Final Report

Project Title (Acronym)
Further Development of Risk Management for the EC listed *Anoplophora* species, *A. chinensis* and *A. glabripennis* (ANOPLORISK-II)

Project Duration:

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2. Executive Summary

Project Summary

Further Development of Risk Management for the EC listed *Anoplophora* species, *A. chinensis* and *A. glabripennis* (ANOPLORISK-II)

There is a high threat of harmful impact arising from material infested by *Anoplophora chinensis* (Citrus longhorned beetle; CLB) and *Anoplophora glabripennis* (Asian longhorned beetle; ALB) within EU territory. While interceptions of CLB with plants for plantings have been reduced in the last years due to recommended measures according to the implementing decision 2012/138/EU of the EU Commission, ALB is intercepted in wood packaging material in continuously high numbers. Measures against these species require import inspections as well as eradication or containment measures demanding high personnel and financial resources. More readily available and validated detection methods are important. This project followed up promising methods for detection and identification that were developed in the previous project ANOPLORISK. In order to connect proper usage of available methods with measures required according to EU legislation, contingency plans for both, ALB and CLB were developed for Germany that could serve as models for other European countries.

Detection methods for wood boring stages

The use of detection dogs was evaluated and further developed. Two sets of experiments in double blind trials were carried out to quantify sensitivity and selectivity of dog detection towards ALB scents (frass, living larva, infested wood) using 10 and 14 dogs, respectively. Under standardized abstract conditions dogs showed a sensitivity of 85-93 % and a specificity of 79-94 %. Experiments under field conditions in a young poplar plantation and an old orchard ascertained an overall sensitivity of 75-88 % and a specificity of 85-96 %. The study demonstrated the ability of well trained dogs to detect ALB scents in both standardized testing ground and realistic conditions and, therefore the feasibility of canine scent detection as one complementary method for detection of woodboring stages of ALB. A guideline for the use of detection dogs was developed and included in ALB and CLB contingency plans for Germany.

To further explore the possibilities of acoustic detection of woodboring stages of ALB new sound recordings and analysis with methods developed in ANOPLORISK were carried out. These showed the limitations of these available methods, mostly due to difficulties with background noise and the need for manual analysis of all material. Therefore, sound recordings were sent to Sejona GmbH (Germany) to test their analytic software. The material was not feasible for automated analysis because the nature of the recordings did not meet the requirements of the software. Further improvements in the method and financial inputs would be required before acoustic detection could be considered a realistic option for either wide-area monitoring or interception monitoring for ALB/CLB at ports of entry.

Temperature recordings to inform models for larval development under UK conditions were performed in three *Salix* species. Temperatures in trunks and branches were very close to ambient air temperature, with extremes being more pronounced in the latter condition. The lack of differences was unexpected given the results from previous analysis in *Betula* trees in the UK, which found higher temperatures in the trunk. This demonstrates the complexity of trying to model potential development rates of xylophagous insects for different localities and host trees.

Detection of adult ALB with pheromone baited traps

Traps and lures for ALB that were developed in North America were tested in outbreak areas in the UK and Austria. Teflon coated cross vane traps baited with a lure consisting of ALB pheromone compounds 4-(n-heptyloxy)butanal, 4-(n-heptyloxy)butan-1-ol, and host tree volatiles were deployed in 2014 and 2015. In the UK, no ALB was caught in 18 traps; in Austria no ALB was caught in 20 traps in 2014 but one female ALB was caught in 27 traps in 2015. Additionally, two native cerambycid species were caught in Austria, none in the UK. The low catches were not unexpected based on

experiences from North America. Due to eradication measures at both sites the ALB populations were likely low or absent. Despite low numbers of collected beetles, traps can be recommended as one additional tool for surveillance in outbreak areas.

Additionally, ALB traps as well as traps for *Monochamus* spp. were deployed at major stone importers, considered high risk sites for introduction. No ALB were caught. On the other hand, the *Monochamus* lure proved its power, and the utility of this technique; four *M. galloprovincialis* were caught in a total of five traps. Additionally, 15 cerambycid species (among them one non native) were caught, suggesting the value of further development of trapping with generic lures in high risk areas. A feasibility study of the use of smart traps for remote monitoring concluded that the technology is available but it is still at too early stage for wider use. A review in a few years is recommended.

Molecular tools for diagnosis

Loop-mediated amplification (LAMP) assays were validated on samples of ALB and CLB as well as native wood borers using CO I as genetic marker and a GENIE II instrument for amplification. Analytical sensitivity of 100 % was achieved for fresh larvae of ALB, CLB, and *Aromia moschata*; assays for *Saperda carcharias* failed. Dry material from beetles was detectable after 45 min but failed after 30 min for ALB and CLB; no detection was achieved for *A. moschata*. The specificity was excellent for CLB with no false positives for other species. The ALB assay showed false positives for *Rosalia alpina*, *Monochamus* spp., and CLB. Analytical repeatability was 100 % for CLB and *A. moschata*. ALB on the other hand, showed weaknesses at the lower detection limit. LAMP assays for ALB and *A. moschata* need some redesign for improvement of sensitivity and specificity. The assay for CLB, however, showed the strength of LAMP assay as a powerful tool for on-site diagnosis. Detection of DNA in wood shavings and frass was problematic because of the low amount of intact beetle material (DNA) vs. wood material.

As a method for quick determination, PCR-RFLP analysis of ALB and CLB had been established in the previous project ANOPLORISK. The analysis was extended to more species. Now RFLP data are available for 5 native cerambycids (including *Lamia textor*, which can be easily confused on visual inspection with ALB or CLB in the larval stage) and 2 xylophagous Lepidoptera that are frequently encountered in trees during ALB surveys, as well as 3 Asian cerambycids that were found in imported wood packaging material.

Development of contingency plans

One important objective of this project was to provide recommendations for measures against ALB and CLB based on literature review and existing eradication plans as well as to explain and concretize measures according to EU legislation for the plant protection services. As a main output, contingency plans for ALB and CLB were developed for Germany, which could serve as examples for other countries. The plans provide details and explanations on measures such as confirmation of infestation, movement of wood for destruction, demarcation of infested zone and buffer zone, criteria for exemption from the obligation to fell trees in the infested zone and alternative measures, monitoring procedures from ground and in the crown, the use of detection dogs, etc. The contingency plans consist of a main part comprising (1) aim, background and legal basis, (2) detection with flow chart from suspicion to diagnosis, and notification of the outbreak, (3) measures to be implemented after confirmation of an outbreak, (4) contacts and addresses, and of (5) annexes with comprehensive and detailed information. The plans serve as basis for planning eradication measures by the plant protection services of the German Federal Länder and have been agreed and accepted on high political level in all 16 responsible ministries in the Länder.

3. Report

Introduction

Longhorn beetles of the Genus *Anoplophora* originating in the Far East (primarily China, Japan and Korea) (Lingafelter and Hoebeke 2002), are wood-boring cerambycid beetles, with larvae feeding in the phloem-cambium region during the early instars and boring into the xylem of trees in later instars. They are highly polyphagous on trees and shrubs, and are able to colonise and kill both weakened and healthy plants. These xylophagous insects are able to complete their development in very small host material within one or two years depending on climatic conditions (Adachi 1994). There is a continuously high threat of harmful impact in EU territory arising from the increase of material infested by *Anoplophora chinensis* (Citrus longhorned beetle; CLB) and particularly *Anoplophora glabripennis* (Asian longhorned beetle; ALB) being imported and subsequently intercepted in nurseries and in wood packaging material. Evidence of ability to establish in the EU is provided by the list of new host plants attacked in Italy, France, The Netherlands, Germany and Austria in nurseries and also in gardens, public parks and woodlands (Haack *et al*., 2010). The EU responded to the increasing threat with Commission Implementing Decisions 2012/138/EU for CLB and 2015/893 for ALB. Both require import inspections as well as eradication or containment measures requiring high personnel and financial resources. More available and validated detection methods are important.

In the EUPHRESCO project on risk management of *Anoplophora* species (ANOPLORISK) from 2010 to December 2012 various methods for detection and diagnosis of *Anoplophora* were developed and tested. Some methods have been very promising; however, it was apparent that it will be important to refine and validate the results. ANOPLORISK-II followed up on selected methods. Additionally, it was one objective to include the new methods in contingency plans for ALB and CLB that were developed for Germany and could serve as example for other member states.

Objectives

The objectives for ANOPLORISK-II were, to refine and validate a number of techniques that will help with detection and diagnosis and to enable a set of management/detection techniques to be incorporated into contingency and management plans.

- Refine and evaluate the use of detection methods for woodboring stages, such as detection dog and acoustic detection methods
- Test traps and lures for detection of adult beetles
- Further develop molecular tools for diagnosis of *Anoplophora* spp. and other cerambycids and evaluate (incl. technology transfer) existing tools developed in other research projects
- Development of contingency plans for ALB and best practice manuals for the developed and validated detection/diagnosis tools based on the research undertaken in the project

Work Plan

The project was organized in four work packages (WP) that were run concurrently. Each WP had clearly defined objectives and required input from more than one partner.

WP 1 Project Management and Co-ordination

WP 2 Detection methods for woodboring stages

WP 3 Detection of adult beetles with pheromone baited traps

WP 4 Molecular tools for diagnosis

WP 5 Development of contingency plans for ALB and CLB

WP1: Project Management and Co-ordination

Objectives

- Ensure the smooth running of the project and encourage cross partner cooperation
- Ensure that milestones & deliverables across WPs are on target
- Ensure that all reporting obligations are met
- Facilitate the interaction with researchers from countries outside of the consortium

Participants
This WP was led by P1 with close interaction of all partners.

Deliverables
D1.1: Final report

Milestones
M1.1: First project meeting
M1.2: Second project meeting
M1.3: Final project meeting
M1.4: Final report

Three consortium meetings were held throughout the project, in which all work package leaders as well as core members of the research team participated. Progress and plans for experiments were discussed, cooperative work was coordinated:

- First project meeting 17 July 2014 at BFW, Vienna
- Second project meeting 26-27 May 2015 at JKI, Braunschweig
- Final project meeting 27-28 January 2016 at JKI, Braunschweig

To ensure smooth running of the project, the project leader was in regular e-mail or telephone contact with the other WP leaders as well as among the consortium members. No formal teleconferences were deemed necessary. Due to the relatively small size of the project consortium, also detailed issues could be discussed at the project meetings. There was one additional meeting for WP4 to transfer LAMP technology from FERA to JKI (see WP4 report). Consortium members established contacts with researchers in other ALB affected areas, such as Bavaria (Germany), Lombardia (Italy), and the U.S.A. The U.S. Forest Service provided valuable information on trapping ALB and established contacts with the company producing traps and lures tested in the project. Results of the research were presented at international conferences as well as national plant protection meetings.

WP2: Detection methods for woodboring stages

Measures to prevent introduction and the spread of CLB and ALB have been defined within the two emergency decisions 2012/138/EU and 2015/893/EU, respectively (European Commission, 2012 and 2015). Import inspections as well as eradication or containment measures require enormous personal and financial resources. Therein the visual detection of infestation is particularly challenging because externally visible symptoms may be hidden or removed due to environmental influences. Intensive surveys have to be carried out in outbreak areas and in high risk areas like importing companies and their vicinity. Visual inspection, both from ground and by tree-climbers, is the standard procedure. Additional methods are desirable.

In this project we further tested and refined two non-destructive detection methods that proofed to be useful under operational conditions in the preceding EUPHRESCO project ANOPLORISK: Canine scent detection and acoustic detection.

Objectives
1. Further development and evaluation of the use of detection dogs
2. Further development acoustic detection methods

Participants
The WP was led by P1 and had significant input from P3. Objective 1 was mostly carried out by P1, objective 2 by P3.

Deliverables
D2.1: Report on the further development and evaluation of detection dogs and acoustic detection methods

Milestones
M2.1: Complete quantitative studies in defined test situations to evaluate sensitivity and specificity of using detection dogs for ALB
M2.2: Complete tests on factors that impact on the sensitivity and specificity of acoustic detection for ALB
M2.3: Complete additional temperature recordings to help inform ALB biology under UK conditions

Objective 1: Further development and evaluation of the use of detection dogs

Anoplophora detection dogs have been trained at BFW since 2009. Dogs proofed to be able to detect scents of all developmental stages of ALB and CLB as well as empty galleries, exit holes and overgrown oviposition sites. Subsequently, *Anoplophora* detection dogs were employed in outbreak areas, in nurseries and at import controls for wood packaging material and plants in Austria, Germany, Italy, Croatia, United Kingdom, the Netherlands and Switzerland and the method has been continuously refined in practice. Until the end of 2015, a total of 77 dogs and 62 dog

handlers from Austria, Germany and Switzerland have been trained and certified by the BFW. NPPOs or ministries partially funded training of dog teams. Despite the regular use of detection dogs in Europe experimental quantitative data have been lacking. In order to close this gap, this study tested the sensitivity and selectivity of trained ALB detection dogs towards different scent sources of ALB (larvae, frass and wood shavings, infested wood) under abstract as well as realistic conditions.

Materials and Methods

Two test series with 14 and 10 dogs, respectively, where carried out in October 2014 and February 2015. All dogs and dog handlers in this test were trained at BFW between 2009 and autumn 2014. Dogs were of various breeds and had different levels of experience in ALB detection work due to the time of initial training (Tab. 2.1).

Table 2.1: Breeds of dogs (number of dogs in parenthesis) employed in the Test series

Hunting dogs	Brandlbracke (3), German shorthaired Pointer (1), German wirehaired Pointer (1), Small Munsterlander (1), Labrador Retriever (3), Lagotto Romagnolo (3), Petit Bleu de Gascogne (1), Tiroler Bracke (1)
Shepherd dogs	Australian Shepherd (1), Border Collie (2)
Others	Crossbreed (1)

All tests followed the same basic setup. In each test, a dog had to examine eight samples, two of which contained positive ALB scent material; six were negative (i.e. without ALB scent material). Positions of the samples were randomized. Each test was repeated three times. For each repetition samples were placed in new random order. For blind testing, scent material was well hidden and positions were unknown for dog handlers and dogs. ALB material originated from the Austrian outbreak area Gallspach. The tests were carried out outdoors; air temperature and wind speed and direction were recorded.

A first test series was carried out under standardized field conditions in Aggstein, Austria, in October 2014. Fourteen dogs and their handlers took part. We tested (1) ALB frass and wood shavings hidden in the grass at the base of young poplar trees in a plantation, (2) ALB frass and wood shavings wrapped in filter paper and put into a ventilated, dark PET tube mounted at 1.8 m height on the stem of a poplar tree (negative samples contained filter paper only), and (3) ALB frass and wood shavings hidden in holes or crevices in the bark of two old orchard trees at approximately 1.8 m height. For each test, two trees were randomly selected for placement of positive samples, six were negative. Air temperatures ranged from 13°C to 22°C; it was dry and partially sunny.

A second test series was carried out under abstract, standardized conditions in Ossiach, Austria, in February 2015. Ten dogs and their handlers took part. Therefore, scent materials were placed in hollow concrete building blocks and covered with a wooden lid with eight 1-cm diameter holes. We tested (1) ALB wood shavings and frass, (2) living ALB larva, and (3) living ALB larva on a piece of infested wood (negatives with uninfested wood). Eight concrete blocks were lined up in a row; two contained positive, six contained negative samples. A search in a more complex environment was performed between test 1 and 2 to allow for refocusing of the dogs; two pieces of ALB infested wood (i.e. wood pieces only with ALB larval galleries, exit holes and/or wood shavings and frass, but without living stages) were hidden under 4-m long, piled spruce logs. Air temperatures ranged from -2°C to 8°C; conditions were dry, but remaining snow existed.

To characterize searching success, the following values were computed:

Sensitivity = number of correctly identified positives / total number of positive samples X 100
Specificity = number of correctly identified negatives / total number of negative samples X 100

Values per dog were based on three runs per test (i.e. total of 6 positives and 18 negatives). The non-parametric Kruskal-Wallis test followed up by pairwise post-hoc Mann-Whitney U tests (corrected for type I error) was used to compare sensitivity and specificity between tests with dogs being the replicate.

Results

Test under standardized field conditions: When searching for ALB frass hidden in the grass at the base of young poplar trees in a plantation, overall sensitivity was 88.1 % and specificity 95.6 %. Frass in test tubes with filter paper (negative samples consisted of test tubes with filter paper only) placed on the stems of young poplars at 1.8 m height was detected with 75.0 % sensitivity and 86.5 % specificity. The lower success occurred because some dogs responded to the visual cue of the tubes on the trees leading to premature termination of the search. In the most complex environment, where frass was hidden in holes and bark crevices of old fruit trees in an orchard, sensitivity was 83.3 % and specificity was 85.0 %. Sensitivity values did not differ significantly between the test situations (Kruskal-Wallis Test: $\chi^2 = 2.967$, df = 2, P = 0.227). Difference in specificity values was more pronounced (Kruskal-Wallis Test: $\chi^2 = 91.988$, df = 2, P = 0.010), due to significantly lower specificity towards frass in the old orchard compared to frass on the ground of the poplar plantation (Fig. 2.1).

