DNA PROBES FOR DISTINGUISHING *PSYCHODOPYGUS WELLCOMEI* FROM *PSYCHODOPYGUS COMPLEXUS* (DIPTERA: PSYCHODIDAE)

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Genomic DNA fragments from males of Psychodopygus wellcomei *were isolated and shown to be useful as sensitive diagnostic probes for positively separating individuals of this species from those of* Ps. complexus. *These two members of the* Ps. squamiventris *series are found sympatrically in foci of cutaneous leishmaniasis in the hill forests of southern Pará State. Of the two species, only* Ps. wellcomei *is thought to be an important vector of* Leishmania braziliensis *sensu stricto, but this is based on circumstantial evidence because of the difficulties of identifying female sandflies within the series. The diagnostic probes were isolated from a library of* Ps. wellcomei *built by ligating short fragments of* Sau 3A-*restricted, genomic DNA into the plasmid vector pUC 18. Differential screening of 1316 library clones with total genomic DNA of* Ps. wellcomei *and* Ps. complexus *identified 5 recombinants, with cross-hybridizing inserts of repetitive DNA, that showed strong specificity for* Ps. wellcomei. *As little as 0.4% of the DNA extracted from an individual sandfly (= ca. 0.5 nanograms) was specifically detected. The diagnostic probes were used to identify as* Ps. wellcomei *a wild-caught female sandfly found infected with* L. braziliensis s. s., *providing only the second positive association between these two species.*

Key words: phlebotomine sandflies – *Psychodopygus wellcomei* – *Leishmania braziliensis s. s.* – diagnostic DNA probes – Brazil

In November 1968, two of us (R. L. & J. J. S.) collected a new species of highly anthropophilic phlebotomine sandfly in the recently explored hill forests of the Serra dos Carajás, Pará State, where forest workers were shown to be at high risk of contracting cutaneous infection with *Leishmania braziliensis sensu stricto* (Lainson et al., 1973; Ward et al., 1973; Lainson & Shaw, 1979). Later described as *Psychodopygus wellcomei* by Fraiha et al. (1971), the male of this sandfly has a uniquely structured paramere, which distinguishes it from closely-related species in the *Psychodopygus squamiventris* series (Martins et al., 1978) and has been shown to be constant in F1 and F2 isofemale broods reared in the laboratory (Ready & da Silva, 1984). However, laboratory rearing of isofemale broods in Belém also revealed that *Psychodopygus complexus* (Man-

gabeira, 1941) is a good biological species (rather than being the male of *Psychodopygus squamiventris* (Lutz & Neiva, 1912) as concluded by Martins et al. (1968, 1978), and that the female is morphologically indistinguishable from the female of *Ps. wellcomei* (Ready et al., 1982, 1984; Ready & da Silva, 1984; Fraiha et al., 1986).

This problem of identification has made it difficult to investigate the transmission cycles of *Le. braziliensis s. s.* and related parasites in southeastern Amazônia, where *Ps. wellcomei* and *Ps. complexus* often occur in the same forests, as is true in the Serra dos Carajás (Ready et al., 1984). To date, natural infection with *Leishmania* has been found in more than 50 females of *Ps. wellcomei/complexus* from the Serra dos Carajás, and 35 of these infections have been positively identified as *Le. braziliensis s. s.* according to growth characteristics in hamsters and in culture, isoenzyme electromorphs or specific binding by diagnostic monoclonal antibodies (Lainson et al., 1973; Lainson & Shaw, 1979; Ryan et al., 1987a, b; Shaw et al., 1987). Unfortunately, only one of the 35

female sandflies found infected with *Le. brazi-liensis s. s.* has been positively identified as *Ps. wellcomei* — by rearing male progeny (Ryan et al., 1987a). Routine separation of the females of *Ps. wellcomei* and *Ps. complexus* has not been possible because (male) progeny are not easily reared and unequivocally diagnostic characters have not been identified by the usual methods of analysis, including multivariate morphological discrimination (Lane & Ready, 1985), electrophoretic separation of allo-enzymes (Ready & da Silva, 1984) and gas liquid chromatography (GLC) of cuticular hydrocarbons (Ryan et al., 1986). The latter analysis was the most promising, with 41 out of 43 (95.3%) laboratory-reared females being correctly identified according to quantitative differences in 15 GLC peaks, but there are doubts about its applicability for the routine identication of the remains of dissected indi-vidual female sandflies (Ryan et al., 1986; Ryan, pers. comm.).

