Taxonomy of the Genus *Leishmania*: Present and Future Trends and Their Implications

Jeffrey J Shaw

Belém Research Projects, Instituto Evandro Chagas, Fundação Nacional de Saúde, Caixa Postal 3, 66017-970 Belém, PA, Brasil and Instituto de Ciências Biomédicas, Universidade de São Paulo, Caixa Postal 66208, 05389-970 São Paulo, SP, Brasil

The application of different taxonomic methods (Cladistic, Evolutionary Taxonomy and Numerical Taxonomy) to the taxonomy of the Genus Leishmania *are reviewed. The major groupings of the most recent classifications obtained using the cladistical approach agree with the major divisions of previous classifications which used traditional taxonomy (Evolutionary Taxonomy). The advantage of the cladistical approach is that it produces cladograms whose branches indicate more accurately levels of relationships between the different taxa. Numerical Taxonomy is useful for identification but not as good as the cladistical approach for classification. The ancient division of this monophyletic genus into two major evolutionary lines supports the use of the subgeneric names* Leishmania *and* Viannia.

Key words: *Leishmania* - classification - Cladistics - Phenetics - Eclectic - Evolution - *Viannia*

In his heart of hearts I believe a taxonomist is a detective, very much like Sir Arthur Conan Doyle's universally famous character, Sherlock Holmes, trying to unravel the family tree of a particular group. As you read this I can hear some of you saying to yourselves "Elementary my dear Watson!" However today's taxonomist certainly has more clues than Sherlock Holmes did, but he still has to use a basic deductive process to unravel his clues, - which are characters, and as in any good detective story this is where the difficulties and controversy begins.

Identification and classification are linked but are not the same thing and the former generally takes priority when dealing with diseases, such as the leishmaniases, which are caused by parasites of the genus *Leishmania* that to the untrained eye appear to be morphological all the same. It may be possible to identify a parasite but we may not be able to classify it because we simply do not know enough about it. This was the case with *Toxoplasma* and *Pneumocystis* for many years. The continual search for better methods of identifying a pathogen lead to the development of a more efficient diagnosis of a disease and such methods have been crucial in giving the taxonomist characters to help him classify the pathogens in question. In leishmaniasis the technological flow in the past was primarily from applied research to basic research, but recently this trend has become reversed. Now tools that were originally developed by scientists interested in the actual parasites are being used to diagnose the disease in both man and animals. This is largely due to the discoveries in the 80's and 90's in molecular biology which were of such a universal nature that it is relatively easy to transfer the successful methods developed for one organisms to another.

Comments on taxonomic methods

In Biology taxonomy is used by scientists with very different interests such as Molecular Systematists, Developmental Biologists and Evolutionary Biologists. Those who are interested in the taxonomy of the *Leishmania* are mostly parasitologists with a leaning towards evolutionary biology.

In 1965 Hennig published his "Phylogenetic Systematics" which is considered to be the starting point of Modern Cladistics, although some workers consider that cladistics has always been a part of systematics. This method is accepted as the method of choice for reconstructing interrelationships between taxa, but their is still some debate about the underlying assumptions of the method.

The purpose of an analytical classification method is to group items of any type into subsets that have similar characters. Parallel to the studies in molecular biology scientists interested in taxonomy developed theoretical techniques to

This work received financial support from the Wellcome Trust, London, the Instituto Evandro Chagas of the Fundação Nacional de Saúde and the Universidade de São Paulo.
Received 1 December 1993
Accepted 29 April 1994

analyse character information more accurately. With these analytical methods it is possible to construct trees whose branches relate the different subsets at different levels.

Different taxonomic methods result in different trees and the branches produced by Numerical Taxonomy, Evolutionary Taxonomy and Cladistics do not follow the same patterns. It is, however, now generally accepted that cladograms give the most reliable phylogenetic trees. Figure 1 illustrates this problem and shows how three taxa can be grouped differently on the absence of characters C-H. Using cladistics (Fig. 1a) the absence of characters is ignored but in Numerical and Evolutionary taxonomy (Fig.1b) it is not, which results in a tendency to form artificial groups.

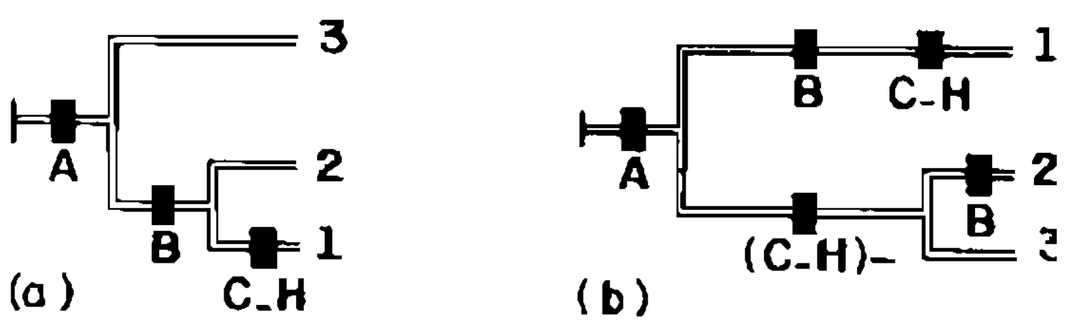

Fig. 1: cladistic (a) and evolutionary (b) trees showing the different association of taxas 1,2,3 related to the absence of characters C-H in taxa 2 and 3 which were considered to be important by the evolutionary method (after Scotland 1992) and thus they become artificially grouped together.

