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Leishmania infections: Molecular targets and diagnosis

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ABSTRACT

Progress in the diagnosis of leishmaniases depends on the development of effective methods and the discovery of suitable biomarkers. We propose firstly an update classification of *Leishmania* species and their synonymies. We demonstrate a global map highlighting the geography of known endemic *Leishmania* species pathogenic to humans. We summarize a complete list of techniques currently in use and discuss their advantages and limitations. The available data highlights the benefits of molecular markers in terms of their sensitivity and specificity to quantify variation from the subgeneric level to species complexes, (sub) species within complexes, and individual populations and infection foci. Each DNA-based detection method is supplied with a comprehensive description of markers and primers and proposal for a classification based on the role of each target and primer in the detection, identification and quantification of leishmaniasis infection. We outline a genome-wide map of genes informative for diagnosis that have been used for *Leishmania* genotyping. Furthermore, we propose a classification method based on the suitability of well-studied molecular markers for typing the 21 known *Leishmania* species pathogenic to humans. This can be applied to newly discovered species and to hybrid strains originating from inter-species crosses. Developing more effective and sensitive diagnostic methods and biomarkers is vital for enhancing *Leishmania* infection control programs.

1. Introduction

1.1. Leishmaniasis is a global problem

Leishmaniases are vector-borne diseases caused by obligate protistan parasites from the genus *Leishmania* (Trypanosomatida: Trypanosomatidae) endemic in large areas of the tropics, subtropics and Mediterranean basin, spanning more than 98 countries. There

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are ~ 350 million people at risk and ~ 12 million cases, with an estimated worldwide annual incidence of 0.7–1.2 million cases of cutaneous leishmaniasis (CL) and 0.2–0.4 million cases of visceral leishmaniasis (VL) (Alvar et al., 2012). Hence Leishmaniases constitute a major public health problem with an increasing burden over the last decade, and among tropical infections ranks as the 2nd and 4th most common cause of death and disease, respectively (Bern et al., 2008).

Leishmania infections have six clinical forms, defined by the location of the parasite in the infected tissues: visceral (VL), post-kala-azar dermal leishmaniasis (PKDL), cutaneous (CL), diffuse cutaneous (DCL), mucocutaneous (MCL) and mucosal (ML) leishmaniasis. The most common form is CL: over 90% of cases are distributed across three main regions: (i) Afghanistan, Iran, Saudi Arabia and Syria; (ii) Algeria and Tunisia; and (iii) Brazil and Peru. Over 90% of cases of the less common but more pathogenic VL occur in another three geographic foci: (i) Bangladesh, India and Nepal; (ii) Ethiopia, Kenya and Sudan; and (iii) northeast Brazil (Alvar et al., 2012).

Currently, 54 *Leishmania* species [excluding synonymous species (the synonymy based on molecular typing results *e.g.,* MLEE (Multilocus Enzyme Electrophoresis), MLMT (Multilocus microsatellite typing) and sequencing) and including *Sauroleishmania*] are known and at least 21 of them are pathogenic to humans (Akhoundi et al., 2016) (Fig. 1). *Leishmania* flagellates are transmitted to vertebrates by the bite of infected female phlebotomine sandflies and are frequently hosted by canids, rodents, marsupials, mongooses, bats and hyraxes; therefore the disease may be zoonoses, anthropozoonoses or anthroponoseses, although few species are strictly anthroponotic.

Due to the wide spectrum of non-human reservoir hosts and the large number of vectors involved in the transmission, improved diagnosis of *Leishmania* parasites is an important feature of control programs (Stauch et al., 2014). Only a minority of infected humans develops the disease: most are infected at a sub-clinical level (Singh et al., 2002). These asymptomatic hosts help sustain VL transmission in endemic areas and represent major challenge for infection control. Given that virulence, pathogenicity and clinical manifestation varies among *Leishmania* species, and that the clinical outcome of the disease also depends on the sandfly vector type and the host's genetic background, highly accurate diagnosis is essential for successful elimination of these versatile parasites because it is the mainstay for bridging molecular epidemiology and therapy choices (local/systemic and drug).