Under abstract, standardized conditions, dogs detected ALB frass or a piece of infested wood plus larva with a mean sensitivity of 91.7 % and 92.6 %, respectively. Specificity was 85.6 % and 94.4 %. Searching ability for living larvae alone was slightly lower; sensitivity was 85 %, specificity 79.4 %. Sensitivity towards the three different scent materials did not differ significantly (Kruskal-Wallis Test: $\chi^2 = 3.484$, df = 2, P = 0.175). Specificity differed significantly ($\chi^2 = 99.751$, df = 2, P = 0.0068) (Fig. 2.1) due to significantly higher specificity towards larvae plus infested wood compared to larvae alone. The search for the infested wood under the piled spruce logs was highly successful; the samples were detected in 95 % of the cases.

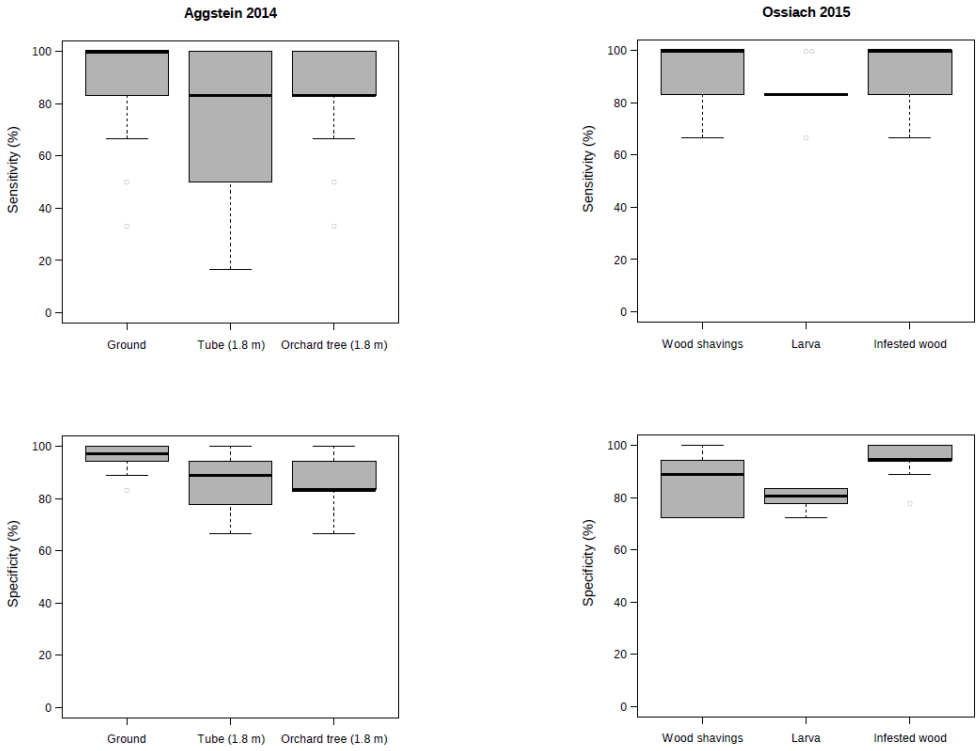

Fig. 2.1: Left: Sensitivity and specificity from tests under standardized field conditions. ALB wood shavings and frass were hidden in the following places on/near tree stems: in the grass at the base of a stem, in a test tube at 1.8 m height, in holes or crevices in the stems of old orchard trees at ca. 1.8 m height. n = 14 dogs. **Right**: Sensitivity and specificity from tests under abstract, standardized conditions. ALB wood shavings plus frass, ALB larvae, and infested wood plus larvae were used as scent materials in the tests. n = 10 dogs.
Figures from Hoyer-Tomiczek U., Sauseng G. & Hoch G. 2016: Scent detection dogs for the Asian longhorn beetle, *Anoplophora glabripennis*. Bulletin OEPP/EPPO Bulletin, **46:** 148-155

Discussion

This study demonstrated that trained dogs are able to detect ALB scents originating from frass, wood shavings or live larvae in abstract as well as realistic situations. The two test series were carried out with a total of 18 dogs (14 and 10 dogs, respectively) of 11 different breeds. This number is much higher than the average of 4.6 dogs reported in a literature review of studies on canine scent detection (Johnen *et al.*, 2013). The tests evaluated sensitivity and specificity as measures of the searching ability. As recommended by Johnen *et al.* (2013), samples in the tests were new to the dogs. Moreover, dog handlers were blinded in order to avoid any influence on the result of the tests (Lit *et al.,* 2011, Johnen *et al*., 2013).

The recorded mean sensitivity from 85.0 % to 92.6 % under abstract and from 75.0 % to 88.1 % under realistic conditions is comparable to values reported for dogs trained to find other insect species, such as red palm weevil, *Rhynchophorus ferrugineus*, with correct positive indications in 78 % (Suma *et al.* 2014). Reported sensitivity is higher towards bed bugs (97.5 %) (Pfiester *et al.,* 2008) or fire ants (98

%) (Lin *et al.,* 2011). The median sensitivity towards ALB frass and wood shavings or infested wood in our study was 100 % under abstract conditions. Also in the complex search situation in the old orchard, 6 out of the 14 dogs showed 100 % and 5 dogs showed 83 % sensitivity.

Placing samples at 1.8 m height did not lead to significantly lower finding success; sensitivity was not significantly different between the search for frass on the ground and in the old orchard. The lower sensitivity in the test with test tubes mounted on 1.8 m height is due to the fact that some dogs responded to the unknown visual cue of tubes on the tree. Five of them began to jump and snap for the tubes leading to premature termination of these tests. We did not test placement of samples higher than 1.8 m. But as is shown from training and from work in outbreak areas, dogs are able to detect infestations in the tree crowns up to 6 m or higher (Hoyer-Tomiczek & Sauseng, 2013 and personal observations). The success in the old orchard demonstrates that dogs handled the complex environment very well. Only the time required to complete the search was longer (5.0 ± 0.3 min vs. 3.3 ± 0.3 min).

The study did not test for the availability to discriminate ALB from other woodboring insects. Only in the situation in the old orchard, many of the negative trees were infested with native insects. They were not incorrectly indicated in a systematic way. One tree was the exception; it was falsely indicated by 64% of the dogs. Both, infestation with native insects producing overlapping scents as well as wind drift of the ALB scent from one adjacent positive tree are possible explanations. The ability for species discrimination has been shown for dogs searching for red imported fire ants, which were distinguished from other ant species (Lin *et al.,* 2011) as well as bed bugs (Pfiester *et al.,* 2008) or termites (Zahid *et al.,* 2012). Experiences from the practical use of dogs in ALB outbreak areas strongly suggest that such discrimination is possible. Trees that are infested with other woodboring insects are typically not indicated by *Anoplophora* detection dogs (Hoyer-Tomiczek, personal observation).

Overall, the test series provide for the first time quantitative data on detection of ALB by trained detection dogs. The dogs were able to detect ALB frass, larvae and infested wood under, both abstract and realistic conditions, illustrating the feasibility of canine scent detection as one complementary method in monitoring and survey for ALB.

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Objective 2: Further development acoustic detection methods

Studies conducted under the previous project, ANOPLORISK resulted in the design and testing of various sensors with different resonant frequencies and amplification factors for the acoustic detection of ALB and CLB. Sensitivity tests were used to determine optimal parameters. A series of 8-channel and 16-channel multiplexed systems were developed for use by Plant Health inspectors under practical conditions on imported Bonsai. Single sensor and USB-based sensors were also developed for potential field deployment and laboratory studies, respectively. A sound library of feeding sounds was created for 11 species of wood boring beetle (some taken during previous research projects) and an assessment made using a custom-developed 'bite discrimination' software. Discrimination between species was considered to be feasible in an earlier project, although the software to do this was no longer available. It was clear from the testing of the available software version that it was not quite fit for purpose. The main issues were (i) the software struggled with the large amounts of data from the Bonsai recordings, (ii) there were stability issues under circumstances when there is a lot of background noise (iii) false positives – the noisy environment of the Bonsai grower nurseries led to a very high percentage of false positives, and (iv) false negatives – many of the 'bites' in the test recordings of ALB larval feeding were not flagged as suspicious by the software. There was no expertise or opportunity for further refinement of the existing software. Hence, the new project would be constrained unavoidably by the availability and suitability of any commercially available software/freeware. A further limitation was the short supply of insect material (ALB/CLB) held under quarantine license conditions at the Fera laboratory (see below). Despite this, progress was made against the majority of the acoustic detection objectives.

Materials and Methods

Culturing of ALB and CLB

Asian (ALB) and Citrus (CLB) Longhorned beetles are quarantine pests in the UK, and were imported and held (cultured) under licence according to specified quarantine conditions at the Fera laboratories, Sand Hutton, York U.K. An initial consignment of 80 ALB larvae had been obtained from the European Biological Control Laboratory (EBCL), Montpellier-sur-Lez, France in June 2011. Only 52 (65%) survived transfer to the UK, and of these only 32 (40%) developed to adults.

From this limited collection, there was only one individual surviving at the commencement of the current project. A second consignment of 90 ALB larvae was collected from EBCL in October 2013. Unfortunately, the majority of these produced either deformed pupae or adults, which soon died, resulting in only a small number being available for acoustic recordings and/or breeding. A third consignment of 45 ALB and ~50 CLB larvae was therefore collected from the EBCL laboratory in October 2014.

Larvae were reared using an artificial diet (according to instructions provided by EBCL, Montpelier, France). This was is based, with some modifications, upon the *A. glabripennis* diet developed by Zhao *et al.* (1998), as specified in Dubois *et al.* (2002). In the modified diet, the phloem-cambium content from *Acer saccharum* trees was omitted, and was replaced by increasing the amount of cellulose fibre (Bio-Serv, New Jersey, U.S.A.) from 70 mg kg^{-1} (Dubois *et al.* 2002) to 195 mg kg^{-1}. The only other change was that ferric phosphate (amorphous, 0.2 mg kg^{-1}) was replaced by using either ferric ammonium sulphate (Sigma-Aldrich) at 0.55 mg kg^{-1}, or ferric citrate (Sigma-Aldrich) at 0.4 mg kg^{-1}. The ALB and CLB larvae were kept individually, in glass or plastic jars – containing the artificial diet. The lids of the jars had holes drilled for ventilation. The cultures were kept in a controlled environment room, protected by a cold corridor (4 °C) and double-door system (air-lock) within the quarantine facility at Fera. The cultures were maintained at 21 ± 2 °C, 65% relative humidity and under a 16 : 8 h LD photocycle. Where necessary (i.e. if not already implemented by the EBCL laboratories prior to dispatch), larvae were subjected to long-term chilling (~ 120 days at 4 °C) to promote diapause prior to pupation. Developing larvae were examined at regular intervals, and when the ratio of media to frass was found to have dropped to about 20% (approximately every 4 – 6 weeks) the larvae were transferred to clean jars with fresh diet. Larvae typically pupated on the surface of the medium, whereupon they were carefully monitored until ready for adult emergence (gradually turning from brown to black). Whenever an adult was about to emerge, it was placed in a larger ventilated plastic tub that contained a fresh twig of box elder maple (*Acer negundo*), and a water source. When the adult cuticle was fully tanned (hardened), the jar of larval medium was removed, and additional *A. negundo* twigs and leaves were added to the tub. Adults were kept isolated for between 7-10 days (sometimes longer) to feed and mature, before being released into a breeding cage (custom-built Perspex cage, see ANOPLORISK report). Fresh twigs of *A. negundo*, *Salix* sp. (willow) or *Acer platanoides* (Norway maple) were provided, standing in a tub of water within the breeding cage as food source, with a rooted cutting of *Salix* sp. for oviposition.

Acoustic monitoring

The various types of apparatus used for acoustic monitoring have been described previously (see ANOPLORISK report) and consisted of a number of single, dual, and multiplex custom-built sensors based on 2 kHz resonant piezoelectric transducers (Chesmore & Scofield, 2010) with digital recorder (Tascam DR-05), memory cards, cables and battery power supply. For longer term recordings (e.g. in the Bonsai tree recording reported previously), a compressed file format (MP3441. kHz, 256 kbps) was used, but for higher quality recordings made in this project an uncompressed 16 bit 44.1 kHz format was utilised. Recordings were transferred to PC for analysis, and were examined using freely available (open source) audio editing software, Audacity® ver. 2.1.1 (www.audacityteam.org). Selected samples from a range of

sound recordings were transferred to USB memory stick for transfer to Sejona GmbH, Kassel, Germany to be analysed further using a commercially developed 'sound analysis' software (courtesy of Martin Brandstetter, BFW, Austria and Sebastian Hübner, Sejona).

Results

Because of problems with insect supply and survival (see below), only a small number of sound recordings could be made in relation to the specified objective and Milestone (M2.2) to determine factors that impact on the sensitivity and specificity of acoustic detection for ALB. Ancillary recordings of additional species (woodlice, vine weevil, CLB) were made, but detailed analysis of these audio files using 'intelligent sound recognition' software awaits the results from the samples sent to Sejona GmbH, Germany.
From the consignment of 90 ALB larvae that was collected from EBCL in October 2013, the majority produced either deformed pupae or adults, which soon died, resulting in only a small number of survivors being available for acoustic recordings and/or breeding. From the consignment of 45 ALB (and ~50 CLB) larvae was collected from the EBCL laboratory in October 2014, 12 (27%) of the ALB larvae died during larval development, 9 (20%) died in the pupal stage; 5 resulted in deformed adults, and only 19 (42%) developed to normal adults. Of the 19 normal and surviving adults, there were 10 males, 4 females, and 4 initially of indeterminate sex (from visual inspection) that emerged over a period of 2.5 months. This rearing procedure yielded only 4 breeding pairs, with much of the effort expended having been concentrated on attempting to establish a viable culture. There were therefore very few ALB larvae and adults available for acoustic recordings during the lifetime of the project. The developmental problems (deformed pupae, adults) and poor survival of the batch of ALB obtained from EBCL in 2013 was believed initially to be in part due to these larvae having been 'overwintered', i.e. kept under chilling conditions for far too long (at least 12 months) before being transferred to the U.K. However, when similar issues (mostly poor progress to the adult stage) occurred with the third batch of ALB (2014), which were chilled in the U.K., and not prior to dispatch from EBCL, the possibility of some other reason needed to be examined. Of the likely alternatives, the dietary components and controlled environment conditions are regarded as most likely. Indeed, an elemental analysis of the agar component conducted at Fera suggests that there may have been issues with one or more of the batches (this is currently being further investigated).

The ALB recording samples that were sent to Germany for automated audio analysis were as follows:

(A) Larval ALB feeding samples (approximately 2-3 weeks after hatch)

These audio samples consisted of simultaneous mono recordings from 2 separate wood samples (both Norway Maple, *Acer platanoides*, i.e. sample 1 recording using the left channel, sample 2 recording on right channel (See photo, Fig. 2.2).

Fig. 2.2: Wood samples set-up for simultaneous audio recordings of ALB larval feeding

Left channel: ALB presumptive larvae feeding in Norway Maple that was placed in with adults 14/10/15 and taken out 30/10/15. Recordings made on 9/11/15

Right channel: ALB presumptive larval feeding in Norway Maple that was placed in with adults 14/10/15 and taken out 30/10/15. Recordings made on 9/11/15

ANO16C_0404.wav
ANO16C_0405.wav
ANO16C_0406.wav
ANO16C_0407.wav
ANO16C_0408.wav
ANO16C_0409.wav

(B) Adult ALB feeding on different tree species (Acer, Salix)

Recordings made from single wood samples with single ALB adult feeding (Fig. 2.3)

Fig. 2.3: Adult ALB feeding showing the recording apparatus attached to the sapling

(i) Recordings of adult feeding on *Acer negundo* (Box elder)

ANO8CH_1092.wav
ANO8CH_1093.wav
ANO8CH_1094.wav
ANO8CH_1095.wav
ANO8CH_1096.wav

(ii) Recordings of adult feeding on *Salix sp.* (Willow)

2CHANA_0036.wav

(C) A series of 'unknowns' for analysis by commercial software (various recordings)

Audio_A.wav
Audio_B.wav
Audio_C.wav
Audio_D.wav[a]
Audio_E.wav[b]
Audio_F.wav[b]
Audio_G.wav
Audio_H.wav
Audio_I.wav[c]

N.B. [a]Audio_D is a cycle of 7 samples. Recordings 1 min/sample per cycle. There is loud click to signal change of sample. The 8th sample is a recording of a watch used for navigation in long recordings.
[b]Audio_E and Audio_F are cycles of 7 samples. Recordings 5 min/sample per cycle. There is a loud click to signal change of ample, and double click 8th to 1st sample.
cAudio_F is a cycle of 15 samples. Recordings 5 min/sample per cycle. There is a loud click to signal change of sample. The 16th sample = silence, used for navigation in long samples. This wav file starts part way through sample 15.

