RESEARCH NOTE

The Evolution of Salivarian Trypanosomes

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CA Hoare (1972 *The Trypanosomes of Mammals*, Blackwell, Oxford, 749 pp.) divided mammalian trypanosomes into two sections, the Salivaria and the Stercoraria. The Salivaria comprises all the African tsetse-transmitted trypanosomes of mammals, while the Stercoraria includes mammalian trypanosomes transmitted by the posterior route. However, the status of other tsetse-transmitted species, e.g. *Trypanosoma grayi* from crocodiles, and of other trypanosomes transmitted by the anterior route, e.g. *T. rangeli* from South America and leech transmitted trypanosomes, remain under debate. To some extent such unknowns are linked to the limitations of the morphological and transmission characters used in early investigations of trypanosome taxonomy and evolution. With the wealth of molecular sequence data now available, particularly for the phylogenetically versatile 18S rRNA gene (ML Sogin et al. 1986 *Proc Natl Acad Sci USA 83*: 1383-1387) some of these questions may now be fruitfully re-addressed. Accordingly, we undertook phylogenetic analysis of published ribosomal RNA 18S sequences, supplemented with data from our own studies, our aim being to re-examine the evolutionary relationships of Salivarian trypanosomes of mammals and other vertebrates.

Fourteen *Trypanosoma* species 18S rRNA sequences were obtained from EMBL/GenBank for phylogenetic analysis: *T. boissoni* U39580; *T. carassii* L14841; *T. rotatorium* U39583; *T. triglae* U39584; *T. brucei brucei* M12676; *T. congolense* (kilifi-type) U22317; *T. congolense* (forest-type) U22319; *T. congolense* (savannah-type) U22315; *T. simiae* U22320; *T. vivax* U22316; *T. cruzi* X53917; *T. cruzi* M31432; *T. avium* U39578; *T. scelopori* U67182; together with three newly available sequences (JR Stevens et al. 1999 *Parasitology 118*: 107-116.); *T. grayi* AJ005278; *T. rangeli* AJ009160; *T. varani* AJ005279. Five additional 18S sequences from a range of other kinetoplastid species were included as outgroups: *Crithidia fasciculata* X03450; *Leishmania amazonensis* X53912; *L. major* X53915; *Trypanoplasma borreli* L14840; *Bodo caudatus* X53910. Sequences were aligned primarily on the basis of their secondary structure (J-M Neefs et al. 1990 *Nucleic Acids Res 18*: 2237-2243). Sub-sections of the alignment, between regions of high homology were sub-aligned using the program Clustal V (DG Higgins et al. 1992 *Comp Applns Biosci 8*: 189-191), before final adjustments were made by eye. Bootstrapped maximum parsimony analysis of the 22 rRNA 18S sequences was performed with 100 replicates using test version 4.0d63 of PAUP*, written by David L Swofford.

The phylogenetic analysis (Figure) places the Salivarian trypanosomes in a monophyletic clade comprising exclusively mammalian trypanosomes of African origin. Within the Salivarian group, the various types of *T. congolense* also constitute a monophyletic group. *T. rangeli*, *T. grayi* and trypanosomes of fish and amphibia are excluded from the Salivarian clade; *T. rangeli* is placed firmly in a separate clade with *T. cruzi*. A third major clade, comprising trypanosomes with aquatic hosts, forms a separate early branch within the monophyletic *Trypanosoma* which is not directly ancestral to either the Salivaria or the Stercoraria. Branches receiving less than 50% bootstrap support are presented as polytomies.

In agreement with a number of previous studies (F Alvarez et al. 1996 *Mol Phylogen Evol 5*: 333-343, J Lukes et al. 1997 *J Mol Evol 44*: 521-527), our analysis confirms the monophyly of the African Salivarian trypanosomes. Within the Salivaria, our results also indicate that the various types of *T. congolense* (forest, kilifi, savannah) share common ancestry. Such a finding contrasts with the results of isoenzyme and RAPD based studies (I Sidibé, cited M Tibayrenc 1998 *Int J Parasitol 28*: 85-104), in which it is suggested that *T. congolense* may be polyphyletic. We propose that this apparent difference in conclusions is due

