

Intraspecific variation in the ability of *Microctonus aethiopoidea* (Hymenoptera: Braconidae) to parasitise *Sitona lepidus* (Coleoptera: Curculionidae)

C. B. PHILLIPS

R. P. CANE

Biocontrol and Biosecurity Group
AgResearch
P.O. Box 60
Lincoln
Canterbury, New Zealand

J. MEE

H. M. CHAPMAN

Plant and Microbial Sciences Department
University of Canterbury
Private Bag 4800
Christchurch, New Zealand

K. A. HOELMER

D. COUTINOT

USDA-ARS European Biological Control
Laboratory
Campus International de Baillarguet
CS 90013 Montferrier-sur-Lez
34988 St. Gély du Fesc Cedex, France

Abstract *Sitona discoideus* (Coleoptera: Curculionidae), a pest of lucerne (*Medicago sativa*), is controlled in New Zealand by the introduced parasitoid *Microctonus aethiopoidea* (Hymenoptera: Braconidae). Unfortunately, a second *Sitona* species, *S. lepidus* (= *flavescens*), which has recently invaded New Zealand and has become a pest of white clover (*Trifolium repens*), is not parasitised by *M. aethiopoidea*. Previous experiments have shown that New Zealand *M. aethiopoidea* will attack *S. lepidus*, but its eggs appear to be killed by the host immune response. In contrast, *M. aethiopoidea* has been observed to successfully parasitise *S. lepidus* in Europe. It is possible either

that New Zealand *S. lepidus* has a more effective immune response to *M. aethiopoidea* than European *S. lepidus*, or that New Zealand *M. aethiopoidea* is less able to evade the *S. lepidus* immune system than European *M. aethiopoidea*. An experiment was conducted to compare the suitability of French and New Zealand *S. lepidus* as hosts for French *M. aethiopoidea*. This provided no evidence of *S. lepidus* intraspecific variation in host suitability for parasitism. Furthermore, amplification of inter simple sequence repeat (ISSR) regions of *M. aethiopoidea* DNA demonstrated clear genetic differences between French and New Zealand *M. aethiopoidea*. It was concluded that intraspecific variation in the ability of *M. aethiopoidea* to evade the immune response of *S. lepidus* is the reason for the low levels of parasitism observed in New Zealand compared with Europe. Development rate data for *M. aethiopoidea* larvae and pupae are reported.

Keywords biological control; host range; host suitability; inter simple sequence repeat; intraspecific variation; development rate; *Microctonus aethiopoidea*; *Sitona discoideus*; *Sitona flavescens*; *Sitona lepidus*

INTRODUCTION

Microctonus aethiopoidea (Hymenoptera: Braconidae: Euphorinae), a solitary, arrhenotokous parasitoid, has a natural Palaearctic distribution, and has been introduced to North America for biological control of weevils in the genera *Sitona* and *Hypera* (Coleoptera: Curculionidae) (Loan 1975), and to Australia and New Zealand for biological control of *Sitona discoideus* (Aeschlimann 1995). The female parasitoid oviposits in an adult weevil, and the larva develops through 4–5 instars within the living, active host. The mature larva then emerges to pupate, while the host dies due to the parasitism (Loan & Holdaway 1961).

Microctonus aethiopoidea was first imported to Australia from Morocco and released in 1977–78 (Cullen & Hopkins 1982; Aeschlimann 1983a, 1995). Further importations of *M. aethiopoidea* from Greece were released in 1979–80 (Aeschlimann 1983a). The Moroccan parasitoids readily established in Australia, but establishment of the Greek parasitoids was not confirmed (Aeschlimann 1995). *M. aethiopoidea* was subsequently collected from Australia and released in New Zealand during 1982–83 (Stufkens et al. 1987) where it effectively suppressed *S. discoideus* (Barlow & Goldson 1993). New Zealand's *M. aethiopoidea* population probably originated from Morocco, although it is possible some Greek specimens were also introduced (Aeschlimann 1995).

