Final Report

Project title (Acronym)
Use of Stable Isotope Ratio Analysis (SIRA) for the identification of invasive species native in alien environments (Invasive species by SIRA)

Project duration:

Start date:	2018-10-01
End date:	2023-03-31

Contents

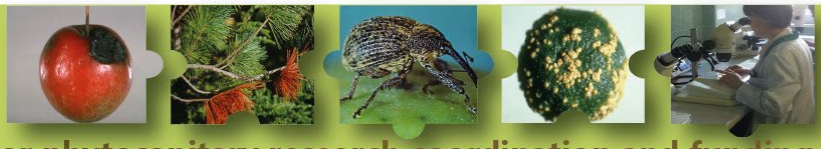
1. Research consortium partners

Coordinator – Partner 1			
Organisation	Fera Science Ltd (Fera)		
Name of Contact (incl. Title)	Katharina Heinrich	**Gender**:	F
Job Title	Senior Scientist		
Postal Address	Sand Hutton, York, YO41 1LZ, United Kingdom		
E-mail	katharina.heinrich@fera.co.uk		
Phone	+44(0)1904-462692		

Partner 2			
Organisation	Department of Lithospheric Research (DLR), Vienna University and: Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH-Linz (AGES)		
Name of Contact (incl. Title)	Dr Micha Horacek	**Gender**:	M
Job Title	Scientific Expert		
Postal Address	Wieningerstr. 8, 4020 Linz, Austria		
E-mail	untertrias@gmail.com		
Phone	+43(0)5 0555-41414		

Partner 3			
Organisation	Bundesforschungszentrum für Wald (BFW)		
Name of Contact (incl. Title)	Dr. Gernot Hoch	**Gender**:	M
Job Title	Department head (Department of Forest Protection)		
Postal Address	Seckendorff-Gudent-Weg 8, 1131 Vienna, Austria		
E-mail	gernot.hoch@bfw.gv.at		
Phone	+43 1 87838 1155		

Partner 4			
Organisation	Université d'Orleans - INRAE- Zoologie Forestiere Orléans (URZF)		
Name of Contact (incl. Title)	Pr Géraldine Roux	**Gender**:	F
Job Title	Professor of Biology		
Postal Address	2163 Avenue de la Pomme de Pin, CS 40001 Ardon, F- 45075 Orléans Cedex 2, FRANCE		
E-mail	geraldine.roux@univ-orleans.fr, geraldine.roux@inrae.fr		
Phone	+33 2 38 41 80 33		



Partner 5			
Organisation	Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA)-Centro di ricerca Difesa e Certificazione		
Name of Contact (incl. Title)	Dr. Giuseppino Sabbatini-Peverieri Dr Sauro Simoni	**Gender**:	M/M
Job Title	Researchers		
Postal Address	Via Lanciola 12a – 50125 Firenze, Italy		
E-mail	giuseppino.sabbatini@crea.gov.it sauro.simoni@crea.gov.it		
Phone	+39 0552492252 +39 349 6491473		

Partner 6			
Organisation	United States Department of Agriculture, Animal and Plant Health Inspection Service; PPQ S&T Forest Pests Methods Laboratory (APHIS-USDA)		
Name of Contact (incl. Title)	Dr Hannah Nadel	**Gender**:	F
Job Title	Supervisory Entomologist – Insect Production		
Postal Address	1398 West Truck Road, Buzzards Bay, MA 02542, USA		
E-mail	Hannah.nadel@usda.gov		
Phone	+1-508-563-0904 (office), +1-508-274-2274 (cell)		

2. Short project report

2.1. Short executive summary

The project 'Use of Stable Isotope Ratio Analysis (SIRA) for the identification of invasive species native in alien environments' aimed to develop SIRA as an arthropod tree-pest decision tool to inform phytosanitary response.

