Final Report

For more information and guidance on completion and submission of the report contact the Euphresco Call Secretariat (bgiovani@euphresco.net).

Project Title (Acronym)
***Epitrix* (flea beetle) species, life cycles and detection methods (*EPITRIX*)**

Project Duration:

Start date:	01/11/13
End date:	31/10/16

1. Research Consortium Partners

Please note that partner numbers differ from the ones used in the original proposal

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In 2014, the consortium was expanded to include the work undertaken by senior scientists Dr. Kees Booij (lead contact: kees.booij@wur.nl) and Antje de Bruin at Wageningen Plant Research, Wageningen UR, the Netherlands.

2. Executive Summary

Project Summary

The genus *Epitrix* Foudras (Coleoptera: Chrysomelidae: Alticinae) comprises nearly 180 species worldwide. Most of the species occur in the neotropics and only 12 and 17 species are known from North America and Europe, respectively. *Epitrix* species feed mainly on plants from the family Solanaceae, though they may feed on other plant families when their preferential host is not available. The adults feed above-ground on the leaves of their host-plants. The females lay eggs at the base of the stem, and the larvae develop underground, on the root system. In the American continent, several *Epitrix* species which are very similar in appearance have been associated with potato damage. In Europe two species, *E. papa* and *E. cucumeris*, were introduced and have since become established in Portugal and Spain which requires control measures to be implemented to prevent economic damage.

The main objectives of the project consortium were:

- To collect and rear *Epitrix* species for supply to project partners
- To make an identification key of North American and European *Epitrix* species and establish a reference collection of different species
- To develop and validate quick molecular detection methods, comprising a general screening assay for the *Epitrix* genus and specific assays for several *Epitrix* species
- To evaluate the overwintering possibilities for potato flea beetles under Northern European winter conditions
- To identify insecticides which are effective in controlling *Epitrix* species
- To investigate the biology, ecology and possibility of controlling *E. papa* and *E. cucumeris* by entomopathogenic nematodes
- To find attractants to use for monitoring of *Epitrix* beetles

The key results of the project consortium are:

- For *E. papa* estimates of the mean developmental times from egg to adult were obtained at 15, 20, 25 and 30 ºC, as were estimates of juvenile mortality, preoviposition period, oviposition period, adult longevity and fecundity. The lower developmental threshold was determined
- Pre-imaginal stages of *E. papa* were found to be susceptible to products of entomopathogenic nematodes, with *Steinernema feltiae* and *Heterorhabditis bacteriophora* showing good promise. A field experiment showed the relevance of soil moisture for studying the efficacy of nematodes under field conditions
- In a choice experiment carried out in the field with potted plants of potential host plants, black night-shade (*Solanum nigrum*) was more attractive to adult *Epitrix* spp. than potato
- *Solanum*-produced secondary leaf volatiles were identified and tested in laboratory olfactometer and field trapping tests for attractiveness for *E. papa* and *E. cucumeris*. A combination of leaf volatiles was found to be sufficiently attractive to have potential for *Epitrix* spp. monitoring
- An identification key of North American and European species of *Epitrix* was developed

- A reference collection of different species was established
- An update of the EPPO diagnostic protocol on *Epitrix* spp. was produced with an identification key based on the morphology of male and female genitalia allowing differentiating the regulated species in the European lists of the European species
- A collection of voucher specimens comprising pinned adults and slide-mounted male and female genitalia was established and could be made available to members of EPPO / NPPOs
- Following the change of taxonomic position of one of the regulated species (*E. similaris* as *E. papa*), specimens of *E. similaris* were collected in their native range (California) and subsequently characterised molecularly in order to update the Qbank database
- A generic qPCR assay based on ITS (internal transcribed sequence) for the genus Epitrix was developed. The screening assay was successfully validated against specimens of *Epitrix* and unrelated flea beetles
- A one-step, rapid and sensitive SybrGreen duplex-assay was developed for the detection and identification of the *E. papa* and *E. cucumeris*; specificity was tested against *Phylotreta atra*, *Chaetocnema concinna* and *E. tuberis*
- Molecular assays were developed for the four regulated *Epitrix* species and five further *Epitrix* species from North America and Europe. Testing against specimens of all 9 species and one South American species revealed high specificity in all cases
- An in-field rapid LAMP assay was developed for *E. papa* and *E. cucumeris*. Preliminary testing validated the *E. papa* assay, but indicated the *E. cucumeris* assay required further development. Barcode sequences were used to produce a phylogenetic tree for *Epitrix* species indicating that COI (cytochrome c oxidase subunit I) gene is an ideal target for diagnostic development.

In conclusion, the results which will be separately published indicate that *E. papa* can find suitable temperature conditions for development in European zones not presently invaded; the results can be used to verify the zones at risk. The fact that *Epitrix* spp. also thrive on other plants beside potato should be taken into account in management strategies. The susceptibility of *Epitrix* spp. to nematodes are encouraging in view of finding non-chemical control means and should be further investigated in terms of finding optimal nematode species, strains, formulations and doses for field use. Suitable traps and attractants are indispensable for monitoring *Epitrix* populations in potato crops and for detecting unwanted *Epitrix* presence in potato exports to non-infested zones. The project consortium identified some attractive substances but further studies are needed to develop effective and selective monitoring tools. The taxonomic results now allow morphological identification of European species and of North American species developing on potato. This together with the different molecular assays developed in the project consortium ensure fast and accurate notification in the event of an outbreak/interception as well as confidence in situations where non-regulated *Epitrix* species are found within crops. Further validation of the assays developed for the detection and identification of the *Epitrix* species would be beneficial.

3. Report

Project Management
Project consortium leaders: Andrew Cuthbertson (FERA) was co-ordinator for the first 2½ years of the project consortium but due to differing end dates in funding resources Annie Enkegaard (AU) took over the project co-ordination for the final year.

The consortium activities and the partners' interactions were defined within the kick-off meeting held at FERA (York, UK) on 11th December 2013. At this meeting all partners agreed on the current structure (ecological, taxonomy and molecular based research) for the project. This was partly due to ongoing research work within Portugal, hence the need to avoid duplication. Since biological research relied heavily upon sustained supply of insects, it was agreed that INIAV would supply both FERA and AU specimens to initiate their own rearing cultures. Tasks were re-defined as presented in the current report.

The consortium progress was verified with a second annual meeting, held at INIAV (Oeiras, Portugal) on 6th November 2014. A third meeting – with some results and conclusions presented – was held at ANSES (Montpellier, France) on the 5th November 2015. A final (4th) meeting was held on the 27th October 2016 again at INIAV (Oeiras, Portugal). This meeting discussed the putting together of the final report.

Consortium management activities ensured smooth running and accomplishment of the activities by definition of Work Package (WP) leaders/roles and by coordination of the exchange of information and obtaining data among WPs, predominately by the use of e-mails and annual meetings.

Close contact with Dr. Baldissera Giovani (the Euphresco Co-ordinator at EPPO) was maintained throughout the life of the project.

Re-classification of beetle species
After the submission of our consortium proposal, the populations of *Epitrix* formerly identified as *E. similaris* Gentner established in Portugal and Spain changed taxonomical position to *E. papa* Orlova-Bienkowskaja (Orlova-Bienkowskaja, 2015). In the text below the species name '*E. papa*' has therefore replaced '*E. similaris*' used in the application.

3.1. **Objectives and tasks of the project**, as stated in the proposal, with degree of achievement

The aims of the consortium were to assist NPPOs with 1) a study of the lifecycle and ecology of tuber flea beetles as a basis for understanding their potential to establish in Northern European regions and the choosing of efficient control methods; 2) the development of detection and identification tools of tuber flea beetles both for growers and NPPOs.

The objectives of the consortium were (I and VI were not specifically mentioned in the original application but are added here for clarity):

I. To maintain live cultures of *E. papa* and *E. cucumeris* (Harris) and to collect field specimens for the supply of insects to project partners. INIAV. *Objective achieved.*

II. To make an identification key of North American and European species of *Epitrix* and establish a reference collection of different species. More specifically the objectives were to produce i) an update of the EPPO diagnostic protocol on *Epitrix* spp. with an identification key based on the morphology of male and female genitalia allowing differentiating the regulated species in the European lists of the European species; and ii) a collection of voucher specimens comprising pinned adults and slide-mounted male and female genitalia. As an additional task, following the change of taxonomic position of one of the regulated species (*E. similaris* as *E. papa*), specimens of *E. similaris* were collected on a field trip from its native range (California) and subsequently characterised molecularly. ANSES. *All objectives achieved.*

III. To develop and validate quick molecular detection methods, comprising a general screening assay for the *Epitrix* genus and specific assays for the four species of most imminent threat to Europe (*E. papa, E. cucumeris, E. tuberis* Gentner*, E. subcrinita* (LeConte)) in addition to other relevant *Epitrix* species, if possible. Tasks undertaken in fulfilment of these objectives were:

- Development of a generic test for *Epitrix* detection. AU
- Development of molecular diagnostic assays for molecular identification of the 4 regulated *Epitrix* species: *E. tuberis*, *E. subcrinita, E. papa and E. cucumeris* as well as 5 *Epitrix* species sourced from crops and weeds in North America and Europe: *E. brevis* Schwarz*, E. hirtipennis* Melsheimer*, E. cf hirtipennis, E. pubescens* (Koch) and *E. similaris*. SASA
- Taking part in validation of tests developed by other partners. SASA
- Contributions to the development of diagnostic assays. FERA
- Validation of already developed FERA taqman assays for flea beetle diagnostics FERA
- Development of a duplex real-time PCR based-method for the simultaneous detection of *E. papa* and *E. cucumeris* captured in infested potato fields. INIAV
- Planning and performing a preliminary in-house validation of the duplex real-time PCR based-method to confirm performance parameters (specificity and LOD). INIAV

All objectives achieved.

IV. To evaluate the overwintering possibilities for potato flea beetles under Northern European winter conditions and evaluate the generation times and population increase capacity of the beetles under Northern European summer conditions. Studies to be done in Denmark based on laboratory reared populations of *Epitrix* sp. This objective was modified after the first consortium meeting, following discussion with project partners, to focus solely on the overwintering possibilities. AU. *Objectives not achieved due to persistent problems with rearing of the flea beetles*.

V. To identify insecticides effective in controlling *Epitrix* species, including an initial literature survey of current and potential control methods. Studies to be done in UK based on laboratory reared populations of *Epitrix* sp. FERA. *Objective only partially achieved due to persistent problems with rearing of the flea beetles.*

VI. To investigate the biology, ecology and possibility of controlling *E. papa* and *E. cucumeris* by entomopathogenic nematodes following work initiated in Portugal in a project funded by the Portuguese Foundation for Science and Technology (FCT - Fundação para a Ciência e Tecnologia): "Pest status, bioecology and sustainable control of two exotic potato pests newly introduced in Portugal: *Epitrix similaris* and *Epitrix cucumeris*" (PTDC/AGR-PRO/120292/2010). INIAV. *Objective achieved.*

In 2014, the consortium was expanded (with permission of the EUPHRESCO coordinator) to include the work undertaken at Plant Research International, Wageningen UR (WUR/DLO), the Netherlands, aiming at finding attractants to use for monitoring of *Epitrix* beetles. *Objective partly achieved, research ongoing.*

3.2. Methods and Results

1. Maintenance of live cultures of *E. papa* and *E. cucumeris* and collection field specimens (INIAV)

Cultures of *E. papa* and *E. cucumeris* were kept in separate premises to prevent cross-contamination. *E. cucumeris* was reared until September 2014, and *E. papa* until July 2015. The first rearing was stopped due to INIAV relocating premises from Lisbon to Oeiras as the climatic chambers were disassembled. The *E. papa* rearing unit was kept until the current project funding stopped. Throughout the life of the project the cultures were refreshed twice a year with field collected insects.

The insects were maintained on aubergine potted plants kept in cages at 22 ºC day and 20 ºC night, with a16 L: 8 D photoperiod, following a protocol developed at INIAV for mass rearing *Epitrix* beetles needed for biological studies (Boavida *et al*., 2014a). Live *E. papa* were supplied to the Danish partner under an official LoA, in May and September 2014. Dead specimens were supplied to SASA, the Danish Plant Health, FERA, and ANSES.

