PCR-based methods for identification of two *Eteobalea* species (Lepidoptera: Cosmopterigidae) used as biocontrol agents of weedy *Linaria* species (Scrophulariaceae)

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Abstract—Two European species of the genus *Eteobalea* Hodges are being tested as classical biological control agents for toadflax (*Linaria* spp.) in Canada. Permits for the release of these species apply only to an Italian population of *E. serratella* Treitschke and a Serbian population of *E. intermediella* Riedl. When these species are imported as larvae or pupae from an area where they are sympatric, such as around Rome, they must later be separated in the laboratory. This step is essential to establish a pure laboratory colony of the correct species, both to increase the rate of breeding success and to comply with conditions of the importation permit. Although the male and female genitalia and the egg chorion have good diagnostic characters, these characters are not useful for identifying immature stages or newly emerged adults before mating and oviposition. This problem has hampered the establishment of rearing colonies of these two species in Canada. We describe the development and application of four polymerase chain reaction – restriction fragment length polymorphism assays that have been used to distinguish between these two species. If and when either of these species becomes established in the field, the tests presented here should also prove useful for monitoring populations.

Résumé—Deux espèces européennes d'*Eteobalea* Hodges sont actuellement à l'essai au Canada comme des agents de lutte biologique contre les mauvaises herbes du genre *Linaria*. Les permis pour le lâcher de ces espèces sont valables seulement pour une population italienne d'*E. serratella* Treitschke et une population serbienne d'*E. intermediella* Riedl. Lorsque ces espèces sont importées d'une région où elles existent en sympatrie, par exemple près de Rome, elles doivent être séparées plus tard dans le laboratoire. Ceci est indispensable pour établir une colonie pure de l'espèce correcte, à la fois pour assurer le succès de la multiplication et pour se conformer aux conditions du permis d'importation. Bien que les organes génitaux des deux sexes et le chorion de l'oeuf possèdent des caractères utiles pour leur identification, ceux-ci ne servent pas pour l'identification des stades immatures, ni des adultes nouvellement émergés avant de l'accouplement et l'oviposition. Ceci a entravé l'établissement des colonies de multiplication de ces deux espèces d'*Eteobalea* au Canada. Nous décrivons le développement et l'application de quatre essais de réaction de polymérisation en chaîne – polymorphismes de longueur de fragments de restriction qui ont été utilisés pour distinguer entre ces espèces. Ces essais seront également utiles pour la surveillance des populations dans le cas que n'importe quelle de ces espèces s'établit dans le champ.

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Introduction

Two European species of *Eteobalea* Hodges (Lepidoptera: Cosmopterigidae) have been approved for field release in Canada as biological control agents for the weedy introduced species common toadflax, *Linaria vulgaris* Mill. (Scrophulariaceae), and Dalmatian toadflax, *Linaria genistifolia* subsp. *dalmatica* (L.) Maire & Petitm. Larvae of both moth species mine the roots of their host plants: *Eteobalea serratella* (Treitschke), referred to in some recent literature as *E. gronoviella* (Scopoli) (see Discussion), attacks primarily common toadflax, whereas *Eteobalea intermediella* (Riedl) accepts both this species and Dalmatian toadflax (Saner *et al*. 1990). Neither *Eteobalea* species is currently established in the field anywhere in Canada, but efforts to establish them are continuing in Alberta, British Columbia, and Nova Scotia (DeClerck-Floate and Harris 2002; McClay and DeClerck-Floate 2002).

