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Molecular identification of *Epitrix* potato flea beetles (Coleoptera: Chrysomelidae) in Europe and North America

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Abstract

Epitrix species (Coleoptera: Chrysomelidae) feed mostly on plants from the family Solanaceae and some of them are major pests of potato crops. All *Epitrix* species are morphologically highly similar, which makes them difficult to identify and limits their study and management. Identification of species is mostly based on the observation of the genitalia and requires a high level of expertise. Here, we propose a tool to reliably identify all developmental stages of the most economically important *Epitrix* species feeding on potato in Europe and North America (*Epitrix cucumeris*, *Epitrix similaris*, *Epitrix tuberis*, *Epitrix subcrinita* and *Epitrix hirtipennis*). We first sequenced two DNA markers (mitochondrial cytochrome *c* oxidase I (*COI*) and nuclear internal transcribed spacer 2 (ITS2)) to test their effectiveness in differentiating among six *Epitrix* species (126 specimens). Morphospecies of *Epitrix* were well-differentiated by both DNA barcodes and no mitochondrial introgression was detected. Then, we developed an RFLP-based diagnostic method and showed that unambiguous species discrimination can be achieved by using the sole restriction enzyme *Taq*I on *COI* polymerase chain reaction products. The tool proposed here should improve our knowledge about *Epitrix* species biology, distribution and host range, three capacities that are particularly important in the detection and management of these pest species. Specifically, this tool should help prevent the introduction of *E. tuberis* and *E. subcrinita* in Europe and limit the spread of the recently introduced *E. cucumeris* and *E. similaris*, with minimal disruption to Solanaceae trade.

Keywords: Alticinae, barcoding, M13-tailed primer cocktails, PCR–RFLP, QBOL, solanaceae

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Introduction

The genus *Epitrix* Foudras (Coleoptera: Chrysomelidae: Alticinae) comprises nearly 180 species worldwide. Most of the species occur in the neotropics (130) and only 12 and 17 species are known from North America and Europe, respectively (Doeberl, 2000).

Epitrix species feed mainly on plants from the family Solanaceae (Doguet, 1994), though they may feed on other plant families when their preferential host is not available (e.g., Chenopodiaceae, Cucurbitaceae, Fabaceae (European and Mediterranean Plant Protection Organization (OEPP/EPPO), 2005, Table 1)). Five North American native species have been reported to feed on potatoes (*Solanum tuberosum* L.): *Epitrix cucumeris* (Harris), *Epitrix tuberis* Gentner, *Epitrix similaris* Gentner, *Epitrix subcrinita* (LeConte) and *Epitrix hirtipennis* (Melsheimer), and the two first are considered serious pests of potato crops (Gentner, 1944; Hoy *et al*., 2007, Table 1).

Adults feed on foliage, producing numerous small round holes (1.0–1.5 mm diameter, 'shot-hole' pattern) (OEPP/EPPO, 2011). Young plants and seedlings are particularly susceptible to attacks and heavy infestations may cause plant stunting. However, damage caused by adults is rarely economically important. When present in large numbers, soil dwelling *Epitrix* larvae, which feed on roots, stolons and tubers, may be responsible for more serious damage and important economic losses (Gentner, 1944; Morrison *et al*., 1967). For example, *E. tuberis* larvae burrow into tubers, leaving roughened trails on the surface, or tiny tunnels extending as far as 1.5 cm into the tuber flesh ('worm track' damage) that are still evident after peeling. Tunnels may cause deep cracks, rough and pimply skin and sometimes distortion of the tuber. One or two larvae can do enough damage to make a tuber unmarketable (OEPP/EPPO, 1989). Furthermore, larvae may act as vectors of bacteria (e.g., *Streptomyces* spp.), viruses (e.g., Andean Potato latent tymovirus, APLV) and fungal pathogens (*Verticillium dahliae*, *Fusarium coeruleum* and *Thanatephorus cucumeris*), which may increase financial losses result from damaged crops (OEPP/EPPO, 2005, 2010a; Vreugdenhil *et al*., 2007, Table 1).

Epitrix species are morphologically highly similar, which makes them difficult to identify for non-specialists. Adults are tiny pubescent beetles, generally ranging in size from 1.5 to 2 mm. Identification of the species is mostly based on the observation of the genitalia of both sexes and requires a high level of expertise. There is no comprehensive recent revision of the genus and consequently only local and partial keys are available (Seeno & Andrews, 1972; Doeberl, 2000; Warchalowski, 2003). No diagnostic characters have been reported for the immature life stages. Difficulties in distinguishing between *Epitrix* species limit our knowledge of their biology and render their management particularly challenging (Boavida & Germain, 2009). *Epitrix* species are often considered a pest complex (OEPP/EPPO, 2010b). In many records, specimens are not identified to species, which makes available data on species distribution and host range not fully reliable. Field identification is generally based on type of damage to tubers or foliage. However, as a systematic survey on *Epitrix* species in North America has not been attempted since Gentner (1944), confusion of symptoms because of species misidentification may exist and pest status of some species may be re-assessed in the future (Boavida & Germain, 2009).

