# Genomic variability within laboratory and wild isolates of the trichostrongyle mouse nematode *Heligmosomoides polygyrus*

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**Abstract**

PCR-RFLP techniques have been used to characterize wild and laboratory isolates of the trichostrongyle nematode *Heligmosomoides polygyrus* from the wood mouse *Apodemus sylvaticus* and the laboratory mouse *Mus musculus* respectively. Both isolates can be distinguished by eight endonuclease digestions of the ITS region of the rDNA repeat namely, *Alu* I, *Dde* I, *Hpa* II, *Hae* III, *Hinf* I, *Hha* I, *Pvu* II and *Sal* I. In two of the digests, *Hinf* I and *Rsa* I, a minor polymorphism was observed in the wild isolate of *H. polygyrus* which has been cultured in laboratory-bred *A. sylvaticus* for several generations when compared with *H. p. polygyrus* from wild *A. sylvaticus*. A minor polymorphism was also identified in further wild isolates of *H. polygyrus* collected from *A. sylvaticus* in a field site in Egham, Surrey. However no evidence of polymorphism was observed in the laboratory isolate of *H. polygyrus* from the CD1 strain of *M. musculus* and the laboratory-bred *A. sylvaticus*. Reasons for this are discussed and further studies on the population genetics of *H. polygyrus* are suggested.

## Introduction

The trichostrongyle nematode *Heligmosomoides polygyrus*, an intestinal parasite of rodents, occurs as the wild subspecies *H. p. polygyrus* in natural populations of the wood mouse, *Apodemus sylvaticus* in Britain and Europe (Lewis, 1968, 1987; Lewis & Twigg, 1972; Gregory *et al*., 1990, 1992; Gregory, 1992). Another subspecies, *H. p. bakeri*, occurs in the laboratory mouse, *Mus musculus*. This mouse–nematode laboratory system has been used as an epidemiological and immunological model for human hookworm disease (Bartlett & Ball, 1972; Keymer, 1985; Behnke, 1990; Keymer & Slater, 1990).

The two subspecies of *H. polygyrus* are morphologically similar, except for some differences in their cuticular ridges at the scanning electron microscope level (Behnke *et al*., 1991), the degree of protein synthesis also differs in both subspecies (Abu-Madi *et al*., 1994a).

Polymerase chain reaction-linked restriction fragment length polymorphism techniques (PCR-RFLP) have previously been used to discriminate between closely-related species of protozoans (Brindley *et al*., 1993), trematodes (Andreson & Baker, 1993), cestodes (Bowles & McManus, 1993) and nematodes (Gasser *et al*., 1994). The present study explores the use of PCR-RFLP, or the internal transcribed spacer which includes ITS1, the 5.8s gene and

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ITS2 as well as partial regions of the large and small rRNA genes, to differentiate *H. p. bakeri* derived from the CD1 strain of the laboratory mouse *M. musculus* and the isolates of *H. p. polygyrus*, derived from laboratory-bred and wild populations of the wood mouse *A. sylvaticus*. This method codes ITS, a highly variable region, with 1100 bp and such variability is ideal for differentiating strains, subspecies and species.

## Materials and methods

### *Experimental design*

Doses of 100 L3 per 0.1 ml of water of *H. p. bakeri* and *H. p. polygyrus* were used to experimentally infect batches of 6-week-old naïve laboratory-bred CD1 mice and *A. sylvaticus* as previously described (Abu-Madi, 1994). Isolates of adult worms of *H. polygyrus* from these experimental mice were grouped as follows: group 1, *H. p. polygyrus* from wild *A. sylvaticus*; group 2, *H. p. bakeri* from the CD1 strain of *M. musculus*; group 3, *H. p. bakeri* from laboratory-bred *A. sylvaticus*; group 4, *H. p. polygyrus* from laboratory-bred *A. sylvaticus*. In addition, adult worms of *H. p. polygyrus* (group 5) from a natural population of *A. sylvaticus* in Egham, Surrey (Abu-Madi *et al*., 1994b) were used for comparison with experimental infections.

### *Worm extraction*

All groups of mice were killed by cervical dislocation under ether anaesthesia. The small intestine was isolated and opened longitudinally in Petri dishes containing PBS at a pH of 7.2 and incubated at 37°C for 1 h. Adult worms which had migrated out of the intestinal wall into the surrounding media were collected and washed several times in fresh PBS, placed in sterilized Eppendorph tubes, and stored at –70°C until required.