Discussion

It has not yet been possible to discuss or derive any definitive conclusions from the acoustic analysis because the results from the commercial software analysis of the data files supplied are still pending. Problems occurred because the material was not feasible for automated analysis because the nature of the recordings did not meet requirements of the software. The only firm evidence at the moment is that manual interpretation of the data files is far too time-expensive (even for a very small number of recordings), as it frequently requires much longer analysis time than the recordings themselves. This is especially so for recordings containing a great deal of baseline noise (typical of field samples versus laboratory-generated), with the inherent difficulties of determining what may be significant sounds (e.g. insect feeding noises) above background.

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Objective 3. Complete additional temperature recordings to help inform ALB biology under UK conditions

Anoplophora sp. larvae develop within the sapwood and heartwood of their host trees. While *A. chinensis* tends to attack the base of the trunk of host trees or exposed roots (Maspero *et al.*, 2008), *A. glabripennis* tends to attack the upper trunk or main branches (Haack *et al*., 2006). At the outbreak of *A. glabripennis* in Kent, the beetle was found to attack branches varying in diameter between 2.1 and 61.5 cm, but the majority were in branches with a diameter between 4 and 10 cm (Straw et al., 2014). In order to evaluate the risks of a pest to a particular country and also the risks at different locations within a country, it is necessary to assess the climatic suitability of different locations. Some climatic models have been created to assess the threat posed by both *A. chinensis* and *A. glabripennis* to Europe and North America (Peterson & Vieglais, 2001); MacLeod *et al*., 2002; Peterson *et al*., 2004; Robinet *et al*., 2012; Ge *et al*., 2014). These models have made use of records of where the species have been found and also temperature response data (Adachi, 1988, 1994; Keena 2002, 2006; Keena *et al*., 2002; Keena & Moore, 2010).

The models mentioned above use air temperature as a predictor of environmental suitability for *Anoplophora*. However, there are differences between air temperature and the temperature within the trunk and branches of trees (Jamieson, 1957; Derby & Gates, 1966; Stockfors, 2000). Measuring and evaluating the temperatures recorded within trees will allow a better understanding on the potential development rates for *Anoplophora* in areas outside the locations where they are currently found.

In the EUPHRESCO ANOPLORISK project, temperature loggers were established with six *Betula* species at Sand Hutton near York, England. Three loggers were established near the base of trees that would be exposed to sunlight and three were set up at the base of trees that would receive little sunlight due to shading from surrounding trees. In that study, loggers were set up for a year and the annual number of day degrees (in excess of base 10°C) based on air temperature was 617 DD, for the shady trees totals ranged from 685-1018 DD and in the exposed trees 1153-1278 DD. Therefore, in all trees the day degree total was higher than that expected by air temperature, but the day degree total in the trees experiencing sunshine was around double the temperature of that expected by air temperature.

In the current study, temperature loggers were used again, but this time in three *Salix* sp. trees with one logger placed close to the base of the tree and three at the base of upper branches. Based on the experience of the previous ANOPLORISK study, the expectation was that the temperature in the tree trunk bases would be higher than that in the branches due to the greater exposure to sunshine.

Method

The method used in this study was similar to that used for the study in ANOPLORISK. Three trees at the National Agri-Food Innovation Campus (Fera) were used in the study. Logging began on 24th July 2015 and continued without any problems until 6th December 2015. On 6th December a fault developed in one of the loggers. This is thought to have been due to the tubing that had been put around the electrical cable which leads from the probe to the logger filling up with rain water.

Temperature data was recorded using Gemini Tinytag Plus 2 temperature loggers and Gemini Thermistor PB-5005-0M6 probes. Five of the loggers were the TGP 4020 model which only recorded the temperature of the temperature probe, a sixth was the TGP4510 which recorded both the probe temperature plus air temperature.

In order to set up the temperature loggers, holes were drilled to a depth of 44 mm into the trunk or branch using 2.5 and 3 mm drill bits. The temperature probes are 8 mm long and 2.5 mm in diameter, therefore the centre of the probe was 40 mm below the surface of the trees. All holes were drilled on the south side of the trees. The holes at the base were drilled c. 20 cm from the base of trees (a site typical for *A. chinensis*) and the holes in the branches were drilled at the base of one of the larger branches (see Fig. 2.4).

Fig. 2.4: Locations of temperature loggers in one of the *Salix* sp. trees

The probes were placed in holes and then the holes were then sealed with silicon door sealant. The leads going in between the probes and temperature loggers were surrounded by plastic tubing in order to protect them from rodent damage. The data loggers were placed in wooden bird nest boxes to keep the connections dry (see Fig 2.5). For the TGP4510 logger, as the air temperature was being measured by an inbuilt probe in the logger, a hole was cut in the side of the nest box and covered with a fabric mesh to allow air flow past this sensor. Loggers were set to record hourly temperature readings. Data from the loggers was downloaded periodically and converted from Tinytag format (TTD) to Microsoft Excel (97-2003) format using the Tinytag software. Degree days were calculated using the method published by (Baker, 1980). The threshold temperature used for the calculations was 10°C which was considered appropriate by Smith *et al.* (2004).

Fig. 2.5: Bird box used to house the temperature logger and lead going into the tree trunk surrounded by plastic as rodent protection

Results

A summary of the results of the recordings is shown in Table 2.2. The mean temperature over the course of the study was almost identical in the tree trunks bases (12.4°C) and the branches (12.5°C), both marginally higher than the recorded air temperature (11.8°C). The mean number of day degrees (in excess of 10°C) was almost identical in the trunk bases (380.3 DD) to the number calculated from air temperature (382.4 DD), the mean for the loggers in the branches was slightly higher (414.5 DD). The extreme high and low temperatures were most extreme for air temperature, followed by branch temperature and lastly by trunk temperature.

Table 2.2: Comparison of the tree temperature loggers set up at the base of three *Salix* sp. trees and in the branches

	Tree trunk base			Tree branches			Air	Trunk Mean	Branch Mean
Tree No	**1**	**2**	**3**	**1**	**2**	**3**			
Mean temperatue beteen 24 July-5 Dec	12.6	12.4	12.1	12.5	12.9	12.0	11.8	12.4	12.5
Extreme max. temperatue beteen 24 July-5 Dec	17.9	19.2	21.8	20.5	21.6	24.9	24.8	19.6	22.3
Extreme min. temperatue beteen 24 July-5 Dec	1.8	1.2	0.3	0.2	0.5	-0.5	-1.8	1.1	0.1
Mean daily maximum temperatue between 24 July-5 Dec	13.7	13.5	15.5	14.7	15.6	15.4	14.8	14.2	15.2
Mean daily minimum temperatue between 24 July-5 Dec	11.1	11.2	8.3	10.3	10.0	8.7	9.6	10.2	9.7
No. day degrees over base 10° C beteen 24 July-5 Dec	375.0	382.2	383.8	415.3	439.4	388.9	382.4	380.3	414.5
Day degrees (base 10) in August	161.2	184.2	194.2	193.1	186.5	194.1	179.9	179.9	191.2
Day degrees (base 10) in November	31.1	19.5	30.6	31.9	42.4	30.1	35	27.1	34.8
Circumference of trunk or branch at logger location (cm)	93.5	101	46	50	37	30	NA	80.2	39.0

The conditions within the trunk and stems show a relationship with the circumference of the trunk or branch, larger diameter branches or trunks experience higher minimums and lower maximum temperature (Fig. 2.6 and 2.7).

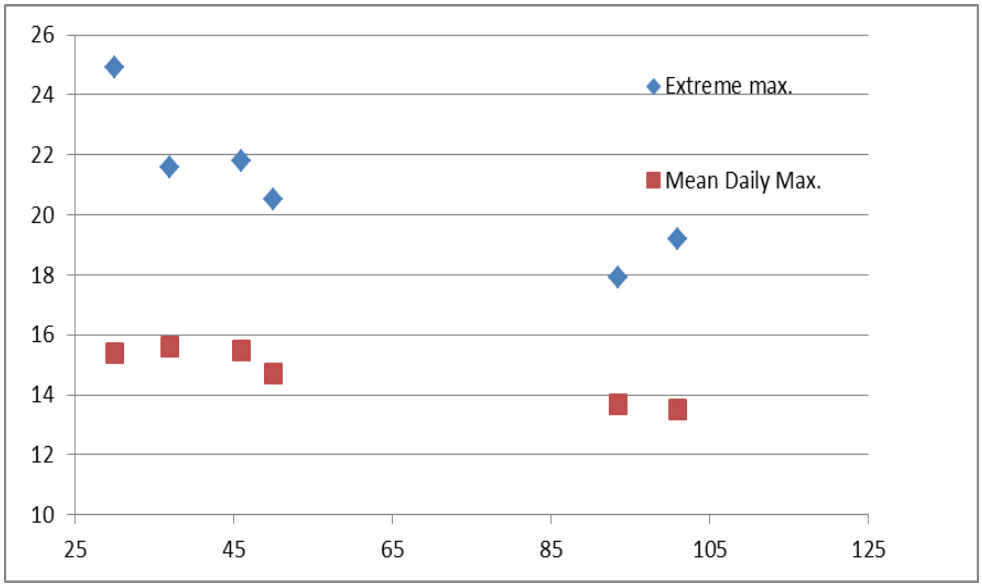

Fig. 2.6: Extreme maximum temperature and mean daily maximum temperature (°C) plotted against trunk or branch diameter (cm)

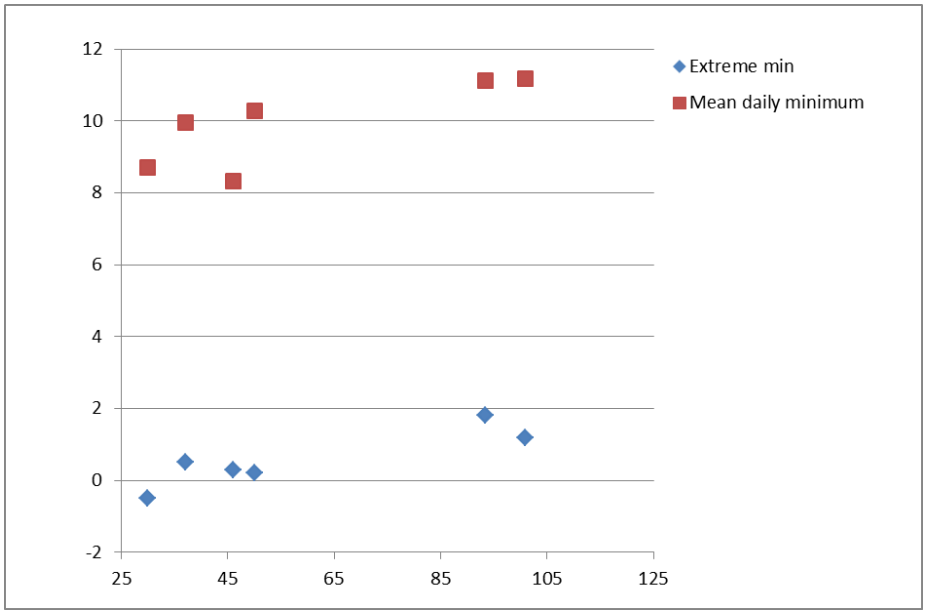

Fig 2.7: Extreme minimum temperature and mean daily minimum temperature (°C) plotted against trunk or branch diameter (cm)

Discussion

The hypothesis that the temperature experienced within trunk bases would be higher than the temperature in the branches was rejected, with very similar conditions being found in both locations. The result in trunk bases contrasted with the results noted in ANOPLORISK when the temperature in trunk bases of *Betula* sp. trees facing south was found to greatly exceed air temperature. Comparing the temperatures within the trunks and branches appears to show a relationship with their circumference, for *Salix* sp. trees this may be as or more important than whether or not the wood is at the base or within the canopy. This is thought to be due to the larger dampening effect within larger volumes of wood.

These results demonstrate the complexity of trying to model potential development rate of xylophagous insects and that some of the differences in development rate may be due to differences in the temperature experienced.

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WP3: Detection of adult beetles with pheromone baited traps

ALB/CLB eradication programs are accompanied by intensive monitoring programs relying mostly on visual identification of feeding signs of larvae and beetles. Adult beetles are only accidentally captured. Pheromone baited traps would complement our available detection tools in outbreak areas but also in high risk areas for introduction. Traps and attractants for ALB have been developed evaluated in outbreak areas in the U.S. Cross traps are baited with ALB pheromone compounds 4-(n-heptyloxy)butanal, 4-(n-heptyloxy)butan-1-ol, and host tree volatiles. In a four-year evaluation period in the infested zone in Worcester, Massachusetts, U.S., a total of 45 ALB was captured in 40 traps from a total of 876 baited traps (= 4.6%). No ALB were caught in 137 unbaited traps. Trap catches decreased with declining numbers of infested trees due to the eradication measures (Nehme *et al*., 2014). We evaluated the use of traps in two European outbreak areas, Paddock Wood (Kent, UK) and Gallspach (Austria). Since both outbreaks are under eradication and numbers of infested trees are much lower than in Worchester, no high trap catches were to be expected. Populations might be higher at the Austrian site where the outbreak was detected in fall 2013. In addition, traps were deployed on storage areas of two big stone importers in Austria, i.e. high risk areas for ALB introduction.

Objectives
Testing of traps and lures for ALB in outbreak areas and in high risk areas (ports, stone importers)

Participants
This WP was be led by P3 and had significant input from P1.

Milestones
M3.1 Deploy pheromone traps at outbreak sites or high risk areas and analyse trap catches
M3.2 Complete feasibility study of use of smart traps for remote monitoring

Deliverables
D3.1: Final report on detection of adult beetles with pheromone baited traps

3.1. Pheromone traps in ALB outbreak areas and high risk areas for introduction

Materials and Methods

Traps and lures were purchased from ChemTica Internacional S.A. (Costa Rica). Traps were cross vane traps with big collection cups optimized for cerambycid trapping. The surface was treated with fluon by the manufacturer. For deployment in Austria, holes were drilled into the collection cups to allow dry catches. A piece of insecticide-impregnated netting, StoraNet (BASF, Germany), containing alpha

cypermethrin was used to kill captured insects. In the UK, collection cups were not drilled, and insects were wet-captured in water containing a small amount of detergent. In all cases, lures consisted of the pheromone compounds 4-(n-heptyloxy)butanal and 4-(n-heptyloxy)butan-1-ol as well as the host tree volatiles linalool, (Z)-3-hexen-1-ol, and trans-caryophyllene, although ratios of the various compounds varied slightly between the different deployment years. Lures were installed and replaced according to the manufacturer's instructions. In 2015, additional traps and lures provided by Witasek Pflanzenschutz GmbH (Austria) were used. Valuable input on trap placement and maintenance was given by Melody Keena (USDA Forest Service, Northern Research Station).

a) Traps in ALB outbreak areas

Paddock Wood, Kent, UK: 18 traps (all ChemTica) were deployed in locations near the outbreak zone (latitude 51°11' N, longitude 0°24' E) in 2014 and 2015, respectively. Trap locations were the same, or similar to those used in 2012 and 2013 (in which no ALB were caught). Single traps were hung in a range of host trees, comprising various species of *Acer*, *Aesculus*, *Betula* and *Salix;* Tab. 3.1, from July to November, and were checked and emptied bi-weekly (over 16 weeks). Individual trap contents were identified to genus or species, where possible – with particular attention to Coleoptera.

Table 3.1: Trap locations at Paddock Wood, UK (2015)

Trap Number	Tree species	Grid Reference	Details
1	*Acer* sp.	TQ 67218 45798	Gabriel Chemie
2	*Acer platanoide* s 'crimson king'	TQ 67212 45865	Gabriel Chemie
3	*Betula* sp.	TQ 67298 46300	Garden Centre
4	*Acer* sp.	TQ 67436 46728	Knowles garden
5	*Salix* sp.	TQ 67394 46721	Knowles garden
6	*Acer pseudoplatanus*	TQ 67732 46712	Knowles Field walk
7	*Acer platanoides* 'crimson king'	TQ 67521 46811	Hoskins garden
8	*Salix* sp.	TQ 67415 47301	Wakeling's garden
9	*Acer platanoides* 'crimson king'	TQ 67334 47299	Hop farm car park
10	*Salix* sp.	TQ 67281 47299	Hop farm boundary
11	Chestnut (*Aesculus hippocataneum*)	TQ 67089 46995	Hop farm land
12	*Acer sacchurum*	TQ 66559 47143	Woodland
13	*Salix* sp.	TQ 66934 47800	Woodland
14	*Acer* sp.	TQ 67109 47962	Woodand by weir
15	*Acer pseudoplatanus*	TQ 67352 48335	Branbridge Ind. estate
16	*Salix* sp.	TQ 67337 48338	Branbridge Ind. estate
17	*Acer platanoides* 'crimson king'	TQ 67724 47718	Tompsetts neighbour garden
18	*Acer*	TQ 65887 46009	Garden nursery

Gallspach, Austria: 20 traps (all ChemTica) and 27 traps (20 ChemTica, 7 Witasek) were deployed in the infested zone in 2014 and 2015, respectively. Traps were hung in the crowns of living host trees (Fig. 3.2) from June to October and checked and emptied bi-weekly. For one trap in the area where most trees were removed because of ALB infestation, an artificial construction holding climbing plants was used. To facilitate easy inspection, all traps were placed on public ground (Fig. 3.1).

