How does *Trypanosoma equiperdum* fit into the *Trypanozoon* group? A cluster analysis by RAPD and Multiplex-endonuclease genotyping approach

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(*Received 16 July 2002; revised 24 October 2002; accepted 20 November 2002*)

SUMMARY

The pathogenic trypanosomes *Trypanosoma equiperdum*, *T. evansi* as well as *T. brucei* are morphologically identical. In horses, these parasites are considered to cause respectively dourine, surra and nagana. Previous molecular attempts to differentiate these species were not successful for *T. evansi* and *T. equiperdum*; only *T. b. brucei* could be differentiated to a certain extent. In this study we analysed 10 *T. equiperdum*, 8 *T. evansi* and 4 *T. b. brucei* using Random Amplified Polymorphic DNA (RAPD) and multiplex-endonuclease fingerprinting, a modified AFLP technique. The results obtained confirm the homogeneity of the *T. evansi* group tested. The *T. b. brucei* clustered out in a heterogenous group. For *T. equiperdum* the situation is more complex: 8 out of 10 *T. equiperdum* clustered together with the *T. evansi* group, while 2 *T. equiperdum* strains were more related to *T. b. brucei*. Hence, 2 hypotheses can be formulated: (1) only 2 *T. equiperdum* strains are genuine *T. equiperdum* causing dourine; all other *T. equiperdum* strains actually are *T. evansi* causing surra or (2) *T. equiperdum* does not exist at all. In that case, the different clinical outcome of horse infections with *T. evansi* or *T. b. brucei* is primarily related to the host immune response.

Key words: *Trypanosoma equiperdum*, characterization, RAPD, multiplex-endonuclease fingerprinting, AFLP.

INTRODUCTION

Dourine, Surra and Nagana are all lethal diseases in horses caused by *Trypanosoma equiperdum*, *T. evansi* and *T. b. brucei*, respectively (Office International des Epizooties, OIE list B). They are all members of the *Trypanozoon* subgenus and have morphologically identical bloodstream forms. *T. equiperdum* and *T. evansi* are transmitted respectively by sexual contact and by blood-sucking flies explaining their worldwide distribution, while the dependence on tsetse flies as the vector limits *T. b. brucei* to sub-Saharan Africa. (Stephen, 1986).

Most research on the genome of pathogenic Salivarian trypanosomes is performed on *T. b. brucei*. The genomes of *T. equiperdum* and *T. evansi* have not been thoroughly studied and most investigations focus on the sequence of variable surface glycoproteins (VSGs) (Baltz *et al.* 1986; Roth *et al.* 1986; Urakawa *et al.* 2001), on expression sites (Florent, Raibaud & Eisen, 1991), and on the kinetoplast DNA (kDNA) (Riou & Saucier, 1979; Frasch *et al.* 1980; Borst, Fase-Fowler & Gibson, 1987; Masiga & Gibson, 1990; Ou, Giroud & Baltz, 1991; Lun, Brun & Gibson, 1992*b*).

Despite numerous attempts, researchers have not been able to differentiate *T. equiperdum* from *T. evansi* consistently, neither at the serological, nor at the molecular level (Baltz, unpublished observations; Hide *et al.* 1990; Lun *et al.* 1992*a*; Lun *et al.* 1992; Biteau *et al.* 2000). Previous studies performed in our laboratory further underline the close relationship between both species (Claes *et al.* 2002). Only 2 of the 10 putative *T. equiperdum* strains, the BoTat 1.1 (Morocco) and the Onderstepoort Veterinary Institute (OVI) strain (South Africa), seem to differ from the rest of the *T. equiperdum* strains in Variable Antigen Repertoire. All other *T. equiperdum* have the same characteristics as *T. evansi* strains. In the present study, we examined the

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Parasitology (2003), **126**, 425–431.
DOI: 10.1017/S0031182003002968 Printed in the United Kingdom

