

Our study suggests that automatic monitoring of the infestation state in bulk grain is feasible in small containers. This kind of service can assist reliable decision making if it can be transferred to larger storage establishments. Very soon and with further technological development (e.g. piezo electric sensors embedded in cables submerged in the grain) the acoustic methodology can provide a quick and easy way, not only of detecting, but also of estimating pest population density in larger establishments of stored grain facilities.

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PHID-Coleo - a database identification tool for wood-boring beetles in plant health interceptions

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Abstract

Recent examples for the introduction of wood-breeding beetles in Europe include the asian longhorn beetles *Anoplophora* spp. and *Aromia bungii* (red-necked longhorn beetle). These and other woodboring beetle species pose a high risk of economic damage to trees and wood products. Smaller beetles like the powderpost beetles from the families Bostrichidae and Lyctidae also have the potential for causing considerable damage. These are often not identified adequately during inspections of wood packaging materials, making it impossible to assess their risk for becoming invasive. This project will aim at closing that gap. Our project PHID-Coleo (= Plant Health Identification of Coleoptera) has the objective to develop new diagnostic tools for the identification of potentially invasive and economically important beetles that can be found in wood packaging materials. The identification methods include classical identification keys based on morphological characters as well as molecular methods based on DNA analysis by PCR (barcoding). The methods for species identification will be supplemented by molecular analyses of introduced populations to clarify within species variations. Such methods will make it possible to determine the taxonomic relationship of samples from different areas and to draw conclusions about the introduction pathways, resulting in more efficient monitoring of the invasive species and preventing their spread. PHID-Coleo will build a freely accessible database of relevant species which are potentially invasive.

Keywords: Cerambycidae, Bostrichidae, morphological diagnostics, molecular diagnostics, population analysis.

1. Global trade: introduction pathways for potentially invasive insect species

An increasing number of imported goods from all over the world are reaching European ports every day. These are often accompanied by untreated wood in the form of wood-packing material (WPM). The international transportation of WPM (e.g. as wooden dunnage, pallets or crates) is recognized as an important pathway for the introduction of non-native harmful insects. For this reason, specific international phytosanitary measures (International Standard for Phytosanitary Measures No. 15 or ISPM15) have been developed by the Food and Agriculture Organization of the United Nations (FAO) within the framework of the International Plant Protection Convention (IPPC) (IPPC, FAO 2013).

To prevent the introduction of harmful species this standard specifies that wooden materials (> 6 mm) used for shipment have to be free from living organisms, when being exported into a country following the ISPM15. In practice, this usually requires WPM to be debarked and then heat treated or fumigated. If such treatments have not been done adequately, wood boring insects may survive in WPM and then be introduced into the importing country. Therefore, inspections still need to be conducted at European ports. These inspections are aimed at an accurate identification of any intercepted species as well as preventing them from entering the country. But infested WPM are not always recognized properly and intercepted during the inspections at the import control and of course only in random samples. In such cases, non-native species can enter the country, may survive under suitable conditions (e.g. the availability of suitable hosts) and become established. This can result in serious damage to crops or forest trees. Recent examples of such introductions into Germany are the Asian long-horned beetle (*Anoplophora glabripennis*) (Mühleisen & Zimmermann 2016) and the rednecked long-horned beetle (*Aromia bungii*) (LfL Bayern 2018).

In case a new species is repeatedly found in the field, several possibilities need to be considered and distinguished, including whether a) the species is already established, b) there have been repeated introductions or c) the samples are related to each other. Identifying which of these possibilities apply with new molecular tools can help to improve the monitoring of invasive species and preventing their spread.

2. The problem of species identification and the necessity of pest risk analysis

The species identification is necessary, because it is the basis for pest risk analysis (PRA) conducted by the regulatory national plant health organization (e.g., the Julius Kühn-Institute for Germany). The PRA analyses the risks and possible consequences of the introduction of a new pest, under inclusion of biological (e.g. climatic tolerance, available hosts) and other scientific information. It also identifies the phytosanitary measures required to protect plant resources against a new

potential pest. In case of an introduction event, the PRA helps decision makers to react quickly and in the most suitable way (JKI - Julius Kühn-Institut 2018).

The morphological identification of non-native species is difficult, since identification keys are often unsuitable for the inspection teams, are unavailable, or are only available in a foreign language (Ohbayashi & Nisato 2007). This is especially the case for the immature stages of frequently imported groups of insects like Cerambycidae and Bostrichidae (Wang 2017, Geis 2002).

Another problem arises, when the sample is in a bad physical condition (e.g., a crushed larva) and identification traits have been lost. A molecular identification based on DNA barcoding analysis can help in such cases (Wu et al. 2017). For this, a genetic marker (a short sequence) of the specimen's DNA is compared with DNA references from an online database. If both DNA sequences match, the examined sample is most likely the species that has been deposited as reference in the database. Unfortunately, in databases such as NCBI there are error rates of up to 20%, because some species had been misidentified before they were barcoded (NCBI 2018). Such erroneous references must be recreated to meet quality standards and accreditation requirements in diagnosis.

