Trypanosoma evansi: Genetic variability detected using Amplified Restriction Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD) analysis of Kenyan isolates.

Daniel K. Masiga[1, 2], Kariuki Ndung'u[3], Alison Tweedie[4], Andrew Tait[4] and C. Michael R. Turner[1,4*]

1. Division of Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK
2. Department of Biochemistry and Biotechnology, Kenyatta University, P.O. Box 43844, 00100 – Nairobi, Kenya
3. Trypanosomiasis Research Centre (KARI), P.O. Box 362, Kikuyu, Kenya.
4. The Wellcome Centre for Molecular Parasitology, The Anderson College, University of Glasgow, Dumbarton Road, Glasgow G11 6NU

Corresponding author:
Division of Infection and Immunity, IBLS, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ.
Phone: +44 141 3306629
Fax: +44 141 3302041
Email: m.turner@bio.gla.ac.uk

Abstract

We compared two methods to generate polymorphic markers to investigate the population genetics of *Trypanosoma evansi*; random amplified polymorphic DNA (RAPD) and amplified restriction fragment length polymorphism (AFLP) analyses. AFLP accessed many more polymorphisms than RAPD. Cluster analysis of the AFLP data showed that twelve *T.evansi* isolates were very similar ('type A') whereas two isolates differed substantially ('type B'). Type A isolates have been generally regarded as genetically identical but AFLP analysis was able to identify multiple differences between them and split the type A *T. evansi* isolates into two distinct clades.

Key words: *Trypanosoma evansi*, polymorphism, AFLP, RAPD, *Trypanosoma brucei*.

1. Introduction

Trypanosoma evansi is the most widespread of the pathogenic salivarian trypanosomes (Luckins and Dwinger 2004). This species is thought to have evolved from *T. brucei* by adaptation to mechanical transmission enabling it to spread beyond the tsetse belt in Africa (Hoare 1972). *T. evansi* can cause significant disease in camels, horses, cattle and water buffalo in particular (Luckins 1988; Lun et al 1993; Reid 2002).

Several studies have shown that *T. evansi* is genetically related to, but distinct from, *T. brucei*. One of the key distinguishing features is the absence of maxicircles in *T. evansi* compared with *T. brucei* (Borst et al 1987; Ou et al, 1991; Songa et al, 1990). *T. evansi* also has essentially homogeneous minicircles (Borst et al 1987; Songa et al, 1990; Masiga and Gibson, 1990; Lun et al 1992). Several studies using isoenzymes found that isolates of *T. evansi* from

many different parts of the world were genetically homogeneous (Gibson et al 1983; Stevens et al, 1989). Similar conclusions have been made using Restriction Fragment Length Polymorphism (RFLP) (Songa et al, 1990), microsatellite (Biteau et al 2000) and Random Amplified Polymorphic DNA (RAPD) analysis (Ventura et al 2002; Lun et al 2004). The *T. evansi* species seems to exist as a single clonal lineage with exceptions to this general finding having been found in only a few isolates from Kenya (Gibson et al 1983; Njiru et al 2005) and Sudan (Boid, 1988). The majority of isolates have been collectively termed 'type A' and the few exceptions in East Africa 'type B' (Masiga and Gibson 1990).

To study further genetic variability in *T. evansi*, we employed two methods, Random Amplified Polymorphic DNA (RAPD) PCR (Welsh and McClelland, 1990; Williams et al, 1990) and the Amplified Restriction Fragment Length Polymorphism (AFLP) (Vos et al, 1995) techniques. Both these methods are genome-wide profiling techniques that do not require prior availability of any sequence data and are therefore applicable to any organism. Each has its strengths and weaknesses. RAPD PCR relies on the use of a single primer for PCR at low stringency; is simple, fast and requires very little DNA template. It can be difficult to generate reproducible data however because of the low PCR stringency. AFLP is a DNA profiling technique based on the amplification of restriction fragments by PCR (Vos et al, 1995; Masiga and Turner 2004) and can detect large numbers of DNA polymorphisms by combining the reliability of restriction enzyme digestion (similar to RFLP) and the robustness of high stringency PCR.