Table 1. Trypanosome populations used in this study

Species	Clone/strain	ITMAS	Origin	Year	Host
T. b. brucei	AnTat 2.2	100297B	Nigeria	1970	Tsetse fly
T. b. brucei	AnTat 5.2	220197	The Gambia	1975	Cattle
T. b. brucei	AnTat 17.1	210596	R. D. Congo	1978	Sheep
T. b. brucei	KETRI 2494	270881	Kenya	1980	Tsetse fly
T. evansi	AnTat 3.1	070799	South America	1969	Capybara
T. evansi	RoTat 1.2	020298	Indonesia	1982	Water buffalo
T. evansi	Merzouga 56	120399D	Morocco	1998	Camel
T. evansi	Zagora I.17	040399B	Morroco	1997	Camel
T. evansi	KETRI 2480	110297	Kenya	1980	Camel
T. evansi	CAN 86 K	140799B	Brazil	1986	Dog
T. evansi	Stock Colombia	150799	Colombia	1973	Horse
T. evansi	Stock Vietnam	101298	Vietnam	1998	Water buffalo
T. equiperdum	AnTat 4.1	210983A	Unknown	Unknown	Unknown
T. equiperdum	Alfort	241199A	Unknown	Unknown	Unknown
T. equiperdum	SVP	241199B	Unknown	Unknown	Unknown
T. equiperdum	ATCC 30019	020301	France	1903 ?	Horse
T. equiperdum	ATCC 30023	280201	France	1903 ?	Horse
T. equiperdum	STIB 818	010999	P. R. China	1979	Horse
T. equiperdum	American	220101	Unknown	Unknown	Unknown
T. equiperdum	Canadian	290101	Unknown	Unknown	Unknown
T. equiperdum	OVI	241199C	South Africa	1975	Horse
T. equiperdum	BoTat 1.1	240982A	Morocco	1924	Horse

characteristics of several *T. equiperdum*, *T. evansi* and *T. b. brucei* populations with 2 molecular techniques, Random Amplified Polymorphic DNA (RAPD) and the multiplex-endonuclease fingerprinting method.

MATERIALS AND METHODS

Trypanosome populations

A collection of 4 *T. b. brucei*, 8 *T. evansi* and 10 *T. equiperdum* populations, derived from strains isolated all over the world, was used in this study (Table 1). All populations were kept as cryostabilates in liquid nitrogen. For the *T. equiperdum* strains, the history is mostly unknown. Only the OVI strain from South Africa, was well documented.

Preparation of trypanosome DNA

Blood-stream form trypanosomes were expanded in mice and rats and were purified from the blood by DEAE chromatography (Lanham & Godfrey, 1970), followed by repeated centrifugation (3 times 20 min, 2000 ***g***) and sediment washes with phosphate-buffered saline glucose (PSG) (38 mM $Na_2HPO_4 . 2H_2O$, 2 mM $NaHPO_4$, 80 mM glucose). Finally, trypanosome pellets were subsequently stored at −80 °C.

Twenty μl of trypanosome pellets (approximately 2×10^7 cells) were resuspended in 200 μl of phosphate-buffered saline (PBS) and the trypanosome DNA was extracted using the QIAamp DNA mini kit (Westburg, Leusden, The Netherlands), resulting in pure DNA in 200 μl of Milli-Q water. The typical yield of DNA extracted from a 20 μl pellet was 150 ng/μl or 30 μg of total DNA. The extracts obtained were diluted in Milli-Q water to a standard concentration of 50 ng/μl and stored at −20 °C.

Random Amplified Polymorphic DNA (RAPD)

Ten μl of extracted DNA (50 ng/μl) were mixed with 40 μl of a PCR-mix containing: 0·5 U *Taq* DNA recombinant polymerase (Promega, UK), PCR buffer (Promega, UK), 3·0 mM $MgCl_2$ (Promega, UK), 200 μM of each of the 4 dNTPs (Roche, Mannheim, Germany) and 0·5 μM of the oligonucleotide 10-mer (Gibco BRL, UK). The different oligonucleotides used were (in 5′-3′ direction): RAPD 606 CGG TCG GCC A (Ventura *et al.* 2001) and RAPD ILO 525 CGG ACG TCG C (Waitumbi & Murphy, 1993).

