



Molecular Evolution and Phylogeny of *Leishmania*

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Gabriele Schönian, Julius Lukeš, Olivia Stark, and James A. Cotton

Abstract

The genus *Leishmania* was first described in 1903 for the parasite *Leishmania donovani*, but many additional species have been described since then. Although recent hierarchical taxonomic schemes have increasingly used molecular or biochemical characters to assign *Leishmania* organisms into different species, they are still heirs of the first classifications based primarily on geographical distribution, vector species, and disease presentations. The current classification system, based on multilocus enzyme electrophoresis, proposes up to 53 species, although molecular phylogenies of *Leishmania* suggest that the number of species may be too large. Very recently this classification system has been revised based on multiple gene phylogenies. For many decades, there has been a controversial discussion on whether the genus *Leishmania* appeared first in the Old World or in the New World. Analyses of whole-genome data led to the supercontinent hypothesis, in which the parasites evolved from a monoxenous ancestor on Gondwana and separated into *Paraleishmania* and all other species around the time when Gondwana split.

Many molecular markers have demonstrated substantial intraspecies diversity and the existence of geographically and genetically isolated populations in all *Leishmania* species tested so far. In particular the idea that *Leishmania* evolve predominantly clonally with only rare sexual recombination has repeatedly been questioned by the detection of hybrids, mosaic genotypes, and gene flow between

G. Schönian (✉) · O. Stark
Charité – Universitätsmedizin Berlin, Institut für Mikrobiologie und Hygiene, Berlin, Germany
e-mail: gabriele.schoenian@t-online.de

J. Lukeš
Biology Centre, Institute of Parasitology, Czech Academy of Sciences, Ceske Budějovice
(Budweis), Czech Republic

J. A. Cotton
Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, UK

populations and strong inbreeding and, finally, the detection of genetic recombination under laboratory conditions.

This chapter reviews the recent (mostly) molecular data that provide new insights into the evolution, taxonomy, phylogenetic, and population genetic relationships of *Leishmania* but also the questions raised by this knowledge. It also discusses the power of modern approaches, such as multilocus sequence analysis, multilocus microsatellite typing, and comparative genomics for studying the inter- and intraspecies variation of *Leishmania* parasites.

2.1 Introduction

Phylogenetics is the study of evolutionary relatedness among various groups of organisms (e.g., species or populations); this relatedness is revealed, for example, through morphological data and molecular data, particularly DNA sequence data. *Taxonomy*, the science of naming and classifying organisms, is enriched by phylogenetics, although both fields remain methodologically and logically distinct. They overlap, however, in the area of *phylogenetic systematics*—the science that reconstructs the pattern of evolutionary events that have led to the distribution and diversity of life.

Modern phylogenetic studies with different molecular data have transformed our knowledge of evolutionary history and, consequently, taxonomy, as phylogenies based on these data have challenged traditional classifications for many groups of organisms. This is particularly the case for the most basal groups, and a new classification system of eukaryotes has been recently proposed based on data from modern morphological approaches, biochemical pathways, and molecular phylogenetics [1].

The genus *Leishmania* has also suffered taxonomic changes. Its position within the family Trypanosomatidae has been revised, the number of species belonging to it is disputed, and geographically defined populations have been identified in many *Leishmania* species. A good definition of *Leishmania* species is crucial for correct diagnosis and prognosis of the disease as well as for making decisions regarding treatment and control measures. This is a fundamental issue since the severity and nature of the clinical manifestation in immunocompetent patients varies with the infecting organism. Different *Leishmania* species cannot be distinguished by morphological criteria and have therefore been assigned to different species primarily based on clinical, biological, geographical, and epidemiological standards and, more recently, on immunological and biochemical data. Accordingly, since the first description of the genus *Leishmania* in 1903, the number of species has increased continuously. While species based on these criteria may be clinically useful, it is unclear that they will reflect the true evolution and diversity of the genus. Although many molecular methods have been recently introduced for unraveling the

phylogeny of *Leishmania* and define its taxonomy, defining a *Leishmania* species or achieving a consensus on the described species is still not easy.

2.2 Molecular Methods for *Leishmania* Phylogenetics, Identification, and Population Genetics

The selection of the molecular method or marker most suitable for its use in phylogenetic studies depends on the question needed to be addressed and the required level of resolution. While trees resulting from molecular studies of *Leishmania* are preferred, they should not be used alone, as evolution of hosts and vectors, as well as climatic and geographical features, should also be taken into account [2]. A comprehensive review has recently listed previously used markers for *Leishmania* diagnosis and strain typing [3].

2.2.1 Molecular Methods for Studying *Leishmania* Phylogeny

Currently, phylogenetic relationships at the level of Kinetoplastea, as well as at the level of the genus *Leishmania*, are mostly based on DNA sequences. Slowly evolving genomic sequences such as small subunit (SSU) rRNA genes and glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) genes have been most widely used for establishing molecular phylogenies of these pathogenic flagellates. Numerous molecular tools have been described that distinguish species and strains of *Leishmania* parasites. Since the genus *Leishmania* is relatively homogenous, as compared to the related genus *Trypanosoma*, techniques that reveal genetic variation at a higher level of resolution are required. Multilocus enzyme electrophoresis (MLEE) has been considered for many years as the reference technique for the identification of *Leishmania* species and subspecies [4]. However, MLEE has drawbacks including the need to cultivate parasites to obtain sufficient amounts of cells for the experiments, as well as the lack of discriminatory power to differentiate the parasites below the species level [5, 6]. On the other hand, molecular approaches based on PCR or other amplification techniques have the advantage of combining high sensitivity for direct detection of the infecting parasites in various human, animal, and sand fly tissues, with the ability to distinguish *Leishmania* parasites at species and intraspecies levels [7]. The PCR-based methods include the amplification and subsequent restriction fragment length polymorphism (RFLP) or DNA sequence analysis of multicopy targets or multigene families (including coding and noncoding regions and PCR fingerprinting techniques), to the recently developed multilocus sequence analysis [8–10] and multilocus microsatellite typing (for review see [11]). These tools have been applied for the identification of the causative agent of leishmaniasis in patient isolates, for epidemiological studies in different foci endemic for the disease, as well as for taxonomic, phylogenetic, and population genetic studies in *Leishmania*.

For phylogenetic studies, differential diagnosis of species by sequencing single-copy gene targets is preferred over methods based on the evaluation of RFLP or fingerprinting patterns, although these latter methods may be useful in epidemiological studies to distinguish between a set of strains known to be circulating in a single focus. Phylogenies based on one gene are often not fully adequate to understand the phylogeny of the Trypanosomatidae or its subgroups, given some instances of recombination, or even different mutation rates between lineages. Instead, several independent genes displaying different evolutionary histories are preferable [12, 13], such as implemented in multilocus sequence analysis (MLSA).

For inferring the phylogenetic relationships and the molecular classification at the level of kinetoplastids, as well as at the *Leishmania* genus, analyses based on DNA sequence comparisons are preferred. Nucleotide sequences or predicted amino acid sequences at specific positions can be used as “characters” in a phylogenetic analysis [14]. As these characters (nucleotides) are the basic units of information encoded in the organism’s genome, the potential number of informative characters is enormous. Furthermore, sequence data are highly reproducible and easy to compare between laboratories.

Multilocus sequence typing was initially developed for bacteria [15] and applied in the same manner as MLEE. In the strict bacterial context, short DNA sequences of 300–500 bp for 7–12 gene targets are generated by direct sequencing in both directions. Each sequence is scored as a haplotype, bacteria being haploid; the combination of the haplotypes for all gene targets constitutes the sequence type (ST). Gene targets must be selectively neutral, given that among the relatively small number of genes, a single gene subject to strong positive or negative selection may disrupt phylogenies. In *Leishmania*, different approaches for MLSA have been developed in which case it is the diploid sequence type that is codified, using the codes for ambiguous nucleotides. However, a publicly available database has not yet been created. The *L. (L.) donovani* complex has been studied by using ten loci for gene coding for enzymes used in MLEE [16, 17]. Five of these ten loci plus two additional conserved loci have been used for studying Chinese isolates representing different *Leishmania* species [10], and four of these loci were applied for getting new insights into the taxonomy and phylogeny of *L. (Viannia)* parasites [8]. El-Baidouri et al. have selected seven other independent loci for their MLSA approach which was applied to different Old World species of *Leishmania* [9]. All these MLSA approaches include at least partial sequencing of the selected loci and further phylogenetic analyses of the concatenated sequences. They all indicate that the same gene targets can be used through the *Leishmania* genus and will enable comparisons of genetic distances between the species but also allow to assess the degree of genetic diversity within species.

2.2.2 Molecular Methods for Population Genetic Studies in *Leishmania*

Population genetic approaches depend on highly polymorphic neutral markers that are not affected by natural selection, which must also be co-dominant to permit the detection of all three possible allele combinations in a diploid genome. Multilocus microsatellite typing (MLMT) may meet the criterion of neutrality better than MLSA. Microsatellite sequences are repeated motifs of 1–6 nucleotides that may vary in length due to the gain or loss of single-repeat units during DNA replication. This variation can easily be detected after amplification with primers annealing specifically to their flanking regions. Microsatellite markers are prone to homoplasy and the evolutionary history of a particular repeat may be uncertain. All analyses should therefore include a panel of 10–20 unlinked sequences to overcome this main obstacle in the use of microsatellite markers. Microsatellite markers have been found to be largely species-specific in *Leishmania* [18, 19] and therefore, MLMT is not suited for phylogenetic studies. In fact, comparison of DNA-based methods of strain typing shows that MLMT and PCR-RFLP of kinetoplast (k) DNA minicircles are most useful to discriminate *Leishmania* parasites at intraspecies level, with both of these methods allowing a fine-grained characterization of parasite diversity, for example, in demonstrating genetic links between remote populations of *L. (L.) infantum* and *L. (L.) donovani* [20, 21]. Given that kDNA PCR-RFLP is not co-dominant and its results are difficult to reproduce and to compare between laboratories, MLMT appears to be the current method of choice for population genetic studies in *Leishmania*.

2.2.3 Next-Generation Sequencing Used for Interspecific and Intraspecific Differentiation in *Leishmania*

New and increasingly cheaper high-throughput sequencing technologies that enable fast sequencing of large numbers of genes have opened the door for genome-wide multilocus genotyping between and within *Leishmania* species. Since the publication of the first *Leishmania* reference genome of *L. (L.) major* [22], reference genomes have been published for many other species, such as *L. (L.) infantum* and *L. (V.) braziliensis* [23], *L. (L.) donovani* [24], *L. (L.) mexicana* [25], *L. (L.) amazonensis* [26], *L. (V.) panamensis* [27], and the *Sauroleishmania L. (S.) tarentolae* [28] and *L. (S.) adleri* [29]. These data, together with unpublished assemblies for many other species—and even multiple strains for some species—are available on the kinetoplastid genome database, TriTrypDB (<http://tritrypdb.org>) [30]. The quality of these assemblies varies in terms of how completely they represent the true genome sequences and how contiguous they are. New sequencing technologies are now being used in *Leishmania* that allow generation of very high-quality genome assemblies more easily and from much longer sequencing reads [31], and improved genome assemblies for many species are likely to be available soon. Accurate annotation of genes and other genome features is required for making

these resources useful to the research community. Although consistent, high-quality automated annotation is now possible [32], and manual inspection and improvement of annotation is still critical, particularly in ensuring that genome resources accurately reflect findings from the literature. Comparison of different leishmanial genomes revealed a remarkable conservation of gene content and synteny in orthologous chromosomes [23, 33]. Using whole-genome information for different species of *Leishmania*, MLSA could be, thus, extended to several hundreds of gene targets [34].