In 1996, a second *Sitona* species, *S. lepidus* (= *flavescens*) (Coleoptera: Curculionidae), was discovered in New Zealand (Barratt et al. 1996). Adult weevils feed on foliage of clovers (*Trifolium* spp.) and larvae feed on the roots. It currently occurs only in the northern half of the North Island, but its vast distribution in the Northern Hemisphere, which includes Europe (Dieckmann 1980), North Africa, Occidental Asia (Hoffmann 1950) and North America (Bright 1994), suggests it will eventually spread throughout New Zealand. *S. lepidus* is regarded as being of European origin (Campbell et al. 1989). The extent of damage to white clover (*Trifolium repens*) in the North Island suggests *S. lepidus* will severely compromise New Zealand farmers' ability to exploit clover, both as a means of fixing atmospheric nitrogen and as food for stock (Willoughby & Addison 1997). Research is underway to develop methods of managing *S. lepidus* in New Zealand, including work on plant resistance, insecticide use, pasture management, and biological control.

The *M. aethiopoidea* established in New Zealand for biological control of *S. discoideus* is ineffective as a parasite of *S. lepidus*. This was first demonstrated using *M. aethiopoidea* collected from Otago (Barratt et al. 1997a) and has since been corroborated using parasitoids from Lincoln, Canterbury (M. McNeill & J. Proffitt, AgResearch, Lincoln, 2002 pers. comm.). Furthermore, dissections of >10 000 *S. lepidus* adults collected from various sites in Waikato did not reveal any parasitism, even though *M. aethiopoidea* was present at these sites infecting *Listronotus bonariensis* and *Irenimus aequalis* (both Coleoptera: Curculionidae) at low rates (P. Addison, AgResearch, Ruakura, 2002 pers. comm.). Laboratory experiments indicated that New Zealand *M. aethiopoidea* attacked *S. lepidus*,

but their eggs appeared to succumb to the *S. lepidus* immune system (McNeill et al. 2000). However, this inability of *M. aethiopoidea* to evade the immune response of *S. lepidus* is not universal, since up to 18% parasitism of *S. lepidus* by *M. aethiopoidea* has been recorded in the field in France and Switzerland (Aeschlimann 1980; Phillips et al. 2000; C. B. Phillips unpubl. data). Furthermore, in laboratory tests in Switzerland, c. 20% of *S. lepidus* were parasitised by *M. aethiopoidea* (L. Reimer, CABI Bioscience, Delémont, Switzerland, 2000 pers. comm.), while almost identical methods in New Zealand yielded <2% parasitism (Barratt et al. 1997a).

Similar variations in the ability of *M. aethiopoidea* to evade immune responses of other hosts have previously been observed. For example, *M. aethiopoidea* collected in France and Sweden were morphologically similar, but were regarded as different strains because the Swedish larvae had a much higher survival rate in *S. cylindricollis* (Loan & Holdaway 1961). A Swedish male and French female *M. aethiopoidea* mated (Loan & Holdaway 1961), and their offspring appeared to inherit the Swedish resistance to the *S. cylindricollis* immune response (Loan 1960).

Variations in host preference have also been observed. For example, while French *M. aethiopoidea* preferred to oviposit in *Hypera postica* (Coleoptera: Curculionidae), it would also parasitise *S. hispidulus*, but it completely rejected *S. lepidus* (Sundaralingam 1986). In contrast, Moroccan *M. aethiopoidea* parasitised only *S. hispidulus* and completely rejected *H. postica* and *S. lepidus* (Sundaralingam 1986).

Due to the presence of populations in Europe that are able to parasitise *S. lepidus*, additional importations of *M. aethiopoidea* to New Zealand are being considered (Goldson et al. 2001). However, the value of further importations is uncertain because the reason for the inability of *M. aethiopoidea* to parasitise *S. lepidus* in New Zealand is unknown. Either New Zealand *M. aethiopoidea* is less able to evade the *S. lepidus* immune system than European *M. aethiopoidea* (in which case further importations could be warranted), or New Zealand *S. lepidus* has a more effective immune response to *M. aethiopoidea* than European *S. lepidus* (in which case further importations may be of dubious value). The work described in this contribution investigated whether variation in *M. aethiopoidea* or *S. lepidus* was responsible for the observed variation in parasitoid efficacy, and also

used molecular DNA methods to seek evidence of genetic variation between New Zealand and French populations of *M. aethiopoidea*.