Amplifications were performed in a Biometra® Trio-block thermocycler. Cycling conditions were as follows: denaturation for 4 min at 94 °C, followed by 40 amplification cycles of 2 min denaturation at 94 °C, 2 min primer-template annealing at 40 °C and 2 min polymerization at 72 °C. A final elongation step was carried out for 5 min at 72 °C.

Twenty μl of the PCR product and 10 μl of a 3 kb size marker (MBI Fermentas, Germany) were subjected to electrophoresis in a 2% agarose gel (90 min at 100 V). Gels were stained with ethidium bromide (0·5 μg/ml) (Sigma, USA) and analysed on an Imagemaster Video Detection System (Pharmacia, UK).

Multiplex-endonuclease fingerprinting method

A fine-scale genotyping approach involving multiplex endonucleases in combination with a pair of cognate adapters was used according to Agbo *et al.* (2003). Briefly, 100–250 ng genomic DNA were digested for 4 h using 10 U each of *Bgl*II, *Bcl*I, *Acs*I and *Mun*I endonucleases in 2 successive double digestion reactions. The final digestion products were precipitated and reconstituted in 10 μl of distilled water. Ten μl of a buffer containing 660 mM Tris–HCl, 50 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM ATP, pH 7·5, and 20 pM of each *Bgl*II (5′-CGGACTAGAGTACACTGTC; 5′-GATCGACAGTGTACTCTAGTC) and *Mun*I (5′-AATTCCAAGAGCTCTCCAGTAC; 5′-AGTACTGGAGAGCTCTTG) adapters were added. The *Bgl*II adapter also ligated to the overhang sites created by *Bcl*I, while *Mun*I adapter also ligated to the *Acs*I site. One μl (400 U) of T4 DNA ligase (New England Biolabs) was added and the mixture incubated for 2 h at 25 °C. Pre-selective amplification was performed in a total volume of 20 μl containing 4 μl of 1 : 1-diluted ligation product, 1 U of *Taq* polymerase (Roche Molecular Biochemicals, Almere, The Netherlands), 10 × PCR buffer (100 mM Tris–HCl, pH 9·0, 50 mM KCl, 1% Triton X-100, 0·1% w/v gelatin), 2·5 mM $MgCl_2$, 200 μM of each dNTP and 5 pM of each *Bgl*II (5′-GAGTACACTGTCGATCT) and *Mun*I (5′-GAGAGCTCTTGGAATTG) primers. The reaction mix was incubated for 2 min at 95 °C, and subjected to 20 cycles of PCR (30 s at 95 °C, 30 s at 56 °C and 2 min at 72 °C). Four μl of 1 : 20-diluted pre-selective products were used as template for selective reaction with *Mun*-0/*Bgl*-A selective primer combination (in which the *Mun* primer was fluorescently labelled). The PCR program was essentially the same as for pre-selective amplification, except that the last cycling step was followed by a 30 min incubation at 60 °C. The final products were diluted 1 : 1 with TE, and Genescan-500 internal lane standard (PE Applied Biosystems) was added. One μl of the mix was resolved in a 7·3% denaturing sequencing gel using a model ABI 373A automated DNA sequencer. Gels were routinely prepared by using ABI protocols and electrophoresed for 5 h. Gel patterns were collected with GenScan software (PE Applied Biosystems) and sample files were transferred to GelCompar II software (Applied Maths, Kortrijk, Belgium).

Cluster analysis

The GelCompar II program was used for cluster analysis of RAPD and AFLP profiles by Unweighted Pair Group Method with Arithmatic Mean (UPGMA) based on the Dice coefficient.

With the obtained data matrices, Wagner Parsimony analysis was performed on bootstrapped data using the Seqboot, Mix and Consense programs from the PHYLIP software (Felsenstein, 1989).

RESULTS

RAPD and AFLP reactions were performed with the same set of samples on different days and by different persons. The DNA banding patterns obtained confirmed the repeatability of both techniques in our laboratory (data not shown).

