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Mitochondrial DNA Variation Among *Muscidifurax* spp. (Hymenoptera: Pteromalidae), Pupal Parasitoids of Filth Flies (Diptera)

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ABSTRACT Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing analyses were used to characterize an amplicon of ≈625 bp in 4 of the 5 nominate species of *Muscidifurax* Girault & Sanders, pupal parasitoids of muscoid flies. A single polymorphic nucleotide site was observed among 2 samples of *M. raptor* Girault & Sanders. No sequence variation was observed among 3 samples of *M. raptorellus* Kogan & Legner. The sequence of *M. uniraptor* Kogan & Legner was identical to that of *M. raptorellus.* Nucleotide divergence among the *Muscidifurax* spp. ranged from 0.14 to 0.18 substitutions per nucleotide. *Muscidifurax zaraptor* Kogan & Legner exhibited multiple haplotypes, 2 of which were characterized by sequencing and 4 others by PCR–RFLP. The sequenced haplotypes differed by 0.08 nucleotide substitutions per site. Restriction site analysis indicated that nucleotide divergence ranged from 0.03 to 0.10 among all 6 haplotypes. Analysis of progeny from individual females indicated that the observed variation in *M. zaraptor* was caused by multiple haplotypes within individuals rather than differentiation among individuals. These results bring to question the specific status of *M. uniraptor* and indicate that the genus is native to the Western Hemisphere, and not introduced with their primary host, *Musca domestica* L., as previously proposed. Heteroplasmy and translocation of a portion of the mitochondrial genome to the nuclear genome are discussed as possible causes for the variation observed in *M. zaraptor.*

KEY WORDS *Muscidifurax*, polymerase chain reaction–restriction fragment length polymorphism, mitochondrial DNA, phylogeny

WASPS IN THE genus *Muscidifurax* are pupal parasitoids of muscoid flies, especially the house fly, *Musca domestica* L., and the stable fly, *Stomoxys calcitrans* (L.), and are among the most promising biological control agents for these flies in the confined livestock environment (Miller and Rutz 1990, Petersen et al. 1990). *Muscidifurax* includes 5 species. *Muscidifurax raptor* Girault & Sanders, the most widespread, is found throughout the temperate and semitropical regions of the world (Kogan and Legner 1970). The remaining 4 species are limited to the New World. *Muscidifurax zaraptor* Kogan & Legner is found sympatrically with *M. raptor* in western North America (Kogan and Legner 1970, Lysyk 1995), *M. raptoroides* Kogan & Legner and *M. raptorellus* Kogan & Legner are found allopatrically in Central and South America, respectively (Kogan and Legner 1970). Two forms of *M. raptorellus* have been reported, one solitary and the other gregarious (Kogan and Legner 1970, Legner 1988). *Muscidifurax uniraptor* Kogan & Legner is a parthenogenic species known only from the island of Puerto Rico (Kogan and Legner 1970).

The geographic origins and phylogenetic relationships of *Muscidifurax* are unclear. Kogan and Legner (1970) proposed 2 alternatives for the origins of the genus. First, they originated in the Ethiopian region and were introduced to the New World along with house flies, or 2nd, they are native to the New World and have secondarily adapted to house flies. Kogan and Legner conclude that the "remarkable preference of *Muscidifurax* spp. for house flies as compared to native Nearctic flies" indicates an Old World origin for the genus. Legner (1983) indicates that the dependence of *Muscidifurax* upon the "barnyard" environment outside of Africa is further evidence that the genus was not native to the New World. However, to accept an Old World origin of the genus and account for the 4 species endemic to the New World, one must accept a very rapid rate of speciation following their introduction.

Despite the interest in these species for biological control of filth flies and the questions concerning their geographic origin, little work has been done on the population genetics and genetic structure of *Muscidifurax*. Propp (1986) used allozymes to ex-

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Table 1. Strains of *Muscidifurax* spp. used in this study

Species	Origin	Date collected	Source[a]
M. raptor	Nebraska	1995	MLIRL
	New York	Unknown	Cornell University
M. raptorellus	Chile	Unknown	Legner via MLIRL
	Nebraska	1991	MLIRL
	Peru	Unknown	Legner via MLIRL
M. uniraptor	Puerto Rico	Unknown	Rochester University
M. zaraptor	Nebraska	1991–1993	MLIRL
	Nebraska	1995	MLIRL

[a] MLIRL, Midwest Livestock Insect Research Laboratory, USDA-ARS, Lincoln, NE; Legner via MLIRL, originally collected by E. F. Legner and transferred to MLIRL in 1989.

amine several populations of *M. raptor* and *M. zaraptor*. He found relatively low levels of differentiation among geographic isolates of the 2 species, but a high level of differentiation was observed between species. Antolin et al. (1996) used RAPD–PCR to examine 3 *Muscidifurax* spp. They were able to differentiate the species and associate the gregarious North American *Muscidifurax* sp. with *M. raptorellus*, but indicated they were unable to explore phylogenetic relationships because of the nature of the RAPD–PCR data.

The purpose of this study was to develop molecular diagnostic characters and examine genetic differentiation among *Muscidifurax* spp. A region of the mitochondrial genome, ≈625 bp, including parts of the cytochrome oxidase I (COI) and II (COII) genes as well as the entire tRNA leucine ($tRNA^{leu}$) gene was examined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) and sequencing. Representative samples of 4 of the 5 species in the genus (samples of *M. raptoroides* were not available) were included in this study.

Materials and Methods

Samples. *Muscidifurax* samples were obtained from established colonies (Table 1). The Chile and Nebraska samples of *M. raptorellus* represented gregarious populations and the Peru sample was solitary. The *Apis mellifera lingustica* L. control sample was collected from an apiary in Lincoln, NE, and the *Cochliomyia macellaria* (F.) samples were from a laboratory colony originating in Fargo, ND. *Muscidifurax* colonies were maintained on freeze-killed *M. domestica* pupae (Petersen and Matthews 1984).

Isofemale lines were initiated by isolating individual females (presumably mated) from the main colony and placing them in small plastic cups (2 cm diameter, 2 cm high) with ≈150 freeze-killed fly pupae. Progeny emerged in 3 wk. Specimens were stored at −80°C.