Our aim in this study was to address three questions. How do these two methods compare in accessing information on polymorphisms in *T. evansi*? Can either or both methods confirm the findings from previous studies (using isoenzyme or RFLP methods) that *T.evansi* isolates comprise a single clonal lineage? Is there any evidence for population sub-structuring within type A isolates?

2. Materials and Methods

2.1 Source of trypanosomes

Fourteen *T. evansi* and two *T.b. rhodesiense* stocks (KETRI 3176 and 3186) were obtained from the Trypanosomiasis Research Centre, KARI (formerly the Kenya Trypanosomiasis Research Institute, KETRI). The origins of each isolated stock from Kenya are given in Table 1. The origins of the three other *T. brucei* isolates used (STIB 247, STIB 386 and TREU 927) have been previously described (Turner et al, 1990).

2.2 Growth of parasites and isolation of DNA

T. evansi isolates, together with KETRI 3176 and 3186, were grown in mice and purified from blood by anion exchange chromatography (Lanham and Godfrey, 1970). STIB 247, STIB 386 and TREU 927 were grown as procyclics in SDM79 medium supplemented with 10% fetal calf serum (Brun and Schonenberger, 1979) and recovered by centrifugation at 1000 g for 5 minutes. Trypanosome DNA was extracted by published methods (Sambrook, Fritsch and Maniatis, 1989).

2.3 RAPD analysis

Six decamer primers of random sequence were made (Cruachem, Glasgow, UK), evaluated and three of them found suitable for use in this study. These were Primer 1 (AACGCGCAAC), Primer 2 (CCCGTCAGCA) and Primer 3 (CCCGTCAGCA).

RAPD analysis was performed in 20µl reaction volumes containing 200µM dNTPs, 0.5 µM primer, 4 mM $MgCl_2$, 1.25 units of *Taq* Polymerase, PCR buffer (Applied Biotechnologies) and 10-20 ng genomic DNA. PCR reactions were carried out in a PE 2400 thermal cycler (Perkin Elmer) using the following profile: 94 ^{o}C for 1 min, 36 ^{o}C for 2 min and 72 ^{o}C for 1 min for 30 cycles, and a final extension at 72 ^{o}C for 5 min. PCR products were size-separated by electrophoresis on a 1.2 % agarose gel, stained with ethidium bromide and visualised under UV illumination.

2.4 AFLP

AFLP analysis was conducted as previously described (Masiga and Turner 2004) using the AFLP analysis system II (Life Technologies, UK) and following manufacturer's instructions, except for the *Mse*I primers which were custom made (Cruachem, Glasgow, UK). Briefly, trypanosome genomic DNA (approximately 250 ng) was digested to completion with *Eco*RI and *Mse*I by incubation for 3 h at 37^{o}C with 5 units of each enzyme in a 20µl reaction volume. Adapters for *EcoR*I and *Mse*I were then added to the reaction and ligated to the restriction fragments at 20^{o}C for 2 h using T4 DNA ligase. The reactions were then heat-inactivated at 70^{o}C for 10 minutes before two rounds of PCR amplification. The first round was carried out with primers specific for the adapters, *EcoR*I (core primer with no selective nucleotides) and *Mse*I (core primer with one cytidine as selective nucleotide). Amplification was performed for 20 cycles by denaturation at 94^{o}C for 30s, annealing at 65^{o}C for 30s and extension at 72^{o}C for 1 min. PCR products were then diluted 5-fold in TE buffer (10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA) and used for selective amplification with a primer specific to the *EcoR*I adapters plus two selective nucleotides (E-TA) and primers specific to the *Mse*I adapters plus two selective nucleotides (M-CA and M-CT). The *Eco*R1 primer was radiolabelled with [γ-^{33}P] dATP. Selective PCR amplification was carried out as follows: two cycles were performed at 94^{o}C for 30s, 65^{o}C for 30s and 72^{o}C for 1 min. The same conditions for denaturation and extension were maintained for 12 cycles, while the annealing temperature was stepped-down by 0.7^{o}C for each cycle, to 56^{o}C. This was followed by 23 cycles, denaturing at 94^{o}C for 30s, annealing at 56^{o}C for 30s while the extension step was at 70^{o}C for 1 min.