Next-generation sequencing allows analyses of different mutation types, such as single nucleotide polymorphisms (SNPs), insertion/deletions (indels), chromosome copy number variations (aneuploidy), and gene copy number variations (CNVs). So far, most studies in *Leishmania* have focused on analyzing SNP data which, depending on the selection criteria, can differentiate parasites at the interspecies and/or intraspecies levels (see paragraph 4.2 for more details).

Sterkers et al. [35, 36] reported that in *L. (L.) major*, chromosomal content varies not only from strain to strain but also from cell to cell creating “mosaic aneuploidy”. This leads to high karyotypic plasticity and conserved intra-strain genetic heterogeneity combined with loss of heterozygosity per cell. Next-generation sequencing has confirmed the existence of remarkable chromosome copy number variations and mosaic aneuploidy for parasites belonging to the same or closely related *Leishmania* species [24, 37, 38]. Recently, Dumetz et al. [31] reported dynamic changes of aneuploidy during the parasite’s life cycle. Whereas chromosome copy numbers were highly variable in a strain during in vitro cultivation, smaller yet consistent karyotype changes were noticed after a passage through a sand fly, and aneuploidy dropped significantly in a strain-specific manner in hamster amastigotes. As a consequence, all DNA-based typing methods employed earlier have the problem that they cannot decide if a cell population (or strain) consists of heterozygous cells or of homozygous cells presenting different allelic and ploidy content. Approaches to study the genomes of single cells are now available but have not yet, to our knowledge, been applied to *Leishmania* [39].

2.2.4 The Importance of Sampling for Phylogenetic and Population Genetic Studies

Sampling is crucial for phylogenetic as well as for population genetic analyses and depends on the question(s) to be addressed. None of the phylogenetic and population genetic studies published so far in *Leishmania* meet all the requirements for optimal sampling, although more recent studies increasingly try to do so. For phylogenetic inference, parasites should be ideally sampled from the whole range of geographical distribution, but most studies have analyzed only one or a few strains per species, normally reference strains that are kept in cryobanks and have been subcultured many times. Population genetic studies often suffer from the drawback that for some geographic areas, only few isolates are available. Analyzing parasites at a finer geographical scale, using sufficient numbers of isolates, has been shown to be

necessary for the detection of hidden substructures within the *Leishmania* species [40]. In addition, *Leishmania* spp. pathogenic for humans are, for understandable reasons, usually overrepresented in the sample collections. It is urgently needed to include more flagellates that are collected from animal hosts or insect vectors or even asymptomatic hosts into phylogenetic and population genetic studies. The availability of parasite isolates in promastigote culture is essential for in-depth study of phenotypic differences between strains, but *Leishmania* parasites can be difficult to isolate [41], and therefore, the use of direct applications in host tissues should be preferred for molecular epidemiological and population genetic studies.

2.3 Molecular Evolution and Origin of the Genus *Leishmania*

Six basic groups of eukaryotes, similarly to the traditional “kingdoms”, have been recognized in the new classification system by Adl et al. [1], and the genus *Leishmania* has been assigned to the supergroup Excavata. While groups at this highest taxonomic level share few distinguishing features, and are largely based on molecular data, excavates are ancestrally flagellated protozoa feeding on small particles via a feeding groove. *Leishmania* are kinetoplastid parasites belonging to the Trypanosomatidae (Table 2.1).

2.3.1 Molecular Phylogeny of Kinetoplastids

Kinetoplastids constitute a remarkable group of morphologically rather simple unicellular organisms that share several unusual features in their genomes. The most prominent unique structure is the kinetoplast DNA, a massive network of thousands of topologically interlocked DNA circles of two types, mini- and maxicircles, corresponding to mitochondrial DNA [44]. Other unique features include mitochondrial RNA editing of the uridine insertion/deletion type, trans-splicing of nuclear-encoded mRNA transcripts, intron poverty, presence of

Table 2.1 Taxonomic position of the genus *Leishmania* according to the classification by Adl et al. [1] and Jirku et al. [42] emended by Maslov and Lukeš [43]

Super-group	Excavata Cavalier-Smith 2002, emend. Simpson 2003 (P?)
Phylum	Euglenozoa Cavalier-Smith 1993, emend. Simpson 1997
Class	Kinetoplastea Honigberg, 1963 emend. Vickerman 1976
Subclass	Metakinetoplastina Vickerman, 2004 (R)
Order	Trypanosomatida Kent, 1880 stat. nov. Hollande, 1952
Family	Trypanosomatidae Doflein, 1951
Subfamily	Leishmaniinae Maslov and Lukeš, 2012 emend. Shaw, Texeira and Camargo 2016
Genus	<i>Leishmania</i> Ross, 1903

(P?) Possibly paraphyletic; (R) group identified by small subunit (SSU) rRNA

Table 2.2 The morphology and hosts of Trypanosomatida modified after Stevens et al. [45]

	Genus	Morphology	Hosts	Vectors
Monoxenous	<i>Blastocrithidia</i>	Epimastigote, amastigote, cyst	Insects, ticks	
	<i>Crithidia</i>	Choanomastigote	Insects	
	<i>Herpetomonas</i>	Promastigote, opisthomastigote	Insects	
	<i>Leptomonas</i>	Promastigote, cyst	Insects, ciliates, nematodes	
	<i>Rhynchoidomonas</i>	Trypomastigote—no undulating membrane	Diptera	
	<i>Sergeia</i>	Promastigote	Diptera	
	<i>Blechomonas</i>	Promastigote	Siphonaptera	
Heteroxenous	<i>Endotrypanum</i>	Amastigote, promastigote, epimastigote, trypomastigote	Sloths	Sandflies
	<i>Leishmania</i>	Amastigote, promastigote	Mammals, lizards	Sandflies
	<i>Phytomonas</i>	Promastigote	Flowering plants	Hemiptera
	<i>Trypanosoma</i>	Amastigote, epimastigote, trypomastigote	Vertebrates	Arthropods, leeches

hypermodified base J, and arrangement of genes in large polycistronic clusters [13]. The kinetoplastid species show a variety of life styles ranging from ubiquitous free-living organisms (some bodonids), through ecto- and endoparasites of fish (e.g., *Cryptobia*, *Trypanoplasma*, *Ichthyobodo*) to obligatory parasites of invertebrates, vertebrates, and plants (for review see [13, 43]). The species parasitizing plants (*Phytomonas*), insects (*Crithidia*, *Herpetomas*, *Leptomonas*, *Blastocrithidia*, *Rhynchoidomonas*, *Strigomonas*, *Angomonas*, *Sergeia*, *Blechomonas*, *Paratrypanosoma*), fish, amphibians, and reptiles (*Trypanosoma*) or mammals (*Trypanosoma*, *Leishmania*, *Endotrypanum*) are comprised in the Trypanosomatidae (Table 2.2) [46].

Originally, the taxonomy of kinetoplastids was based on their morphology and life cycles. With the initial molecular biological studies, it became clear that the so far existing taxonomy does not reflect the true genetic relationships of these organisms. These early molecular phylogenetic studies suffered, however, (a) from inappropriate sampling (i.e., mainly medically important trypanosomatids were included in the analysis and the diverse bodonids were ignored) and (b) from troubles with the first gene target sequence used (the SSU rRNA gene of kinetoplastids have several large fast-evolving regions which, if not removed, lead to artifacts in tree construction and, if removed, result in a faint phylogenetic signal in the alignments obtained).

A broad sampling of kinetoplastid diversity and the introduction of additional informative markers like heat-shock proteins (HSPs) and glycosomal glyceraldehyde-3-phosphate dehydrogenases (gGAPDH) revealed that the Kinetoplastea consist of Prokinetoplastina and Metakinetoplastina. The former brings together intracellular endosymbionts of fish-pathogenic amoeba, *Perkinsela* spp., and ectoparasites of fish, *Ichthyobodo* spp. [46, 47]. The latter clade is further subdivided into four subclades: the trypanosomatids (Trypanosomatida) and three clades of bodonids (Neo-, Eu-, and Parabodonida) [48]. Branching of the trypanosomatids from within the bodonids is now strongly supported [49]. As a whole, the wealth of new sequence data makes the old division of the kinetoplastids into bodonids and trypanosomatids artificial. The trypanosomatids were considered to be most closely related to the mostly free-living Eubodonida (e.g., *Bodo saltans*), a result which is congruent with an earlier study based on a partial mitochondrial DNA sequence [50]; however, the recently described *Paratrypanosoma confusum* represents the most basal branching trypanosomatid, which likely retains numerous ancestral features [49].

The current molecular phylogeny of the Trypanosomatidae is mainly based on the analyses of SSU rRNA genes [51–56] and gGAPDH genes, although to a lesser extent [53, 54, 57, 58], as well as the spliced-leader (SL) RNA gene [59]. Neither gene is, however, suitable for inferring a robust phylogeny across the entire family, and additional phylogenetic markers should be used for the trypanosomatids, such as DNA and RNA polymerase genes [60]. The current picture that has emerged from SSU rRNA and gGAPDH genes is that the genus *Trypanosoma* represents a large monophyletic clade in a sister-group relationship with the rest of the family (e.g., see Fig. 2.1). The monoxenous lineages of insect parasites currently assigned to the genera *Blastocrithidia*, *Crithidia*, *Leptomonas*, *Sergeia*, and *Wallaceina* are intermingled with dixenous lineages of parasites of mammals or reptiles (*Leishmania*) and plants (*Phytomonas*). Only *Leishmania* and *Phytomonas* form monophyletic clades, whereas all monoxenous flagellate genera have been found to be paraphyletic and widely interspersed in the phylogenetic trees.

In the SSU rRNA tree (Fig. 2.1), the root is located between the clades of trypanosomes and “non-trypanosomes”. However, the recently discovered *Paratrypanosoma confusum* likely constitutes the most basal flagellate that acquired the parasitic life style [49]. While it is difficult to rigorously exclude dixenous life cycle, the available data strongly point to the fact that *P. confusum* is a monoxenous parasite of dipteran insects (Skalický et al. unpubl. data). The branching order of the main clades within Trypanosomatidae is not well supported; hence, more data is needed to confirm the basal branching of *Blechnomonas* [64].