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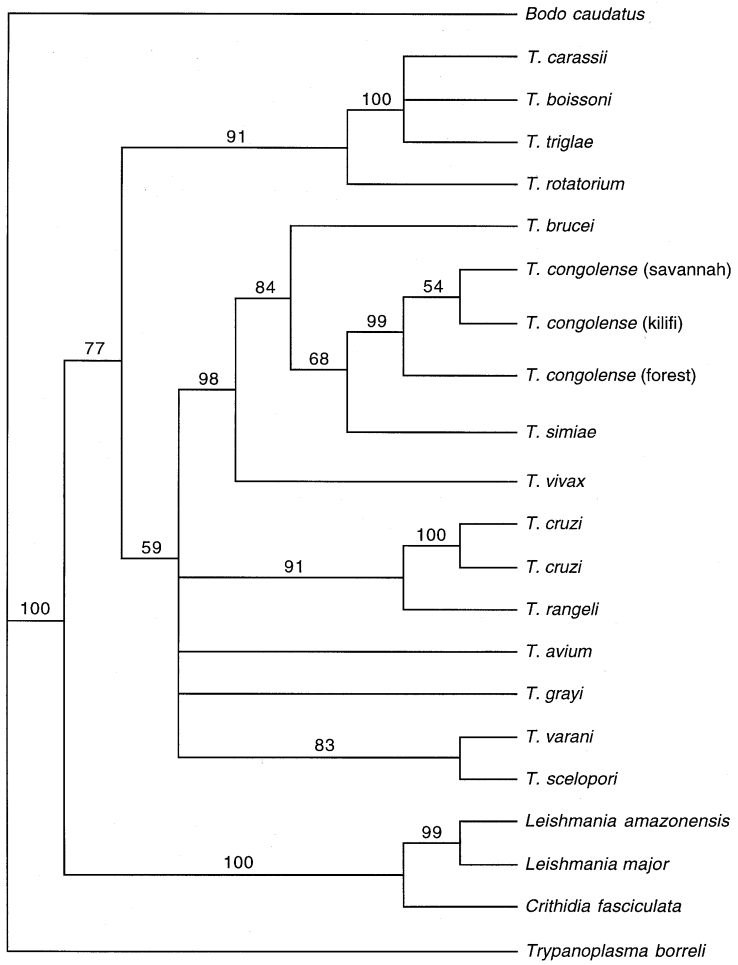

Phylogram constructed by bootstrapped (100 replicates) maximum parsimony analysis of 22 kinetoplastid 18S ssu rRNA sequences, based on a standard alignment of 1809 nucleotide sites (JR Stevens et al. 1999 *Parasitology 118*: 107-116). Bootstrap values for all major nodes are given and all branches receiving bootstrap support values >50% are shown; relationships failing to achieve this level of support are shown as polytomies.

to the differing clock speeds of the markers used in the two studies, highlighting the potential limitations of using fast evolving 'population genetics' markers for evolutionary studies.

The taxonomic and evolutionary status of *T. rangeli* has long been debated (A D'Alessandro & NG Saravia 1992 *Trypanosoma rangeli*, p. 1-54. In JP Kreier & JR Baker (eds), *Parasitic Protozoa*, 2nd ed., vol. 2, Academic Press, San Diego) and, even following the application of molecular tools (for example, MI Amorim et al. 1993 *Acta Tropica 53*: 99-105), has remained unresolved. The results of this study, however, indicate a close evolutionary relationship between *T. rangeli* and *T. cruzi*, suggesting *T. rangeli* be placed within the Stercorarian subgenus *Schizotrypanum*. Similarly, the non-Salivarian status of the posteriorly tsetse-transmitted *T. grayi* is also confirmed; the evolutionary significance of its position separate from either the Salivaria or the *Schizotrypanum* species (i.e. *T. cruzi* and *T. rangeli*) remains to be explored.

Finally, the distinct nature of the clade comprising trypanosomes with aquatic hosts, which forms a separate early branch within the monophyletic *Trypanosoma*, suggests that these taxa are not directly ancestral to either the Salivaria or the Stercoraria. The position of this 'aquatic' clade may have fundamental consequences for hypotheses concerning the evolution of the Salivaria and parasitism in the Kinetoplastida as a whole.