Eteobalea serratella is recorded from Spain, Portugal, France (including Corsica), Italy (including Sardinia and Sicily), Switzerland, Malta, Greece, Austria, Poland, Hungary, the Czech Republic, Slovakia, the former Yugoslavia, Bulgaria, the European part of the former USSR, Syria, Kazakhstan, and Mongolia (Riedl 1967, 1969, 1978, 1996). *Eteobalea intermediella* is broadly sympatric with *E. serratella* but has a more southern distribution, occurring in Morocco, Algeria, Tunisia, Spain, Portugal, France, Italy (including Sardinia and Sicily), Greece (including Crete), Austria, Hungary, Slovakia, the former Yugoslavia, Romania, Bulgaria, the European part of the former USSR, Malta, Turkey, Lebanon, Syria, Iran, and Mongolia (Riedl 1978, 1983, 1996). Under the permits granted for release of these species in Canada, only insects from the populations that were subjected to host-specificity tests may be released. For *E. serratella* this population is found near Rome, Italy, and for *E. intermediella* the approved population is from Belgrade, Serbia. Thus, when these species are imported from an area of Europe where they are sympatric, it is important to ensure that rearing colonies are established from a pure population of the correct species.

The insects are usually imported as larvae or pupae in field-collected toadflax roots from collection sites in Europe. When adults emerge in the laboratory, they are paired up and placed in oviposition cages. Eggs are collected from the cages and placed on potted toadflax plants to establish a rearing colony for use as a source for field release. However, because adults of these two species are very similar morphologically, it is at best difficult, and often impossible, to correctly pair up conspecific adults when they emerge from roots harboring mixed populations. The male and female genitalia have diagnostic features (Riedl 1975), the sculpturing of the egg chorion is distinctive in each species (Rizza and Pecora 1979; Saner *et al*. 1990; Fig. 1), and adults in perfect condition can be separated via a forewing character (Koster and Sinev 2003): in *E. intermediella* the distal costal mark is entirely white, whereas in *E. serratella* it is white on the costal margin and golden interiorly. However, this last character is not completely reliable for separating adults because the wing scales are easily lost, and none of the above-mentioned characters is useful for identifying larvae or pupae. This problem has hampered establishment of rearing colonies of these two *Eteobalea* species in Canada.

Riedl (1975) places *E. intermediella* closest to *E. beata* (Walsingham) and *E. sumptuosella* (Lederer) while stating that *E. serratella* is the easiest species to identify within the *E. beata* species group; thus, *E. intermediella* and *E. serratella* are not expected to be sister species, and we have no reason to question their current taxonomic status as separate species. The purpose of this study, therefore, was only to provide a rapid and reliable means for distinguishing between these two species given any life stage of these insects. Such a method would be useful both in establishing pure breeding colonies and in identifying the two species if they eventually become established in mixed populations in Canada.

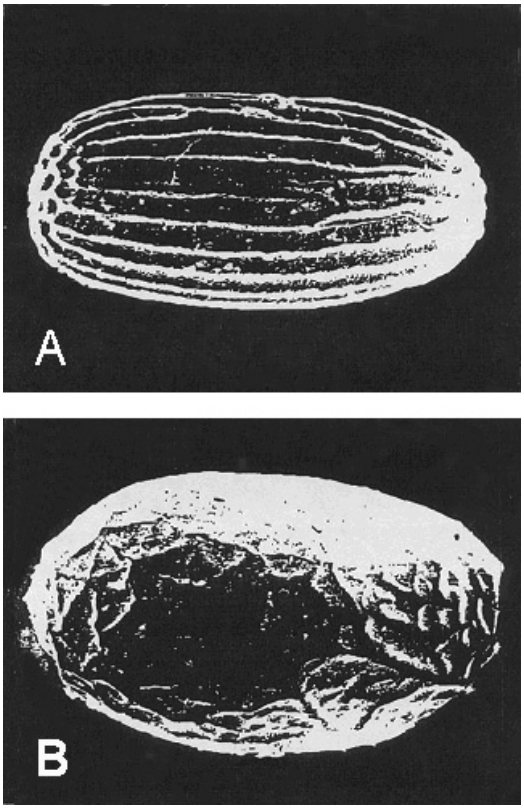
Materials and methods

Material examined

First series (1999)

A shipment of field-collected *L. vulgaris* roots containing larvae of one or more *Eteobalea* species was received from Italy on 10 June 1999. The collection site was Bracciano, 35 km NE of Rome. From the adults that emerged, 32 oviposition tubes were set up, each with a single female and one to three males, depending on availability. Twenty-one of these "pairs" laid eggs and of these, 15 lots were *E. serratella*

