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David B. Taylor
University of Nebraska-Lincoln, dave.taylor@ars.usda.gov

Allen L. Szalanski
University of Nebraska-Lincoln

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Identification of *Muscidifurax* Spp. by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism

David B. Taylor* and Allen L. Szalanski†

**USDA, ARS Midwest Livestock Insects Research Laboratory, Department of Entomology, and †Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska 68583*

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Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of the nuclear ribosomal ITS-1 region was used to differentiate *Muscidifurax* (Hymenoptera: Pteromalidae) species which are parasitoids of filth fly pupae. Three restriction enzymes, *Dpn* II, *Mse* I, and *Taq* I, produced restriction patterns which were diagnostic for the four species analyzed, *M. raptor, M. raptorellus, M. uniraptor,* and *M. zaraptor.* Seven other restriction enzymes were able to differentiate one or more of the species and can be used alone, or in combination with other enzymes, to verify identifications. No intraspecific variation was observed among the populations examined. The utility of the PCR-RFLP technique compared with other molecular and biochemical diagnostic procedures is discussed. © 1999 Academic Press

Key Words: **PCR-RFLP; molecular diagnostics; *Muscidifurax;* filth fly parasitoids; Pteromalidae.**

INTRODUCTION

Wasps in the genus *Muscidifurax* are among the most promising biological control agents for flies breeding in the confined livestock environment (Miller and Rutz, 1990; Petersen *et al.,* 1990). The genus contains five species, four of which have been used for biological control. Because of their small size, differentiating the species can be difficult. In fact, only one species, *M. raptor* Girault & Sanders was recognized prior to 1970 (Kogan and Legner, 1970). Although morphologically similar, each of the *Muscidifurax* species has unique biological characteristics, which make accurate identification important. In addition, differentiating released species from native wasps is important for evaluating the impact and efficacy of augmentive releases for biological control.

Several techniques have been used for molecular diagnostics of insect species, each with its own intrinsic advantages and disadvantages. Among the most popular techniques are randomly amplified polymorphic DNA (RAPD-PCR) (Kambhampati *et al.,* 1992; Wilkerson *et al.,* 1995; Antolin *et al.,* 1996), polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) of nuclear (Slade *et al.,* 1993; Pfeifer *et al.,* 1995) and mitochondrial (Sperling *et al.,* 1994; Taylor *et al.,* 1996; Roehrdanz, 1997) genomes, and species-specific PCR primers (Scott *et al.,* 1993) and probes (Haymer *et al.,* 1994). We chose PCR-RFLP for this study because the technique is simple and inexpensive, very reliable and repeatable, and provides discrete character states, which can be used for phylogenetic analyses as well as diagnostics.

PCR-RFLP and sequencing analyses of a portion of the mitochondrial genome of *Muscidifurax* species (Taylor *et al.,* 1997) revealed several restriction patterns diagnostic between *M. raptor* and *M. raptorellus* Kogan & Legner. However, *M. uniraptor* Kogan & Legner was identical to *M. raptorellus* and multiple (>20) restriction patterns were observed from individual *M. zaraptor* Kogan & Legner, apparently caused by multiple or repeated translocations of the mitochondrial sequence to the nuclear genome.

The nuclear ribosomal intergenic transcribed spacer (ITS-1) has proved useful for differentiating species (Pfeifer *et al.,* 1995; Powers *et al.,* 1997; Szalanski *et al.,* 1997). This region diverges at a rate 3–4 times faster than mitochondrial DNA in reproductively isolated populations (Marçon *et al.,* 1999), but, in general, the repeats remain relatively homogeneous within individuals and populations (Zimmer *et al.,* 1980; Elder and Turner, 1995). Rapid divergence and concerted evolution make the ITS region ideal for molecular diagnostics.

The purpose of this study was to identify species diagnostic characters for the four species of *Muscidifurax* used for biological control of filth flies. The fifth species, *M. raptoroides* Kogan & Legner, has not been used for biological control in North America nor has it been colonized.

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TABLE 1

Strains of *Muscidifurax* spp. Used in This Study

Species	*N*	Origin	Date colony established	Source
M. raptor	3	Nebraska	1995	MLIRL[a]
	3	New York	Unknown	Cornell University
	5	Alberta, Canada	1995	Ag Canada, Lethbridge
M. raptorellus	3	Chile	Unknown	Legner via MLIRL[b]
	3	Nebraska	1991	MLIRL[a]
	3	Peru	Unknown	Legner via MLIRL[b]
M. uniraptor	3	Puerto Rico	Unknown	Rochester University
M. zaraptor	3	Nebraska	1993	MLIRL[a]
	7	Alberta, Canada	1995	Ag Canada, Lethbridge

[a] Midwest Livestock Insect Research Laboratory, USDA-ARS, Lincoln, NE.