METHODS

New Zealand source of *Sitona lepidus*

Sitona lepidus adults were collected from pasture in Waikato, New Zealand, and air-couriered to the United States Department of Agriculture, Agricultural Research Service, European Biological Control Laboratory's quarantine facility situated near Montpellier in Hérault (Department 34) in southern France. The weevils were maintained in cages at 22°C and 14:10 L:D photoperiod and fed with bouquets of white clover pending use in experiments. Bouquets of white clover were prepared from plants collected in the field by wrapping the bases of c. 10 stems together in moist tissue paper and enclosing them in a small plastic bag. The clover was arranged so the leaves protruded from the bag, and the top of the bag was sealed around the stems with adhesive tape.

French sources of *Sitona lepidus* and *Microctonus aethiopoidea*

Sitona lepidus and *S. hispidulus* were collected from white clover in Ariège (9), Gard (30), and Hérault (34) in southern France using a modified leaf-blower as has been previously described (e.g., Phillips et al. 2000). The weevils were maintained in separate, two-chambered cages according to collection site and species at 20°C and 14:10 L:D photoperiod, and supplied with white clover bouquets. The weevils were restricted to the upper, mesh-floored chamber of each cage, while the lower chambers were lined with tissue paper. The weevils were maintained for at least 1 month to allow any braconid parasitoid larvae that were present in the weevils at the time of collection to emerge. After emerging, parasitoid larvae dropped through the mesh floor (2 × 2 mm grid) of the upper chamber and pupated underneath the tissue in the lower chamber. Once parasitoid larvae had spun their cocoons, they were transferred to Petri dishes containing a moistened dental wick to maintain high RH until the adults eclosed.

Whenever possible, each newly emerged *M. aethiopoidea* female was enclosed in a Petri dish with a male *M. aethiopoidea*, and observed until mating occurred. Females were then provided with

50% honey solution and kept for up to 24 h before use in the experiment. Each of the nine males used in the experiment were reared from *S. hispidulus*. Six of the 11 females used in the experiment were also reared from *S. hispidulus*, while the remaining five were reared from *S. lepidus*. Two of the females reared from *S. lepidus* were not mated because no males were available when they emerged.

Comparison of the suitability of French and New Zealand *Sitona lepidus* as hosts for *Microctonus aethiopoidea*

Each French *M. aethiopoidea* female was confined for two periods of 24 h in two separate cages, one containing 20 *S. lepidus* from New Zealand and the other containing 20 *S. lepidus* from southern France. The sequence in which weevils from each country were exposed to *M. aethiopoidea* females was alternated in different tests. Cages were circular (115 mm diam. × 60 mm deep), with gauze-covered lids, and each was provisioned with two bouquets of white clover, and a 5 × 5 mm piece of filter paper soaked in honey solution as food for the parasitoid. After 24 h, the parasitoid was removed, and the weevils were transferred to the upper section of a two-chambered cage pending the emergence of *M. aethiopoidea* larvae. The weevils were maintained like this for c. 1 month at 22°C, 14:10 L:D, and the clover in each cage was replaced as required (c. every 2–4 days). Cages were checked every day and any weevils that had died were transferred to vials of 70% alcohol. Parasitoid cocoons were transferred to Petri dishes as described above. After 1 month, all of the remaining weevils were killed by freezing, and stored in 70% alcohol. All weevils were subsequently dissected to search for immature parasitoids and for evidence of host immune responses to parasitism.

Statistical analysis

The numbers of parasitoid offspring produced were fitted to a generalised linear model with Poisson errors and compared by analysis of deviance using the chi-square distribution for probability estimates (McCullagh & Nelder 1983). Data for durations of the parasitoid larval and pupal stages were assessed by analysis of variance.