E. subcrinita and *E. tuberis*, which have not been detected in Europe so far, are listed as quarantine pests (A1 list) by OEPP/EPPO (1989). Following the recent report of *E. cucumeris* and *E. similaris* in Portugal (Boavida & Germain, 2009), those two species have been listed as quarantine pests (A2 list) by EPPO. Pest risk analysis has shown that *E. cucumeris* and *E. similaris* could spread and find environmental factors suitable for establishment in most European countries (OEPP/EPPO, 2010b). Finally, *E. hirtipennis*, which was introduced to Europe in 1984 (Italy), is not considered a serious pest of potato crops in the European countries where it is established (Boavida & Germain, 2009).

With 62 millions tons produced per year (2008 data), potato production in the European Union (EU, 27 member states) is ranked second in the world after China (Schwartzmann, 2010). Specifically, in the EU-5 zone comprising the United Kingdom, the Netherlands, Belgium, Germany and Northern France, the production and international trade of ware potatoes (usually as washed commodities) generate a sales value of about $4.3 billion a year (Schwartzmann, 2010).

By reducing the quality and marketable yield of ware potatoes and requiring additional insecticide applications for pest control, tuber-damaging flea beetles represent a considerable threat for the European agricultural economy and environment. For example, once *Epitrix* species have established, economic losses for English main crop potato growers could be as high as $15–60 millions per year (Fera, 2012). Those estimates include increased costs of additional insecticide applications and revenue losses because of reduction in quality and marketable yield. However, loss of export markets for ware and seed potatoes are not included, hence losses may even be underestimated. Human-mediated spread seems more likely than natural spread, but flight distances are poorly known (OEPP/EPPO, 2010b). The most likely pathways for introduction and spread are through soil attached to roots or tubers where larvae, pupae or overwintering adults may occur (Cusson *et al*., 1990; OEPP/EPPO, 2010b). Currently, the EU Standing Committee on Plant health is working altogether with member state plant protection services on emergency measures to prevent the introduction of *E. subcrinita* and *E. tuberis* and the spread of *E. cucumeris* and *E. similaris* in Europe (J.-F. Germain, personal communication). Therefore, a tool to reliably identify all developmental stages of potato flea beetles occurring in Europe and North America is urgently needed.

Here, we compared the effectiveness of the mitochondrial *cytochrome c* oxidase I (*COI*) standard barcode fragment (Hebert *et al*., 2003) and the nuclear internal transcribed spacer 2 (ITS2) in differentiating among six morphospecies of *Epitrix* occurring in Europe and North America. Indeed, mitochondrial introgression, which can mislead species identification (see e.g., Frezal & Leblois, 2008) has been reported in several groups of Chrysomelidae (e.g., Gomez-Zurita & Vogler, 2006; Campbell *et al*., 2011). As no mitochondrial introgression was detected, we then used *COI* sequences and tested the potential of the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) approach to rapidly distinguish among *Epitrix* species.

Materials and methods

Taxonomic sampling and morphological identification

We sampled 126 specimens of *Epitrix* from several localities across Canada (British Columbia and Prince Edward Island), Costa Rica, Portugal (including the Azores, which lies some

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Table 1. Summary of available data on the five *Epitrix* species reported to feed on potatoes (*S. tuberosum*) in North America and Europe.