The taxonomic approaches used for the genus *Leishmania* during the last 20 years fall into three main catagories:

Phenetics (Numerical Taxonomy) - The results depend on the choice of the clustering alogrithims and there is a tendency to produce clusters based on the absence of characters. This method has been used by in the past by workers at Montepellier between 1981 and 1984, at the London School of Hygiene and Tropical Medicine (Le Blanq et al. 1986), Richard Kreutzer's group in the USA and Colombia (Kreutzer et al. 1987) and more recently by workers at the Instituto Oswaldo Cruz (Cupollilo et al. 1994) to classify zymodemes and is still used in various disciplines to examine in particular species groups. The danger is that it groups organisms that appear to be similar regardless of the possibility of parallel or convergent evolution and so groups can be artificial.

Eclectic or Evolutionary Taxonomy - This is also called the classical or traditional form of Taxonomy and employs the expert's intuition. There is a tendency to weight characters which often leads to disagreement among specialists and the danger is that characters used to define groups may not be evolutionary significant which again can lead to groups being artificial. Basically this is the method that Ralph Lainson and myself followed between 1972 and 1987. In our evolving classifications of the *Leishmania* (Lainson & Shaw 1987) we introduced the idea of species complexes and in 1987 we created the subgenus *Viannia* for a group of very distinctive parasites of the New World that had a developmental phase in the sandflies hind gut.

Cladistics - The basis is that speciation is a dichotomous process and species at nodes become extinct. At the heart of the method is finding the maximally parsimonious tree or the tree with the least branches. If up to 20 taxa are used, exact algorithms are used but for larger quantities heuristic methods are used which may not necessarily find all the optimal trees (Kitching 1992). Within the Cladistic school there are four different approaches to parsimony that are named after the scientists who first used them - Wagner Parsimony, Fitch Parsimony, Dollo Parsimony and Camin-Sokal Parsimony.

Workers at Montepellier have produced cladograms from isoenzyme data for the genus *Leishmania* since 1984 (Lanotte et al. 1986, Thomaz-Soccol et al. 1993) and conclude that the genus has a monophyletic origin.

The origin of the genus Leishmania

The genus *Leishmania* belongs to the family Trypanosomatidae whose major lineages, according to comparisons of k-DNA maxicircles, are considered to diverged at an early period. Saf'janova (1986) suggested that *Leishmania* probably branched off from a basic trypanosomatid stock during the Mesozoic period, often known as the age of the reptiles, before the separation of the two continents, when the sandflies emerged as group. Thomaz-Soccol et al. (1993) proposed that New World members of the subgenus *Leishmania* separated from their Old World sisters some 27-55 million years ago in the early Cenozoic period and were introduced into the Americas by the migration of rodents from the Old World. Based on the monophyletic evidence produced in the cladogram of these workers this would presently seem to be the most reasonable explanation.

Beverley et al. (1987) estimated that nuclear DNA sequence divergence amongst the major lineages of *Leishmania* was comparable to that of animal species that had separated 10-80 million years ago. This agrees with the empirical estimate of Saf'janova. The very antiquity of the groups indicates that they are very successful parasites that can readily adapt to changing environments and hosts. What are man's chances of combating such successful parasites?

The evolutionary process

The process by which *Leishmania* evolve is not understood but is considered most likely to be predominantly by asexual methods. Cibulskis (1986) considered that more than one mutational process may be responsible for genetic variation in the genus *Leishmania*. He drew attention to the fact that enzyme analysis only detects enzyme differences that have a different net charge and not differences at the allelic level. Electrophoresis may not detect differences in base pair sequence variation that are below 30%. Thus some genotypes could be considered as identical by electrophoresis when they in fact are not. Mutations in asexually reproducing organisms also tend to favour the accumulation of null alleles which are not detectable by electrophoresis which poses another problem when trying to assess the major method responsible for genetic variation.

Natural occurring leishmanial populations are considered to be clones (Tibaryenc et al. 1990) and this concept linked to asexual reproduction is important when considering evolution of the genus.

A species may result from two different processes. At a certain point in time descendants of a species may differ and eventually give rise to a new species thus one branch in the tree becomes two. This is termed cladogenesis. A new species may also arise by the hybridization of two species and this is termed reticulate speciation (Wiley et al. 1991).

It is quite likely that both processes are involved in speciation with the genus *Leishmania*. For instance *L.(L)arabica* most probably arose by reticulate speciation and there is some evidence that this is also occurring in Venezuela and Central America (Darce et al. 1991, Bonfante et al. 1992).