[b] Originally collected by E. F. Legner and transferred to MLIRL in 1989.

MATERIALS AND METHODS

Techniques used for this study were similar to those used by Taylor *et al.,* 1996. DNA was isolated from frozen and alcohol-preserved specimens using the phenol/chloroform extraction with ethanol precipitation. The ITS-1 region was amplified with the primers $rDNA_2$ (TTGATTACGTCCCTGCCCTTT; Vrain *et al.,* 1992) and $rDNA_{1.58s}$ (ACGAGCCGAGTGATCCACCG; Cherry *et al.,* 1997). The PCR protocol was 35 cycles of 94°C for 45 s, 52°C for 1 min, and 72°C for 2 min. Amplicons were digested with 27 restriction enzymes (RE, *Alu* I, *Apo* I, *Ase* I, *Ava* I, *Ban* II, *Bfa* I, *Bsr* I, *Dde* I, *Dpn* II, *Dra* I, *Ecl*136 II, *EcoR* I, *EcoR* V, *Hae* III, *Hinc* II, *Hind* III, *Hinf*I, *Hpa* I, *Mse* I, *Msp* I, *Pvu* II, *Rsa* I, *Sau*96 I, *Scrf*I, *Ssp* I, *Taq* I, and *Xba* I). Digest products were separated by polyacrylamide (10%) and agarose (2.5% MetaPhor) gel electrophoresis. Gels were stained with ethidium bromide and photographed with ultraviolet illumination. A 50-bp ladder was included on each gel. Fragment lengths were estimated with the computer program GEL-JML (LaCroix, 1994). Wasp samples examined are presented in Table 1.

RESULTS

The ITS-1 amplicon was approximately 980 bp long for all four species of *Muscidifurax*. Thirteen restriction enzymes, *Alu* I, *Ase* I, *Ava* I, *Ban* II, *Bfa* I, *Ecl*136 II, *EcoR* I, *EcoR* V, *Hinc* II, *Hind* III, *Hpa* I, *Pvu* II, and *Xba* I, failed to cut this amplicon. Fragment patterns for the remaining 14 REs are presented in Table 2. No intraspecific variation was observed in the samples examined. Digest patterns for four REs, *Apo* I, *Msp* I, *Sau*96 I, and *Ssp* I, were similar for all four species. Three REs, *Dpn* II, *Mse* I, and *Taq* I, produced patterns which differentiated all four species (Fig. 1). Three REs produced unique digest patterns for one species, *Dde* I for *M. raptor,* *Bsr* I for *M. raptorellus,* and *Dra* I for *M. uniraptor.* *Hinf*I, *Rsa* I, and *Scrf*I differentiated *M. raptor* and *M. zaraptor* from *M. raptorellus* and *M. uniraptor.* *Hinf*I was also able to differentiate *M. raptorellus* from *M. uniraptor.*

DISCUSSION

Muscidifurax species can be easily differentiated with *Dpn* II, *Mse* I, and *Taq* I digests of the nuclear ribosomal ITS-1 region. Species identification can be verified by the use of several other enzymes either alone or in combination. *Mse* I is the most useful enzyme for differentiating these species because all

TABLE 2

Restriction Fragment Lengths Estimated on 2.5% Metaphor Agarose Gels for *Muscidifurax* Species

RE	*M. raptor*	*M. raptorellus*	*M. uniraptor*	*M. zaraptor*
Apo I	404,[a] 353, 187, 32	404, 353, 187, 32	404, 353, 187, 50	404, 353, 187, 32
Bsr I	903, 84	620, 280, 84	903, 84	903, 84
Dde I	510, 470	980	980	980
Dpn II	335, 245, 135, 112, 92, 60	335, 245, 135, 112, 82, 62	150,[a] 135,[a] 112, 103, 54	335, 245, 135, 112, 103, 60
Dra I	716, 257	716, 257	626, 364, 97, 48	716, 257
*Eco*R I	980	980	980	980, 590, 390
Hae III	390, 306, 163, 101	390, 306, 163, 101	390, 306, 163, 101	510, 306, 160
Hinf I	337,[a] 202, 97	491, 285, 202	337, 202,[a] 155, 97	337,[a] 194, 97
Mse I	275, 215, 195, 181, 65	370, 192, 192, 90, 62, 55	416, 194, 108,[a] 62, 43	522, 194, 171, 64
Msp I	344, 286, 246, 117	332, 283, 254, 117	341, 283, 256, 117	338, 286, 260, 117
Rsa I	450, 426, 73, 24	450, 392, 75, 26, 24	450, 389, 76, 26	450, 426, 79, 24
*Sau*96 I	560, 406	560, 406	560, 406	560, 406
Scrf I	965	854, 115	854, 115	965
Ssp I	718, 243	718, 243	718, 243	718, 243
Taq I	543, 263, 156	503, 283, 156, 36	522, 283, 156, 46	355, 283, 156, 79, 72, 37

[a] Interpreted as double bands.