To visualise the products, each amplification reaction was mixed with an equal volume (20µl) of formamide dye (98% formamide, 10 mM EDTA, pH 8.0 with bromophenol blue and xylene cyanoll as tracking dyes). The mixtures were heat-inactivated for 3 min at 95^{o}C and chilled on ice. Three microlitres of each sample was loaded on a 6 % denaturing polyacrylamide gel (Sequagel XR solution, National Diagnostics, USA). Electrophoresis was carried out at 55 W with 100 mM Tris/100 mM Boric acid/2 mM EDTA as running buffer. The gels were transferred on to Whatman 3MM paper and dried for 2 h at 80^{o}C under vacuum. The dry gel was then exposed to x-ray film for at least 12 h at -70^{o}C and autoradiograms were then read manually. Gels were then re-exposed for a longer time period (at least 3 days) to overexpose the films for definitive scoring.

To investigate the degree of relatedness of isolates using AFLP markers we measured Jaccard's similarity index using an unweighted arithmetic average clustering method in the Clustering Calculator software (http://www2.biology.ualberta.ca/jbrzusto/cluster.php). Dendrograms were plotted in TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.5 PCR analysis

A PCR assay for kDNA maxicircles was used to distinguish *T. brucei* from *T. evansi*. Primers were designed to the 9S ribosomal RNA subunit (kDNA 12: 5'-TTAATGCTATTAGATGGGTGTGC-3' and kDNA 13: 5'-CTCTCTGGTTCTCTGGGAAATCAA-3'). PCRs were performed as previously described (MacLeod et al 1999) except that cycling conditions were 95 ^{o}C for 50s, 55 ^{o}C for 50s and 65 ^{o}C for 50s for 30 cycles. As controls, PCRs were also conducted on the triosephosphate isomerase (TIM) gene using primers E and F (MacLeod et al 1997).

3. Results

3.1 RAPD

The results of RAPD analysis are shown in Fig 1 for Primers 1 and 2. Fig 1A shows the RAPD analysis of mouse DNA and four *T. evansi* stocks. The banding profile for mouse DNA is different from that of *T. evansi*, indicating that there was no contamination of the trypanosome samples with mouse DNA. Fig 1B and C show results of RAPD analysis of 13 *T. evansi* isolates compared with three *T. brucei* isolates. The differences between stocks appear as the presence/absence of amplified bands and for both primers there are several polymorphic bands that enable the three *T. brucei* and a number of the *T. evansi* stocks to be distinguished from each other. The overall impression is of few polymorphisms amongst the *T. evansi* stocks and little genetic distinction between *T. evansi* and *T. brucei*. Ten bands polymorphic amongst the *T. evansi* stocks are indicated, but four of these result only from the presence of KETRI 2737 in the population sample. A number of the *T. evansi* samples appear to be identical; KETRI 3271 and 3292 and KETRI 2458, 3109 and 3295.

3.2 AFLP

The use of just two sets of primer pairs for AFLP resulted in 66 polymorphic markers thus demonstrating the capability of this technique to identify polymorphisms on a scale difficult to achieve using RAPD. Fig 2 illustrates a representative AFLP separation. A number of monomorphic bands are marked that are common to all *T. evansi* and *T. brucei* isolates and indicate the close phylogenetic relationship of these two species. These monomorphic bands act as internal controls verifying the reproducibility of the AFLP technique. 30 markers discriminated *T. brucei* from *T. evansi* or were polymorphic amongst the four *T. brucei* isolates whilst 36 markers were polymorphic within the group of *T. evansi* isolates. 14 of these are shown in Fig 2; the other 22 were identified using the second primer combination.