Fig. 3.1: Locations of the traps in 2014. Areas where infested trees had to be removed earlier are covered by traps 1 to 12 (excl. 11) and traps 18 to 19.

Fig. 3.2: Placement of trap in the crown of a maple tree.

b) Traps in high risk areas for introduction

Two stone importers with large storage yards receiving significant amounts of shipments with granite stone from China where selected for our test. Additionally to ALB traps (ChemTica), traps for *Monochamus* spp., vectors of the pine wood nematode, were set up. Therefore, we used Teflon-coated multifunnel traps (Econex SL, Murcia, Spain) baited with the *Monochamus* lure Galloprotect-2D (Sociedad Española de Desarrollos Químicos (SEDQ), Barcelona, Spain). The lure consists of the *Monochamus* pheromone 2-undecyloxy-1-ethanol (monochamol), as well as the bark beetle kairomones 2-methyl-3-buten-2-ol and ipsenol. In 2015, α-pinene (SEDQ) was added to the above mentioned lure to increase the attractivity for other woodborers of coniferous hosts.
Stone importer I is mostly surrounded with residential areas with high numbers of ALB host trees in the gardens and along the streets. Five ALB traps and three *Monochamus* traps were set up. Stone importer II is mostly surrounded by agricultural land with lower number of trees in close vicinity but as small forest ca. 500 away. Ten ALB traps and two *Monochamus* traps were set up.

Results

a) Traps in ALB outbreak areas

Paddock Wood, Kent, UK: No ALB were caught in the UK traps in 2014 or 2015 (likewise, no ALB had been caught in similar trap deployments in the previous years, 2012 and 2013). All traps contained non-target catches of various insects and other arthropods, including at least 18 species of Coleoptera, none of which were Cerambycidae. The most commonly caught non-target species was *Byrrhus pilula* (Coleoptera: Byrrhidae) (Pill beetle, n = 314), together with a variety of ladybird beetles (Coleoptera: Coccinellidae) (n = 15, ~ 6 species). One trap was found to contain numerous specimens of *Phosphuga* (*Sipha*) *atrata* (Coleoptera: Silphidae) (European carrion beetle, n = 26), with 4 other specimens were found in two other traps. The beetle is known to feed on insects, as well as carrion feeder, hence likely to be incidental.

Gallspach, Austria: No ALB were caught in the traps in the Austrian infested zone in 2014. One ALB female was caught in 2015. The specimen was found in a ChemTica trap on August 12. Two infested trees had been detected and removed within 50 m of the trap site in 2014. After the trap catch, inspections of trees were repeated in this area and one tree with an exit hole and one dead beetle in another exit hole was detected. Numbers of other cerambycids in the traps were low. None were caught in 2014; three specimens were caught in 2015: one *Phymatodes testaceus*, one *Leptura rubra* and one part of a Lepturinae, likely also *L. rubra* (Tab. 3.2). Interestingly, three specimens of the scarabaeid *Osmoderma eremita* (1 in 2014, 2 in 2015) were caught in the ALB traps (2 in ChemTica and 1 in Witasek traps, respectively).

Table 3.2: Trap catches in ALB outbreak areas. Number of deployed traps, number of ALB beetles and number of other cerambycids caught throughout the entire trapping period

		Traps	ALB	Other Ceramb.
Paddock Wood, UK	2014	18	0	0
	2015	18	0	0
Gallspach, Austria	2014	20	0	0
	2015	27	1	3

b) Traps in high risk areas for introduction

No ALB were caught in the ChemTica traps at the two stone importers in 2014 and 2015. A total of 4 *Monochamus galloprovincialis* were caught in the Econex/SEDQ traps. Numbers of other cerambycids were markedly higher (total of 52 specimens) than in the outbreak areas. Nine species were caught in the ALB traps and 11 species in the *Monochamus* traps; 5 of the species were caught in both trap types (Tab. 3.3).

Table 3.3: Trap catches of ALB, *Monochamus galloprovincialis* and other cerambycids at the sites of the two stone importers (S I and S II) caught in ALB traps (ChemTica) and *Monochamus* traps (ECONEX/SEDQ).

	ALB		*Monochamus*	
	S I	S II	S I	S II
Anoplophora glabripennis	-	-	-	-
M. galloprovincialis	-	-	3	1
Acanthocinus griseus	-	-	-	1
Anisarthron barbipes	1	-	-	-
Aromia moscata	1	-	3	-
Arhopalus rusticus	-	2	-	-
Chlorophorus figuratus	-	-	-	1
Chlorophorus sp.	-	-	-	1
Hylotrupes bajulus	-	7	-	-
Leptura rubra	-	-	-	1
Lepturinae	1	-	2	1
Obrium brunneum	-	-	1	-
Phymatodes testaceus	-	3	-	1
Rhopalopus clavipes	-	2	-	1
Spondylus buprestoides	1	1	15	3
Trichoferus campestris	-	-	1	-
Xylotrechus sp.	-	1	-	-

Discussion

Placement of ALB traps in two outbreak areas that are subject to eradication measures did not allow any experimental approach, such as comparison of trap types or lures, because of the expectedly low catch based experiences from North America (Nehme *et al*., 2014), due to the eradication measures that had been taken previously – particularly in the UK outbreak area. However, our study illustrates the feasibility of integrating pheromone trapping into surveys during the eradication measures in ALB infested zones. Only one ALB female was caught in a trap in Austria; the finding was valuable for the eradication measures. After receiving the report, inspectors surveyed the area around the trap and detected a tree with an emergence hole (and an additional dead beetle). This tree had been inspected earlier in 2014; symptoms of ALB infestation had been overlooked.

Trap catches of ALB were low. One specimen in a total of 46 traps over two years is in a comparable range than reported from the big outbreak area in Worchester, Massachusetts where 45 beetles were caught in 876 traps over four years (Nehme *et al*., 2014). It may be possible that ALB does not rely on chemical signal to the same extent as other Cerambycidae for mate or host tree location. It may also be that the current lure does not represent the full signal needed. Recent research has shown that members of the genus *Monochamus* (like ALB in the subfamily Lamiinae) readily respond to lures consisting of *Monochamus* pheromone and bark beetle pheromone compounds functioning as kairomones resulting in high trap catches (e.g., Sanchez-Husillos *et al.*, 2015; Pajares *et al*., 2016). However, the response to the *Monochamus* pheromone alone is limited and the synergistic effect of the bark beetle pheromone compounds is required for significant catches. The full blend will still be a source of volatiles that is distinct from the background bouquet in a forest. The ALB blend consists of the ALB pheromone and volatiles of broad leaved trees. Placing such trap in the crown of a tree will make a less distinctive source of volatiles. Overall, we believe there is potential for improvement of ALB lures; but also with the currently available lures, traps will be a valuable compliment to other survey measures in ALB infested zones.

Besides placement in outbreak areas, we conclude that traps can also be useful for surveillance in high risk areas. Particularly, the *Monochamus* traps demonstrated their suitability for surveys for the vectors of the pine wood nematode. Although the traps were not located near pine forests, a total of 4 specimens were collected in 5 traps. Overall, both trap types caught a diversity of cerambycid beetles in Austria, although there were no cerambycids caught in the UK. Species like *Spondylus buprestoides* responded significantly to the lure in the *Monochamus traps*, particularly to the host tree volatile α-pinene. We made one remarkable catch in the high risk areas: One specimen of *Trichoferus campestris* was caught in a *Monochamus* trap. This Asian woodborer has been frequently intercepted in wood packaging material from China (Krehan, 2014). This is the first interception of a beetle outside wood packaging material in Austria. The beetle is reported to be present in Romania, Hungary, Slovakia and Czech Republic (Dascalu *et al*., 2013).

3.2. Feasibility study of use of smart traps for remote monitoring

Pheromone traps for monitoring of pest insect populations are usually relatively inexpensive, typically comprising just a few sections of water-resistant cardboard (e.g. delta and cross-vane traps), or mostly simple pre-formed plastic containers (e.g. funnel traps), together with fixings and fittings, chemical lures (pheromones, kairomones, plant extracts, etc.), and the means to retain (if necessary) the target pest (e.g. by adhesive strip or other mode of killing). Delta traps, for example, would typically cost approximately € 7-10 (~ £ 5-8), whereas cross-vane and funnel traps may be 3-4 times as expensive. Many of the lures and other chemicals or extracts are also relatively inexpensive, although there are some exceptions. Some traps may require, or benefit from an additional light or UV source, or other adaptation (e.g. suction device) that may increase the base cost, but not by a tremendous amount.

By contrast, the manual deployment and regular monitoring of traps, which inevitably requires a great deal of personnel time (travelling, placement, checking, collecting, identifying, recording, refreshing, etc.) is inordinately more expensive, typically costing several hundred or thousands times more than the traps themselves. Hence, any system that can reduce the level or complexity of human input should have the potential to provide significant cost savings. This is the essence of the concept of 'Smart' or remote trapping, being a system that in some way obviates much of the labour time involved in (typically) the monitoring / recording aspects.

Until recently, the means to do this in anything remotely resembling an efficient and cost-effective way simply did not exist. However, we now have available several newer technologies that can be combined to provide some potentially realistic and beneficial alternatives. In order to evaluate such opportunities for monitoring pests such as ALB, the basic requirements of such a system must be specified. In the simplest form, assuming that the trap itself is providing at least some degree of selectivity or discrimination is a means to count or record remotely, together with a means to store or preferably transmit the information gained from a remote location to a central point. If the trap is highly selective, then the information to be stored or transmitted may simply numerical, but if somewhat less selective (most traps, particularly those for many wood-boring beetles) then visual (i.e. image-capture) information will need to be stored and/or transmitted. For both of these (simple numerical and image-capture), some form of energy supply is required, and the greater the resolution or temporal complexity of the image capture may lead inexorably to an exponential increase in data storage and transmission complexity, with a concomitant increase in required energy provision.

In recent years, several different prototype 'smart systems' for automated remote monitoring of a variety of pest species have been developed and experimentally tested in the field (e.g. Beerwinkle, 2001; Tabuchi *et al.*, 2006; Jiang *et al.*, 2008; Kim *et al.*, 2011; Fukatsu *et al.*, 2012; Lopez *et al.*, 2012; Selby *et al.*, 2014; Potamitis & Rigakis, 2015; Potamitis *et al.*, 2015). These have typically been based on some form of camera-based system using Wi-Fi or GSM to transmit the images. To date, none of these seems to have been fully commercialised, making the detailed analysis of their potential utility for monitoring ALB somewhat difficult, at least on a cost-effectiveness basis. Most have tended to use 'off-the-shelf' components, such as digital cameras or wireless image sensors that are assembled into bespoke units. A recent report by Hardwick *et al*. (2014) reviews the potential of systems developed for the (human) security and surveillance industries to be adapted for use in insect

pest surveillance and biosecurity in New Zealand. After eliminating 39 of 45 available security system cameras for technical reasons (size incompatibility, lack of weather resistance, not standalone power requirements, absence of data storage / transmission), they compare detailed specifications of six self-reporting cameras available in 2010 that utilised technologies with the potential to be used in pest management monitoring. The conclusion, however, was that none of the security cameras examined could be used in conjunction with invertebrate traps without considerable further development. The low level of optical performance (image resolution, distortion) at the required focal distance (inside traps) being the primary limitation with these devices for the discrimination of small targets such as pest insects.

One custom-made automated remote monitoring system that has been developed and fully commercialised for pest insect detection is the iTrap / TrapVIEW® from EFOS d.o.o., Hruševje, Slovenia (http://www.TrapView.com) in conjunction with Pessl Instruments Group, Weiz, Austria (http://www.metos.at). According to the Pessl Instruments sales literature this is a patented combination of hardware and software with integrated electronics and sticky trap (delta trap) that is light enough to be hung were needed, and self-sufficient, being powered by a solar panel and battery. [http://www.findri.hr/Vijesti/Download/Product%20Catalogue%20Pessl%20Instruments%202014-2015.pdf] Multiple cameras take high-resolution pictures of the sticky plate, and images are sent via GPRS to the TrapVIEW web platform. Accumulated results can be visualised on web or mobile devices. Control is real-time a collected data is available for further analysis. The specifications are as follows: Memory 4MB, Internet connectivity GSM – GPRS, EDGE, HDSPA, GPS receiver, Internet contact up to 4x per day, Lithium battery, Solar panel, 4 x 2 megapixel cameras; size 180 cm x 130 cm x 35 cm, weight 0.93 kg. The pests currently listed as 'catchable' with TrapVIEW include: Codling moth (*Cydia pomonella*), Cotton bollworm/Corn earworm (*Helicoverpa armigera*), Diamondback moth (*Plutella xylostella*), European corn borer (*Ostrinia nubilalis*), Tomato leafminer (*Tuta absoluta*), several other moths, plus Spotted-wing Drosophila (*Drosophila suzuki*), but in theory almost any pest species for which a suitable attractant is available, and which may be retained by delta traps could be captured and monitored using such a system. Of significance for tree pest monitoring, there is already a customised version of the trap, called TrapVIEW FOREST, developed for monitoring of European spruce bark beetle (*Ips typographus*) and spruce wood engraver (*Pityogenes chalcographus*) on spruce [http://www.trapview.com/v2/en/ tab FOREST]. A further variant is Trapview AURA, which combines a light source to attract insects by UV light (e.g. night flying Lepidoptera, such as European corn borer).

Though not yet developed for use in other trap designs (such as the cross-vane or multi-funnel traps typically required for monitoring ALB and other tree pests) it is easy to see how current equipment and technology could be readily adapted – although some engineering would be required (e.g. different camera positioning, and potentially shape and size). An indication of what may be required can be obtained from a comparison of the TrapVIEW installation video [https://www.youtube.com/watch?v=gCukfzO57rI] and details of ALB traps, e.g. [http://ento.psu.edu/news/2011/using-traps-to-detect-asian-longhorned-beetle].

One important consideration is that the low efficacy and low specificity of the recommended ALB trap/lure/tree volatile combinations (23 beetles caught per 450 traps deployed in 2011; 6 beetles caught per 130 traps in 2012; Nehme *et al*., 2014) means inevitably that a high ratio of traps per suspected pest population is required,

and that careful discrimination of the visual images obtained (= significant operator time) may also be needed.

Although a formal price quotation for the TrapVIEW system has not been requested from the suppliers for the purposes of this review, there is information available courtesy of an Australian-based partner /distributor for TrapVIEW (ADAMA Australia) to suggest that individual devices (units) would cost AUS$1370 + GST (10%) (~ €1000; £850), together with ongoing Annual User Licence, AUS$300 + GST (10%) (~ €223; £187). Hence the estimated setup cost of deploying 100 such smart traps would be in excess of €100,000 (£85,000), with annual repeat cost of €23,300 (£18,700).

This needs to be considered against the cost of perhaps 5-10 operators for 8 days each (4 visits per 2 months, ~ 20 traps per two people per day) for non-automated monitoring, and with the knowledge that at least initial trap set-up and 6-8 weekly lure refresh also requires manual intervention, as well as potentially considerable staff time in evaluating the images obtained / transmitted from the traps. The inevitable conclusion, if only provisional, would seem to suggest that this form of 'smart trapping' would not be cost-effective for this particular pest.

A second type of commercialized 'smart trap', is the b2m-sola-001 smart trap by Korean company M. cucurbit farming corporation Co., Ltd. (see https://www.google.com/ smart trap – Gobizkorea). This is a solar powered suction trap that can be used either with titanium dioxide lamp or pheromone to attract different insects. It has wireless transceiver camera (CMOS) as option, but the image quality is quite low (0.25 – 0.35 megapixel). It is basically an automated, ground positioned 'suction' trap for monitoring relatively small insects (aphids, gnats, Lepidoptera), and hence would appear to be unsuitable for ALB monitoring.

There are perhaps other 'smart traps' in development and commercialisation, although the technology would appear to be at a relatively early stage. It is recommended that the situation should be reviewed again within a few years.

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WP4: Molecular tools for diagnosis

Unambiguous determination of developmental stages of ALB and CLB are crucial before carrying out delimiting surveys or eradication measured. Many of the native woodboring insects are rare or endangered and therefore often protected species. To improve available diagnostics more phylogenetic information of wood boring insects is needed and the detection reliability of diagnostic methods has to be known. Molecular tools for diagnosis of ALB and CLB have been developed in preceding projects. New and promising techniques have been introduced in recent years. Particularly, LAMP assays deserve a thorough evaluation for their usefulness for quick determination of immature stages or frass of ALB/CLB. The work package was split into three sub-sections with the following objectives.

Objectives
1. Validation of Genie-LAMP assays on field samples of larvae
2. Validation DNA barcoding on frass from samples negative for ALB/CLB
3. Extend PCR-RFLP analysis to further *Anoplophora* species as well as other cerambycids regularly intercepted in wood packaging material.

Participants
This WP was led by P3 and with significant input from P1 and P2. A close cooperation between P2 and 3 was established for validation of Genie-LAMP assays. P1 extended the PCR-RFLP analysis.