Modern analytical technologies, used in other fields can be applied to entomological research and pest control. Wood-boring larvae assimilate the isotope ratios of the wood they feed on, and isotope signatures can provide clues to the geographic origin of the emerged adults. The Asian longhorn beetle (ALB) *Anoplophora glabripennis* is among the most damaging invasive tree-pest insects in Europe and North America, and its detection can trigger an eradication response. Knowing if the source of detected specimens is local or foreign can influence the degree of response. A captured beetle with a local isotope signature probably developed from an egg laid locally one or two years earlier. The response to this would involve much effort and destruction of trees to locate further adults, larvae and exit holes made by adults in order to eradicate the pest. A captured beetle with a foreign (e.g. Chinese) isotope signature is more likely to be the only beetle present and an opportunity exists to trace the imported host wood to determine whether other adults may have emerged. In this case, only lower-cost localised monitoring for additional adult beetles would be required.

A method based on the measurement of stable isotopes in adult ALBs to identify the origin of the wood from which they emerged was developed in the framework of the project activities. The method can detect that an ALB has emerged from European wood in at least 95% of cases. The method was calibrated with ALBs from 11 locations in China (n=55) and 6 locations in Europe (Italy, n=28).

Additionally, samples of *Monochamus galloprovincialis*, *Monochamus sutor*, and *Monochamus sartor* – potential vectors of the pine wood nematode – from nine European countries (49 locations) were analysed.

To examine whether SIRA of one pest can instruct about another pest with a similar feeding habit, six pest species (n = 5 adults each) and timber from which they emerged 20 to 1047 days after seizure at US ports (between 2012 and 2016) were analysed for hydrogen and carbon isotope ratios. We found a positive offset for carbon from timber to beetle on all but one species on *Populus* wood.

Rearing beetles with lifecycles of 2 or more years in the laboratory is challenging. A most up to date guideline has been disseminated by USDA. However, to achieve higher survival rates from larvae to adult and create larger data sets, a more natural but controlled setting is recommended. While we demonstrated that SIRA can have a promising use in directing control efforts, more data may improve its power as an arthropod tree-pest decision tool.

2.2. Project aims

The overall goal of the project was to develop Stable Isotope Ratios Analysis (SIRA) as an arthropod tree-pest decision tool to inform phytosanitary response. The phytosanitary measures should be scientifically justified. Hence, amongst other things they should be underpinned by scientifically based, reliable information about the geographic origin of intercepted exotic pest species. Previous investigation has shown the use of SIRA to determine whether a non-native-species of arthropod (e.g. *Anoplophora glabripennis* – Asian Longhorn Beetle (ALB)) that has been caught in Europe, developed in Europe or whether the adult arrived from elsewhere. This determination could form part of the decision-making process on the actions to be taken, in case of interceptions or outbreaks of non-native species. The objectives of the project were: (1) sourcing of tree pest specimens, e.g. *Anoplophora glabripennis* (ALB), *Monochamus* spp., etc. from different geographic regions in sufficient numbers for SIRA and statistical evaluation of the results; (2) combining the analysis of insect specimen and host tree wood/bark for the investigation of correlation between their stable isotopes.

2.3. Description of the main activities

The main project activities were:

Sourcing of specimens

Sufficient numbers of specimens (adult beetles and timber) were sourced to allow for statistical evaluation of data. ALB specimens were sourced from China and other localities (Italy, USA). *Monochamus* specimens were sourced from various European localities. Specimens were also sourced for rearing studies.

SIRA of specimens

SIRA of specimens was coordinated between Fera (GB) and BFW/DLR (AT), alongside the sharing of analytical standards (in-house reference materials). Samples (adult beetles and timber) were analysed by SIRA and data were statistically evaluated, to gain preliminary understanding of isotopic signature correlation between host timber and pest specimens.

2.4. Main results

2.4.1. Technical assessment of instrumentation used

The technical performance of the stable isotope instrumentation was assessed and deemed satisfactory by the usage of in-house reference materials which are linked to IAEA certified materials.