2. Insecticides (FERA)

Establishing viable cultures of *Epitrix* species

Specimens of *E. cucumeris* and *E. papa* were originally imported under specific license from Portugal (INIAV) in 2011. These were cultured within FERA's Insect Quarantine Unit following the method of Boavida *et al*. (2014a).

The cultures of *Epitrix* spp. did not take off and even though much time was spent endeavouring to improve the culture, it failed.

Literature review and selection of potential insecticides for testing

A full literature survey was undertaken using search engines such as Web of Knowledge, SCOPUS and Cab Extracts. Grey literature and personal scientific

contacts were likewise sourced for information covering past, current and potential control measures for *Epitrix* spp.

A literature review was published outlining current and potential control methods (chemical and ecological) of *Epitrix* spp. (Cuthbertson, 2015). Very little information exists in regards to the control options available for potato flea beetles. Synthetic pyrethroids are the weapon of choice for the beetles. However, the impetus in integrated pest management is to do timely (early-season) applications with something harsh which will give long-term protection at a time when there are not a lot of beneficials in the field. Finding the balance for control of *Epitrix* spp. is proving difficult.

Based on the literature review and discussion with consortium partners (at the second annual meeting), the following insecticides were selected for testing as control agents. The products Chloropyrifos 1b, Thiacloprid 4A, Acetamiprid 4A, Thiamethoxam 4A and Cypermethrin 3A, which are generally used for Colorado beetle control within potato crops, were deemed worthy of investigation. However, *Epitrix* spp. continued to prove extremely difficult to culture under laboratory conditions. As a result, chemical screening of products against life stages of *Epitrix* spp. could not be undertaken.

3. Overwintering possibilities (AU)

Specimens of *E. papa* were imported from Portugal and reared under approved quarantine conditions at Research Centre Flakkebjerg, AU, using methods adapted from Boavida *et al*. (2014a). The first shipment (May 2014) failed to produce a new generation of beetles in adequate numbers to sustain the rearing and a new shipment was consequently imported in September 2014. This resulted in a viable, albeit small and unsynchronised rearing which sustained itself over a period of several months though never yielding enough specimens for initiation of the planned experiments on tolerance of the beetles to temperatures mimicking Danish winter conditions. Multiple efforts to improve the rearing and increase the output failed.

4. Biology, detection and control studies

4.1 Biological parameters of Epitrix papa *(INIAV)*

Laboratory studies were carried out with insects reared at the INIAV insectary. In order to determine developmental times at different temperatures (15, 20, 25 and 30 ºC) cohorts of eggs were established by exposing clean potato plants to caged populations of adults. After 24 hours the plants inoculated with eggs were separated from the adult insects and transferred to a climate controlled chamber at a constant temperature to allow the insects to develop into adults. The adults emerged were daily removed with an aspirator and counted. To determine the pre-oviposition and oviposition periods, and the longevity and fecundity of the females, one-day-old females were reared, together with males, on a potato leaflet in a Petri dish until death. Adult mortality was daily recorded, and the eggs counted and removed. To determine the egg incubation period, eggs were transferred to moist filter paper in Petri dishes and incubated at room temperature until hatching. To assess the juvenile mortality, a known number of eggs was collected, transferred to the soil of clean potted plants, and incubated until the adult's emergence. The total number of adults emerged was recorded.

Estimates of the mean developmental times from egg to adult were obtained for the temperatures 15, 20, 25 and 30 ºC, and were used to calculate the lower developmental threshold (LDT). Estimates of the duration of the embryonic development, preoviposition, and oviposition periods, and adult longevity were obtained, as well as estimates for the average female fecundity. The juvenile mortality was estimated.

4.2 Development time of Epitrix cucumeris *(INIAV)*
The above described methods were used. The disabling of the climate controlled chamber dedicated to *E. cucumeris* caused the loss of the experiments.

4.3 Susceptibility of Epitrix papa *to entomopathogenic nematodes (INIAV)*
In 2014, the susceptibility of larvae, pre-pupae, pupae and adults of *E. papa* to commercial formulations of *Steinernema carpocapsae* (Weiser), *Steinernema feltiae* (Filipjev) and *Heterorhabditis bacteriophora* (Poinar) applied at a rate of 50 IJ/cm^2 were tested under laboratory conditions. To complement this, a higher nematode dose (300 IJ/cm^2) was tested on beetle larvae. The insects were collected in the rearing unit. The entomopathogenic nematode products were supplied by e-nema GmbH. A rearing method for testing the larvae was developed: after the larvae were collected from the soil by a flotation method they were transferred to small rearing tubes containing soil and rooted aubergine plantlets and allowed to settle on the roots for 2 days. The nematode suspensions were then applied directly on the soil. For testing pre-pupae, pupae and adults, the insects were confined in Petri dishes with the bottoms lined with filter paper, and the nematode suspensions were applied on the filter paper. Insect mortality was assessed after a fixed number of days.

Insect mortality varied according to the insect developmental stage, the entomopathogenic nematode species, and the dose rate applied. Larvae, pre-pupae and pupae were susceptible to the three nematode species, while adults were only susceptible to *S. carpocapsae*. Overall, the most effective species against the pre-imaginal stages were *S. feltiae* and *H. bacteriophora* (Boavida *et al.,* 2014b).

4.4 Field experiment to determine the efficacy of entomopathogenic nematodes (INIAV)
In 2015 an experiment was carried out in a farmer's field to compare the efficacy of commercial formulations of *S. carpocapsae*, *S. feltiae* and *H. bacteriophora* to control *Epitrix* populations and tuber damage. The nematode formulations were supplied by e-nema GmbH. The potato field had a uniform distribution of *Epitrix* adults. The products were applied after tuber initiation, at the end of the day (7 pm), and were prepared according to the instructions of the supplier, after a thorough irrigation was undertaken. The experimental lay-out was a randomized block design with 4 treatments (the three nematode species and a water control) and four replicates. The net plot size was 1.6 x 4.5 m = 7.2 m^2 (2 rows). The evaluation was carried out by comparing the number of adults emerged per treatment, and by comparing the length of galleries tunnelled by the larvae in the tuber surface. To count the number of adults emerged in each treatment, one bottomless emergence cage was placed over each plot for one week, to retain the adult *Epitrix* emerging from the soil at the end of the experiment. To assess the tuber damage caused by the larvae, a sample of 30

tubers/plot was collected, and the length of the tunnels present on the tuber's surface was measured in the laboratory.

No differences were observed between the nematode products and the water treated control neither in terms of tuber damage nor in the number of adults emerging, indicating no efficacy of the nematode products. Nevertheless, these negative results must be taken with caution, since the nematode's survival and activity may have been hampered by a presumable lack of soil moisture. Indeed, the drip irrigation installed in this field proved to be inadequate to assure a uniform moisture level of the soil, with the irrigation pipes getting clogged after the passage of the machines, as shown by the high incidence of tubers showing symptoms of either excess or lack of water in the soil at the end of the experiment.

4.5 Attractants for the detection of Epitrix *spp. (INIAV and WUR/DLO)*

Host-plant preference studies

A choice experiment was carried out by INIAV in 2014 in the field to determine the preferences of natural populations of *Epitrix* spp. in relation to five selected plant species, namely potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), aubergine (*Solanum melongena*), fat hen (*Chenopodium album*) and black night-shade (*Solanum nigrum*). The plants were grown in small pots (9 x 9 x 9 cm) in a glasshouse and brought to the field where they were distributed in a 5 x 5 randomised block design. Each plot had 10 plants of each of the five plant species. The number of *Epitrix* adults per plant species was weekly monitored during one month.

Solanum nigrum was the most attractive plant for *Epitrix* spp. confirming previous results (Boavida *et al*., 2013).

Collecting plant and insect volatiles to attract Epitrix

Following these results, samples of volatile substances from both insects and clean and infested aubergine and black-nightshade plants were collected in 2014 and in 2015, by headspace-sampling, and by extraction of adults in dichloromethane, at the INIAV laboratory. The samples were chemically analysed at Wageningen (WUR/DLO) in order to identify promising attractive substances that could be used for monitoring. From this analysis and literature derived information potentially attractive compounds were selected to be tested as an attractant for *Epitrix*. Behavioural response to these substances was assessed in olfactometer studies in the lab and in traps in a preliminary field test. In both cases significant differences were found in attractivity of different volatiles. One mixture has the potential as a monitoring substance in the field, which should be further tested.

Field testing of traps and attractants

In 2015, 30 C*Solomon* "hat"raps (KLP) with a volatile dispenser were distributed in a potato field, uniformly infested with *Epitrix* beetles the day after the potato haulms were cut for harvest. There were four treatments: 1, 3-butanediol; Z3-hexenylacetate + Linalool 1:1; E ß-farnesene; and a blank control. The experimental lay-out was a block design with 4 treatments x 7 replicates. After one week, the traps were collected and the number of *Epitrix* trapped by treatment was counted at the INIAV laboratory separately for each *Epitrix* species.

Both species were trapped. The mixture Z3-hexenylacetate + Linalool 1:1 caught significantly more flea beetles than the blank control; the other two compounds were not attractive. This mixture was retained as a contrast for future experiments.

Olfactometer studies

Elaborating on the preliminary field experiment 7 other potentially attractive plant compounds were selected from the head-space analysis and literature data and tested in combination with the Hexenylacetate / Linalool blend for attractivity. One of them improved the attraction of *Epitrix papa* considerably. Further field testing is required to show their potential to be used in field monitoring.

5. Identification key and reference collection (ANSES)

Morphological studies

Adult specimens of the twelve target species (*E. abeillei* (Bauduer)*, E. atropae* Foudras*, E. caucasica* Heikertinger*, E. cucumeris, E. dieckmanni* Mohr*, E. hirtipennis, E. intermedia* Foudras*, E. papa, E. pubescens, E. similaris, E. subcrinita, E. tuberis*) were collected from fresh material or collections borrowed from various institutions (Basel Museum, Instituto Nacional de Investigação Agrária e Veterinária (INIAV I.P.), Muséum national d'histoire naturelle (MNHN), Natural History Museum (NHM), Smithsonian National Museum of Natural History (NMNH), California Department of Food and Agriculture (CDFA)).

Their genitalia, female spermatheca and male aedeagus, were dissected and slide-mounted according to the following method:

Dry specimens are softened in warm water for approximately 30 minutes, until the abdomen can be removed. The whole abdomen is removed under binocular microscope with forceps and placed in warm potassium hydroxide solution (approx. 10%) for approx. 20 minutes to macerate muscles and fat tissues. The abdomen is rinsed and dissected in distilled water under a binocular microscope (X20), opening the abdomen along one side with fine scissors or pins, cleaning out the remaining tissues and carefully severing the genitalia from the apical segments. The abdomen and genitalia are rinsed in distilled water or ethanol and placed into lavender oil. Then the genitalia (aedeagus or spermatheca) are placed in a drop of Canada balsam on a slide for permanent storage. The genitalia were subsequently studied under high power microscope (X200).

The study of the genitalia allowed the development of a morphological identification key which served as a basis for updating the EPPO diagnostic protocol (EPPO, 2011, see appendix 2). In addition, a reference collection has been made. Slide-mounted genitalia of the studied species constitute a part of this reference collection. A second part consists of pinned adults (field records and museum specimens) of European *Epitrix* species and North American ones developing on potato. This collection of voucher specimens is maintained at ANSES and could be made available to members of EPPO / NPPOs.

Molecular characterization

Following the morphological identifications, DNA barcode sequences were obtained for every regulated *Epitrix* species as well as for *E. similaris*. Genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol without destruction of the specimens, to allow subsequent examination of morphology. The standard 658 bp region of the COI gene was amplified by PCR and sequenced following the EPPO protocol for DNA barcoding of arthropods (EPPO, 2016).

The obtained COI sequences were used to (i) support the recognition of *E. papa* as a species distinct from *E. similaris* (Mouttet *et al*., 2016b), (ii) produce a corrigendum to existing studies on molecular identification of *Epitrix* potato flea beetles (Mouttet *et al.*, 2016a) and (iii) update the QBank database (http://www.q-bank.eu/) and the EPPO diagnostic protocol (EPPO, 2011).