Taxonomic characters

Theoretically any homologous character can be used to study the taxonomy of the *Leishmania*. The basic concept behind homology is common ancestry and if a character was acquired from a source other than an ancestral form then it is taxonomically not useful. Examples of this are the wings of birds and bats which are not homologous or DNA sequences independently acquired from bacteria or viruses at different times. This problem is particularly important when dealing with nucleotide sequences and characters need to be tested for true homology. Thus sequence similarity does not necessarily mean homology and unlike morphological characters it is difficult to submit a nucleotide base to further examination. In this respect it is important to use sequences whose function is known as these give a better idea as to homology. For instance it is known that heat shock proteins are conservative in their structure throughout a very wide range of animals, but that there are specific areas in the C-terminal region that could be taxonomically useful.

Some characters may be better for discriminating between organisms than others but if they do not fulfil requirements of homology they cannot be used for taxonomy. Recently Cupolillo et al. (1993) noted that monoclonal antibodies were more discriminatory than enzymes and that using 18 instead of 10 enzymes did not increase the discriminatory capacity of these characters.

There are differences of opinion as to suitability of characters such as susceptibility and virulence. Recent studies have shown that such attributes can be related to the presence or absence of certain surface molecules, such as LPG and GP63 (Blackwell 1992). In the future it will probably be possible to define them at the molecular level which may make them more attractive as characters to those who are presently against their use.

The following groups of characters can be used to identify and classify *Leishmania* and some have been used in more extensive taxonomic studies: (a) morphology, developmental patterns in sandflies and mammals, host specificity; (b) restriction products of genomic DNA (includes rDNA) and kinetoplast DNA - determination of base pair sequences, Karyotypes; (c) enzyme characterization (includes isoenzyme studies); detection (with monoclonal antibodies) and structure of proteins, lipids, carbohydrates, surface lipophosphoglucans (LPG), glycoconjugates, glycoinoaitol phospholipids (GIPLs), glycoproteins (GP63), heat shock proteins (HSp70).

A few characters have been more extensively used by some groups to examine relatively large numbers of isolates and have resulted in parasites being grouped into zymodemes (isoenzymes), schizodemes (restriction enzyme patterns of k-DNA), and serodemes (reactions with monoclonal antibodies). Only the data relating to zymodemes has been analyzed extensively by different taxonomic methods.

Other characters such as enzyme activity measured by radiorespirometry and DNA buoyant densities are no longer used because the techniques involved are not now readily available.

The characters used to classify and identify a *Leishmania* may not necessarily be the same. Thus some may be more useful for identifying specific populations or subsets of individuals that occur in the developmental cycle while others may be better suited for taxonomic studies. Basically the choice of which characters or group of characters are used depends at what level you

want to distinguish or associate organism. For example some regions of the ribosomal DNA (rDNA) are conserved throughout taxonomically very different organisms and are useful in determining relationships at higher levels (Sogin et al. 1986). On the other hand other regions, such as the rDNA internally transcribed spacer sequences, are more useful for separating more closely related groups, such as trypanosomes and leishmania but they show little variation within species (Uliana et al. 1991).

Karyotyping of *Leishmania* has not proved as useful a taxonomic tool as one might have hoped and differences between strains and species of the same complex were not good enough to make positive identifications (Tavares et al. 1992). Within a species there is a variability of up to 2/3 rds while between species it is in the order of 1/3 rd (Giannini et al. 1986). Karyotype data has so far not been analysed by any of the taxonomic method.

The named species of *Leishmania* in 1993

At the time of writing there are 30 named species of *Leishmania* of which 10 occur in the Old World and 20 in the New World. All the Old World species belong to the subgenus *Leishmania* and 7 are known to infect man. There are also 11 named New World species of the same subgenus and of these 5 have so far not be found in man. Parasites of the subgenus *Viannia* only occur the New World and of the 9 named species 8 are known to infect man.

The named species of subgenus *Leishmania* are listed in chronological order with the year in which they were described (O=Old World, N=New World, H=infects man): *L. (L.)donovani* (OH) 1903; *L.(L.)tropica* (OH) 1906; *L. (L.)infantum* (OH) 1909; *L.(L.)major* (OH) 1914; *L.(L.)archibaldi* (OH) 1919; *L.(L.)chagasi* (NH) 1937; *L.(L.) enriettii* (N) 1948; *L.(L.) mexicana* (NH) 1953; *L.(L.) pifanoi* (NH) 1959; *L. (L.) gerbilli* (O) 1964; *L.(L) hertigi* (N) 1971; *L.(L.) amazonensis* (NH) 1972; *L.(L.)aethiopica* (OH) 1973; *L.(L.)deanei* (N) 1977; *L.(L.)garnahmi* (NH) 1979; *L.(L.)venezuelensis* (NH) 1980; *L.(L.)killicki* (OH) 1986; *L.(L.)arabica* (O) 1987; *L.(L.)aristedesi* (N) 1987; *L.(L.)turanica* (O) 1990, *L.(L.)forattinii* (N) 1993.

The named species of the subgenus *Viannia* are: *L.(V.)braziliensis* (NH) 1911; *L.(V.) peruviana* (NH) 1913; *L.(V.)guyanensis* (NH) 1954; *L.(V.)panamensis* (NH) 1972; *L.(V.) lainsoni* (NH) 1987; *L.(V.)naiffi* (NH) 1989; *L.(V.) shawi* (NH) 1989; *L.(V.)colombiensis* (NH) 1991; *L.(V.)equatorensis* (N) 1992.