Species	Distribution*	Host range (wild)	Damage (on *S. tuberosum*)
E. cucumeris (Harris) 'Potato flea beetle'	**North America:** Widely distributed **South America:** Bolivia, Colombia, Ecuador and Venezuela **Europe:** Introduced to Portugal	**Preferred host:** *S. tuberosum* (potato) **Alternative hosts:** Other Solanaceae [e.g., *Capsicum* spp. (pepper), *Datura stramonium* (thorn apple), *Lycopersicon esculentum* (tomato), *N. tabacum* (tobacco) and *Solanum melongena* (eggplant)]. Occasionally other plant families (Asteraceae, Brassicaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae and Poaceae)	**Adult:** Foliage injury, characteristic 'shot-hole' pattern **Larvae:** Superficial tunnelling on the tuber surface (occasionally)
E. similaris Gentner	**North America:** Reported only from California **Europe:** Introduced to Portugal and possibly Spain	**Preferred host:** *S. tuberosum* **Alternative hosts:** Other Solanaceae [e.g., *D. stramonium*, *L. esculentum*, *Solanum melongena*, *Solanum nigrum* (European black nightshade), *Solanum trifolium* (cutleaf nightshade) and *Solanum jasminoides*]	**Adult:** Foliage injury, 'shot-hole pattern' **Larvae:** Shallow sub-epidermic sinuate furrows and recent report of serpentine tunnelling on the tuber surface in Europe ('worm track' damage)
E. tuberis Gentner 'Tuber flea beetle'	**North America:** From California to British Columbia **South America:** Ecuador and Costa-Rica	**Preferred host:** *S. tuberosum* **Alternative hosts:** Other Solanaceae (e.g., *Capsicum* spp., *L. esculentum*, *N. tabacum* and *S. melongena*). Occasionally other plant families (Chenopodiaceae, Cucurbitaceae and Fabaceae)	**Adult:** Foliage injury, 'shot-hole pattern' **Larvae:** 'Worm track' damage and deeper holes in the tuber flesh that are still evident after peeling
E. hirtipennis (Melsheimer) 'Tobacco flea beetle'	**North America:** Widely distributed **Europe:** Introduced to Italy, Portugal (the Azores), Greece, Bulgaria and Turkey	**Preferred host:** *N. tabacum* **Alternative hosts:** Other Solanaceae (e.g., *S. tuberosum*)	**Adult:** Not known **Larvae:** Not known
E. subcrinita (LeConte) 'Western potato flea beetle'	**North America:** From South California to British Columbia **South America:** Peru	Poorly know Mostly Solanaceae [e.g., *Capsicum* spp., *Ipomoea batatae* (Sweet potato), *S. melongena* and *S. tuberosum*]. Occasionally other plant families (Chenopodiaceae)	**Adult:** Foliage injury, 'shot-hole pattern' **Larvae:** Poorly known, occasionally burrow just under the peel causing little damage

Epitrix species being morphologically very similar, they are often considered a pest complex. Therefore, data on species distribution, host range and damage should be taken with caution.
* Detailed distributions can be found in OEPP/EPPO (2010b).

1500 km west of the coast of Portugal) and the United States (California, Kansas and Wisconsin) between April 2010 and August 2011. Samples were mostly collected on *S. tuberosum* L., though several specimens were collected on *Physalis longifolia* Nutt. (Long-leaf groundcherry) and *Capsicum* sp. (table 2). Specimens were collected alive and stored in 95% ethanol. Specimens were mostly collected as adults, though we also assessed the reliability of our molecular tools by testing assignment to species of a few larvae (table 2). Adult specimens were identified to species by J.-F.G. based on examination of habitus and genitalia, following Seeno & Andrews (1972), Doeberl (2000), Warchalowski (2003) and OEPP/EPPO (2011). Preparations of genitalia were made using the following protocol: genitalia were dissected from specimens using two needles, gently heated in a 10% KOH solution for 20 min, transferred to water (20 min), dehydrated using 70% EtOH and 100% EtOH (5 min each), transferred to lavender oil (for at least 10 min) and slide-mounted in a drop of Canada balsam for permanent storage. Slides were deposited at ANSES-LSV, Montferrier-sur-Lez, France.

DNA extraction, amplification and sequencing

Genomic DNA was isolated using the Qiagen DNeasy kit (Hilden, Germany) following the manufacturer's protocol without destruction of the specimens, to allow subsequent examination of morphology.

This study is part of an ongoing project (Quarantine Barcode of Life, QBOL), which aims at developing diagnostic resources to enable molecular identification of quarantine arthropod pests for Europe (Bonants *et al.*, 2010). About 300 species from seven different arthropod orders are sequenced not only on the standard 658 bp region of the *COI* gene (Hebert *et al.*, 2003) but also on ITS2. The choice of ITS2 was motivated by the fact that primers could be designed in regions that were conserved across many taxa (i.e., 5.8S and 28S rRNA) and that ITS2 often exhibits more intra-specific variability than other nuclear markers.

To achieve our general goal, efforts were taken to amplify all species following a single protocol. Primers were designed as follows: for both *COI* and ITS2, we aligned as many *COI* and ITS2 sequences as possible from arthropod species, found on GenBank. For *COI*, we started from the classical Folmer *et al.* (1994) primers and designed more optimal primers by degenerating appropriate sites to increase amplification success. ITS2 primers were designed in regions that were conserved across all taxa, with appropriate degenerate sites to increase amplification success. Furthermore, instead of using pairs of conventional degenerate primers (one forward and one reverse), *COI* and ITS2 were amplified using primer cocktails,

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Table 2. List of *Epitrix* specimens included in this study.