Voucher specimens from each of the *Muscidifurax* samples have been placed in the collection of the University of Nebraska State Museum, Lincoln.

DNA Extraction and Amplification. Pools of 5–25 wasps were used for each DNA extraction. DNA was extracted with the chloroform–phenol technique as outlined in Taylor et al. (1996).

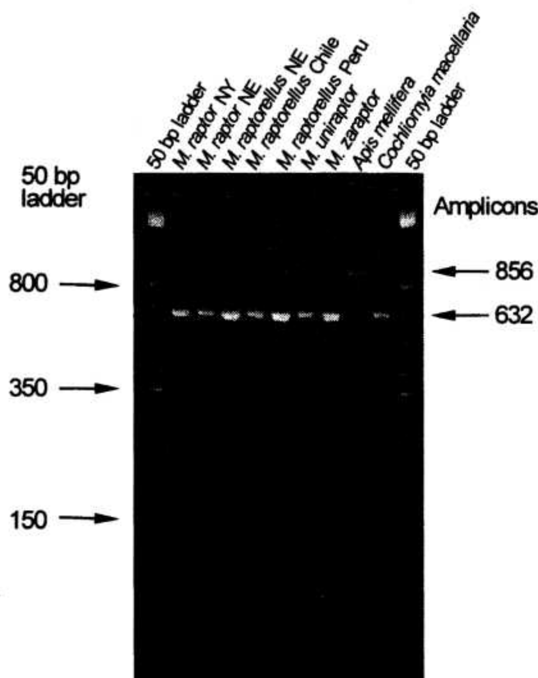

Fig. 1. Intact amplicon from 4 *Muscidifurax* spp., *A. mellifera*, and *C. macellaria* on 2.5% MetaPhor agarose gel. Primers were 5′ ATACCTCGACGTTATTCAGA 3′ and 5′ TCAATATCATTGATGACCAAT 3′.

A region of mtDNA, ≈625 bp long, was amplified using the primers 5′ ATACCTCGACGTTATTCAGA 3′ (= S2792; Bogdanowicz et al. 1993) and 5′ TCAATATCATTGATGACCAAT 3′ (K. Pruess, personal communication). The 5′ ends of these primers were located at bp 2773 and 3400 of the *Drosophila yakuba* mtDNA map (Clary and Wolstenholme 1985), respectively.

For amplification, 1 μl of sample DNA was added to a reaction mixture containing 2.5 μl of reaction buffer (Perkin-Elmer, Norwalk, CT), 2 μl of dNTP mix (10 mM each—dATP, dTTP, dCTP and dGTP), 1 μl of each primer (20 mM), 1.0 unit of Taq polymerase (Perkin-Elmer) and deionized water to a volume of 25 μl. Amplifications were done in a Perkin Elmer Cetus Model 9600 thermocycler programmed for 35 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 or 2 min. Amplification products were stored at 4°C.

Restriction Endonuclease Digests. Twenty-seven restriction enzymes—*Alu I, Apo I, Ase I, Ava I, Ban II, Bfa I, Bsr I, Dde I, Dpn II, Dra I, EcoR I, EcoR V, Hae III, Hinc II, Hind III, Hinf I, Hpa I, Mse I, Msp I, Pvu II, Rsa I, Sac I, Sau96 I, ScrF I, Ssp I, Taq I,* and *Xba I*—were screened. Digests for polyacrylamide gel electrophoresis (PAGE) were done using 1.5 μl of PCR product, 0.2 μl enzyme (New England Biolabs, Beverly, MA), 1× buffer (New England Bio-

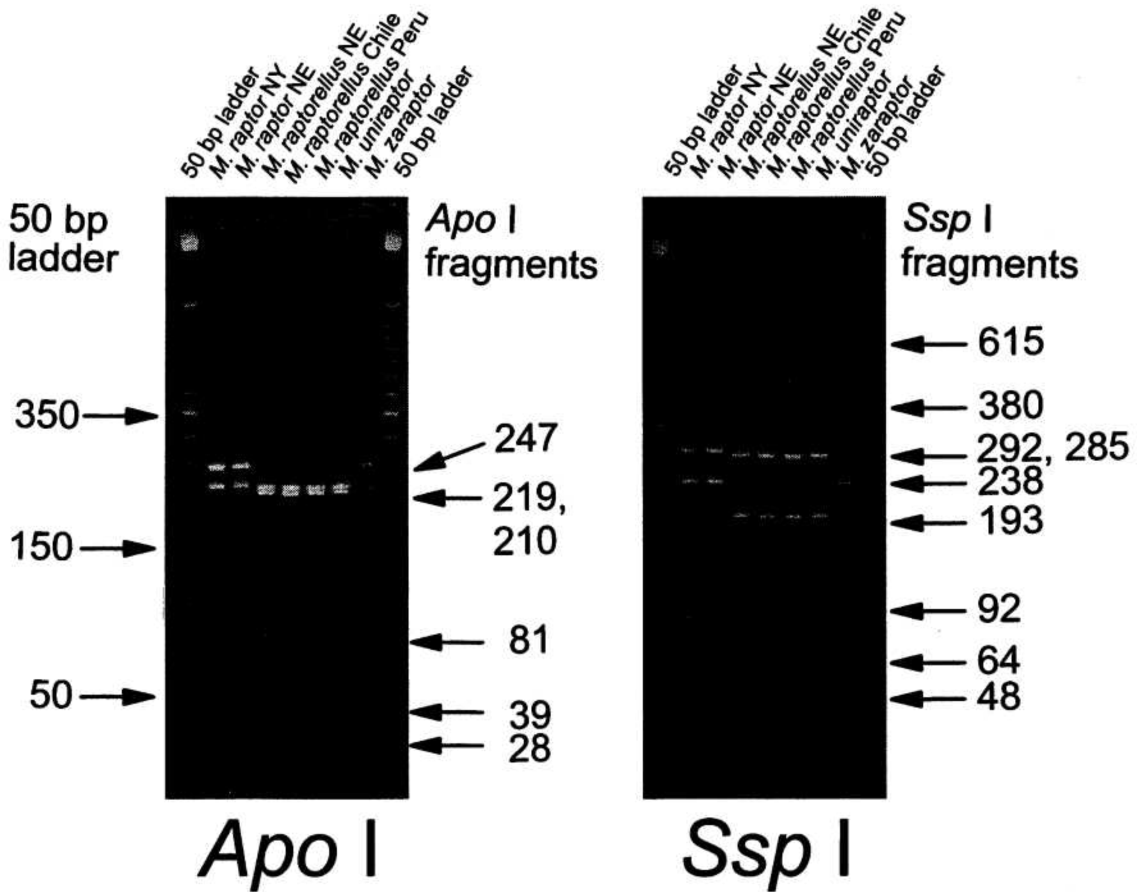

Fig. 2. *Apo* I and *Ssp* I digests of *Muscidifurax* spp. on 2.5% MetaPhor agarose gels.

