A simple PCR-RFLP method to distinguish between species and strains of *Microctonus* parasitoids found in New Zealand

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Abstract Two strains of the hymenopteran parasitoid *Microctonus aethiopoides* have been released in New Zealand for the biological control of *Sitona* weevil species. One attacks *Sitona discoideus*, a pest of lucerne, and the other attacks *Sitona lepidus*, a pest of clover. Two other *Microctonus* species also attack weevils in pasture; *M. hyperodae* was released for the biological control of *Listronotus bonariensis* and the native *M. zealandicus* attacks *Irenimus* spp. These *Microctonus* species can attack non-target weevil hosts and the identification of the larvae of the different *Microctonus* species and the separation of adults of *M. aethiopoides* strains can only be achieved by molecular methods. This paper describes a simple polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method for distinguishing between the two *M. aethiopoides* strains, *M. hyperodae* and *M. zealandicus*. This PCR-RFLP method requires minimal molecular equipment and is cheaper and/or faster than other molecular methods.

Keywords restriction fragment length polymorphism, PCR-RFLP, cytochrome *c* oxidase subunit 1 (COI), *Microctonus*.

INTRODUCTION

Microctonus species (Hymenoptera: Braconidae) have been successfully introduced to New Zealand for the biological control of weevil pasture pests (Barlow & Goldson 1993; Goldson et al. 1998; Kean & Barlow 2001; Barker & Addison 2006; Gerard et al. 2011). Two strains of the parasitoid *Microctonus aethiopoides* Loan, 1975 have been released in New Zealand for the control of *Sitona* species. One strain of *M. aethiopoides* attacks *Sitona discoideus* Gyllenhal, 1834, a pest of lucerne, and was introduced from Morocco via Australia (Aeschlimann 1995; Vink et al. 2012). Three cytochrome *c* oxidase subunit 1 (COI)

haplotypes of this strain have been recorded in New Zealand, although one of the haplotypes has only ever been recorded once (Vink et al. 2003) and has not been recorded in any subsequent sampling (Vink et al. 2003; Phillips et al. 2006; C.J. Vink, unpublished data). The other strain of *M. aethiopoides* attacks *Sitona lepidus* Gyllenhal, 1834, a pest of clover, and was introduced to New Zealand from Ireland (Gerard et al. 2006). Two COI haplotypes of this strain have been released in New Zealand (Phillips et al. 2006). The two strains of *M. aethiopoides* are referred to as the Irish strain and the Moroccan strain,

respectively, although phylogenetic studies have shown that intraspecific genetic variation in *M. aethioides* is more strongly associated with weevil host species than geographic origin (Phillips et al. 2008a; Vink et al. 2012).

Another *Microctonus* species, *M. hyperodae* Loan, 1974, was released for the biological control of *Listronotus bonariensis* (Kuschel, 1955). There are two COI haplotypes of *M. hyperodae* in New Zealand, one originated from the west of South America and the other from the east, which is much more common in New Zealand (Phillips et al. 2008b). The native *M. zealandicus* Shaw, 1993 is also found in New Zealand pasture and attacks endemic weevils in the genus *Irenimus*, but has only been found in Canterbury (Shaw 1993; Vink et al. 2003) and only one COI haplotype has been found.

All of the *Microctonus* species introduced into New Zealand to control forage pests can also attack non-target weevil hosts (Barratt et al. 1997; Barratt et al. 2006; Barratt et al. 2007; Barratt et al. 2012). The identification of the larvae of the different *Microctonus* species and the separation of adults of *M. aethioides* strains cannot be achieved morphologically with reliability (Vink et al. 2003), but several molecular methods can be used.