The idea of complexes introduced by Ralph Lainson and myself in 1972 has been useful and later workers have confirmed their existence as groups by other methods (Thomaz-Soccol et al. 1993, Cupolillo et al. 1994). As more information has become available, some of the complexes have been further subdivided. Complexes are not a formal taxonomic unit, but are useful for grouping *Leishmania* that are similar but whose specific status may be questionable.

The use of *L.(L.)chagasi* as a specific name has been controversial for sometime and the French workers at Montpellier consider it to be a synonym of *L.(L.)infantum* while we (Lainson & Shaw 1987) have preferred that this parasite retains its specific rank. Besides Jackson et al.'s schizodeme evidence mentioned by us (Lainson & Shaw 1987) it has also been shown that antigenically *L.(L.)chagasi* differs from *L.(L.)infantum* (Santoro et al. 1986) and more detailed molecular studies may show other differences. If the specific status of *L.(L.)chagasi* is changed then it could well be treated as a subspecies of *L.(L.)infantum*, but certainly not as a subspecies of *L.(L.)donovani*, as some workers have done in the past.

Phylogenetic trees

So far the only attempt to produce an evolutionary tree based on molecular data is that of Beverley et al. (1987) and their results are summarized in Fig. 2. The tree is based on the DNA fragment relationships of the products of six restriction enzymes hybridized against probes of three different loci. By this method it was impossible to determine relationships between the complexes, but its particular attraction is that it offers an inbuilt molecular time clock which is very useful in estimating evolutionary events for organisms that have no fossil records.

The recently published cladogram of Thomas-Soccol et al. (1993) is shown in Fig.3. It basically conserves the phenetic and phyletic complexes determined previously by ourselves and the Montpellier group and defines a total of 15 complexes. What it does that the others did not do, however, is produce evidence that shows that the *Leishmania* are definitely monophyletic in origin. Various other points of interest are shown in this cladogram, especially the ancient division of the two subgenera and the division of the subgenus *Viannia* into the four complexes *braziliensis*, *guyanensis*, *naiffi* and *lainsoni*. Newly described parasites such as *L.(V.)colombiensis* and *L.(V.)equatorensis* are not included and it will be interesting to see which complexes they belong to.

In general there is good agreement between trees and levels of divergence based on different character sets but Beverley et al. (1987) found more genomic DNA divergence within the *major* complex than in the *tropica* complex whereas isoenzyme studies show the reverse.

