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## Genetic Variation in North American Black Flies in the Subgenus *Psilopelmia* (*Simulium*: Diptera: Simuliidae)

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### Abstract

Resolution of the genetic heterogeneity of closely related insect species depends on the selection of reliable genetic markers derived from representative specimens. We report the results of a survey of genetic variability in nine species of black flies in the subgenus *Psilopelmia* Enderlein. Three regions of the mitochondrial genome and an amplicon including the internal transcribed spacer 1 of the nuclear ribosomal RNA gene cluster (ITS1) were amplified using the polymerase chain reaction (PCR), and the amplicons were examined for intraspecific and interspecific polymorphisms. Six of the seven *Psilopelmia* species that yielded PCR products in the ITS1 PCR reaction were found to generate products that were indistinguishable on the basis of size. Similarly, little interspecific variation was noted in the 16S rRNA amplicon among nine species of *Psilopelmia* assayed by heteroduplex analysis. In contrast, the remaining regions of the mitochondrial genome exhibited both intra- and inter-specific variation when analyzed by heteroduplex analysis. Information collected from the five amplicons could be employed to develop a classification scheme capable of distinguishing the nine species of *Psilopelmia* examined.

## Introduction

*Psilopelmia* Enderlein is one of 14 subgenera of *Simulium* Latreille that are restricted primarily to the Neotropical region (Coscaron 1987; Crosskey 1987). The preimaginal stages are found in a variety of stream types ranging from small streams to medium-sized rivers. Adults of these species are best known for their bright colors, which range from orange to black, with white, yellow, or silvery blue striations or markings.

Female *Psilopelmia* are mammalophilic, and a few species are important pests of livestock in western North America. For example, vesicular stomatitis virus was isolated from *Simulium bivittatum* Malloch in Colorado and Utah (Francy et al. 1988; Kramer et al. 1990). Anderson and Voskuil (1963) reported a significant reduction in milk production by cattle in Merced County, California, caused by *S. trivittatum* when 500–800 females were observed feeding on a single animal in the late afternoon. Thus, reliable identification of the females of these species is of medical and agricultural importance. However, it has not been possible to definitively classify females of the different species of *Psilopelmia* by means of morphological characters.

Identification of female *Psilopelmia* is complicated, as the taxonomy of the subgenus remains unresolved. Crosskey (1987) assigned 50 species to *Psilopelmia* and 14 species to the closely related subgenus *Ectemnaspis* Enderlein. However, Coscaron and co-workers have restricted the limits of *Psilopelmia*, and in doing so have decreased the number of species assigned to it by one-half (Coscaron 1987; Coscaron et al. 1996). The majority of species removed from *Psilopelmia* were transferred to various species-groups of *Ectemnaspis* sensu Coscaron (1984, 1987).

Peterson (1993) and Coscaron et al. (1996) have recently revised the classification of the Nearctic and Neotropical species of *Psilopelmia*. Peterson (1993) described two unusual species, *Simulium labellei* and *S. robynae*, from the Rio Grande River system and provided keys to all life stages after the egg for North American species. Coscaron and co-workers have constructed keys to larvae, pupae, and adults for seven Neotropical species (Coscaron et al. 1995). Moulton has recently collected *S. notatum* Adams and *S. longithallum* Diaz Najera and Vulcano in Arizona, and reports data demonstrating that *S. clarum* (Dyar and Shannon) is a valid species (Moulton 1998). Therefore, 10 species of *Psilopelmia* are now known to exist in America north of Mexico.