Deliverables
D4.1 Final report on evaluation of Molecular tools for diagnosis of ALB/CLB

Milestones
M4.1: Complete validation of genie lamp assays on field samples of larvae
M4.2: Complete analysis of DNA barcoding on frass from samples negative for ALB/CLB
M4.3: Extend PCR-RFLP analysis to further *Anoplophora* species and other cerambycids

4.1. Validation of Genie-LAMP for detection of *Anoplophora glabripennis* (ALB) and *A. chinensis* (CLB)

The aim of this work package was to verify the application specificity, sensitivity and reliability of Loop-mediated amplification (LAMP) assays using the GENIE II instrument for the laboratory and on-site detection of invasive cerambycid beetles ALB, CLB and two indigenous European species, *Aromia moschata* (musk beetle) and *Saperda carcharias* (large poplar longhorn). The indigenous target species were chosen due to morphological similarities of their larva and their early instars sharing the same habitat as the invasive species (phloem and xylem of living deciduous trees). Whilst the ALB LAMP assay was building on previous work from FERA (Ian Adams, unpublished), the assay for CLB detection was designed by FERA (Jenny Tomlinson) in co-operation with JKI (Stephan König). Both assays for the detection of

European cerambycid species were generated by JKI. The technology transfer (advice, training) from P3 (Fera) to P2 (JKI) was completed in advance of the assay development at JKI, including an exchange visit by Stepan König to Fera in 2014, and further discussion over the life of the project.

Loop-mediated isothermal amplification (LAMP) allows DNA amplification without thermal cycling, being more rapid than PCR but with a comparable degree of specificity and sensitivity (Tomlinson *et al.*, 2010). The method enables on site screening of suspect beetles material using the GENIE II instrument (OptiGene Ltd., West Sussex, UK). The chosen genetic marker region for LAMP assay generation was the mitochondrial cytochrome C oxidase subunit I (COI). The PCR primers for this region were designed early in the 1990s (Folmer *et al.,* 1994). In comparison to ribosomal genes, such as SSU (small ribosomal subunit) or ITS (internal transcribed spacer), COI combines both the advantages of conserved protein-coding regions suitable for generic primer design (Folmer *et al.,* 1994) together with a certain degree of variability present in unconstrained sites intercepting these genes (Simon *et al.,* 1994).
To estimate usability of the assays, the performance criteria analytical sensitivity, analytical specificity and repeatability/ reproducibility were determined according to EPPO Standard PM 7/98(2) (EPPO, 2014). Reference method for the testing of LAMP in all performance criteria was the DNA barcoding method combining PCR and Sanger sequencing.

Material and Methods

Database and primer design

Primer design for specific detection of CLB, *A. moschata* and *S. carcharias* (Tab. 4.1) has based on a reference COI sequence database containing 671 sequences of 12 genera including 29 species in total. Sequences were obtained from public databases GenBank (National Center of Biological Information) and Barcoding of Life Database (BOLD) as well as from own Sanger sequences of preserved and fresh specimen from South East Asia and Europe. To this end, the database comprised other available *Anoplophora* species (*asuanga*, *beryllina*, *birmanica*/ *stanleyana*, *chinensis* including fo. *malaysiaca*, *davidis*, *elegans*, *flavomaculata*, *freyi*, *granata*, *horsfieldi*, *lurida*, *macularia*, *nobilis*) and species (not necessarily cerambycid beetles) which could be morphologically confused with ALB/ CLB or such sharing the same habitat as there are *Cossus cossus*, *Zeuzera pyrina*, four *Monochamus* spp., *Sesia apiformis*, *Dorcus paralellipedus*, *Rosalia alpina*, two *Dolichoprosopus* spp. and the indigenous targets *Saperda carcharias* and *Aromia moschata* (for species COI phylogeny see Fig. 4.1). Unfortunately Sanger sequencing of *Aromia bungii* an invasive wood boring species from Italy failed to work, but sequence information was available from GenBank. Isothermal amplification by LAMP uses three sets of primers (internal, external and loop primers) which have different physical requirements in primer design. While external, part one of the internal and loop primers should be designed to keep a melting temperature of 60°C the second part of the internal primer should have 65°C melting temperature (e.g. Tomlinson *et al.,* 2010). Data analysis was as follows: separate alignments for CLB, *A. moschata* and *S. carcharias* were assembled into clusters of closely related taxa using a cut-off

homology of 92% considering the genetic variation within COI. Remaining clusters were used to identify convenient primer target sites. Virtual separation for sequence homology, alignment of related species sequences, primer target site identification, and specificity adjustment in comparison to the remaining sequences were performed using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Table 4.1: Oligonucleotide sequences reference method PCR and LAMP assays.

Primer	Sequence
LCO1490 Forward (Folmer *et al.* 1994)	GGTCAACAAATCATAAAGATATTGG
HCO2198 Reverse (Folmer *et al.* 1994)	TAAACTTCAGGGTGACCAAAAAATCA
ALB-F3	TACTACTAATAAGAAGAWTTGTAGATAG
ALB-B3	CCTGCTGGATCAAAAAATGAAG
ALB-FIP	ATTACAGTTGTAATAAAATTAACTGCYCCTAAGGAACAGGATGAACAGTTTATC
ALB-BIP	TATAGATCAATTACCTTTATTTGTATGAGCAGTTATTTAAATTTCGATCTGTTAAAAGTAT
ALB-F-loop	GAAGAACCTCTATGTGCAACATT
ALB-B-loop	CCAGTTCTTGCTGGAGCAATT
CLB-F3	TTATCCACCATTAGCTGCTAATG
CLB-B3	AAATGTTGATAAAGAATTGGATCAC
CLB-FIP	AACTGCTCATACAAATAAAGGTAATCGAGATTTAGCTATTTTCAGATTACATCTT
CLB-BIP	AGCAATCACAATACTTCTTACAGATCGCTCCTCCTGCTGGATCAAAG
CLB-F-loop	AATAAAATTAATTGCTCCTAAAATTGAG
Ar-mos F3	TTTAATACTCGGAGCACCAGATATA
Ar-mos B3	GAGATAATAACAGAAGAATTGCTGT
Ar-mos FIP	AGCAATRTTTCTAGAAAGTGGGGGGCCCTTCATTAACTTTTTTAATTCTGAGT
Ar-mos BIP	TCAGGAATAAGCCYAGACCGAATGAACAACAGCCCACACAAATAAAG
Ar-mos F-Loop	AGTTCCGGCACCYCTTTCTA
S-car F3	TATCATAATTGGYGGATTTGGAAAC
S-car B3	TGTAATTTTAACTGCTCAGACGAAT
S-car FIP	AATTAAGAGTCTTAAGGATGGGGGTAAAAGGCTCCAGATATAGCCTTTCCC
S-car BIP	GCTCCATTTAGCTGGAATTTCCTCTGGGGTATTCGATCTAYTGATATG
S-car B-Loop	AACTGTYATTAATATGCGACCCC

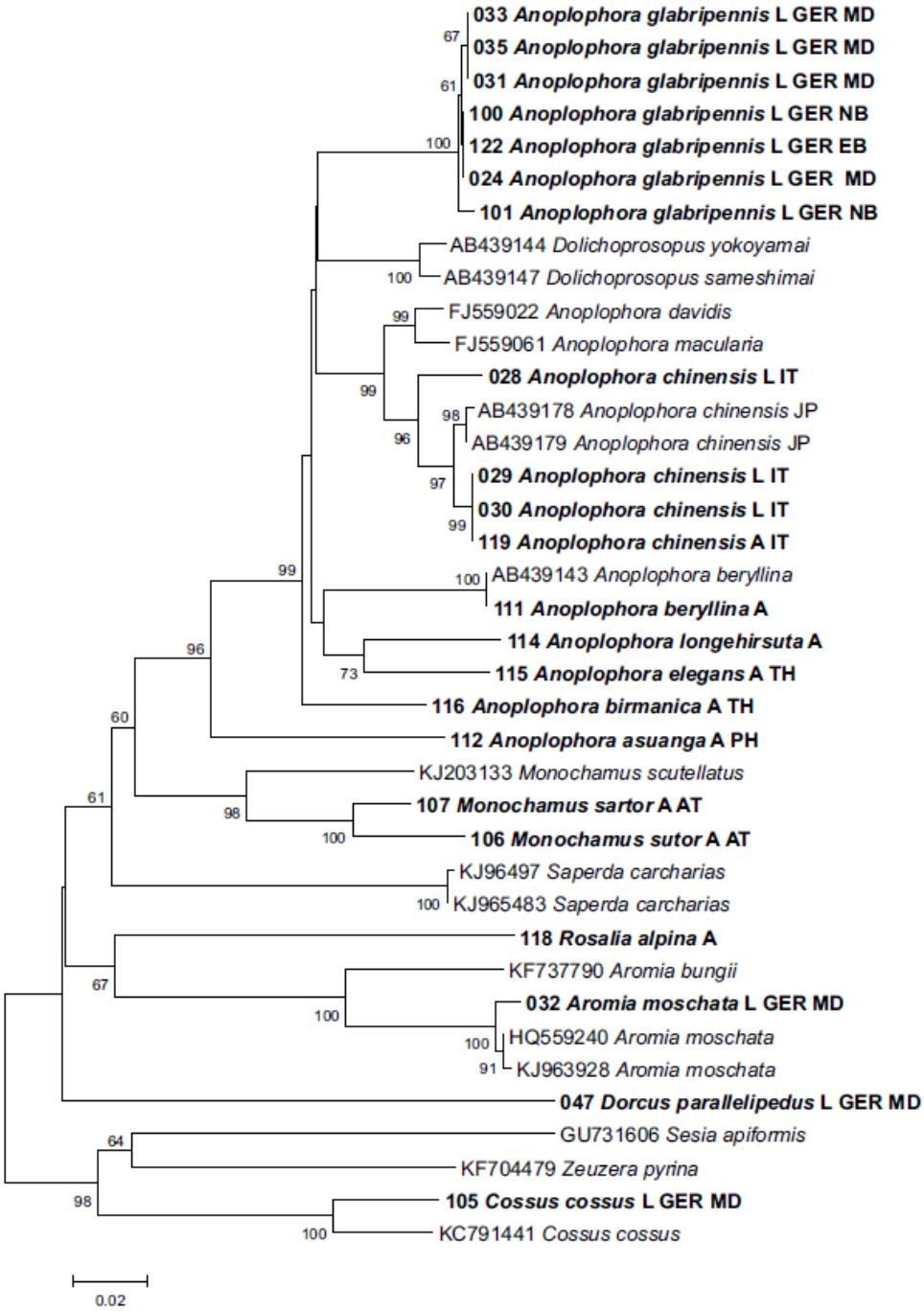


Fig. 4.1: Neighbor Joining phylogram of partial COI gene sequences of *Anoplophora* spp. and related species based on alignment performed in MAFFT (http://mafft.cbrc.jp/alignment/server/). Bootstrap values higher than 50% are shown. Sanger sequences from own reference material are given in bold. Developmental stage of reference material is indicated by L (larva) and A (adult individual); country of origin is given as two letter country code (IT = Italy, JP = Japan, TH = Thailand, PH = Phillipines, AT = Austria, or for origin within Germany (GER) the following two letters showing place of origin (MD = Magdeburg/ Saxony Anhalt, EB = Ebersberg/ Bavaria, NB = Neubiberg/ Bavaria).

Collection material and DNA extraction

For validation of the different assays, we tested fresh larvae from Germany, preserved adult beetles' reference material from all over the world and wood shavings/ frass from a rearing of ALB (thanks to Gerhard Renker, Chamber of Agriculture, North Rhine Westphalia). Fresh larval Reference Material of ALB was derived from several outbreaks in Germany (Magdeburg/ Saxony Anhalt, three locations in Bavaria), fresh material of CLB originated from Italy (thanks to Matteo Maspero) and indigenous material was collected in the vicinity of Magdeburg. All of the adult beetles tested were only available under preserved conditions from the collection of Thomas Schröder (see Fig. 4.1).

DNA of fresh and preserved insect material was extracted according to the protocol for total DNA purification from animal tissue as implemented in the Qiagen DNeasy Blood and Tissue Kit with a slight modification for the amount of proteinase K added in two steps of 20 µl and 10 µl. DNA of wood shavings and frass was extracted by two different kit systems, InviMag Plant DNA Mini Kit (magnetic beads approach, Stratec Molecular GmbH, Berlin, Germany) and a test series kit improved for DNA extraction of wood shaving material containing high amounts of phenolic substances (Analytik Jena, Jena, Germany) that is presently not commercially available.

Isothermal amplification in GENIE II and COI Barcoding PCR/ Sanger Sequencing

When not stated otherwise, Loop-mediated isothermal amplification was conducted in 25 µl reactions containing 15 µl of isothermal reaction mix (OptiGene), 6.5 µl molecular grade water, 0.5 µl of external primers (final concentration 0.2 µM) and internal primers (2.0 µM), 0.25 µl of loop primers (1.0 µM) and 1.0 µl template DNA. The running protocol in GENIE II comprised two phases, 30 min amplification at 65°C and template annealing measurement between 95°C and 75°C with a ramp rate of 0.05°C/ Sec. An extended time span of 45 min for amplification was only used for the *Aromia moschata* assay. Conventional PCR was conducted using the arthropod specific COI primers LCO1490/ HCO2198 according to the protocol of Folmer *et al.* (1994). Sanger forward and backward sequencing was performed by MacroGen Europe (Amsterdam, The Netherlands).

Validation Plan following EPPO Standard PM 7/98(2)

Analytical sensitivity the measure of target amount which can be reliable identified was tested for all developmental stages of ALB and CLB, larvae of the indigenous target species and all material conditions (fresh vs. preserved material). Therefore the Limit of detection (LOD) was tested twice, for biological units in a dilution series of proteinase K digested tissue in separate DNA extractions of each single dilution step and a simple DNA dilution series. 11-stepped tissue dilution series ranged between ca. 70 mg to 0.05 mg/ 100 µl elution buffer (Qiagen) with remaining DNA concentrations between about 70 and 0.06 ng/ µl.

Analytical specificity was tested intra specific for different origins of ALB and CLB (European and South East Asian) and inter specific for reference species shown in Table 4.2.

Repeatability of all LAMP assays was tested for dilution series step 2 (ca. 35 mg digested tissue eluted in 100 µl elution buffer) and step 8 (ca. 1.4 mg tissue/ 100 µl). Reproducibility was only tested for ALB and CLB LAMP assay using the same dilution levels. Two independent test persons carried out this test. Calculations of all parameters were carried out according to the EPPO Standard PM 7/98 (2).

Results

Analytical sensitivity was determined for all assays and all specified matrix materials (fresh larvae, preserved dry adult beetles, wood shaves/ frass). For comparison with reference method barcoding PCR (Fig. 4.2), we used a volume of 2.5 µl template DNA and 25 µl reaction mix. Analytical sensitivity of 100% was achieved for larval material using LAMP assays for detection of ALB, CLB and with the restriction of an extended run time of 45 Min also for the *Aromia moschata* assay. The assay for detection of *Saperda carcharias* failed completely and needs to be redesigned. The target DNA detection in dilution series showed a nearly linear regression for CLB (R^2 = 0.92); ALB (R^2 = 0.9002; Fig. 4.3) and *Aromia moschata* (R^2 = 0.9843). Linear correlation of reaction peaks of the assays is necessary for predictable and reliable assay performance within a certain range of target DNA concentrations due to amplification inhibiting effects which limits assay reliability (e.g. for amplification in PCR see Polz & Cavanaugh, 1998). The amplification of preserved dry material from adult beetles failed for all assays in conventional run time of 30 Min and was only detectable using extended run times of 45 Min for ALB and CLB assay but not for the *Aromia moschata* assay. For this type of material the LAMP assay was not reliable (sensitivitiy < 50%) and showed no linear regression. Reference method COI barcoding PCR confirmed by Sanger sequencing was successful for 3 out of 5 dry beetle material samples. Unfortunately the performance of LAMP as well as PCR failed for the extracts derived from wood shaves/ frass. None of the used DNA extraction methods enabled amplification of cerambycid DNA from this material.

