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ScienceDirect
Molecular Phylogenetics and Evolution 43 (2007) 1178–1184
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Short communication

Genetic differentiation between body size biotypes of the parasitoid fly *Pseudacteon obtusus* (Diptera: Phoridae)

Marcus R. Kronforst [a,*], Patricia J. Folgarait [b], Richard J.W. Patrock [a,c], Lawrence E. Gilbert [a,c]

[a] *Section of Integrative Biology, University of Texas, Austin, TX 78712, USA*
[b] *Centro de Estudios e Investigaciones, Universidad Nacional de Quilmes, Roque Saenz Peña 180, Bernal B1876BXD, Buenos Aires, Argentina*
[c] *Brackenridge Field Laboratory, University of Texas, Austin, TX 78712, USA*

Received 11 May 2006; accepted 1 September 2006
Available online 9 September 2006

1. Introduction

The fly family Phoridae is a massive taxonomic group comprising as much as 2% of the Earth's animal species (Disney, 1990). However, due to their very small body size, the biology of this diverse group remains largely unexplored (Disney, 1990, 1994). One group of phorids that has begun to receive considerable attention recently is the ant-parasitoid genus *Pseudacteon*. While many phorid species are parasitoids (Disney, 1990, 1994), and ants commonly serve as hosts (Disney, 1990, 1994), flies in the genus *Pseudacteon* stand out because they parasitize fire ants of the genus *Solenopsis*, two species of which are major invasive pests in the United States. Interest in utilizing *Pseudacteon* flies as biological control agents against red (*Solenopsis invicta*) and black (*Solenopsis richteri*) imported fire ants has spurred recent research into the natural history, biogeography, ecology, and behavior of these phorids (reviewed in Morrison, 2000). Field observations and lab experiments indicate that *Pseudacteon* flies attack and effectively control fire ants by distracting foragers, thereby shifting the competitive balance in favor of native ant species (Mehdiabadi and Gilbert, 2002; Orr et al., 1995).

One aspect of this biological control effort that is critical for its long-term success is the careful matching of potential *Pseudacteon* parasitoids to the local environment in which they are to be released (Gilbert and Patrock, 2002). This includes specificity testing (Simberloff and Stiling, 1996), to verify that they will attack the ant species they are intended to control and not attack native species (Gilbert and Morrison, 1997; Porter and Alonso, 1999; Porter and Gilbert, 2005a,b), as well as matching to local environmental conditions such as temperature, average rainfall, and humidity (Gilbert and Patrock, 2002). Perhaps one of the most important conditions is matching fly size to local host size (Gilbert and Patrock, 2002). The interactions between fly size and host ant size are 3-fold. First, adult fly size is largely a function of host ant size, with larger flies emerging from larger hosts (Morrison and Gilbert, 1998; Morrison et al., 1999). Second, females of some *Pseudacteon* species have specific host size preferences; females choose the same size host for oviposition regardless of the distribution of host sizes available (Folgarait et al., 2005a). Finally, and perhaps most importantly from a biocontrol perspective, host size determines the sex of the developing fly in some *Pseudacteon* species. In such species, females develop from larger ants and males develop from smaller ants (Folgarait et al., 2005a, 2006; Morrison et al., 1999). If fly size is not carefully matched to host size in the introduced range, phorid females may be reluctant to attack if the preferred host size is not available, or the sex-ratio of the introduced phorid population may quickly become skewed if only large or small hosts are available.

Size matching has proven to be an important issue in Texas (Gilbert and Patrock, 2002), where the red imported fire ant is a major pest. While most of the southern United States is dominated by single-queen, or monogyne, *S. invicta* colonies which produce relatively large workers, colonies in Texas are largely polygynous (Porter et al., 1991, 1992) and have comparably smaller workers (Greenberg et al., 1985). For phorid species like *Pseudacteon tricuspis*, the size distribution of ants from monogyne colonies

* Corresponding author. Present address: Department of Ecology and Evolutionary Biology, Rice University, Houston, TX 77005, USA. Fax: +1 713 348 5232.
E-mail address: mkronforst@mail.utexas.edu (M.R. Kronforst).

1055-7903/$ - see front matter
doi:10.1016/j.ympev.2006.09.003

produces sufficient females to maintain captive and wild populations but the smaller size of polygyne ants results in phorid sex ratios heavily biased toward males. Finding a *Pseudacteon* species with the appropriate host specificity, environmental tolerance, and size requirements to match the local *Solenopsis* is critical. *Pseudacteon obtusus* is a promising candidate.