Molecular methods have proved helpful in resolving taxonomic relationships and can be fruitful as tools for developing methods of classifying morphologically similar species. Several methods have been developed that are based upon the analysis of the amplification products (amplicons) of the polymerase chain reaction (PCR). These include direct DNA sequence analysis of amplicons, restriction site polymorphism analysis of amplicons, and conformation-based techniques such as single-strand conformational polymorphism and heteroduplex analysis (HDA). In HDA, a universal-probe DNA is annealed to a target amplicon to form two homoduplex and two heteroduplex molecules, which can be separated on a partially denaturing acrylamide gel. The mobility of the heteroduplex products is retarded relative to that of the homoduplex products. The degree of retardation of the heteroduplex molecule is a function of the number, type, and position of mismatches between the probe and target molecules (Zimmerman et al. 1993, 1995). Because HDA is simple and

rapid to perform, it can be used to effectively assess the degree of intra- and inter-specific variation in candidate DNA molecules that might be used for phylogenetic purposes, or to develop assays for distinguishing closely related species (Delwart et al. 1993; Tang et al. 1996a, 1996b).

In previous studies, HDA has been used in conjunction with selective DNA sequence analysis to classify several species of *Psilopelmia*, including *S. bivittatum* and *S. ochraceum* s.l. (Tang et al. 1996a). In the experiments described below, we applied HDA and PCR amplicon size polymorphism to evaluate the degree of intra- and inter-specific variation in nine species of the subgenus *Psilopelmia*.

## Materials and methods

### *Sample collection and preparation of DNA*

Nine species of black flies in the subgenus *Psilopelmia* were examined in this study. The sites and dates of collection of the specimens are summarized in Table 1. The African black fly *S. damnosum* s.l. Theobald served as a source of probe DNA for the HDAs. Three additional North American species and one Australian species outside of the subgenus *Psilopelmia* were also included as reference samples in some studies. These included *Cnephia dacotensis* Dyar and Shannon, *Simulium (Psilozia) enciso*i Vargas and Diaz Najer, *Simulium (Psilozia) vittatum* s.l., and *Austrosimulium bancrofti* Taylor. Individual specimens stored in 100% ethanol were washed in 95% ethanol, air-dried for 20 min, and homogenized in 15  $\mu$ L of a solution containing 10 mM Tris-HCl (pH 8.0), 312.5 mM EDTA, 1% sodium lauryl sarcosyl, and 1% polyvinylpyrrolidone (Steiner et al. 1995). Following homogenization, 20  $\mu$ L of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE) was added to each sample. The samples were incubated at 90°C for 20 min and centrifuged at 13 000  $\times$  g for 5 min. The supernatant was collected and diluted 1:40 with TE before use in the PCRs.

**Table 1.** Collection data on specimens used in this study

	<i>n</i>	Collection site	Collection date
Subgenus <i>Psilopelmia</i>			
<i>S. bivittatum</i> Malloch	1	Calamus R., Loup Co., Nebr.	12 Oct. 1990
	1	Sybille Creek, Platte Co., Wyo.	9 June 1992
	6	Verde R., Coconino Co., Ariz.	16 June 1994
<i>S. clarum</i> Dyar & Shannon	3	Mokolumne R., San Joaquin Co., Calif.	2 Oct. 1993
<i>S. griseum</i> Coquilett	2	Belle Fourche R, Crook Co, Wyo.	21 May 1994
<i>S. longithallum</i> Diaz Najera & Vulcano	2	San Pedro R., Cochise Co., Ariz.	28 April 1993
<i>S. mediovittatum</i> Knab	1	Paluxy R., Somervell Co., Tex.	5 June 1995
<i>S. ochraceum</i> Walker	8	Guatemala	NR
	3	Guatemala	November 1988
<i>S. notatum</i> Adams	8	Arivaipa Creek, Pinal Co., Ariz.	NR
	3	Arivaipa Creek, Pinal Co., Ariz.	11 July 1993
	8	Santa Maria R., Yavapai Co., Ariz.	14 April 1994
	8	Gila R., Pinal Co., Ariz.	18 Nov. 1994
<i>S. robynae</i> Peterson	10	Rio Grande R., Brewster Co., Tex.	17 March 1993
Outgroup species			
<i>S. trivittatum</i> Malloch	5	Paluxy R., Somervell Co., Tex.	5 June 1995
<i>Austrosimulium bancrofti</i> Taylor	1	Cotter R., New South Wales	27 Feb. 1993
<i>Cnephia dacotensis</i> Dyar & Shannon	1	Bellamy R., Strafford Co., N.H.	4 May 1994
<i>Simulium (Psilozia) enciso</i> Vargas & Diaz Najera	6	San Pedro R., near Sierra Vista, Ariz.	NR
<i>Simulium (Ewardsellum) damnosum</i> s.l. Vajime & Dunbar	1	Bong Co., Liberia	July 1987
<i>Simulium (Psilozia) vittatum</i> s.l. Zetterstedt	2	Pedernales R., Gillespie Co., Tex.	16 Dec. 1989