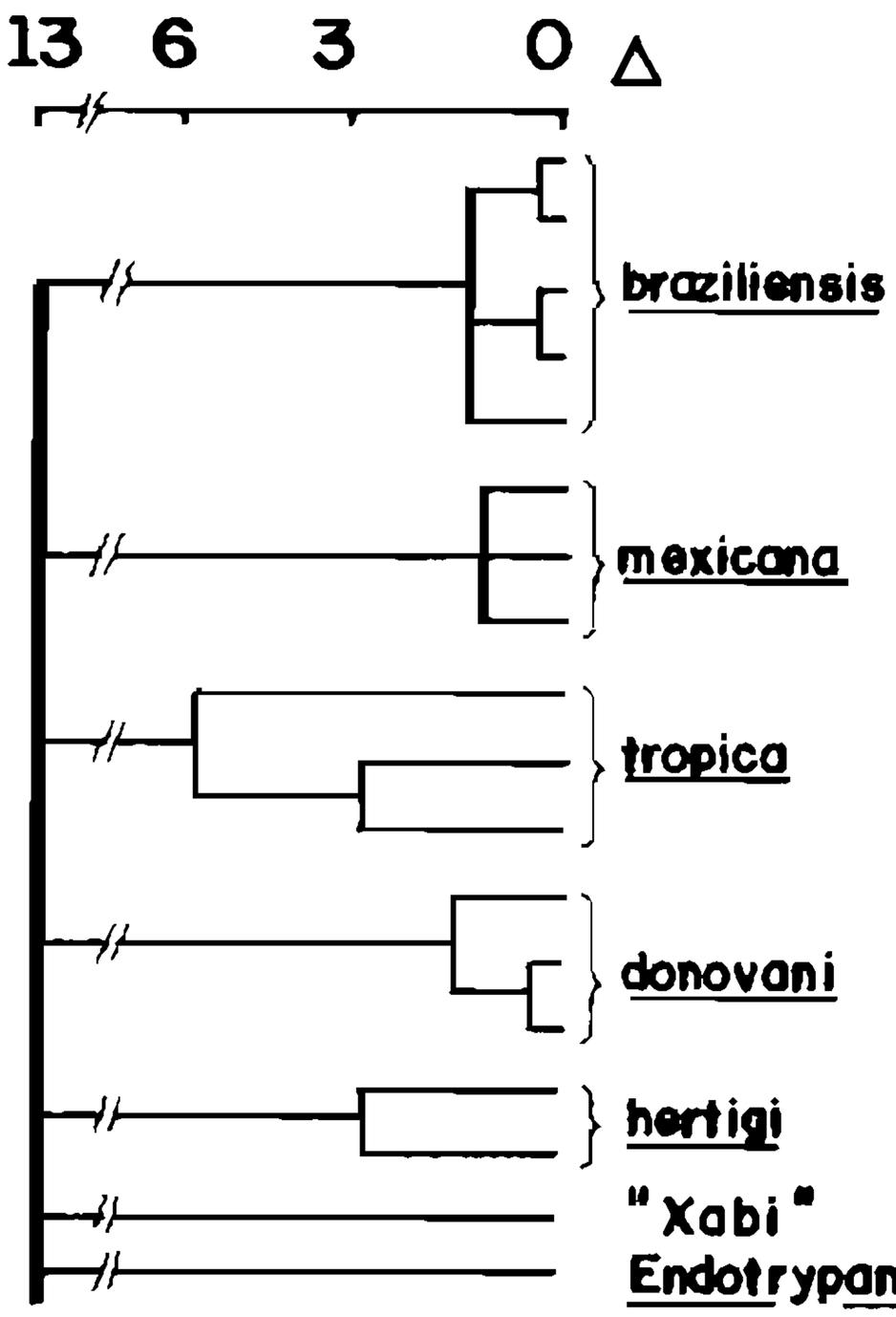

Fig. 2: a molecular tree depicting five *Leishmania* complexes based on the products of six restriction enzymes hybridized with probes against three loci. The thicker vertical lines indicate ambiguous relationships (after Beverley et al. 1987).

Identification and taxonomy: relationships and recent advances

Identification and taxonomy are obviously related, but like the chicken and the egg, which comes first? To me it is identification, as once you have identified an organism you become interested in classifying it. Also in general identification techniques concentrate on one character and later it has to be seen if it is suitable for taxonomy.

Subgeneric classification was originally based on the development in the sandfly, but now it has also been defined using rDNA intergenic nontranscribed spacer divergence sequences (Ramirez & Guevara 1987, Guevara et al. 1992). If a trypanosomatid produces only amastigotes in a hamster and grows as promastigotes in culture it can safely be considered to be *Leishmania*. However not all strains isolated from mammals infect hamsters and generic identification may be difficult. Uliana et al. (1991) partly helped to solve this problem when they discovered a domain of the 18S rDNA that was specific for the genus *Leishmania* and *Crithidia*. As the latter does not occur in mammals it is potentially a useful diagnostic tools, but there are still problems with parasites isolated from sandflies. One wonders what is the phylogenetic relationship between these two genera.

Heterogeneity for the genes coding for GP63 (Blackwell 1992) has been detected for