DNA sequence data can readily distinguish all species and strains of *Microctonus* found in New Zealand (Vink et al. 2003; Phillips et al. 2006; Phillips et al. 2008a; Vink et al. 2012), as can esterase isozymes and aldehyde oxidase allozymes (Iline & Phillips 2003, 2004; Phillips et al. 2006). Recently, DNA melting analysis and high-resolution DNA melting analysis have been explored for their utility in identifying pasture pests and their biological control organisms (Monk et al. 2011; Winder et al. 2011) and DNA melting analysis methods have been developed to distinguish the species and strains of *Microctonus* (C.J. Vink, unpublished data). Although all of these methods work very well, they do have their drawbacks. DNA sequencing usually takes 2 or more days to complete from DNA extraction to the analysis of sequence data. Isozyme and allozyme analysis requires specialist skills and equipment not often available in many molecular laboratories and the

DNA melting analyses can only be achieved with expensive real-time PCR equipment and software.

In order to meet the demand for a relatively quick, simple and cheap molecular method, a polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method for distinguishing between the two *M. aethioides* strains, *M. hyperodae* and *M. zealandicus* has been developed and is described in this paper.

MATERIALS AND METHODS

Sequences from all of the cytochrome *c* oxidase subunit 1 (COI) haplotypes known to occur for *Microctonus* spp. in New Zealand (Vink et al. 2003; Phillips et al. 2006; Phillips et al. 2008a; Vink et al. 2012; C.J. Vink, unpublished data) were assembled and imported into the Sequencher 4.6 (Gene Codes Corporation). The “Cut Map” function was used in Sequencher to display a “restriction map” for each haplotype; this showed which restriction enzymes would cut the sequence and where the cuts would occur. Sequences were compared and a restriction enzyme was selected that would provide different sized fragments for the two strains of *M. aethioides*, for *M. hyperodae* and for *M. zealandicus*. The restriction enzyme *AseI* was selected and the sizes of the fragments are shown in Table 1.

Seven specimens were selected for which the COI sequences were known (Table 2). DNA had been extracted in previous studies (Phillips et al. 2006; Phillips et al. 2008a; C.J. Vink, unpublished data) from three legs per specimen using a DNeasy™ Tissue kit (Qiagen) or a ZR Genomic DNA™-Tissue MiniPrep kit (Zymo Research). The primers used to amplify and sequence a 676 base pair (bp) COI fragment were LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (Folmer et al. 1994) plus C1-N-2191-aethio (5'-CCTGGTAAATTTAAATRTATACCTC-3') (Vink et al. 2003). Each 25 µl PCR reaction constituted 0.25 µl *i*-StarTaq™ DNA Polymerase (iNtRON Biotechnology), 2.5 µl 10x PCR buffer (iNtRON Biotechnology), 0.5 µl dNTP mixture (10 mM), 0.5 µl LCO1490 (10 µM), 0.5 µl C1-N-2191-aethio (10 µM), 1 µl DNA template and

Table 1 Predicted sizes (bp) of PCR fragments from different *Microctonus* strains and species following digestion with AseI.

Species	Strain	COI haplotype ¹	Fragment sizes (including primer annealing sites)
<i>M. aethioides</i>	Moroccan	6	80, 184, 222, 241
<i>M. aethioides</i>	Moroccan	7	80, 184, 222, 241
<i>M. aethioides</i>	Irish	3	80, 184, 463
<i>M. aethioides</i>	Irish	15	80, 184, 463
<i>M. hyperodae</i>	East	East	117, 610
<i>M. hyperodae</i>	West	West	117, 610
<i>M. zealandicus</i>			44, 220, 463

¹COI haplotype numbers were arbitrarily assigned in Vink et al. (2003) and Phillips et al. (2006).