**Note:** *n* is the number of samples tested in this study; NR, no record.

### PCR amplification

Portions of the mitochondrial small (12S) and large subunit (16S) rRNA genes, the cytochrome oxidase subunit II gene (COII), and the 3' end of the nuclear 18s rRNA gene and its adjacent internal transcribed spacer (ITS1) were used as PCR templates in separate experiments. Amplifications were carried out in a total volume of 20  $\mu$ L in a solution containing 60 mM Tris-HCl (pH 8.5 at 20°C), 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each of dATP, dGTP, dCTP, and TTP, 400 nM of each primer, 0.8 unit of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, Connecticut), and 0.4  $\mu$ L of black fly template DNA, prepared as described above. The sequence of the primers used in this study and the annealing temperatures used to amplify each locus are summarized in Table 2. The cycling conditions included 38 cycles of denaturation at 95°C for 30 s, annealing for 50 s at various temperatures (Table 2), and extension at 72°C for 1 min. All PCR products were visualized by agarose gel electrophoresis followed by staining in 2 mg/mL ethidium bromide.

**Table 2.** Primers and annealing temperatures used to amplify mitochondrial and nuclear DNA loci

Locus amplified	Size of PCR product (bp)	PCR annealing temp.	Primer designation <sup>a</sup>	Sequence (5'-3')
16s	552	45°C	LR-J-12883	CTCCGGTTTGAACTCAGAT
			LR-N-13417	CGCCTGTTTATCAAAAACATG
12s	398	45°C	SR-J-14233	AAGAGCGACGGGCGATGTGT
			SR-N-14588	AAACTAGGATTAGATACCCTATTAT
COII	325	42°C	C2-J-3407	CATCAATGATACTGAAGTTATGA
			C2-N-3686	CAATTGGTATAAAAATGATTGTC
ITS1	900	48°C	18s/sd5'	TGGTGCATGGCCGTTCTTAG
			5.8s/sd3'	GTCGATGTTTCATGTGCTCTGC

a. For mitochondrial sequences, primer designations are as described by Simon et al. (1994).

### Heteroduplex assay (HDA)

To increase the sensitivity of the assay, probe DNAs were derived from *S. damnosum* s.l., an African black fly species not closely related to the test samples (Tang et al. 1996a; Zimmerman et al. 1995). Equal volumes (4  $\mu$ L) of the target and probe PCR products were mixed with 8  $\mu$ L of TE and overlaid with 10  $\mu$ L of mineral oil. The mixture was denatured for 2 min at 98°C and heteroduplex products were allowed to form by slow cooling to room temperature over a 30-min period. An aliquot (14  $\mu$ L) of each heteroduplex solution was mixed with 6  $\mu$ L of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). A total of 10  $\mu$ L of this mixture was loaded onto a 5% polyacrylamide/urea gel (19:1 acrylamide : bisacrylamide, 2.7 M urea) prepared in 1.2 $\times$  TBE (1 $\times$  TBE = 90 mM Tris-boric acid (pH 8.3), 2 mM EDTA). Electrophoresis was performed on a 20  $\times$  20 cm Protean II Xii system (Bio-Rad, Melville, New York) at 12 mA per gel for 18 h in 1 $\times$  TBE buffer. Gels were stained in 2  $\mu$ g/mL ethidium bromide and homoduplex and heteroduplex patterns were visualized under UV light. In some cases the two heteroduplex products co-migrated in the assay. In contrast, in assays employing distantly related probe and target DNAs, the homoduplex products derived from the probe and target DNAs were often separated, yielding two homoduplex bands. As a result, between two and four bands could be visualized in a given HDA.