Species	Sampling localities	Collection date	Stage (No. of specimens)	Primary host plant	GenBank accession numbers	
					COI haplotypes (No. of specimens)	ITS2 variants (No. of specimens)
E. cucumeris	Canada, Harrington (PEI)	16 July 2010	Adults (14)	*S. tuberosum*	JQ947968, JQ947971 (12), JQ947972	JQ947954
	Portugal, São Miguel Island, Lomba São Pedro	20 August 2010	Adults (2)	*S. tuberosum*	JQ947971	JQ947954
	Portugal, Cinfaes	13 July 2010	Adults (3)	*S. tuberosum*	JQ947971	JQ947954
	Portugal, Mira, Carapelhos	17 May 2011	Adult (1)	*S. tuberosum*	JQ947971	JQ947954
	Portugal, Paredes	13 July 2010	Adults (4)	*S. tuberosum*	JQ947971	JQ947954
	Portugal, Penafiel	13 July 2010	Adults (2) Larvae (2)	*S. tuberosum*	JQ947971	JQ947954
	Portugal, Porto	21 April 2011	Adults (3)	*S. tuberosum*	JQ947971	JQ947954
	Portugal, Vidago	15 July 2010	Adults (4)	*S. tuberosum*	JQ947971	JQ947954
	Portugal, Vila do Conde, Aveleda	17 May 2011	Adult (1)	*S. tuberosum*	JQ947971	JQ947954
	USA, Boscobel (WI)	1 August 2011	Adults (10)	*S. tuberosum*	JQ947969, JQ947970, JQ947971 (8)	JQ947954
E. hirtipennis	Portugal, São Miguel Island, Achadinha	19 August 2010	Adults (4)	*S. tuberosum*	JQ947986	∅
	Portugal, São Miguel Island, Lomba São Pedro	20 August 2010	Adults (4)	*S. tuberosum*	JQ947986	∅
E. similaris	Portugal, Alpiarça	17 May 2011	Adults (3)	*S. tuberosum*	JQ947962 (2), JQ947963	JQ947950
	Portugal, Cinfaes	13 July 2010	Adults (2)	*S. tuberosum*	JQ947961, JQ947962	JQ947950
	Portugal, Maia, Travessa Nova de Lagielas	12 July 2010	Adults (5)	*S. tuberosum*	JQ947960, JQ947962 (4)	JQ947949, JQ947950 (4)
	Portugal Mira, Carapelhos	17 May 2011	Adults (2)	*S. tuberosum*	JQ947962	JQ947950
	Portugal, Paredes	13 July 2010	Adults (3)	*S. tuberosum*	JQ947962 (2), JQ947963	JQ947950
	Portugal, Salvaterra de Magos	17 May 2011	Adults (6)	*S. tuberosum*	JQ947962 (3), JQ947963 (3)	JQ947950
	Portugal Vila do Conde-modivas, Travessa de Real	17 May 2011	Larvae (11)	*S. tuberosum*	JQ947962 (9), JQ947963 (2)	JQ947949, JQ947950 (10)
E. subcrinita	USA, Irvine (CA)	4 June 2011	Adults (2)	*Capsicum* sp.	JQ947976	JQ947955
E. tuberis	Canada, Abbotsford (BC)	6 June 2010	Adults (10)	*S. tuberosum*	JQ947973 (9), JQ947974	JQ947957
	Canada, Agassiz (BC)	21 June 2010	Adults (18)	*S. tuberosum*	JQ947973	JQ947957
	Costa Rica, Volcan Irazu	19 April 2010	Adults (2)	*S. tuberosum*	JQ947975	JQ947956
E. fasciata	USA, nr Waterville (KS)	30 July 2011	Adults (8)	*P. longifolia*	JQ947979, JQ947980, JQ947981 (6)	JQ947948
Outgroup						
D. barberi	USA, Clinton County (IA)	n/a (GenBank)	n/a	n/a	AF278544	AF278559
D. longicornis	USA, Nuckolls County (NE)	n/a (GenBank)	n/a	n/a	AF278547	AF278562

Abbreviations used: BC, British Columbia; CA, California; IA, Iowa; KS, Kansas; NE, Nebraska; PEI, Prince Edward Island; WI, Wisconsin.

to further increase amplification success (Ivanova *et al.*, 2007). Finally, as PCR products were amplified from primer cocktails, primers were M13-tailed to allow sequencing (table 3, tails being highlighted in pale grey). Indeed, M13-tailed primers have proven more effective, allowing longer sequencing reads with more overlap (Ivanova *et al.*, 2007).

All PCRs were performed using the following reagents in a 25 µl reaction volume: 4 µl of genomic DNA (25–200 ng), 15.875 µl of ultra pure water, 2.5 µl of 10 × PCR buffer (final concentration = 1 ×), 0.5 µl of 25 mM $MgCl_2$ (0.5 mM), 0.5 µl of each 10 µM primer cocktail (0.2 µM), 1 µl of each 2.5 mM dNTP (0.1 mM), 0.125 µl of 5 units Taq DNA Polymerase (Qiagen, Hilden, Germany) (0.625 unit). PCR conditions for *COI* were: 94°C for 2 min, five cycles of 94°C for 30 s, 45°C for 40 s and 72°C for 60 s, followed by 35 cycles of 94°C for 30 s, 51°C for 40 s and 72°C for 60 s, with a final extension at 72°C for 10 min. PCR conditions for ITS2 were: 94°C for 2 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. PCR products

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Table 3. PCR primer cocktails used in this study.