Monoxenous trypanosomatids of insects are not only extremely diverse but developed distinct life strategies. One clade represented by the genera *Strigomonas* and *Angomonas* invariably contains endosymbiotic bacteria in their cytoplasm [65]. It was shown that all bacteria parasitizing these globally distributed trypanosomatids are derived from a single acquisition event of a betaproteobacterium by a flagellate [66] that developed into a tight endosymbiotic relationship involving targeting of proteins from one partner into another

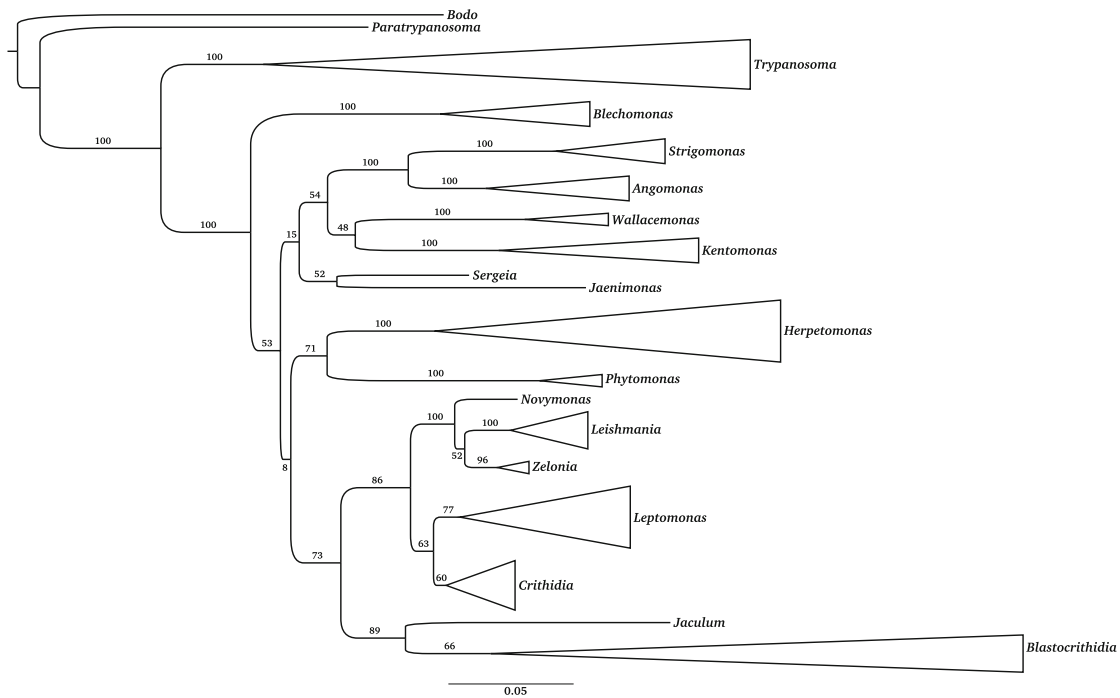


Fig. 2.1 Sequences for phylogenetic analyses were received from publicly available sources for both SSU rRNA and gGAPDH genes. The datasets for each gene were aligned by MUSCLE [61] separately and selection of relevant positions with subsequent concatenation was performed using Gblocks [62]. Phylogenetic model selection with ModelGenerator using four Γ rate categories favored GTR+ Γ model and ML trees were constructed using RAxML 8.27 [63] with 1000 bootstrap replicates

[67]. Interestingly, a trypanosomatid in hemipteroid bugs captured in Ecuador hosts yet another bacterium that was acquired in an independent endosymbiotic event [68].

Another example of unique features being found in monoxenous trypanosomatids is the case of *Blastocrithidia* sp. In an unprecedented step, this flagellate repurposed all three stop codons into sense codons, and its translation machinery, therefore, has to distinguish between a multitude of in-frame stop codons and the genuine one that indeed terminates its genes [69]. These two examples demonstrate that trypanosomatids in insects constitute a group of dexterous parasites capable of altering under certain conditions their molecular and biochemical capacities.

Therefore, it is not surprising that the monoxenous parasites underwent repeated transitions to dixenous parasitism [13, 70], at least once in the *Trypanosoma* clade and once in each of the lineages leading to *Leishmania/Endotrypanum* and *Phytomonas*. The phylogenetic position of *Leishmania* within insect trypanosomatids as a relatively late emerging group, supports the classical “insect-first” hypothesis postulating that dixenous parasites evolved from primary insect parasites via acquisition of hematophagy [71, 72]. The discovery of two larval sand flies in Early Cretaceous Burmese amber parasitized by trypanosomatids led to the hypothesis that these protists were ingested by sand fly larvae, carried through the pupal and into the adult stage and introduced into a vertebrate during blood feeding [73]. The establishment of trypanosomatids in the vertebrate and subsequent

reacquisition by sand flies finally resulted in a dixenous life cycle. Some infections of animals and humans, often immunosuppressed patients, with monoxenous trypanosomatids have been reported recently showing that acquisition of mammals as hosts by primarily insect flagellates is not a rare event of the past [74–77].

2.3.2 Molecular Phylogeny of the Genus *Leishmania*

The first phylogenetic trees of the genus *Leishmania* were based on MLEE data analyzed by phenetic and cladistic techniques [4, 78, 79]. These analyses confirmed, at the time, the monophyletic origin of the genus and its subdivision into two subgenera: *L.* (*Leishmania*) comprising all species from the Old World (OW), *L.* (*L.*) *mexicana* and *L.* (*L.*) *amazonensis* from the New World (NW), and *L.* (*Viannia*) consisting of only NW species. The lizard species were, however, excluded from these studies because the *Sauroleishmania* were then considered to be a separate genus. A concept of species complexes was proposed and later modified to group *Leishmania* species based on biological and biochemistry characteristics [72, 80]. The validity of this classification began to be questioned when the species status of some representatives of both *L.* (*Leishmania*) and *L.* (*Viannia*) subgenera as well as the concept of species complexes as a whole [81] were not supported by molecular analyses. In addition, recently discovered putative new species may belong to separate groups (for more details see paragraph 3.3).

As for the kinetoplastids, the SSU rRNA gene and mitochondrial gene sequences are most widely used for the inference of deep phylogenetic relationships within the genus *Leishmania*. The variation in the SSU rRNA gene was, however, insufficient to robustly resolve any internal branching within *Leishmania* [82], and the extensive editing of most mitochondrial genes in *Leishmania* [83] may cause problems in phylogenetic studies.

During the past 20 years, several DNA sequences have been used to investigate the phylogeny of the genus *Leishmania*. These have included single-copy genes encoding the catalytic polypeptide of DNA polymerase α (*polA*) [60], the largest subunit of RNA polymerase II (*rpoIIIS*) [60], the 7SL RNA gene [84], the noncoding multicopy ribosomal internal transcribed spacer (ITS) [85–87], the *N*-acetylglucosamine-1-phosphate transferase (NAGT) gene [88], the mitochondrial cytochrome *b* gene (*cytb*) [89], and, more recently, sequences of the heat shock protein 70 gene (*hsp70*) subfamily [90]. Sequence analyses of these different targets have been consistent in that the subgenera *L.* (*Leishmania*) and *L.* (*Viannia*) each forms a distinct monophyletic clade and that the OW and NW species are separated within the *L.* (*Leishmania*) subgenus (Figs. 2.2 and 2.3). When *Sauroleishmania* were included, they branched off in between the *L.* (*Leishmania*) and *L.* (*Viannia*) subgenera as an independent taxon. This result suggests that lizard-hosted *Leishmania* might be derived from mammalian parasites [60] and that they should be regarded as a subgenus of *Leishmania* rather than an independent genus [91]. However, RNA and DNA polymerase genes were shown to evolve faster in the lizard *Leishmania* than in the mammalian *Leishmania* making it difficult to define the exact

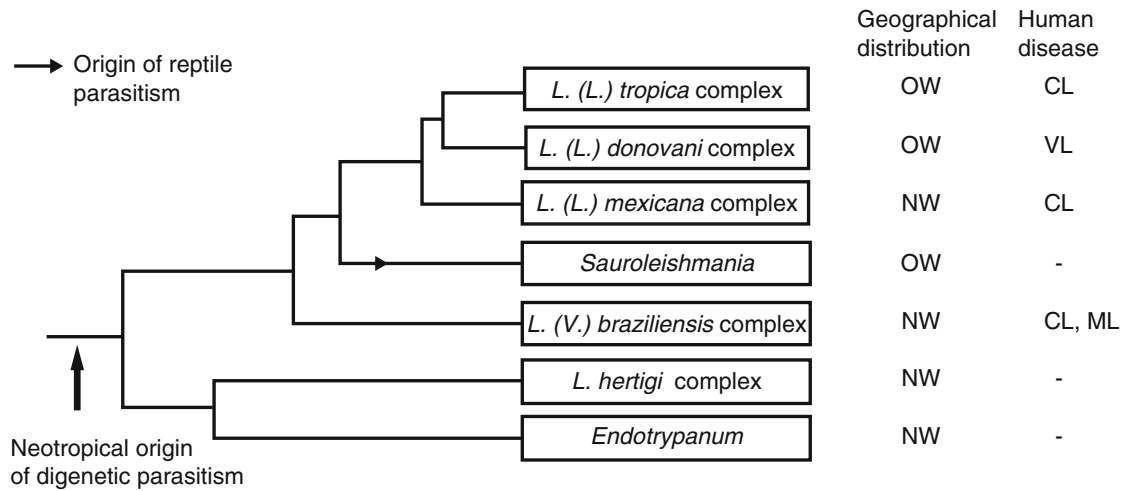


Fig. 2.2 Schematic tree showing the evolution of the *Leishmania/Endotrypanum* subtree of the Kinetoplastida based on POLA/ROPIILS nucleotide sequences (Croan et al. [60]). The *L. (L.) tropica* complex, as shown here, comprises sequences of *L. (L.) tropica*, *L. (L.) major*, *L. (L.) aethiopica*, and *L. (L.) arabica*; *L. (L.) donovani* complex those of *L. (L.) donovani* and *L. (L.) infantum*; *L. (L.) mexicana* complex those of *L. (L.) mexicana* and *L. (L.) amazonensis*; *L. (V.) braziliensis* complex those of *L. (V.) braziliensis* and *L. (V.) panamensis*; *L. hertigi* complex those of *L. hertigi* and *L. deanei*; and *Endotrypanum* those of *E. monterogeii* and *L. herreri*. *Sauroleishmania* were represented by the species *L. hoogstraali*, *L. tarentolae*, *L. adleri*, and *L. gymnodactyli*. For each taxon, an indication of the geographical distribution (OW, Old World; NW, New World) and typical disease pathology (CL, cutaneous; VL, visceral; MC, mucocutaneous) observed following infection is shown on the right. (Reprinted from Croan et al. [60] © 1997, with permission from Elsevier)

taxonomic position of lizard parasites [60]. In all studies, the *L. (Viannia)* subgenus was closest to the root, while *L. (Leishmania)* and *L. (Sauroleishmania)* formed the crown of the trees.