At the same time other databases like the Barcode of Life Database, BOLD, with higher quality standards are yet often incomplete (Ratnasingham & Hebert 2007). Also, the Q-BOL Project which covered a broad range of organism groups of pests and diseases and resulted in the database Q-Bank, still does not include e.g. important Cerambycid beetles such as *Batocera lineolata* or *Saperda candida* (Q-Bank 2017). In Europe the important PM7 diagnostic protocols for identification are usually limited to some more relevant species that are already regulated (EPPO 2018). Available molecular references for species associated with WPM are currently very limited. Therefore, relevant species that have been found in WPM so far and are likely to be mistaken with similar species have to be barcoded to fill that gap and to build up reliable datasets for their use by diagnostic laboratories.

3. The project activities of PHID-Coleo

The project PHID-Coleo (**Plant Health IDentification of Coleoptera**) has been designed in Germany as a cooperative project between the plant protection service of Baden-Württemberg in Karlsruhe (LTZ) and the University of Hohenheim (UHOH). The project was launched in 2017 and runs for three years until June 2020 (Bauer & Zimmermann 2018).

PHID-Coleo aims at developing new diagnostic tools for the identification of potentially invasive and economically important beetles which are associated with WPM. In addition, it aims to develop new molecular methods for the analyses of already established exotic species. Introduction pathways and relationships between existing populations of invasive species need to be investigated as fast as possible to predict the invasive potential and to prevent economical damages.

The activities of the project are divided into three sections, with sections one and three being implemented by the Centre for Agricultural Technology Augustenberg (LTZ), Department 33, Zoological Diagnostics in Karlsruhe, Germany and section two by the Departments of Live Stock Population Genomics and Applied Entomology at the University of Hohenheim, Germany.

Project activities - Section 1: Morphological and molecular key

The activities under section one aim at new identification tools for potentially invasive and economically important species of false powderpost beetles and long-horned beetles (Coleoptera: Bostrichidae and Cerambycidae, respectively). According to the European phytosanitary alert system EUROPHYT these are the most important groups of insects that can be found in WPM (EUROPHYT 2018). For this, classical identification keys based on morphological characters are being developed for the relevant species of these groups. The keys will not only consider the adult beetles but also the immature stages, because usually only the larvae are found in WPM.

The classical keys will be supplemented by molecular methods for species identification. This will make it possible to deal with the smallest tissue samples and physically damaged specimens. Thus, species can be identified quickly and easily in the laboratory.

In collaboration with entomologists and according to recently published species lists (Geis 2014, Eyre and Haack 2017), the project partners have identified more than 100 species, which were confirmed to be associated with WPM. These are currently being documented photographically and molecular references are being developed. The keys will also be available in a printed version and molecular data will be published as well in established online barcoding databases.

Project activities - Section 2: molecular analysis of insect populations

The activities under section two aims at developing a detailed understanding of the population dynamics of invasive beetles and their dispersal. *Anoplophora glabripennis*, the Asian long-horned beetle (ALB) is serving as a model example. Investigations of intraspecific nucleotide variations should help to understand the relationship between populations found at different infestation sites, e.g. in Germany.

Genetic markers for the molecular comparison of individual populations of invasive species will be selected and provided for diagnosis (Hasselmann et al. 2015). They should help plant protection services to trace and identify new infestation events and to understand introduction pathways. A higher resolution of the ALB population structure will be achieved by using a larger amount of genetic markers for mitochondrial and genomic DNA. A set of molecular tools such as sequencing, conventional microsatellite analysis and state-of-the-art single-nucleotide polymorphism screening (Nolte et al. 2005, Gruber et al. 2013) will provide a broader spectrum of genetic markers than available now. For the genotype analysis specific software for measuring genetical differences will be used (Pritchard et al. 2000).

The project partners are collecting genetical material of the ALB from different infestation sites in Germany, as well as from other countries for comparisons, including Europe and the native habitat of this species in Asia. Research results about the intraspecific genetic differences of ALB populations indicate that there are variations in the D-Loop region and the cytochrome oxidase subunits 1 and 2 of the mitochondrial genome, as well as in the microsatellite regions of the genomic DNA. A preliminary analysis with microsatellites in the PHID-Coloe project showed promising results. The genome of the ALB was 'screened' and approximately 700 regions with tandem repeats have been found, of which 25 microsatellites had been tested, that have not been used in ALB-studies so far. After sequencing, eight of them showed considerable differences in length and in the number of so-called repeats in comparison to the provisional reference genome (McKenna et al. 2016).

Project activities - Section 3: open identification keys and an expert network for identifying beetles intercepted during plant health inspections

Under section three, the results obtained under the previous two sections will be published as booklets, printed identification keys, and as well as online in the form of a freely accessible database for interested scientists, plant health services and zoological diagnostic laboratories. Diagnostic workshops will also include training for companies that provide barcoding services. Workshops and single training will be offered during the project, but also beyond the term of the project. Interested parties may contact the PHID-Coleo partners.

A further aim of the project is to establish a long term collaborative network in the field of molecular pest diagnosis which will include plant protection professionals, entomologists, research scientists (e.g. from state institutes and museums) as well as commercial companies. This collaborative network should continue its activities after the end of the project for a quick and safe identification of future interceptions of unknown insect species.

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