### *DNA preparation*

Single worms were crushed with a pipette tip in 1.5 ml Eppendorf tubes containing 10 $\mu$l of lysis buffer (50 mM KCl; 10 mM Tris, pH 8.3; 2.5 mM $MgCl_2$; 0.45% Tergitol NP-40; 0.45% Tween 20; 0.01% gelatin; 60 mg ml$^{-1}$ proteinase K). The homogenate was frozen at –80°C for 10 min, followed by incubating at 65°C for 1 h followed by 95°C for 10 min.

### *PCR amplification*

PCR reactions contained 10 $\mu$l 10× buffer (500 mM KCl; 100 mM Tris-HCl, pH 8.3; 15 mM $MgCl_2$; 0.1% gelatin (w/v); 1.0% Triton X-100), 1.0 $\mu$l of 20 mM dNTPs, 1.0 $\mu$l of 1000 $\mu$g ml$^{-1}$ forward and reverse primers, 4 units of *Taq* polymerase and 5 $\mu$l of worm lysate in a total reaction volume of 100 $\mu$l. The primer sets used were either Vrain *et al*. (1992):

18S (forward primer)
5′ TTGATTACGT CCCTGCCCTT T 3′
26S (reverse primer)
5′ TTTCACTCGC CGTTACTAAG G 3′

or Ferris *et al*. (1993):

forward
5′ CGTAACAAGG TAGCTGTAG 3′
reverse
5′ TCCTCCGCTA AATGATATG 3′

The product contained the ITS region flanked by short regions of the small and large ribosomal subunit genes. Amplification was carried out in a Techne PCH-3 thermocycler with a heated lid as follows: 2 min denaturation at 94°C, followed by 40 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min and a final 5 min extension at 72°C.

### *Restriction endonuclease digestion of PCR products*

PCR products were digested to completion using the following restriction endonucleases; *Alu* I, *Bst*O I, *Dde* I, *Dra* I, *Eco*R I, *Hae* III, *Hha* I, *Hin*d III, *Hinf* I, *Hpa* II, *Kpn* I, *Pst* I, *Pvu* II, *Rsa* I, *Sal* I, *Sau*3A I, *Sau*96 I, *Taq* I and *Xba* I in buffers supplied by the manufacturer. For all digestions, a sample of 5 $\mu$l of PCR product was digested with 0.5 $\mu$l restriction enzyme, 1 $\mu$l 10× enzyme buffer and 3.5 $\mu$l $dH_2O$. Endonuclease-digested PCR products were prepared for size fractionation on 1.5% (w/v) agarose gel containing 0.5 $\mu$g ml$^{-1}$ ethdium bromide by adding 1 $\mu$l of loading buffer to each digested DNA sample. The gel was run at 100 volts for 3 h and photographed under a UV transilluminator using a Polaroid camera.

## Results

PCR products of *H. p. polygyrus* from wild *A. sylvaticus* (group 1) and *H. p. bakeri* from CD1 strain *M. musculus* (group 2) digested with 19 endonuclease enzymes yielded a number of visible bands (fig. 1). From the 19 digests used, eight restriction enzymes (*Alu* I, *Dde* I, *Hae* III, *Hha* I, *Hinf* I, *Hpa* II, *Pvu* II and *Sal* I ) showed differences in the pattern of bandings between the both subspecies. This shows some evidence of polymorphism between both subspecies of *H. polygyrus* occurring in their respective natural hosts. The remainder of the digests namely: *Bst*O I, *Dra* I, *Eco*R I, *Hin*d III, *Kpn* I, *Pst* I, *Rsa* I, *Sau*3A I, *Sau*96 I, *Taq* I and *Xba* I all shared identical bands between both subspecies.

The DNA profiles of all 19 digests in *H. p. bakeri* from CD1 *M. musculus* (group 2) and laboratory-bred *A. sylvaticus* (group 3) were similar, indicating that no evidence of polymorphism existed in cross infected mice (data not shown). However, a minor polymorphism was observed between the DNA isolate of *H. p. polygyrus* (fig. 3) from laboratory-bred *A. sylvaticus* (group 4) compared with that (group 5) observed by Abu-Madi *et al.* (1994b). In the latter two isolates the patterns of banding were similar except with *Hae* III and *Rsa* I digests where minor changes were observed (fig. 2).