Analysis of genetic variation between French and New Zealand *Microctonus aethiopoidea*

Inter simple sequence repeat (ISSR) bands were used to determine the degree of genetic

differentiation within and among the *M. aethioides* populations. ISSR bands are highly variable, but are more repeatable than randomly amplified polymorphic (RAPD) DNA bands because the method employs an "anchored" primer comprising degenerate bases attached to multiple nucleotide repeats (Wolfe et al. 1998).

Eight *M. aethioides* reared from *S. discoideus* collected in Canterbury, New Zealand, and nine *M. aethioides* reared from *S. lepidus*, *S. discoideus*, and *S. hispidulus* collected in Hérault (34) and Gard (30) in southern France were used in the analysis. Parasitoid genomic DNA was extracted following the method of Henry et al. (1990). Insect heads were used to avoid contamination from gut bacteria DNA. The heads were placed in 1.5 ml microfuge tubes, frozen by dipping them into liquid nitrogen, then crushed with a sterile micropestle. One ml of TENT buffer (10 mM Tris-HCL (pH 7.4), 25 mM EDTA, 10 mM NaCl, and 0.5% Triton X-100) was added to each tube, and centrifuged at 13 000 g for 5 min. Each pellet was resuspended in 600 µl of TEN buffer (10 mM Tris-HCl (pH 7.4), 25 mM EDTA, and 10 mM NaCl), 20 µl of 25 mg/ml proteinase-K (final concentration 0.7 mg/ml), and 68 µl of 10% sodium dodecyl sulphate (SDS) (final concentration 1%), then incubated for 4 h at 37°C. Following incubation, 60 µl of 5 M NaCl was added to each reaction tube. The mixture was washed twice with 500 µl of phenol/chloroform. Following each wash, the tubes were centrifuged for 10 min at 13 000 g and the aqueous phases transferred to clean microfuge tubes. DNA was precipitated 30 min after adding 300 µl of isopropanol, and then pelleted by centrifuging for 15 min at 13 000 g. The supernatant was aspirated, the pellets were air dried for 30 min, then resuspended in 50 µl of TE (tris acetate; 10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

Primers selected for amplification of ISSR regions in the DNA of *M. aethioides* were based on those used by McCall (2000). Thirteen primers were initially screened for reproducible bands. From these, seven primers (see Table 2) that gave clear and consistent banding patterns were selected for analysis of the 17 specimens. Primers were obtained from the University of British Columbia Biotechnology Laboratory (UBC Primer Set #9, Vancouver, Canada).

Polymerase chain reaction (PCR) was performed in 25 µl reaction mixtures (2.5 µl 10 × PCR buffer, 3 µl 25 mM/l MgCl₂, 1.25 µl dNTP mix (10 mM each), 0.1 µl 100 µM primer, 0.25 µl 5 U/µl

Taq DNA polymerase (Roche Molecular Biochemicals), 16.9 µl sterilised distilled H₂O, and 1 µl genomic DNA of unknown concentration) overlaid with 20 µl of paraffin oil. Amplifications were conducted in a PTC-200 thermal cycler (MJ Research) using the following reaction conditions: (i) 93°C for 3 min, 1 cycle; (ii) 93°C for 20 s, 52°C for 1 min, 72°C for 20 s, 40 cycles; (iii) 72°C for 4 min, 1 cycle. After PCR, products were kept at 4°C until being loaded into a 2% agarose gel for separation by electrophoresis (70 V for 80 min.) in 1 × TBE buffer. Gels were stained in ethidium bromide for 30 min, washed briefly in water, and banding patterns were recorded with a Kodak DC 120 digital camera mounted over a UV illumination table.

PCR amplification products were scored as either present or absent for each specimen. Only bands that were clear and reproducible were included in the analysis. Every third reaction was repeated to confirm reproducibility. The Nei & Li (1979) similarity index (S) was calculated using Multivariate Statistical Package version 3.1 (Kovach Computing Services Pentraeth, UK 1998) to compare the presence/absence of ISSR fragments: $S = 2N_{ab}/(N_a + N_b)$, where N_{ab} is the number of bands shared by both lanes, N_a is the number of bands unique to lane a, and N_b is the number of bands unique to lane b. The distance values were analysed using the unweighted pair group method analysis (UPGMA) and displayed graphically in the form of a dendrogram. The individuals were too similar to be clearly resolved with principal coordinate analysis.