A dendrogram of the similarity matrix of the AFLP data showed that 12 of the *T. evansi* samples clustered together tightly whereas the other two, KETRI 2737 and 3116, appeared more similar to the four *T. brucei* samples. Of the 12 clustered samples, two have been identified previously as type A *T. evansi* – KETRI 2454 (Ngaira et al 2005) and KETRI 2439 (Njiru et al 2005). The bootstrap values for many nodes of the dendrogam were rather low however which is a common difficulty in dendrogram construction that combines groups of samples that are very different with groups that are very similar. A cluster analysis using only the *T. evansi* samples generated a more robust dendrogram as shown in Fig 3. Two isolates, KETRI 2737 and 3116, were markedly different from each other and separated from the type A isolates by 26 AFLP markers. The genetic differences between these two isolates and the main *T. evansi* group raised the possibility that these were two *T. brucei* isolates that had been misidentified. To test for this possibility we undertook PCR amplification using a maxicircle-specific marker. No PCR product was detected in either KETRI 2737 or 3116 however, indicting that they were not misidentified *T. brucei* isolates (Fig 4). The marker was present in two *T. brucei* isolates and absent from two type A *T. evansi* isolates acting as biological positive and negative controls respectively. A fragment of the TIM gene amplified

successfully from all six samples as a positive control for the presence and quality of DNA in each case. Thus, these data show the absence of the kinetoplast maxicircles in KETRI 2737 and 3116 establishing that they are *T. evansi* isolates.

The most interesting finding was that 10 of the 36 markers that detected polymorphisms amongst all *T. evansi* isolates were able to discriminate amongst the 12 type A isolates and split them into two robust clades (Fig 3).

4. Discussion

We have compared AFLP and RAPD methods to detect genetic polymorphisms in *T. evansi* and used the former to determine the genetic relatedness of 14 isolates from northern Kenya. The RAPD method was able to access only limited numbers of polymorphisms in agreement with previous studies (Waitumbi and Murphy 1993; Watanapokasin et al 1998; Ventura et al 2002; Claes et al 2003; Lun et al 2004) whereas AFLP was markedly more successful. We are aware of only one other report using AFLP with *T. evansi* but it is unclear how much genetic variability was accessed amongst the four isolates studied as part of an extensive phylogenetic analysis of several *Trypanosoma* species (Agbo et al 2002).

Our study has shown using AFLP that there is considerable genetic diversity amongst *T. brucei* isolates compared with *T. evansi* in agreement with Agbo et al (2002) and other investigations using different methods; for example isoenzymes (Gibson et al 1983), microstallites (Biteau et al 2000) and RAPDs (Lun et al 2004). AFLP identified two of the 14 *T. evansi* stocks as being very different from the others. The clear genetic relatedness of these two isolates to four East African *T. brucei* isolates raised the possibility that they were either type B *T. evansi* or *T. brucei* isolates from camels that had perhaps been misnamed (Njiru et al 2005). The most extensive evidence for type B has come from Kenya (Gibson et al 1983; Ngaira et al 2005; Njiru et al 2005) where tsetse flies are also present, potentially transmitting *T. brucei*. PCR of kDNA maxicircles indicated however that these isolates were not *T. brucei* and lacked maxicircles. In view of the overwhelming evidence in favour of two types of *T. evansi*, A and B, that are genetically quite different, the possibility has to be considered that this species is polyphyletic. We would hypothesise that there were two independent origins of *T. evansi* from *T. brucei*. Type A then spread to its current worldwide distribution whereas type B remains more local to East Africa.