6. Quick molecular detection methods

6.1 Development of a generic test for Epitrix *detection (AU)*

Based on an alignment of *Epitrix* ITS sequences with the ITS from other insect species (Figure 1) primers and a probe were designed as

EpitrixFwd TCG TTT AAT AAT ARC GAG CAA GAC A
EpitrixRev ACT TAA GAC GCG CAC TGA TCA G
EpitrixProbe FAM-TCT CGG ACA CGA CAA TCG TGC ACA- BHQ1 (reverse strand)

DNA extractions from specimens obtained from project partners were carried out using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's instruction.

Real-time PCR was carried out using a ViiA^{Tm} 7 real-time PCR system (Life Technologies) using 384 well plates. A reaction mixture of 13 µl was prepared containing 0.9 µM of each of the forward and reverse primers, 0.25 µM of probe and 7.5 µl of Bio Probe Mix LoRox (PCR Biosystems, London, UK). Water and 2 µl of DNA template were added to a total volume of 15 µl. Thermal cycles in the real-time PCR consisted of an initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Two technical replicates were prepared for each reaction and the average threshold cycle (Ct) value was calculated. Ct values below 38 (cut off value) were considered positive; Ct values above 38 were not consistent between the two technical replicates and were thus considered negative.

First, an assay was tested on two batches of *E. papa* DNA isolated from live populations of insects reared at AU, using two different Master mixes "TaqMan Universal" from Life Sciences and "qPCRBIO" from PCR Biosystems, which gave similar results (Table 1).

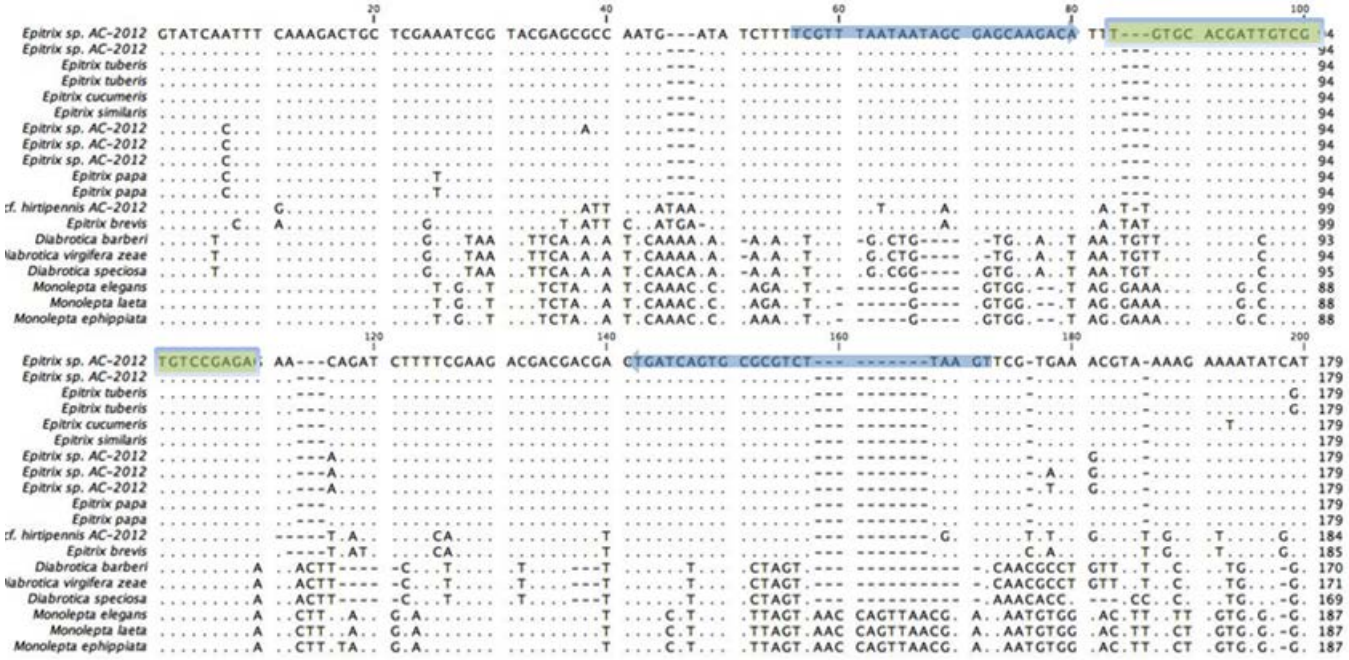

Figure 1. Alignment of ITS sequences from *Epitrix* and non-target species (as available from Genbank prior to January 2017). Primers and probes are indicated in blue and green, respectively.

Table 1. Ct values obtained using two different qPCR master mixes (TaqMan Universal and qPCRBIO). Two samples ("1" and "2") were amplified undiluted and a ratio of 1:10

TaqMan Universal	
Sample	Ct
1	15.02
1	14.87
2	13.66
2	13.43
1 1:10	18.32
1 1:10	18.15
2 1:10	16.25
2 1:10	16.62
H_2O	Undetermined
H_2O	Undetermined

qPCRBIO	
Sample	Ct
1	14.95
1	14.38
2	13.18
2	12.96
1 1:10	17.96
1 1:10	17.84
2 1:10	16.66
2 1:10	16.48
H_2O	Undetermined
H_2O	Undetermined

Next, the assay was tested at SASA, against their *Epitrix* species collection. All gave positive amplifications.

Finally, the assay was tested against a number of non-target insects (Chrysomelidae, Galerucinae, Alticini), commonly found in *Epitrix* surveys, obtained from ANSES: *Altica oleracea* L., *Psylliodes chrysocephala* (L.), *Podagrica decolorata* Duvivier,

Chaetocnema tibialis Illiger*, Chaetocnema confinis* Crotch.*, Phyllotreta nemorum* L.*, Phyllotreta undulata* Kutschera and *Aphthona lutescens* (Gyllenhal). Of these, only *A. lutescens* gave a positive but relatively weak signal in the qPCR (Ct 28.4).

6.2 Development of a duplex real-time PCR based-method (INIAV)
In addition to the target species (*E. cucumeris* and *E. papa*), *E. tuberis, Phyllotreta atra* (Fabricius) and *Chaetocnema concinna* (Marsham) were included in the experiments for verification of method specificity.

Prior to the method development, COI gene was amplified and sequenced using the LCO1490 and HCO2198 primers (Folmer *et al.,* 1994). Based on an alignment of *E. cucumeris and E. papa* COI gene sequences either available on the BoldSystems database or obtained at INIAV for the Portuguese specimens, two pairs of primers were developed during this study to amplify two regions within the COI gene specific for *E. cucumeris* and *E. papa*, with 109 bp and 82 bp, respectively. The respective melting temperatures were 83 ºC and 79 ºC. *In silico* base pairing of the pairs of primers confirmed the specificity of the detection/identification assay. Full confirmation was obtained using mixtures with different proportions of specimens of the five different species under evaluation by means of SybrGreen real-time PCR. The maximum sample intake was 10 specimens and DNA was extracted with Quick-gDNA™ Blood MiniPrep (Zymo Research, USA).

Real-time PCR measurements were performed in triplicate in an optical reaction rotor-disc 100 and run on a Rotor-Gene Q 5-Plex HRM System (Qiagen, Hylden, Germany) with the following thermal cycling protocol: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 10 s and 62 °C for 1 min followed by amplicon melting temperature analysis. The latter was done in closed tubes after the PCR was completed in a range of temperatures varying from 55 °C to 90 °C with 0.02 °C increments.

The reaction volume was set at 15 µL and each reaction contained 1 x SyBR®Green PCR Master Mix, without ROX, (ThermoFisher Scientific) containing 0.17 µM of each of the *E. cucumeris* primers, 0.83 µM of each of the *E. papa* primers and 3 µL of DNA template (equivalent to 20 ng of DNA).

The increase of fluorescence crossing the threshold line at a specific cycle together with the expected amplicon melting temperature was only possible for the targeted species. All mixtures having either *E. cucumeris* or *E. papa* or both exhibited positive amplification of the expected melting temperature. Agarose gel electrophoresis confirmed the presence of band(s) of the expected amplicon size (done only one time). Mixtures not having the target species did not record the expected melting temperature, independently of showing or not positive amplification.

Real-time PCR performed on DNA extracted from specimens' mixtures, corresponding to serial dilutions of *E. cucumeris* and *E. papa* with specimens of the other species, exhibited consistent amplification up to 1 individual of *E. cucumeris* or *E. papa* in a total of 10.

6.3 Development of assays for molecular identification of 9 Epitrix *species (SASA)*

Confirmation of species identification

Specimens of beetles of eight species were obtained from ANSES, France (*Epitrix tuberis, E. cucumeris, E. papa, E. yanazara* Bech*, E. similaris, E. subcrinita, E. hirtipennis* and *E. pubescens*), INIAV, Portugal (*E. papa* and *E. cucumeris*) and Agriculture and Agri-Food, Canada (*E. tuberis*). Further DNA extractions were obtained from INRA (*E. brevis, E. cucumeris, E. hirtipennis, E. cf hirtipennis, E. tuberis, E. papa* and *E. similaris*).

The four regulated species (*E. tuberis, E. subcrinita, E. papa, E. cucumeris*) were considered the main targets and five further species sourced from crops and weeds in North America and Europe (*E. hirtipennis, E. cf hirtipennis, E. similaris, E. pubescens, E. brevis*) were considered secondary targets as rapid positive identification would be very useful if found within trade or national surveys. A limited amount of *E. yanazara* was available so an assay was not developed for this species.

Non-destructive DNA extractions were carried out using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's instruction with additional steps (available on request). Specimens were punctured in the abdomen and incubated overnight at 56 °C in a Thermomixer (Thermofisher) at 800 rpm. Their identity was then confirmed by DNA sequencing of the standard COI barcoding region (Hebert *et al*., 2003) using primer cocktail LCO1490puc_t1, HCO2198puc_t1- (Cruaud *et al*., 2010), LCO1490Hem1_t1, HCO2198Hem2_t1, HCO2198Hem1_t1 (Germain *et al*., 2013). PCRs were performed on a Veriti thermal cycler (Life Technologies) in a 20 µl reaction: 1 µl genomic template, 10 µl Type-it x2 mastermix (qiagen), 0.5 µl of 10 pmol/µl primer cocktail (Eurofins) and 8.5 µl RNase free water (Sigma). PCR conditions were: 95 °C for 5 min, five cycles of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 60 s, followed by 35 cycles of 95 °C for 30 s, 51 °C for 60 s and 72 °C for 60 s, with a final extension at 72 °C for 10 min. PCR product were then sequenced 'in-house' using BigDye v3.1.

Identity of samples was confirmed by COI sequence using BOLD and NCBI databases where possible and comparing to previous samples.

Development of species specific assays

ITS2 was amplified using 5.8S_cbgp_F1_t1, 5.8S_cbgp_F2_t1, 5.8S_cbgp_F3_t1, 28S_cbgp_R1_t1, 5.8S_cbgp_F1_t1, 5.8S_cbgp_F2_t1, 5.8S_cbgp_F3_t1, 28S_cbgp_R1_t1 primer cocktail (Germain *et al*., 2013). Using cycling conditions 94 °C for 5 min, five cycles of 94 °C for 30 s, 45 °C for 60 s and 72 °C for 90 s, followed by 35 cycles of 94 °C for 30 s, 55 °C for 60 s and 72 °C for 90 s, with a final extension at 72 °C for 10 min. ITS2 was also amplified using Epi_ITS2_F-GATACTCGTTCCCGGACCAC, Epi_ITS2_R-AGTCTCACCTGCTCTGAGGT (this study). ITS1 was amplified with Epi_ITS1_1F-TCGTCGATGCTTCGGAAAGA, Epi_ITS1_1R-TGCAGTTCACAAGTTGACGC primers (this study). RpL27A was amplified with Rpl27a_F-GTGACTTGTGTTCGTGTTTTCTCG, Rpl27a_R-ACCACCAGCATTACCTCTACCTCC and rpS7-3 with rpS7-3_1F-CAAAGCCAAGGAGATCGAGGT and rpS7-3_1R-ATTTGAGCTCGTTGTGGCATG (previous 'in-house' study). The PCR conditions for these sets of primers were: 95 °C

for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 60 s and 72 °C for 2 min, with a final extension at 72 °C for 10 min. 1µl genomic template, 10µl Type-it x2 mastermix (Qiagen), 1µl of 5 pmol/µl of each primer (Eurofins) and 7µl RNase free water (Sigma).