labs) and water to bring the volume to 5 μl in 200-μl tubes. For agarose gel electrophoresis, all quantities were increased 2.5 times. Samples were incubated at 37°C for 3–16 h and stored at 4°C until further analysis.

Electrophoresis. For PAGE, 1.5 μl of loading buffer (10% Ficoll 400 [Sigma, St. Louis, MO], 0.25% Bromophenol Blue [Sigma], 50 mM EDTA, 10 mM Tris-HCl pH 7.5) was added to the 5-μl digest product. The entire digest product (6.5 μl) was loaded onto a 10% acrylamide gel (1× Tris:borate:EDTA (TBE), 0.5% Photoflo-200 [Kodak, Rochester, NY], 0.15% TEMED, and 0.05% ammonium persulfate). A molecular size standard, pGEM (Promega, Madison, WI), was included on each gel. Hoefer (Hoefer Scientific Instruments, San Francisco, CA) SE 600 electrophoresis units with gels (16 by 20 cm by 0.75 mm) and 28 well combs were used for electrophoresis. Gels were run in 1× TBE buffer at a constant 300 V (15 mA/gel) for 1.5 h at 20°C. Gels were stained

Table 2. Restriction fragment lengths estimated on 2.5% Metaphor agarose gels for *Muscidifurax* species

Restriction enzymes	*M. raptor* NE	*M. raptorellus*	*M. uniraptor*	*M. zaraptor*
Apo I	247, 219, 52, 42, 28	219, 210, 81, 39	219, 210, 81, 39	247, 219, 210, 189, 98, 40
Ase I	327, 297	387, 165, 59	387, 165, 59	454, 234, 180, 167
Dpn II	373, 239	628	628	373, 239
Dra I	321, 243, 65	245, 239, 80, 37, 31	245, 239, 80, 37, 31	328, 248, 184, 149, 38, 33, 27
Hae III	614	477, 147	477, 147	631
Hinf I	600, 34	454, 133, 34, 24	454, 133, 34, 24	585, 537, 446, 257, 179, 136, 41, 31
Mse I	149, 98, 94, 71, 53, 33, 14	147, 92[a], 58, 32, 25, 17	147, 92[a], 58, 32, 25, 17	149, 117, 69, 36, 27
Ssp I	292, 239, 98	285, 193, 64, 48	285, 193, 64, 48	615, 380, 329, 287, 270, 236, 222, 190, 184, 99
Taq I	452, 162	618	618	463, 411, 336, 295, 164, 108, 93

[a] Possibly double bands.

```
                   ------------------------------CO I-------------------------------
M. raptor                                  Y  P  D   S  Y  L   C  W  N  M   I  S  S
M. raptorellus                             .  .  .   .  .  .   .  .  .  V   .  .  .
M. zaraptor I                              .  .  .   .  .  .   .  .  .  .   .  .  .
M. zaraptor II                             .  .  .   .  .  .   .  .  .  .   V  .  .
M. raptor        1 atacctcgac gttattcaga TTATCCGGAT TCATATTTAT GTTGAAATAT AATTTCTTCC
M. raptorellus   1                       ......A... .......... ........G. ...C..A..A
M. zaraptor I    1                       ......A... .......... .......... .........A
M. zaraptor II   1                       ......T... .......... .......... .G........

                   ------------------------------CO I-------------------------------
M. raptor           L  G  S   F  I  S  M   M  S  T   M  L  F   F  F  I  I   W  E  S
M. raptorellus      M  .  .   I  .  .  .   .  .  .   L  F  .   .  .  .  .   .  .  .
M. zaraptor I       .  .  .   I  .  .  .   V  .  .   I  F  .       L  L   F  E  K  E
M. zaraptor II      .  .  .   I  .  .  .   V  .  .   I  F  .   .  Y  .  .   .  .  .
M. raptor       61 CTGGGAAGAT TTATTTCTAT AATAAGAACA ATATTATTTT TCTTCATTAT TTGAGAAAGA
M. raptorellus  61 A.A......A ....C..A.. .........T T....T.... ....T..... ..........
M. zaraptor I   61 T.A......A .......... .G........ ..T..T..-- --..T..... ..........
M. zaraptor II  61 T.A..G...A .......A.. .G........ ..T..T..C. .T.A...... .........G

                   ------------------------------CO I-------------------------------
M. raptor           M  I  S   N  R  T  V   I  F  N   K  N  M   N  N  S  I   E  W  I
M. raptorellus      I  .  .   .  .  I  I   .  .  .   .  S  I   .  .  .  V   .  .  .
M. zaraptor I        L  F  L   I  E  L   *
M. zaraptor II      I  .  .   .  .  I  .   .  .  .   .  S  .   .  .  .  .   .  .  V
M. raptor      121 ATAATTTCTA ATCGTACAGT AATTTTTAAT AAAAATATAA ATAATTCAAT TGAATGAATT
M. raptorellus 121 ..T....... .C..A.TTA. T......... ....GA..C. .......TG. A........C
M. zaraptor I  117 ..T....... ....A.TTA. .......... ....G..... .......T.. ..........
M. zaraptor II 121 ..T....... ......TT.. .......... ....G..... .......T.. .......G..

                   ------------------------------CO I----------------------------**--
M. raptor           M  S  F   P  P  S  F   H  S  F   S  E  I   P  K  I  Y   K  *
M. raptorellus      .  A  .   .  .  .  .   .  .  .   .  .  .   .  .  .  F   N  *
M. zaraptor I
M. zaraptor II      .  N  .   .  .  .  M   .  .  .   N  .  .   .  .  L  .   Y  *
M. raptor      181 ATATCTTTTC CACCTTCATT TCATTCATTT AGAGAAATTC CAAAAATTTA TAAATAATTT
M. raptorellus 181 ...G.A.... .C..A..... ......T... .......... .T.......T C..T...A..
M. zaraptor I  177 ....GC.... ........A. A.....T... .AT....... .....C.... .T.T......
M. zaraptor II 181 ...AAC.... .......TA. G.....T... .AT....... .T...C.... .T.T......

                   -----------------------------tRNA Leu-----------------------------
M. raptor
M. raptorellus
M. zaraptor I
M. zaraptor II
M. raptor      241 TTAAAATGGC AGATTAGTGC AATAGACTTA AATTCTATAT ATATAATAAT TAGTATTATT
M. raptorellus 241 .......... .......... ......T... .......... ........T. A.AC......
M. zaraptor I  237 .......... .......... ......T... .......... ........-- ..-A......
M. zaraptor II 241 .......... .......... ......T... ..C......A ........-- ..AA......
```