Dendrograms from the RAPD results, analysed by pairwise fragment comparison using the Dice coefficient and by data clustering using UPGMA, are shown in Figs 1 and 2. In RAPD 606, all *T. evansi* strains cluster out in 1 homogenous group with a 95–100% similarity level. Also, in this cluster 8 out of the 10 tested *T. equiperdum* strains are found. All *T. b. brucei* and the 2 remaining *T. equiperdum* isolates (BoTat 1.1 and OVI) cluster out in a more hetereogenous way (with 72–88% similarity coefficiënt). The similarity level between these two *T. equiperdum* strains is 75% with, respectively, a 76 and 74% similarity coefficient with the *T. evansi*/ *T. equiperdum* cluster. *T. equiperdum* BoTat 1.1 shares the highest similarity with *T. b. brucei* AnTat 2.2, while the OVI strain is distinct from the rest of the group.

In RAPD ILO 525, the *T. evansi* strains are grouped in 1 cluster with 90–100% similarity. This cluster harbours the same 8 out of 10 *T. equiperdum* strains. *T. b. brucei* forms a more heterogenous groups (Dice coefficients ranging from 74 to 83%) including the *T. equiperdum* Botat 1.1 and OVI. With this RAPD *T. equiperdum* BoTat 1.1 relates most to *T. equiperdum* OVI and *T. b. brucei* KETRI 2494 and AnTat 2.2; OVI is highly similar to KETRI 2494.

In the UPGMA clustering data obtained from the modified AFLP analysis branches of the homology tree are longer, indicating the higher resolution power of this technique (Fig. 3). All *T. evansi* are grouped in 1 cluster with a similarity of 85–95%, together with the same 8 *T. equiperdum* strains. Also with this technique the *T. b. brucei* group appeared as a heterogenous cluster, including the BoTat 1.1 and OVI strains. Based on the modified AFLP data, the level of similarity of these two latter strains was calculated at 74%. In this analysis, OVI seems closely related to *T. b. brucei* KETRI 2494, while BoTat 1.1 shares more homology with *T. b. brucei* AnTat 2.2.

Neither with the RAPD nor the modified AFLP, the position of the strains amongst the clusters seemed to be related to their geographical origin, original host species or the year of isolation. In RAPD 606, *T. evansi* and *T. equiperdum* from different regions and hosts (RoTat 1.2, AnTat 3.1, CAN 86K, stock Vietnam, STIB 818, American stabilate, ATCC 30019 and ATCC 30023) gave a 100% similarity coefficiënt (Fig. 1). On the other hand, with RAPD ILO 525 (Fig. 2), *T. evansi* stocks from different origins (RoTat 1.2, stock Colombia, Zagora I.17) showed exactly the same pattern.