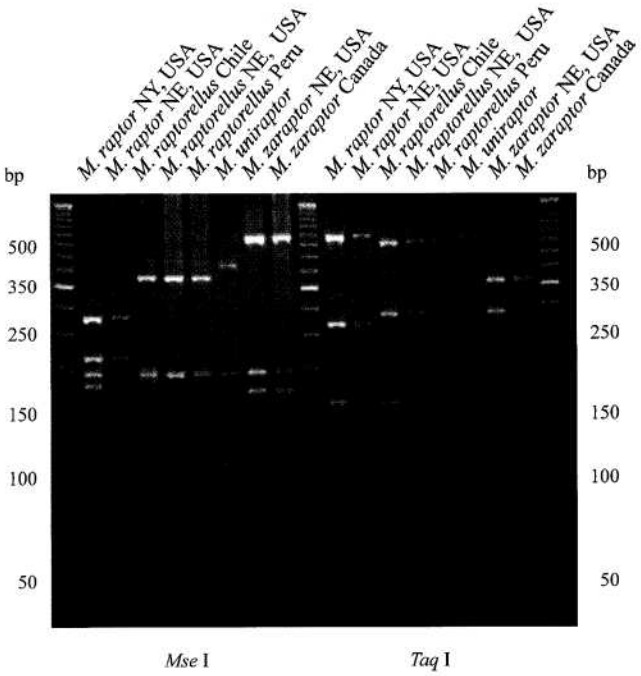

FIG. 1. *Mse* I and *Taq* I digests of *Muscidifurax* spp. ITS-1 amplicon on 2.5% MetaPhor agarose gels.

four species can be characterized by unique fragments larger than 100 bp. Differentiating *M. raptorellus* and *M. uniraptor* with *Dpn* II and *Taq* I is primarily dependent upon smaller fragments, which can be difficult to visualize under less than optimal PCR and electrophoretic conditions.

Because our sample sizes are relatively small, it is possible that undetected intraspecific variation may compromise some of the diagnostic restriction sites. However, one of the major advantages of the PCR-RFLP technique is that multiple discrete characters are available for diagnostic purposes. Even if undetected variation reduces the usefulness of one restriction site, others can be used to verify the identification. Additional advantages of the PCR-RFLP technique are that fragment patterns will be observed for all species, whether they have been previously characterized or not. These patterns can be associated with the correct species at a later date, without having to reanalyze the specimen. This characteristic is also valuable for detecting previously unrecognized cryptic species. Like PCR-RFLP, RAPD-PCR produces specific patterns that may be used for later identification of uncharacterized species. However, RAPD-PCR banding patterns can vary with DNA quality and quantity as well as amplification protocols and even thermocyclers (Black, 1993). Problems with reproducibility of banding patterns among samples of varying quality and among laboratories make RAPD-PCR a less than optimal choice for general species diagnostics. Other diagnostic techniques, such as species-specific PCR primers, probes, and ELISA (Ma *et al.*, 1990), require that unidentified samples be reanalyzed with new reagents as they become available to reach a positive identification. Also, because PCR-RFLP involves two steps, amplification followed by digestion, false negative identifications due to sample or reagent degradation are avoided. Failure of the PCR step indicates that the specimen is unidentifiable, probably because of sample degradation. Other techniques, such as species-specific primers and probes and ELISA, rely upon the quality of the sample for the identification step of the analysis. The lack of the positive reaction may be due to either the sample not being the target species or the sample being degraded. In ecological studies, in which a large number of samples are available and individual errors are not critical, these techniques may be acceptable. However, when identifying quarantine pests, forensic samples, or in other situations in which the number of samples is limited and error, especially false negative, is unacceptable, we believe that the PCR-RFLP technique is superior. PCR-RFLP can be used to identify samples preserved with most commonly used preservatives as well as dried-pinned specimens (Taylor *et al.*, 1996).

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