Based on a variety of molecular criteria, Cupolillo et al. [92] have proposed the separation of the genus *Leishmania* into two sections: Euleishmania comprising the subgenera *Leishmania*, *Sauroleishmania*, and *Viannia*, and Paraleishmania consisting of *L. hertigi*, *L. deanei*, *L. colombiensis*, *L. equatoriensis*, *L. herreri*, and strains of *Endotrypanum*. *L. hertigi*, and *L. deanei* have only been found in Neotropical porcupines and an unknown sand fly vector, and do not, or only transiently, infect humans [93]. *L. herreri* was isolated from sloths and different sand fly species in Costa Rica [94]. Comparison of DNA and RNA polymerase sequences [60] as well as PCR-RFLP of the SSU rRNA gene [93, 94] revealed that these three species are closely related to *Endotrypanum*, a parasite of Neotropical tree sloths. In the resulting trees, these species represented the most basal branches.

Several *Leishmania* isolates have been described that could not be assigned to any of the known species. Noyes et al. [95] identified a parasite, *L. martiniquensis*, isolated from human cutaneous lesions in Martinique by MLEE and sequencing of different targets, as the most divergent member of the genus *Leishmania*. Recently, a new species of *Leishmania* has been reported from a focal CL outbreak in Ghana [96] as well as from VL cases in immunocompetent and immunosuppressed patients

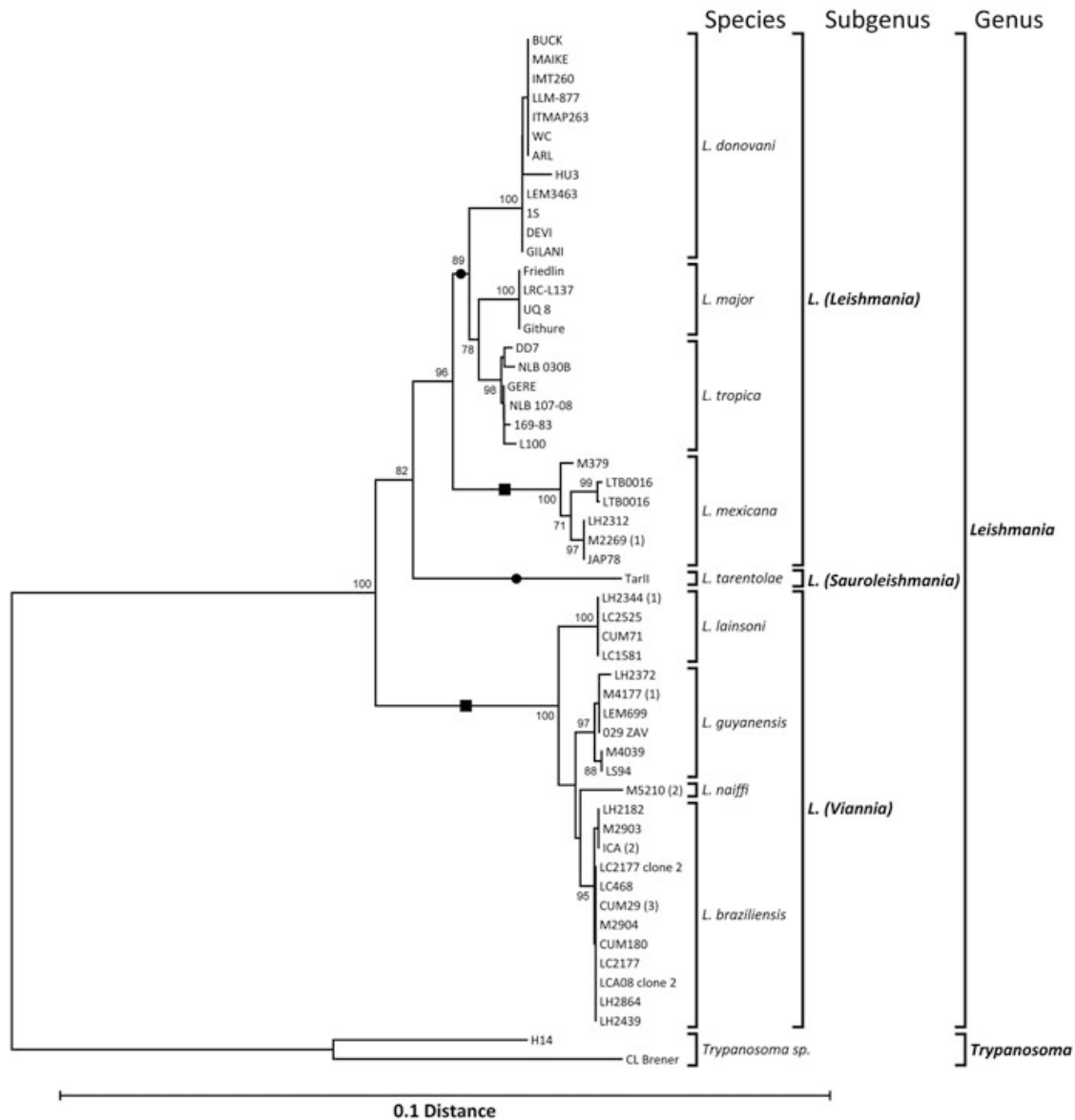


Fig. 2.3 Neighbor-joining phylogeny of hsp70 sequences of 52 strains representing 17 *Leishmania* and 2 *Trypanosoma* species, based on an alignment of 1380 nucleotides (Fraga et al. [90]). Distances were estimated using the Kimura-2 parameter model, thereby excluding all 10 sites with ambiguous nucleotides. Bootstrap support of the branches was inferred from 2000 replicates and is given in percentages at the internodes when exceeding 70%. The tree is drawn to the scale at the bottom, expressed as distance per nucleotide. Supported monophyletic species and subgenera are depicted at the right, irrespective of the species classification presented in Table 2.1 but reflecting the observations from Sect. 2.4. Old World clusters are indicated by a dot on the branch leading to the cluster, while a square is used for New World groups. The tree was rooted with the two *Trypanosoma* sequences found most related to *Leishmania* hsp70. Numbers between brackets following the strain names indicate the number of ambiguous nucleotides in the sequence. (reprinted from Fraga et al. [90] © 2010 with permission from Elsevier)

in Thailand [97], named *Leishmania* sp. *siamensis*. In those cases, parasites were, however, not isolated in culture, and the identification was based on microscopy

and/or PCR using DNA extracted from clinical samples. Surprisingly, locally acquired CL was also detected in kangaroos, wallaroos, and wallabys, living in captivity in the Northern Territory of Australia, a region that was considered free of *Leishmania* parasites [98, 99]. DNA sequence analyses revealed that the parasites were genetically indistinguishable and possibly represent a novel *Leishmania* species. Autochthonous cases of CL in German and Swiss horses and in a Swiss cow have been associated by DNA sequence analyses with *Leishmania* parasites that could be classified neither as OW nor NW *Leishmania* species but were most closely related to *L. sp. siamensis* [100, 101]. Finally, another novel trypanosomatid has been isolated from the native Australian black fly, *Simulium (Morops) dycei* [102].

Two recent studies have analyzed different DNA sequences such as coding for RNA PolIII, HSP70, gGAPDH, and V7V8 SSU rRNA and included not only parasites that fall within the *L. (Leishmania)*, *L. (Viannia)*, and *L. (Sauroleishmania)* but also parasites earlier classified as *Paraleishmania* and, so far, unclassified ones. Based on their results, Espinosa et al. [103] propose a taxonomic revision of the trypanosomatids currently known as *Leishmania* and *Endotrypanum*. They adopt the principle of the subfamily Leishmaniinae within the family Trypanosomatidae [42] and define new genera and subgenera which are supported by their phylogenetic analyses (Table 2.3, see also Box 2.1). Four genera were identified within the new subfamily. The genus *Leishmania* consists of the already known subgenera *L. (Leishmania)* comprising mainly human pathogens from the Old and New Worlds, *L. (Viannia)* including exclusively NW parasites many of which are pathogenic to humans, and *L. (Sauroleishmania)* consisting of reptilian parasites occurring only in the OW. The fourth subgenus, *L. (Mundinia)*, was newly created for the *L. enrietti* complex. It also includes *L. (M.) martiniquensis*, the parasites isolated from a kangaroo now defined as the species *L. (M.) macropodum* [102] as well as some so far unnamed parasites. The parasites isolated from Central and South American sloths and transmitted by sand flies that eventually infect humans are assigned to the genus *Endotrypanum*. Three new genera are proposed: *Porcisia* accommodating the NW parasites isolated from porcupines, *Zelonia* comprising trypanosomatids from Neotropical hemipterans [103] and from an Australian black fly [102], and *Novymonas* harboring so far only a monoxenous trypanosomatid from the digestive tract of a hemipteran (Rhopalidae) from Ecuador [68].

The by far greatest number of taxa of the genus *Leishmania* has been included in a phylogenetic analysis based on the *hsp70* gene [90]. Several strains per species were sequenced trying to cover the geographical distribution of different species. Species of the new subgenus *L. (Mundinia)* were, however, not analyzed. The trees were rooted using *hsp70* sequences of the two most closely related *Trypanosoma* species (Fig. 2.3). The resulting phylogeny supported the existence of three monophyletic groups representing the subgenera *L. (Leishmania)*, *L. (Sauroleishmania)*, and *L. (Viannia)* and the basal branching of the latter. The two mammalian subgenera include 4 monophyletic clusters each corresponding to a different species or species complex.