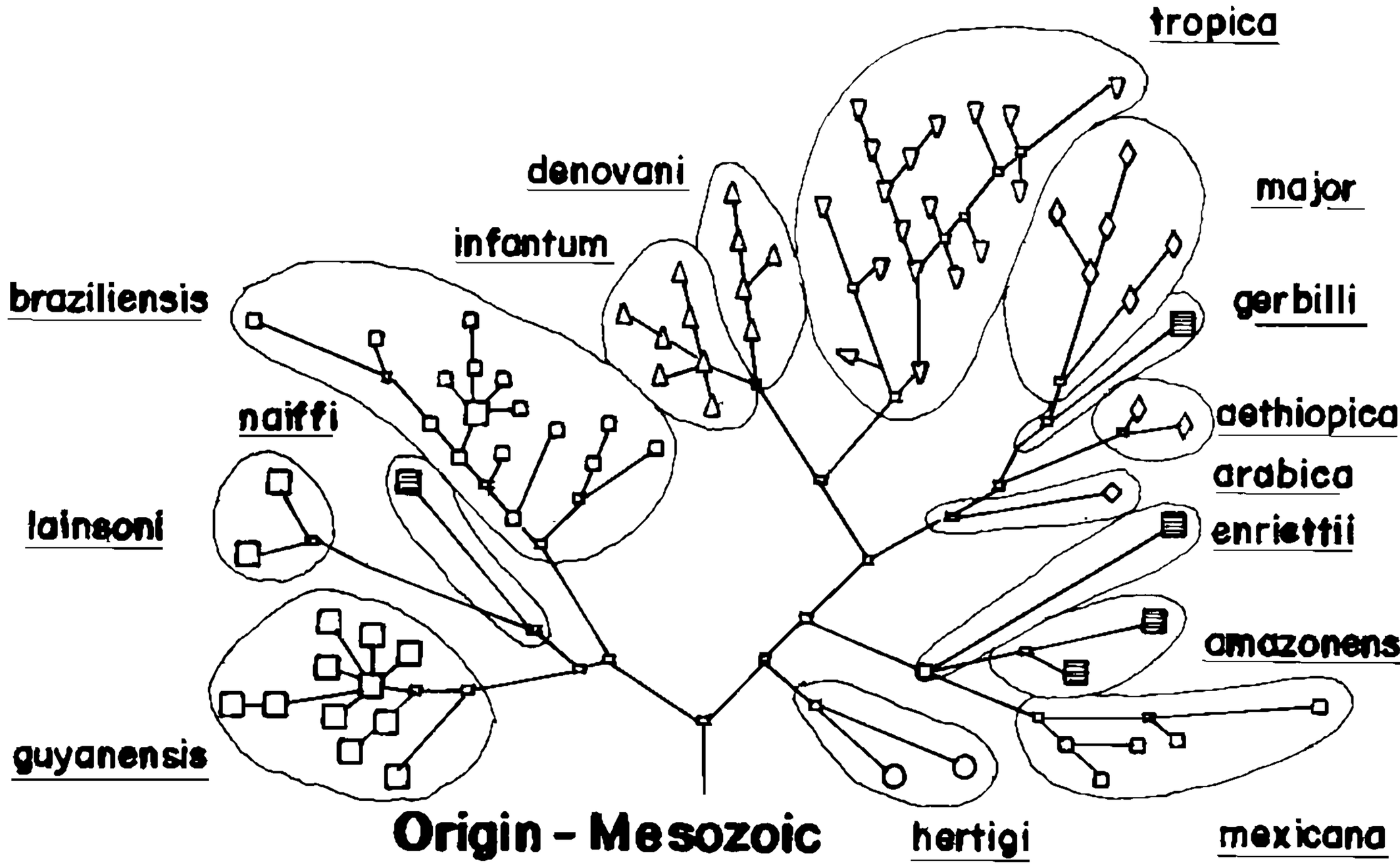

Fig. 3: a cladogram showing the phylogenetic relationships of the 15 complexes of the genus *Leishmania* based on the analysis of 80 zymodemes visualized using 13 different enzymes showing the monophyletic origin of the genus (after Thomaz-Soccol et al. 1993).

L(L)donovani, L(L)major and *L(V.)peruviana*. This is a beginning but data on more species is needed before their taxonomic usefulness can be assessed.

Interest in producing diagnostic tools can also contribute to taxonomy. For example Dr Douglas Barker and his colleagues (de Bruijn & Barker 1992) have extensively sequenced k-DNA minicircles to enable them to produce synthetic oligonucleotide primers for specific diagnosis with the polymerase chain reaction (PCR). Their sequencing data is suitable for taxonomic studies and it is presently being analysed for this purpose.

Some years ago Huntsman (1968) showed the value of human haemoglobin variants as "racial markers" and this stuck in my mind for a long time. What particularly fascinated me was that there was strong evidence that Haemoglobin D is centred in the Punjab and that at that time it only occurred very rarely in native English and Irish populations. It was suggested that it was introduced into the United Kingdom because of marriages between British and Indian women during the Sikh war in the Punjab. With the advent of leishmanial monoclonal antibodies in 1981 (McMahon-Pratt & David 1981) I wondered if they could be used in a similar way to follow *Leishmania*.

Below are some observations on three different monoclonal antibodies which in my opinion pose some interesting phylogenetic questions.

Some time ago I thought that it was possible to prepare monoclonal antibodies that were specific for a *Leishmania* species, but I am presently wondering if there is such a thing as truly species specific leishmanial monoclonal. In 1991 we described (Hanham et al. 1991) a monoclonal antibody that we called N3 which only reacted with the promastigotes of *L(V.)naiffi*. Since it is so useful diagnostically we included it in our routine monoclonal antibody panel and one day it unexpectedly reacted with a strain of *L(V.)braziliensis* that we had received from Peru. Subsequently we have tested it against over 200 strains of *L(V.)braziliensis* (Shaw,Ishikawa,Lainson,Silveira, Hanham unpublished observations) and we have so far only found the N3 epitope in *L(V.)braziliensis* strains that come from west of the Andean foothills. This suggests that this epitope was present in the ancestral form and was only retained by *L(V.)naiffi* and the ancestors of the western Andean strains of *L(V.)braziliensis*. So far we have failed to find the N3 epitope in *L(V.)peruviana* strains. Will other data support an east/west taxonomic division of *L(V.) braziliensis*?

Recently we (Shaw, Ishikawa, Lainson, unpublished observations) observed that a *L(L)tropica* monoclonal called T3 (Jaffe & McMahon-Pratt 1983) reacts with *L(L)gerbilli, L(L)major, L(L)tropica, L(L)mexicana, L(L)hertigi, L(L)enriettii* and *Endotrypanum schaudinni* but not with *L(L)amazonensis* when tested by immunofluorescence. Here it would seem that we are seeing an epitope that has been inherited by most of the New and Old world rodent *Leishmania* but how did it get into *Endotrypanum* and how does this parasite relate to the phylogeny of New World *Leishmania?*

The monoclonal B18 (McMahon-Pratt & David 1981) was thought to be specific for *L(V.)braziliensis* (Shaw et al. 1986, Grimaldi et al. 1987) but recent results (Shaw, Braga, Ishikawa, Lainson, Braga, Silveira unpublished observations) show that it also reacts with *L(V.) peruviana* and some members of the *braziliensis* complex species group that occur in the Iquitos area. The latter is an example of an epitope that has not been inherited by all members of a species.

To date monoclonal antibody studies suggest that they are useful for identification in defined geographical areas. The results also pose some tantalizing questions such as how certain epitopes are inherited and more analyses are needed to see if they are taxonomically useful. One serious problem with a monoclonal antibody is that often the function and identification of the epitope that it recognizes is unknown.