19.75 µl water. PCR amplifications were performed in a Mastercycler® (Eppendorf) thermocycler with a cycling profile of 35 cycles of 94°C denaturation (30 s), 45°C annealing (30 s), 72°C extension (1 min) with an initial denaturation of 3 min and a final extension of 5 min. An aliquot of each PCR product was checked for presence and quantity by agarose gel (0.8% TBE) electrophoresis and ethidium bromide fluorescence over UV. PCR products (5 µl) were each combined with 1 µl AseI (BioLabs), 2.5 µl NE Buffer 3 (BioLabs) and 16.5 µl water, and incubated at 37°C for 2 h. Samples were then mixed with 2 µl 6× DNA loading dye (Fermentas) and loaded onto a 2% agarose gel along with a GeneRuler™ DNA 100–10,000 bp ladder (Fermentas). The gel was electrophoresed using 90 V for 2 h and fragments were detected by ethidium bromide fluorescence over UV light. Photographs were taken using an UVIdoc HD2 gel doc (UVItech).

RESULTS

The restriction enzyme AseI cut the PCR products into fragments (Figure 1) that corresponded to those predicted by the Sequencher analysis (Table 1). The initial PCR amplification for one of the *M. hyperodae* specimens (East haplotype) was not strong and the 117 bp fragment is barely visible (Figure 1). The 44 bp fragment expected for *M. zealandicus* is not visible in Figure 1, which is not surprising as small (<100 bp) fragments are usually not visible on 2% agarose gels (Sambrook & Russell 2001).

DISCUSSION

All *Microctonus* species and *M. aethioides* strains could be differentiated using the AseI PCR-RFLP method outlined in this paper. Although the 117 bp fragment for one of the *M. hyperodae* specimens (East haplotype) was barely visible, the specimen can be readily diagnosed by the

Table 2 *Microctonus* strains and species digested with AseI. Two-letter locality codes follow Crosby et al. (1998).

Species	COI haplotype	Host	Collection locality	GenBank accession no.
<i>M. aethioides</i>	6	<i>Rhinocyllus conicus</i>	DN, Hakataramea	DQ525081
<i>M. aethioides</i>	7	<i>L. bonariensis</i>	MC, Rakaia	DQ525082
<i>M. aethioides</i>	3	<i>S. lepidus</i>	HB, Patoka	JQ778311
<i>M. aethioides</i>	15	<i>S. lepidus</i>	Ireland, Oakpark	DQ525080
<i>M. hyperodae</i>	East	<i>L. bonariensis</i>	NN, Motupiko	EU078360
<i>M. hyperodae</i>	West	<i>L. bonariensis</i>	MC, Lincoln	JQ801634
<i>M. zealandicus</i>		<i>Irenimus</i> sp.	MC, Yaldhurst	EU078361

Figure 1 Photograph of agarose gel using ethidium bromide fluorescence to visualise the RFLP analysis of the COI PCR products from *Microctonus* species and their strains.

463 bp fragment, which is larger than any *Asel* PCR-RFLP fragment from other species and smaller than an uncut fragment would be (727 bp, including primer annealing sites). The PCR-RFLP of *M. zealandicus* is similar to the Irish strain of *M. aethiopoides*, but the fragments are different sizes and can be differentiated provided the gel is run for long enough (at least 1.5 h).

Cost and time estimates (C.J. Vink, unpublished data) were used to compare the PCR-RFLP method, DNA sequencing and DNA melting analysis. The cost of PCR-RFLP was less than a third of the cost of DNA sequencing and less than half of that of DNA melting analysis, which also requires expensive real-time PCR equipment and software and specialist knowledge. Preparation time for DNA sequencing is approximately equivalent to that of the PCR-RFLP method, but a result for the latter can be achieved within a day, whereas the former usually requires samples to be sent away for sequencing, which can take 2–5 days before a result is received. DNA melting analysis can produce results quicker than any other molecular methods, but PCR-RFLP requires less technical training and much cheaper equipment.

Despite having been replaced by more modern molecular methods, PCR-RFLP methods

have been successfully developed and used to identify fruit fly (Tephritidae) and tussock moth (Lymantriidae) species intercepted at the New Zealand border (Armstrong et al. 1997; Armstrong et al. 2003). The method reported in this paper can be confidently used to identify species and strains of *Microctonus* found in New Zealand.

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