The very close genetic relatedness of the type A *T. evansi* isolates we observed is consistent with previous findings (Gibson et al 1983; Waitumbi and Murphy 1993; Biteau et al 2000; Watanapokasin et al 1998; Ventura et al 2002; Ngaira et al 2005). Importantly however, AFLP accessed polymorphisms that distinguished amongst type A isolates in a way that was not possible using other methodologies or was not apparent due to limited sample sizes. These data are still completely consistent with type A *T. evansi* having originated from a single strain of *T. brucei* and having spread beyond the tsetse belt by mechanical transmission. It indicates that mutations will have continued after the original speciation event. All polymorphisms we have accessed are assumed to have resulted from mutations as *T. evansi* stocks, by virtue of being not transmitted by tsetse, are in recombinational isolation. In this context, the identification of two clades is interesting with a genetic distinction of the four isolates from Galana/Rumuruti from those in other parts of Kenya. Such differences within 'type A' *T. evansi* has the potential to be of practical importance. The use of RoTat 1.2 variable surface glycoprotein (VSG) expression as a basis for *T. evansi* diagnosis has been employed in a number of recent surveys (Ngaira et 2003, 2004; Delafosse and Doutoun 2004; Njiru et al 2004) but some type A stocks have been identified that do not contain this gene

(Ngaira et al 2005; Njiru et al 2005). For a number of reasons, VSG genes per se are inappropriate population genetic markers, but it would be interesting to investigate if RoTat 1.2 presence correlated with the clade structure identified using AFLP.

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Figure Legends

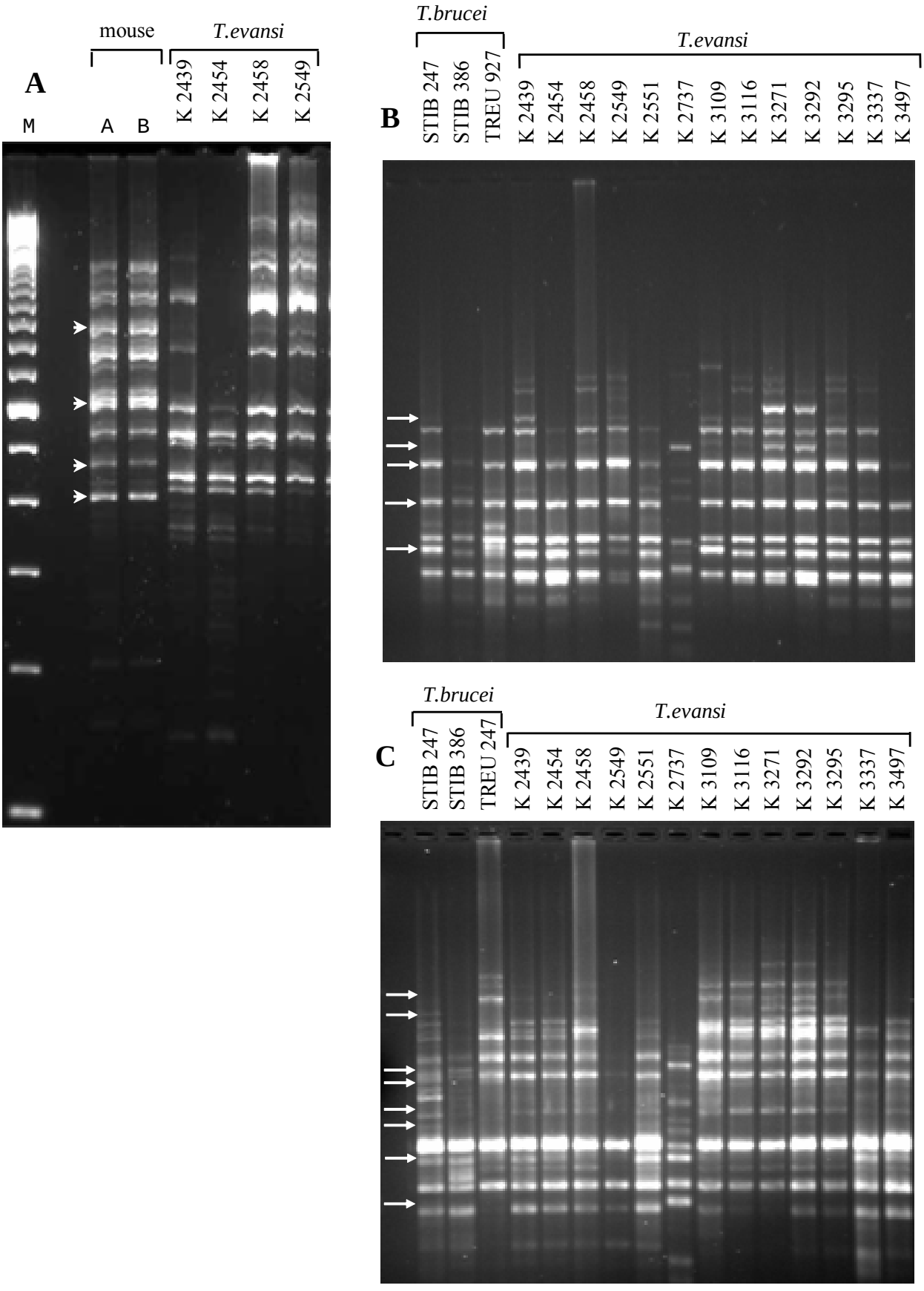
Figure 1. (A) Banding patterns produced by RAPD analysis of mouse DNA and four isolates of *T. evansi* with primer 1. Arrowheads show mouse DNA specific bands that are absent from *T. evansi*. M = markers. (B) RAPD products with primer 2 for DNA from a panel of *T.brucei* (lanes 1-3) and *T.evansi* isolates (lanes 4-16). (C) RAPD products for the same DNA samples with primer 3. Arrows indicate bands polymorphic between *T. evansi* isolates.