2.4.2. SIRA of adult beetles and timber specimens

To examine whether SIRA of one pest can inform about another pest with a similar feeding habit, six pest species (n = 5 adults each) and timber from which they emerged 20 to 1047 days after seizure at US ports (between 2012 and 2016) were analysed for hydrogen and carbon isotope ratios. The beetles had not been intended for isotope studies, so the host wood was moistened weekly with sprays of tap water to enhance their survival.

The species were: *Anastrangalia dubia* (Scopoli), *Arhopalus rusticus* (Linnaeus), *Arhopalus* sp., *Chlorophorus diadema* (Motschulsky), *Hylotrupes bajulus* (Linnaeus) and *Trichoferus*

campestris (Faldermann*)*. For carbon isotopes, we noted a tentative correlation with a positive offset from timber to beetle for all samples, except for *Chlorophorus diadema* on *Populus* wood. For hydrogen, the offset differences from timber to beetle ranged from -12.3 to 71.5 ‰, with no apparent pattern. The non-correlation for hydrogen isotopes may have been caused in the laboratory by the tap-water sprays.

2.4.3. SIRA of ALB samples

The aim of the analysis was to explore the extent to which we can use isotope ratio measurements to diagnose whether an Asian longhorn beetle (beetle) that was caught in Europe, developed and emerged in Europe or was adventitiously imported from China through infested wood. If it is believed that a beetle has emerged from European wood, a great amount of effort and destruction of trees will be required to eliminate the infestation and monitor for further larvae and exit holes; because this means that the adventive population has produced at least one generation in the new environment. If the beetle is believed to have emerged from Chinese wood and been adventitiously imported, then it is more likely that it is the only beetle present (or one of the few specimens accidentally introduced into the environment). This creates an opportunity to trace the source of the import and define the status of the early find, and successively determine whether additional adults may have been introduced or emerged. In this case, only localised monitoring for additional adult beetles and fresh oviposition would be required. Hence an analytical method for discrimination between European-emerged beetles and imported beetles may be valuable to reduce the cost of subsequent action in some cases and to support decision makers.

For our diagnosis, a finding that a beetle emerged from European wood in Europe represents a *positive* detection of an invasive population, while a finding that a beetle was adventitiously imported in wood from China represents a *negative* test result for an invasive population. The diagnostic method was based on setting threshold values for the results of hydrogen isotope ratio measurements (or a combination of different isotope ratio measurements) when deciding whether a beetle was invasive or not. We statistically analysed results generated from European and Chinese beetles.

We estimated:

(i) the false positive rate (proportion of Chinese beetles identified as European) and diagnostic sensitivity (proportion of European beetles identified as European) for several thresholds,
(ii) the uncertainty associated with the estimates, and
(iii) the performance of a similar approach based on a linear combination of hydrogen, carbon and nitrogen isotope ratio measurements.

Figure 1 shows the estimated false negative rate for beetles (proportion of European beetles identified as Chinese) that emerged in Europe (turquoise curve) and false positive rate (proportion of Chinese beetles identified as European) (red curve) for detecting the presence of a beetle that emerged in Europe. Detection is declared for hydrogen isotope ratio measurements at or more positive than the chosen x-axis value.
The tolerance intervals (shaded areas) show the uncertainty associated with the estimated probabilities. We used the curves to choose a threshold to detect that an ALB has emerged

from European wood in *at least*[1] 95% cases. On this basis, a threshold of -102.1‰ was selected (panels 1 and 2 of Figure 1), for which less than 5% of European beetles will have a more negative isotope ratio.