Specificity testing has shown that *P. obtusus* females regularly attack both exotic *Solenopsis* species (Folgarait et al., 2002, 2005a) but avoid the native tropical fire ant, *Solenopsis geminata* (Morrison and Gilbert, 1999; L.E.G and C. Estrada, unpublished data). Furthermore, *P. obtusus* is among the most widely distributed of any *Pseudacteon* species in South America (Calcaterra et al., 2005; Folgarait et al., 2005b) suggesting it is tolerant of a wide range of environmental conditions. Finally, *P. obtusus* exhibits striking variation in adult body size. In parts of Argentina, two distinct size classes, or biotypes, of *P. obtusus* co-occur. Adult females of the large biotype have a mean thorax width of 0.45 mm (SD = 0.04 mm, $N = 271$; P.J.F., unpublished data) while small biotype females have a mean width of 0.34 mm (SD = 0.06 mm, $N = 82$; P.J.F., unpublished data). *P. obtusus* females from Brazil, which do not exhibit this size variation, are approximately 25% smaller than the large females from Argentina (Folgarait et al., 2005a). Such extensive intraspecific size variation is unprecedented in *Pseudacteon* (Folgarait et al., 2005a) and may indicate that these two, otherwise morphologically similar groups, are actually distinct species. From a biological control perspective, identifying the source of this variation is important. If the two size classes represent distinct species, the small form could be well suited to areas dominated by polygyne fire ant colonies. On the other hand, if the observed size variation is a result of polymorphism, or is simply environmental variation, it is unlikely to lend itself to new biocontrol strategies. In this study, we use DNA sequence variation from two loci to determine whether body size biotypes of *P. obtusus* are genetically differentiated.

2. Materials and methods

2.1. Samples

We collected specimens of eight phorid species from various locations in North and South America (Table 1). Among them we collected multiple large and small *P. obtusus* from two provinces in Argentina where the two biotypes co-occur, Santiago del Estero and Corrientes (Fig. 1). We also obtained two *P. obtusus* samples from Campinas, Brazil (Fig. 1), where the large biotype is not known to occur. Adult female flies, which were caught in the field as they responded to host ants at baits or around disturbed mounds, were preserved in 95% ethanol and identified to species based on phenotypic characters such as ovipositor morphology (Porter and Pesquero, 2001).

2.2. DNA preparation, polymerase chain reaction (PCR) amplification, and sequencing

We extracted total genomic DNA from individual flies using a DNeasy tissue kit (Qiagen, Valencia, CA). From these DNA extracts we PCR amplified and sequenced two genomic regions, part of the nuclear gene *wingless* (*wg*) and a portion of the mitochondrial genome. The *wg* region consisted of a portion of exon 4, all of intron 4, and a portion of exon 5 (positions 1151–1763 in *Drosophila melanogaster wg* mRNA reference sequence NM_078778). This region was amplified and sequenced with primers LepWG1 (GARTGYAARTGYCAYGGYATGTCTGG) and LepWG2a (ACTICGCARCACCARTGGAATGTRCA) from Brower and DeSalle (1998). The mitochondrial region (*CO*) consisted of the 3′ end of *Cytochrome Oxidase I*, the 5′ end of *Cytochrome Oxidase II*, and the intervening tRNA-Leu (positions 2793–3388 in the *D. melanogaster* complete mitochondrial genome sequence AF200828). This region was amplified and sequenced with a forward primer similar to C1-J-2797 of Simon et al. (1994) (ATACCTCGACGTT ATTCAGA) and reverse primer C2-N-3389 (TCATAAG TTCARTATCATTG).

PCR products were amplified in 10 μl reaction volumes using a touch-down thermal cycling profile which consisted of an initial denaturing step at 94 °C for 2 min followed by 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min for 15 cycles with the annealing temperature reduced 1 °C/cycle, then 25 cycles at an annealing temperature of 50 °C. All PCR products were sequenced in both directions using Big Dye version 3 (PE Applied Biosystems, Foster City, CA) and were analyzed with an ABI Prism 3100 automated sequencer (PE Applied Biosystems). Chromatograms were edited with the program BioEdit version 7.0.2 (Hall, 1999) and sequences were aligned by eye. Six large *P. obtusus* samples had *wg* haplotypes that differed in size due to a variable indel. In each case we used the overlaid chromatogram produced by direct sequencing along with sequences from individuals that were homozygous for *wg* haplotypes to identify the two haplotypes present. *CO* was not amplified for nine samples and *wg* was not amplified from one sample.