In the present paper, we report the molecular cloning of short diagnostic DNA fragments of *Ps. wellcomei* that are highly repeated in the genome of this species but cannot be detected in either females or males of *Ps. complexus.*

MATERIALS AND METHODS

Phlebotomine populations — From 22 May to 2 June 1989, towards the end of the rainy season, males and females of *Ps. wellcomei* and/or *Ps. complexus* (together with numerous other phlebotomine species) were collected at night in Shannon traps and in CDC miniature light traps (Ready et al., 1983) set at ground level in different types of primary forest on the Serra dos Carajás property of the Companhia Vale do Rio Doce in southern Pará State ($6°S,50°20'W$) (Ward et al., 1973; Ready et al., 1984). The sites sampled (with altitudes above sea-level (a. s. l.)) were: N2 (700 m), Bahia (665 m, ca. 30 km west of N2), Parauapebas (350 m, on easterly descent to river of this name), and Paranapanema (200 m, in the valley of the river Parauapebas). All male sandflies and many of the females caught were snap-frozen in liquid nitrogen, whilst many other females were dissected during the field trip (see below).

Females of *Ps. complexus* were also captured, in April 1989, in CDC miniature light traps set at ground level in a patch of residual primary forest on the outskirts of Chiquita, municipality of Salvaterra, Island of Marajó, Pará State (Lainson et al., 1990). Some were allowed to engorge on a hamster and the progeny raised in the Instituto Evandro Chagas, Belém. First generation (F1) males (all *Ps. complexus*) and females were snap-frozen in liquid nitrogen.

All material for DNA studies was taken to London on cardice (= solid carbon dioxide) and then stored in a − 80 °C freezer.

Identification of sandflies and extraction of DNA — A binocular microscope with cold-light illumination was used at 60-120x magnification to sort sandflies placed in petri dishes on cardice. Members of the *Ps. squamiventris* series were easily identified by the characteristic external genitalia (males) and the combination of short fifth palpal segments together with straw-coloured abdomen and thoracic pleura contrasting with lightly-infuscated pronotum and darker prescutum, mesonotum and head (with its long subequal labrum). Each specimen used for molecular studies was individually dissected in a drop of TE solution [10mM Tris.HCl, 1mM EDTA pH 7.5] on a microscope slide placed on cardice: the head and terminalia were slide-mounted in Berlese fluid for morpho-logical and morphometric identification, and the thorax and anterior abdomen were trans-ferred to a 1.5ml snap-cap (Eppendorf) micro-centrifuge tube which was then placed in cardice.

DNA was extracted from each sample by the method of Ish-Horowicz (1982). The disposable plastic tip of a Gilson pipette was used to grind the sandfly remains in 15 μl of a neutral Tris buffer [10mM Tris.HCl pH 7.5, 60mM NaCl, 10mM EDTA, 0.15mM spermine, 0.15mM spermidine, 5% (w/v) sucrose], and the homo-genate then mixed with a further 85 μl of this grinding buffer together with 10 μl of a sodium dodecylsulphate (SDS) buffer [1.25% (w/v) SDS, 0.3M Tris.HCl pH 9.0, 0.1M EDTA, 5% (w/v) sucrose, 0.34% (v/v) diethylpyrocarbo-nate], before incubation at 65 °C for 45 min in order to lyse cells and denature proteins. After cooling the sample on ice, 30 μl of 8M potassium acetate was mixed with the homogenate, which was incubated on ice for 45 min before pelleting the contaminating proteins for 2 min at 14Kr. p. m. in a micro-centrifuge. The nucleic acids in the supernatant were then ethanol precipitated and washed three times in 70% ethanol (spinning

at 14Kr.p.m. in a micro-centrifuge), before drying the final pellet under vacuum and dissolving it in 15 μl of TE. Extracts were stored at 4 °C.