Individual isolates of *H. p. polygyrus* in wild *A. sylvaticus* from a field site at Egham, Surrey showed identical banding patterns, yielding two major bands at 691 and 564 bp. However, individual worms from the Egham wild mouse population showed a polymorphism with additional bands at 501 and 389 bp (fig. 3), although the methodology here utilized Vrain *et al.* (1992) rather than Ferris *et al.* (1993) primers. When this experiment was conducted the only primer set available to us was

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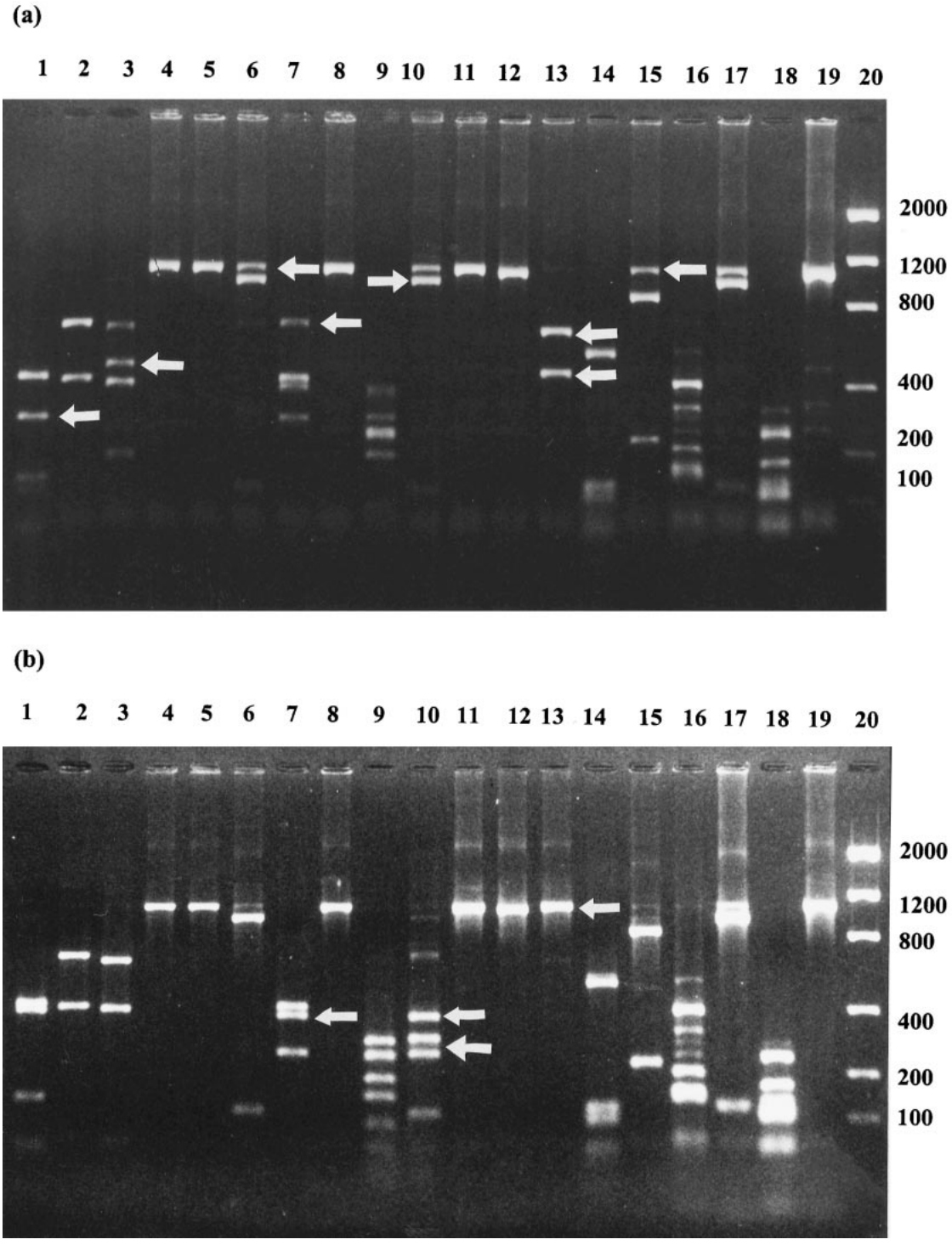


Fig. 1. Agarose-gel separation gel of ITS PCR product in *Heligmosomoides p. polygyrus* from *Apodemus sylvaticus* (a) and *H. p. bakeri* from CD1 *Mus musculus* (b), with eight restriction enzymes indicating differences in the pattern of banding (arrowed). Size in kilobases (kb) indicated at right. (1, *Alu* I; 2, *Bst*O I; 3, *Dde* I; 4, *Dra* I; 5, *Eco*R I; 6, *Hae* III; 7, *Hha* I; 8, *Hin*d III; 9, *Hinf* I; 10, *Hpa* II; 11, *Kpn* I; 12, *Pst* I; 13, *Pvu* II; 14, *Rsa* I; 15, *Sal* I; 16, *Sau*3A I; 17, *Sau*96 I; 18, *Taq* I; 19, *Xba* I; 20, marker.)