Table 2.3 Revised taxonomy of Leishmaniinae parasites [102, 103]. The table was created based on the data taken from Espinosa et al. [103] and Barratt et al. [102]

Sub-family	Leishmaniinae						Porcisia	Endotrypanum	Zelonia	Novyomonas
	Leishmania									
Genus	<i>L. (Leishmania)</i>	<i>L. (Viannia)</i>	<i>L. (Sauroleishm.)</i>	<i>L. (Mundinia)</i>						
Sub-genus	<i>L. (L.)</i>	<i>L. (V.)</i>	<i>L. (S.)</i>	<i>L. (M.)</i>	<i>L. (M.)</i>			<i>E. schaudinni</i>	<i>Z. costaricensis</i>	<i>N. esmeraldas</i>
Species	<i>donovani</i> ^a	<i>braziliensis</i> ^a	<i>tarentolae</i>	<i>enriettii</i>				<i>P. hertigi</i>		
	<i>L. (L.) infantum</i> ^a	<i>L. (V.) peruviana</i> ^a	<i>L. (S.) adleri</i>	<i>L. (M.) martiniquensis</i> ^a				<i>P. deanei</i>	<i>E. monterogei</i>	<i>Z. australiensis</i>
			<i>L. (S.) agamae</i>	(syn: "siamensis")					<i>E. colombiensis</i> ^a	
	<i>L. (L.) tropica</i> ^a	<i>L. (V.) guyanensis</i> ^a	<i>L. (S.) ceramodactyli</i>	<i>L. (M.) macropodium</i>					<i>E. equatoriensis</i>	
	<i>L. (L.) aethiopica</i> ^a	<i>L. (V.) panamensis</i> ^a	<i>L. (S.) davidi</i>	<i>L. (M.) spp. Ghana</i>					<i>E. herreri</i>	
	<i>L. (L.) major</i> ^a		<i>L. (S.) gulikae</i>	<i>L. (M.) spp. Trang (TL)</i>						
	<i>L. (L.) gerbilli</i>	<i>L. (V.) lainsoni</i> ^a	<i>L. (S.) gymnodactyli</i>							
	<i>L. (L.) arabica</i> ^a		<i>L. (S.) helioscopi</i>							
	<i>L. (L.) turanica</i>	<i>L. (V.) shawi</i> ^a	<i>L. (S.) hemidactyli</i>							
			<i>L. (S.) hoogstraali</i>							
	<i>L. (L.) mexicana</i> ^a	<i>L. (V.) naiiffi</i> ^a	<i>L. (S.) nicollei</i>							

(continued)

Table 2.3 (continued)

Sub-family	Leishmaniinae				
<i>L. (L.) amazonensis</i> ^a					<i>L. (S.) phrynocephali</i>
<i>L. (L.) aristidesi</i>	<i>L. (V.) lindenbergi</i> ^a				<i>L. (S.) platycephala</i>
<i>L. (L.) venezuelensis</i> ^a					<i>L. (S.) senegalensis</i>
<i>L. (L.) pifanoi</i> ^a	<i>L. (V.) utingensis</i>				<i>L. (S.) sofieffi</i>
<i>L. (L.) waltoni</i> ^a					<i>L. (S.) smeevi</i>
<i>L. (L.) garnhami</i> ^b					<i>L. (S.) zuckermanni</i>
<i>L. (L.) forattini</i> ^b					<i>L. (S.) henrici</i> ^c
					<i>L. (S.) chamaeleonensis</i> ^c

Type species are given in bold

^aSpecies associated with human leishmaniases

^bSpecies status requires conformation

^cMost probably intestinal flagellates

2.3.3 Molecular Taxonomy of *Leishmania*

Leishmania flagellates have been assigned to different species primarily based on clinical, biological, geographical, and epidemiological criteria and, later, immunological and biochemical data, recently reviewed by Akhoundi et al. [104]. Hierarchical taxonomic schemes have been proposed using the categories of species complexes, species, subspecies, and subgenus. Compared to all other methods, MLEE has been applied to the most varied and largest number of *Leishmania* isolates in the past 25 years. The classification system resulting from the application of numerical taxonomy and cladistic techniques to electrophoretic data [4, 72, 92, 105, 106] has only very recently been replaced by new systems based on analyses of different concatenated DNA sequences [8, 9, 102, 103].

Molecular phylogenies of *Leishmania* have largely confirmed the taxonomy of the genus *Leishmania* by MLEE suggesting, however, that the number of species may be too large. The analysis of Hsp70 sequences identified only nine monophyletic groups which, according to the phylogenetic species concept, represent different species in the subgenera *L. (Leishmania)* and *L. (Viannia)* [90]. This is in good agreement with two studies that have investigated representative sample sets for the OW *L. (Leishmania)* [9] and the NW *L. (Viannia)* [8], respectively, by MLSA. For instance, two of the species earlier included in the *L. (L.) donovani* complex, namely, *L. (L.) chagasi* and *L. (L.) archibaldi*, are not supported by any molecular analyses. Strains of *L. (L.) chagasi* (NW) are undistinguishable from strains of *L. (L.) infantum* (OW) and, in fact, represent South American strains of *L. (L.) infantum* [107, 108]. *L. (L.) archibaldi* could also not be confirmed as a valid species [9, 109]; only a single nucleotide polymorphism in the glutamate oxaloacetate transaminase gene was causing the different MLEE phenotype [5]. Even more, most of the molecular phylogenies did not produce monophyletic groups for the other two species *L. (L.) donovani* and *L. (L.) infantum* [60, 86, 87, 89, 90].

Another discrepancy concerns the status of *L. (L.) killicki*, which was classified as a separate species by MLEE [4] but shown to be *L. (L.) tropica* by molecular analyses [9, 89, 110, 111]. MLSA suggests that *L. (L.) killicki* emerged from a single founder event and evolved independently from *L. (L.) tropica* but does not support a distinct taxon status for *L. (L.) killicki* [112]. According to most of the DNA-based phylogenies, *L. (L.) tropica* cannot be distinguished from *L. (L.) aethiopica* as both form a single cluster [84, 86, 89, 90]. Whether they are different subspecies of the species, *L. (L.) tropica* is debatable and needs to be investigated with a larger number of strains. The MLSA results are rather in favor of a progressive genetic isolation between the clusters representing *L. (L.) tropica* and *L. (L.) aethiopica*, suggesting that the latter might be descendants of ancestral populations that led to the *L. (L.) tropica* cluster [9].

As far as the species of the *L. (L.) mexicana* complex are concerned, most of the DNA-based phylogenies included only strains of *L. (L.) mexicana* and *L. (L.)*

amazonensis. In the hsp70 trees, which include one strain of *L. (L.) garnhami*, none of these species could be distinguished as a monophyletic clade, and *L. (L.) mexicana* was the only recognized species [90]. This is supported by some phylogenetic studies [58, 60, 87] but contrasts with others [84, 86, 89]. More strains of all species of the *L. (L.) mexicana* complex, also including *L. (L.) venezuelensis* and *L. (L.) aristidesi*, representing their whole area of distribution should be studied to evaluate the species or possible subspecies status within this complex.

Four monophyletic groups were clearly observed in an MLSA of 96 strains of the *L. (Viannia)* subgenus representing basically *L. (V.) naiffi* and *L. (V.) lainsoni*, the most divergent groups, and *L. (V.) braziliensis* and *L. (V.) guyanensis* [8]. Strains of *L. (V.) peruviana* always grouped with the strains of *L. (V.) braziliensis* and, thus, did not appear as a discrete typing unit distinct: this is in contrast to the results of MLEE, RAPD, and hsp70 phylogenies [90, 113] but is in agreement with the results of studies based on monoclonal antibodies [114] and analysis of microsatellite variation [115]. The latter study showed that strains of *L. (V.) peruviana* were intermingled with strains of *L. (V.) braziliensis* from Peru and from the Acre State, a Brazilian region bordering Peru. Strains of *L. (V.) guyanensis* and *L. (V.) panamensis* formed in different phylogenetic trees a monophyletic cluster divided into two monophyletic sub-clusters suggesting that there are two subspecies in the species *L. (V.) guyanensis*. The inclusion of *L. (V.) shawi* in the *L. (V.) guyanensis* group was demonstrated by MLEE analysis [105] and corroborated by molecular markers, as PCR-RFLP of ITS rDNA [116] and PCR-RFLP and sequence analysis of the hsp70 gene [8, 117]. Molecular phylogenies have indicated that species status is justified for *L. (V.) naiffi* and that *L. (V.) lainsoni* is a separate and the most divergent species inside the *L. (Viannia)* subgenus [8, 90, 117]. Finally, hsp70 gene analysis [117], MLMT [118] and MLSA [8], showed that the species *L. (V.) lindenbergi* [119] and *L. (V.) utingensis*, the last being represented by only one sample isolated from a *Lutzomyia tuberculata* sand fly, are quite distinct from the other *L. (Viannia)* species, although only the reference strains were analyzed.

In conclusion, the concept of species complexes for grouping *Leishmania* species which was initially proposed based on biological and biochemistry characteristics and later modified [72] is not supported by molecular phylogenies and has been abandoned in the newly defined classification scheme (Box 2.1) where the assignment to major groups across the entire genus *Leishmania* was based on gene sequences. For classification within the major groups, highly discriminatory markers such as MLST, microsatellites, or genome-wide SNPs are probably better suited.

Box 2.1 Revised classification and nomenclature of Leishmaniinae species based on molecular phylogenies of the organisms [102, 103]. Type species are underlined

The subfamily Leishmaniinae (Maslov and Lukes in [42]) consists of the genera *Leishmania*, *Porcisia*, *Endotrypanum*, *Zelonia* and *Novymonas*.

GENUS *LEISHMANIA* ROSS 1908

Subgenus *L. (Leishmania)* Safjanova, 1982 consists of the following Old World (OW) and New World (NW) species:

OW: *L. (L.) donovani* Layeran & Mesnil, 1903; *L. (L.) infantum* Nicolle 1908; *L. (L.) tropica* Wright, 1903; *L. (L.) aethiopica* Bray, Ashford & Bray, 1973; *L. (L.) major* Yakimoff & Shokhor, 1914; *L. (L.) gerbilli* Wang, Qu & Guan, 1964; *L. (L.) arabica* Peters, Elbihari & Evans, 1986; *L. (L.) turanica* Strelkova et al. 1990;

NW: *L. (L.) infantum* Nicolle 1908 (syn. *L. (L.) chagasi* Cunha & Chagas, 1937); *L. (L.) mexicana* Biagi, 1953; *L. (L.) amazonensis* Lainson & Shaw, 1972; *L. (L.) aristidesi* Lainson & Shaw, 1979; *L. (L.) venezuelensis* Bonfante-Garrido, 1980; *L. (L.) pifanoi* Medina & Romero, 1959; *L. (L.) waltoni* Shaw, Pratlong & Dedet, 2015.

Species status not yet confirmed: *L. (L.) garnhami* Scorza et al. 1979; *L. (L.) forattinii* Yoshida et al 1993.

Subgenus *L. (Sauroleishmania)* Ranque, 1973 consists of reptilian parasites only found in the Old World:

L. (S.) tarentolae Wenyon 1921; *L. (S.) adleri* Heisch 1954; *L. (S.) agamae* David 1929; *L. (S.) ceramodactyli* Adler & Theodor 1929; *L. (S.) davidi* Strong 1924; *L. (S.) gulikae* Ovezmuchammedov & Safjanova 1987; *L. (S.) gymnodactyli* Khodukin & Sofiev 1929; *L. (S.) helioscopi* Khodukin & Sofiev 1940; *L. (S.) hemidactyli* Mackie et al. 1923; *L. (S.) hoogstraali* McMillan 1965; *L. (S.) nicollei* Khodukin & Sofiev 1940; *L. (S.) phrynocephali* Khodukin & Sofiev 1940; *L. (S.) platycephala* Telford 2008; *L. (S.) senegalensis* Ranque 1973; *L. (S.) sofieffi* Markov et al. 1964; *L. (S.) zmeevi* Andruchko & Markov 1955; *L. (S.) zuckermani* Paperna et al. 2011;

Species status not yet confirmed: *L. (S.)* sp. I Telford 1979; *L. (S.)* sp. II Telford 1979.