Primer name	Primer sequence (5′–3′)	References
COI		
Forward		
LCO1490puc_t1-	TGTAAAACGACGGCCAGTTTTCAACWAATCATAAAGATATTGG	Cruaud *et al.* (2010)
LCO1490Hem1_t1	TGTAAAACGACGGCCAGTTTTCAACTAAYCATAARGATATYGG	This study
Reverse		
HCO2198puc_t1	CAGGAAACAGCTATGACTAAACTTCWGGRTGWCCAAARAATCA	Cruaud *et al.* (2010)
HCO2198Hem2_t1	CAGGAAACAGCTATGACTAAACYTCAGGATGACCAAAAAAYCA	This study
HCO2198Hem1_t1	CAGGAAACAGCTATGACTAAACYTCDGGATGBCCAAARAATCA	This study
ITS2		
Forward		
5.8S_cbgp_F1_t1	TGTAAAACGACGGCCAGTTCGATGAAGAACGCAGCDAAHTG	This study
5.8S_cbgp_F2_t1	TGTAAAACGACGGCCAGTTCGATGAAGAMCGCAGYTAACTG	This study
5.8S_cbgp_F3_t1	TGTAAAACGACGGCCAGTTCGATGAAAGACGCAGCAAAYTG	This study
Reverse		
28S_cbgp_R1_t1	CAGGAAACAGCTATGACGATATGYTTAAATTCRGSGGGT	This study

M13 tails from Ivanova *et al.* (2007) are highlighted. Original references for the untailed versions of each primer are provided.

Table 4. Kimura two-parameter pairwise distances (percentage) between specimens of *Epitrix* species.

	E. cucumeris	*E. hirtipennis*	*E. similaris*	*E. subcrinita*	*E. tuberis*	*E. fasciata*
E. cucumeris	0.00–0.31 / 0.00	17.79–18.19	20.73–21.37	11.47–11.66	10.25–10.92	18.44–19.26
E. hirtipennis	n/a	0.00 / n/a	22.55–24.09	18.60	18.48–19.22	17.45–17.65
E. similaris	6.93–7.11	n/a	0.00–1.39 / 0.00–0.17	18.49–19.10	18.54–20.28	20.76–21.80
E. subcrinita	0.70	n/a	6.93–7.11	0.00 / 0.00	7.29–7.60	19.63–20.04
E. tuberis	0.54–0.73	n/a	6.59–6.78	0.36–0.54	0.00–0.92 / 0.00–0.18	18.06–19.03
E. fasciata	24.86	n/a	24.41–24.61	24.36	22.36–22.62	0.00–0.31 / 0.00

COI distances are reported in the upper triangular matrix and ITS2 distances (calculated using the 'pairwise deletion' of gaps option) are reported in the lower triangular matrix. Off-diagonal entries: ranges of pairwise distances between samples among *Epitrix* species (inter-specific divergence); on diagonal entries: ranges of pairwise distances within *Epitrix* species (intra-specific divergence). n/a: non applicable.

were visualized on a 2% agarose gel using an E-Gel96 Precast Agarose Electrophoresis System (Invitrogen, Paisley, UK). Unpurified PCR products were sent to Eurofins MWG Operon (Ebersberg, Germany) for sequencing using M13F (−21) 5′-TGTAAAACGACGGCCAGT-3′) and M13R (−27) 5′-CAGGAAACAGCTATGAC-3′ primers (Ivanova *et al.*, 2007), which correspond to the 'tails' added to the PCR primers.

Both strands for each overlapping fragment were assembled using CodonCode Aligner v 3.7.1.1 (CodonCode Corporation, Dedham, Massachusetts, USA). Divergent haplotypes obtained for each marker were deposited in GenBank (table 2).

Sequence data analyses

Sequence alignment

All gene regions were aligned with MAFFT 6.864 (Katoh *et al.*, 2005) using the L-INS-i option. *COI* alignment was translated to amino acids using MEGA 4 (Tamura *et al.*, 2007) to detect frame-shift mutations and premature stop codons, which may indicate the presence of pseudogenes.

Distance analyses

Pairwise nucleotide sequence divergences were calculated using a Kimura 2-parameter model of substitution (Kimura, 1980) in MEGA 4, using the 'pairwise-deletion' of gaps option.

