisticated and species-specific method. Molecular investigations using DNA guarantee enough specificity for species identification. Next Generation Sequencing (NGS) technology has already been established for several years, with applications in food and feed authenticity testing increasing. DNA metabarcoding exploiting the high-throughput capacity of NGS platforms is a powerful tool for characterising biodiversity. Within this project, an NGS method for differentiation of insect species in feed was established.

METHODOLOGY

The protocol proposed in this project is based on the amplicon sequencing of a small barcode region included in the COI gene, a mitochondrial gene well known to be able to discriminate animals at species level and already tested successfully on insects. The planned work comprised: exhaustive *in silico* and phylogenetic literature research using library and gene databases (e.g. the National Center for Biotechnology Information, NCBI; and the Barcode of Life Database, BOLD) to derive sequence information and alignments with appropriate software; sample collection, for the creation of an extensive reference set of the German National Reference Laboratory for animal protein in feed (NRL-AP); preparation of admixtures of insects and feeds; DNA extraction from pure materials and compound matrices; end-point PCR for the setting up of the best operative conditions and evaluation of the DNA quality; setting up of the NGS-based metabarcoding protocol and of the bioinformatic pipeline for the analysis of the sequence; sequencing of the barcoding regions of selected insect species; testing on model and commercial feeds formulated with insects.

RESULTS

Two sets of primers, covering different parts of the COI barcode and producing amplicons with different lengths, were used in combination. The simultaneous employment of both primer sets increased the amount and improved the quality of the information collected during the analysis. The tested insect species in fact demonstrated a different ability to produce amplicon reads according to the employed primer. When DNA isolated from pure insects was used as a template, one or more species were always recognised, and the longer amplicon resulted overall in the most informative one in terms of number of correctly assigned reads. Even when DNA was extracted from commercial feeds not containing insects as a main ingredient, several reads were assigned to insects or other arthropods, most probably present as environmental contaminants in the feed. When unapproved insect species were used for the preparation of model feeds, they were identified down to a certain level of incurrence, which depended on the species.

DISCUSSION

NGS-based DNA metabarcoding is a powerful tool for characterising the biodiversity. The protocol developed showed a high sensitivity and a good ability to discriminate insects at species level. Due to the availability of extensive reference databases, the target COI represents the best choice of barcode. However, the barcoding region is highly variable and the primers show differential affinity, which is reflected in the number of reads assigned to a given species. This number is not representative of the starting quantity of template; therefore the protocol is not quantitative. The co-presence in the sample of species for which the primers have differential affinity may lead to underrepresentation or even to the loss of some species. Another major limitation is that the unintentional presence of small amounts of insects contaminating the feed cannot be easily distinguished from a fraudulent formulation using unauthorised insect species. Since the current EU Regulation does not indicate a maximum tolerated amount of prohibited insect material, which could be translated into a zero-tolerance policy, this represents an issue for the application of the method.