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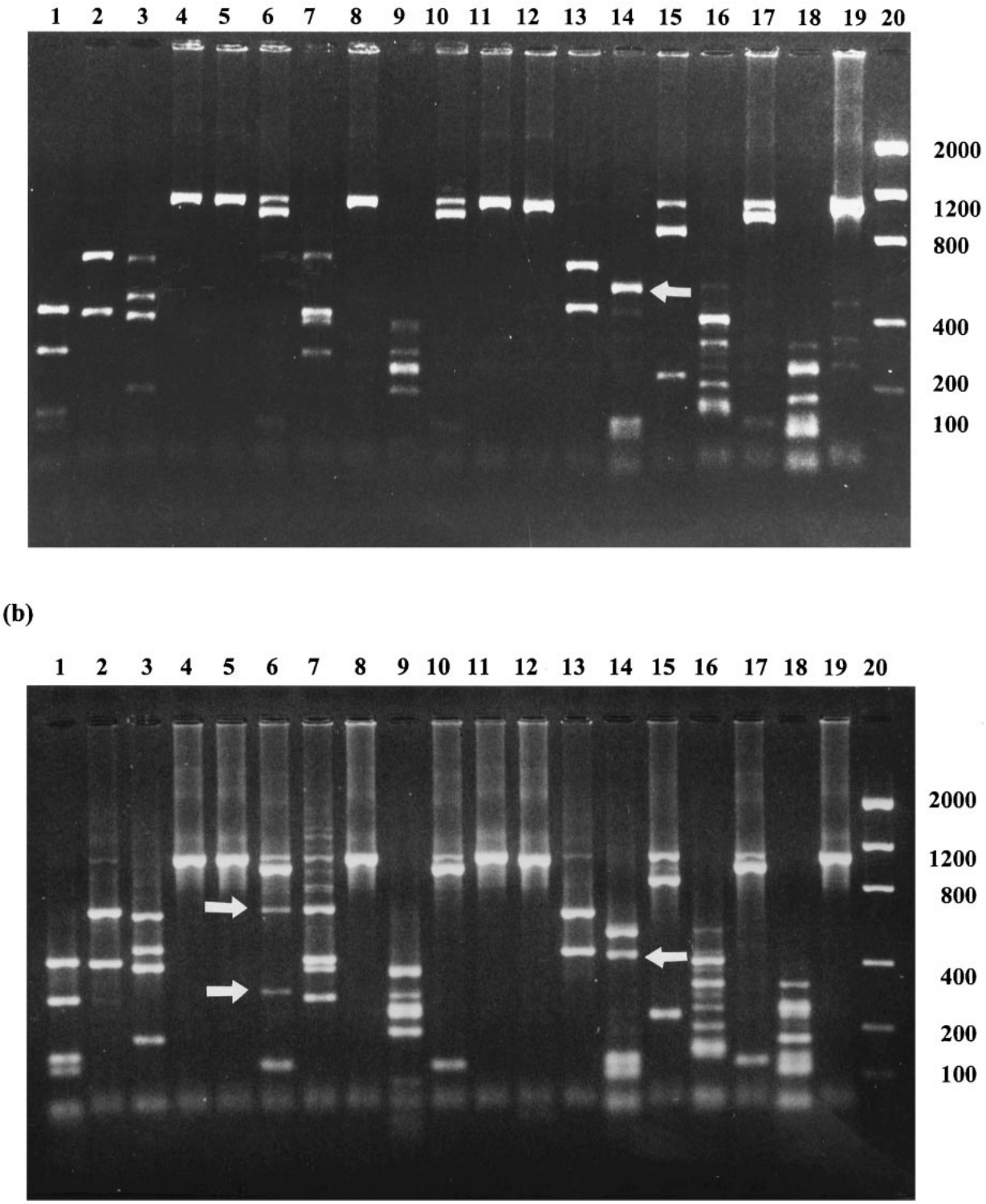