Phylogenetic reconstruction

The most appropriate model of evolution for each gene region was identified using the Akaike information criterion implemented in MrAIC.pl 1.4.3 (Nylander, 2004). We performed maximum likelihood (ML) analyses of the two gene regions using MPI-parallelized RAxML 7.2.8 (Stamatakis, 2006a). GTRCAT approximation of models was used for ML bootstrapping (Stamatakis, 2006b) (1000 replicates). Analyses were conducted on a 150 cores Linux Cluster at CBGP. *COI* and ITS2 sequences from *Diabrotica barberi* Smith & Lawrence, 1967 and *Diabrotica longicornis* (Say, 1824) were downloaded from GenBank and used as outgroups (table 2).

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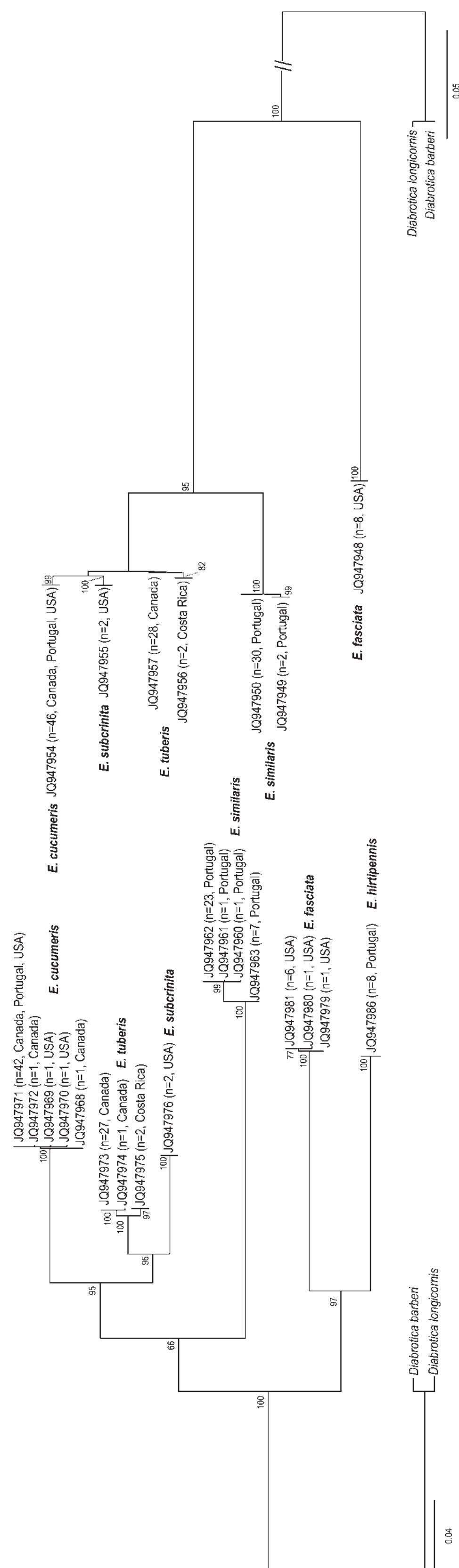

Fig. 1. Ml trees from the analyses of *COI* (left) and ITS2 (right) sequences. Specimens with identical haplotypes are collapsed to a single terminal node (GenBank accession numbers, number of specimens and geographical origin are indicated for each haplotype; details are provided in table 2). Bootstrap supports higher than 60% are indicated at nodes.

PCR–RFLP analyses

Owing to failure to amplify orthologous ITS2 sequences for *E. hirtipennis* and given that no mitochondrial introgression was detected between *Epitrix* species, PCR–RFLP analyses were carried out on *COI* only. Restriction patterns were predicted using BioEdit (Hall, 1999) and *Taq*I was selected to allow discrimination of the six species. *COI* amplicons from all tested species and haplotypes were subjected to *Taq*I restriction activity at 65°C for 3 h. Ten microlitres of PCR product were digested with 5.0 units of *Taq*I (Promega, Madison, Wisconsin, USA), 1 × buffer E and sterile distilled H_2O in a 15 µl reaction volume. The restriction fragments were separated by electrophoresis on 1.5% agarose gel at 110 V for 90 min and stained with ethidium bromide.

Results

Taxonomic sampling and morphological identification

Adult specimens were sorted into six morphospecies (table 2). Specimens collected in Portugal (49) were identified either as *E. cucumeris* (41%), *E. similaris* (43%) or *E. hirtipennis* (16%, collected only in the Azores).

Amplification success and sequence data

COI (658 bp) and ITS2 (691 aligned bp) were successfully amplified from all adult specimens and larvae. Alignment of *COI* was straightforward owing to a lack of length variation and no stop codons or frame shifts were detected. Alignment of ITS2 revealed that a divergent paralog was sequenced from all specimens of *E. hirtipennis*. A second attempt to amplify orthologous sequences led to the same result and ITS2 sequences from *E. hirtipennis* were consequently excluded from the analysis.