Fig. 3. Sequence comparison for *Muscidifurax* spp., represents the same amino acid or nucleotide is present as that in *M. raptor;* −, indicates a deletion. The *M. raptorellus* sequence represents both that species and *M. uniraptor*. Lower case letters in the *M. raptor* sequence represent primer sequences.

```
                    -------**---------------------CO II-----------------------------
M. raptor                    M   S  M  W   S  Q  L  M   L  Q  D   S  N  S   P  I  M  E
M. raptorellus               .   A  L  .   N  .  M  .   F  .  .   .  .  .   .  .  .  .
M. zaraptor I                .   .  .  .   N  .  I  .   F  .  .   .  .  .   .  .  .  .
M. zaraptor II               .   .  L  .   N  .  I  .   F  .  .   .  .  .   .  .  .  .
M. raptor        301 TTTAAAAATT TCAATATGAA GTCAATTAAT ATTACAAGAT AGTAATTCTC CTATTATAGA
M. raptorellus   301 .......... G..T...... A....A.... ...T...... ..A.....A. .C........
M. zaraptor I    294 .......... .......... A....A.T.. ...C...... ..A.....A. ..........
M. zaraptor II   299 .......... ...T...... A....A.T.. ...T.....C ..G..C.... .A........

                    ------------------------------CO II-----------------------------
M. raptor             M  M  I   Y  F  H   D  H  S  M   M  V  I   M  V  I   I  S  L  I
M. raptorellus        S  .  .   M  .  .   .  .  G  .   L  I  .   I  M  .   .  .  M  .
M. zaraptor I         S  .  .   M  .  .   .  .  G  .   L  .  .   I  I  .   .  .  .  .
M. zaraptor II        S  .  .   M  .  .   .  .  G  .   L  .  .   I  I  .   .  .  .  .
M. raptor        361 AATAATAATT TATTTTCATG ATCATAGAAT AATAGTTATT ATAGTTATTA TTAGTTTAAT
M. raptorellus   361 ..G....... ATA....... .C...G.... .T..A..... ..TA.A.... .....A....
M. zaraptor I    354 ..G....... ATA....... .....G.... .T....A... ..CA...... .C..A.....
M. zaraptor II   359 ..G....... ATA..C.... .....G.... .T....A... ..TA...... ....A.....

                    ------------------------------CO II-----------------------------
M. raptor             M  Y  I   I  L  F   M  F  F  N   N  L  M   N  R  F   M  L  E  G
M. raptorellus        L  .  .   .  M  .   .  .  I  .   T  .  I   .  .  .   .  .  .  .
M. zaraptor I         L  .  .   .  M  .   .  .  V  .   T  .  I   .  .  Y   .  .  .  .
M. zaraptor II        L  .  .   .  M  .   .  .  L  .   M  .  I   .  .  .   .  .  .  .
M. raptor        421 TATATATATT ATTTTATTTA TATTTTTTAA TAATTTAATA AATCGATTTA TATTAGAAGG
M. raptorellus   421 .C.T...... ...A....C. .....A.... ..CA.....T .....G.... ..........
M. zaraptor I    414 .T........ ...A...... .....G.C.. ..CA.....T .......A.. ..........
M. zaraptor II   419 .T....C... ...A....C. .......A.. ..TA..G..C .......... ..........

                    ---------------------- -------CO II-----------------------------
M. raptor             Q  M  I   E  I  I   W  T  I  I   P  I  F   F  L  I   I  L  A  I
M. raptorellus        .  .  .   .  .  .   .  .  V  .   .  .  .   .  .  V   F  .  .  .
M. zaraptor I         .  .  .   .  .  .   .  .  V  .   R  .  .   .  .  .   F  .  .  .
M. zaraptor II        .  .  .   .  .  .   .  .  V  .   .  .  .   .  .  .   F  .  .  .
M. raptor        481 TCAAATAATT GAAATTATTT GAACAATTAT TCCAATTTTT TTTTTAATCA TTTTAGCAAT
M. raptorellus   481 C......... .......... .....G.A.. ...T...... ......G.AT ..........
M. zaraptor I    474 ...G...... .......... .....G.A.. ..GT...... ........TT ..........
M. zaraptor II   479 ...G...... .......... ....TG.A.. ...T...... ........TT ..........

                    ------------------------------CO II-----------------------------
M. raptor             P  S  L   K  I  L   Y  M  T  D   E  M  N   L  P  N   L  S  I  K
M. raptorellus        .  .  .   .  .  .   .  .  .  .   .  L  .   I  .  .   .  T  M  .
M. zaraptor I         .  .  .   .  .  .   .  L  .  .   .  S  .   .  .  .   .  .  .  .
M. zaraptor II        .  .  .   .  .  .   .  L  .  .   .  .  .   .  .  .   .  T  .  .
M. raptor        541 TCCTTCATTA AAAATTCTTT ATATAACTGA TGAAATAAAT TTACCTAATT TATCTATTAA
M. raptorellus   541 ......T... ......T.A. .C..G..... ....T..... A.T..A.... ..A....A..
M. zaraptor I    534 ...G...... .......... ..T....... ...GTC.... .......... ....A.....
M. zaraptor II   539 ...A..C... .......... ..T....... .......... .......... ..A.A.....

                    ----------CO II-------------
M. raptor             I
M. raptorellus        V
M. zaraptor I         V
M. zaraptor II        V
M. raptor        601 AATTattggt catcaatgat attga
M. raptorellus   601 .G.A
M. zaraptor I    694 .G.A
M. zaraptor II   699 .G.A
```