Construction of genomic library and screening for repetitive sequences of Ps. wellcomei — Genomic DNA was extracted from 36 males of *Ps. wellcomei* captured in Shannon traps set at ground level in evergreen seasonal forest (665m a.s.l.) at Km 33 of the Bahia-N1 road, on the Serra dos Carajás. The samples were pooled, phenol-chloroform extracted and the DNA ethanol precipitated overnight at − 20 °C in the presence of 0.3M sodium acetate. The DNA pellet was dried under vacuum, dissolved in 20 μl TE and digested overnight at 37 °C with the restriction endo-nuclease *Sau 3A* (Boehringer). The digestion products were phenol-chloroform extracted, ethanol precipitated, and then checked for molecular size and concentration by fraction-ating an aliquot, alongside standards, on a 1.0% agarose gel incorporating ethidium bro-mide. Employing standard techniques (Sam-brook et al., 1989), 40-50 ng of the restricted genomic DNA (mostly of molecular size less than 500 base pairs (bp)) was ligated into *Bam HI*-cut, phosphatased pUC 18 plasmid (Pharma-cia), using 375 ng of this vector in order to give a molar ratio of target-to-vector DNA of approximately 1:1 and, thereby, to reduce the likelihood of multiple insertions. Religated DNA was transformed into the recipient strain *Escherichia coli* XL1 and recombinant colonies were identified by their inability to degrade the lactose analogue X-Gal (Sambrook et al., 1989; Bullock et al., 1987).

Recombinant colonies were collected (using sterile toothpicks) on gridded, 9 cm-diameter L-ampicillin agar plates (to a maximum of 150 colonies/plate), and colony DNA was transferred to roundels of Hybond-N (Amersham) hybri-dization membranes by conventional methods (Sambrook et al., 1989). Transferred DNA was hybridized with 0.25 μg of nick-translated (Rigby et al., 1977) high molecular weight genomic DNA of *Ps. wellcomei* (from library source) and, following high stringency post-hybridization washes and overnight auto-radiographic exposure, colonies that gave strong or moderate signal were amassed on two duplicate master plates.

Identification and amplification of diagnostic clones, and screening against DNA of other species (by Southern and dot-blot hybridi-zations) − Recombinant colony DNA on the master plates was transferred to Hybond-N membranes and hybridized with nick-translated genomic DNA extracted either from *Ps. wellco-mei* (from source of library) or from *Ps. com-plexus* (from Chiquita, Marajó). Using standard techniques (Sambrook et al., 1989), some of the recombinant clones identified (by this differential screening process) as being diagnostic for *Ps. wellcomei* were amplified by the small-scale method of alkaline lysis. The "mini-prep." plasmid DNA so obtained was digested (singly and doubly) with the restriction endonucleases *Eco R1* and *Hind 111* before fractionation on agarose gels, bidirectional Southern blotting and differential hybridization with nick-translated total genomic DNA of *Ps. wellcomei* and *Ps. complexus* (sources as above) − in order to check the size and diagnostic value of the inserted sandfly DNA. The recombinant DNA of a smaller number of promising clones was then amplified by a large-scale method using alkaline lysis and caesium chloride-gradient purification (Sambrook et al., 1989). The diagnostic value of the inserted sandfly DNA was analysed by restriction digests and agarose-gel fractionation followed by: Southern blotting and differential hybridization with nick-translated total genomic DNA of *Ps. wellcomei* and *Ps. complexus;* or hybridization of the oligo-labelled inserts (Feinberg & Vogelstein, 1984) to the blotted genomic DNA of various phlebotomine populations and species.

DNA transfer to nylon hybridization mem-branes (Genescreen Plus, DuPont) was carried out by standard techniques: Southern blotting was by the capillary method; dot blotting was performed manually by direct application to the dry membrane of a series of 2 μl volumes from a Gilson pipette (each being allowed to dry), followed by *in situ* alkali lysis and neutral-ization; following transfer, the membranes were rinsed briefly in 2x SSC [0.3M NaCl, 0.03M tri-Na citrate] and air-dried (Sambrook et al., 1989; Ready et al., 1988; Smith et al., 1989). DNA probes were radio-labelled to a specific activity of greater than 10^8 c.p.m. μg^{-1} (Rigby et al., 1977; Feinberg & Vogelstein, 1984) and the solutions for pre-hybridization (4 h at 42 °C) and hybridization (overnight at 42 °C) consisted of 50% (v/v) formamide, 5x SSC, 5x Denhardt's solution, 0.5% (w/v)

SDS and, for hybridization, 100 μg/ml denatured sonicated salmon sperm DNA as well as 25 ng/ml denatured probe. Post-hybridization washes were either at low stringency (2x SSC, 0.5% (w/v) SDS, 65 °C) or at higher stringency (0.1x SSC, 0.5% (w/v) SDS, 65 °C), and autoradiography was at − 80 °C using Fuji-SX X-ray film and intensifying screens.

Identification of naturally-infected phlebotomines − Sandflies were dissected and the alimentary tracts searched for flagellate infections using phase contrast microscopy; infecting trypanosomatids were isolated by direct spreading on microscope slides and by inoculation into hamsters and culture media − to permit typing by zymodeme and monoclonal antibody analyses (Lainson & Shaw, 1987; Shaw et al., 1987). The thoracic and abdominal remains of infected flies were snap-frozen in liquid nitrogen, stored at not less than − 70 °C, and the DNA extracted and analysed by dot blotting as described above.