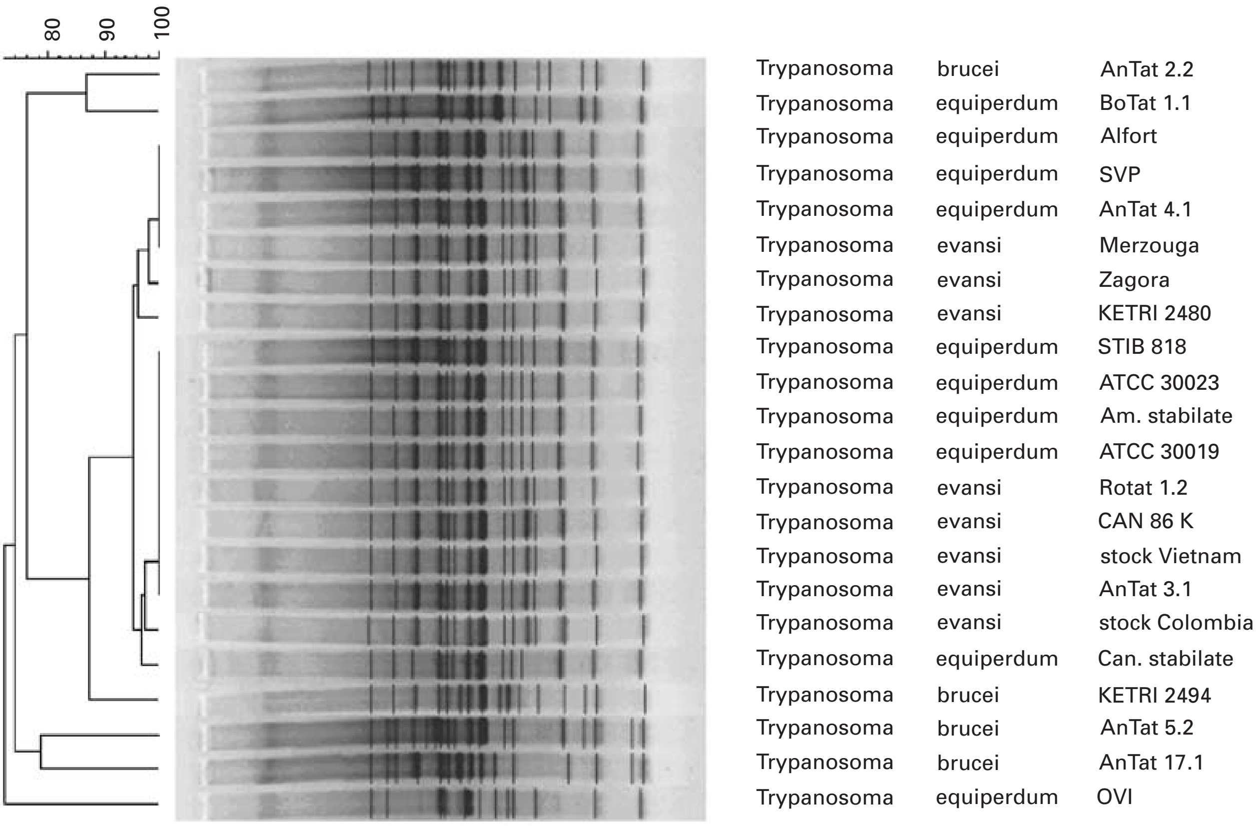

Fig. 1. UPGMA Cluster analysis based on the RAPD results with primer 606.

When mixed parsimony analysis was performed on bootstrapped data from both RAPDs and the modified AFLP, the homogenous *T. evansi*/*T. equiperdum* cluster differed from the more heterogenous group with an 80 % and 100 % probability coëfficient, respectively for the modified AFLP and both RAPD's (data not shown).

DISCUSSION

Comparison of the RAPD 606 results in the present study with those from Ventura *et al.* (2001), reveals a similar close genetic relationship between *T. evansi* populations from different origins, and approximately the same distance between *T. equiperdum* BoTat 1.1 and the *T. evansi* cluster (76 % similarity versus 60 %, respectively).

With RAPD ILO 525, Waitumbi & Murphy (1993) were able to divide the *Trypanozoon* subgenus into 3 groups: (1) *T. b. brucei* and *T. b. rhodesiense*, (2) *T. b. gambiense* and (3) *T. evansi*. No *T. equiperdum* was included in their analysis.

Other previous characterization studies mainly focused on the *T. brucei* subspecies or on *T. evansi* and only few *T. equiperdum* were included. Hide *et al.* (1990), analysed 42 *T. brucei* by repetitive DNA probes, together with only 1 *T. equiperdum* and 1 *T. evansi*. A separate *T. b. gambiense* type I cluster was found while *T. b. brucei* and *T. b. rhodesiense* were more heterogenous. The *T. equiperdum* and *T. evansi* appeared to have a dissimilarity level of 56 % with the *T. brucei* group and a dissimilarity level of 45 % between each other. Unfortunately from their paper, it is not clear which *T. equiperdum* and *T. evansi* strains were used. By both kDNA and isoenzyme analysis, Lun *et al.* (1992 *a*, *b*) could not find differences which would distinguish 12 stocks of *T. evansi* from 1 *T. equiperdum* (STIB 818). Agbo *et al.* (2002) included 2 *T. evansi* (AnTat 3.1 and RoTat 1.2) and 2 *T. equiperdum* (AnTat 4.1 and STIB 818) in their AFLP analysis of *Trypanosoma* spp., again without conclusive results on the differentiation between *T. evansi* and *T. equiperdum*.