Fig. 1. Eggs of *Eteobalea* species. (A) *Eteobalea serratella*; (B) *E. intermediella*. From Rizza and Pecora (1979).



type and 6 were *E. intermediella* type. Four egg lots of each type were fertile, the rest infertile. Females that produced fertile eggs were assumed to have mated with males of their own species. Only individuals from such pairs were used for the DNA extractions and, in the case of *E. serratella*, for further rearing. When oviposition was complete, adults were stored in 70% ethanol at –15 °C. The individual moths that laid or sired infertile eggs were discarded. Sixteen adult male and female *E. serratella* and *E. intermediella* identified in this manner were used for DNA extraction and the development of the polymerase chain reaction – restriction fragment length polymorphism (PCR–RFLP) diagnostic tests. These specimens included three males and four females of *E. serratella* and four males, two females, and three of unknown gender of *E. intermediella*.

Second series (2001)

A second shipment of field-collected *L. vulgaris* roots infested with larvae of one or more *Eteobalea* species was received from Italy on 8 June 2001. These roots were collected on the road to Ardeatina at km 24, 10 km south of Rome. The PCR–RFLP tests developed with the first series were used at the Alberta Research Council, Vegreville, Alberta, in an effort to identify the *Eteobalea* species present in this shipment, to facilitate setting up a pure culture of *E. serratella*. During dissection of the imported roots in quarantine, a number of freshly dead or moribund *Eteobalea* larvae were found. Most of these larvae were apparently paralyzed as a result of being stung by adult females of a gregarious ectoparasitic braconid that was abundant in the shipment. Molecular diagnostic tests were carried out with these larvae to conserve as many of the healthy individuals as possible for the foundation of a laboratory breeding colony.

Examination of genitalic structures

Identities of the adult specimens from the first series were confirmed by comparison of the genitalia with published figures (Riedl 1969, 1975). Genitalia were prepared by clearing the abdomens in 10% KOH at 90 °C for 5 min and were examined under a dissecting microscope. The abdomens of two *E. serratella* females and three *E. intermediella* of unknown gender were consumed by the DNA extraction process and identities could not be confirmed with genitalia.

DNA extraction, PCR, and DNA sequencing

First series

DNA was extracted from heads and thoraces of specimens using the QIAGEN DNeasy Tissue Kit (QIAGEN Inc., Valencia, California). Whenever possible, the wings and abdomens were kept as vouchers. PCR was performed in 50-μL volumes, following the protocol of Sperling and Hickey (1995). For one specimen of each species, the entire COI–COII region (cytochrome-*c* oxidase subunit I to subunit II; approximately 2.3 kbp) was PCR amplified using the end primers TY-J-1460 (= K698) and TK-N-3782 (= Eva) (see Table 1 for primer sequences) and a variety of internal primers. PCR fragments were sequenced in both directions using the DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Cleveland, Ohio) and fractionated on an ABI PRISM® 377 automated DNA sequencer (Applied Biosystems, Foster City, California).

Table 1. PCR primers used in PCR–RFLP assays of *Eteobalea* spp.

Primer	Primer sequence (5′–3′)
K698 (TY-J-1460)	TAC AAT TTA TCG CCT AAA CTT CAG CC
K699 (C1-N-1840)	AAG AAG ATA AAC AGT TCA YCC
Ellen (C1-J-2531)	TTT ACT GTA GGA GGA TTA ACW GG
Barbara (C2-N-3661)	CCA CAA ATT TCT GAA CAT TGA CCA
Marlon (C2-J-3408)	CAA TGA TAT TGA AGT TAT GA
Eva (TK-N-3782)	GAG ACC ATT ACT TGC TTT CAG TCA TCT

Sequences were assembled into contiguous arrays using Sequencher™ (Gene Codes Corporation, Ann Arbor, Michigan). Alignment of the two consensus sequences was trivial because of the rarity of insertions and deletions; therefore, it was performed by hand using the software Se-Al (Rambaut 2000). The K698–K699 and Marlon–Eva fragments (see below) were sequenced in one direction only for 10 additional specimens of *Eteobalea* (*i.e.*, 5 specimens of each species) to assess DNA sequence variation. The DNA sequence for the K698–K699 fragment could not be obtained for a single specimen of *E. intermediella* (DNA No. 1277) although the Marlon–Eva fragment was obtained for this specimen.