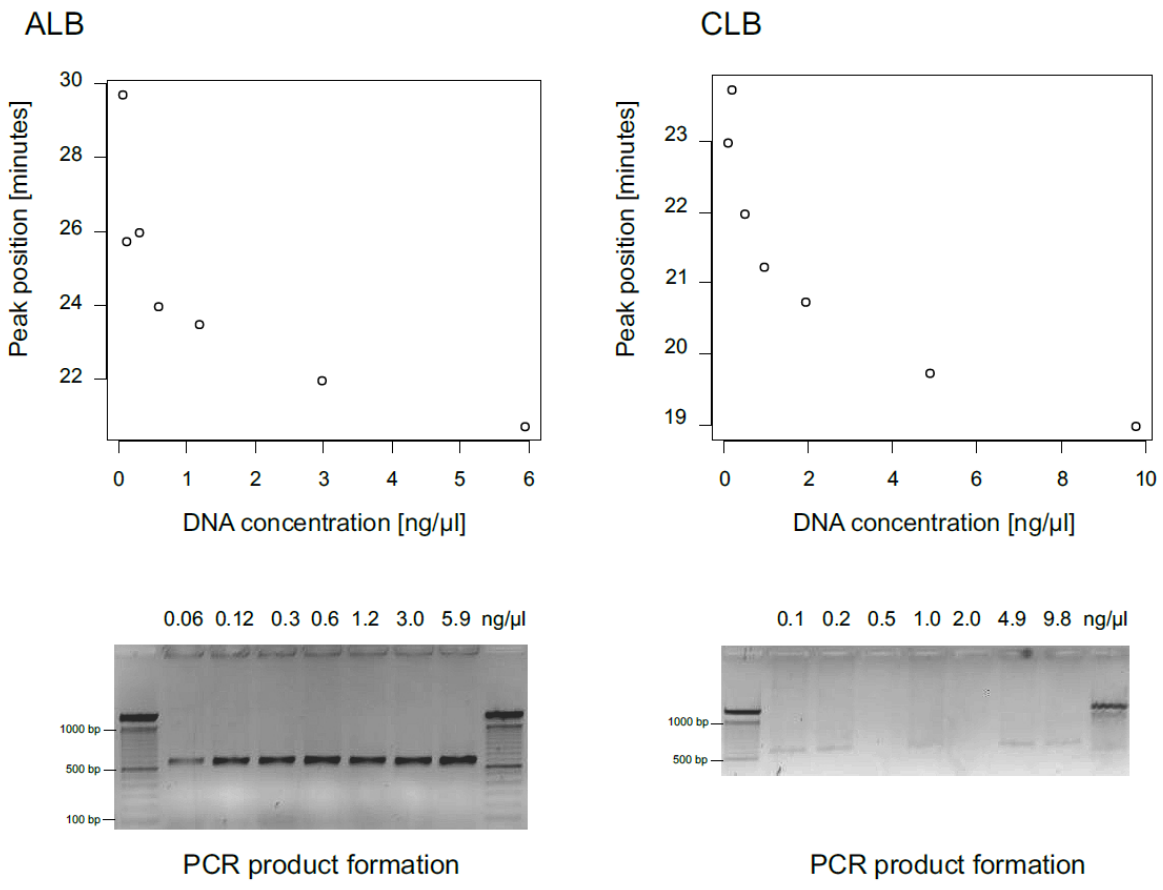


Fig. 4.2: Analytical sensitivity of ALB and CLB LAMP assay in comparison to reference method COI PCR.

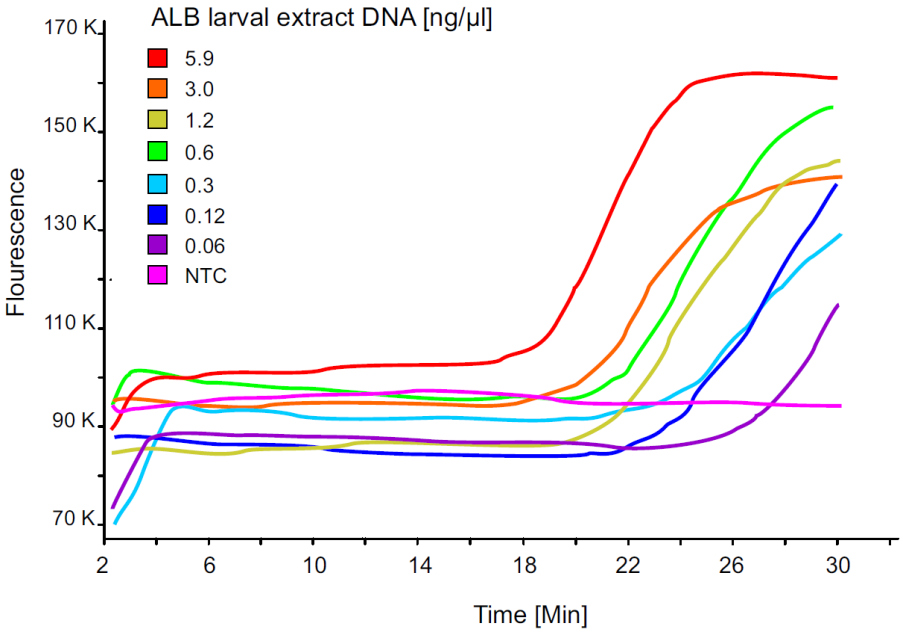


Fig. 4.3: Serial DNA dilution (DNA concentrations see Fig. 4.2) of ALB fresh larval material detected by the respective LAMP assay.

Analytical specificity was tested using fresh larval material or appropriate concentrations of PCR products derived from dried beetle material. COI of 10 out of 16 preserved specimens from different reference species was amplifiable in PCR. Because PCR products mainly contain COI DNA of the respective organism a dilution series was carried out to determine the appropriated dilution level in comparison to larval and PCR amplified extracts of assay target species. The determined dilution levels ranged between E-04 and E-06, and therefore both levels were used to perform standardized intra- and inter-specific cross-reference tests. Intra-specific variability ranged between 99.8 and 96.1% in CLB while the differences within ALB and *A. moschata* were lower (100 to 99.1%). All of the different European and Asian origins of ALB and CLB could be detected successfully. Cross reference tests of non-target species showed deficiencies especially for *Aromia moschata* assay using the extended run time of 45 Min (69.3%) and ALB (80%; Fig. 4.4). ALB showed false positive signals for *Monochamus* sp., *Rosalia alpina* (at the higher level of DNA concentration) and *Anoplophora chinensis* (CLB) fresh larval DNA. On the other hand the CLB assay showed no false positive cross reactions (Tab. 4.2). However, although the PCR products used showed clear readable Sanger sequences with no cryptic background noise, a contamination of the DNA extracts cannot be ruled out definitively.

Table 4.2: Specificity performance of the LAMP assays using DNA templates of target and non-target reference species

Sample parameters					Results (+/-; Peak [mm])					
Species	**Develop. Stage** [a]	**Template** [b]	**DNA-concn. [ng µl^{-1}]**	**Dilution** [c]	**ALB-Assay**		**CLB-Assay**		**Ar-mos Assay**	
									30 min [d]	**45 min** [d]
ALB	L	Extract	47.5	No	+ (16)	+ (16)	-	-	-	(+) (>45)
ALB	A	PCR	7.1	E-06	+ (23)	+ (25)	-	-	-	NA
ALB	A	PCR	7.1	E-04	+ (18)	+ (17)	-	-	-	NA
CLB	L	Extract	78	No	+ (28)	+ (28)	+ (19)	+ (21)	-	(+) (>45)
CLB	A	PCR	4.3	E-06	-	NA	+ (24)	+ (25)	-	NA
CLB	A	PCR	4.3	E-04	-	NA	+ (22)	+ (21)	-	NA
Ar-mos	L	Extract	36.7	No	-	-	-	-	+ (29)	+ (29)
A-asu	A	PCR	1.1	E-06	-	-	-	-	-	(+) (>45)
A-asu	A	PCR	1.1	E-04	-	-	-	-	(+) (>30)	+ (41)
A-ber	A	PCR	0.8	E-06	-	-	-	-	-	(+) (>45)
A-ber	A	PCR	0.8	E-04	-	-	-	-	(+) (>30)	+ (39)
A-bir	A	PCR	1.2	E-06	-	-	-	-	-	NA
A-bir	A	PCR	1.2	E-04	-	-	-	-	-	NA
A-ele	A	PCR	0.5	E-06	-	-	-	-	-	NA
A-ele	A	PCR	0.5	E-04	-	-	-	-	-	NA
A-lon	A	PCR	0.6	E-06	-	-	-	-	-	NA
A-lon	A	PCR	0.6	E-04	-	-	-	-	-	NA
C-cos	L	Extract	29.7	No	-	-	-	-	-	-
D-par	L	Extract	25.2	No	-	-	-	-	-	-
M-sar	A	PCR	0.5	E-06	-	-	-	-	-	(+) (>45)
M-sar	A	PCR	0.5	E-04	+ (24)	+ (25)	-	-	-	+ (42)
M-sut	A	PCR	1.5	E-06	+ (27)	+ (28)	-	-	-	-
M-sut	A	PCR	1.5	E-04	+ (22)	+ (22)	-	-	-	-
R-alp	A	PCR	1.6	E-06	-	-	-	-	-	NA
R-alp	A	PCR	1.6	E-04	+ (23)	+ (23)	-	-	-	NA
S-car	L	Extract	NA	No	-	-	-	-	-	-

[a] Developmental stage L = Larva; A = Adult
[b] Template describes the source of material used as sample input into LAMP –
Extract = genomic DNA derived from fresh material extracted by the Qiagen DNeasy Blood and Tissue Kit;
PCR = generic COI primer LCO1490/HCO2198 PCR amplified DNA derived from preserved adult specimen (see text for further explanation)
[c] Extracts from larval fresh material were used without dilution; the dilution level of the pure target DNA derived from PCR references was chosen to met the same ratios (measured as reaction peak generation) as compared to LAMP performance (reaction peak generation) of **ALB and CLB undiluted larval fresh material** (see text)
[d] LAMP Assay designed for the detection of *Aromia moschata* (Ar-mos) requires a longer runtime of 45 min to fulfil the criteria regarding sensitivity
(+) positive reaction, generated peak was not completely established during run time

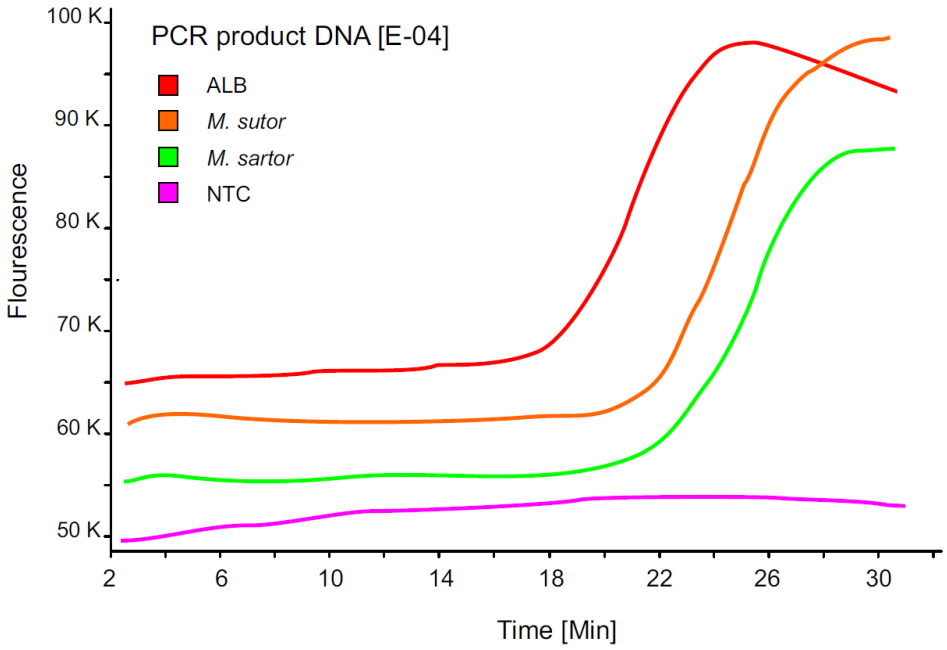
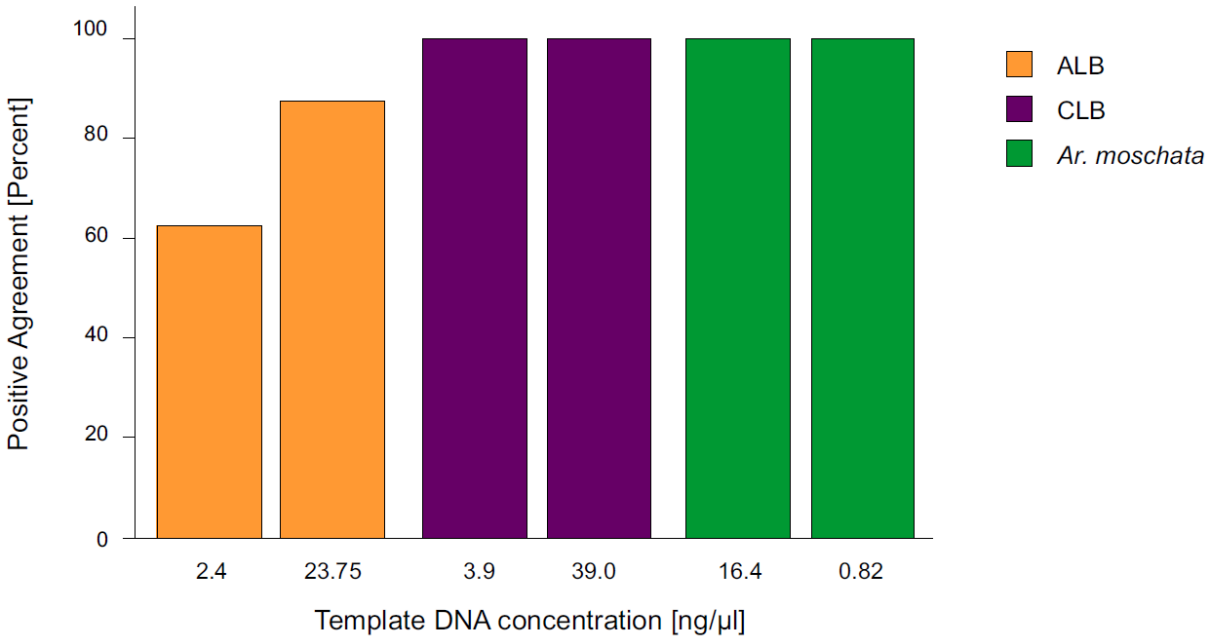


Fig. 4.4: ALB LAMP amplification plot in GENIE II indicating false positive reactions for *Monochamus sutor* and *M. sartor* in reference material PCR products diluted E-04

Analytical repeatability of target species detection was 100% for the CLB and *Aromia moschata* assays. The ALB assay showed weaknesses for the lower detection limit at DNA concentrations of 0.6 ng μl^{-1} (Fig. 4.5a). Analytical reproducibility depended on the test person and here the ALB LAMP assay was also slightly more error prone than the CLB assay (Fig. 4.5b).
Reference method barcoding PCR fulfilled 98 percent of all required criteria with a minor exception for CLB sensitivity test (Fig. 4.2).

a)



b)

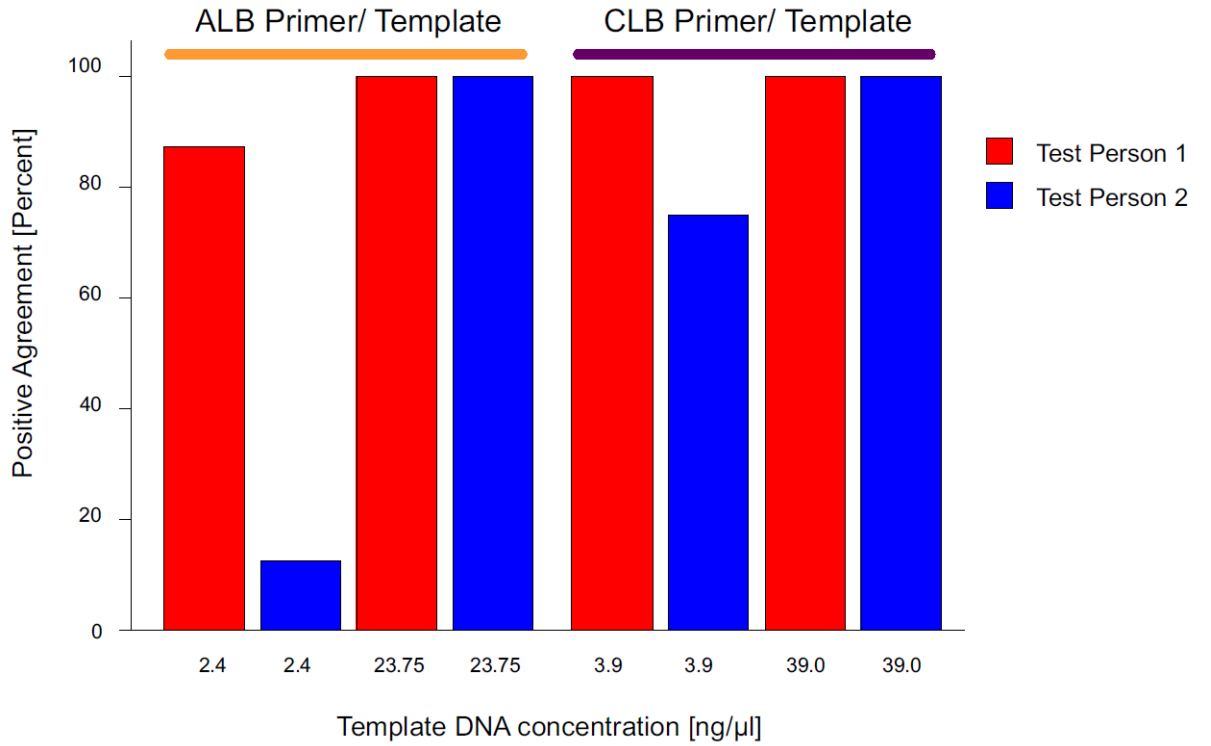


Fig. 4.5: Analytical repeatability of ALB, CLB, and *Ar. moschata* LAMP assays (**a**) and analytical reproducibility of ALB and CLB assays (**b**).