Using microsatellite markers on 3 *T. equiperdum* (BoTat 1.1, STIB 818 and a South African strain), Biteau *et al.* (2000) observed heterogenous patterns amongst them and concluded that 'previous interpretation of the close relationship of *T. evansi* and *T. equiperdum* by isoenzyme and RFLP analysis might have been simplisitic'.

Only Zhang & Baltz (1994) found some differences between *T. equiperdum* and *T. evansi* stocks using repetitive DNA probes. BoTat 1.1 and a South African strain were separated from the *T. evansi* group. They were more similar to *T. b. brucei* than to the *T. evansi* cluster which contained a third *T. equiperdum* (STIB 818). Zhang & Baltz concluded that this outlier *T. equiperdum* STIB 818 could reflect the limit of sensitivity of the RFLP technique used or could be due to the misclassification of this

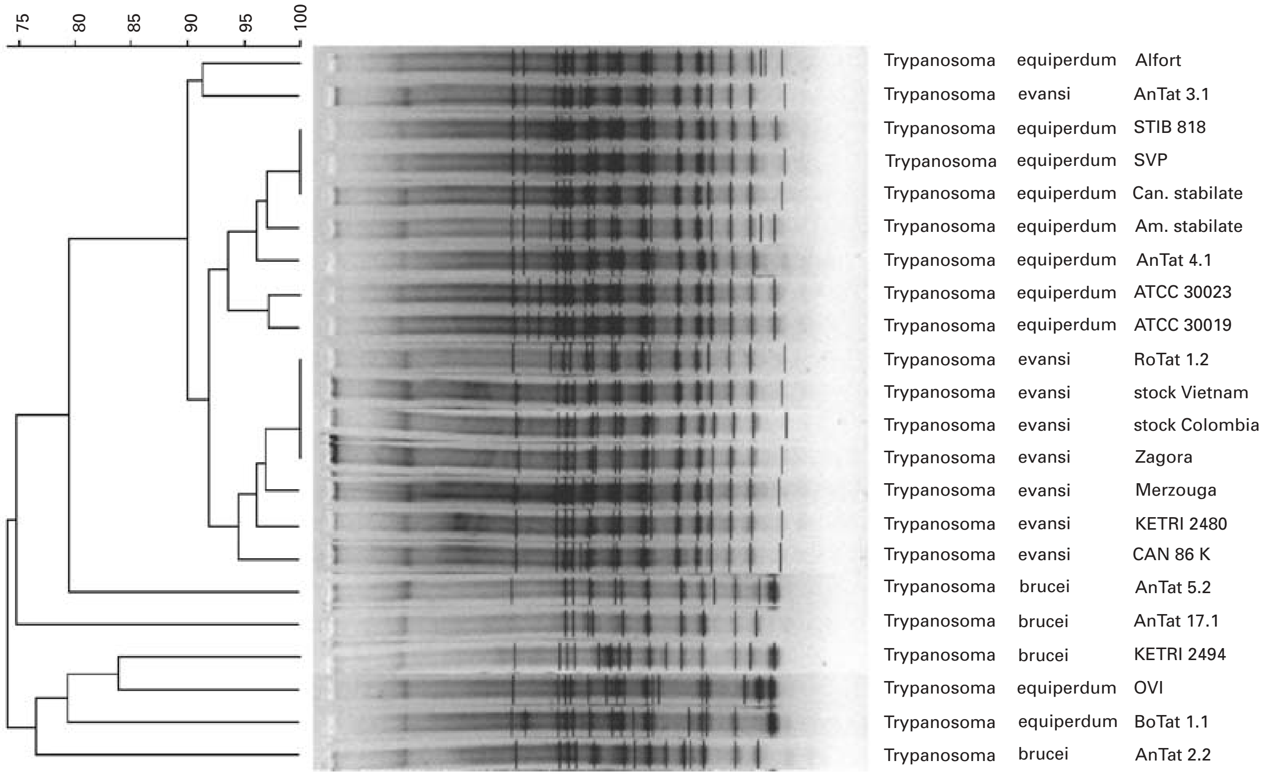

Fig. 2. UPGMA Cluster analysis based on the RAPD results with primer ILO 525.

strain. To our knowledge, the South African strain is the Onderstepoort Veterinary Institute (OVI) strain (T. De Waal, personal communication).