Second series

Twelve dead or paralyzed larvae (without attached parasitoid eggs or larvae) from the June 2001 shipment were preserved in ethanol and used for DNA extraction. Whole larvae were macerated in 300 µL of a DNA extraction buffer (2% cetyltrimethylammonium bromide, 1.4 mol/L NaCl, 0.1 mol/L Tris, 20 mmol/L EDTA, pH 8.0). DNA was obtained by phenol–chloroform extraction, precipitated in isopropyl alcohol, washed in 70% ethanol, and resuspended in 50 µL of Tris–EDTA. DNA extracts from specimens 1256 and 1258 from the first series were used as positive controls for *E. intermediella* and *E. serratella*, respectively. PCR reactions were performed as for the first series.

Design of PCR–RFLP assays

The software program tacg (Mangalam 1997) was used to search the two complete COI–COII DNA sequences for restriction sites that would yield species-specific diagnostic banding patterns. These analyses were performed from the command line with the following options: -C2 -D0 -f1 -L -r* -s -S -V, where * is the name of the restriction enzyme used. PCR fragment – restriction enzyme combinations were selected using the following criteria: (*i*) there should be at least one restriction site within a PCR fragment for both species to serve as a positive control for the correct functioning of the restriction enzyme; (*ii*) the difference in the sizes of the restriction fragments should be large enough that they can be differentiated on agarose gels; (*iii*) if possible, the PCR fragment should be short enough (<600 bp) that the test can be used even with degraded DNA, although there is a trade-off between this criterion and the previous one; and (*iv*) less expensive restriction enzymes are preferred. Three primer pairs were selected for further analysis (primer sequences in Table 1): the K698–K699 pair amplifies the 5′-most region of the COI gene; the Ellen–Barbara pair amplifies a region spanning the 3′ end of COI, the tRNA leucine gene, and the 5′ end of COII; and the Marlon–Eva pair amplifies the 3′ end of COII. Two restriction enzymes (*Alu*I and *Taq*I) and four primer pair – restriction enzyme combinations were selected. Table 2 shows the expected relative positions of restriction sites within each PCR fragment and the sizes of the resulting PCR–RFLP fragments.

Restriction digests

Restriction digests were performed as described in Sperling and Hickey (1995), with the following exceptions. Total reaction volume was 15 µL, containing 5 µL of sterile H_2O, 1.5 µL of 1× enzyme buffer, 0.5 µL of enzyme (10 U/µL; 1 U ≈ 16.67 nkat), and 8 µL of PCR product. The *Alu*I reactions were incubated for 90 min at 37 °C and the *Taq*I reactions were incubated for 90 min at 65 °C, following the manufacturer's instructions. Restriction fragments were visualized on 2.2% agarose gels stained with ethidium bromide (0.25 µg/mL). For samples from the second series, restriction digests of the Marlon–Eva products were performed using *Taq*I and the K698–K699 products were digested with *Alu*I. Because the

Table 2. Expected restriction digestion patterns in PCR–RFLP assays of *Eteobalea* spp.

		E. intermediella		*E. serratella*	
Primer pair	Enzyme	Positions of cuts (bp)	Fragment lengths (bp)	Positions of cuts (bp)	Fragment lengths (bp)
K698–K699*	*Alu*I	104, 305	104, 115, 201	293, 305	12, 115, 293
Marlon–Eva†	*Taq*I	96, 258	96, 149, 162	114	114, 290
Ellen–Barbara‡	*Alu*I	270, 722, 965, 1127	41, 162, 243, 270, 452	724, 811, 985	87, 174, 182, 724
Ellen–Barbara‡	*Taq*I	34, 112, 259, 603	34, 78, 147, 344, 565	34, 82, 112, 605	30, 34, 48, 493, 562

*Length of PCR fragment was 420 bp in both species.
†Length of PCR fragment was 407 bp in *E. intermediella* and 404 bp in *E. serratella*.
‡Length of PCR fragment was 1168 bp in *E. intermediella* and 1167 bp in *E. serratella*.