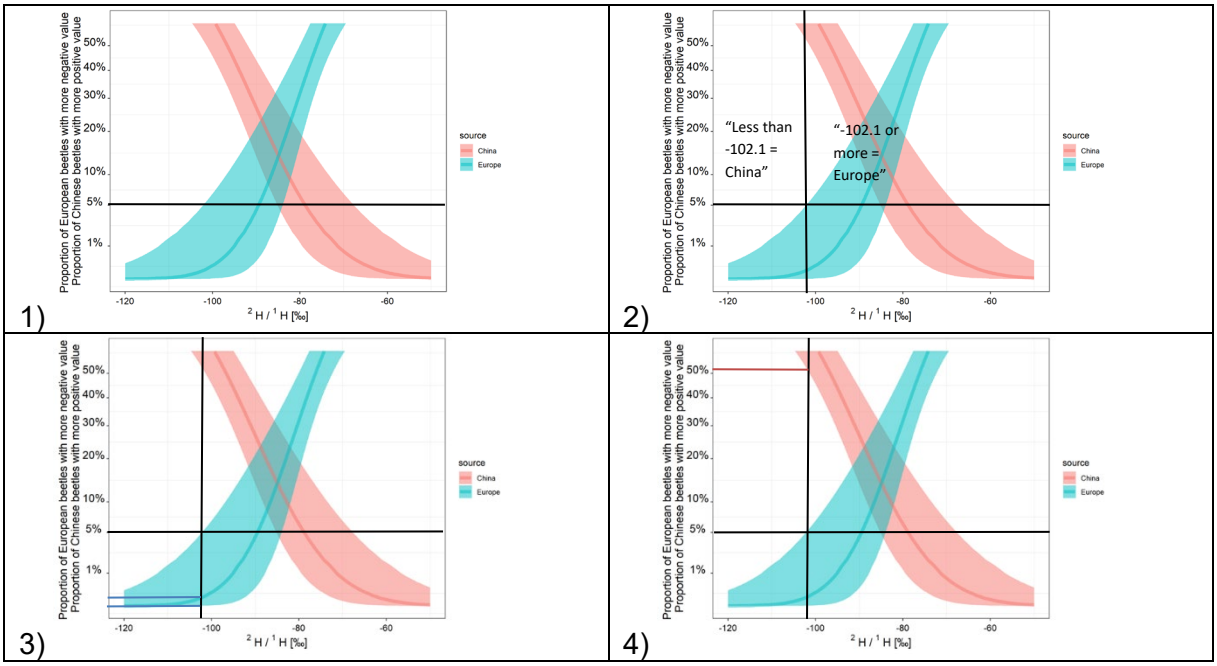


Figure 1: Tolerance intervals for selecting a decision threshold for hydrogen isotope ratio measurements with a low false negative rate for European beetles and the false positive rate for Chinese beetles.

Panel 3 of Figure 1 (blue horizontal lines) shows how the European curve provides the central estimate for the false negative rate (as the proportion at which the threshold line hits central estimate of the false negative rate), and lower limit for the false negative rate (as the proportion at which the lower estimate of the false negative rate hits the threshold line).
Assigning beetles with a hydrogen isotope ratio more negative than -102.1‰ as 'Chinese' gives a correct 'Chinese' identification in somewhere between 17 to 48% of cases (Panel 4 of Figure 1 – red horizontal line- shows the lower limit for the proportion of Chinese beetles that will produce a result that is lower than -102.1 ‰ as 52%; whilst the upper limit with 83% is not shown).
Hence, this method provides an opportunity to reduce control costs for a proportion of findings: up to 48% of beetles adventitiously imported from China, while maintaining the control of invasives in somewhere between 95% and over 99.9% of cases. The use of hydrogen, carbon and nitrogen isotope measurement results in a linear discriminant function was estimated to provide somewhat improved performance, although this would require further validation.
Furthermore, it must be taken into account that all investigated beetles from European locations came from Italy, even if they were from different outbreak sites and different years. Any ALB samples developed in trees further north in Europe are expected to possess more negative δ^2H-values.

[1] With 95% confidence

2.4.4. SIRA of Monochamus samples

One detailed analysis of the potential application of SIRA focused on the genus *Monochamus*, particularly the species *Monochamus galloprovincialis*, the vector of the pine wood nematode in Europe.