RESULTS

Differential screening of genomic library of Ps. wellcomei − Of 1316 recombinant clones screened with total genomic DNA of *Ps. wellcomei*, 122 gave strong or moderate signal after high stringency washes and overnight autoradiography and, therefore, were assessed as containing sequences that are highly or moderately repeated in the genome of this sandfly.

Differential screening of colony DNA with total genomic DNA of *Ps. wellcomei* and *Ps. complexus* demonstrated that the sequences of 18 out of the 122 "repetitive" clones were not detectable in *Ps. complexus*, whereas the sequences of just 5 clones were abundant in both species.

Genomic DNA probes diagnostic for Ps. wellcomei − Five of the 18 putatively diagnostic recombinant clones were selected for large-scale amplification, based on the marked differential hybridization of Southern-blotted "mini-prep." DNA with total genomic DNA of *Ps. wellcomei* and of *Ps. complexus*. Four of these clones contained an insert of ca. 370 base pairs (bp) and the insert of the fifth clone was ca. 185bp long. There was strong cross-hybridization between these 5 inserts (data not shown) and, therefore, only 2 were chosen for probing genomic DNA.

The insert DNA of the smaller recombinant (pPsw.105) and of one of the large recombinants (pPsw.33) was oligo-labelled and used to probe Southern-blotted and dot-blotted genomic DNA extracted from individual sandflies of various species. For males from several populations of *Ps. wellcomei* collected in the Serra dos Carajás, each probe detected 2 naturally-occurring *Sau 3A* restriction fragments that corresponded in size to the cloned lengths of the 2 probes (Fig. 1; Table); the tandem arrangement of these sequences is

TABLE

Origins of sandflies of *Psychodopygus squamiventris* series tested with diagnostic probes
(pPsw.33 and pPsw.105)

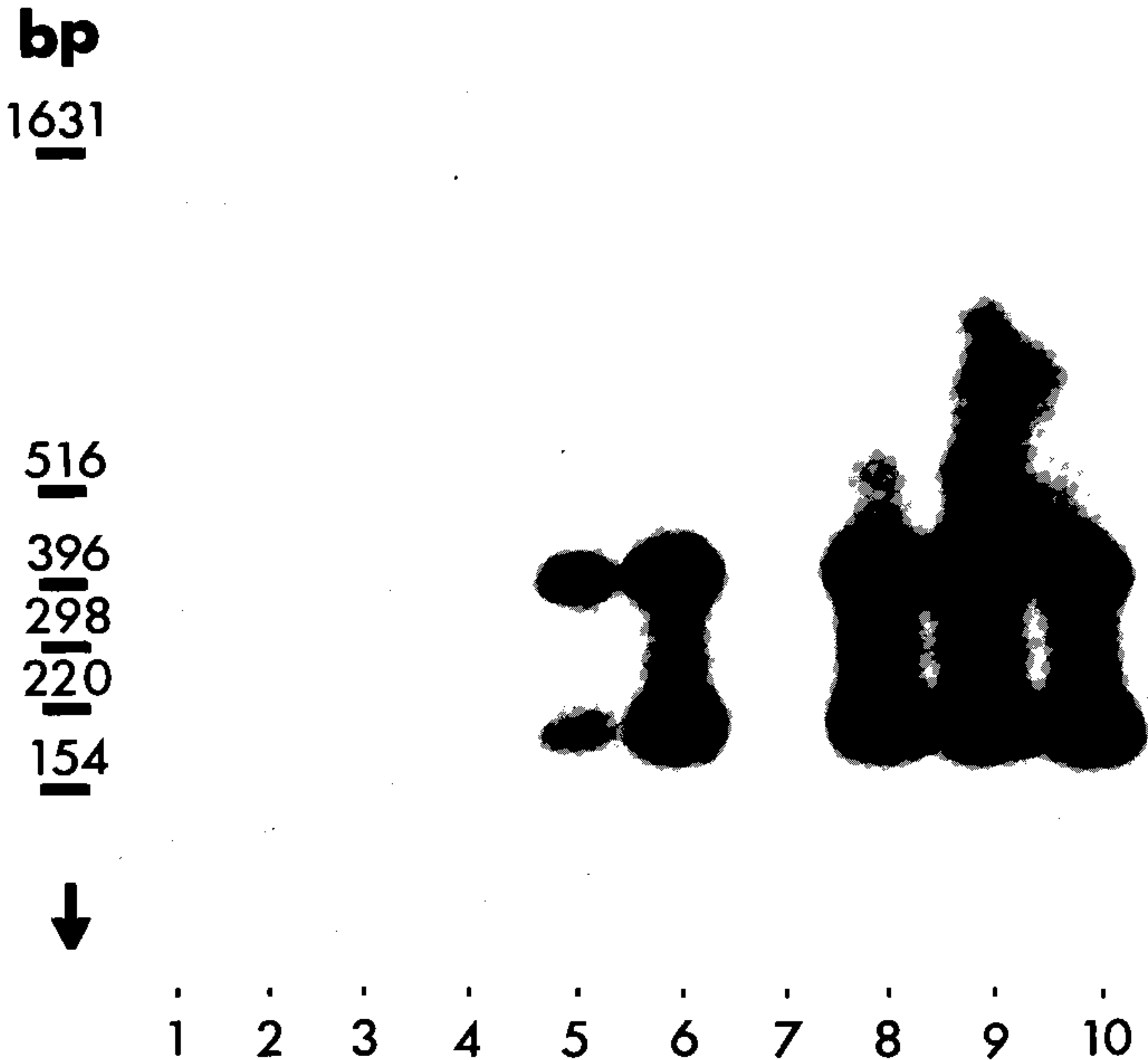|	Island of Marajó (Chiquita)	Serra dos Carajás (200 m a.s.l.)	Serra dos Carajás (350-700 m a.s.l.)
Ps. wellcomei males			
N⁰ + ve with probes	−	1	15
N⁰ − ve with probes	−	0	0
Ps. complexus males			
N⁰ + ve with probes	0	0	0
N⁰ − ve with probes	7	10	1
Ps. complexus link-reared females			
N⁰ + ve with probes	0	−	−
N⁰ − ve with probes	6	−	−
Wild-caught females			
N⁰ + ve with probes	−	−	7
N⁰ − ve with probes	−	−	2