2.3. Analyses

We identified all unique haplotypes for each locus and used MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001) to infer gene trees and estimate posterior probabilities for each node based on the default GTR + I + Γ model. Using PAUP* version 4.0b10 (Swofford, 2002), we tested the congruence of the two datasets with a partition-homogeneity test (100 replicates). We then combined the *wg* and *CO* datasets and inferred a phylogeny among all individuals. For this full analysis, the *Microselia texana wg* haplotype that was sequenced from sample No. 153 was combined with the *M. texana CO* haplotype that was sequenced from sample No. 155 (Table 1). For each analysis we ran four Metropolis-Coupled Markov chains for

Table 1
Sample information and GenBank Accession numbers

Sample ID	Species	Biotype	Collection location	GenBank Accession No.	
				wg	*CO*
51	*Apocephalus feeneri*	—	Travis County, Texas, USA	DQ448548	DQ448517
52	*Apocephalus feeneri*	—	Travis County, Texas, USA	DQ448549	DQ448518
153	*Microselia texana*	—	Travis County, Texas, USA	DQ448550	
155	*Microselia texana*	—	Travis County, Texas, USA		DQ448519
59	*P. litoralis*	—	Santiago del Estero, Argentina	DQ448551	
172	*P. pradei*	—	Campinas, Brazil	DQ448552	
170	*P. nudicornis*	—	Campinas, Brazil	DQ448553	
35	*P. curvatus*	—	Campinas, Brazil	DQ448554	DQ448520
164	*P. curvatus*	—	Campinas, Brazil	DQ448555	DQ448521
4	*P. tricuspis*	—	Campinas, Brazil	DQ448556	DQ448522
5	*P. tricuspis*	—	Campinas, Brazil	DQ448557	DQ448523
23	*P. tricuspis*	—	Campinas, Brazil	DQ448558	DQ448524
24	*P. tricuspis*	—	Campinas, Brazil	DQ448559	DQ448525
57	*P. obtusus*	Large	Corrientes, Argentina	DQ448560	DQ448526
				DQ448561	
58	*P. obtusus*	Large	Corrientes, Argentina	DQ448562	
538	*P. obtusus*	Large	Corrientes, Argentina	DQ448563	DQ448527
				DQ448564	
539	*P. obtusus*	Large	Corrientes, Argentina	DQ448565	DQ448528
				DQ448566	
540	*P. obtusus*	Large	Corrientes, Argentina	DQ448567	
541	*P. obtusus*	Large	Corrientes, Argentina	DQ448568	DQ448529
542	*P. obtusus*	Large	Corrientes, Argentina	DQ448569	DQ448530
543	*P. obtusus*	Large	Corrientes, Argentina	DQ448570	DQ448531
				DQ448571	
544	*P. obtusus*	Large	Corrientes, Argentina	DQ448572	
				DQ448573	
550	*P. obtusus*	Small	Corrientes, Argentina	DQ448574	DQ448532
551	*P. obtusus*	Small	Corrientes, Argentina	DQ448575	
552	*P. obtusus*	Small	Corrientes, Argentina	DQ448576	DQ448533
553	*P. obtusus*	Small	Corrientes, Argentina	DQ448577	DQ448534
554	*P. obtusus*	Small	Corrientes, Argentina	DQ448578	
555	*P. obtusus*	Small	Corrientes, Argentina	DQ448579	DQ448535
533	*P. obtusus*	Large	Santiago del Estero, Argentina	DQ448580	DQ448536
534	*P. obtusus*	Large	Santiago del Estero, Argentina	DQ448581	DQ448537
				DQ448582	
535	*P. obtusus*	Large	Santiago del Estero, Argentina	DQ448583	DQ448538
536	*P. obtusus*	Large	Santiago del Estero, Argentina	DQ448584	DQ448539
537	*P. obtusus*	Large	Santiago del Estero, Argentina	DQ448585	DQ448540
545	*P. obtusus*	Small	Santiago del Estero, Argentina	DQ448586	DQ448541
546	*P. obtusus*	Small	Santiago del Estero, Argentina	DQ448587	DQ448542
547	*P. obtusus*	Small	Santiago del Estero, Argentina	DQ448588	DQ448543
548	*P. obtusus*	Small	Santiago del Estero, Argentina	DQ448589	DQ448544
549	*P. obtusus*	Small	Santiago del Estero, Argentina	DQ448590	DQ448545
987	*P. obtusus*	Small	Campinas, Brazil	DQ448591	DQ448546
988	*P. obtusus*	Small	Campinas, Brazil	DQ448592	DQ448547

750,000 generations, following 250,000 burn-in generations, sampling every 100 generations, starting from a random tree. Trees were drawn with MEGA version 3.1 (Kumar et al., 2004).