Samples of *Monochamus* spp. were sourced from several European countries. *Monochamus galloprovincialis* specimens were received from Portugal, Spain (two countries where the PWN has been detected), France, Serbia, North Macedonia, and Bosnia-Herzegovina. *Monochamus sutor* was available from Sweden. Some of the *Monochamus. galloprovincialis* were provided by partner INRAE (FR).

A rearing of *Monochamus sartor* from captured adults was started at BFW (AT) in an outdoor insectary during summer 2019. All adults and extracted larvae from 3 time points were stored at -20°C. Only one adult beetle emerged from the logs in summer 2020. Since no further offspring emerged afterwards, all logs were dissected in winter 2021/22 and living larvae were extracted and stored for analysis.

All *Monochamus* samples were prepared and analysed at BOKU - University of Natural Resources and Life Sciences, Tulln, Austria.

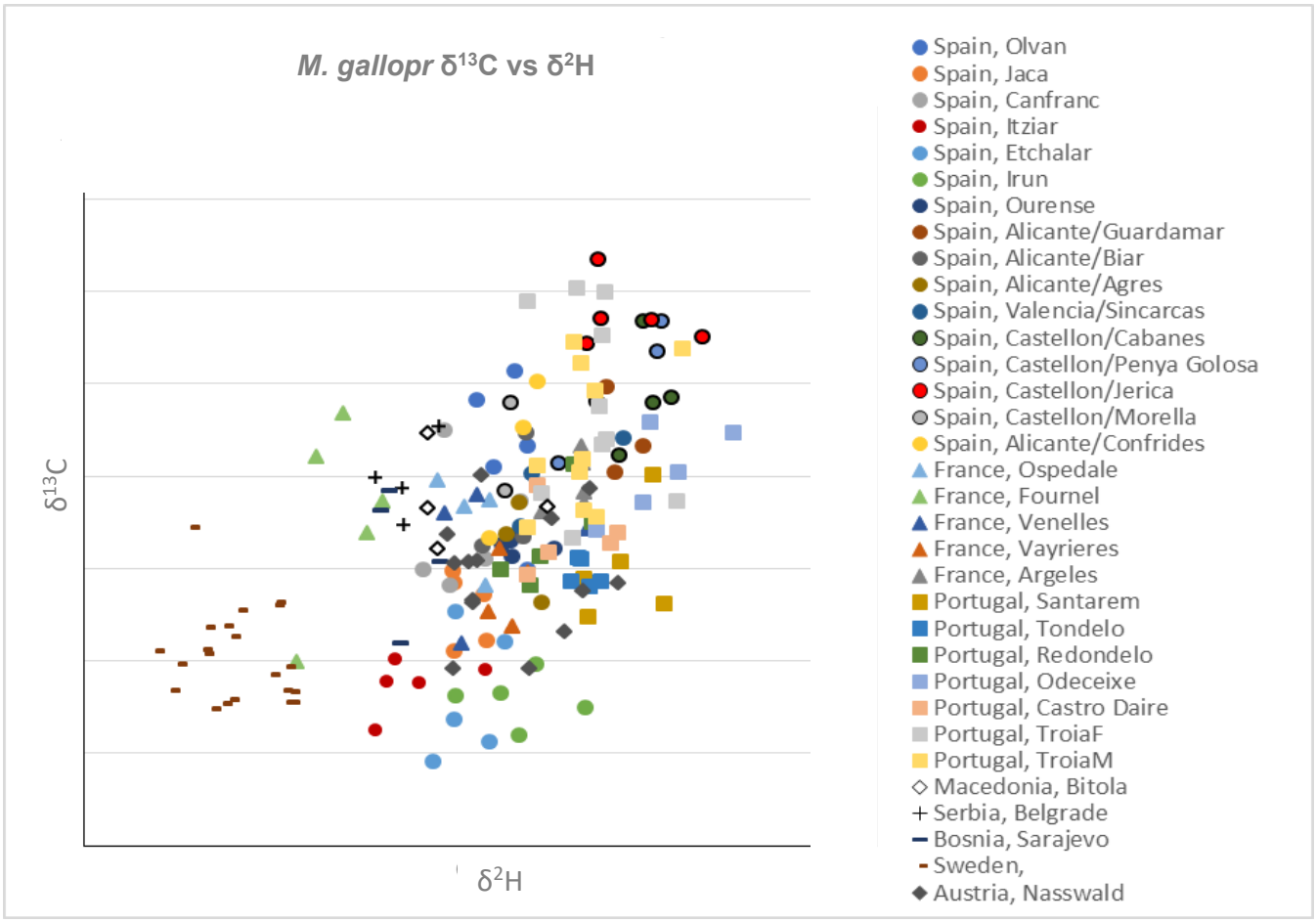


Figure 2: Correlation of $\delta^{13}C$ and $\delta^{2}H$ values of European Monochamus *specimens.*

Considering the *Monochamus* results from all of Europe (Figure 2), the samples from Scandinavia stand out. Their $\delta^{2}H$ values were significantly more negative than from any other investigated European region. These more negative $\delta^{2}H$ values are expected for regions with higher latitude and lower temperature. Also, samples from the West Balkan and the French Alps showed relatively negative $\delta^{2}H$ values. This could be due to an influence of continental climate, but also to an important contribution of snow to the water supply to the host trees. Samples from Spain and France showed a broad range of $\delta^{2}H$ of 40 ‰, which likely reflects the variation of the sampling sites from the coastal to the mountainous (higher altitude) forests. Results for Portuguese and Austrian samples fell in a much smaller range of 20 ‰ for $\delta^{2}H$.