DISCUSSIONS

Dear Drs Stevens and Gibson

Thank you for your very interesting presentation. I found your results very interesting but I would also like to ask two further questions: (i)

The level of bootstrap support for your different clades. I did not find this important information at your website either; (ii) What strain you used for *T.rangeli*. This is a polymorphic species and its strong association with *T. cruzi* (another highly polymorphic species) in your study could be strain dependant.

Hooman Momen

Answer (i): apologies for not giving the bootstrap values, this was a serious oversight. Support for the major clades were as follows: rangeli/cruzi clade >91%; Salivarian clade 98%; aquatic clade >91%. I hope this goes some way to convincing you of the robustness (and our associated enthusiasm) of these results.
Answer (ii): *T. rangeli* strain RGB(Basel). Perhaps some of you could furnish us with more history and results relevant to this strain.

Dear Drs Stevens and Gibson

Did you investigate alternative topologies from the point of view of how much "worse" they are? Thank you for your presentation.

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Answer: I have begun to explore the tree with the various branch swopping tools available in MacClade and, thus far, all the major clades (Salivarian, Schizo and aquatic) are robust. The early branching of the aquatic clade will require further investigation, but, whatever our findings, it will not affect significantly our conclusions relating to the relationship of the Salivarian and Schizo clades. I hope to explore clade robustness further in the near future using Bremer support measures.

Dear Jamie and Wendy

I was very interested by your phylogenetic study. However, I was surprised that you conclude *T. rangeli* is a Schizotrypanum from the fact that it is closer to *T. cruzi* than to African trypanosomes. Hoare (1972) classified other species as stercorarian, such as *T. lewisi*, *T. musculi* (subgenus Herpetosoma) and *T. theileri* and *T. melophagium* (subgenus Megatrypanum). Couldn't it be that *T. rangeli* is closer to these species than to *T. cruzi* ? Or do you have evidence that *T. rangeli* is phylogenetically closer to *T. cruzi* than are other Schizotrypanum species such as *T. dionisii* or *T. vespertilionis*?

Sylvain Brisse

Answer: as noted above, support for the close relationship of *T. rangeli* and *T.cruzi* (based on this 18S marker) is good (>91%) and suggests a genuine phylogenetic relationship with *T.cruzi*. Whether or not *T. rangeli* is phylogenetically closer to *T. cruzi* than are some other Schizotrypanum species remains to be seen, but even if it is not, it does not rule against its Schizotrypanum status ... in any phylogenetic or taxonomic system, some taxa will be obviously be more or less integral to the clade.

COMMENTS

The taxonomic position of *T. rangeli* has been a point of contention for many years. However, this species can be distinguished from members of the subgenus Schizotrypanum by a number of methods (reviewed by Grisard et al). It is well known that these two species occur in the same hosts. There is the famous case when a mixed infection of the two species was described as a new species. We must be open to new data but we need to be certain that the DNA that is being analysed is from the correct parasite. What other data is available to say that the strain used by Drs Stevens and Gibson was *T. rangeli*? It would be interesting to analyse the actual strain further with other methods.

We must remember what emphasis was popular in biology at the time that a particular classification system was proposed. When Dr Hoare made his division of the trypanosomes into hind and forgut transmission biological characters were begining to gain ground against classical morphology. Such characters are indeed a summation of many different complex characters which may or may not be homologous. Thus although a development site is a definitely may be useful for characterizing an organisms it may or may not be useful in classification depending on its homology within the group. My point is that the character stercoraria or salivaria is useful for describing where the infective forms are found or leave the vector but we must be careful in using it in classification. Thus to me it is quite acceptable that *T. rangeli* and *T. brucei* have a salivarian development since the infective forms are found in the mouthparsts. However, that does not mean that we have to classify them in the same group. I hope this goes some way to answering Roberto's question and clarrifying the use of the terms salviarian and stercorarian.