To directly test whether genetic variation in *P. obtusus* was structured by geography or individual body size, we performed two analyses of molecular variance (AMOVA) using Arlequin version 2.0 (Schneider et al., 2000). First, we grouped *P. obtusus* haplotypes by collecting location, Corrientes or Santiago del Estero, and calculated the proportion of variation within and between groups. Next we grouped haplotypes by individual body size, large or small biotype, and calculated the proportion of variation within and between groups. For each AMOVA we estimated the probability of obtaining a larger between-group variance component by permuting haplotypes between groups 1000 times.

3. Results

From a total of 45 *wg* and 31 *CO* sequences (Table 1), we identified 17 unique *wg* haplotypes and 13 unique *CO* haplotypes (Fig. 2). The aligned, 633 bp *wg* dataset contained 102 variable sites (81 parsimony-informative) and five indels. The aligned, 595 bp *CO* dataset contained 191 variable sites (154 parsimony-informative) and three indels.

Fig. 1. *Pseudacteon obtusus* collection locations and size variation. (A) Large and small biotype *P. obtusus* specimens were collected at two locations in Argentina, Santiago del Estero and Corrientes. Small biotype *P. obtusus* were also collected near Campinas, Brazil. (B) Example of size variation between *P. obtusus* size biotypes. The small fly (top left) was collected at Campinas while the large fly (bottom right) was collected at Corrientes.