Sequences were aligned and assays designed using Geneious software (Biomatters). Sequences for primer and probes for all the assays designed to date can be found in Table 2. The assays were carried out on a 7900HT (Life Technologies) real-time platform with each reaction comprising of 1 µl genomic DNA template, 5 µl JumpStart™ Taq ReadyMix™ for Quantitative PCR (Sigma), 0.5 µl of each 5 pmol/µl primer, 2.5 µl 5 pmol/µl probe (Eurofins) of and 1.75 µl RNase free water (Sigma). Reaction conditions were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Table 2. Primer and probe sequences of assays

Species	Primer	Sequence	Reporter	Region
E. cucumeris	Ecuc_1F	CAGTACTAGCCGGAGCCATC		COI
	Ecuc_1P	TGACCGAAACCTGAATACTTCCT	Fam/BHQ	
	Ecuc_1R	GGATCTCCTCCCCCAATTGG		
E. hirtipennis	Ehirt_3F	AGCTATGGTTGGTGAGTATACT		rpL8-2
	Ehirt_3P	CGGTCACTTATTTGGCAATTTTTG	FAM/BHQ	
	Ehirt_3R	TGATGTGAAATGAACCAATTGTTT		
E. pubescens	Epub_2F	TATAAAAGATCGCGCCCGCT		ITS1
	Epub_2P	ACTGTTGTCGTTTAAACAAGCACT	Fam/BHQ	
	Epub_2R	TTAAGGCTGACCACGCCAAT		
E. papa	Epap_F	CTCATAATCGGAGCCCCTGA		COI
	Epap_P	ACCCCCTTCACTAACTCTTCT	Fam/BHQ	
	Epap_R	AAACAGTTCAACCGGTTCCG		
E. similaris	Esim_F	ATATCCTTCGACCGCATGCC		COI
	Esim_P	GTGTGGGCAGTAGTAATTACAGC	Fam/BHQ	
	Esim_R	AGAATTGGGTCTCCTCCTCCA		
E. brevis	Ebrev_F	TGGAGCCCCTGATATAGCCT		COI
	Ebrev_P	TGACTTCTTCCACCTTCCTT	Fam/BHQ	
	Ebrev_R	CTGTTCATCCTGTTCCGGCT		
E. cf. hirtipennis	E cf hirt_F	GGTTGAACGGTTTATCCTCCCT		COI
	E cf hirt_P	GGTTCATCAGTAGACCTGGCA	Fam/BHQ	
	E cf hirt_R	GTCATTCCTGCGGGTCGTAT		
E. tuberis	Etub_3F	CGAGCCAGATAGTCTAGAGA		rpS7-3
	Etub_3P	CGCACTGCCTATCAATGAAT	Fam/BHQ	
	Etub_3R	CCTTCCGAGATCCATTACTG		
E. subcrinita	Esub_3F	CGAACTTAAGACGCGCACTG		ITS2
	Esub_3P	TCGTCTTGTTCGCAATTTAAC	FAM/BHQ	
	Esub_3R	AATGTCGGAGCGTGTTGGAT		

Assays were tested against other *Epitrix* species in the SASA collection. Due to limited availability of *Epitrix* samples only 2 replicates were used during testing and some assays have not been tested against every species due to this restriction (Table 3).

Table 3. Assays performed and the *Epitrix* species which they have been tested against. Y (Yes) = assay picks up species. X = assay did not pick up this species when tested. Blank indicates species not tested with this assay

	Assay									
Species	Dutch	Ecuc_1	Ebrev	Ehirt_3	EcfHirt	Epub_2	Epap	Esim	Esub_3	Etub_3
E. cucumeris	Y	Y	X	X	X	X	X	X	X	X
E. brevis	Y	X	Y	X	X	X	X	X	X	X
E. hirtipennis	Y	X	X	Y	X	X	X	X	X	X
E. cf hirtipennis	Y	X	X	X	Y	X	X	X		
E. pubescens	Y	X	X	X	X	Y	X	X	X	X
E. papa	Y	X	X	X	X	X	Y	X	X	X
E. similaris	Y	X	X	X	X	X	X	Y	X	X
E. subcrinita	Y	X	X		X	X	X	X	Y	X
E. tuberis	Y	X	X	X	X	X	X	X	X	Y
E. yanazara	Y		X		X		X	X		

6.4 Contribution to the development of diagnostic assays (FERA)

Dead *Epitrix* specimens from the failing laboratory culture were frozen and kept for diagnostic assays. A range of *Epitrix* specimens were also received from ANSES. DNA was extracted from these samples using the Qiagen DNeasy Blood and Tissue Kit (Germany). The DNA was then amplified with the primers LCO1490 and HCO2198 and the resulting cytochrome oxidase I fragments cycle sequenced by Eurofins (Germany). Table 4 details the samples successfully barcoded and sequenced. The barcode sequences were used to produce the phylogenetic tree described below.

Table 4. *Epitrix* samples barcoded as part of this project

LSV reference	Identified as	Country of origin	Successful DNA extraction and barcode produced
1003154	*Epitrix cucumeris*	Canada	Partial barcode
1001673	*Epitrix tuberis*	Canada	Y
1200014	*Epitrix cucumeris*	-	Y
1001545	*Epitrix tuberis*	Canada	Y
0901965	*Epitrix papa*	Portugal	Y
1001544	*Epitrix tuberis*	Canada	Y
1200013	*Epitrix brevis*	USA	Y
1003154	*Epitrix cucumeris*	Canada	Y

6.5 Validation of FERA taqman assays for flea beetle diagnostics (FERA)

Based on discussions within FERA as to the requirement for diagnostics for *Epitrix* it was decided to develop two LAMP assays specific for *E. cucumeris* and *E. papa.* LAMP assays have the advantage over real time assays in that they are faster, and being less sensitive to inhibitors can be run with crude extracts. This makes them ideal for field or near to field use and when coupled with the Optigene Genie, or equivalent devices, they are ideal for running by non-laboratory staff such as plant health inspectors.

Figure 2 shows a Neighbour-Joining Tree constructed (pre-July 2016) using Cytochrome Oxidase I (COI) gene sequences from members of the genus *Epitrix* both from Genbank and sequenced as part of this project. It shows that the individual species are clearly separated using the COI gene making this gene an ideal target for diagnostic assay development.

Regions of COI conserved for either *E. cucumeris* or *E. papa* but distinct from the other species were used to design LAMP assays specific for each species:

Epitrix papa specific:

F3 ATATTAATCCGTACTGAATTGGGGAACCC

F1 ATCGAGGGAAGGCTATATCAGGGGCTCCGATTATGGATCAAATTTATAATG
P TTATTGTAACTGCCCATGCTTTCATTATAA

FL GTCAGTTACCAAATCC**Y**CCAATTATAATCGGTATT

B3 GATATGCCATGTGGTCGTATATTAATAATAGTAGTAATGAAATTAATAGC

B1 CTCTTCTTATTATAAGAAGAATAATTGAAAGAGGGGCCGGAACCGGTTGAG
P AAATTCCTGCTAGATGTAGCCTAAAGATTGC

BL CTTCTAATATTGCCCATGAGGGRTCTTCAG

Epitrix cucumeris specific:

F3 TTCTAATCCGCACCGAATTAGGAAATCCC

F1 TGGAAAAGCCATATCTGGTGCTCCGATTATTAGGGGATCAAATTTATAATGTTATT
P GTTACTGCCCATGCCTTTATTATAA

FL GTCAATTTCCGAATCCCCCAATTATAATTGGTATA

B3 ATATTCCGTTAGGTCGTATGTTAATTACTGTGGTAATAAAGTTGATAG

B1 TATTATTAATTATAAGCAGAAGAGTAGAAAGAGGAGCTGGGACAGGATGAACAGG
P AGAGATTCCTGCTAAATGTAGTCTAAAAATAGCT

BL CTTCTAATATTGCCCATGAAGGATCATCGG

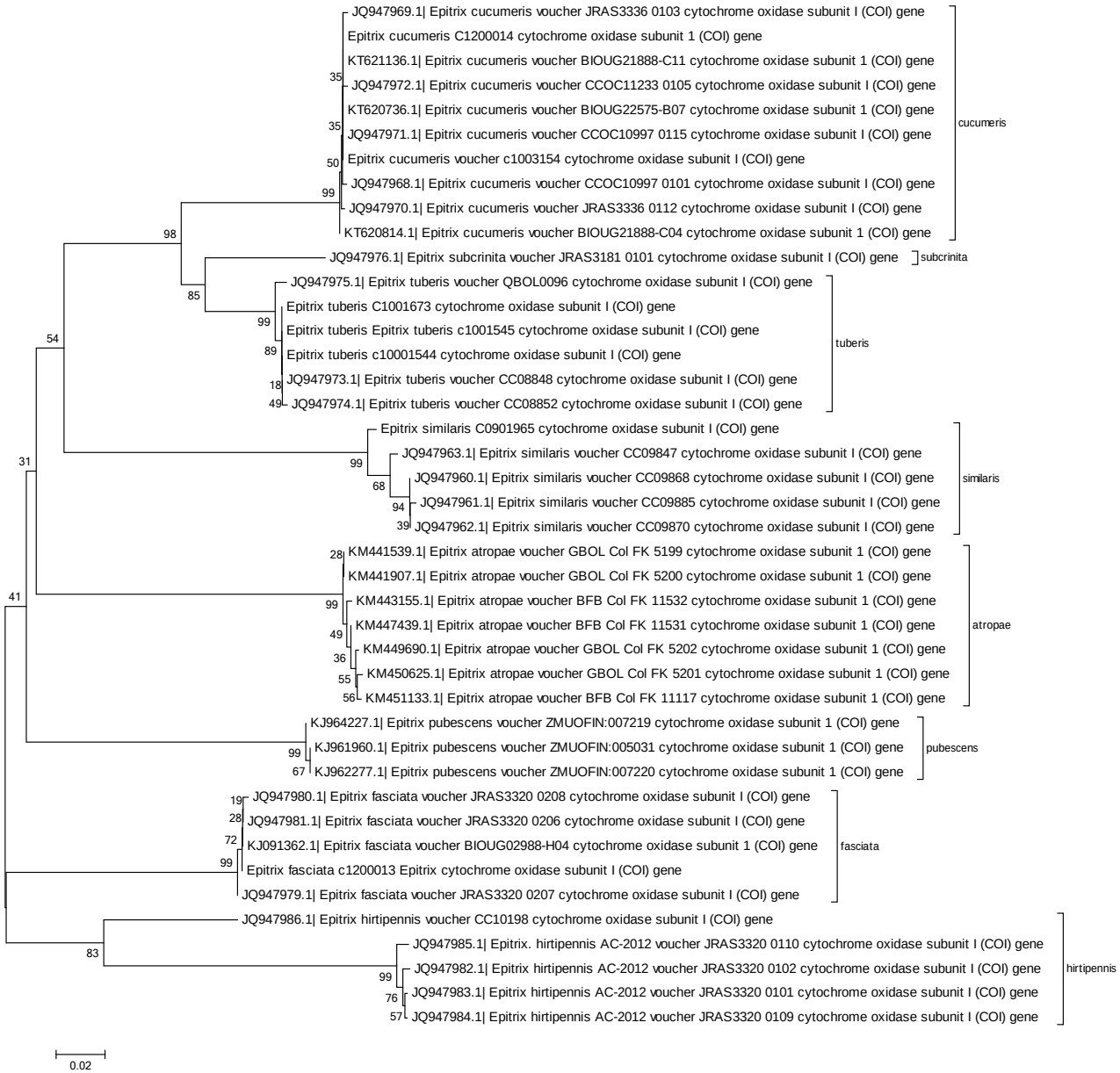

Figure 2. Neighbour-Joining Tree of COI gene from *Epitrix* species (as available from Genebank prior to July 2016).