Identification is clearly linked with taxonomy and if we gazed into a crystal ball we might well see *Leishmania* being identified by comparing particular rDNA or DNA sequences with data contained in gene bank libraries. A number of questions come up when you are crystal ball gazing, however, such as whether or not the technology involved is too complicated and costly for the practical problem. You may look at a few strains to test the methods, but would it be practical to do this routinely in say a public health laboratory? Only the future will give us the answers, but I am sure that a few years ago many people thought that Western Blots might never be used routinely, but now they are.

Concluding comments

Because the very methods used to construct phylogenetic trees are based on theories every tree is a *hypothesis* and so there is no such thing as a definitive taxonomy. Like the *Leishmania*, taxonomy is also evolving and besides depending on the analytical methods employed it will also depend on how we handle the new characters as they become available. One might suggest that the ideal tree should be based on the DNA sequence of the whole genome. At the present time and possibly for a long time to come, however, this is clearly an impossible goal, and even then their will be variable sequences whose functions are unknown or that may not be expressed, or may have been secondarily acquired.

It is satisfying to me personally that the cladograms based on enzymes support the basic taxonomy that Ralph Lainson and myself suggested using an entirely different approach, that of the evolutionary biologist. Besides this we used other characters, including morphology and biological characters, such as development and pathology, to define our complexes and subgenera. This strongly supports the opinion that it is both useful and desirable to base a classification on more than one type of character. A major difficulty is at what point should a strain or group of *Leishmania* strains be given a taxonomic rank. This decision must be made by the individual scientists involved according to the characters that they consider to be of distinguishing value.

In discussing their results obtained with leishmanial genomic restriction patterns Beverley et al. (1987) drew attention to the fact that molecular divergence may be uncoupled from morphological and taxonomic divergence. Thus the differences between men and chimpanzees is of the same molecular order as that between sibling species of *Drosophila*. Similarly David (1986) warned that allozymic polymorphism, such as associated with enzyme patterns, may be maintained by selective pressures or it is neutral and of less evolutionary importance, being more of a by-product, yet it is still a useful tool for studying population structures.

These words of caution are not only important in relating to our choice of taxonomic characters, but also indicate how we should handle the results that more sophisticated methods, such as sequencing, are now producing.

I am sure we will see changes in the taxonomy of the genus *Leishmania* but the basic concepts of subgenera composed of different complexes has stood the test of various different types of taxonomic analysis and will most likely remain a stable feature. I think we will, however, see more changes at the specific and subspecific levels where characters are being analysed and their taxonomic importance is in the process of being assessed.

Cladograms need to be prepared for characters other than enzymes and different data character sets should be combined and analysed. The data and information generated by these studies could lead to advances in diagnostic methods and even control strategies. Identifying and grouping *Leishmania* more accurately will undoubtedly help us to understand the many different epidemiologic situations that are being discovered.

ACKNOWLEDGEMENTS

To Dr Rupert Quinnell and Prof. Ralph Lainson for their helpful discussions.

REFERENCES

Beverley SM, Ismach RB, McMahon-Pratt D 1987. Evolution of the genus *Leishmania* as revealed by comparisons of nuclear DNA restriction fragment patterns. *Proc Natl Acad Sci USA 84:* 484-488.

Blackwell JM 1992. Leishmaniasis epidemiology: all down to the DNA. *Parasitology 104 (Suppl):* S19-34.

Bonfante-Garrido R, Meléndez E, Barroeta S, Mejía de Alejos MA, Momen H, Cupolillo E, McMahon-Pratt D, Grimaldi Jr G, 1992. Cutaneous leishmaniasis in western Venezuela caused by infection with *Leishmania venezuelensis* and *L.braziliensis* variants. *Trans R Soc Trop Med Hyg 86:* 141-148.

Cibulskis R 1986. Mutation and recombination in the Trypanosomatidae, p.297-303. In J-A Rioux *Leishmania Taxonomie et phylogenése. Applications éco-épidémiologiques.* Colloque International. IMEEE, Montpellier.

Cupolillo E, Grimaldi Jr G, Momen H 1993. Discrimanatory ability of typing systems in *Leishmania. Trans R Soc Trop Med Hyg 87:* 116-117.

Cupolillo EG, Grimaldi Jr G, Momen H 1994. A general classification of New World *Leishmania* using numerical zymotaxonomy. *Amer J Trop Med Hyg* (in press).

Darce M, Moran J, Palacios X, Belli Z, Gomez-Urcuyo F, Zamora D, Valle S, Gantier JC, Momen H, Grimaldi Jr G, 1991. Etiology of human cutaneous leishmaniasis in Nicaragua. *Trans R Soc Trop Med Hyg 85:* 58-59.

David J 1986. La théorie neutraliste du polymorphisme enzymatique confrontée au cas de la déshydrogénase alcoolique chez la Drosophile, p.289-296. In J-A Rioux *Leishmania Taxonomie et phylogenése. Applications éco-épidémiologiques.* Colloque International. IMEEE, Montpellier.

De Bruijn MHL, Barker DC 1992. Diagnosis of New World leishmaniasis: specific detection of species of the *Leishmanian braziliensis* complex by amplification of kinetoplast DNA. *Acta Trop 52:* 45-58.

Giannini SH, Schittini M, Keithly JS, Warburton PW, Cantor CR, Van Der Ploeg LHT 1986. Karyotype analysis of *Leishmania* species and its use in classification and clinical diagnosis. *Science 232:* 762-765.