Jeffrey Shaw

It is well known that *T. rangeli* and *T. cruzi* share a large number of triatomine vectors and vertebrate hosts, including man. For *T. cruzi*, many studies describing such type of variables have been published. Until the finding of *T. rangeli* in south-

ern Brazil, the distribution area of this parasite was restricted in Brazil to the Amazon region. Unfortunately, I don't know specific papers describing (or identifying) variables for such approach, probably due to the non-pathogenic characteristic of *T. rangeli* to humans. Among several characteristics, both of these human trypanosomes are transmitted by triatomines, however, through different ways. Thus, the expected distribution for *T. rangeli* is correspondent to the distribution of susceptible triatomine vectors. These triatomines in which *T. rangeli* can invade and develop in the hemolymph and salivary glands and are transmitted by bite mainly belongs to the Genus Rhodnius. As you see, this expected distribution for *T. rangeli* is overllaping the *T. cruzi* distribution area. I agree with your comment that studies targeting variables to predict the distribution of *T. rangeli*, or the possible spreading area of this parasite, will be very helpful and useful to better understand the biology of this parasite.

Concerning the discussions and questions about *T. rangeli* taxonomic position: Dr Hoare classified trypanosomes into Salivaria and Stercoraria sections based in their development mode, primarily in the vector and secondarily in the mammalian hosts. In his monograph published in 1972, he defined as Stercoraria those trypanosomes which development in the vector is completed (with formation of metatrypanosomes) in the posterior station and transmission is contaminative (in *T. rangeli* also in anterior station, with inoculative transmission). He defined as Salivaria those trypanosomes which development in the vector is completed (with formation of metatrypanosomes) in the anterior station (except in mechanical inoculators) and transmission is inoculative. There are some remarkable facts that we have to keep in mind since Dr Hoare initial description. First, there is no description of successful transmission of *T. rangeli* through forms present in triatomine feces, despite the presence of this parasite in both naturally and experimentally infected triatomines. *T. rangeli* metacyclic trypanosomes only develop inside salivary glands of triatomine bugs and are transmitted to the vertebrate host through inoculation with saliva during blood sucking. For these reasons, Dr Hoare mentioned *T. rangeli* as an exception in the Stercoraria section, even sharing some other biological characteristics with the type species (*T. cruzi*). As very well noted by Dr Shaw, we think that this nomenclature was based on biological characteristics and focused primarily on the site of development of the infective forms inside the vector. More studies should be addressed to better understand the particular evolutionary correlation of these different transmission pathways. The use of modern biochemical, immunological and molecular methods for taxonomic purposes of trypanosomatids should be treated with special care. The number of strains, their biological behavior, isolation methods, geographical regions and original fonts as well as the high polymorphism detected among strains or stabilates within same species should be considered in such analysis. Also, relying on single or even a few methods or markers can result in dubious categorizations due to the narrow biological point of view of these analyses. Phylogenies intended to resolve the classification of these closely related organisms should be based on markers more relevant to the processes of transmission. Trypanosome strains should be very well characterized prior to analysis using such refined methods, since contaminations might be expected, especially in labs that cultivate both parasites (*T. cruzi*/*T. rangeli*) or in regions were these parasites coexists. Recently, Dr H Noyes (*Parasitol Today 14*: 49-50, 1998) reviewed the credibility of trypanosome trees. Different mechanisms that can affect and/or distort these trees, giving rise to misinterpretations are discussed in this very interesting paper. Despite *T. rangeli* sharing characteristics with different subgenus of both Salivaria and Stercoraria sections, including the 18s SSU rRNA sequence described in this meeting, it is transmitted through triatomine bite, i.e. through anterior station. In reference to other points raised in relation to the paper of Stevens and Gibson, *T. rangeli* forms present in triatomine faeces are, at least until now, non-infective for vertebrate hosts. Thus, due to this biological characteristic we believe that *T. rangeli* is not properly classified in the Stercoraria section. Moreover, the suggestion to treat *T. rangeli* as a Schizotrypanum trypanosome due to the close relationship with *T. cruzi* revealed only by SSU rRNA analysis of a single strain is too dangerous. Our aim was not to propose the change of *T. rangeli* taxonomic position, but to evaluate the heterogeneity among different isolates and bring up a very old a controversial question that remains unanswered. We probably should use as many well characterized representative strains and characteristics as possible to make evolutionary or phylogenetic inferences.

Edmundo Grisard

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