Figure 2. AFLP analysis of a panel of *T.evansi* and *T.brucei* isolates using selective primers E-TA and M-CA. Arrows on the left of the autoradiograph denote eight monomorphic markers present in all samples and arrowheads on the right denote 14 markers polymorphic amongst *T.evansi* isolates.

Figure 3. A dendrogram showing similarities between *T. evansi* isolates. Bootstrap values are shown for key nodes.

Figure 4. (A) PCR of 9S ribosomal subunit on the kDNA maxicircle. (B) PCR of TIM as control.

Figure 1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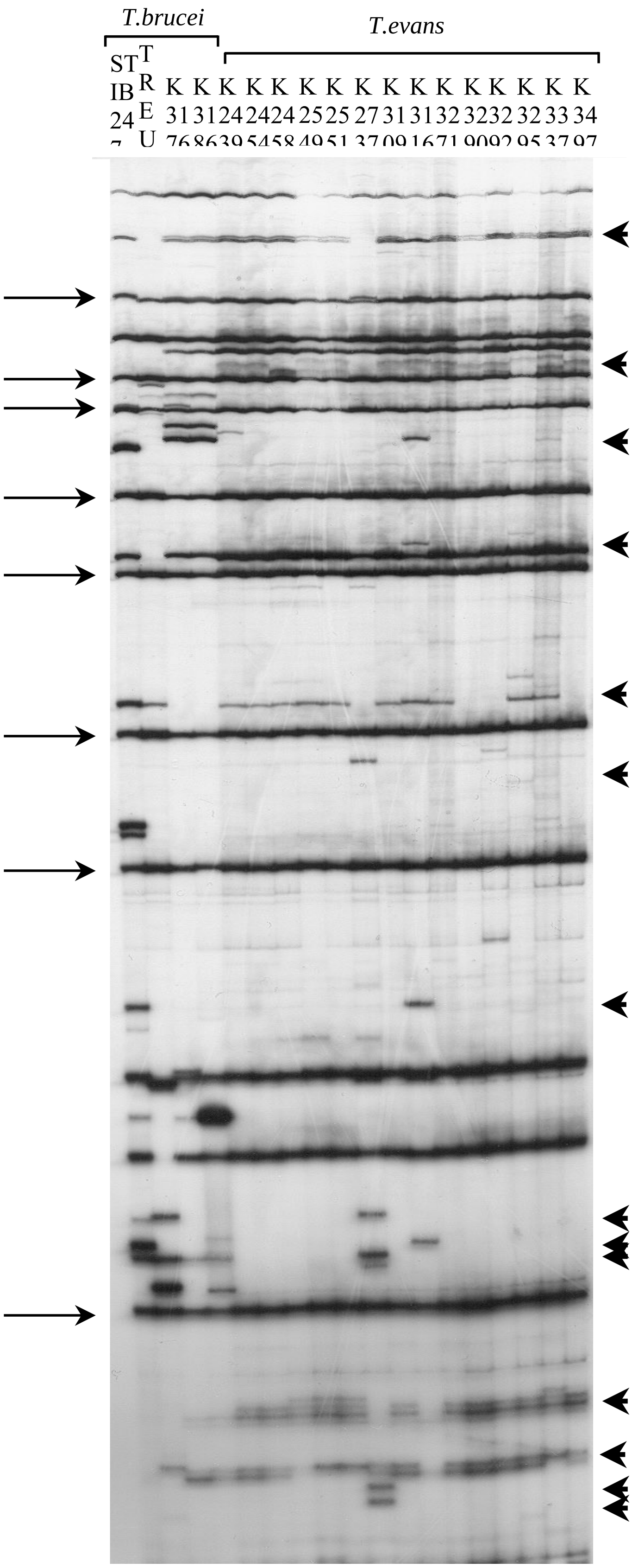
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Figure 3

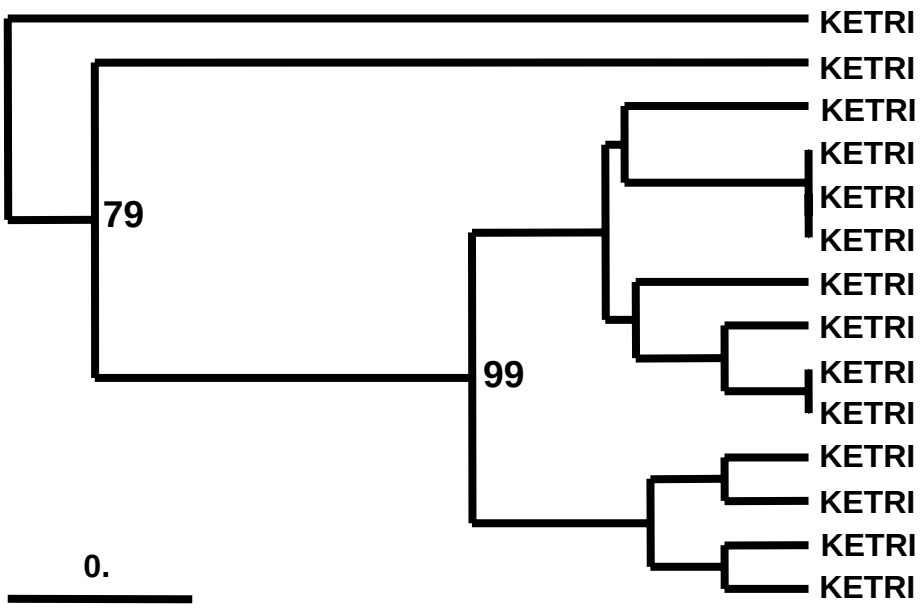
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Figure 4