Fig. 1: autoradiogram of Southern-blotted genomic DNA extracted from individual sandflies, following: — digestion with *Sau 3A*, size fractionation on a 1.2% agarose gel, capillary blotting, hybridization with the oligo-labelled insert of pPsw.105 (= diagnostic probe), low stringency washes and autoradiography for 2 days at — 80 °C. Size markers (bp = base pairs) = restriction fragments of plasmid pAT 153 digested with *Hinf 1*. Lanes: 1,2 = male *Ps. complexus* from Marajó; 3,4 = male *Ps. complexus* from Paranapanema, S. d. Carajás; 5 = male *Ps. wellcomei* and 6, 7 = female *Ps. wellcomei/complexus* from Parauapebas, S. d. Carajás; 8 = male *Ps. wellcomei* and 9,10 = female *Ps. wellcomei/complexus* from N2, S. d. Carajás.

indicated by the ladder pattern given by partially-digested DNA (e.g. Fig. 1, lane 9). Unfortunately, link-reared females of *Ps. wellcomei* were not obtained from blood-engorged females collected in the Serra dos Carajás in May 1989, because the eggs or larvae in all broods entered a state of dormancy and then died. Nevertheless, the specificity of the probes for *Ps. wellcomei* is clear because of their qualitative (all-or-nothing) reaction with DNA extracted from individual females (morphologically *wellcomei* or *complexus*) caught in the high altitude forests (350-700 m a. s. l.) of the Serra dos Carajás, where *Ps. wellcomei* predo-minates (Ready et al., 1984; Table): of 7 females tested, 2 gave no detectable reaction with the probes (e.g. Fig. 1, lane 7), whilst 5 gave a strong reaction with two *Sau 3A* fragments of the same size obtained from males (Fig. 1, lanes 6, 9, 10; Table).