RESULTS

Comparison of the suitability of French and New Zealand *Sitona lepidus* as hosts for *Microctonus aethioides*

Microctonus aethioides produced a mean (± SEM) of 6.8 ± 1.7 offspring per cage (Table 1). The mean number of offspring produced by parasitoids in the first 24-h exposure (6.7 ± 2.4) was similar to the number produced in the second 24-h exposure (6.9 ± 2.5, $P = 0.46$). The country of origin of the host weevils affected neither the number of *M. aethioides* adults produced ($P = 0.98$), the total number of offspring (i.e., adults produced plus larvae found during dissections, $P = 0.53$); the larval development time ($P = 0.92$), nor the pupal development time ($P = 0.15$) (Table 1).

During the larval stage, *M. aethiopoulos* females developed more rapidly than males ($P < 0.001$), while during the pupal stage, females developed more slowly than males ($P < 0.001$; Table 1). When the larval and pupal durations were added, development times of males (520 ± 5 h, $n = 33$) and females (528 ± 4 h, $n = 41$) were similar ($P = 0.42$).

None of the 74 *M. aethiopoulos* larvae found during weevil dissections exhibited any signs of encapsulation or other host immune responses.

Analysis of genetic variation between French and New Zealand *Microctonus aethiopoulos*

ISSR analysis of 17 insects produced 37 ISSR bands, 10 (27%) of which were polymorphic (Table 2). The frequency of these polymorphic bands ranged between 24 and 94%. Of these, bands 886_1, 886_3, 848_3, 842_2, 842_4, and 822_2 were fixed in the French specimens. No polymorphic bands were fixed in the New Zealand specimens, although

one low frequency band was found only in New Zealand specimens. Of the six polymorphic bands fixed in the French samples, numbers 848_3, 842_2, and 842_4 were totally absent in the New Zealand samples. A general pattern evident in the banding was a lack of bands in New Zealand specimens at loci that were fixed, or nearly fixed, in French specimens (Table 3). Amplification products of samples from the two countries were, therefore, clearly distinct.

The UPGMA dendrogram separated the parasitoids into two groups according to country of origin (Fig. 1). The total mean genetic distance calculated using Nei & Li's (1979) similarity index was 0.068, with a maximum distance between countries of 0.114. The mean genetic distances among parasitoids from France and New Zealand were 0.009 and 0.024 respectively. In a bootstrap analysis, the separation of parasitoids into country clusters occurred in 97 of 100 iterations.

Table 1 Mean number of *Microctonus aethiopoulos* offspring per cage, and mean duration of immature parasitoid development (h) when reared in *Sitona lepidus* collected from France and New Zealand (\pm SEM).

	French <i>S. lepidus</i>	<i>n</i>	New Zealand <i>S. lepidus</i>	<i>n</i>	Overall	<i>n</i>
Adult offspring per cage	3.0 \pm 1.3	11	4.6 \pm 1.3	11	3.8 \pm 0.9	22
Total offspring per cage	5.9 \pm 2.5	11	7.7 \pm 2.4	11	6.8 \pm 1.7	22
Development (h)						
Female larvae	329 \pm 7	21	321 \pm 5	22	325 \pm 4	43
Male larvae	334 \pm 8	9	342 \pm 6	24	340 \pm 5	33
All larvae	334 \pm 5	33	335 \pm 4	50	334 \pm 3	83
Female pupae	205 \pm 4	19	205 \pm 6	22	205 \pm 4	41
Male pupae	184 \pm 6	9	179 \pm 4	24	180 \pm 3	33
All pupae	198 \pm 3	28	191 \pm 4	46	194 \pm 3	74

Table 2 Inter simple sequence repeat (ISSR) bands scored in the analysis of *Microctonus aethiopoulos* from New Zealand and France.

