Typing of *Simulium* using mitochondrial DNA PCR products with restriction endonuclease digestion

Several pathogenic agents have been identified (and the diseases they cause diagnosed) by using the polymerase chain reaction (PCR) to amplify some of their DNA and then subjecting this DNA to restriction endonuclease digestion (Yagita and Endo, 1990; Tachibana *et al.*, 1992).

In the present study, the cytochrome c oxidase subunit I (COI) region of blackfly mitochondrial DNA (mt DNA) was subjected to this method of analysis.

Three species of blackfly, *Simulium ochraceum*, *S. metallicum* and *S. colvini*, were collected in

Fig. 1. Electrophoretic profiles of PCR products digested with *AccII*, *HaeIII*, *HhaI*, *HinfI*, *MspI*, *RsaI*, and *Sau3AI*. c, *Simulium colvini*; m, *S. metallicum*; o, *S. ochraceum*; M, DNA size marker.

Fig. 2. Partial sequence (409 bp) of the COI region of mitochondrial DNA from a single female *Simulium metallicum* (Sm), compared with the sequence of the corresponding region (from 2358 to 2766) of *Drosophila yakuba* (Dy). Boxes show interspecific differences.

Guatemala and stored at -80°C until required. Pools of 10 frozen flies of each species were each homogenized in chilled buffer (0.25 M sucrose, 10 mM EDTA, and 30 mM Tris-HCl; pH 7.5) in a microhomogenizer to ensure that most of the cells were broken. Samples of mt DNA were then partially isolated (Tamura and Aotsuka, 1988) from these homogenates.

A single *S. metallicum* was also homogenized. The homogenate was treated with phenol/chloroform (1:1) twice and chloroform once and the DNA (including nuclear DNA) in it was then precipitated with alcohol.

The DNA samples were amplified in a thermal cycler (Perkin-Elmer Cetus) using CO-I-3 (5'GTNTGRGCNCAYCAYATRT-

TYAZNGT3') and CO-I-6 (5'GGRTAR-TCNZWRTANCGNCGNGGYAT3'; N = A, C, T or G; W = A or T; Y = C or T; Z = G or C) primers and 30 cycles of denaturation (95°C for 1 min), annealing (50°C for 2 min) and extension (72°C for 3 min). The PCR products were treated with PEG solution to remove residual primers and then subjected to digestion with nine restriction endonucleases: *AccII*, *AluI*, *HaeIII*, *HhaI*, *HinfI*, *MspI*, *RsaI*, *Sau3AI* and *Sau96I*. The double-stranded DNA fragments were then sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Excess dye terminators were removed with spin columns (Pharmacia)

and the termination fragments loaded onto a 373A DNA Sequencer (Applied Biosystems).

An approximately 470-base pair fragment of mt DNA from each species was amplified. The electrophoretic resolutions for *AluI* and *Sau96I* were poor and are not discussed further. *AccII*, *HhaI* and *Sau3AI* each gave a single band identical to that of undigested DNA but the remaining four enzymes gave useful banding patterns. It was possible to differentiate all three *Simulium* spp. using the patterns from *HaeIII*, *RsaI* and *MspI* and to separate *S. ochraceum* from the other two species using *HinfI* (Fig. 1).

Figure 2 shows a partial sequence of amplified mt DNA from a single *S. metallicum*, together with that of the COI region of *Drosophila yakuba* (Clary and Wolstenholme, 1985). The >80% homology between the two sequences indicates that the PCR blackfly product was amplified from the COI region.

Cytogenetic studies of Guatemalan *S. metallicum* and *S. ochraceum* have indicated that both are species complexes containing many sibling species and/or geographical strains (Hirai, 1983, 1987; Conn *et al.*, 1989). The results of the present, preliminary study indicate that PCR and endonuclease digestion could be applied to identifying these strains/sibling species.

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