Fig. 3. *Continued*

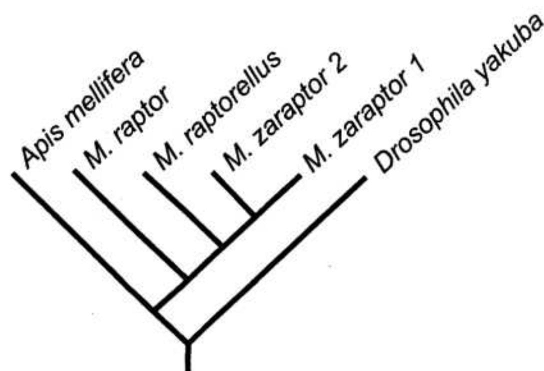

Fig. 4. Cladogram based upon sequence data of *Muscidifurax* spp., *A. mellifera,* and *D. yakuba.* All branches were supported by ≥97 of 100 bootstrap replicates.

Table 3. Nucleotide substitution (*d*) rates (below the diagonal) and transition to transversion ratios (above the diagnonal) among *Muscidifurax* spp., *A. mellifera*, and *D. yakuba*

Species	Species					
	1	2	3	4	5	6
1 *A. mellifera*	—	0.41	0.33	0.33	0.36	0.37
2 *D. yakuba*	0.53	—	0.27	0.31	0.28	0.32
3 *M. raptor*	0.46	0.53	—	0.50	0.52	0.56
4 *M. raptorellus*	0.53	0.61	0.19	—	0.66	0.82
5 *M. zaraptor* 1	0.47	0.62	0.14	0.15	—	1.22
6 *M. zaraptor* 2	0.54	0.60	0.17	0.18	0.08	—

for 5 min with ethidium bromide (1 μg/ml). MetaPhor agarose (FMC Bioproducts, Rockland, ME) gel electrophoresis was used to estimate fragment lengths. Gels (2.5%) were run for 4 h in Gibco-BRL (Gaithersburg, MD) Horizon 11·14 gel boxes at 80 V with 1× TBE buffer. A 50-bp ladder (Gibco-BRL, Grand Island, NY) was used as a size standard. Ethidium bromide was added to the gel, 0.06 μg/ml final concentration, and 8 μg of ethidium bromide was added to the buffer tray at the anodal end. Gels were interpreted on an ultraviolet (312 nm) transilluminator. Fragment sizes were calculated with the computer program GEL-JML (LaCroix 1994).

DNA Sequencing. Amplified DNA was purified using Geneclean II (Bio 101, Vista, CA) following manufacturer protocols and resuspended in 30 μl of TE (pH 7.5). DNA was blunt-ended using New England Biolabs (Beverly, MA) reagents and ligated into pBluescript sk+ plasmid using Stratagene (La Jolla, CA) reagents (Sambrook et al. 1989). Competent *E. coli* were transformed and positive colonies were verified by picking colonies with a sterile toothpick, replating and dipping the toothpick into a 50-μl PCR reaction mixture (Sambrook et al. 1989). PCR reaction was performed as before but for only 20 cycles. Two μl of PCR product was run on an agarose gel to determine if the insert was present. Positive clones were sequenced by the University of Nebraska-Lincoln Center for Biotechnology DNA Sequencing Laboratory (Lincoln, NE) using a LI-COR Model 4000 DNA Sequencer (LI-COR, Lincoln, NE). Two primers, T3 and T7 promoters (Gibco-BRL, Gaithersburg, MD), were used for sequencing. Two clones were sequenced in both directions for each sample. Published sequences for *A. mellifera* (Crozier and Crozier 1993) and *D. yakuba* (Clary and Wolstenholme 1985) were used for comparisons with those species.

Nucleotide sequences for *M. raptor, M. raptorellus,* and *M. zaraptor* (sequences 1 and 2) have been deposited in Genbank with accession numbers U97506–U97515.

Statistical Analyses. Sequence divergence (*d*) was calculated from restriction site data using The restriction enzyme analysis package (REAP) (McElroy et al. 1992) following the procedures of Nei and Tajima (1981) and Nei and Miller (1990). The DNADIST procedure of PHYLIP 3.5 (Felsenstein 1993) was used to calculate *d* values from the sequence data using the Kimura 2-parameter model (Kimura 1980). The consensus dendrogram of the mtDNA haplotypes was derived using the Wagner parsimony method (DNAPARS and CONSENS procedures of PHYLIP). Bootstrap replicates (100) of the sequence data were generated with the SEQBOOT procedure of PHYLIP.

Results

The primer pair produced an amplicon estimated to be 632 bp for all of the *Muscidifurax* spp. and *C. macellaria* with a 2.5% metaphor agarose gel (Fig. 1). The *A. mellifera* amplicon was estimated to be 856 bp.

Restriction Fragment Length Patterns. The restriction enzymes *Alu* I, *Ava* I, *Ban* II, *Bfa* I, *Bsr* I, *Dde* I, *EcoR* I, *EcoR* V, *Hinc* II, *Hind* III, *Hpa* I, *Msp* I, *Pvu* II, *Rsa* I, *Sau*96 I, *Sac* I, *Scrf* I, and *Xba* I did not cut the amplicon from any of the *Muscidifurax* samples. Twenty-four restriction sites were identified among the 4 *Muscidifurax* spp. for the remaining 9 restriction enzymes. The NY and NE samples of *M. raptor* gave identical restriction fragment patterns for all REs except *Apo* I. One of 2 wasps from the NY colony had an 81-bp band and lacked the 28-bp band. These bands were absent and present, respectively, in both of the NE and the other NY *M. raptor* (Fig. 2). The 3 samples of *M. raptorellus* and *M. uniraptor* gave identical restriction fragment patterns (Fig. 2; Table 2). Several of the restriction digests for *M. zaraptor* had faint bands in addition to the stronger bands, and the sum of the lengths of the bands consistently exceeded the length of the intact amplicon. Although these bands appeared to be caused by incomplete digests, they were not observed for the other species under similar digest conditions and could not be eliminated by increasing enzyme concentration or the duration of the digest. Because of these difficulties in interpreting the *M. zaraptor* restriction digests, this species was not included in the restriction site analysis. The

Table 4. Restriction sites in *Muscidifurax* spp.