Beetles from Sweden showed also more negative $\delta^{13}C$ values. This may be an indicator for a minor influence of drought stress in this region. Spanish samples support this assumption; as more negative values were recorded from humid regions in the North and Northwest (e.g. Irun, Itziar, Etchalar) compared to more positive values in Castellon, Alicante and Valencia.

Overall, the isotope analysis of *Monochamus* specimens from Europe shows some differentiation among regions but with notable overlap, except for the Scandinavian samples.

The analysis from reared *Monochamus sartor* was inconclusive: Isotope analysis of larvae from the rearing gave highly variable results and extreme outliers. As C:N ratios indicated

insufficient lipid extraction, we concluded that the sample matrix was not suitable. Therefore, no valid results are available from this experiment to compare adult beetle, offspring larvae or wood samples.

2.4.5. Guideline for rearing of beetles

Work was undertaken by partner USDA (US) on improving the rearing of longhorn beetles. The findings were:

1. Larvae originating from temperate climates benefitted from chilling at 10°C for 11 weeks starting in autumn or early winter.

2. Larvae survived at a higher rate if left undisturbed in the wood in which they were found.

3. Larvae that had separated from their host wood through an opening benefitted from being returned to their galleries. To prevent them from leaving again, a second piece of wood was placed against the opening and the two wood pieces were strapped together with string or rubber bands.

4. Survival improved when moisture was increased inside the incubation barrels. The interiors of the barrels were sprayed weekly with water from a hand sprayer.

5. A greater percentage of larvae survived to the adult stage in their host wood (34%) than in an artificial diet developed for ALBs (20%).

6. Species that were reared to adult in the ALB diet included members of these genera: *Aeolesthes, Anoplophora, Arhopalus, Clytus, Coptops, Megopis, Monochamus, Phoracantha, Plagionotus, Pseudastylopsis, Trichoferus, Xylotrechus,* and *Xystrocera*.