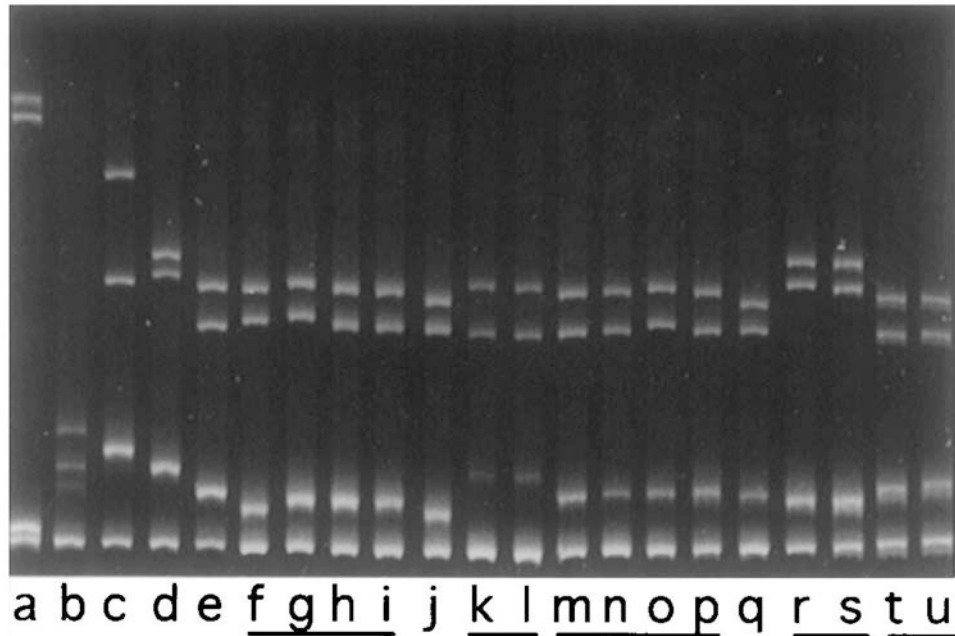
### Results

As mentioned in the Introduction, the taxonomic limits between *Psilopelmia* and *Ectemnaspis* remain unclear; therefore, the species of *Psilopelmia* analyzed in this study (except the *S. ochraceum* Walker complex) were chosen because they belong to *Psilopelmia* in the strictest sense (Coscaron 1987; Coscaron et al. 1996; Crosskey 1987).

Initially, an amplicon derived from the mitochondrial 16S rRNA gene was examined for intra- and inter-specific heterogeneity among the different species of *Psilopelmia*. This sequence was chosen because previous studies had demonstrated its utility in differentiating a number of American and African black fly species (Tang et al. 1996b, 1995). Furthermore, preliminary studies using this sequence had demonstrated that some degree of intraspe-

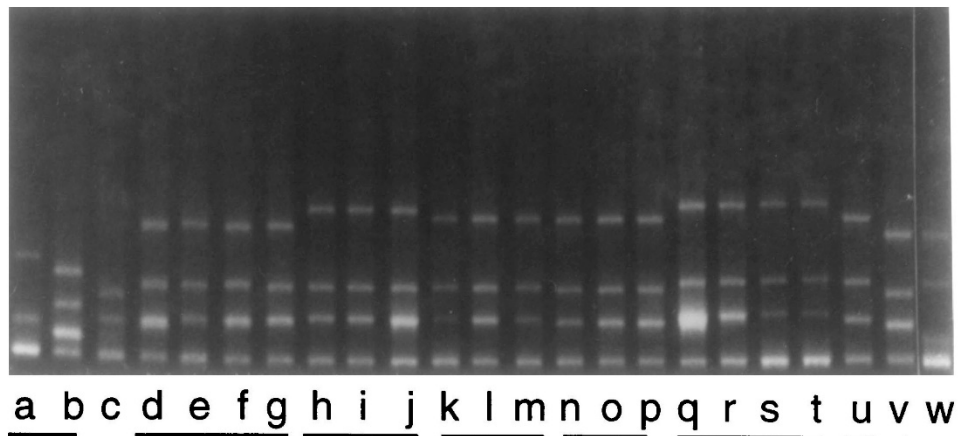
cific variability existed in the 16S sequence of *S. ochraceum* s.l. (Tang et al. 1996a). Representative results of HDA assays using the 16S sequence are shown in Figure 1. Little variation was found in the 16S rRNA gene among the eight remaining *Psilopelmia* species (Fig. 1).