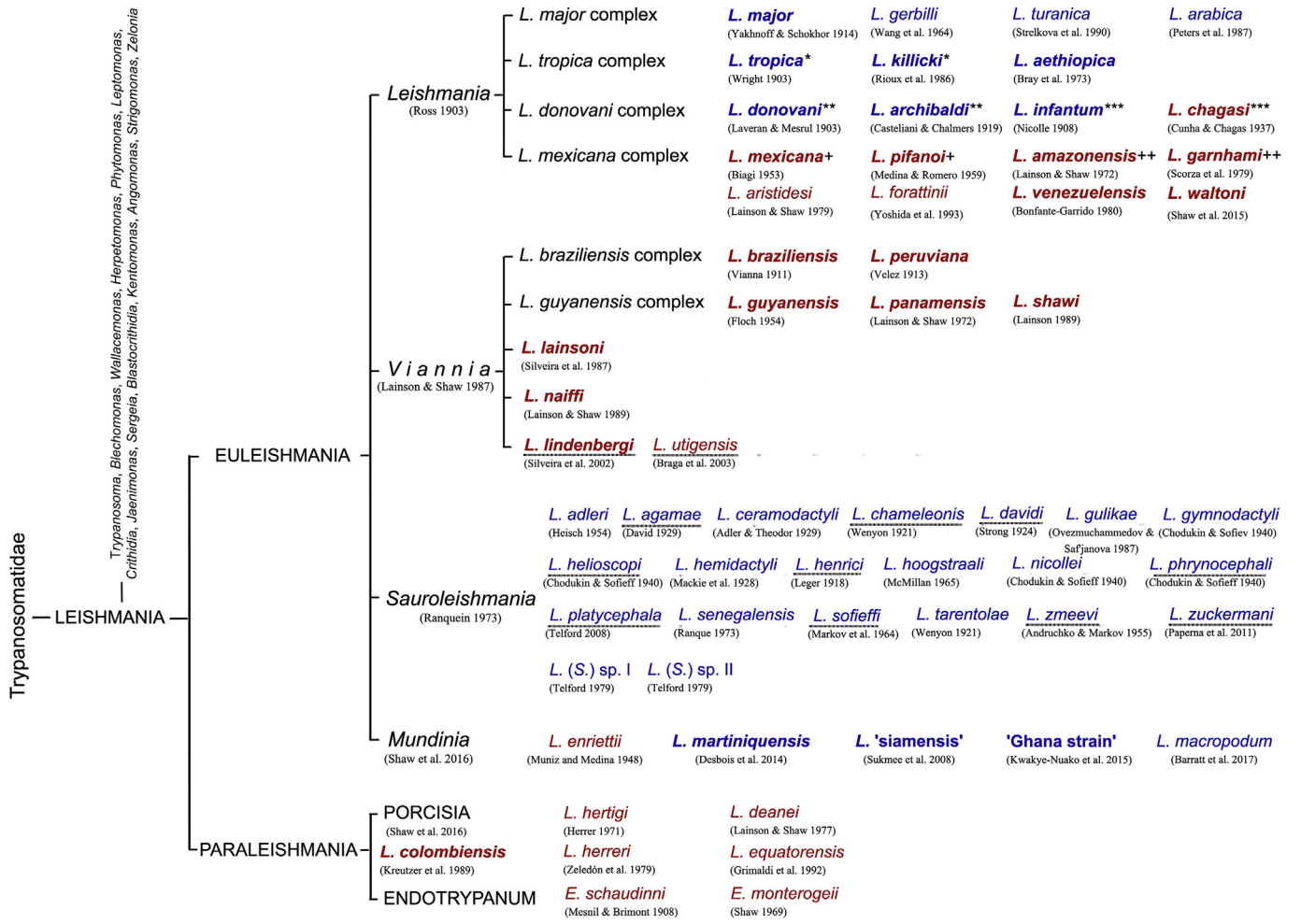

Fig. 1. Updated classification of *Leishmania* species

*, +: Synonym; different numbers of star (*) and plus (+) signs mean which species name is the synonym for which original species, Underlined: No final classification. *L.* 'siamensis' and *L. martiniquensis* have been found also in the New World. *Leishmania* names in quotation mark are unofficial names without formal descriptions. The human pathogenic species are written in bold. Old and New world species are highlighted in blue and red respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1.2. Diagnosing leishmaniases