In comparison, even with low stringency washes, it was uncommon to detect any signal when either of the diagnostic probes was hybridized with DNA extracted from link-reared or wild-caught *Ps. complexus* — from Marajó or the Serra dos Carajás, respectively (Fig. 1, 2; Table); weak signal given by only 2
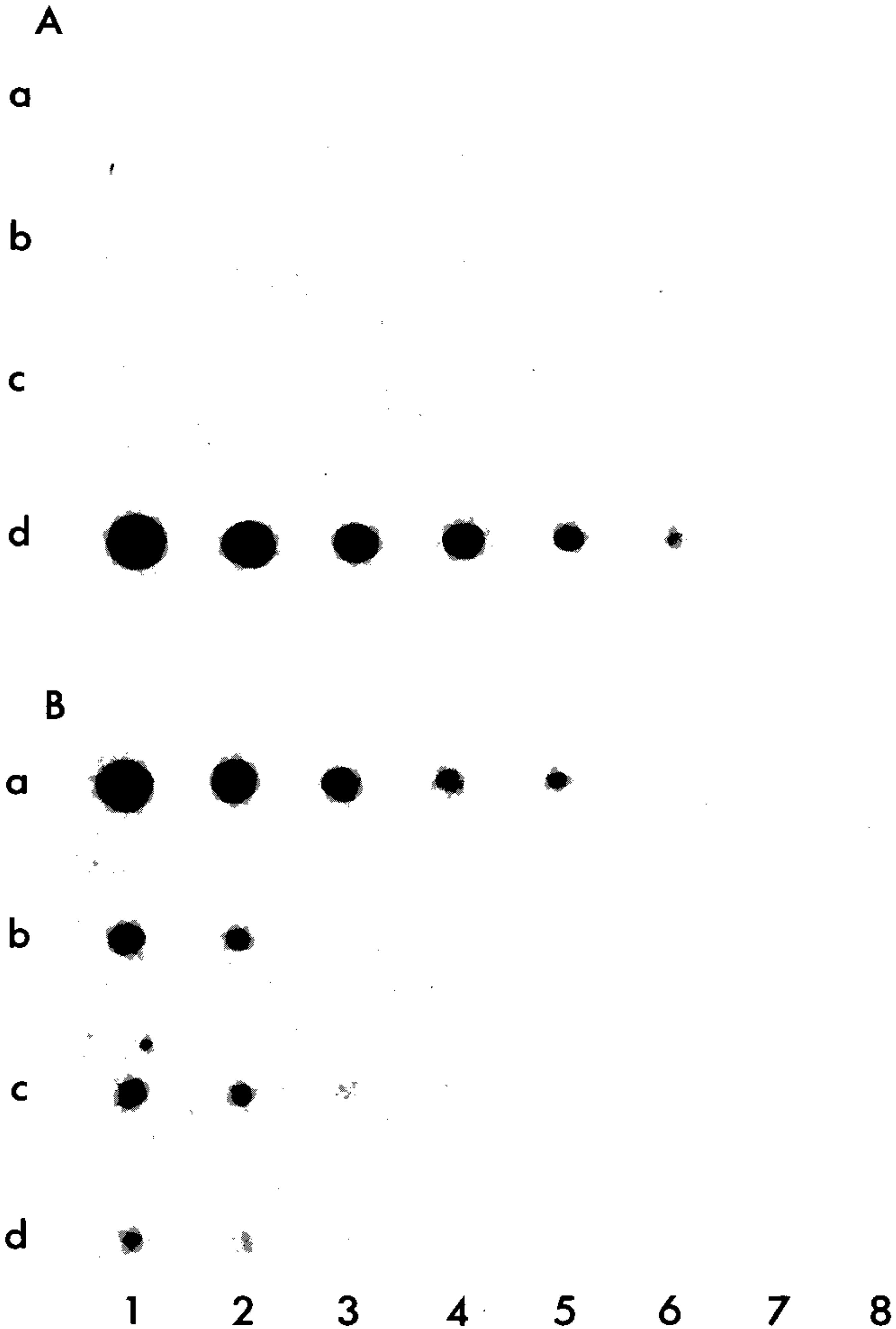
Fig. 2: autoradiogram of serial dilutions of dot-blotted genomic DNA extracted from individual sandflies, following: – sequential hybridization with the oligo-labelled insert of (A) pPsw.33 (= diagnostic probe) then (B) pPp.5.2 (= conserved 18S ribosomal DNA probe), low stringency washes and autoradiography for 2 days at – 80 °C. Dots 1-8 = 1:2 serial dilutions, starting with 50% of DNA extracted from each fly. a = female *Ps. complexus* from Marajó; b, c = male *Ps. complexus* and d = male *Ps. wellcomei* from Paranapanema, S. d. Carajás.