(continued)

Box 2.1 (continued)

Subgenus *L. (Viannia)* Lainson & Shaw, 1987 consists of species exclusively endemic in the New World:

L. (V.) braziliensis Vianna, 1911; *L. (V.) peruviana* Velez, 1913; *L. (V.) guyanensis* Floch, 1954; *L. (V.) panamensis* Lainson & Shaw, 1972; *L. (V.) shawi* Lainson et al. 1989; *L. (V.) lainsoni* Silveira et al. 1987; *L. (V.) naiffi* Lainson & Shaw, 1989; *L. (V.) lindenbergi* Silveira et al. 2002; *L. (V.) utingensis* Braga et al. 2003.

Subgenus *L. (Mundinia)* Shaw, Camargo & Texeira 2016 consists of worldwide distributed species:

L. (M.) enrietti Muniz & Medina 1948; *L. (M.) martiniquensis* Desbois et al. 2014 (syn. *L. siamensis*); *L. (M.) macropodum* Barratt et al. 2017; *L. (M.) spp.* Ghana [MHOM/GH/2012/GH5] (LV757); *L. (M.) spp.* Trang, Thailand [MHOM/TH/2012/PVM2].

GENUS *PORCISIA* SHAW, CAMARGO & TEXEIRA 2016

Consists of parasites occurring in porcupines in the NW, previously assigned to the *L. hertigi* complex:

P. hertigi Herrer, 1971; *P. deanei* Lainson & Shaw 1977.

GENUS *ENDOTRYPANUM* MESNIL & BRIMONT 1908

Consists of NW parasites from sloths, sand flies and humans:

E. schaudinii Mesnil & Brimont 1908; *E. monterogei* Shaw 1969; *E. colombiensis* Kreutzer et al. 1991, *E. equatorensis* Grimaldi jr. et al. 1992; *E. herreri* Zeledon, Ponce & Murillo, 1979.

GENUS *ZELONIA* SHAW, CAMARGO & TEXEIRA 2016

Consists of parasites obtained from predatory hemipterans in the NW Equatorial regions:

Z. costaricensis Yurchenko et al. 2006; *Z. costaricensis* strain G755 Noyes et al. 2002; *Z. costaricensis* strains TCC169E, 504 and 2696 Espinosa et al. 2016; *Z. australiensis* Barratt et al. 2017.

GENUS *NOVYMONAS* KOSTYGOV & YURCHENKO 2016

Contains so far only a trypanosomatid from the digestive tract of a hemipteran (Rhopalidae) from Ecuador:

N. esmeraldas Votypka, Kostygov, Maslov & Lukeš 2016.

2.4 Intraspecific Differentiation in *Leishmania*

2.4.1 Multilocus Microsatellite Typing (MLMT)

Very recently, MLMT and, to a lesser extent, MLSA provided evidence for considerable genetic structure for different *Leishmania* species at the intraspecies level. So far, microsatellite loci with high discriminatory power and suitable for characterizing closely related strains have been published for population studies in the *L. (L.) donovani* complex [120, 121], *L. (L.) major* [18, 122], *L. (L.) tropica* [110], and for species of the subgenus *L. (Viannia)* [115, 123, 124]. Furthermore, a searchable database of microsatellite loci within the genome has been established at <http://www.genomics.liv.ac.uk/tryps/Microsatellites.V1.html>, which allows the development of additional microsatellite markers for the *L. (L.) donovani* complex, *L. (L.) major*, and *L. (V.) braziliensis* [125]. Indeed, novel microsatellite loci can be successfully identified from even very incomplete draft genome assemblies (e.g., [126, 127]) and microsatellite polymorphisms at those loci inferred from sequence data [128]) before further testing.

Most of the MLMT studies published so far have addressed epidemiological and population genetic questions related to the *L. (L.) donovani* complex. When strains of *L. (L.) donovani* and *L. (L.) infantum* isolated from the main regions endemic for VL were investigated, seven main genetically distinct populations were identified: three populations of *L. (L.) infantum* from the Mediterranean area, South America, and Asia comprising the strains representing the zymodeme (MLEE type) MON-1 (v, vi, and vii) and one (iv) of other zymodemes (taken together as non-MON-1), as well as two populations of *L. (L.) donovani* from East Africa (ii and iii) and one of *L. (L.) donovani* MON-2 from India (i) (Fig. 2.4). The highest microsatellite diversity was observed for *L. (L.) infantum* from the Mediterranean Basin. MLMT distinguished strains of *L. (L.) infantum* belonging to the predominating isoenzyme type, MON-1, and revealed the existence of genetically different populations, often with geographical associations, on different hierarchical levels [6, 129–134]. MON-1 strains from the Western Mediterranean differed from those of the Eastern Mediterranean and North Africa. Different genetic groups within MON-1 strains were also prevalent in the Spanish mainland and the Balearic Islands, respectively, as well as in Israeli and Palestinian foci. MLMT detected gene flow between different populations of *L. (L.) infantum* and hybrids between populations representing different zymodemes. New World strains of *L. (L.) infantum* (syn. *L. (L.) chagasi*) were less diverse and most closely related to the strains from southwest Europe [108].

Microsatellite diversity was also remarkable for East African strains of *L. (L.) donovani*. Two main populations have been identified, one comprising strains from Sudan and northern Ethiopia and the other strains from southern Ethiopia and Kenya, which were both further divided into two subpopulations. The presence of two geographically and genetically isolated populations of *L. (L.) donovani* in Ethiopia is supported by differences in clinical behavior and biology of the strains from the two foci [135]. Four putative hybrids detected in this study were retyped

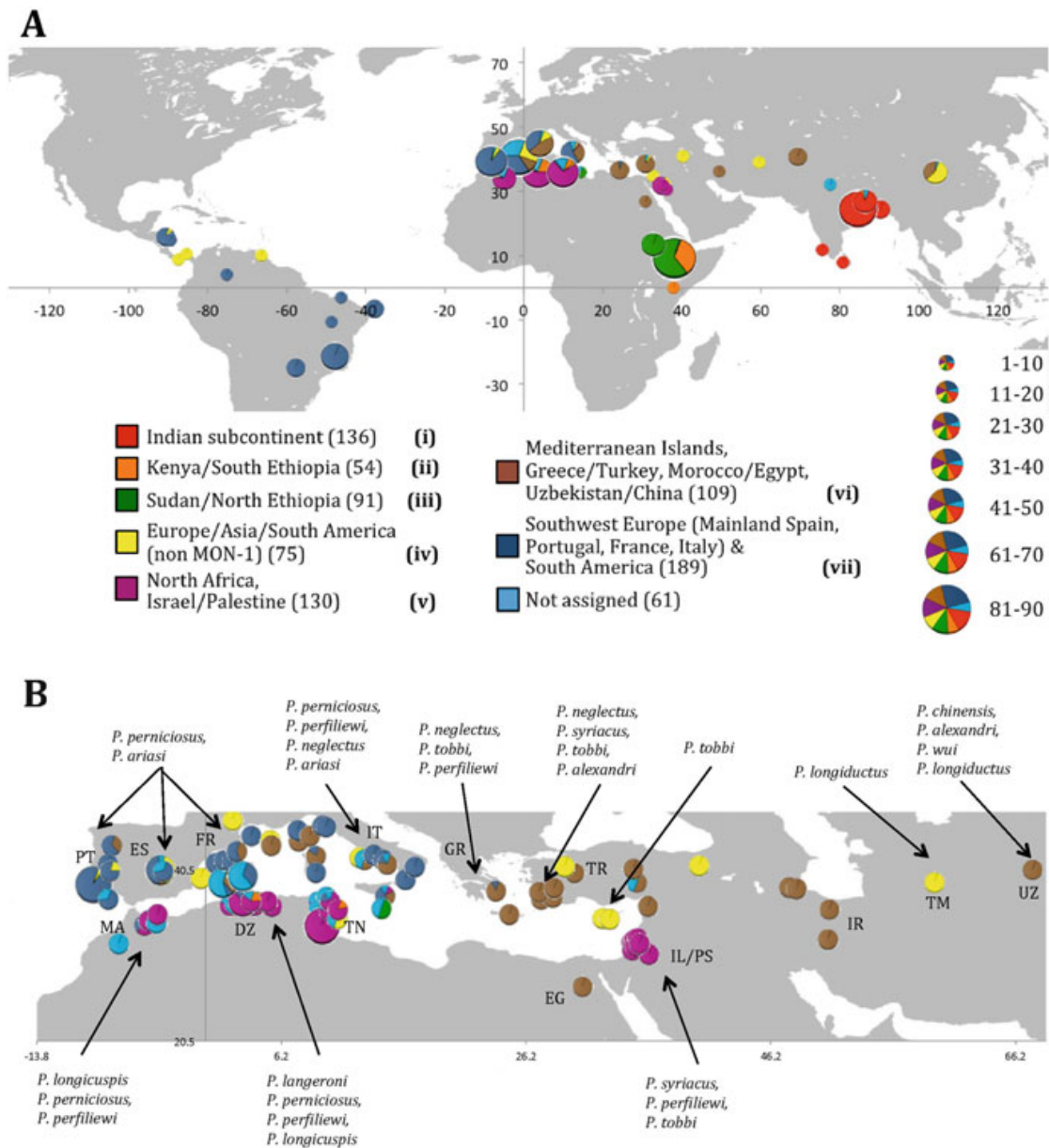


Fig. 2.4 (a) Geographical distribution of the identified population clusters inferred by MLMT for 845 individual isolates, of which 784 were clearly assigned to clusters (i) to (vi) and 61 were “not assigned” (Stark, Schönian et al. unpublished data). Colors refer to the population assignments obtained by BCA. Pie chart sizes are classified to illustrate the relative frequency of samples in the respective focus. (b) Focus on the Mediterranean Basin. Arrows indicate the phlebotomine sand fly species present in these regions. Country abbreviations are PT Portugal, ES Spain, FR France, IT Italy, GR Greece, TR Turkey, IR Iran, TM Turkmenistan, UZ Uzbekistan, IL Israel, PS Palestine, EG Egypt, TN Tunisia, DZ Algeria, MA Morocco

using a combination of MLMT and MLSA and shown to be true genetic hybrids. Each of them possessed heterozygous markers consistent with inheritance of divergent alleles from genetically different Ethiopian *L. (L.) donovani* lineages [136]. MLMT of 124 Sudanese strains of *L. (L.) donovani* revealed significant genetic diversity, minor structuring between years, and highlighted the role of

dogs as important local reservoirs of visceral leishmaniasis [137]. In contrast, strains of *L. (L.) donovani* from the Indian subcontinent were surprisingly homogenous with over 80% of the strains tested sharing an identical MLMT profile regardless of their geographical origin, clinical manifestation, and whether they presented in vitro or in vivo susceptibility to antimonial drugs (Sb^{V}) [138, 139].