Taken together, the above-mentioned results correspond well with the present results based on a larger collection of *T. evansi* and *T. equiperdum* strains. Irrespective of the DNA amplification method, 2 major groups can be formed: 1 homogenous group including all *T. evansi* and most of the *T. equiperdum* strains and 1 heterogenous group including all *T. b. brucei* and 2 *T. equiperdum*, the BoTat 1.1 clone and the OVI strain.

Previous serological and molecular studies on the same collection yielded similar results: all hitherto tested *T. evansi* share the presence and expression of the RoTat 1.2 VSG gene, while for the screened populations of *T. equiperdum* only BoTat 1.1 and OVI, as well as all tested *T. b. brucei*, do not express nor contain this VSG gene (Claes *et al.* unpublished observations).

Combining these data, 2 hypotheses can be formulated. Firstly, BoTat 1.1 and the OVI strain are the only genuine *T. equiperdum* while all other *T. equiperdum* actually are misclassified *T. evansi*, thus extending the view of Zhang & Baltz (1994). Indeed, in experimental infections with the OVI strain by Barrowman (1976), clinical signs of dourine were observed in the infected horses, while in experimental infections with the American and Canadian stabilates, which in our study are both found in the *T. evansi* cluster, only general signs of trypanosomiasis were observed (Hagebock *et al.* 1993). Unfortunately, for most *T. equiperdum* strains, including BoTat 1.1, similar clinical experiments have not been performed.

The question whether BoTat 1.1 and OVI are 'real' *T. equiperdum* strains could be solved by following the clinical outcome of horses experimentally infected with both strains and by comparing the result with infections with *T. evansi*-like *T. equiperdum* strains. However, one should keep in mind that strains that have undergone multiple passages in laboratory animals might have lost or changed their pathogenicity and virulence. Alternatively, specific serological or molecular markers could be identified which can differentiate *T. equiperdum* from *T. b. brucei*. In the absence of a conclusive *T. b. brucei* marker this remains a challenging issue.

An alternative hypothesis is that the species *T. equiperdum* actually does not exist but that dourine is caused by particular strains of *T. evansi* and/or *T. b. brucei*. Then the clinical outcome of the infection would merely depend on the virulence or the tissue tropism of the infective strains or the immunological response of the individual host to the trypanosome infection.

Indeed, in horses both acute, subacute and chronic forms of nagana (*T. b. brucei*) have been described, sometimes with clinical signs such as oedema of prepuce and legs, and sporadically the appearance of urticarial plaques. Also for surra (*T. evansi*) in horses, both acute and chronic infections have been mentioned. Here also, oedema is evident as plaques on the ventral surface of the body or as