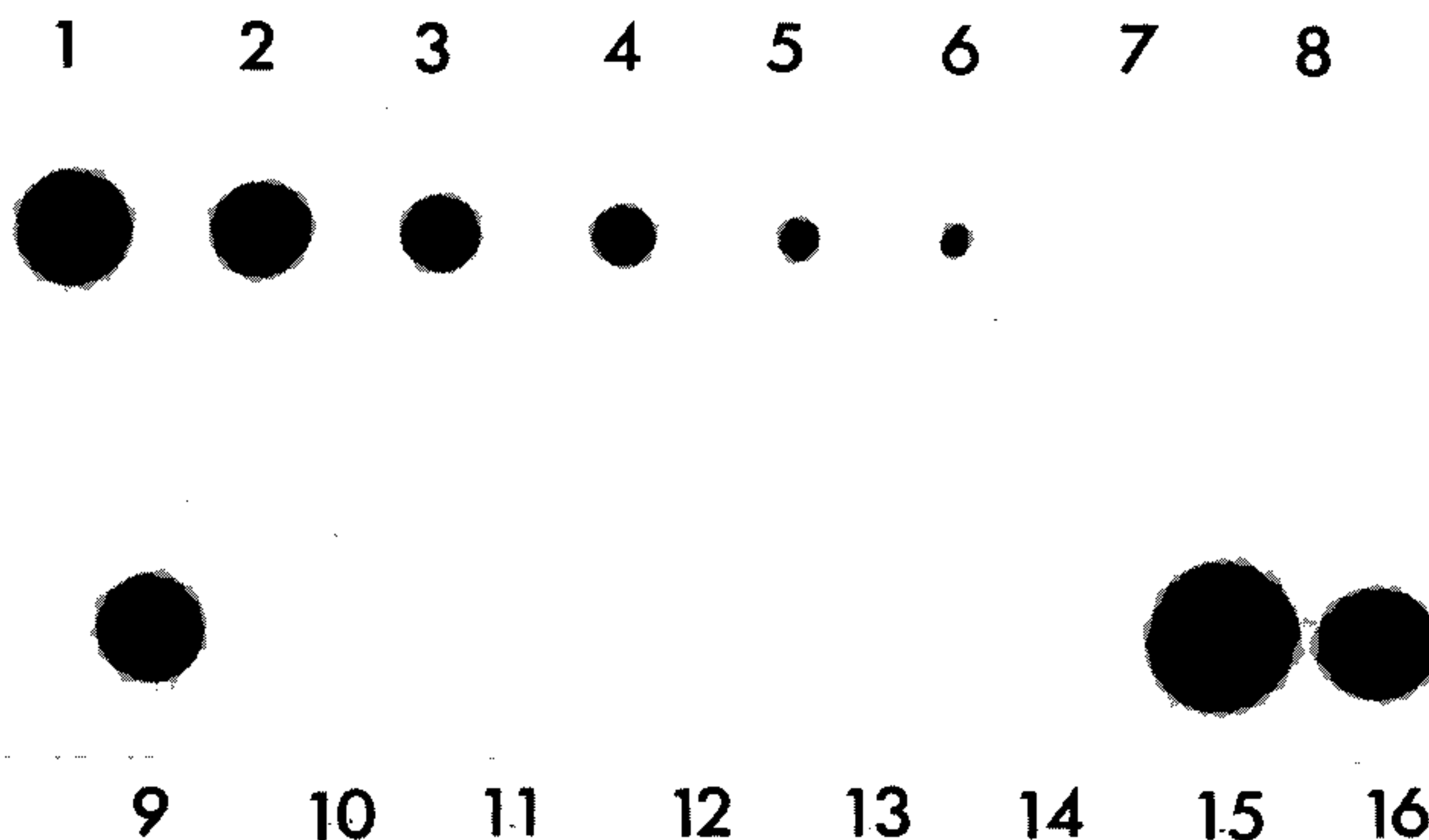
Fig. 3: autoradiogram of dot-blotted genomic DNA extracted from individual sandflies, following: – hybridization with the oligolabelled insert of pPsw.33 (= diagnostic probe), low stringency washes and overnight autoradiography at – 80 °C. Dots 1-8 = 1:2 serial dilution of DNA (starting with 50% of total extracted) from a female *Ps. wellcomei* from Parauapebas, S. d. Carajás; 9-14 = total DNA extracted from a male *Ps. wellcomei* from the same location, a male *Ps. amazonensis*, a female *Ps. hirsutus*, a male *Ps. geniculatus*, a female *L. longipalpis* and a female *L. furcata*; 15,16 = total DNA extracted from each of two infected female *Ps. wellcomei* from N2, S. d. Carajás.