The 16S rRNA HDA detected interspecific variation only in the case of *S. griseum* Coquillett and *S. mediovittatum* Knab. *Simulium griseum* produced heteroduplex products with diagnostic mobilities compared with the other species (Fig. 1, lanes r–s), while *S. mediovittatum* was differentiated from the remaining species by the unique mobility of its homoduplex band (Fig. 1, lanes k–l). Moreover, intraspecific variation was observed in *S. notatum* (Fig. 1, compare lane g with lanes f, h, and i) and *S. clarum* (Fig. 1, compare lanes m and o with lanes n and p). Some of these individuals produced heteroduplex patterns that were similar to those seen in *S. bivittatum* (Fig. 1, lane e), *S. trivittatum* (Fig. 1, lane q), and *S. robynnae* (Fig. 1, lanes t and u). On the other hand, black flies from other genera and subgenera produced HDA patterns that differed from those seen in flies in the subgenus *Psilopelmia*. For example, *A. bancrofti* (Fig. 1, lane a), *C. dacotensis* Dyar and Shannon (Fig. 1, lane b), *S. vittatum* s.l. (Fig. 1, lane c), and *S. encisoii* (Fig. 1, lane d) all produced heteroduplex patterns that were quite distinct from those seen within *Psilopelmia*.



**Figure 1.** Heteroduplex analysis of amplicons derived from the mitochondrial 16S rRNA gene of black flies in the subgenus *Psilopelmia*. The probe DNA was derived from *Simulium damnosum* s.l. Underlining groups individuals of the same species. Samples are as follows: *A. bancrofti* (lane a); *C. dacotensis* (lane b); *S. vittatum* s.l. (lane c); *S. encisoii* (lane d); *S. bivittatum* (lane e); *S. notatum* (lanes f–i); *S. longithallum* (lane j); *S. mediovittatum* (lanes k–l); *S. clarum* (lanes m–p); *S. trivittatum* (lane q); *S. griseum* (lanes r–s); and *S. robynnae* (lanes t–u).

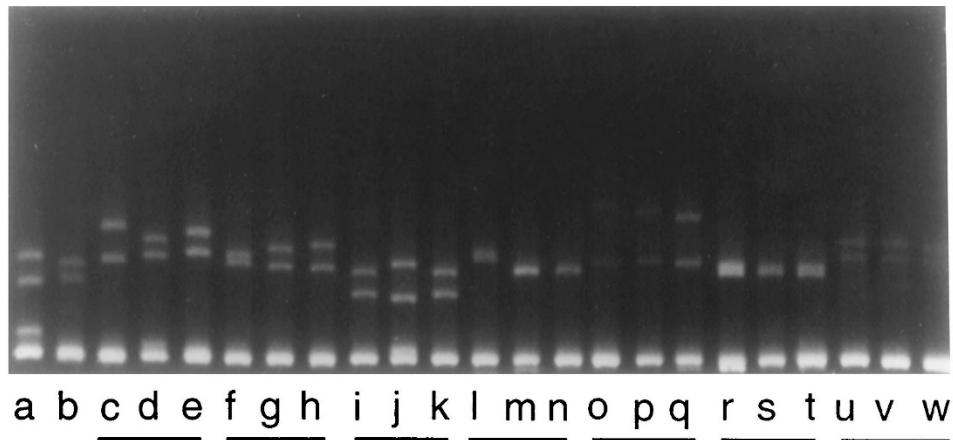
Additional tests were then performed using PCR products generated from the 12S rRNA gene. This amplicon is highly conserved in the sibling species of *S. damnosum* s.l. (J. Tang, unpublished data) and serves as a useful adjunct to the 16S rRNA amplicon in assays to distinguish some North American black fly species (Tang et al. 1996b). Representative results employing the 12S rRNA amplicon are presented in Figure 2. In contrast to the 16S-HDA, intraspecific variation was detected in *S. griseum* as well as in *S. ochraceum* s.l. No intraspecific variation was seen in the other species examined (Fig. 2). The 12S HDA was able to differentiate *S. bivittatum* from all other species, Fig. 2, lane *c*) and was capable of differentiating *S. trivittatum* (Fig. 2, lanes *q-t*) and *S. longithallum* (Fig. 2, lanes *h-j*) from the other *Psilopelmia* species (Fig. 2).