Restriction enzyme site	*M. raptor*	*M. raptorellus*	*M. zaraptor* I	*M. zaraptor* II	*M. zaraptor* clones					
					1	2	3	4	5	6
Apo I										
183	−	−	−	−	+	−	−	+	−	+
215	+	+	+	+	+	+	+	+	+	+
224	+	+	−	−	−	−	−	−	−	−
236	−	+	−	−	−	−	−	−	−	−
270	±	+	+	−	−	−	+	−	−	−
306	+	−	+	+	+	+	+	+	+	+
343	−	+	+	−	−	−	+	−	−	−
552	+	+	+	+	−	+	+	+	+	−
577	+	−	+	+	+	+	+	+	+	+
586	−	+	−	−	−	−	−	−	−	−
Ase I										
234	−	−	+	+	−	+	+	−	+	+
326	+	−	−	−	−	−	−	−	−	−
392	−	+	−	−	−	−	−	−	−	−
415	−	−	+	+	−	+	+	−	−	+
455	−	+	+	−	?	−	?	?	−	?
458	−	−	+	−	?	−	?	?	−	?
*Dde*I										
60	−	−	−	+	−	+	−	−	+	+
Dpn II										
379	+	−	+	+	+	+	+	+	+	+
455	−	−	−	+	−	+	−	−	+	−
Dra I										
242	+	+	+	+	+	+	+	+	+	+
269	−	+	+	+	+	+	+	+	+	+
303	+	+	+	+	+	+	+	+	+	+
446	−	−	−	+	+	+	−	+	+	+
549	−	+	−	−	+	−	−	−	−	+
Hae III										
480	−	+	−	−	−	−	−	−	−	−
Hinf I										
28	+	+	+	+	+	+	+	+	+	+
155	−	+	−	−	−	−	−	−	−	−
176	−	+	−	−	−	−	−	−	−	−
552	−	−	−	−	+	−	−	−	−	+
571	−	−	+	−	−	−	+	−	−	−
Mse I										
146	+	+	+	+						
209	−	−	+	+						
234	−	+	+	+						
241	+	+	+	+						
268	+	+	+	+						
289	−	+	−	−						
302	+	+	+	+						
326	+	−	−	−						
392	−	+	−	−						
416	+	−	+	+						
447	+	−	−	+						
455	+	+	+	−						
458	−	+	+	−						
524	+	−	+	+						
548	+	−	+	+						
562	−	−	+	+						
575	−	+	−	−						
590	−	+	−	+						
597	+	−	+	+						
Ssp I										
92	+	−	−	−	−	−	−	−	−	−
287	−	+	−	−	+	−	−	+	−	−
330	+	+	−	−	−	−	−	−	−	−
390	−	+	+	+	−	+	+	+	+	+
580	−	+	−	−	−	−	−	−	−	−
Taq I										
6	+	+	+	+	+	+	+	+	+	+
129	−	−	+	−	−	−	+	−	−	−
170	−	−	−	−	+	−	−	−	−	−
463	+	−	+	+	+	+	+	+	+	+

Site positions are based on *M. raptor* map (Fig. 3). *M. uniraptor* was identical to *M. raptorellus* for all restriction sites. Site presence (+) and absence (−) were based on the sequences and verified by RFLP analysis for *M. raptor*, *M. raptorellus*, *M. uniraptor*, and *M. zaraptor* types I and II. Data for clones 1–6 of *M. zaraptor* are based on RFLP data only.

Table 5. Restriction fragment lengths estimated on 2.5% MetaPhor agarose gels for 6 *M. zaraptor* clones

Restriction enzymes	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5	Clone 6
Apo I	271, 186, 95, 50	245, 216, 95, 50	214, 214, 62, 53	247, 189, 99, 51	247, 219, 98, 51	271, 221, 98, 51
Ase I	456, 170	231, 209, 184	229,184, 170	443, 168	377, 234	234, 184, 168
Dde I	617	532, 66	617	617	536, 66	543, 66
Dpn II	377, 246	373, 170, 84	368, 246	373, 243	373, 168, 83	371, 243
Dra I	245, 146, 106, 78, 60	243, 180, 146, 33[a]	321, 240, 33[a]	240, 180, 146, 34[a]	240, 177, 146, 34[a]	240, 146, 105, 81, 60
Hinf I	525, 76	571	518, 56	576	576	511, 76
Mse I	149[a], 67[a]	149, 117, 82, 67	145, 117, 68[a]	149[a], 68[a]	150,[a], 82, 68	151, 119, 68
Ssp I	330, 291	381, 236	368, 234	284, 231, 99	281, 234	372, 234
Taq I	293, 164[a]	442, 160	325, 162, 122	447, 160	447, 160	447, 160

[a] Possibly double bands.

estimated number of nucleotide substitutions per nucleotide, *d*, calculated from restriction site data (Nei and Tajima 1981) was 0.23 between *M. raptor* and *M. raptorellus–M. uniraptor*. All 9 restriction enzymes gave restriction patterns that were diagnostic between *M. raptor* and *M. raptorellus–M. uniraptor*.