Primer	Sequence (5'-3')	No. of bands per primer	No. of polymorphic bands	Percentage polymorphic bands (%)
UBC 886	5'-VDV(CT) ₇ -3'	8	2	25
UBC 866	5'-(CTC) ₆ -3'	7	2	28.6
UBC 860	5'-(TG) ₈ RA-3'	4	0	0
UBC 848	5'-(CA) ₈ RG-3'	4	1	25
UBC 842	5'-(GA) ₈ YG-3'	6	3	50
UBC 822	5'-(TC) ₈ A-3'	3	2	66.7
UBC 808	5'-(AG) ₈ C-3'	5	0	0
Total		37	10	27

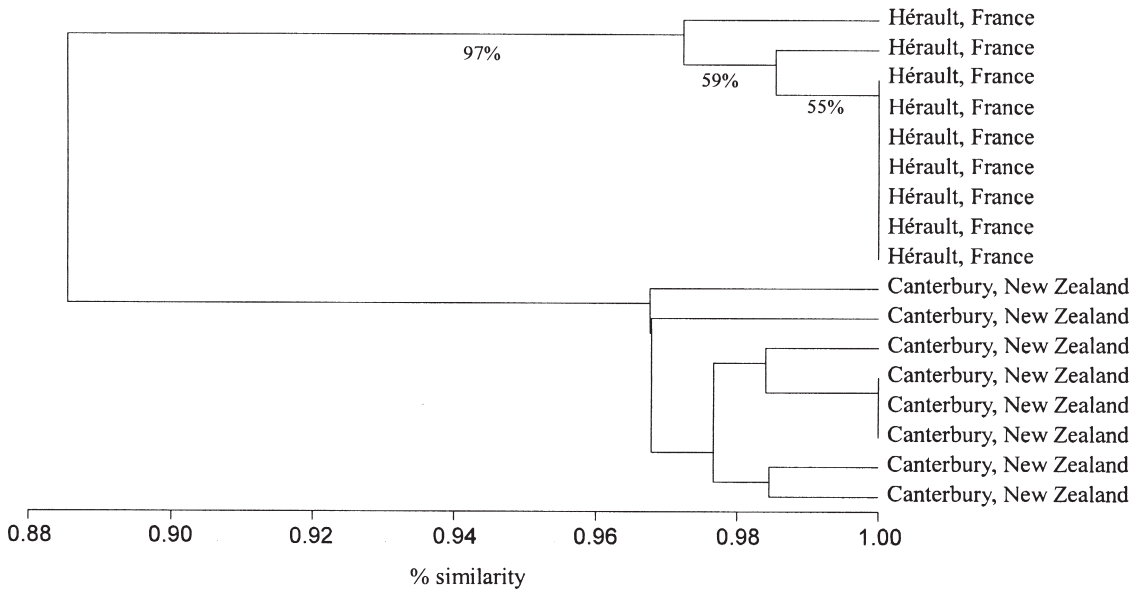


Fig. 1 Unweighted pair group method analysis (UPGMA) dendrogram showing relationships among the New Zealand and French *Microctonus aethioides*. Bootstrap values for branches recorded in > 50% of replications are shown as percentages below the branches.

DISCUSSION

The results indicated there is no difference between *S. lepidus* collected from France and New Zealand in host suitability for French *M. aethioides*. Therefore, the greater ability of European over New Zealand *M. aethioides* to parasitise *S. lepidus* appears to be due to differences between populations of *M. aethioides*, rather than differences between populations of *S. lepidus*. Support for genetic variation between French and New Zealand populations of *M. aethioides* was obtained in this study using ISSR analysis. This has recently also been corroborated by results from sequencing a 415 base pair (bp) region of the mitochondrial

cytochrome oxidase 1 (COI) gene because parasitoids from Waikato, Hawke's Bay, Canterbury, and Otago had sequences distinct from those of European *M. aethioides* reared from *S. lepidus* (Vink, Phillips & Mitchell unpubl. data). In addition, the similarity of the weevil populations has been backed up by a genetic comparison of French and New Zealand *S. lepidus* because they only differed by 2–4 bp in a 400 bp fragment of the COI gene (T. Glare & N. Richards, AgResearch, Lincoln, New Zealand, 2002 pers. comm.).