Guevara P, Alonso G, da Silveira JF, de Mello M, Scorza JV, Anez N, Ramirez JL 1992. Identification of new world *Leishmania* using ribosomal gene spacer probes. *Mol Biochem Parasit 56:* 15-26.

Hanham CA, Shaw JJ, Lainson R 1991. Antibodies that react with *Leishmania* (*Viannia*) *naiffi*. *J Parasit 77:* 680-687.

Huntsman RG 1968. Haemoglobin in Man and Animals, p.21-28. In JG Hawkes *Chemotaxonomy and Serotaxonomy*. Systematics Association Special Volume No. 2, London.

Jaffe CL, McMahon-Pratt D 1983. Monoclonal antibodies specific for *Leishmania tropica*. I. Characterization of antigens associated with stage- and species-specific determinants. *J Immunol 131*:187-1993.

Kitching IJ 1992. Tree-building techniques, p.44-71. In PL Forey, CJ Humphries, IJ Kitching, RW Scotland, DJ Siebert, DM Williams (eds). *Cladistics. A practical course in systamatics*. Systamatics Association Publication No. 10, Oxford.

Knuetzer RD, Souraty N, Semko ME 1987. Biochemical identities and differences among *Leishmania*

species and subspecies. *Amer J Trop Med Hyg 36*:22-32.

Lainson R, Shaw JJ 1987. Evolution, classification and geographical distribution, p.1-120. In W Peters, R Killick-Kendrick (eds). *The Leishmaniases in Biology and Medicine, Volume I, Biology and Epidemiology*. Academic Press Inc., London.

Lanotte G, Rioux J-A, Serres E 1986. Approche cladistique du genre *Leishmania* Ross, 1903. A propos de 192 souches originaires de l'Ancien Monde. Analyse numérique de 50 zymodèmes identifiés par 15 enzymes et 96 isoenyzymes. p. 269-288. In J-A Rioux *Leishmania Taxonomie et phylogenése. Applications éco-épidémiologiques*. Colloque International. IMEEE, Montpellier.

Le Blancq SM, Cibulskis RE, Peters W 1986. *Leishmania* in the Old World: 5. Numerical analysis of isoenzyme data. *Trans R Soc Trop Med Hyg 80:* 517-524.

McMahon-Pratt D, David JR 1981. Monoclonal antibodies that distinguish between New World species of *Leishmania*. *Nature 291:* 581-583.

Ramirez JL, Guevara P 1987. The ribosomal gene spacer as a tool for the taxonomy of *Leishmania*. *Mol Biochem Parasitol* 22:117-183.

Saf'janova VM 1986. The problems of classification and phylogeny of the genus *Leishmania*, p.247-255. In J-A Rioux *Leishmania Taxonomie et phylogenése. Applications éco-épidémiologiques*. Colloque International. IMEEE, Montpellier.

Santoro F, Lemesre J, Rizvi F, Afchain D, Sadigursky M 1986. Spécificité au niveau des protéines de surface des promastigotes de *Leishmania donovani* (Laveran et Mesnil, 1903) *Leishmania infantum* Nicolle, 1908 et *Leishania chagasi* Cunha et Chagas, 1937, p.71-75. In J-A Rioux *Leishmania Taxonomie et phylogenése. Applications éco-épidémiologiques*. Colloque International. IMEEE, Montpellier.

Shaw JJ, Lainson R, McMahon Pratt D, David JR 1986. Serodemes of the *Leishmania braziliensis* complex, p. 179-183. In J-A Rioux, *Leishmania Taxonomie et phylogenése. Applications éco-épidémiologiques*. Colloque International. IMEEE, Montpellier.

Sogin ML, Elwood HJ, Gunderson JH 1986. Evolutionary diversity of eukaryotic small subunit rRNA genes. *Proc Natl Acad Sci USA 83:* 1383-1387.

Tavares CC, Grimaldi Jr G, Traub-Cseko YM 1992. Molecular karyotype analysis and mapping of housekeeping genes to chromosomes of selected species complexes of *Leishmania*. *Mem Inst Oswaldo Cruz 87:* 477-486.

Thomaz-Soccol V, Lanotte G, Rioux JA, Pratlong F, Martini-Dumas A, Serres E 1993. Monopyletic origin of the genus *Leishmania* Ross, 1903. *Ann Parasit Hum Comp 68:* 107-108.

Tibayrenc M, Kjellberg F, Ayala FJ 1990. A clonal theory of parasitic protozoa: the population structure of *Entamoeba*, *Giardia*, *Leishmania*, *Plasmodium*, *Trichomonas* and *Trypanosoma* and their medical and taxonomical consequences. *Proc Natn Acad Sci USA 87:* 2414-2418.

Uliana SRB, Affonso MHT, Camargo EP, Floeter-Winter LM 1991. *Leishmania*: Genus Identification Based on a Specific Sequence of the 18S Ribosomal RNA sequence. *Exp Parasitol 72:* 157-163.

Wiley EO, Siegel-Causey D, Brooks DR, Funk VA 1991. *The Compleat Cladist. A Primer of Phylogenetic Procedures*. The University of Kansas, Museum of Natural History, Lawrence, Kansas, 158 pp.