Ellen–Barbara amplifications were unsuccessful for the second series, no restriction digests were performed on these products.

Results and discussion

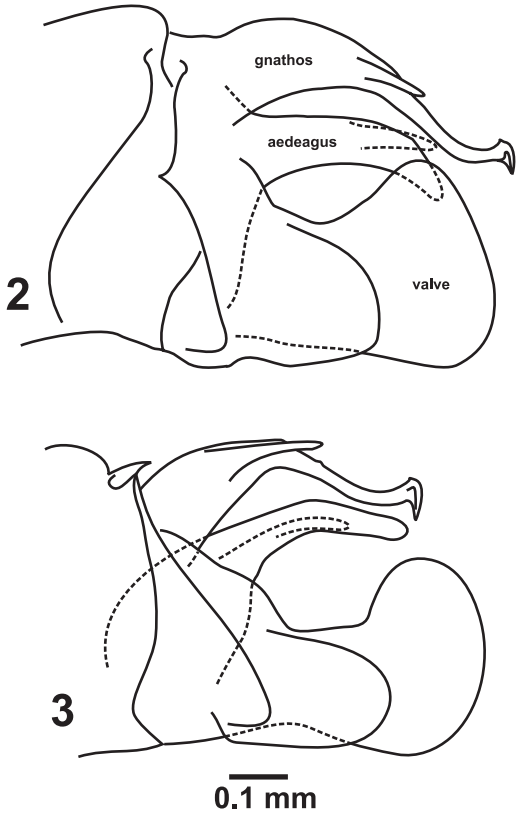
Taxonomy of *Eteobalea*

To head off potential confusion, we note that the original author of *Eteobalea* (Hodges 1962) later decided to synonymize the genus with *Stagmatophora* Herrich-Schäffer; thus, the species treated in this study are sometimes referred to the latter genus in the literature. However, the biological control literature has never used the latter genus name for these species, and a recent checklist of European Lepidoptera kept the European species in *Eteobalea*. We have therefore used *Eteobalea*. The same checklist noted that *E. serratella* is a junior synonym of *E. gronoviella* and used the latter name instead (Riedl 1996). However, this synonymy was rejected by Koster and Sinev (2003) because none of Scopoli's material survives and his original description of *gronoviella* is too vague and could refer to any of the dark species of the genus *Eteobalea*, or even to a species of gelechiid, *Eulamprotes wilkella* (L.) (J.C. Koster, personal communication).

Genitalic characters

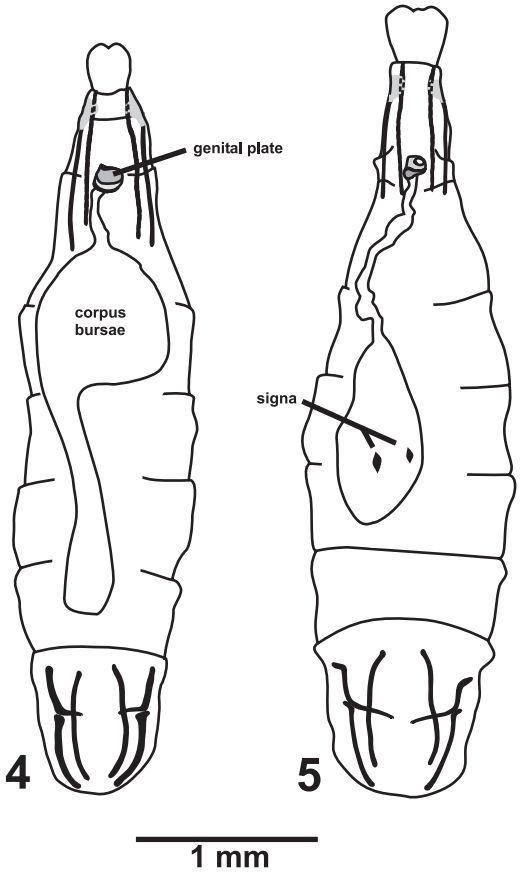
In all cases, the observed genitalia corroborated the results of PCR–RFLP analysis. Diagnostic characters are as follows: in *E. serratella* males (Fig. 2), the distal margin of the valve meets the ventral margin at an 80°–90° angle, and the widest portion of the valve is very near the distal margin; in *E. intermediella* males (Fig. 3), the distal margin of the valve meets the ventral margin at an obtuse angle, and the widest portion of the valve is near the midpoint. Although there are slight differences in the shapes of the gnathos and aedeagus, these structures are very complex and apparent differences may be due to slight differences in angle of rotation. In *E. serratella* females (Fig. 4) the