Distance and phylogenetic analyses

With 17 haplotypes identified, *COI* was more variable than ITS2, for which only seven variants were detected (table 2). K2P pairwise distances between *Epitrix* specimens (as per cent sequence divergence) are summarized in table 4. The intra-specific K2P distance range for *COI* was 0.00–1.39% (mean 0.15%), while the inter-specific distances ranged from 7.29% to 24.09% (mean 17.55%). The intra-specific K2P distance range for ITS2 was 0.00–0.18% (mean 0.01%) while the inter-specific distances ranged from 0.36% to 24.86% (mean 8.23%). Whatever the marker used, the minimum inter-specific divergence exceeded the maximum intra-specific divergence for all species.

Models chosen by MrAIC were as follows: GTR + I + Γ for *COI* and GTR + Γ for ITS2. Given that α and the proportion of invariable sites cannot be optimized independently from each other (Gu, 1995) and following Stamatakis' personal recommendations (RA × ML manual, 2006a), we used GTR + Γ with four discrete rate categories for both *COI* and ITS2. Phylogenetic analyses of *COI* and ITS2 (fig. 1) recovered the same well-supported clusters of sequences, which corresponded to morphologically delineated species. *E. tuberis* was the sole species to show two geographical clusters of haplotypes (Canada *versus* Costa Rica).

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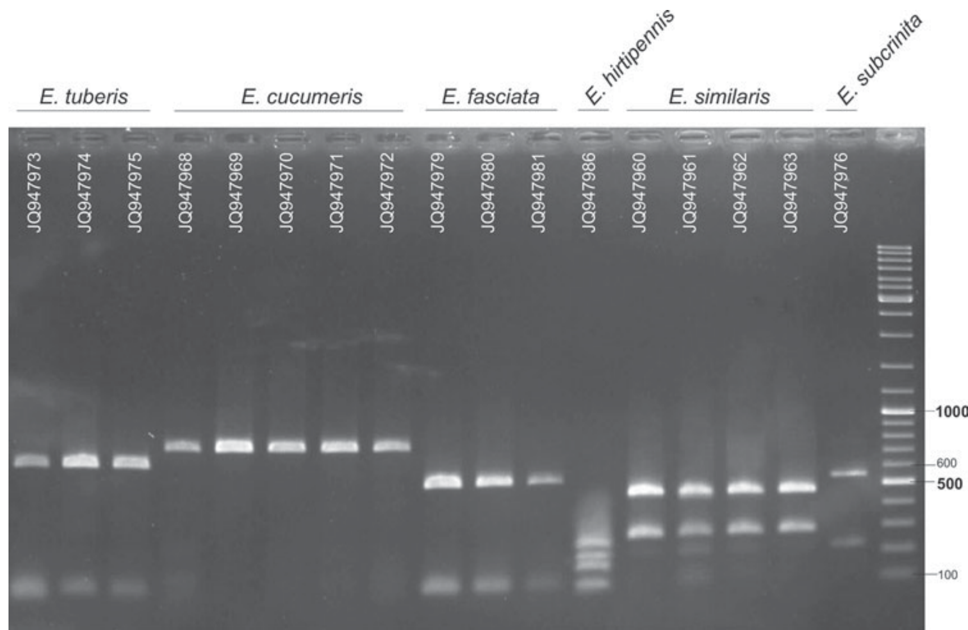

Fig. 2. RFLP profiles for a 658 bp fragment of *COI* digested with the restriction enzyme *Taq*I in the six species of *Epitrix*. GenBank IDs of the different haplotypes are indicated on lanes (see table 2 for details). Far right lane contains GeneRuler™ DNA ladder Mix (Fermentas, Walthman, Massachusetts, USA), with the first 11 fragments ranging in size from 100 bp to 1000 bp in 100 bp increments.

PCR–RFLP analyses

RFLP profiles for the 658 bp fragments of *COI* digested with *Taq*I are shown in fig. 2. In all species, alternate haplotypes produced unique RFLP patterns. *Taq*I activity produced four restriction fragments for *E. hirtipennis* (338, 260, 251 and 151 bp), three restriction fragments for *Epitrix fasciata* (609, 154 and 151 bp), and two restriction fragments for *E. similaris* (505 and 325 bp), *E. subcrinita* (573 and 257 bp) and *E. tuberis* (676 and 154 bp). No *Taq*I cleavage was detected for *E. cucumeris*. The two smallest fragments for *E. fasciata* (154 and 151 bp) were hardly distinguishable on the gel, though without precluding species identification.

Discussion

Since reference libraries validated by taxonomists are available, *COI* DNA barcodes (Hebert *et al.*, 2003) have been proposed as efficient diagnostic tools for biosecurity (e.g., Armstrong & Ball, 2005; de Waard *et al.*, 2010; Floyd *et al.*, 2010). Successful barcode identification requires intra-specific variability being markedly lower than inter-specific variability (Hebert *et al.*, 2004). When mitochondrial introgression occurs, which has been reported in several groups of Chrysomelidae (e.g., Gomez-Zurita & Vogler, 2006; Campbell *et al.*, 2011), this assumption is broken. Intra- and inter-specific variation can also overlap when species are not reciprocally monophyletic, for example when there is incomplete lineage sorting owing to the retention of ancestral polymorphism (Funk & Omland, 2003).