Discussion

Specificity of LAMP diagnostic assays and potential hetero duplex formation depends on (i) the number of base pair differences between FIP/BIP primers and template DNA (especially exchanges of A/T to C/G avoid stable hetero duplex formation); (ii) differences in melting temperature between both molecules, and (iii) probably most important the position of those mismatches at the extension end of the primer. Nevertheless, a longer reaction time and/or high amount of non-target template DNA triggers false positive reactions although mismatches between primer and template are present. As a consequence, the ALB, as well as the *Aromia moschata* assay, needs at least a partial redesign to improve specificity and sensitivity. Detection of cerambycid beetle DNA in wood shaves/ frass seems to be a problem of low amount of beetle body parts (e.g. head capsules) and excrement in high volumes of wood particles without beetles' DNA. Here a method for density dependent accumulation of beetle material prior to DNA extraction could be helpful. Despite current problems regarding specificity of ALB and sensitivity of indigenous cerambycid beetle assays the CLB assay shows explicitly the strength of the loop-mediated amplification method as a powerful tool for on-site detection of wood-boring insects and other pathogens.

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Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P, 1994. Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene-Sequences and a Compilation of Conserved Polymerase Chain-Reaction Primers. *Annals of the Entomological Society of America* **87**, 651-701.

Tomlinson JA, Dickinson MJ, Boonham N, 2010. Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. *Phytopathology* **100**, 143-9.

4.2 Validation DNA barcoding on frass from samples negative for ALB/CLB

For reasons evident from these results of Objective 1 it was not possible to fully deliver on this objective. Both the LAMP and PCR assays developed by partner 2 (JKI) had failed for the extracts derived from wood shaves/ frass. None of the DNA extraction methods used enabled amplification of cerambycid DNA (and presumably other insect DNA) from this material.

Likewise, parallel experiments conducted in the UK by Partner 1 (Fera) on frass originating from a ALB in culture at Fera, a *Monochamus* species deriving from Italy,

a sample from *Cossus cossus*, and two samples of ALB from the Paddock Wood, Kent, UK outbreak, all failed to yield amplification with the ALB1 LAMB assay (Ian Adams, unpublished). In this instance, the frass had been extracted using the Qiagen (UK) blood and tissue kit. Hence, 3 different extraction methods (two at JKI, one in the UK) had all failed to extract sufficient frass DNA suitable for LAMP or DNA barcoding analysis, indicating that a considerable refinement of the extraction procedure would be required. The experimental difficulty associated attempting with refining the extraction procedure is such that an extension or separate project would be required to resolve this issue.

4.3 Extend PCR-RFLP analysis to further *Anoplophora* species as well as other cerambycids regularly intercepted in wood packaging material

Early development stages of ALB and CLB are not always clearly determinable by morphological characters. Therefore the PCR-RFLP analysis was developed in a former project by the Department for Forest Protection of the BFW in co-operation with the Department for Forest Entomology, Forest Pathology and Forest Protection of the University of Natural Resources and Life Sciences Vienna (BOKU). Within the previous EUPRESCO project ANOPLRISK the RFLP patterns of different *Anoplophora* species were established with this method. In ANOPLORISK-II other cerambycids regularly intercepted with wood packaging material as well as native Cerambycidae species were investigated. The work was carried out by Partner 1 (BFW, U. Hoyer-Tomiczek and C. Hüttler).

Material and methods

The basis of the PCR-RFLP method is the amplification of two different parts of the COI (Cytochrome Oxidase I) gene of the mitochondrial DNA of the beetles (or eggs, larvae, pupae) after extraction of total DNA using the DNeasy^{TM} Tissue Kit from QIAGEN following the manufacturers' instructions of the protocol for "animal tissue". The polymerase chain reaction (PCR) – restriction fragment length polymorphism (RFLP) method creates with selected differentiating restriction endonucleases species-specific restriction patterns for various species of *Anoplophora*. The amplification of two PCR fragments (650 bp and 920 bp, respectively) of different parts of the mitochondrial COI gene and the following digestion of each fragment with five different restriction endonucleases increase the certainty of the determination. For the establishment of the species-specific molecular patterns of a species several beetle specimens in good conditions are necessary.
Other *Anoplophora* species than already investigated during the EUPHRESCO project ANOPLORISK were not available during the project period. But not only *Anoplophora glabripennis* is intercepted repeatedly with wood packaging material of different goods, especially of stones, form Asia, especially China into the EU, but also other Cerambicidae species. During inspections of wood packaging material of stones from China in Austria according to the EU decision 2015/474/EU the inspectors of the BFW found also the longhorn beetles *Apriona germari*, *Trichoferus campestris* and *Batocera lineolata.* These species were often intercepted in the larval

stage, making determination difficult. Therefore these species were established with the PCR-RFLP method during this project. As reference species *Anoplophora glabripennis* collected in an Austrian outbreak area was used. *Apriona germari*, *Trichoferus campestris* and *Batocera lineolata* originated from Chinese wood packaging material and were identified at the BFW according to morphological characters (Fig. 4.6).

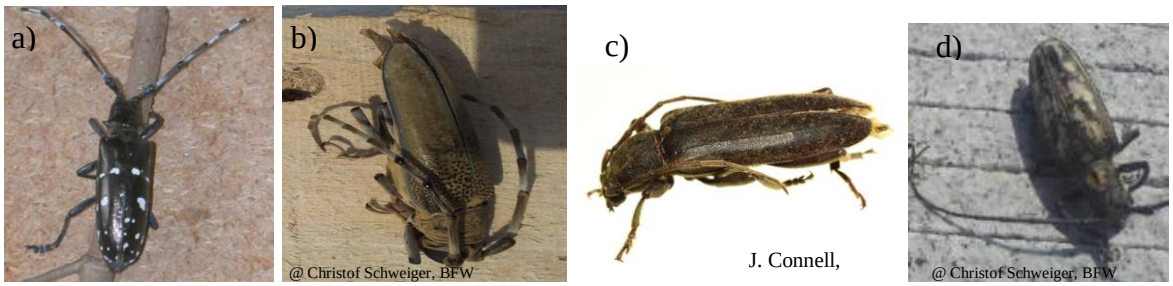

Fig. 4.6: Longhorn beetles used for PCR-RFLP analysis of a) *Anoplophora glabripennis*, b) *Apriona germari*, c) *Trichoferus campestris*), d) *Batocera lineolata*.

In outbreak areas of *Anoplophora glabripennis* or *Anoplophora chinensis,* it is also important to differentiate between these quarantine pests and native wood boring insect species. RFLP-patterns of *Saperda carcharias*, *Saperda octopunctata*, *Saperda perforata* were previously established at the BFW (Hoyer et al., 2003). In this project, native insect species were analysed with the PCR-RFLP method like *Aromia moschata* (Col., Cerambycinae), *Zeuzera pyrina* and *Cossus cossus* (Lep., Cossidae). These were often encountered during surveys in and Austrian *A. glabripennis* outbreak area. All native insect specimens were collected in Austria.
Furthermore the cerambycid *Lamia textor* was included in this analysis. Several larvae of this species were found in the base of the stem of a young poplar in Vienna and were presented to the BFW with the suspicion on *Anoplophora* spp. The larva of *Lamia textor* is morphological very similar to the larva of *Anoplophora glabripennis* or *chinensis*. Therefore a molecular determination method is particularly helpful in this case. One larva could be reared to emergence of the beetle allowing morphological determination (Fig. 4.7).

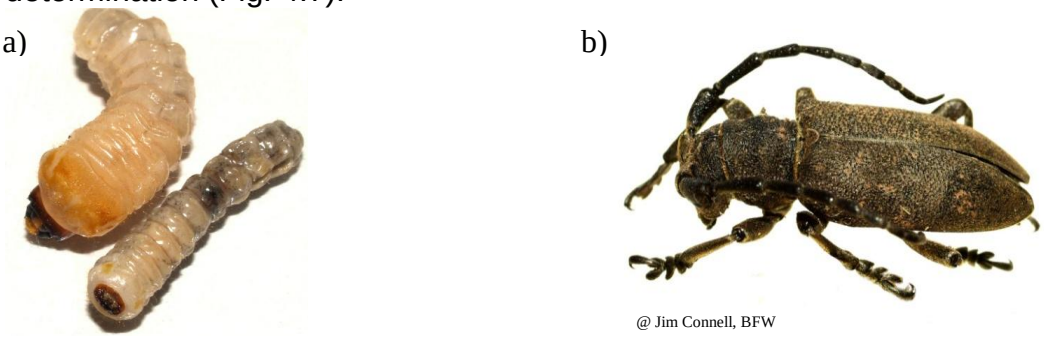

Fig. 4.7: a) Larvae and b) adult of *Lamia textor*.

Results

PCR-RFLP analysis resulted in clearly discernible, species-specific PCR-RFLP patterns for *Apriona germari* and *Trichoferus campestris*, two Asian cerambycid species intercepted in wood packaging material in Austria, and *Anoplophora glabripennis* (Fig. 4.8), as well as for the native *Lamia textor* (Fig. 4.9).

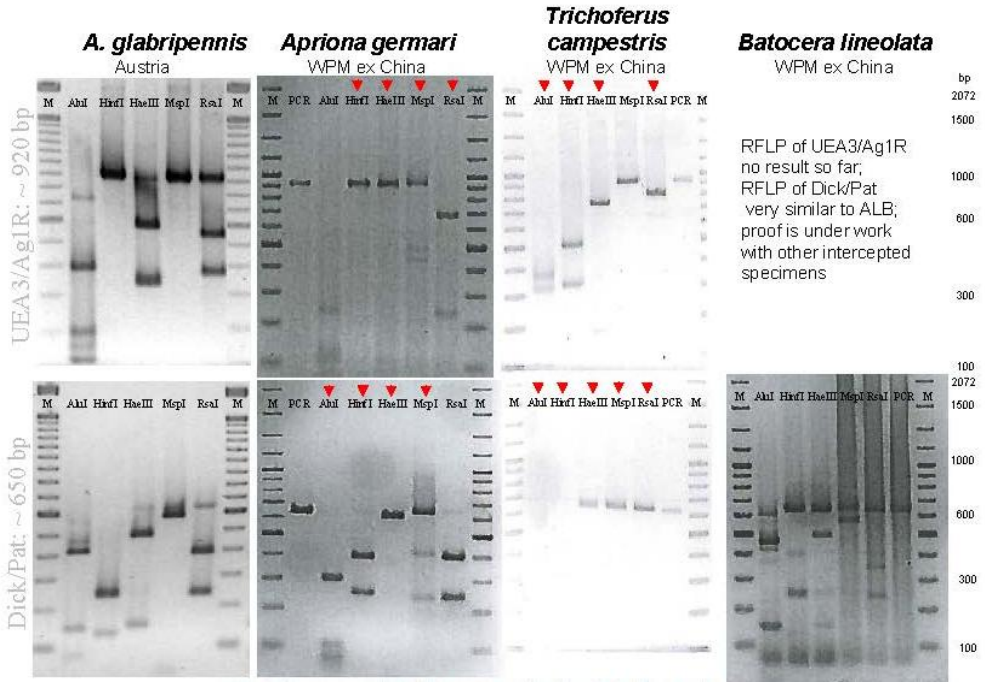

Fig. 4.8: Differentiation of *Anoplophora glabripennis*, *Apriona germari*, *Trichoferus campestris* and *Batocera lineolata* based upon the PCR-RFLP analysis of two different PCR fragments of the COI gene. The red marks indicate differences to *Anoplophora glabripennis*.
bp: base pairs (unit for DNA); *Alu*I, *Hin*fI, *Hae*III, *Rsa*I, *Msp*I: restriction endonucleases for digestion of the PCR fragments; M: DNA size marker (100 bp ladder), Dick/Pat, UEA3/Ag1R: primer pairs for the amplification of the PCR fragments, PCR: not digested PCR fragment.

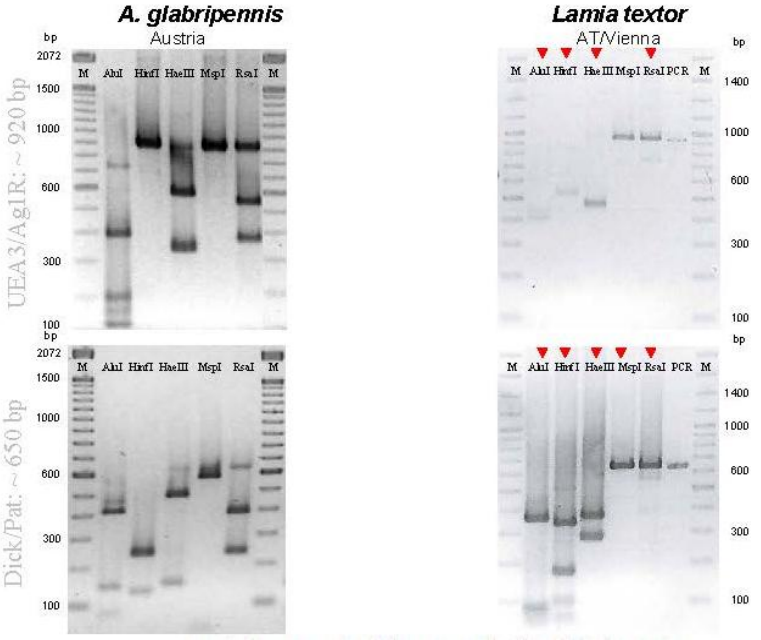

Fig. 4.9: Differentiation of *Lamia textor* upon the PCR-RFLP analysis of two different PCR fragments of the COI gene. The red marks indicate differences to *Anoplophora glabripennis*.
bp: base pairs (unit for DNA); *Alu*I, *Hin*fI, *Hae*III, *Rsa*I, *Msp*I: restriction endonucleases for digestion of the PCR fragments; M: DNA size marker (100 bp ladder), Dick/Pat, UEA3/Ag1R: primer pairs for the amplification of the PCR fragments, PCR: not digested PCR fragment.

The PCR-RFLP patterns of the other native insect species *Saperda carcharias*, *Saperda octopunctata* and *Saperda perforata* as well as those of *Zeuzera pyrina*, *Cossus cossus* and *Aromia moschata* are shown in Fig. 4.10.

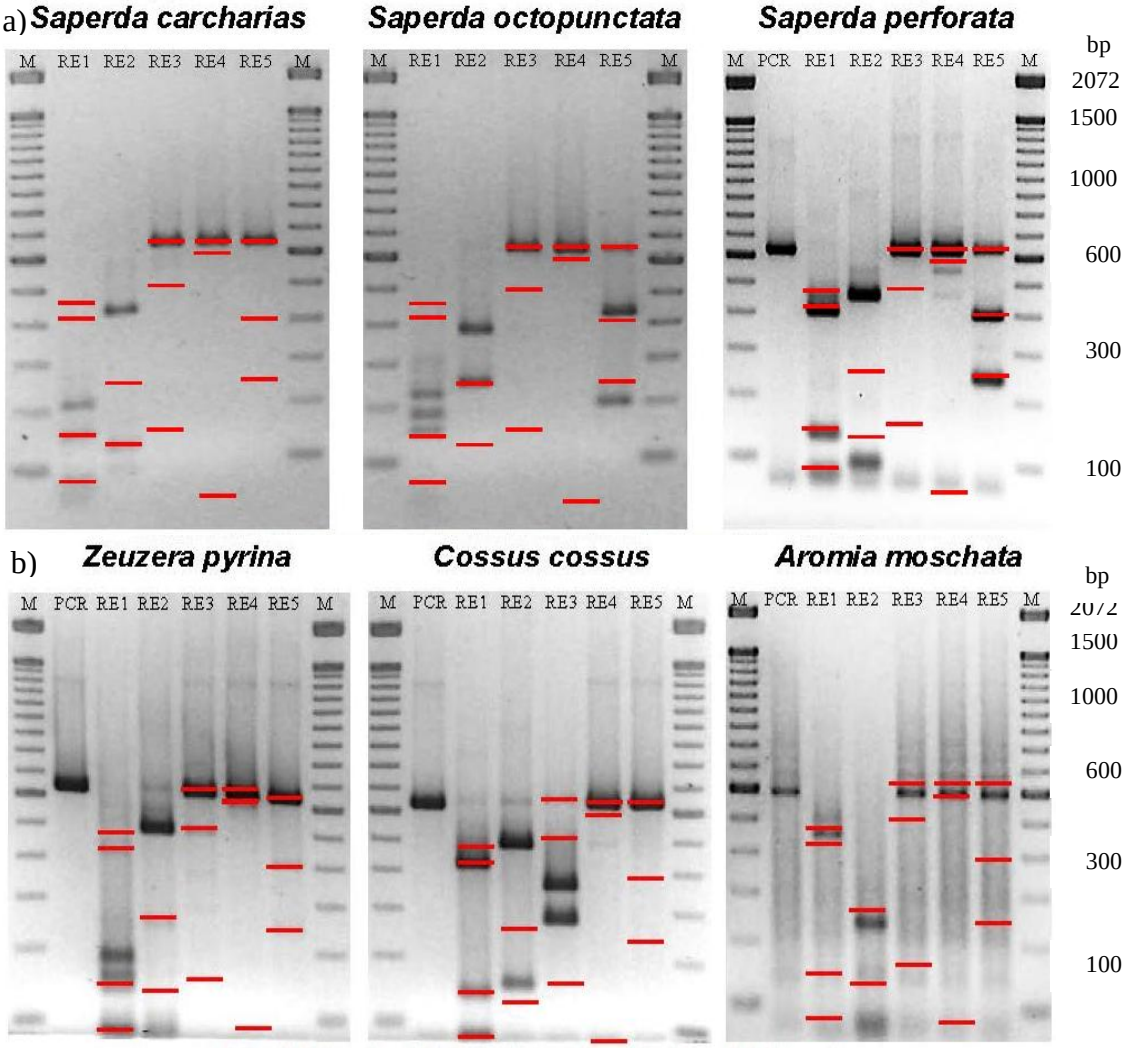

Fig. 4.10: Differentiation of a) *Saperda carcharias*, *Saperda octopunctata*, and *Saperda perforata* as well as of b) *Zeuzera pyrina, Cossus cossus* and *Aromia moschata* based upon the PCR-RFLP analysis of the PCR fragment obtained with the primer pair Dick/Pat of the COI gene. For comparison the red bar code indicates the PCR-RFLP patterns of *Anoplophora glabripennis*.
RE1 = *Alu*I, RE2 = *Hin*fI, RE 3 = *Hae*III, RE 4 = *Rsa*I, RE 5 = *Msp*I: restriction endonucleases for digestion of the PCR fragments; M: DNA size marker (100 bp ladder), bp: base pairs (unit for DNA), PCR: not digested PCR fragment.