Figs. 2–3. *Eteobalea* spp. male genitalia, left lateral aspect: 2, *E. serratella*, *ex*. lab culture, Vegreville, Alberta, Canada, 2000; 3, *E. intermediella*, *ex*. lab culture, Vegreville, Alberta, Canada, 2000.



corpus bursae has no signa, whereas in *E. intermediella* females (Fig. 5) it has two signa. In addition, there are differences in the shape of the genital plate.

Figs. 4–5. *Eteobalea* spp. female genitalia, ventral aspect: 4, *E. serratella*, Bracciano, 35 km NE of Rome, Italy, *ex*. root of *Linaria vulgaris*, larva collected 10–12 June 1992, adult emerged 3 August 1992; 5, *E. intermediella*, Novi Beograd, Belgrade, Yugoslavia [now Serbia], *ex*. root of *Linaria genistifolia*, larva collected May 1992, adult emerged 28 June 1992.



DNA sequence analysis

The full-length COI–COII sequences for *E. intermediella* and *E. serratella* were deposited in GenBank (accession Nos. AY423064 and AY423065, respectively). The sequences are 2294 and 2293 bp in length, respectively; relative to sequences of *E. intermediella* and other Lepidoptera, the *E. serratella* sequence has a 3-bp insertion and a 1-bp deletion in the tRNA leucine gene and a 3-bp (one codon) deletion in the COII gene. The uncorrected pairwise divergence between the *E. intermediella* and *E. serratella* sequences is 5.8%. The base compositions of the two sequences are almost identical and are typical of insect COI–COII sequences, with a mean of 33.4% A, 13.5% C, 12.1% G, and 41.0% T. The highest-scoring hits obtained in BLAST searches conducted with both sequences were COI–COII sequences from various species of the genus *Choristoneura* Lederer (Lepidoptera: Tortricidae), with the closest sequence being 10% divergent from *E. serratella* (score = 1027 bits, E = 0.0).

For within-species comparisons, all K698–K699 fragment and Marlon-Eva fragment sequences were identical to the full-length COI–COII sequences obtained earlier, with one exception: for both PCR fragments, the sequence for DNA 1274 (supposedly a specimen of *E. serratella*) was in fact identical to the other *E. intermediella* sequences. This conclusion was supported by PCR–RFLP analysis of the Ellen–Barbara fragment (see below). Subsequent examination of the genitalia confirmed that this specimen was an *E. intermediella* male. Males were originally identified only by association with females that laid fertile eggs, *i.e.*, they were assumed to be of the same species as the female if the eggs they sired were fertile. Because there were at times up to three males in each oviposition tube with a single female, we initially thought that this could explain the misidentification. However, examination of laboratory notebooks revealed that all the matings that resulted in fertile eggs were from tubes containing only a single male. The only other possible explanations are that either a male *E. intermediella* managed to mate with a female *E. serratella* and sire fertile eggs or there was a labelling error at some stage of the process from rearing adults through to DNA sequencing. We conclude that the latter explanation is more likely.