**Figure 2.** Heteroduplex analysis of 12S rRNA amplicons of black flies in the subgenus *Psilopelmia*: The probe was derived from amplicons generated from *S. damnosum* s.l. Underlining groups individuals of the same species. Samples are as follows: *S. ochraceum* s.l. (lanes *a* and *b*); *S. bivittatum* (lane *c*); *S. clarum* (lanes *d-g*); *S. longithallum* (lanes *h-j*); *S. robynae* (lanes *k-m*); *S. notatum* (lanes *n-p*); *S. trivittatum* (lanes *q-t*); *S. griseum* (lanes *u* and *v*); and *S. mediovittatum* (lane *w*).

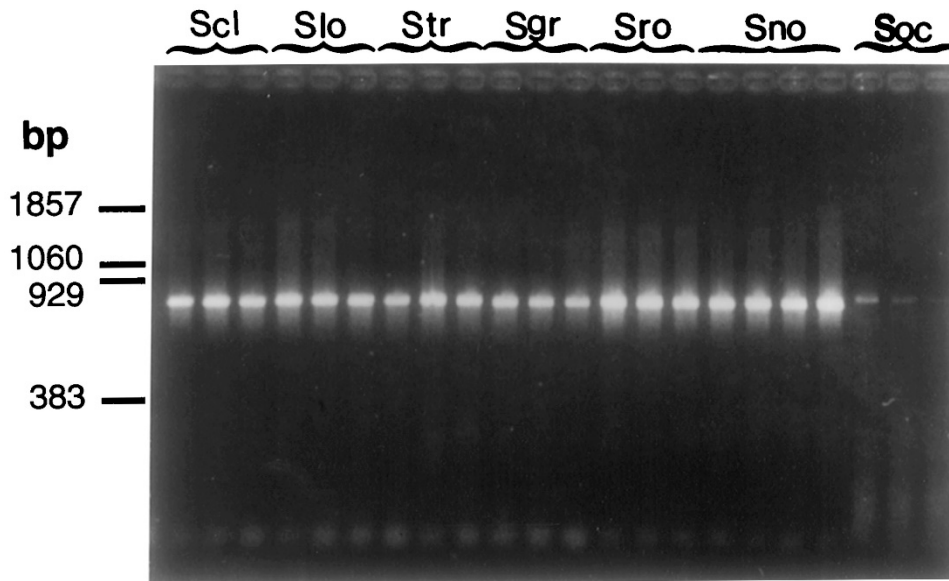
Based upon the results presented above, it was not possible to distinguish all of the *Psilopelmia* species on the basis of the 16S and 12S rRNA HDAs alone. Additional genes were therefore surveyed to identify an amplicon capable of distinguishing the species that were inseparable by means of either the 16S or the 12S HDA. An HDA based upon an amplicon derived from the COII was found to be suitable for this purpose. In the COII HDA, *S. robynae* (Fig. 3, lanes *i-k*) and *S. trivittatum* (Fig. 3, lanes *o-q*) could be differentiated from all other species by the distinct positions of their heteroduplex bands, despite the fact that intraspecific variation was evident in the *S. robynae* and *S. trivittatum* individuals tested. In contrast, *S. griseum* (Fig. 3, lanes *r-t*) and *S. mediovittatum* (Fig. 3, lanes *u-w*) did not show intraspecific variation in the COII HDA. The banding pattern of *S. griseum* was similar to that of *S. notatum* (Fig. 3, compare lanes *m* and *n* with lanes *r-t*), and the pattern produced by *S. mediovittatum* was similar to that exhibited by some of the *S. clarum* samples (Fig. 3, compare lane *d* with lanes *u-w*).