Here, we show that *COI* DNA barcodes should be valuable in routine identifications of all developmental stages of potato flea beetles occurring in Europe and North America. Morphospecies of *Epitrix* were indeed well differentiated by *COI* DNA barcodes. Minimum inter-specific divergence largely exceeded maximum intra-specific divergence and all species were recovered as reciprocally monophyletic by our phylogenetic analyses. Furthermore, comparison with ITS2 genetic clusters revealed no mitochondrial introgression between species. It is noteworthy that, while widely distributed in North America and established in the Azores, Italy, Greece, Bulgaria and Turkey, *E. hirtipennis* was sampled from the Azores only. One could therefore expect slight differences between sequences from our samples and those from other geographic regions, but this should not preclude species identification, though this needs to be formally established. Indeed, intra-specific distances were overall very low. For example, for *E. cucumeris*, which was sampled in Portugal (including the Azores), Canada and the United States intra-specific distances did not exceed 0.32% for both *COI* and ITS2.

While amplification and sequencing of *COI* were straightforward for both adults and larvae in every species, we failed to amplify orthologous ITS2 sequences for *E. hirtipennis*. This indicates that *COI* should be preferred over ITS2 when developing a reference barcode database for identification and monitoring of *Epitrix* species. Providing a molecular diagnostic tool for the identification of potato flea beetles occurring in other parts of the world or other *Epitrix* species was not the purpose of this study. However, primer cocktails and PCR conditions provided here should help in developing such tools. As mentioned in the Materials and methods section, this study is part of the QBOL project, which aims at developing diagnostic resources to enable molecular identification of quarantine arthropod pests for Europe (Bonants *et al.*, 2010). Information regarding barcoded specimens (including those used in the present study) as well as barcode sequences themselves are deposited in an Internet-based database system, Q-bank (http://www.q-bank.eu/arthropods/). A BLAST tool allows online identification of unknown specimens by querying the sequence database.

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To best match the available lab equipment and management objectives, we also developed an RFLP-based diagnostic method and showed that unambiguous species discrimination can be achieved by using the sole restriction enzyme *Taq*I on *COI* PCR products. Thanks to significant reduction in processing time and cost, this RFLP-based identification tool may allow for more extensive sampling strategies and hence more efficient field monitoring surveys.

Prior to this study, identification of *Epitrix* species was based on examination by expert taxonomists of morphological characters of adult specimens, especially genitalia. No diagnostic characters were available for larvae and non-specialists relied exclusively on plant damage to identify species. However, plant damage-based diagnosis can be misleading as species misidentification might have resulted in confusion of symptoms. For example, Boavida & Germain (2009), recently raised the possibility that *E. tuberis* and *E. similaris*, which are sympatric in California (Seeno & Andrews, 1972) and resemble each other in their external morphology (Gentner, 1944), may have been confused. Indeed, in Portugal, where it has been recently introduced, *E. similaris* seems responsible for 'worm track damage' to tubers usually attributed to *E. tuberis* (R. Oliveira, personal communication). By enabling the identification of larvae, molecular diagnostic tools make possible linking *Epitrix* species to observed damage, which should improve our knowledge of species biology. Furthermore, the tool provided here should clarify *Epitrix* distribution and host range, two capacities that are also particularly important in the management of these pest species in Europe and North America. Our study confirms that *E. tuberis* and *E. subcrinita* have probably not been introduced to Europe yet (Boavida & Germain, 2009). Indeed, including this study some hundreds of *Epitrix* specimens have already been sampled in Europe, but not one specimen of these two species has been detected so far. Besides potato flea beetles, we also sampled *E. fasciata*, the Southern tobacco flea beetle, which preferentially develop on *Nicotiana tabacum* L. *E. fasciata* has been recently introduced to the Azores but is not considered a pest of potato (Boavida & Germain, 2009). However, *E. fasciata* and *E. hirtipennis* being morphologically highly similar, molecular barcodes should facilitate distinguishing these two species.

During the final stages of review of this manuscript, the European commission published a decision (212/270/EU) to prevent the introduction of *E. tuberis* and *E. subcrinita* in Europe and limit the spread of *E. cucumeris* and *E. similaris* (Official Journal of the European Union, 2012). By enabling the identification of *Epitrix* specimens at different periods of the life cycle, this diagnostics tool should help member states conducting survey for the presence of potato flea beetles on potato crops as well as other host plant species, as recommended by the European commission. Fast and accurate detection of *Epitrix* potato flea beetles would help study their potential spread and contribute to their management with minimal disruption to Solanaceae trade.

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