Sequence Analysis. Sequencing the amplicon revealed that it was 625 bp long for *M. raptor*, *M. raptorellus*, and *M. uniraptor* (Fig. 3). Sequences for Nebraska and New York *M. raptor* samples were identical except for nucleotide bp 273 (Fig. 3), which exhibited a T to G transversion in 1 of the 2 *M. raptor* from New York. Sequences for Chile, Peru, and Nebraska *M. raptorellus* and *M. uniraptor* were identical. Two distinct sequences were obtained from 2 clones of *M. zaraptor*: clone 1 was 618 bp long and clone 2 was 623 bp. The 4-bp deletion observed in clone 1 (bps 99–102 in Fig. 4) imparted a frame shift in the COI gene. This frame shift changed 12 amino acids before reaching a stop codon and truncating the final 31 amino acids of the subunit. *A. mellifera* differed from *D. yakuba* and the *Muscidifurax* spp. by a 30-bp insert at the 3′ end of the COI gene (after base 234 of the *M. raptor* sequence) and a 192-bp A + T-rich insert between $tRNA^{Leu}$ and

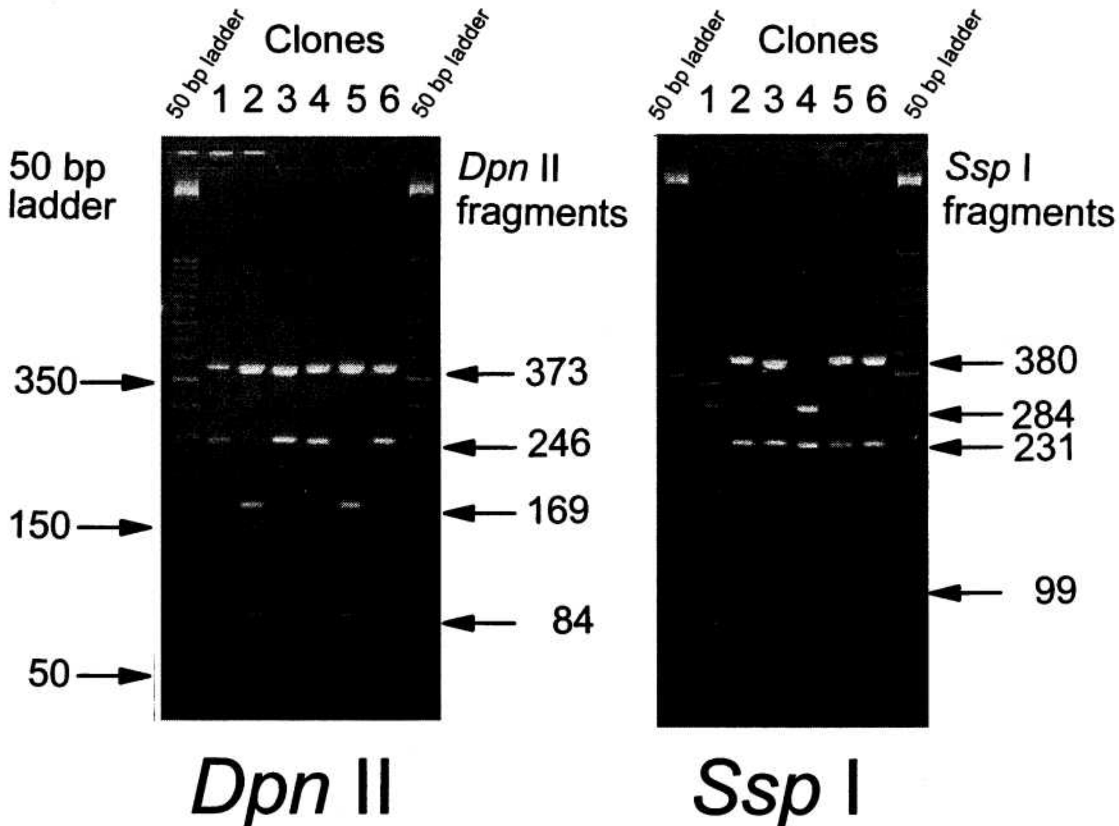

Fig. 5. *Dpn* II and *Ssp* I digests of amplicon from 6 *M. zaraptor* clones on 2.5% MetaPhor agarose gels.

COII (after base 307 of the *M. raptor* sequence) (Fig. 3). These inserts were ignored in the comparisons with *A. mellifera*. *Muscidifurax* spp. differed from *A. mellifera* by 0.50 nucleotide substitutions per site and from *D. yakuba* by 0.59 substitutions per site. Divergence among the *Muscidifurax* spp. ranged between 0.14 and 0.19 substitutions per site (Table 3). The 2 *M. zaraptor* sequences differed by 0.08 substitutions per site. Transition to transversion ratios ranged between 0.50 and 1.22 among the *Muscidifurax* spp., with the lower values being associated with higher *d* values (Table 3). The A + T content of the amplicon was 81–84% in the *Muscidifurax* sp. compared with 75 and 84% in *D. yakuba* and *A. mellifera*, respectively.

Parsimony analysis resulted in a single most parsimonious cladogram with 467 state changes (Fig. 4). The 2 *M. zaraptor* sequences clustered together. All branches of the cladogram were supported by ≥97 of 100 bootstrap replicates of the dataset.

Sequences were scanned for restriction enzyme sites with the computer program DIGEST (written by Ramin Nakisa). All of the restriction patterns observed in the RFLP analysis were supported by restriction enzyme sites in the sequences (Table 4).

The *M. zaraptor* colony used for the study described study was discarded before realizing that it contained distinct mitochondrial haplotypes. In an effort to see if both haplotypes were present in the new *M. zaraptor* colony, amplification products from the new colony were cloned as for sequencing. DNA from individual clones was amplified by PCR, and amplicons from the 6 positive clones were digested with 9 restriction enzymes (Table 5). These enzymes were chosen because the sequence data indicated they were diagnostic between the 2 *M. zaraptor* sequences. Each of the 6 clones resulted in a unique composite digest pattern (Fig. 5). Two of the patterns, clones 3 and 2, matched the digest patterns predicted for the *M. zaraptor* I and II sequences. Restriction site analysis indicated that *d* values ranged from 0.03 to 0.10 among the clones.

To isolate *M. zaraptor* lines with each of the observed haplotypes for morphological comparisons and further genetic studies, we initiated 10 isofemale lines from the *M. zaraptor* colony. DNA was isolated from F_1 wasps (5 wasps pooled and individual wasps) from each line, amplified, and digested with the diagnostic restriction enzymes *Apo* I, *Ase* I, *Dde* I, *Dpn* II, *Hinf* I, *Mse* I, *Ssp* I, and *Taq* I. Restriction digest patterns were identical for the pooled and individual DNA isolations, although amplifications were stronger from the pooled samples. Banding patterns were identical for 9 of the 10 lines and the same as those observed with the earlier pooled samples from the discarded colony (Fig. 2). The sum of the estimated lengths of the observed bands greatly exceeded the length of the amplicon, and the banding patterns appeared to be composites of the patterns observed in the 6 clones combined. The banding pattern of the 10th line lacked several of the bands observed in the other 9, but no new bands were observed.