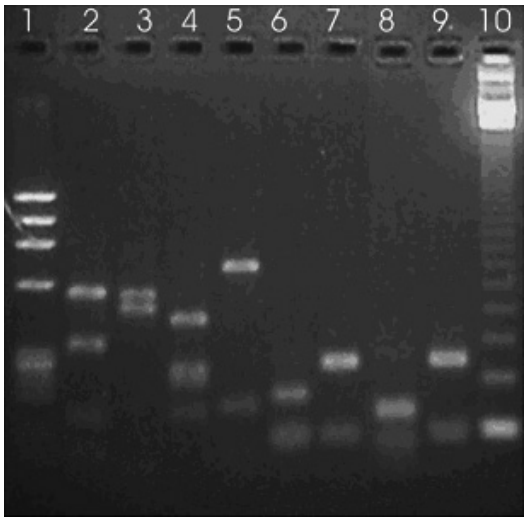
It is noteworthy that we observed no mtDNA sequence variation within each of these two species. Nevertheless, one might expect to find intraspecific mtDNA sequence variation when further specimens are collected in Europe; therefore, it is prudent to design more than one PCR–RFLP assay to distinguish between these species. This we have done, maximizing the probability that at least one PCR fragment – restriction enzyme combination will provide useful results.

RFLP analyses

The restriction fragment patterns predicted in Table 2 were confirmed in the laboratory (Fig. 6). We performed 28 restriction digests with the first series of specimens. Table 3 shows the specimens used for the restriction digests. With the single exception mentioned earlier, which occurred during the development of the assay, all PCR–RFLP assays performed as expected and unambiguously identified specimens as belonging to one of the two species of *Eteobalea* being tested. Restriction digestion of the Ellen–Barbara PCR fragment with *Alu*I gives the most easily distinguishable banding pattern. Use of this PCR fragment has the added advantage that digestion with *Taq*I also gives clear results, so that if the first test is inconclusive, the second test can be performed immediately without the added time and expense of repeating the PCR step. Use of the Ellen–Barbara PCR fragment with either *Alu*I or *Taq*I should therefore be the preferred method. However, the Ellen–Barbara fragment is almost 1200 bp long, meaning that PCR amplification may be unsuccessful with preserved specimens unless they have been frozen at –70 °C or preserved in 95% ethanol. In such cases, either of the other two PCR fragments should amplify successfully because both are approximately 400 bp long.

Ten of the 12 larvae of the second series (the 2001 shipment) yielded PCR amplification products with the Marlon–Eva primer pair, and 9 of these 10 yielded products with the K698–K699 primer pair. Digestion of the 9 K698–K699 amplification products with *Alu*I gave patterns matching the *E. serratella* positive control, with strong bands near the predicted lengths of 115 and 293 bp. The Marlon–Eva products from 5 of these 9 larvae gave clear bands at approximately 300 bp when digested with *Taq*I, matching the *E. serratella* positive control; the predicted bands for *E. serratella* in this digest are at 114 and 290 bp. These 9 larvae were identified as *E. serratella*. The Marlon–Eva fragment from the remaining larva showed no band at 300 bp when digested with *Taq*I but a faint band at <200 bp, matching a similar band in the *E. intermediella* positive control. The predicted bands for this digest of *E. intermediella* are at 96, 149, and 162 bp. This larva was tentatively identified as *E. intermediella.* Of the 12 larvae, 9 were thus identified as *E. serratella,* 1 was identified as *E.intermediella*, and 2 could not be identified.

Fig. 6. Visualization of restriction-digested PCR fragments. Even-numbered lanes are *Eteobalea intermediella*, odd-numbered lanes are *E. serratella*. Lane 1, 500 ng of ΦX174 RF DNA / *Hae*III fragments (Invitrogen Corporation); lanes 2–3, Ellen–Barbara PCR product digested with *Alu*I; lanes 4–5, Ellen–Barbara PCR product digested with *Taq*I; lanes 6–7, K698–K699 PCR product digested with *Alu*I; lanes 8–9, Marlon–Eva PCR product digested with *Taq*I; and lane 10, 500 ng of 123-bp DNA ladder (Invitrogen Corporation).