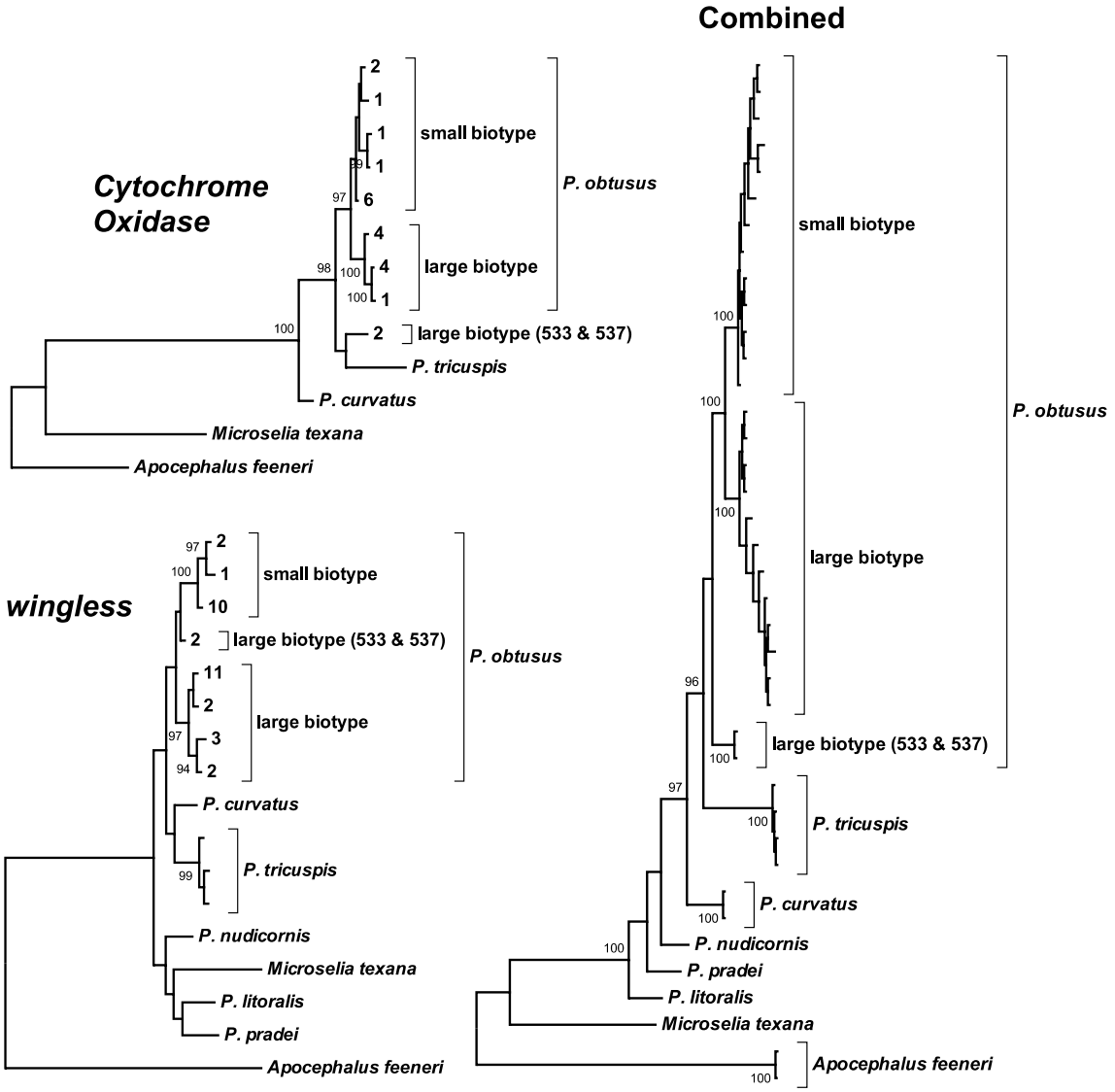

Fig. 2. Bayesian phylogenies of phorid flies based on the mitochondrial gene *Cytochrome Oxidase I* and *II*, the nuclear gene *wingless*, and the combined dataset. Gene trees for *Cytochrome Oxidase* and *wingless* are based on unique haplotypes–terminal *P. obtusus* branches are labeled with numbers to indicate the frequency of that haplotype in the population sample. The combined phylogeny shows the relationships among all sampled individuals. Nodes with Bayesian posterior probabilities >90% are labeled.

Table 2
Results of analyses of molecular variance (AMOVA) comparing the distribution of *P. obtusus* genetic variation between body size classes or geographic collecting locations

	% Variation between groups	*P*-value
Body size—large vs. small biotype		
wg	54.53	<0.001
CO	54.06	<0.001
Collecting location— Corrientes vs. Santiago del Estero		
wg	3.86	0.174
CO	8.15	0.108

Phylogenetic analysis of each gene resulted in haplotype trees consistent with genetic differentiation between large and small *P. obtusus* specimens (Fig. 2). For *CO*, all five small biotype *P. obtusus* haplotypes formed a clade, as did three of the four large biotype haplotypes. A single large biotype haplotype, which was isolated from two specimens (533 and 537), fell outside the *P. obtusus* clade. For *wg*, all *P. obtusus* haplotypes formed a clade, and they were further structured by body size; all three small biotype haplotypes formed a clade and four of five large biotype haplotypes formed a clade. A single large biotype haplotype, which was again isolated from specimens 533 and 537, fell outside the large haplotype clade. The two datasets were not significantly incongruent ($P = 0.84$, partition-homogeneity test) so we combined them and estimated a phylogeny among all individuals (Fig. 2). The combined analysis revealed relationships consistent with the results from the individual genes. All *P. obtusus* samples formed a clade, which was further divided into three clades; an ancestral clade consisting of samples 533 and 537, a clade consisting of all other large biotype samples, and a small biotype clade.

The results of the analyses of molecular variance supported the conclusion that genetic variation in *P. obtusus* was structured by body size (Table 2). For both *wg* and *CO*, approximately 54% of the DNA sequence variation that was present in *P. obtusus* was due to differences between body size biotypes. In contrast, only 4% of the variation at *wg* and 8% of the variation at *CO* was explained by differences between collecting locations.

4. Discussion

Our analyses of DNA sequence variation at two unlinked loci strongly support the hypothesis that large and small forms of the phorid fly *P. obtusus* are genetically distinct and likely represent separate species. At both *wg* and *CO*, haplotypes clustered by individual body size (Fig. 2) and genetic differentiation between the two size classes was highly significant (Table 2). Assuming an evolutionary rate of 1.1–1.2% per lineage per million years for *CO* (Brower, 1994), the average 2.3% divergence (SE = 0.47%) between large and small biotypes suggests that these two groups split approximately 1 million years ago. Since that time the biotypes have diverged in both body size and one other morphological feature; the presence (large biotype) or absence (small biotype) of aristae on the antennae (Porter and Pesquero, 2001).