Fig. 1 shows greater variability in the New Zealand parasitoids (mean genetic distance 0.024) than in the French parasitoids (mean genetic distance 0.009). The New Zealand specimens could be more genetically diverse than the French specimens, perhaps because New Zealand's original introduced population was genetically diverse (originating from Morocco and perhaps Greece), or because it was subject to founder effects (e.g., Mayr 1954; Mayr 1963; Golubstov et al. 1993). However, the mean genetic distance of 0.068 between French and New Zealand parasitoids is insufficient to infer differentiation to the extent of species or subspecies, since numerous other studies have recorded greater intraspecific variation between populations (e.g., Lenney Williams et al.

Table 3 Comparison between *Microctonus aethioides* from New Zealand and France of inter simple sequence repeat (ISSR) bands scored in the analysis.

	France	New Zealand
No. of invariable bands	33	25
No. of variable bands	1	9
Total no. of bands	34	34
No. of unique bands	3	3
Percentage of variable bands	2.9	26.5

1994; Dowdy & McGaughey 1996; Haymer et al. 1997; Pornkulwat et al. 1998; McCall 2000).

The *M. aethiopoidea* development times recorded in this study compared well with those previously measured by Sundaralingam (1986) for Moroccan *M. aethiopoidea* parasitising *S. hispidulus* and French *M. aethiopoidea* parasitising *H. postica*. However, they differed from those measured by Morales & Hower (1981) for *M. aethiopoidea* parasitising *H. postica*, mainly because in their study pupae developed more slowly than larvae. Male pupae have previously been recorded as having shorter development times than female pupae for French (Sundaralingam 1986) and Moroccan (Aeschlimann 1983a; Sundaralingam 1986) populations of *M. aethiopoidea*, but not Greek ones (Aeschlimann 1983a). However, earlier authors have not differentiated between male and female larvae, and the longer development time of female larvae has not previously been observed.

The results suggest further importations of *M. aethiopoidea* from Europe to New Zealand could assist in biological control of *S. lepidus*. However, assessing the potential benefits and risks of such importations is likely to be complex. The *M. aethiopoidea* currently established in New Zealand has been recorded parasitising 13 non-target weevil species there (Barratt et al. 1997b), and this has contributed to concerns about possible impacts of introduced biological control agents on non-target insect populations (e.g., Follett & Duan 2000). Therefore, the effect on the parasitoid's host range of increasing the genetic diversity of New Zealand *M. aethiopoidea* by making further introductions from Europe will need to be considered. Furthermore, *M. aethiopoidea* is currently a very valuable biological control agent of *S. discoideus* in New Zealand (Goldson et al. 1993), and the effects of crossing between New Zealand and European *M. aethiopoidea* on efficacy in parasitising *S. discoideus* and *S. lepidus* will also require attention.

Evidence of significant *M. aethiopoidea* intraspecific variation in morphology, phenology, and host range has previously been reported (e.g., Loan & Holdaway 1961; Aeschlimann 1983b; Adler & Kim 1985; Sundaralingam 1986; Phillips et al. 1993), although support for a genetic, rather than environmental, basis for this variation has often been lacking. The results of this study have provided some of the first direct evidence there is significant genetic variation between geographic and host-associated populations of *M. aethiopoidea*, and a more comprehensive study of this is underway

(Vink, Phillips & Mitchell unpubl. data). The difference between the results of the present study, where French *M. aethiopoidea* was found to be an effective parasite of *S. lepidus*, and those of Sundaralingam (1986), where French *M. aethiopoidea* totally rejected *S. lepidus* as a host, suggests that more than one biotype of *M. aethiopoidea* occurs in France. The results provide support for the hypothesis that an important determinant of success in biological control is correct matching between populations of pests and biological control agents, involving careful assessments of intraspecific variation in pest and natural enemy species (e.g., Messenger et al. 1976).

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