MLMT exposed three main populations of *L. (L.) major*, in Central Asia, the Middle East, and Africa [122]. Studies in Iran and Pakistan describe three and two clusters of *L. (L.) major*, respectively, most of which differ from the three main populations [140, 141]. This might be related to the existence of different transmission cycles involving different vector and/or reservoir host species. In Tunisia, MLMT of *L. (L.) major* revealed genetically differentiated populations of the parasites which spread according to a geographical gradient most probably resulting from human activities [142].

The existence of genetically different populations with geographical associations was also shown for *L. (L.) tropica* [110]. The population structure of *L. (L.) tropica* was found to be more complex, with genetically isolated sympatric populations in rather small territories, e.g., in Israel and the Palestinian Authority, and in Morocco, and the emergence of new variants and foci. In Israel and the Palestinian Authority, increased prevalence of human CL could be linked by MLMT to the recent emergence of genetically similar strains of *L. (L.) tropica* [143]. The expansion of these strains seems to result from the reemergence of a previously existing genotype. A second cluster of strains in this study differed from all other *L. (L.) tropica* in their serological, biochemical, and molecular parameters and by the involvement of a new vector species and was closely related to African strains of *L. (L.) tropica*. More strains from this area fell into different genetic entities mostly related to Asian strains of *L. (L.) tropica*. These many locally encountered genetic variants in the Israeli-Palestinian region have been, most likely, imported during numerous migrations of humans and, eventually, infected animal reservoirs from the past until now. Moroccan strains of *L. (L.) tropica* were separated into two phylogenetic clusters independent from their geographical origin [144]. MLMT has, thus, confirmed the intrafocal distribution of genetic variants of *L. (L.) tropica* observed earlier in MLEE studies [145]. Indian strains of *L. (L.) tropica* regardless whether they were isolated from human cases of CL or VL grouped always together and with strains from other Asian foci [146]. The dermatotropic and viscerotropic strains were, however, not genetically identical. Whether this reflects their different pathogenicities remains to be established. A recent MLMT study of *L. (L.) killicki* (syn. *L. (L.) tropica*) supports its assignment to the *L. (L.) tropica* complex and reveals strong structuring in the parasites between Tunisia and Algeria and within different Tunisian regions, suggesting low dispersion of these parasites [147]. MLMT of *L. (L.) aethiopica* confirmed their close relationship to *L. (L.) tropica* but was unable to answer the question whether these parasites represent two separate species or rather different variants of the same *Leishmania* species [148].

MLMT analyses have been applied to different sample sets of the *L. (Viannia)* subgenus. Variation in 15 microsatellite markers has been studied in 120 strains of *L. (Viannia)* from different Brazilian foci of CL [118]. The strains of *L. (V.)*

braziliensis isolated along the Atlantic coast and those of *L. (V.) guyanensis*, mainly from the Amazonas region, formed two clearly separated populations both exhibiting significant levels of recombination. MLMT identified an epidemic clone inside the Atlantic coast population consisting of 13 strains from a CL outbreak in Minas Gerais. Strains of *L. (V.) braziliensis* from northern Brazil did not group with those from the Atlantic coast but were found to be very polymorphic. They seemed to be more closely related to strains of other subgenus *L. (Viannia)* species, such as *L. (V.) shawi*, *L. (V.) naiffi*, and *L. (V.) lainsoni*, also isolated in northern Brazil CL foci. Bias due to inadequate sampling strategies cannot be excluded for the analyses of strains derived from northern Brazil. Using the same MLMT approach, high genetic diversity, with multilocus genotypes strongly differentiated from each other, were observed for 24 strains of *L. (V.) braziliensis* from Peru [149]. The sample set consisted of strains for which the in vitro susceptibility toward antimonial drug or the clinical treatment outcome was known. No correlation could, however, be found between genotypes and resistance phenotypes.

The MLMT of Bolivian and Peruvian *L. (V.) braziliensis* revealed a strong population structure at a microgeographical scale as the populations within the different countries were genetically heterogenous [40]. The substantial heterozygote deficiency and extreme inbreeding found in this study is not consistent with strict clonal reproduction as previously proposed [150] but rather point to frequent sexual crosses of genetically related parasites or even of individuals from the same strain (endogamy). A high level of sexual recombination and substantial endogamy together with strong Wahlund effects (sampling strains from different subpopulations) was reported in a study of 153 strains of *L. (V.) guyanensis* from French Guyana investigating variation in 12 microsatellite loci [151]. The significant isolation observed suggests an important role for natural hosts and/or vectors in the dispersion of parasites across the country.

2.4.2 Whole-Genome Sequencing

So far, only a few studies have investigated whole-genome SNPs, chromosome, and gene copy number variations for a significant number of strains of the same *Leishmania* species that would allow conclusions on intraspecific diversity. Imamura et al. [37] have recently investigated the history of VL on the Indian subcontinent (ISC) by analyzing whole-genome sequences of 204 *L. (L.) donovani* isolated from VL cases in Nepal, India, and Bangladesh. They reported that most of these parasites first appeared in the nineteenth century which is matching the first historical records of VL epidemics in the area. As shown earlier, the parasite genomes are indeed genetically similar, but whole-genome SNP analyses identified three divergent genetic lineages circulating on the ISC: a core group of 191 closely related parasites occurring in the lowlands of all three countries, a small group of 12 strains from Nepalese highlands, and a single divergent isolate from Nepal (Fig. 2.5). The core population could be clustered into six discrete monophyletic groups which first appeared in the 1960s. Thus, whole-genome analyses confirm

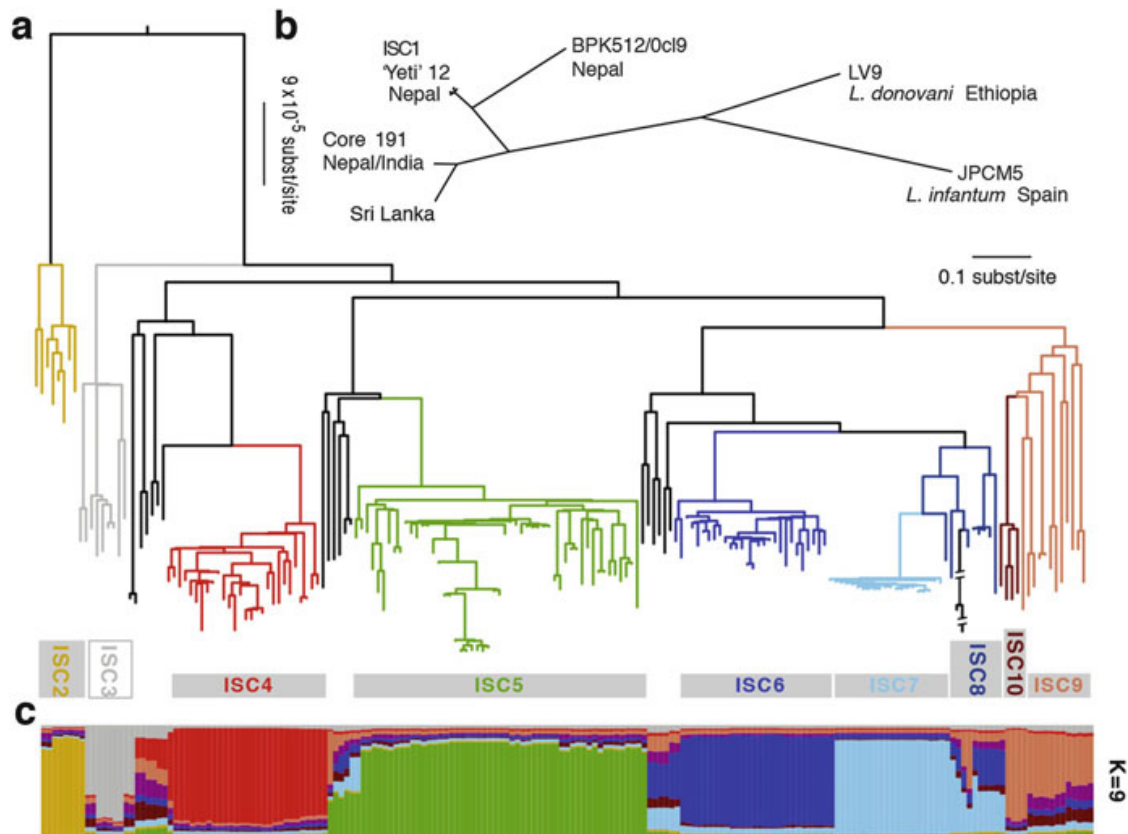


Fig. 2.5 Genealogical history of *L. (L.) donovani* from the ISC (Imamura et al. [37]). (a) Maximum-likelihood tree based on SNPs called for 191 strains from the core population in the Indian subcontinent. Samples are colored by population assignment, with putative hybrid strains not clustered in the main groups in black. Further analysis confirms the hybrid ancestry of some of these isolates. (b) Unrooted phylogenetic network of the *L. (L.) donovani* complex based on split decomposition of maximum-likelihood distances between isolates described here, reference genome isolates, and two published Sri Lankan isolates (Zhang et al. 2014). (c) Model-based clustering of 191 isolates from the core population reveals six discrete monophyletic groups and some groups and other samples of less certain ancestry. Colored bars show the fraction of ancestry per strain assigned to a given cluster, with colors assigned to the population most closely related to each cluster. Reproduced under CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

earlier hypotheses of sustained and ancient reproductive isolation from other *L. (L.) donovani* lineages due to a recent bottleneck event on the ISC related to the insecticide spraying under the Malaria Control Program in the 1960s. Parasites in one of genetically distinct groups were found to be frequently resistant to antimonial treatment. High plasticity was observed for these *L. (L.) donovani* genomes, gene copy number variants cover $\sim 11\%$ of the genome, most of the isolates were aneuploid, and almost all chromosomes show some variations.

Using double-drug resistance markers, genetic recombination among *Leishmania* parasites was unequivocally demonstrated to occur in the sand fly vector under laboratory conditions [152]. The detection of natural hybrids and mosaic genotypes [16, 37, 129, 134, 136, 153–157], gene flow between populations [129, 134], and strong inbreeding [40, 151] have repeatedly posed questions about the role and extent of sexual recombination in natural populations of *Leishmania*. Rogers et al.

[158] have applied whole-genome sequencing to 12 *L. (L.) infantum* isolated in a CL focus in the Cukurova province of southeast Turkey, mainly from sand fly vectors, to investigate the frequency of sexual reproduction in these parasites. They observed a genome-wide pattern of patchy heterozygosity both within individual strains and across the whole group symptomatic of hybrid ancestry. Comparisons with other *L. (L.) donovani* and *L. (L.) infantum* genomes led to the assumption that the Cukurova isolates derived from a single relatively recent cross of two diverse strains with subsequent recombination within the population. After the original hybridization event, the population reproduced primarily clonally, but some recombination also occurred. The frequency of mating has been estimated as ca. 1.3×10^{-5} meioses per mitosis suggesting that sexual crosses might be rare in natural populations of *Leishmania*.