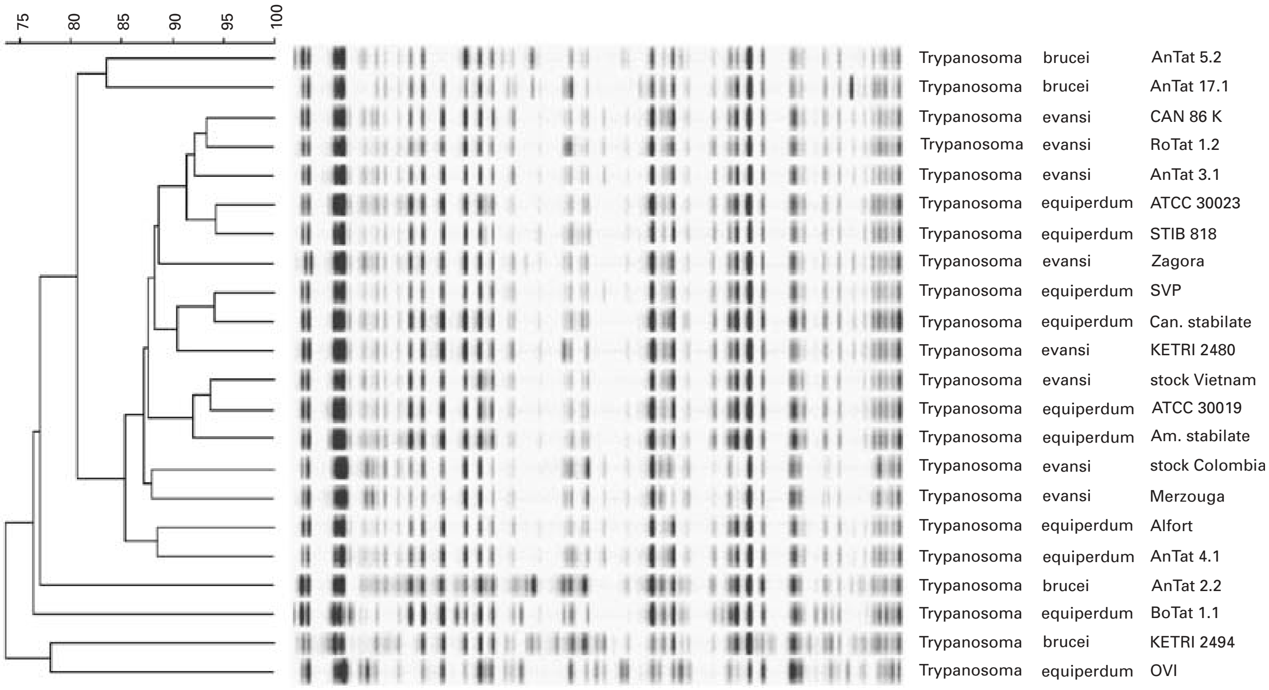

Fig. 3. UPGMA Cluster analysis based on the modified AFLP results.

swelling of the sheath or prepuce (Stephen, 1986). Altogether, some clinical signs of nagana and surra are shared with dourine, i.e. urticarial plaques and genital swellings. Therefore, it might be that differential diagnosis based only on clinical signs is not conclusive for the infecting trypanosome species and certain chronic cases of nagana or surra might have been considered as dourine or *vice versa*. This enigma would be solved if one considers dourine as the chronic form of both diseases.

Both hypotheses should be checked against the other biological characteristics of the 3 trypanosome species. Until now, *T. b. brucei* is considered to be only cyclically transmitted by tsetse flies, while *T. evansi* and *T. equiperdum* are transmitted respectively mechanically and sexually. However, we have no evidence to exclude sexual or mechanical transmission of *T. b. brucei*. The transmission route could even be linked to host specificity and virulence or tissue tropism. Also for *T. evansi*, sexual transmission might occur. Hagebock *et al.* (1993) indeed were able to infect horses by urogenital inoculation with the American and Canadian *T. equiperdum* strains, which in our study cluster together with *T. evansi*. Thus, if these strains are considered to be *T. evansi*, a first proof of sexual transmission of *T. evansi* in horses is obtained. Nevertheless, to prove the possibility of sexual transmission of *T. b. brucei* and *T. evansi*, more experimental infections with both species should be conducted.

Based on the available clinical, serological and molecular data, we propose that there is not sufficient evidence for the existence of *T. equiperdum* as a separate species.

To further clarify the confusion about *T. equiperdum*, we propose to isolate new trypanosome strains from well defined dourine, surra and nagana cases in horses, to analyse them with the most performant serological and molecular techniques and to study their pathogenicity and transmission routes in horses.

This study received financial support from the Institute for the Promotion of Innovation by Science and Technology in Flanders, Belgium (IWT). *Trypanosoma* spp. stabilates were kindly provided by Dr Reto Brun, Swiss Tropical Institute Basel, Switzerland; Dr Peter-Henning Clausen, Free University Berlin, Germany; Dr Joyce Hagebock and Dr David Kinker, National Veterinary Services Laboratories, United States Department of Agriculture, USA; Dr Lun, Zhongshan University, P.R. China; and Dr Zablotsky, All-Russian Research Institute for Experimental Veterinary Medicine (VIEV), Russia. We also want to thank Dr Rigouts (ITM, Mycobaterium laboratory) for the use of the Gelcompar II program.

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