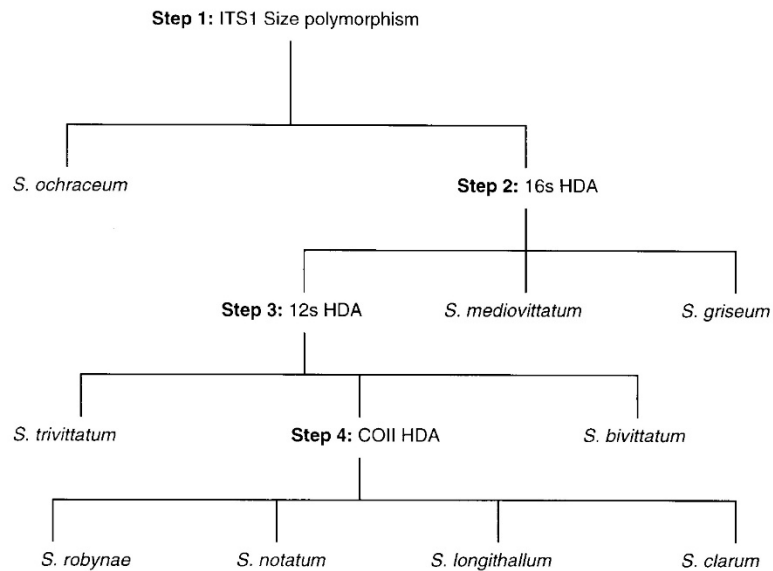
**Figure 3.** Heteroduplex analysis of COII amplicons of black flies in the subgenus *Psilopelmia*. The probe was derived from amplicons generated from *S. damnosum* s.l. Underlining groups individuals of the same species. Samples are as follows: *S. ochraceum* s.l. (lane a); *S. bivittatum* (lane b); *S. clarum* (lanes c–e); *S. longithallum* (lanes f–h); *S. robynae* (lanes i–k); *S. notatum* (lanes l–n); *S. trivittatum* (lanes o–q); *S. griseum* (lanes r–t); and *S. mediovittatum* (lanes u–w).

Previous studies have suggested that size differences in the internal transcribed spacer domain of the ribosomal gene cluster (ITS) might also be useful in distinguishing closely related black fly species (Brockhouse et al. 1993; Tang et al. 1996c). Because of this, the ITS1 domain was amplified using flanking primers derived from the 18S and 5.8S rRNA genes. The resulting amplicons were assayed for diagnostic size polymorphisms. Of the nine species tested, seven produced detectable PCR products (Fig. 4). Six of these species yielded a PCR product roughly 830 base pairs (bp) in size, while *S. ochraceum* s.l. produced an 870-bp fragment (Fig. 4).



**Figure 4.** Size polymorphisms in the ITS1 of the nuclear rRNA gene cluster in different *Psilopelmia* species: amplification of the ITS1 domain was carried out as described in "Materials and methods." The products were separated on a 1% MetaPhor™ agarose gel (FMC, Rockland, Maine) and stained in 2 µg/mL ethidium bromide. Lanes are labeled as follows: Scl, *S. clarum*; Slo, *S. longithallum*; Str, *S. trivittatum*; Sgr, *S. griseum*; Sro, *S. robynae*; Sno, *S. notatum*; Soc, *S. ochraceum* s.l.

The lack of interspecific variation in some sequences, together with the presence of intraspecific variability, made it impossible to rely on a single test to distinguish all nine species of *Psilopelmia* studied. However, a combination of HDA and ITS1 patterns could be used to develop a four-step process to distinguish all nine species. This classification scheme is outlined in Figure 5.