of the 24 *Ps. complexus* tested (e. g. Fig. 2A b) was easily removed by high stringency washes. No signal was detected with DNA extracted from each of 2 males and 2 females of *Ps. amazonensis*, *Ps. davisi*, *Ps. geniculatus*, *Ps. hirsutus*, *Lutzomyia (Lutzomyia) longipalpis* or *L. (Viannamyia) furcata* (in part, Fig. 3).

As well as showing good specificity, each probe has a high sensitivity: in dot-blotted serial dilutions of genomic DNA it was possible to detect as little as 0.4% of the total DNA extracted from the thorax and anterior abdomen of an individual male of *Ps. wellcomei* (Fig. 2A); this was equal to approximately 0.5 nanograms (ng) of DNA (or about 1/250 of the total 125 ng extracted), as estimated by comparing sandfly extracts with DNA standards on ethidium bromide-stained agarose gels.

Figure 2 illustrates the sensitivity of one of the diagnostic probes (pPsw.33) compared with a 1.5 kb fragment of the 18S ribosomal (r) gene of *Phlebotomus papatasi* that is highly conserved between species of higher eukaryotes (Ready et al., 1988; unpublished results). A direct comparison of the signals in rows d of Fig. 2A and Fig. 2B indicates that in *Ps. wellcomei* the insert sequence of pPsw.33 is at least

30x as abundant as the ribosomal sequence, giving a copy number of not less than 5,000 per haploid genome.

Identification of naturally-infected Ps. wellcomei – From the remains of 2 female sandflies that had been caught on 30 May 1989 in the N2 site of Serra dos Carajás (see Ward et al., 1973), DNA was extracted, dot-blotted and hybridized with the diagnostic probe pPsw.33. Both females were positively identified as *Ps. wellcomei*: from one (Fig. 3, lane 15) a *Trypanosoma* sp. had been isolated, and from the other (Fig. 3, lane 16) the intestinal infection had been identified as *L. braziliensis s. s.* following screening with diagnostic monoclonal antibodies (Shaw et al., 1987).

DISCUSSION

Differential screening of genomic libraries has been used successfully to isolate DNA sequences diagnostic for individual species within several complexes of haematophagous Diptera, including the *Anopheles gambiae* complex (Gale & Crampton, 1987), the *An. dirus* complex (Panyim et al., 1988) and the *Simulium damnosum* complex (Post & Crampton, 1988). The morphological differences

between members of the *Ps. squamiventris* series are far greater than those within the other complexes and, therefore, it was not surprising that DNA sequences diagnostic for *Ps. wellcomei* were isolated relatively easily by the differential screening of a small number of recombinant clones containing short inserts.

These diagnostic DNA probes will be invaluable for assessing the relative vectorial importance of *Ps. wellcomei* and *Ps. complexus* in the hill forests of southern Pará, where these species are the only known representatives of the *Ps. squamiventris* series (Ready et al., 1984). Such assessments will entail not only the positive identification of infected female sandflies, but also studies on the biting habits and ecological niches of the two species. Routine, rapid DNA identifications of sandflies and infecting *Leishmania* will rely on simple "squash blotting" of flies, or parts of them (e. g. abdominal remains after dissection), as has been successfully performed with *P. papatasi* using a diagnostic rDNA probe that is considerably less sensitive than those produced for *Ps. wellcomei* (Ready et al., 1988). We have already used our diagnostic probes to identify as *Ps. wellcomei* a female sandfly found naturally infected with *L. braziliensis s. s.*, and clearly it was not necessary to load all of the DNA extracted from the thoracic and abdominal remains (Fig. 3, lane 16). This is only the second positive association of this sandfly species with *L. braziliensis s. s.*

The DNA probes that distinguish *Ps. wellcomei* from *Ps. complexus* have so far proved to be highly specific: homologous sequences have not been detected in sandflies of two neotropical subgenera of *Lutzomyia (Lutzomyia* and *Viannamyia)* nor in other species of *Psychodopygus* that frequently bite man in the forests where *Ps. wellcomei* abounds: when using external characters for rapid sorting of field catches, the females of *Ps. amazonensis*, *Ps. davisi*, *Ps. geniculatus* or *Ps. hirsutus* can each be confused with those of *Ps. squamiventris sensu lato* and, therefore, it is important to note that the diagnostic probes produced for *Ps. wellcomei* do not cross-hybridize with the genomic DNA of any of these species (in part, Fig. 3). We are investigating whether or not these probes cross-hybridize with the DNA of other members of the *Ps. squamiventris* series that are sympatric with *Ps. wellcomei* elsewhere in Amazônia.

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