Discussion

Apriona germari and *Trichoferus campestris* can be clearly distinguished from *Anoplophora glabripennis* as well as between each other based on the two species-specific PCR-RFLP patterns. For *Batocera lineolata* the results are not so obvious. On one hand the PCR fragment with the primer pair UEA3/Ag1R could not be amplified. This could be caused either by destroyed DNA in the target region of one or both primers or by the fact that the primer Ag1R developed for *Anoplophora*

glabripennis is too specific and does not match to the target region of *Batocera lineolata*. On the other hand the PCR-RFLP pattern of the fragment obtained with the primer pair Dick/Pat shows similarities to the pattern of *Anoplophora glabripennis* making a differentiation difficult. Further individuals have to be analysed to obtain clear results.

The PCR-RFLP patterns of both PCR fragments of *Lamia textor* allow a doubtless distinction from *Anoplophora glabripennis*. This is very important because the larva of *Lamia textor* is morphologically very similar to those of *Anoplophora glabripennis* and *A. chinensis*.

The three *Saperda* species and also *Aromia moschata* occur in tree species that are also host species for *Anoplophora glabripennis* and infested trees are often encountered in ALB surveys in European countries. Normally the larvae of *Saperda* species and *Aromia moschata* can be determined by morphological features and differentiated from *Anoplophora glabripennis.* But not in all cases complete larvae, but only parts of them, or only pupae or eggs can be prepared out of a tree for the diagnosis. In these cases the molecular determination is important. Adults and larvae of the lepidopteran *Zeuzera pyrina* and *Cossus cossus* are easily distinguishable from those of *Anoplophora glabripennis*, but the symptoms on the tree are very similar. Since is not always possible to get complete insect instars out of the tree a molecular diagnosis of parts or tissue of insect instars can be useful.

For *Saperda carcharias*, *S. octopunctata* and *S. perforata,* for *Aromia moschata*, as well as Zeuzera *pyrina* and *Cossus cossus* only the PCR fragment with the primer pair Dick/Pat could be amplified because also in these cases the primer Ag1R of the other primer pair is too specific and does not match to the target DNA of the COI gene of the investigated insect species. As shown by the red bar code of the pattern of *Anoplophora glabripennis* in Fig. 4.10, *Saperda carcharias*, *Saperda octopunctata*, *Saperda perforata*, *Aromia moschata*, *Zeuzera pyrina* and also *Cossus cossus* can be clearly differentiated from *Anoplophora glabripennis* based on the PCR-RFLP patterns of one PCR fragment.

M4.3 Extend PCR-RFLP analysis to further *Anoplophora* species and other cerambycids

Other *Anoplophora* species than in the previous project ANOPLORISK were not available.

Overall, PCR-RFLP analysis data are now available for the following insect species for comparison:

1) Asian Cerambycidae

Apriona germari
Batocera lineolata (partially, need to be confirmed)
Trichoferus campestris

2) European Cerambycidae

Saperda carcharias
Saperda octopunctata
Saperda perforata
Lamia textor
Aromia moschata

3) European xylophagous Lepidoptera

Zeuzera pyrina
Cossus cossus

Refernces

Hoyer, U., Brandstetter, M., Stauffer, C., Tomiczek, C., 2003: Cultivation, breeding and diagnosis of *Anoplophora glabripennis* in the Laboratory. 2nd International Symposium on Plant Health in Urban Horticulture, 27.-29.08.2003, Berlin. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft*, 394: 230.

WP 5: Development of contingency plans

Both *Anoplophora* species are listed as quarantine pests by EPPO and European Union *A. glabripennis* (ALB) and *A. chinensis* (CLB) are listed in the current EU quarantine legislation (Directive 2000/29/EC) in Annex I which contains harmful organisms whose introduction in and spread within the EU member states is prohibited. In addition for both *Anoplophora* species emergency measures have been implemented in the EU (CLB: implementing decision 2012/138/EC; ALB: 2015/893/EC). While the implementing decision for CLB was in place since 2008, the decision for ALB was only published recently in June 2015. The phytosanitary measures for eradicating ALB or CLB in main parts rely on EPPO "National Regular Control Systems" (NRCS, PM 9) for procedures for official control of ALB and CLB.
Though both, the EU legislation as well as the NRCS of EPPO seem to be a sound basis for national eradication measures for countries which have an ALB or CLB outbreak or as basis for contingency plans for countries with no current outbreak, there is a lack of details (e.g. how to carry out monitorings) which need interpretation on a sound scientific basis.
Measures for eradicating a harmful organism need to be implemented quickly. In the framework of ALB and CLB one had to realize that eradication measures including necessary monitoring activities are labor intensive and therefore very expensive. In addition as the pests primarily are introduced into urban areas many different groups of people are affected and need to work together. To avoid delays contingency plans developed and agreed by all potential stakeholders may be helpful.

Objectives
The objective of the current WP was to provide recommendations, based on literature review and existing eradication plans as well as research undertaken in the project, to the EU and other countries for incorporation into contingency plans already in existence and, if necessary, to other countries in order to encourage the development of contingency plans. Because in the meantime for both pests EU emergency measures exist, the scope was enlarged in a sense to interpret and concretize the measures required in the EU legislation. Outcome of this WP were contingency plans for ALB and CLB using Germany as an example.

Deliverables
D5.1: Example of a contingency plan for ALB and CLB each (Annex 1 and 2)

Milestones
M5.1: Review of literature, existing eradication plans and related legislation carried out (June 2015)
M5.2: Contingency plans developed (February 2016)

Participants
This WP was led by P2 and had significant input from P1, P3 and was reviewed by all partners.

Program of work

At the beginning of the project P1 and P2 took part at the EPPO "Workshop for Phytosanitary Inspectors Contingency Planning" from 18th to 20th November 2014 in London. Basis for the scientific development of the contingency plans was a comprehensive literature review concerning eradication options with a special focus on ALB in the US. Especially the results of WP 7 of the previous project ANOPLORISK were taken into account. For better explanation of measures listed in the contingency plans, chapters with detailed information on the biology, detection and experience from other outbreak areas were put in annexes.

The following documents were used to build the structure of the contingency plans:

- Generic contingency plan of the Forestry Commission/ UK for Serious Pest Outbreaks in British Trees (FC 2011),
- Contingency plan of the Swedisch Board of Agriculture to manage an outbreak of *Bursaphelenchus xylophilus* (unpublished),
- EU-Commission Implementing Decision for ALB and CLB 2012/138/EU and 2015/893/EU (EU 2012, 2015),
- EPPO National Regulatory Control System ALB and CLB (EPPO 2013a, 2013b),
- Example of a contingency plan used in civil protection (unpublished),
- Guideline for eradicating ALB in Germany

The contingency plans consist of a core text which contains the principle measures. The structure follows the sequence of the Implementing Decisions. Details and additional information are listed in 26 annexes in the case of ALB. This structure of a core part and annexes is based on a contingency plan used in civil protection. This has the benefit that information to special topics easily can be found. Also amendments are easy to include without changing the core text. The size of the document of around 100 pages is still clear and manageable in a way that a database as used in a contingency plan of civil protection is not deemed to be necessary. A database version may be useful in future for an online version which also allows better enquiry possibilities.

The development of the contingency plans took place in close cooperation and constant feedback with the German plant protection services of the Federal Laender (NPPOs) and the project partners of ANOPLORISK-II. Draft versions of the contingency plans were circulated and commented by the NPPOs in July, September and November 2015. A first evaluation of the ANOPLORISK-II consortium took place in September 2015. Building on this progress on 7th and 8th December 2015 an ALB Contingency Plan Workshop at the Julius Kühn Institute with the participation of 47 persons of the plant protection services and local forestry authorities of the federal states and the Federal Ministry of Food and Agriculture (BMEL) was carried out. The new consolidated version of the ALB contingency plan was discussed at the final meeting of the project partners ANOPLORISK-II on 27th and 28 of January 2016. Based on the project results of the use of detection dogs (WP 2) further additions were included.

This was followed by a fourth consultation of NPPOs. Thus, the emergency plan / guideline to eradicate ALB is coordinated at the technical level.

Since the measures under EU Implementing Decision to CLB are largely identical to those of ALB, the ALB contingency plan has been rewritten to the situation of CLB. Since in some areas considerably less experience from other countries to CLB exist (e.g., no insecticide application in trees, no specific attractant traps etc.), these aspects have been deleted in both the core text and in the annexes, so that the CLB contingency plan includes only 23 annexes.

The ALB contingency plan was agreed by the heads of department of agricultural production of the related ministries of the German Federal Laender on 18th march 2016. The CLB contingency plan was agreed by the liaison officers plant health of the the German Federal Laender on 19th May 2016. This step of the integration of the relevant ministry level is important, in order that (as required in the contingency plan) appropriate funds can be planned for the worst case scenario.
Currently both contingency plans are under final editorial review to be prepared for publication in the German Federal Gazette. The final published versions of both contingency plans will be submitted to be attached for publication with the ANOPLORISK-II report.

Content of contingency plans on the example of the ALB emergency plan

Part I: Aim, background information, legal basis (p. 6 - 8)
Part II: Detection with flow chart from suspicion to diagnosis, and notification of the outbreak (p. 9 - 15)
Part III: Measures to be implemented after confirmation of an outbreak (p. 16 - 50)
Part IV: Contacts and addresses (p. 50 - 51)
Part V: Annexes (p. 52 - 114).

The implementing decision is very detailed in respect to the measures to be taken to eradicate ALB. Nevertheless, there are several points that required interpretation or explanation in order to provide a harmonized approach and which have been addressed in the emergency plan:

- confirmation of an infestation based on ALB stadiums (live / dead) or based on symptoms
- movement of wood obtained in the framework of eradication measures with the objective of destruction as opposed to movement of wood as commercial commodity,
- demarcation of an infested zone and a buffer zone,
- criteria for an exemption from the obligation to demarcate an area,
- criteria for an exemption from the obligation to fell trees in a radius of 100 m around an infested tree and alternatives,
- survey to determine an infestation and designation of a demarcated area,
- regular monitoring in a demarcated area,
- adaptation to the situation in the public green, private gardens and parks in contrast to the forest,
- monitoring in tree crown versus monitoring from the ground,
- use of ALB-detection dogs.

For the first time in the management of an introduced organism in Germany the use of a task force has been highlighted as a key element of a contingency plan.

Respective responsibilities must be defined depending on the scope of the outbreak to ensure that corresponding competence team can be implemented and grow up depending on the situation. This concept has been proven itself in the USA and has already been used in Bavaria since 2014. This is derived from e.g. a civil protection staff as it is used to manage natural disasters.
Since the emergency plan has been written from the perspective of a federal authority and is intended to serve the plant protection services of the federal states as a basis for planning, additional checklists can be found in the annexes such as the structure of ALB-management team or a schedule of reporting an attack suspected to implementation of measures based on the relevant personnel. They must be supplemented by the plant protection services of the federal states. The same applies for lists of equipment or companies which are necessary for the control measures.

The complete emergency plans are attached in Annex 1 and 2.

Literature

EPPO (2013a): National regulatory control system PM 9/15 (1) *Anoplophora glabripennis*: procedures for official control. *EPPO Bulletin* 43(3): 510-517.
EPPO (2013b): National regulatory control system PM 9/16 (1) *Anoplophora chinensis*: procedures for official control. *EPPO Bulletin* 43(3): 518-526.
EU (2012): Durchführungsbeschluss 2012/138/EU der Kommission vom 1. März 2012 über Dringlichkeitsmaßnahmen zum Schutz der Union gegen die Einschleppung und Ausbreitung von *Anoplophora chinensis* (Forster) in der aktuellen Fassung. ABl. L 64: 38-47.
EU (2015): Durchführungsbeschluss (EU) 2015/893 der Kommission vom 9. Juni 2015 über Maßnahmen zum Schutz der Union gegen die Einschleppung und Ausbreitung von *Anoplophora glabripennis* (Motschulsky). ABl. L 146: 16-28.
FC (2011): Contingency Plan for Serious Pest Outbreaks in British Trees. 15 pp. (http://www.forestry.gov.uk/pdf/TH_Strategy_Annex2.pdf/$FILE/TH_Strategy_Annex2.pdf, accessed 18.02.2016).

Dissemination

Contingeny plans for ALB and CLB in Germany

See WP 5 and annexes for details on the contingency plans

Workshop on ALB Contingency Plan, 7th and 8th December 2015 at the Julius Kühn Institute with the participation of 47 persons of the plant protection services and local forestry authorities of the federal states and the Federal Ministry of Food and Agriculture (BMEL)

Scientific presentations

IUFRO WP 7.03.10 Meeting, San Michele all'Adige, 22.-26.6.2015
Hoyer-Tomiczek U., Sauseng G., Menschhorn P., Hoch G.: Evaluation of the dog detection method for *Anoplophora glabripennis* and *A. chinensis*

USDA Research Forum on Invasive Species, Annapolis, 12.-15.1.2016
Hoch G.: ALB in Europe – further development of detection methods and risk management (Project ANOPLORISK II)

Upcoming: Deutsche Pflanzenschutztagung, German Plant Protection Conference, Section 14-3, Halle/Saale, 20.-23.09.2016
König S., van Capelle C., Wilstermann A., Schröder T.: Loop-mediated isothermal amplification (LAMP) for the detection of invasive and indigenous cerambycid beetles – strengths and weaknesses of the method

Presentations to Plant Protection Organizations and plant protection specialists

EPPO Workshop for inspectors on contingency planning, London, 18.-20.11.2014
Schröder, T.: Lessons learnt from outbreaks of *Anoplophora glabripennis* in Germany

Workshop on emerald ash borer and Asian longhorn beetle in urban areas, Toronto, 15-20.6.2015
Eyre, D. (Defra, formerly Fera): presentations on pathways for pest movement and one on public engagement activities in the UK

EPPO Council Technical Colloquium, Riga, 17.9.2015
Hoch G.: Further development of risk management for the EC listed species, *Anoplophora chinensis* and *A. glabripennis* (ANOPLORISK-II)

International Plant Protection Convention (IPPC), session of the Commission on Phytosanitary Measures (CPM), Rome, 4.-8.4.2016
Hoyer-Tomiczek U.: Detection dogs for the surveillance of the Asian longhorn beetles *Anoplophora glabripennis* and *Anoplophora chinensis*

Webinar at New York State monthly invasive species meeting, 25.5.2016
Hoch, G.: Presentation on *Anoplophora glabripennis* scent detection dogs and trapping within the project ANOPLORISK-II

Scientific journals

Hoyer-Tomiczek U., Sauseng G. & Hoch G. 2016: Scent detection dogs for the Asian longhorn beetle, *Anoplophora glabripennis*. Bulletin OEPP/EPPO Bulletin, 46, 148-155

Industry journals

Hoch G. & Hoyer-Tomiczek U. 2016: Spürhunde und Lockstofffallen gegen ALB. Forstzeitung, Wien, 127(5), 14-15,

Internet

EUPHRESCO Website
Project slide
http://www.euphresco.net/media/project_slides/anoplorisk_II_2.pdf
Success story
http://www.euphresco.net/media/success_stories/euphresco_success_story_anoplorisk_II_2.pdf

BFW Website
Scent detection dogs for the Asian longhorn beetle
http://bfw.ac.at/db/bfwcms.web?dok=10131

Contact to stakeholders

Results of the project were communicated to officers and inspectors of national plant protection organizations at various meetings at national or regional level by all partners.