**Figure 5.** A proposed procedure for distinguishing nine species of black flies in the subgenus *Psilopelmia*.

## Discussion

Reliable methods are required to identify members of the *Psilopelmia* subgenus, especially adult females, which are difficult to distinguish from one another using morphological criteria. The most diagnostic life-cycle stage for *Psilopelmia* is the adult male, which may be separated using the shape and color pattern of the scutum (Moulton 1998). However, adult females are imperfectly separated using these criteria. For example, female *S. bivittatum* and *S. clarum* are not reliably separated morphologically. This is also true of the species pairs *S. griseum*/*S. notatum* and *S. longithallum*/*S. trivittatum* (Moulton 1998). Pupae of all species except *S. griseum* and *S. notatum* can be separated using the overall shape of the gill and the branching pattern and number of the gill filaments (Moulton 1998). Furthermore, larvae of *Psilopelmia* are extremely homogeneous. Peterson (1993) has published a method for keying *Psilopelmia* larvae. However, his key is useful for identifying only mature larvae, and one of the characters he uses, the number of primary head fan rays, is likely subject to environmental selection (Moulton 1998).

In contrast to morphological characters, molecular methods may be applied equally well to both sexes and to all stages of the insect's life cycle. The results presented above suggest that HDA in combination with size polymorphism in the ITS1 may be used to differentiate members of the subgenus *Psilopelmia*. Because of differences in the degree of intra- and inter-specific variation in the DNA sequences used in this study, it was not possible to rely upon a single assay to distinguish all species of *Psilopelmia*. However, as summarized in Figure 5, a combination of four assays was capable of distinguishing all nine species.

Practically, it may not always be necessary to carry out all four steps in the classification scheme. For example, as mentioned above, female *S. bivittatum* and *S. clarum* are not reliably separated morphologically but may be separated on the basis of the 12S HDA alone. In California these two species may be sympatric, and at least one of them (*S. clarum*) is a pest of humans and cattle (Anderson and Voskuil 1963; Peters and Womelfork 1966). The ability to distinguish adults of these species should permit further studies to be performed to reveal the most important pest of humans and domestic animals, allowing more efficient targeting of control measures.

The phylogenetic relationships among the nine *Psilopelmia* species examined in this study remains unclear. Coscaron and co-workers (Coscaron et al. 1996), using structural characters, attempted to deduce the relationships among species that they consider belong to *Psilopelmia*. This phylogeny was rooted by outgroup comparison with the four species-groups in the subgenus *Ectemnaspis* and with *Chirostilbia* Enderlein. Although distinct lineages within *Psilopelmia* were observed, formal species-groups were not designated. Based upon their phylogeny, the relationships among the species included in this study are as follows: *S. mediovittatum*/*S. bivittatum* + *S. trivittatum*/*S. longithallum*. The remaining species treated in this paper were not included in Coscaron's analysis. Three species-pairs, *S. bivittatum*/*S. clarum*, *S. griseum*/*S. notatum*, and *S. longithallum*/*S. trivittatum*, are more closely related to each other than to any other species, according to morphological evidence (Moulton 1998). The relationships among the species in this presumed group are unclear beyond those of the three species-pairs noted above.

It may be possible to use molecular data to help resolve the phylogenetic relationships among the members of the subgenus *Psilopelmia*. To accomplish this, candidate sequences that contain phylogenetically informative information need to be identified. Such sequences must exhibit minimal intraspecific variation but must also contain sufficient interspecific variation. HDA provides a rapid means of assaying for inter- and intra-specific variability within such candidate sequences. It can be used as a preliminary screening tool to identify potentially informative sequences prior to time-consuming DNA sequence analysis. For example, the minimal variation in the 16S HDA suggests that this sequence is not likely to contain sufficient phylogenetic information to resolve the phylogenetic relationships among the different *Psilopelmia* species studied here. In contrast, both the 12S and the COII HDA revealed considerable interspecific variation, suggesting that these sequences may be more promising candidates for phylogenetic analysis.

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