Fig. 2. Agarose-gel separation gel of ITS PCR product in *Heligmosomoides p. polygyrus* from laboratory-bred *Apodemus sylvaticus* (a) and wild *A. sylvaticus* (b) in group 5 mice, with two restriction enzymes indicating differences in the pattern of banding (arrowed). Size in kilobases (kb) indicated at right. (1, *Alu* I; 2, *Bst*O I; 3, *Dde* I; 4, *Dra* I; 5, *Eco*R I; 6, *Hae* III; 7, *Hha* I; 8, *Hin*d III; 9, *Hinf* I; 10, *Hpa* II; 11, *Kpn* I; 12, *Pst* I; 13, *Pvu* II; 14, *Rsa* I; 15, *Sal* I; 16, *Sau*3A I; 17, *Sau*96 I; 18, *Taq* I; 19, *Xba* I; 20, marker.)

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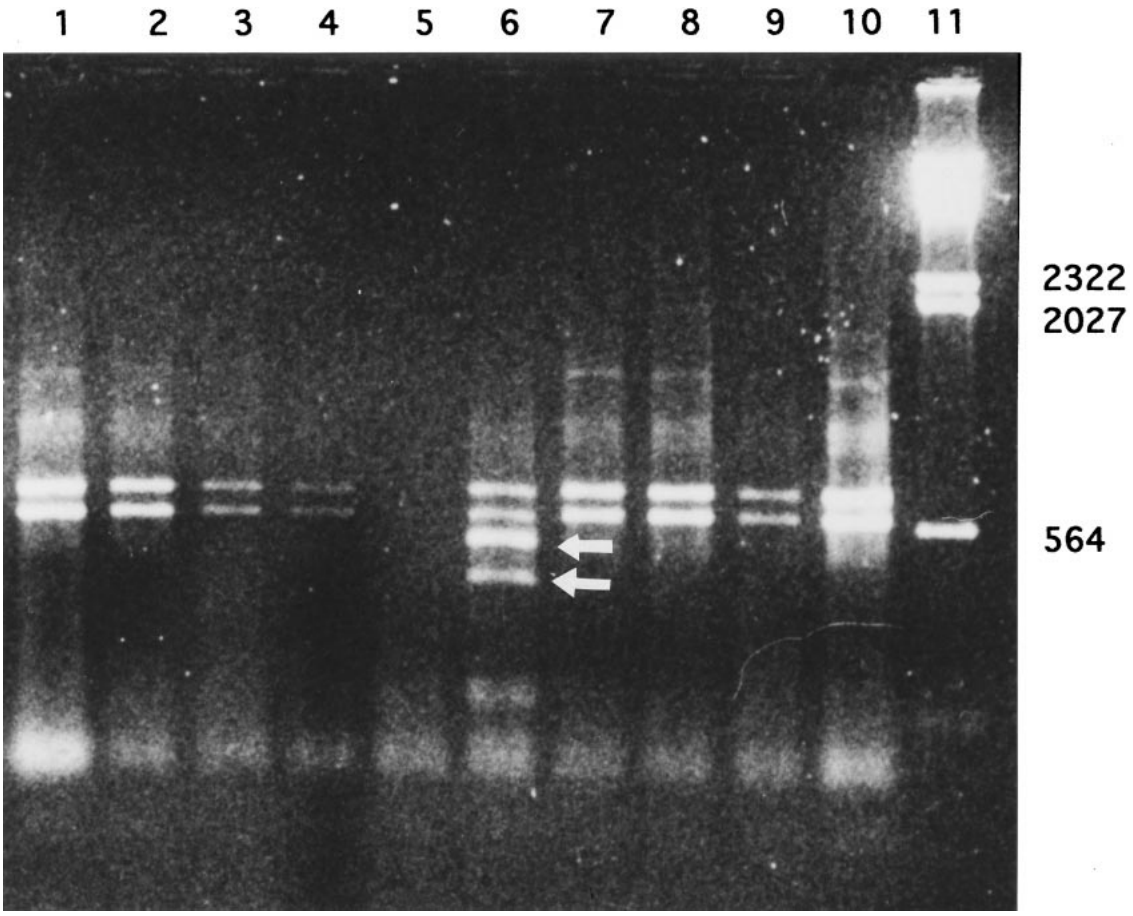


Fig. 3. Agarose-gel separation gel of ITS PCR product in *Heligmosomoides p. polygyrus* from wild *Apodemus sylvaticus* at Egham, with a single worm indicating differences in the pattern of banding (arrowed). Size in kilobases (kb) indicated on right. PCR products from ten individual worms digested with *Pvu* II (lanes 1–10). The primer set used was that of Vrain *et al*. (1992).

that of Vrain *et al*. (1992). Subsequent investigations showed that the Ferris primer set gave a much higher product yield for *H. polygyrus* and for this reason all other amplifications were carried out using these primer sets.