Diagnosis of leishmaniasis is complicated by the clinical pathology of the disease, varying from simple cutaneous to visceral forms and within each of them. Moreover, "localized leishmanial lymphadenopathy (or lymphadenitis)" was described being frequent in Kala-azar (and very rare in Mediterranean VL) as an atypic clinical manifestation from the patients (Daneshbod, 1978; Dabiri et al., 2014; Horrillo et al., 2015). CL lesions vary in their severity (*e.g.,* lesion size), numbers, clinical appearance (*e.g.,* dry or wet lesion) and duration (*e.g.,* the time for spontaneous cure). In addition, similar clinical pathologies can be caused by highly divergent species: VL may be caused not only by the typical viscerotropic *L. donovani* species complex (*L. infantum* and *L. donovani*), but also by *L. tropica* (Magill et al., 1993), and *vice versa* CL may be caused by *L. donovani* complex species in addition to typical dermotropic species such as *L. major* and *L. tropica* (*e.g.,* Pratlong et al., 2004; Alborzi et al., 2006; Svobodová et al., 2009). It also depends on the immunstatus – *e.g.*, many dermotropic *L. infantum* zymodemes are found in HIV positive patients with visceral leishmaniasis (Pratlong et al., 1995; Alvar et al., 1997; Chicharro et al., 2002). Similar clinical symptoms may originate from non-leishmaniasis infections common to tropical regions or cosmopolitan. Some of furuncular myiasis, and bacterial tropical ulcers (similar to acute CL), as well as leprosy, keloid, lupus vulgaris, discoid lupus erythematosus and sarcoidosis (similar to chronic CL) have the common clinical signs with CL symptoms (Convit et al., 1993; Gradoni, 2016). Furthermore, some clinical symptoms observed in chronic malaria, disseminated histoplasmosis, hepatosplenic schistosomiasis, typhoid fever, brucellosis, tuberculosis, endocarditis, relapsing fever, and African trypanosomiasis are similar to VL symptoms in tropics. Additionally, some diseases like syphilis, lymphomas, chronic myeloid leukemia, sarcoidosis, malignant histiocytosis, and liver cirrhosis are also indicative of VL in cosmopolitan regions (Gontijo and Melo, 2004; Van den Bogaart et al., 2012; Gradoni, 2016). Complicating this, all of these diseases generally have a high incidence in leishmaniasis endemic areas. Therefore, diagnostic decisions for the clinical treatment of leishmaniasis can be presumptive in some cases, or evidence based, and identification of *Leishmania* species is only deduced from epidemiological and clinical backgrounds.

Since the initial description and discovery of the causative agent of the disease in 1898 and 1901 by P. Borovsky, W. B. Leishman and C. Donovan and the first official description of the genus *Leishmania* by R. Ross in 1903, concepts and methodologies used to discriminate *Leishmania* species have evolved considerably (Weyers, 2016). Initially, only extrinsic characters such as clinical manifestations, geographical distributions, epidemiological cycles and the behaviour of *Leishmania* in their sandfly vectors, were used to discriminate *Leishmania* species. Accordingly, the *Leishmania* genus was subdivided into two sub-genera: *Leishmania* and *Viannia* (Saf'janova, 1982; Lainson and Shaw, 1987). In the early 1970s, intrinsic characters related to immunology, biochemistry and molecular typing were introduced to type *Leishmania*. Isoenzyme electrophoresis was developed in the late 1970s, followed by the establishment of the widely used MON zymodeme system in the 1990 (Rioux et al., 1990; Akhoundi et al., 2016) and it was considered as the gold standard for a long period. Another system (IOC-Z) that is currently used in the New World was developed by Cupolillo et al. (1994). It is still a reference technique for *Leishmania* typing allowing even differentiation within species, yet it cannot be considered a straightforward and high-throughput method in clinical care services, since it is based on cultured parasites (it cannot be used for clinical samples).