The results of the PCR–RFLP tests on larvae were useful in indicating that at least the bulk of the material included in the 2001 shipment was *E. serratella*. It was therefore less urgent to be able to identify individuals to species to be able to set up correctly mated pairs of *E. serratella* for rearing. Using the 49 live adults that emerged from this shipment, 17 mated pairs were placed in oviposition tubes. Of these, 9 pairs produced eggs, of which 8 lots were striate (*E. serratella* type) and 1 was reticulate (*E. intermediella* type), again confirming that the shipment was predominantly *E. serratella*.

If the larval PCR results had indicated a substantially mixed shipment of the two species, individual identification would have become more important for successful rearing. In that case, DNA samples would have had to be obtained nondestructively from individual moths, before or after adult emergence, and identified by PCR–RFLP so that male and female *E. serratella* could be paired for mating. We

Table 3. PCR–RFLP tests conducted on *Eteobalea* spp.

Species	DNA/voucher No.	PCR fragment		
		Ellen–Barbara	K698–K699	Marlon–Eva
E. serratella	1257*	Seq. + RFLP	Seq. + RFLP	Seq. + RFLP
E. serratella	1258	RFLP	RFLP	RFLP
E. serratella	1271	RFLP	Seq.	Seq.
E. serratella	1272	RFLP	Seq.	Seq.
E. serratella	1273	RFLP	Seq. + RFLP	Seq. + RFLP
E. serratella	1275	RFLP	Seq. + RFLP	Seq. + RFLP
E. intermediella	1255*	Seq. + RFLP	Seq. + RFLP	Seq. + RFLP
E. intermediella	1256	RFLP	RFLP	RFLP
E. intermediella	1274†	RFLP	Seq.	Seq.
E. intermediella	1276	RFLP	Seq.	Seq.
E. intermediella	1277	RFLP	—‡	Seq.
E. intermediella	1278	RFLP	Seq.	Seq.
E. intermediella	1279	RFLP	Seq. + RFLP	Seq. + RFLP
E. intermediella	1280	RFLP	Seq. + RFLP	Seq. + RFLP

Note: Seq., DNA sequence determined; RFLP, restriction digestion performed.
*Full-length COI–COII sequences (~2.3 kbp) determined for these specimens.
†Specimen originally identified as *E. serratella* but DNA sequence data and genitalic characters identified it as *E. intermediella* (see Results and discussion).
‡DNA sequence could not be obtained.

were unable to obtain usable DNA from larval exuviae but were able to successfully extract and amplify DNA from a single mesothoracic tibia cut from a live female moth. Because of the small size of *Eteobalea* larvae and adults, we did not attempt to obtain haemolymph samples. However, it should be possible to extract sufficient DNA for PCR from a small piece (approximately 1 mm^2) of wing tissue (Lushai *et al*. 2000). Thus, full application of the method for individual identification of *Eteobalea* adults would probably require slight mutilation of the adults. The effects of such damage on mating success and fecundity have not been investigated.

The PCR–RFLP methods we have developed could be applied to determine the species composition of future field collections of *Eteobalea* species from Europe, provided that the slight mutilation involved in obtaining DNA samples from individual adults does not hamper their reproduction. If both species eventually become established in the field in Canada, these assays will also be useful in confirming the identity of material collected during monitoring of release sites, without the need for dissection or rearing through to the egg stage.

Alternative assays

Alternative PCR-based assays are possible. For example, denaturing gels could be used to detect conformational polymorphisms in PCR products, and the existence of full-length COI–COII sequences allows one to search for other restriction sites if there are reasons (*e.g.*, cost effectiveness) to favor them. The two 3-bp indels found in the alignment of the complete COI–COII sequences of the two species suggest that an even better diagnostic assay, one based on multiplex PCR, could be designed. With such a test, the whole restriction digestion step, and perhaps even the DNA extraction step, could be avoided (Grevelding *et al*. 1996).

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