Interestingly, phylogenetic analysis of the individual genes, as well as the combined analysis, revealed unexpected evidence of genetic differentiation within the large *P. obtusus* biotype. Two large specimens, both collected from Santiago del Estero, had *wg* and *CO* haplotypes that were distinct from the other large samples. While there was no *a priori* reason to suspect genetic differentiation within the large size class, this result is not surprising. Only two other studies to date have examined the distribution of genetic variation within phorid species and both discovered evidence for the existence of cryptic species. Morehead et al. (2001) found that the species *Apocephalus paraponerae* is actually a complex of at least four distinct species while Cook and Mostovski (2002) found evidence of genetic differentiation within what was believed to be a single population of *Phora holosericae*. As studies of phorid systematics increasingly incorporate DNA sequence data and multiple specimens per species, there is no doubt that a variety of phorid species will be revealed as groups of distinct but morphologically similar species.

While *P. obtusus* clearly consists of multiple, genetically distinct lineages, it is not clear how that occurred. Because they are parasitoids, speciation in *Pseudacteon* might be expected to be a consequence of either co-speciation with hosts or host shifts. Importantly, *P. obtusus* is one of only two *Pseudacteon* species in which males respond to ant mounds to locate and mate with ovipositing females (Calcaterra et al., 2005; P.J.F. and R.J. P., unpublished data). Such host associated mating is highly conducive to sympatric speciation (Berlocher and Feder, 2002; Feder et al., 1994). In fact, cryptic speciation in *A. paraponerae* appears to be associated with shifts in ant hosts (Morehead et al., 2001). *Pseudacteon* is different however. While *Pseudacteon* species are distributed along a variety of niche dimensions including host species (Disney, 1994; Folgarait et al., 2002), the size of ants that are attacked (Folgarait et al., 2002, 2005a, 2006; Morrison and Gilbert, 1998), daily timing of activity (Pesquero et al., 1996; Wuellner and Saunders, 2003), seasonal timing of activity (Folgarait et al., 2003), and whether they attack at nest mounds or on foraging trails (Orr et al., 1997), multiple *Pseudacteon* species all utilize the same individual host species. For instance, all *Pseudacteon* flies analyzed in this study were collected while responding to *S. invicta* at baits or around disturbed mounds. Such overlap in host use is inconsistent with both a hypothesis of co-speciation between host and parasitoid lineages and a hypothesis of sympatric speciation due to host shifts. Interestingly, there is some evidence that *S. invicta* itself is a cryptic species complex; the species is separated into two geographically distinct sub-clades (Ahrens et al., 2005), populations within one of these subclades exhibit substantial genetic differentiation over relatively small geographical distances (Ahrens et al., 2005),

and in at least one instance, nests from a single collecting location were found to represent two genetically distinct populations (Ross and Shoemaker, 2005). Combined with our results, these observations for *S. invicta* suggest an intriguing scenario of recent and hidden parallel radiations between fire ants and their phorid parasitoids.

Regardless of how these forms originated, our finding of genetic differentiation between large and small *P. obtusus* biotypes provides a new tool in the fight against imported fire ants. Although this hypothesis remains to be tested, it is likely that females of the small *P. obtusus* biotype will be more effective at attacking the relatively small ants produced by polygyne ant colonies. Furthermore, unlike the heavily male-biased sex ratios that result when larger *Pseudacteon* species utilize polygynous *S. invicta*, small ants should yield substantially more female phorid offspring when used as hosts by the small *P. obtusus* biotype. A greater proportion of female phorids will lead to higher rates of increase for phorid populations, higher and more stable phorid densities, and ultimately a strong, negative impact on the population densities of imported fire ants.

Acknowledgments

We thank Ulrich Mueller for providing lab facilities. The Lee and Ramona Bass Foundation, the Robert J. Kleberg and Helen C. Kleberg Foundation, Texas Fire Ant Research and Management Project (FARMAAC), CONICET and the Universidad Nacional de Quilmes each contributed support for this study. The staff of ECAS provided permits and logistical help to collect the phorids in Argentina. Brazilian specimens of *Pseudacteon* were exported under permits from IBAMA during 1995–1997, with the critical assistance of Dr. Woodruff Benson, University of Campinas, S.P. Brazil.

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