LAMP assays were performed as described in Tomlinson *et al*. (2016). These assays were tested on the Genie platform but neither gave any amplification with target species DNA even after 40 minutes of amplification. As a backup and in order to confirm that the extracted DNA was amplifiable a series of Realtime TaqMan assays were developed: one specific for *E. cucumeris* and two specific for *E. papa*.

Epitrix cucumeris specific:

Forward GTGTGAGCAGTACTAATTACAGCTATTCTAT

Reverse GGAAGTATTCAGGTTTCGGTCAGT

Probe Fam-ATTATTATCATTACCAGTACTAGCCGGAGCCATCACT-BHQ1

Epitrix papa specific Assay 1:

Forward ATAATTGGAGGATTTGGTAACTGACTAGT

Reverse TTCCGGCCCCTCTTTCA

Probe Fam-AGCCCCTGATATAGCCTTCCCTCGATTAAATAACATA-BHQ1

Due to concerns in finding a region of DNA where a specific *E. papa* assay could be placed a second backup assay was also designed.

Epitrix papa specific Assay 2:

Forward ACCACATGGCATATCTATAGATCGTAC

Reverse TGAGGTATTTAAATTTCGGTCTGTGAG

Probe Fam-CCCCTTATTTGTGTGAGCTGTAATAATTACTGCAATTCTA-BHQ1

These assays were tested using the method described in Woodhall *et al*. (2013) with DNA from the *Epitrix* species: *papa, cucumeris, tuberis, brevis* and *pubescens.* The *cucumeris* assay gave no amplification with any target but the two *papa* assays amplified *papa* DNA but not any of the other species tested. Figure 3 shows the amplification plot for *papa* assay 1. This assay would appear to be specific.

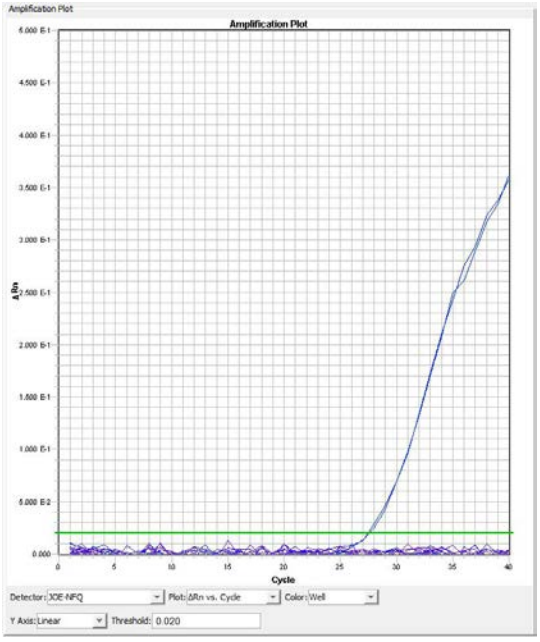

Figure 3. Amplification plot produced by *E. papa* real time assay 1 amplifying *E. papa* DNA but not DNA from *E. cucumeris, E. tuberis, E. brevis* or E. *pubescens.*

3.3 Discussion of results and their reliability

Ecology work

Rearing technique

The development of a rearing technique and the maintenance of insect cultures was crucial, since it allowed the production of the insects needed to carry out the laboratory studies on the biology of the *Epitrix* species, on the susceptibility of *E. papa* life stages to entomopathogenic nematode species, and also to collect samples of volatile compounds needed for the investigation of *Epitrix* attractants.

Still, the culturing technique is very labour intensive, and requires experience, given the difficulty of objectively evaluating and controlling the variability of plant factors such as the soil moisture content and the root condition, that affect the survival and development of eggs and larvae, and therefore the productivity of the rearing. In consequence of this difficulty in controlling these factors, together with problems affecting the functioning or the availability of the climate controlled facilities, some studies were lost or interrupted at INIAV, and local cultures failed to succeed at FERA and AU.

Biological parameters and host-plant preferences

One of the *Epitrix* species present in Portugal and Spain was re-identified as a new species, *E. papa*, and its origin, biology and ecology are unknown. Because of the previous misidentification of *E. papa* as *E. similaris*, the EPPO regions at risk of invasion by *E. papa* were established during a PRA carried out by EPPO on the basis of their climatic similarity with the zones of distribution of *E. similaris*. The estimates of the mean developmental times of *E. papa* which were obtained at INIAV for the temperatures of 15, 20, 25 and 30 ºC, together with the estimate for the lower developmental threshold (LDT), can be now considered to verify the zones at risk of invasion by *E. papa*.

The choice experiment carried out in the field showed that black night-shade *(S. nigrum)* was more attractive for the adults of both species of *Epitrix* than potato (*S. tuberosum*) and aubergine (*S. melongena*) plants, confirming previous laboratory results (Boavida *et al*., 2013).

Control of Epitrix *spp. with entomopathogenic nematodes*

The laboratory tests carried out to compare the mortality caused by three entomopathogenic nematode species (EPN) in the different developmental stages of *E. papa* are promising since they revealed that larvae, pre-pupae and pupae were susceptible to the three nematode species, and that the most effective species against the pre-imaginal stages were *S. feltiae* and *H. bacteriophora.* Similar tests with different EPN strains and doses should be tested in order to increase *Epitrix* mortality rates. The failure of the field experiment carried out was attributed to deficient experimental conditions, namely the irrigation technique installed by the farmer. Given the need for finding non-chemical control approaches to control *Epitrix* populations in the field, in view of the obligations set by the European regulation and caused by ecological and health reasons, the possibility of using EPN formulations under certain climates and soil moisture situations should be considered.

Attractants for the detection of Epitrix spp.
The “hat” traps installed in an infested potato field after harvest proved to be an adequate tool for the present study. Both species of *Epitrix* were trapped. The mixture Z3-hexenylacetate + Linalool 1:1 caught significantly more flea beetles than the blank control which is a promising result in view of finding an attractant for monitoring the flea beetle populations in the potato crops and for detecting its unwanted presence in potato exports to non-infested zones. One additional compound found in behavioural experiments has potential to improve the attractiveness of this blend. It should be noticed that in real field situations the traps have to “compete” with natural host plant odours and other species may be attracted as well. Therefore the effectivity and selectivity of such traps should be further tested.

Identification key and reference collection
The identification key developed within the project allows differentiating the four EU-regulated *Epitrix* species from the European ones. As such, it is reliable only in the context of the study of European species that can be confused with the American species developing on potato. The morphological key does not consider the South American species which account for most of the diversity of the genus *Epitrix* and include some pests which could of concern if they were to be introduced into Europe.

Molecular work
The methods developed provide rapid, reliable and accurate identification to species level for a number of practical uses. Diversity of methods allows a suitable methodology for the majority of molecular laboratories. A comparison of methods and their uses are displayed on Table 5 below.

DNA extraction is similar between methods and does not necessarily require destruction of the specimen (which allows confirmation by a taxonomist in accordance with the EPPO diagnostic protocol, Appendix 2); molecular tests can be conducted to confirm the presence of *Epitrix* spp. and provide rapid and accurate identification either using specific assays of suspected species or by sequencing if there is no likely candidate. Overall, from the limited number of samples tested, the methods appear to be relatively robust, but further specimens and ring testing would help to confirm this. Specific reliability issues are addressed below.

The generic screening assay showed that all examined *Epitrix* species could be detected while all examined non-targets were not detected, except *A. lutescens.* Not all species have nucleotide sequences available in Genbank, making it very difficult to design primers that are specific – this can only be tested empirically, and thus it cannot be confirmed whether some species will give a positive amplification using the primers. It still remains to validate the assay in more detail and to include more non-target species in the validation (and also more *Epitrix* species / specimens).

E. cucumeris and *E. papa* are the known species affecting potato production in Portugal which could delay or even hamper international European shipments due to their quarantine status. We developed a one-step, rapid, specific and sensitive SybrGreen duplex-assay for the detection and identification of these two *Epitrix* species obtained from potato fields. It shows high practicability as it is easy to

perform, did not require additional equipment or training, and shows low sample costs as there is no need for TaqMan chemistry.

Table 5. Comparison of the molecular methods employed in the project consortium

Institute/Country	DNA Extraction	Assay Target	Method used	Species
ANSES - France	Qiagen DNeasy blood & tissue kit	CO1	Sequencing (available in Q-bank)	*E. cucumeris*, *E. papa*, *E. subcrinita*, *E. tuberis*, *E. similaris*
AU – Denmark	Qiagen DNeasy blood & tissue kit	ITS	rtPCR assay (Taqman)	*Epitrix spp.* to genus
INIAV – Portugal	Zymo Quick – gDNA blood miniprep	CO1	Melt curve analysis rtPCR (Sybrgreen duplex reaction)	*E. cucumeris*, *E. papa*
SASA – U.K.	Qiagen DNeasy blood & tissue kit	ITS1, ITS2, CO1, rpS7-3, rpL8-2	rtPCR assays (Taqman) Sequences available from SASA	*E. cucumeris*, *E. brevis*, *E. hirtipennis*, *E. c.f. hirtipennis*, *E. pubescens*, *E. papa*, *E. similaris*, *E. subcrinita*, *E. tuberis*
FERA – U.K.	Qiagen DNeasy blood & tissue kit	CO1	LAMP assay Sequences available from FERA	*E. papa*, *E. cucumeris*

Whilst all assays developed at SASA are still to be fully validated results to date are very promising with all assays showing high specificity. Unfortunately, due to limited availability of *Epitrix* specimens, validation cannot be completed until further material becomes available. If further specimens can be obtained, a full ring test of this and other methods should be considered for validation purposes. Further work to duplex some reactions would also be worthwhile to reduce associated costs and time.

Barcoding of additional *Epitrix* samples provided data broadly in agreement with similar barcoding data publically available. Using this data, it was possible to phylogenetically separate the existing species (Figure 2). The initial intention of producing LAMP assays capable of detecting *E. papa* and *E. cucumeris* did not prove possible within the confines of this project. It was, however, possible to produce a real time assay specific for *E. papa* and this could be taken for further validation. Further examination of why the initial assays failed was also not possible in the confines of the existing project but, in principle, it should be possible to develop LAMP or real time assays for both species

3.4 Main conclusions

Rearing technique
A rearing technique was developed for the mass production and study of *E. papa* and *E. cucumeris*; this technique can also be applied to the multiplication of other species of tuber flea beetles.

Biology and ecology
The estimates of the mean developmental times of E. papa obtained at INIAV for the temperatures of 15, 20, 25 and 30 ºC, together with the estimate for the lower developmental threshold (LDT), could be considered to verify the zones at risk of invasion by E. papa (Climex). The results indicate that *E. papa* can find suitable temperature conditions for development in other cropping zones of Europe (outside of Portugal), not presently invaded.

In a field choice experiment black night-shade *(S. nigrum)* was more attractive for the adults of both species of *Epitrix* than potato (*S. tuberosum*) and aubergine (*S. melongena*) plants confirming previous laboratory results (Boavida *et al*., 2013). The fact that potato attacking *Epitrix* species also thrive on other (mainly Solanaceous) crops and can maintain populations on for example aubergine and black night-shade should be taken into account in management strategies even if spread is mainly due to trade and transport of potato tubers.

In laboratory tests the EPN species *S. feltiae* and *H. bacteriophora* showed promise as killing agents of the pre-imaginal stages of *Epitrix papa.* This result is encouraging in view of finding non-chemical control means, in compliance with Directive 2009/128/EC of the European Parliament and of the Council, and similar studies aiming at finding more virulent species, strains and EPN doses should be continued.

An attractant mixture of Z3-hexenylacetate + Linalool 1:1 caught in the field significantly more flea beetles of both species than the blank control. This is a promising result in view of finding an attractant for monitoring the flea beetle populations in potato crops and for detecting its unwanted presence in potato exports to non-infested zones, and the investigation should be continued. This mixture was selected as the reference in the olfactometer choice experiments carried out in 2016. For surveillance and pest management, suitable traps and attractants are indispensable. Though some attractive substances were already identified further studies are needed to develop an effective and selective monitoring tool.