Discussion

The estimated length of the amplicons was close to that predicted by the *D. yakuba* sequence (Clary and Wolstenholme 1985). The 193-bp A + T-rich, noncoding insert found between the $tRNA^{leu}$ and COII genes by Crozier and Crozier (1993) in *A. mellifera* was not present in *Muscidifurax* sp. As in other non-*Apis* hymenopterans (Cornuet et al. 1991), no nucleotides are present between the $tRNA^{leu}$ and COII genes.

Four restriction enzymes, *Apo I*, *Ase I*, *Mse I*, and *Ssp I*, resulted in digest patterns which were diagnostic among *M. raptor*, *M. raptorellus–M. uniraptor*, and *M. zaraptor*. An additional 5 enzymes, *Dpn II*, *Dra I*, *Hae III*, *Hinf I*, and *Taq I*, differentiated *M. raptorellus–M. uniraptor* from the other species. RFLP analysis could not differentiate the geographic isolates of *M. raptor* or *M. raptorellus* nor could they separate *M. raptorellus* from *M. uniraptor*. Use of PCR–RFLP patterns for species diagnostics of *Muscidifurax* spp. must be used with caution until the questions regarding the multiple, divergent sequences in *M. zaraptor* is resolved.

The sequencing analysis supported the variation detected by PCR–RFLP analysis. Predicted restriction sites were observed in all sequences to account for the observed RFLP patterns. Although a single polymorphic nucleotide sight was observed in the New York sample of *M. raptor*, no fixed differences were observed among the sequences from the geographic isolates of *M. raptor* and *M. raptorellus*. The *M. uniraptor* sequence was identical to that of *M. raptorellus*. These data support the characterization of the Nebraska isolate of *M. raptorellus* as being true to that species, with the probable origin being an introduction from South America (Antolin et al. 1996).

The status of *M. uniraptor* is not resolved. The mtDNA amplicon sequence for this species was identical to that of *M. raptorellus*. Kogan and Legner (1970) indicated that *M. uniraptor* was "extremely difficult" to distinguish from *M. raptor* and "almost impossible" to differentiate from *M. raptoroides*. However, a fringe of setae on the margins of *M. uniraptor* wings "easily" distinguish it from *M. raptorellus* and *M. zaraptor*. Fringe setae were apparent on the wings of *M. uniraptor* from our colony. Legner (1969) indicated that *M. uniraptor* males, on occasion, were able to fertilize *M. raptor* females successfully, but he did not observe successful hybridization between *M. uniraptor* and *M. raptorellus*. Based upon further hybridization data, Legner (1987) suggested "the evolution of *M. uniraptor* from *M. raptor*." Isozyme data (Kawooya 1983) appear to support the relationship between *M. uniraptor* and *M. raptor*. However, extremely high, within-species genetic distances (Nei [1978] unbiased genetic distances of 0.40, 0.50, and 0.76 for *M. raptor*

from North Carolina, Utah, and Israel) were observed in that study. These distances are far larger than expected for within-species variation (Ayala et al. 1974), making Kawooya's results difficult to interpret. Our mtDNA data clearly indicate that *M. uniraptor* is more closely related to *M. raptorellus* than to *M. raptor.*

Currently, our data are insufficient to evaluate the specific status of *M. uniraptor.* Thelytokous reproduction was the primary characteristic used by Kogan and Legner (1970) to distinguish this species. Subsequent studies have implicated *Wolbachia,* a cytoplasmically inherited microorganism, as the cause for thelytokous reproduction in *M. uniraptor* (Stouthamer et al. 1993). *Wolbachia* infections usually can be eliminated with antibiotics or high temperatures (Stouthamer and Luck 1988), rendering thelytokous strains arrhenotokous. Crosses between *Wolbachia*-free *M. uniraptor* and *M. raptorellus* will be needed to confirm the specific status of *M. uniraptor* (Stouthamer et al. 1990).

The levels of sequence divergence observed among *M. raptor, M. raptorellus,* and *M. zaraptor* were higher than expected. Dowton and Austin (1995) indicate that a higher rate of mtDNA divergence may be associated with parasitism in Hymenoptera. Nevertheless, the levels of divergence observed between the species indicate divergence times of several millions of years (Powell et al. 1986); not the 400 yr required if *Muscidifurax* were of Old World origin. These data indicate that the presence of *Muscidifurax* in the New World predates the arrival of house flies and stable flies, their preferred hosts. The phylogeographic structure of the group in the Americas adds further support to this argument. One question which remains to be resolved is whether *M. raptor* was a Holarctic species, or if its introduction to the Old World was a recent event.

Our data for *M. zaraptor* raise several questions. Digests from progeny of individual females indicate that multiple haplotypes are present within each line and within individual wasps. Hence, the mtDNA variation observed in *M. zaraptor* is caused by variation within individuals rather than population differentiation indicative of multiple cryptic species. Further complicating the issue is the frame shift deletion in the *M. zaraptor* 1 sequence. We must assume that a COI subunit with the C-terminal 43 amino acids modified or truncated is nonfunctional. Two explanations for the multiple haplotypes are as follows: (1) the species has a high level of heteroplasmy with extremely high levels of differentiation among the mtDNA haplotypes, or (2) a portion of the mitochondrial genome has been translocated to the nuclear genome where it has formed tandem repeats (Lopez et al. 1994, Zhang and Hewitt 1996a). Heteroplasmy has been reported for several insect species (Boyce et al. 1989, 1994; Harrison 1989). However, in nearly all cases, the heteroplasmy has been the result of length variation in the A + T-rich region, not substitutions in the coding regions. Recent translocation and amplification of mitochondrial sequences in the nuclear genome has been reported for the desert locust (Zhang and Hewitt 1996b). In future studies, the variant digest pattern observed in the exceptional *M. zaraptor* isofemale line will be useful for determining the mode of inheritance.

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