## Discussion

The polymerase chain reaction (PCR) has been used in recent years as an important tool for the identification of many parasite species. This molecular approach was taken in the present study because both subspecies of *H. p. polygyrus* and *H. p. bakeri* are similar morphologically (Durette-Desset *et al*., 1972) and hence are difficult to identify. However, some features can be used to be separate the two subspecies (Behnke *et al*., 1991) and these include the cuticle of *H. p. polygyrus* possessing fewer ridges than *H. p. bakeri*, but the latter possesses more cephalic ridges.

Abu-Madi *et al*. (1994a) used metabolic labelling of the proteins of *Heligmosomoides* with [$^{35}$S]-methionine to differentiate both subspecies. Coomassie blue profiles of the two isolates were similar, but a greater protein content was observed in *H. p. bakeri* when compared with *H. p. polygyrus*. The latter also incorporated less [$^{35}$S]-methionine into proteins than did *H. p. bakeri*.

The detection of RFLP's by DNA hybridization has previously been used to distinguish and isolate parasitic species including the genus *Schistosoma* (McCutchan *et al*., 1994; Rollinson *et al*., 1986), *Trichinella* (Dick *et al*., 1985; Chambers *et al*., 1986), *Echinococcus* (McManus & Rishi, 1989) and *Steinernema* (Hominick *et al*., 1997). Abu-Madi *et al*. (1994b) showed that RFLP analysis using DNA/DNA hybridization probed with pSn derived from clones of the entomopathogenic nematode *Steinernema* (Reid & Hominick, 1993), was a suitable method for the identification of both subspecies of *H. polygyrus*. This study showed that of eight digests used, only one digest when probed with rDNA clones *Pvu* II showed a clear difference between the two isolates. Abu-Madi *et al*. (1994b) also observed that, by using the same method with total genomic DNA hybridization, both subspecies demonstrated a further polymorphism, which was detected in the *Hin*d III digest.

In the present study a more advanced and sensitive PCR-RFLP technique, previously used by Dupouy-Camet *et al*. (1991) and Dick *et al*. (1992) in identifying *Trichinella* isolates, identified polymorphisms between the subspecies

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of *Heligmosomoides*. From the 19 endonucleases digests used, eight digests showed clear differences in the profiles of both subspecies of *H. polygyrus*. It was therefore possible to distinguish between the closely related wild and laboratory strains, *H. p. polygyrus* and *H. p. bakeri* by the digests *Alu* I, *Dde* I, *Hae* III, *Hha* I , *Hinf* I, *Hpa* II, *Pvu* II and *Sal* I which generated polymorphisms within rDNA repeat units of the two subspecies.

This technique, using *Hae* III and *Ras* I digests, also detected minor polymorphisms between DNA isolates of *H. p. polygyrus* which had been cultured in laboratory-bred *A. sylvaticus* for three years in comparison with the original *H. p. polygyrus* isolated from wild *A. sylvaticus* (Abu-Madi *et al*., 1994b). There are two possible explanations for these differences. Firstly, a genetic drift might have taken place in the laboratory-bred strain over the intervening years due to the effect of subculturing. Secondly, Abu-Madi (1994), using Vrain *et al*. (1992) rather than Ferris *et al*. (1993) primers, showed that individual worms in wild mice from the Egham site demonstrated some polymorphism (fig. 3), suggesting that the original laboratory-bred *H. p. polygyrus* strain might have arisen from a mixed population.

Recent studies have shown that the population genetic structure of parasitic nematodes is intimately related to the life history and ecology of the parasite and the ecology of the host (Blouin, 1998). Future studies on *H. polygyrus* should therefore focus on its population genetics in wild *A. sylvaticus* from field sites within the UK and Europe and to determine whether or not parasite mixing is promoted or not by host movement as reported by Blouin *et al*. (1995) for intestinal nematodes in *Peromyscus maniculatus*. Further work is also required in constructing a genomic library for *H. polygyrus* so that homologous probes may be sought to detect whether or not further polymorphisms exist. The use of other polymorphic markers, as an alternative to the advanced RFLP technique used in the present study, may require further investigation to characterize the genome of *H. polygyrus* and these include amplified fragment length polymorphism (AFLP) and/or the use of microsatellites (Roos *et al*., 1998).

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