2.5. Conclusions and recommendations to policy makers

We have shown how the measurement of hydrogen isotope ratios (SIRA) of ALBs can be used to detect whether beetles found in Europe may have developed and emerged in Europe. There are a number of ways we can use this information. Four options are:

1. We can decide not to use isotope measurements and:
    a. assume all beetles we find may have developed in European wood;
    b. assume all the beetles that we find did not develop in European wood.
2. We can use isotope measurements and:
    a. assume that beetles developed in European wood unless the isotope measurement show that this is unlikely;
    b. assume that beetles developed in foreign wood (e.g. Chinese wood) unless measurement results show that this is unlikely.

	Option	Test beetles	Relative cost of unnecessary action	Relative cost of undetected outbreaks
1a	**Assume European origin**	No	100%	0%
2a	Unless hydrogen IRMS test proves[1] not Unless test with increased samples proves not[2]	Yes Yes	53 to 83% 37 to 60%	0.07 to 5% 0.02 to 5%
1b	**Assume foreign (e.g. Chinese) origin**	No	0%	100%
2b	Unless hydrogen IRMS test proves[1] not Unless test with increased samples proves not[2]	Yes Yes	0.07 to 5% 0.15 to 5%	66 to 96% 57 to 81%

[1]: with 95% confidence. [2]: Illustration showing predicted potential performance, where the distribution of beetles from each source has been estimated with double the number of current samples.

Option 2a may give an appreciable reduction in the cost of unnecessary action compared with the strict option 1a while maintaining a high efficacy against invasive beetles that developed and emerged in Europe. Option 2b maintains the low cost of unnecessary action associated with the more permissive option 1b, but provides evidence for action to be taken in a proportion of cases where invasive beetles are observed. Any reduction in mitigation efforts has some value. "Occasionally" detecting invasive presence is not functionally better than never detecting it, therefore this analytical method (SIRA) is recommended for use with option 2a.

The test applies a detection threshold for European beetles being, for option 2a, more positive than -102.1 ‰ and for option 2b more positive than -67.7 ‰. Other thresholds can be applied which balance the sensitivity and specificity. However, there is no threshold that provides both high sensitivity and high specificity.

There is a large uncertainty associated with the detection power of the methods. For example, we will correctly identify Chinese beetles in somewhere between 17% and 48% of cases. Collecting more observations may reduce this uncertainty. If estimates of mean SIRA results and their variation remain as they are now, then doubling the number of observations projects the test performance given in „test with increased samples" in the table.

2.6. Benefits from trans-national cooperation

This project benefited significantly from trans-national cooperation. This included receiving samples collected in China, nine European countries and from ports in the USA. This project relied entirely on analyzing insects from a broad geographical range therefore it would not have been possible without trans-national cooperation.

We received and analysed *Anoplophora glabripennis* from eleven Chinese provinces and six locations in Italy.

We received and analysed *Monochamus galloprovincialis*, *Monochamus sutor*, and *Monochamus sartor*, (potential) vectors of the pine wood nematode, from 49 European locations. We also received samples of six species of wood-boring beetles from the USA:

Anastrangalia dubia (Scopoli), *Arhopalus rusticus* (Linnaeus), *Arhopalus* spp., *Chlorophorus diadema* (Motschulsky), *Hylotrupes bajulus* (Linnaeus), Trichoferus campestris (Faldermann). In addition, we received and analysed timber which had been collected with the sample from the USA. This was important in understanding the relationship between the stable isotope ratios of the beetles and the trees they developed in.

Trans-national cooperation was also beneficial to the project in sharing sample collection and preservation, and analytical methodologies. We also gained an understanding of the issues created for different countries and the requirements for detecting and controlling these pests and vectors.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

Manuscript in preparation: Horacek M., Hoch G., Kacprzyk M., Akulov E., Kirichenko N., Naves P.: Stable isotope analysis as a promising tool to discriminate between the geographic populations of *Monochamus* species (Coleoptera, Cerambycidae), the potential vectors of the pinewood nematode.

4. Open Euphresco data

None.