Identification key and reference collection
The targeted program has been reached, allowing the morphological identification of European species and of North American species developing on potato. The update of the EPPO diagnostic protocol will be made available to EU quarantine laboratories as well as the voucher reference collection.

Molecular work
A generic screening *Epitrix* assay as well as species specific molecular assays for all four regulated species and some species found in crops and weeds in North America and Europe have been developed for rapid identification of *Epitrix* beetles found in trade and/or crops in countries where *E. papa*/*E. cucumeris* have not established.

This will allow fast and accurate notification in the event of an outbreak/interception, and confidence in situations where non-regulated species of *Epitrix* are found within crops.

A one-step, rapid, specific and sensitive SybrGreen duplex-assay was developed for the detection and identification of the two *Epitrix* species. It shows high practicability, as it is easy to perform, did not require additional equipment or training, and shows low sample costs as there is no need for TaqMan chemistry.

The molecular assays undertaken performed well. Further validation of their specificity and repeatability in inter-laboratory situations would be beneficial.

The flow charts below (Fig. 4) illustrate how the methods can be applied in connection with interception, surveillance and routine monitoring and advice.

Future work
Suggestions for future work:

- Further research to identify insecticides effective in controlling *Epitrix* species
- Further research into sustainable, non-chemical control options against *Epitrix* species namely entomopathogenic nematodes
- Further research into the ability of different *Epitrix* species to survive Northern European winter conditions
- Predicting potential distribution of *Epitrix* species using Climex
- Trapping methods for monitoring the populations are needed. These are also necessary at international entry points, to increase the efficiency of *Epitrix* detection. For this purpose, the investigation on semiochemical-based traps for *Epitrix* should be continued
- There is a need to develop knowledge on the unstudied South American *Epitrix* species
- Perform further validation of assays developed for the detection and identification of the *Epitrix* species

The above points could be the subject of a future EUPHRESCO research project.

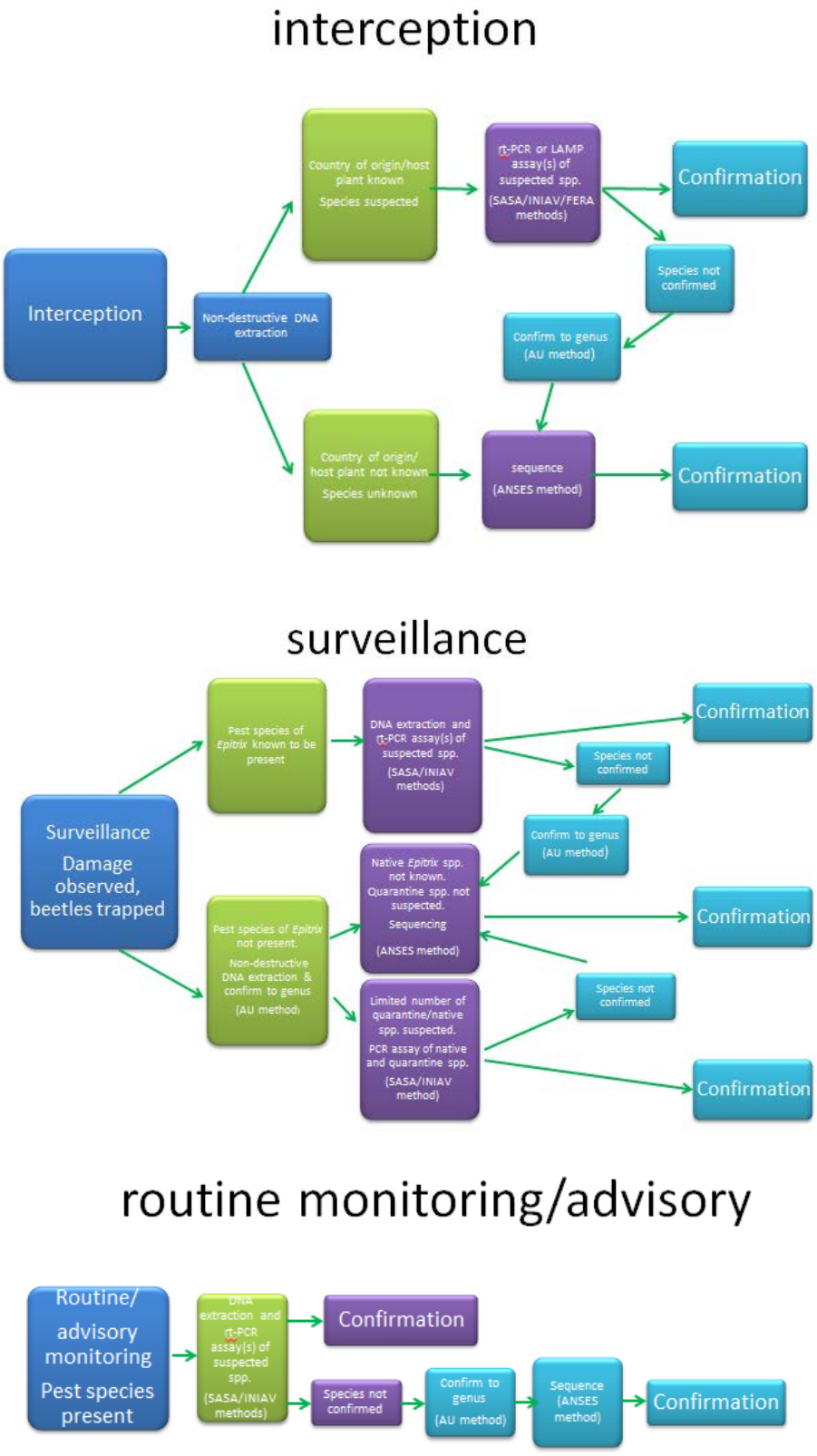

Figure 4. Flow charts illustrating application of molecular methods in connection with interception, surveillance and routine monitoring and advice.

3.5 Acknowledgements

The work undertaken in UK was funded by The Department for Environment, Food and Rural Affairs (Defra, UK) (FERA) and by the Scottish Government Rural and Environment Science and Analytical Services Division (RESAS, SG). SASA are grateful to Dr. Jean-François Germain (ANSES), Dr. Astrid Cruaud (INRA), C. Boavida (INIAV I.P.) and Dr. Bob Vernon (AGR-GC) for providing beetle specimens for the development of assays.

ANSES are grateful to Dr. David Furth (NHNH, Smithsonian Institution), Serge Doguet (MNHN) and M. Geiser (NHM) for loaning material, to the Entomology Laboratory of the Plant Pest Diagnostic Center (CDFA) for giving North American specimens, to the Basel Museum for allowing the consultation of the collections, to C. Boavida (INIAV I.P.) for supplying fresh material from Portugal and to Jean-Claude Streito (INRA-CBGP) and Guénaëlle Genson (INRA-CBGP) for their active participation in collecting *Epitrix similaris* specimens in California.

The research undertaken by INIAV was funded by the Portuguese FCT (project PTDC/AGR-PRO/120292/2010). The work carried out in collaboration between INIAV and WUR/DLO on semiochemicals for *Epitrix* was funded by the Dutch foundation DLO-WUR. INIAV is grateful to Dr. Ralf-Udo Ehlers (e-nema GmbH) for supplying the entomopathogenic nematodes, to the farmers Reinaldo Costa and Vitor Gomes for the utilisation of their fields, to Dr. Luisa M. Oliveira (University of Azores) for her participation in the laboratory entomopathogenic nematodes bioassay, and to Márcia Santos and Clara Fernandes for their efficient assistance and active collaboration.

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Appendix 1
Problems encountered, publications and dissemination activities

1) Problems encountered

During the consortium meetings problems were openly discussed and solutions sought.

Both FERA and AU suffered from loss of *Epitrix* cultures and as a consequence milestones were unable to be met. Both partners, for example FERA, exhausted all avenues possible in regards to getting in more specimens from the project partners (i.e. Portugal). However, efforts were met with a closed door in regards to transporting live quarantine insects from commercial Carriers. Some price quotes that were received were approximately £3,000; something the current project could not support. The possibility of a staff member travelling to Portugal to collect specimens was also investigated. However, following investigating the issue, no airline would allow the transporting of live insects as hand luggage. The possibility of land travel has also discussed. However, land travel between York and Lisbon is not the most practical. All was openly discussed with FERA's funding customer (Defra) who understood and appreciated the difficulties incurred and were happy that all appropriate steps were taken to try and resolve the problem. The funding body was happy that all other objectives progressed appropriately.

It would appear that the transporting of live quarantine insects for research work is a problem for research institutes. Unless the issue of commercial carriers refusing to transport live insects (fully under license) is addressed, research work in regards to gaining knowledge of specific invasive species will be hindered.

Also, during this period the INIAV rearing and laboratorial equipment moved from Lisbon to Oeiras and this, together with national budgetary restrictions, brought a reduction in the number of climate controlled chambers available and also interruptions in the function of the remaining ones, causing the loss of experiments and the impossibility to reach some milestones.

2) Scientific papers and other published articles from the current study

Boavida, C., Santos, e M., Ferreira, M.E. 2014. A rearing Technique for Producing *Epitrix similaris* Gentner (Coleoptera: Chrysomelidae:Alticinae). In: "Proceedings VII Congreso Ibérico de Agroingeniería y CienciasHortícolas: Inovar y Producir para el Futuro" (F. G. UPM, ed.) pp.1849-1854, Madrid, Spain.

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Mouttet, R., Streito, J-C., Genson, G., Germain, J-F. 2016b. Molecular evidence supports the recognition of *Epitrix papa* as a distinct species from *Epitrix similaris.* Bulletin OEPP/EPPO Bulletin 46(3), 583-587.

Santos, M., Boavida, C. 2014. Ciclo biológico de *Epitrix similaris,* uma praga da batateira. 1º Simpósio SCAP e 7º Congresso da SPF, Auditório INIAV 20-21 Novembro 2014.

Other dissemination activities

OEPP/EPPO Protocol of diagnostic for *Epitrix cucumeris, E. papa, E. subcrinita, E. tuberis*. Presented to the EPPO panel for entomology. EPPO 'in-country' consultation, November 2016. (Germain J.-F., Mouttet R.).

Project success story 'Biology of *Epitrix* (flea beetle) and techniques for detection' posted on Euphresco website and Twitter account (October 2015): http://www.euphresco.net/projects/success, https://twitter.com/euphresco

Reference collection with pinned specimens of both European and North American *Epitrix* species developing on potato and with slide-mounted female spermatheca and male aedeagus of the studied species. This collection of voucher specimens is maintained at ANSES and could be made available to members of EPPO / NPPOs.

'In-house' workshop dealing with morphology, molecular biology and rearing techniques of Epitrix for all project partners held at INIAV during the second annual project meeting (2014).

Appendix 2

Diagnostics
Diagnostic

PM 7/109 (2) *Epitrix cucumeris, Epitrix papa, Epitrix subcrinita, Epitrix tuberis*

Specific scope

This Standard describes a diagnostic protocol for adults of *Epitrix cucumeris*, *Epitrix papa*, *Epitrix subcrinita* and *Epitrix tuberis*.

Specific approval and amendment

First approved in 2011-09.
Revision approved in 2016-01.

1. Introduction

Epitrix subcrinita, *Epitrix tuberis* and *Epitrix cucumeris* are flea beetles belonging to the North American species of *Epitrix* developing on *Solanum tuberosum* (Solanaceae). *Epitrix cucumeris* is established in the Azores Islands (Portugal) where it was collected for the first time in Faial Island around 1979 (Boavida & Germain, 2009). In mainland Portugal, *E. cucumeris* was detected with another species new to science, *Epitrix papa*, in approximately 2004 when unusual damage to potato crops was observed (Oliveira *et al.*, 2008). *Epitrix papa* was initially misidentified as *Epitrix similaris*, another North American species (Orlova-Bienkowskaja, 2015). Both species have also been detected in Spain. To date, *E. tuberis* and *E. subcrinita* have not been detected in the EPPO region.