2.5 Origin of *Leishmania* Parasites

Whether the genus *Leishmania* appeared first in the Old World or in the New World has been controversially discussed during the last decades. Tuon et al. [159] have pointed out that regardless of its origin, the spread of *Leishmania* most likely followed the migration of vectors and hosts together, although *Leishmania* are quite capable of jumping hosts. The earliest fossil sand flies (ca. 120 Mya) were reported in Lebanon [160], which formed part of Gondwana, and reptiles or primitive mammals may have been the hosts of primitive *Leishmania*. The different vector-parasite-host theories of dissemination are summarized in Table 2.4.

The Palaeartic origins hypothesis suggests that the first association of the parasites with vertebrates occurred in the Old World with Cretaceous reptiles. Infections of Old World rodents then appeared in the Palaeocene and were carried by vertebrate hosts and sand fly vectors across Beringia to the Neartic in the Eocene. During the Pliocene, infected sigmodontine rodents brought the parasites to the Neotropics via the Panamanian land bridge. There, endemic vectors introduced the parasites to caviomorph rodents, sloths, armadillos, and anteaters [2, 161].

Alternatively, it has been proposed that *Leishmania* originated in the Neotropics during the Palaeocene with sloths as the first vertebrate hosts. After adaptation to rodents in the Eocene, infected porcupines would have carried the parasites across the Panamanian land bridge to the Neartic. From there the parasites were transported by other mammals across Beringia during the Miocene [162]. This hypothesis is supported by host-based area cladograms which use patterns of origination and dispersal of hosts and vectors to infer the phylogeny of the parasites. However, *Leishmania* are often not host- or even vector-specific. Recently, the first apparent fossil member of the genus *Leishmania*, the ~100 my old *Paleoleishmania proterus*, was detected in reptilian blood which was inside the body of the extinct sand fly, *Palaeomyia burmitis*, in Early Cretaceous Burmese amber [73, 163, 164]. Thus, protozoan-vector associations seem to have been established by the Early Cretaceous (100–110 my), reptiles were early hosts of *Leishmania*-like parasites, and the adaptation to mammals occurred later when reptiles declined

Table 2.4 Geological timescale and the theory of the evolution of *Leishmania* adapted from Tuon et al. [159] and modified

Eon	Era	Period	Epoch	Duration	Evolution			
Phanerozoic	Cenozoic	Neogene	Holocene	0–11.5 ty	<i>Leishmania (L.)</i> spread to Neotropic through primitive rodents from Neartic			
			Pleistocene	11.5–1.806 ty	Isthmus of Panama formation and physical unification between Neartic and Neotropic allowing further spread of <i>Leishmania (L.)</i> to South America			
			Pliocene	1.8–5.3 my				
			Miocene	5.3–23.0 my	<i>Leishmania (L.)</i> into Neartic after Bering Strait formation by primitive rodents from Paleartic, appearance of genus <i>Phlebotomus</i> , confirmed vector of <i>Leishmania</i> Placental mammals, ancestral hosts of <i>Leishmania</i> First <i>Leishmania</i> descendant in a reptile host, first hematophagous winged insect, separation of Africa and South America, split between Paraleishmania and the other <i>Leishmania</i> species (~90–100 my) The first digenetic protozoan, a possible ancestor of <i>Leishmania</i>			
			Oligocene	23.0–33.9 my				
			Eocene	33.9–55.8 my				
			Paleocene	55.8–65.5 my				
			Upper	65.5–100 my				
				Lower		100–145 my		
								145–199 my
								199–251 my
								251–299 my
								299–360 my
		Jurassic		Division of Trypanosomatidae following the evolution of Hemiptera and Diptera (vector of the <i>Leishmania</i>) First winged insect				
		Triassic						
		Permian						
		Carboniferous						

(continued)

Table 2.4 (continued)

Eon	Era	Period	Epoch	Duration	Evolution
			Devonian	360–416 my	Formation of first digenetic protozoan, ancestor of other <i>Trypanosoma</i> , not <i>Leishmania</i> . Parasite of a primitive fish
			Silurian	416–444 my	
			Ordovician	444–488 my	
			Cambrian	488–542 my	
Proterozoic		Neoproterozoic		542–1000 my	
		Mesoproterozoic		1–1.6 by	Possible origin of the Phylum Protista
		Paleoproterozoic		1.6–2.5 by	
Archean				3.5–2.5 by	

by, billions of years; my, millions of years; ty, thousands of years

during the Eocene to Oligocene transition [161, 165]. This hypothesis implies that *Sauroleishmania* form a sister clade to all other leishmanial species [166, 167]. In contrast, rooted sequence-based phylogenetic trees of currently known *Leishmania* parasites favor a neotropical origin, showing the New World species branching off close to the base of the trees and the Old World species being at the crown of the subgenus *L. (Leishmania)* [45, 58, 60, 84, 89, 90, 102]. In these phylogenies, NW species emerged 46–34 mya and are ancestral to the OW species [109, 167]. The parasites were then dispersed by their hosts to the Nearctic via the Panamanian land bridge and further to the Palaeartic via the Bering land bridge. This view is further supported by the higher diversity found in the New World species of *Leishmania* [72], as well as by latest analysis of trypanosomatids from Australia [102]. The Neotropical origins hypothesis is, however, in discordance with the position of Old World *L. (Sauroleishmania)* closer to *L. (Leishmania)* than to *L. (Viannia)* but branching off within the New World taxa. It further suggests that reptilian species are derived from mammalian parasites which is in contrast to the Palaeartic hypothesis, and assumes two intercontinental migrations, first of the ancestral *Leishmania/Sauroleishmania* to the Palaeartic and then of a member of *L. (Leishmania)* subgenus back to the Neotropics [167].

The multiple origins hypothesis considers the great genetic difference between the parasites assigned to Euleishmania, comprising the parasites of the genus *L. (Leishmania)*, and Paraleishmania [92], according to the new taxonomy (see Box 2.1 and Table 2.3), and favors an ancient divergence between these two groups. It has been speculated that the two sections of the genus *Leishmania* became separated before the split of Gondwana [166]. The same authors concluded that, with the separation of Gondwana in the Mesozoic, the Euleishmania evolved into *L. (Leishmania)* and *L. (Sauroleishmania)* in the OW and *L. (Viannia)* in the NW. This conclusion is supported by the great genetic distance between the *L. (Leishmania)* and *L. (Viannia)* subgenera and the high genetic diversity within *L. (Viannia)* [116]. This theory, however, does not explain why the American branches of the subgenus *L. (Leishmania)* appear more ancient than the OW branches.

The supercontinent hypothesis is a variation of the multiple origins theory discussed earlier by Yurchenko et al. [58] but received phylogenetic support more recently in the study published by Harkins et al. [167]. These authors applied a phylogenomic approach analyzing more than 200,000 variable sites and 49 genes from across the genome for 24 leishmanial species. In their scenario, Leishmaniinae evolved from monoxenous ancestor on Gondwana, and the split between the Paraleishmania and all other species occurred ~90–100 mya, around the time when Gondwana split. This is in agreement with earlier speculations, that parasites adapted to mammals during the radiation of the latter around 90 mya [168]. Genetic diversification between the OW and NW parasites reflects the vicariance after the separation of South America and Africa [167, 169]. Only the migration of the NW lineage in the *L. (Leishmania)* subgenus is needed by this hypothesis, which took place 30 mya during the mid-Miocene when temperatures were warm enough for sand fly survival. The results of Harkins et al. are consistent with the early Cretaceous fossils of *Paleoleishmania proterus* found in sand flies trapped in Burmese

amber ~100 mya [73] and with the finding that parasites isolated in different geographical regions, such as South America, Australia, Africa, and Asia, are members of the newly defined subgenus *L. (Mundinia)* [103]. Finally, a new Australian species, *Zelonia australiensis*, was found to be related to a parasite isolated in Costa Rica, *Zelonia costaricensis* (earlier *Leptomonas costaricensis*), suggesting a divergence time between the two of ~40 mya when Australia and South America became completely separated. Using this vicariance event for calibrations, it was confirmed that the common ancestor of the Leishmaniinae emerged around 90 mya on Gondwana [102].

The New World species of *L. (Leishmania)* most likely have originated in the Old World. *L. (L.) mexicana* has many similarities to Asian *L. (L.) major* and has been proposed to have dispersed to the Nearctic together with its rodent reservoirs during the Eocene via the Bering land bridge [78, 161] and could then have entered the Neotropics during the Pliocene either via island hopping or after the Panamanian land bridge had been formed. There, further speciation could have taken place leading to the occurrence of the currently known species related to *L. (L.) mexicana*, namely, *L. (L.) amazonensis*, *L. (L.) aristidesi*, *L. (L.) venezuelensis*, and *L. (L.) forattinii* [166].

The etiological agent of New World visceral leishmaniasis, named *L. (L.) chagasi*, has been introduced relatively recently in the American continent, by the European conquistadores, along with multiple, and perhaps ongoing, introductions [107, 108, 170]. Numerous molecular studies have revealed a very restricted diversity within strains of *L. (L.) chagasi* and could not distinguish them from *L. (L.) infantum* indicating a very recent geographical separation. Studies on microsatellite variation have finally proven that strains of *L. (L.) chagasi*, or better of South American *L. (L.) infantum*, were most similar with populations of *L. (L.) infantum* from southwest Europe and arrived in the New World about 500 years ago [108, 171].

In conclusion, a revised classification scheme and nomenclature of *Leishmaniinae* species has been proposed based on molecular phylogenies of the organisms [102, 103]. It represents a useful simplification of the parasites' taxonomy, particularly for the clinician, without losing the detailed knowledge built up over the last 20 years, which is particularly relevant for epidemiological studies. In the future, assignment to and within major groups across the entire genus should be based on whole-genome analyses which are congruent and uncontroversial and explore the significance of variable aneuploidy for the biology and evolution of the parasites. In *Leishmania*, changes in aneuploidy are likely adaptive and depending on the life stage [31]. Clinical samples with only minimal in vitro passaging or, preferentially, without passaging at all should be, therefore, used in future studies linking genomic adaptations to treatment failure, drug resistance, immune, and other environmental pressures.

Acknowledgments We thank the many authors that have contributed to what we know today about the molecular evolution and phylogeny of *Leishmania* and whose work is not cited herein, in particular those who have worked with us, and all of those with whom we have had discussions

about this subject. We acknowledge the financial support of the European Union (grants QLK2-CT-2001-01810, INCO-CT2005-015407, FP7-222895); of Deutsche Forschungsgemeinschaft (SCHO 448/6 and 448/8); of Wellcome Trust (078742/Z05/Z and core support of the WT Sanger Institute WT098051 and WT206194); and of the Czech Grant Agency (16-18699S) and the European Research Council (CZ LL1601).

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