Nine species of *Epitrix* were recorded as present in the EPPO region before these detections (Warchalowski, 2003), these are *Epitrix abeillei*, *Epitrix allardi*, *Epitrix atropae*, *Epitrix caucasica*, *Epitrix dieckmanni*, *Epitrix hirtipennis*, *Epitrix intermedia*, *Epitrix priesneri* and *Epitrix pubescens*. Until recently, among the seven *Epitrix* species present in Europe (Warchalowski, 2003) none was causing damage on *S. tuberosum*, although *E. hirtipennis*, known as a pest of tobacco in Italy (Sannino *et al.*, 1984), is occasionally recorded on potato. This species also has a North American origin. Two other species which are present in the non-European part of the EPPO region, *Epitrix allardi* and *Epitrix priesneri*, are not covered in this diagnostic protocol.

Further information on the host range, geographical distribution and biology of the four species recommended for regulation can be found in the EPPO Global Database (https://gd.eppo.int/). This protocol focuses on detection of *Epitrix* species in potato.

2. Identity

Name: *Epitrix cucumeris* (Harris)
Taxonomic position: Coleoptera: Chrysomelidae: Alticinae
EPPO code: EPIXCU
Phytosanitary categorization: EPPO A2, EU Commission decision 2016/1359/EU

Name: *Epitrix papa* Orlova-Bienkowskaja
Taxonomic position: Coleoptera: Chrysomelidae: Alticinae
EPPO code: EPIXPP
Phytosanitary categorization: EPPO A2, EU Commission decision 2016/1359/EU

Name: *Epitrix subcrinita* (LeConte)
Taxonomic position: Coleoptera: Chrysomelidae: Alticinae
EPPO code: EPIXSU
Phytosanitary categorization: EPPO A1, EU Commission decision 2016/1359/EU

Name: *Epitrix tuberis* Gentner
Taxonomic position: Coleoptera: Chrysomelidae: Alticinae
EPPO code: EPIXTU
Phytosanitary categorization: EPPO A1, EU Commission decision 2016/1359/EU

3. Detection

Symptoms on potato

Adults of *Epitrix* species may be found on all above- ground parts of the plant as well as on the soil surface.

They mainly feed on the upper surface of leaves, and less often on the lower surface. Adult beetles cut characteristic shot-like holes (1.0–1.5 mm in diameter) (Fig. 1).

Epitrix cucumeris: larvae inhabit the soil around potato roots; occasionally they may enter the tubers, leaving roughened trails or tiny tunnels filled with corky tissue.

Epitrix papa and *Epitrix tuberis*: larvae are more injurious, affecting potato tubers which then show long sinuous corky lesions and small holes. These lesions are caused by larvae which feed under the epidermis, digging galleries that usually remain superficial and do not affect the flesh of the tuber (Fig. 2).

Epitrix subcrinita: adults can cause damage on foliage similar to that caused by other *Epitrix* species, However, there is a great uncertainty concerning tuber damage caused by larvae.

4. Identification

Identification is commonly based on the examination of adult specimens. A protocol for DNA barcoding based on the mitochondrial cytochrome *c* oxidase I (COI) gene is described in PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2016), and allows the identification of *E cucumeris, E. papa, E. subcrinita* and *E. tuberis*

Fig. 1 Shot-like holes on potato leaves (courtesy: Germicopa, FR).

Fig. 2 Potato tuber with corky lesions, small superficial warts and a larva (courtesy: Germicopa, FR).

4.1 Morphological identification

Morphological identification requires the examination of both male and female adults. A stereomicroscope and a compound microscope are needed for this purpose. For reliable identification the habitus of the female spermatheca and the male aedeagus should be examined. (See Appendix 1 for the preparation of male genitalia and female spermatheca.)

For a key to the families of Coleoptera see Lawrence *et al.* (2002).

For a key to the subfamilies of Chrysomelidae and the *Epitrix* genus see Warchalowski (2003).

Epitrix species (based on Doeberl, 2000):

Elytra have puncture rows, intervals are entirely covered with erect setae, the pronotum basally is slightly narrower than the elytral base and narrowed anteriorly. A pronotal ante-base transverse impression is developed and the two basal short longitudinal furrows are distinct. Procoxal cavities are closed posteriorly with the first abdominal sternite as long as following three combined.

4.1.1 Eggs

Minute, whitish, approximately elliptical (0.5 mm long) (see Neilson & Finlayson, 1953).

4.1.2 Larvae

Whitish, slender, cylindrical, mature larvae are about 5 mm long with a brown head (Fig. 2).

Fig. 3 Appearance of pupae of *Epitrix* sp. (courtesy: Boavida, INIAV, I.P.).

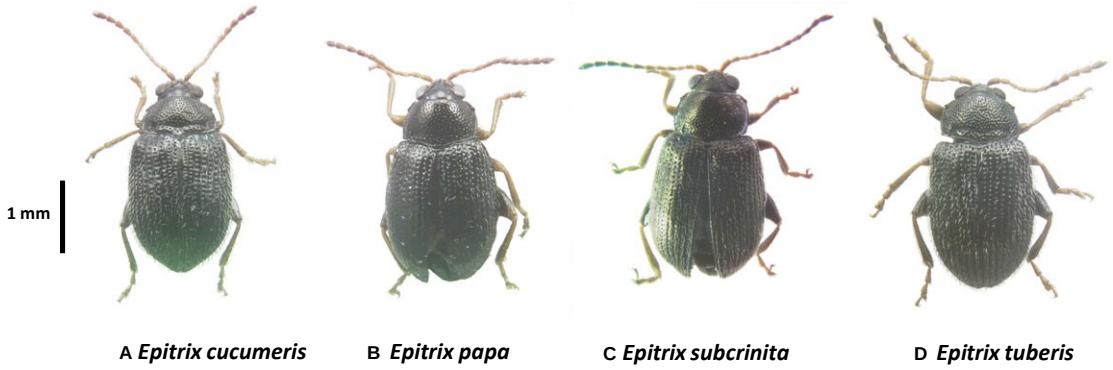

Fig. 4 Adult habitus.

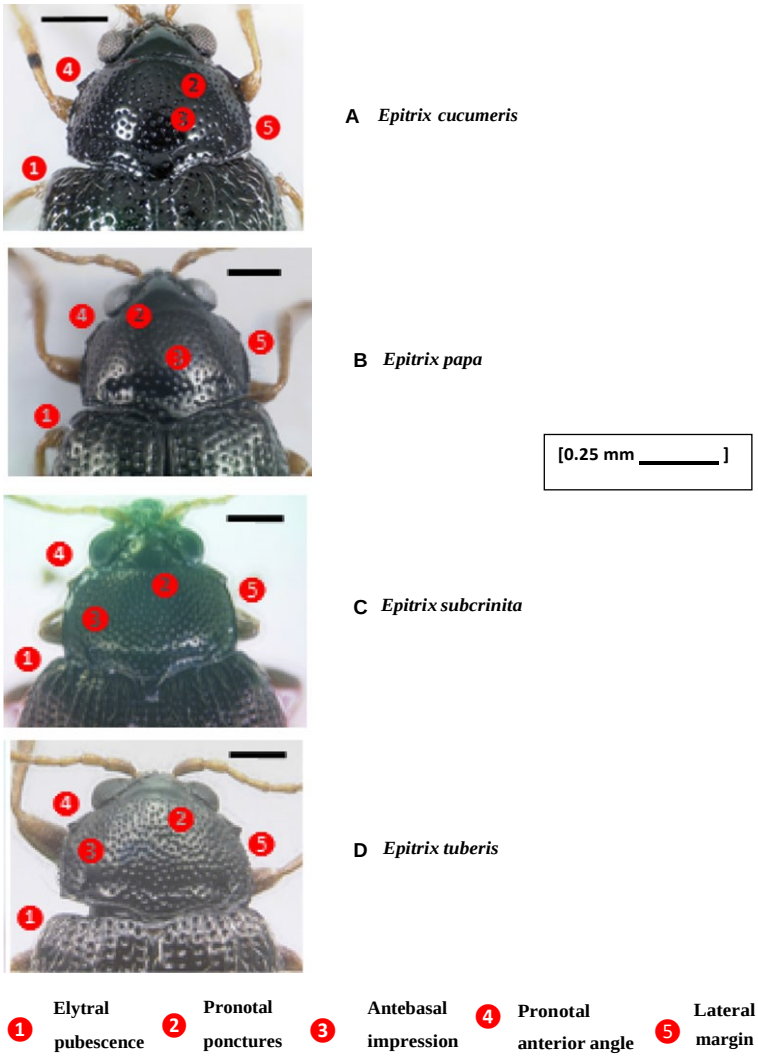

Fig. 5 Prothorax.

4.1.3 Pupa

The pupal stage is in the soil. Pupae are uniformly white, about 2.5 mm long and 1.5 mm wide across the mesothorax (Fig. 3).

4.1.4 Adults

Epitrix cucumeris (based on Gentner, 1944; Orlova-Bienkowskaja, 2015) (Fig. 4):

- Body length from 1.56 to 1.92 mm
- The upper side is always shiny black
- The antennae, tibia and tarsi are pale testaceous; femora are dark testaceous and often lighter towards the apex
- The pronotum shows a tight punctuation with well-marked points, separated by a distance greater than their diameter; anterior sides are angular, dentate, with rather denticulate edges. The ante-basal impression is well marked and deep (Fig. 5a). Elytra are often more densely pubescent than in the other three species
- The spermatheca is narrowed in its central part. It is slightly curved and the ductus is very long and forms a large curve very clearly detached (Fig 6a)
- The median lobe of the aedeagus is distinctly narrowed at the apex; its tip is spatulate (Fig. 7a).

Epitrix papa (based on Orlova-Bienkowskaja, 2015):

- Body length from 1.7 to 2.2 mm
- Dorsal body surface has a weak bronze reflection
- Antennae, tibia and tarsi are yellow to pale testaceous and the 11th antennomere is slightly darkened. Femora are dark, becoming lighter towards the apex
- A less tight pronotal punctuation than in the three other species. Punctures on the pronotum with an interval twice as wide as the diameter. Ante-basal impression is significantly less deep. Anterior angles of the pronotum are weakly dentate (Fig. 5b)
- Elytral hairs are sometimes a little less abundant and less dense than in the other three species. The sutural puncture row of elytra ends at the basal third of elytra length, and with a less tight pronotal punctuation. Points are separated by a distance greater than their diameter
- The central part of the spermatheca is strongly narrowed in its centre and the ductus is relatively short (Fig. 6b)
- The apex of the median lobe of the aedeagus is regularly narrowed, lancet-shaped and its tip is very shortened (Fig. 7b).

Epitrix subcrinita (based on Seeno, 1972, Deczynski, 2014):

- Body length from 1.76 to 2.27 mm
- Dorsal body surface generally of a dark reddish–dark brown colour with a brassy metallic lustre, although it may be practically black there is always a brassy sheen visible
- Antennae yellowish brown, often darkened towards the apex. Tibia and tarsi are testaceous and all femora testaceous to piceous
- The pronotum has a shallower transverse ante-basal impression than the other three species, a more depressed form. Especially on the pronotum there is a brassy sheen visible. In the black species the ante-basal impression is distinctly sinuate and quite deep (Fig. 5c)
- Elytral hairs of are of medium density
- Spermatheca with the upper side of the receptacle concave and the lower side convex; spermathecal duct curved

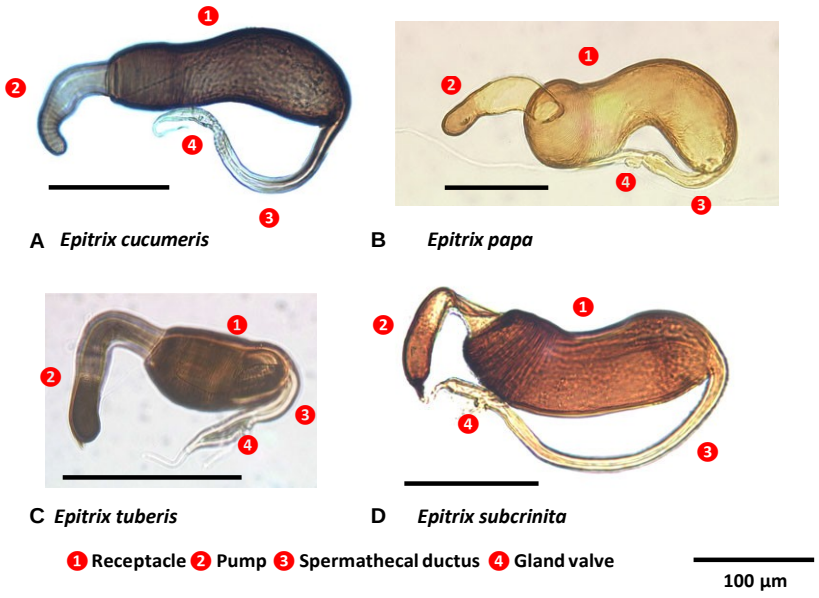

Fig. 6 Spermatheca.

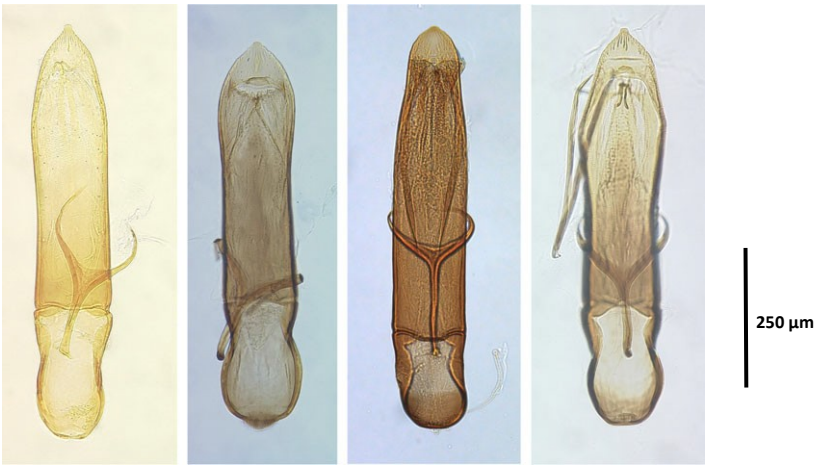

Fig. 7 Aedeagus (ventral view).

Table 1. Key to species of North American *Epitrix* developing on *Solanum tuberosum* including Western Palaearctic species[1]

1 Both sides of the spermatheca receptacle convex Aedeagus apex lancet	*Epitrix hirtipennis* (Melsheimer)
1^0 Both sides of the spermatheca receptacle concave	2
1″ Upper side of the spermathecal receptacle concave, lower side convex	4
1‴ Both sides of the spermathecal receptacle straight	6
1⁗ Upper side straight/lower side convex The apex of the median lobe of the aedeagus has its greatest width in the upper third, its tip has a rectangular aspect	*Epitrix tuberis* Gentner
2 Ductus as long as receptacle, distal part of the ductus a quarter of its length The median lobe of the aedeagus is distinctly narrowed at the apex; its tip is spatulate	*Epitrix cucumeris* (Harris)
2^0 Ductus shorter than receptacle	3
3 Distal part of ductus half of its length, pump no longer than the diameter of the receptacle in its largest part The apex of the median lobe of the aedeagus is regularly narrowed, lancet-shaped, its tip is very shortened	*Epitrix papa* Orlova-Bienkowskaja
3^0 Distal part of the ductus a third of its length, pump longer than the diameter of the receptacle in its largest part Aedeagus apex stretched tip	*Epitrix similaris* Gentner
4 Ductus shorter than spermatheca receptacle Aedeagus in tapered tip	*Epitrix atropae* Foudras
4^0 Ductus longer than spermatheca receptacle Aedeagus with a slight constriction at its ¼ apical part, barely marked tip truncated tab	*Epitrix subcrinita* (LeConte)
4″ Ductus almost as long as the spermatheca receptacle	5
5 Pump with a short neck Aedeagus with a blunt apex	*Epitrix intermedia* Foudras
5^0 Pump with a long neck Aedeagus abruptly round apically to small rounded projection	*Epitrix pubescens* (Koch)
6 Pump almost as long as receptacle which looks quite compact Aedeagus slender, subparallel sides and sharp apex	*Epitrix caucasica* (Heikertinger)
6^0 Pump shorter than receptacle which appears tapered	7
7 Receptacle larger at the pump side Aedeagus slender, sharp apex, slightly narrowed at mid-length	*Epitrix abeillei* (Bauduer)
7^0 Sides of the receptacle parallel or almost parallel Aedeagus short and broad	*Epitrix dieckmanni* (Mohr)

[1]Two other species which are present in the non-European part of the EPPO region, *Epitrix allardi* and *Epitrix priesneri*, are not covered in this diagnostic protocol. For these species see drawings in Warchalowski (2003).

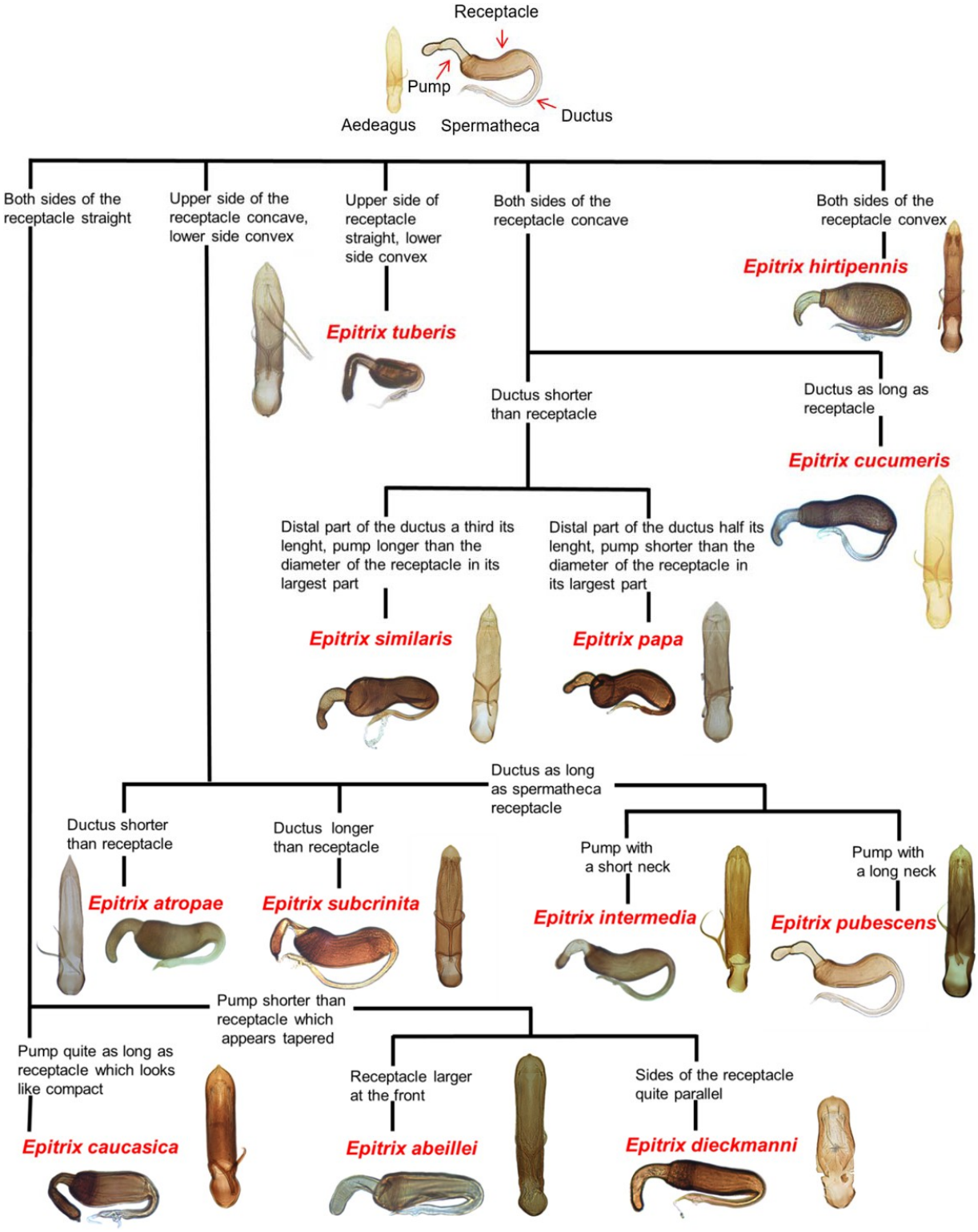

Fig. 8 Pictorial key.

and at least as long as the receptacle. The distal end of the pump has a small appendix (Fig. 6c)
- The aedeagus has a slight constriction at its ¼ apical part, barely marked tip truncated tab (Fig. 7c).

Epitrix tuberis (based on Gentner, 1944, Seeno, 1972):
- Body length from 1.60 to 2.04 mm
- The upper side is less shiny than in the other three species
- Antennae are brownish yellow near the base, becoming darker near the apex. Legs are reddish brown becoming lighter towards the tarsi
- The elytral pubescens is thick
- The pronotum presents a very marked, deep, coarser punctuation, each point separated by a distance less than its diameter, anterior sides angular, clearly dentate. The ante-basal impression is deep and winding, also with a coarse punctuation (Fig. 5d)
- The spermatheca presents an oval without any constriction; the pump is longer than the receptacle (Fig. 6d)
- The apex of the median lobe of the aedeagus has its greatest width in the upper third and its tip has a rectangular aspect (Fig. 7d).

A key to species of North American *Epitrix* developing on *Solanum tuberosum* including Western Palaearctic species (based on female spermatheca and male aedeagus observation) [external morphology can be confirmed with Seeno & Andrews (1972), Doeberl (2000), Doguet (2009), Warchalowski (2003), Biehkowski & Orlova-Bienkowskaja (2016), Deczynski (2016)] is presented in Table 1 and should be used with the pictorial key presented in Fig. 8.

4.2 Molecular methods – sequencing

A protocol for DNA barcoding based on COI is described in Appendix 1 of PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests: DNA barcoding arthropods* (EPPO, 2016) and allows the identification of *E cucumeris, E. papa, E. subcrinita* and *E. tuberis.* Sequences are available in databases including Q-bank (http://www.q-bank.eu/arthropods/). A PCR-restriction fragment length polymorphism (RFLP) test has been described (Germain *et al.*, 2013); however, this test does not cover *E. subcrinita. In silico* testing on COI sequences of *E. subcrinita* shows that the test would not allow the distinction between *E. subcrinita* and *E. cucumeris*.

5. Reference material

Anses-LSV, Unitè Entomologie et Plantes Invasives, CBGP, 755 Avenue du Campus Agropolis, CS 30016, FR-34988 Montferrier-sur-Lez (FR).

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis.*

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on these organisms can be obtained from:

J.-F. Germain/R. Mouttet. Anses-LSV, Unitè Entomologie et Plantes Invasives, CBGP, 755 Avenue du Campus Agropolis, CS 30016, FR-34988 Montferrier- sur-Lez (FR).

jean-francois.germain@anses.fr/raphaelle.mouttet@anses.fr.

9. Feedback on this diagnostic protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press, this will also be marked on the website.

11. Acknowledgements

This protocol was originally drafted by: J. F. Germain and R. Mouttet. Anses-LSV, Unitè Entomologie et Plantes Invasives, Montferrier-sur-Lez (FR).

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12. References

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Appendix 1 – Preparation of male genitalia and female spermatheca for study under compound microscope (3200).

If the specimen is dry soften it in warm water for approximately 30 min until the abdomen can be moved. Remove the whole abdomen with forceps under a stereomicroscope and place it in warm potassium hydroxide solution (approximately 10%) for approximately 20 min to macerate the muscles and fat tissues. Rinse and dissect the abdomen in distilled water under a stereomicroscope (920), opening the abdomen along one side with fine scissors or pins. Clean out the remaining tissues and carefully sever the genitalia from the apical segments. Rinse the abdomen and genitalia in distilled water or ethanol and place into glycerine on a cavity slide for study and temporary storage. For permanent storage, place the genitalia (aedeagus or spermatheca) in a drop of Canada balsam on a slide.