Since the 1980s, the invention of DNA amplification via PCR has enabled the development of fast and highly sensitive detection coupled to molecular typing of *Leishmania* in biological samples. Species discrimination generally involves either direct sequencing of a PCR product (Van Eys et al., 1992), application of single strand conformation polymorphism (SSCP) (Van Eys et al., 1992), use of species-specific restriction sites via restriction fragment length polymorphism analysis (PCR-RFLP) (Cupolillo et al., 1995; Victoir et al., 1998; Schönian et al., 2001), PCR fingerprinting (Sreenivas et al., 2004), random amplified polymorphic DNA (RAPD) (Bañuls et al., 1999; Mauricio et al., 1999; Montalvo et al., 2008), or high-resolution melting (HRM) (Talmi-Frank et al., 2010).

Of these methods only PCR-RFLP and sequence analysis coupled to the appropriate target in the genome are suitable for discrimination of all *Leishmania* species – in many cases also a combination of different markers has to be applied to achieve a final species resolution (Harris et al., 1998; Srivastava et al., 2011; Odiwuor et al., 2011; Van der Auwera et al., 2014). Only a handful of the PCR-based assays targets almost all human-infecting *Leishmania* species (Wortmann et al., 2001; Castilho et al., 2008; Tupperwar et al., 2008), and most are considered non-quantitative (Harris et al., 1998; Odiwuor et al. 2011; Srivastava et al., 2011). Essential is the right combination of the appropriate target within the *Leishmania* genome and the analytical method, to achieve a resolution at the desired taxonomical level.

1.3. Diversity within the genus Leishmania

The taxonomical structure of the genus *Leishmania* is conventionally arranged at the levels of subgenus, species complex and (sub) species. The aim of the guide outlined below provides a context for this review and updates and synthesizes extensive work published previously (Schönian et al., 2011; Van der Auwera et al., 2014; Van der Auwera and Dujardin, 2015; Harkins et al., 2016). It reflects current interpretation of the available phylogenetic data, and is limited to taxonomic units for which markers are available or where admixture has been investigated.

The best-characterized part of the genus *Leishmania* is the section *Euleishmania* composed of at least four subgenera: *Leishmania*, *Viannia*, *Sauroleishmania* and *Mundinia* (including *L. enriettii, L. martiniquensis*, *L.* 'siamensis','Ghana strain' and *L. macropodum*). The subgenus *Leishmania* has four main species complexes: *L. donovani, L. major, L. mexicana* and *L. tropica* (Akhoundi et al., 2016). Determining distinct species complexes within the subgenus *Viannia* is less straightforward and is influenced by insufficient sampling. However, if the scale of genetic diversity between and within complexes can be correlated with that established for the *L.* (*Leishmania*) subgenus, it is possible to classify the subgenus *Viannia* into the species complexes *L. braziliensis*, *L. guyanensis*, as well as the species *L. lainsoni* and *L. naiffi* (Akhoundi et al., 2016) (Fig. 1).

Recently, a new subgenus *Leishmania* (*Mundinia*) was created, composed of guinea-pig infecting *L. enriettii (*Muniz and Medina, 1948), *L. martiniquensis* (Mouri et al., 2014), *L.* 'siamensis' (Leelayoova et al., 2013), 'Ghana strain' (Kwakye-Nuako et al., 2015) and *L. macropodum* (Barratt et al., 2017) - (a specimen isolated from a kangaroo with CL in Australia) (Rose et al., 2004). The latter together with *L.* 'siamensis' are considered as *nomem nudum* (Espinosa et al., 2016). Based on the study carried out by Mouri et al. (2014) reporting the experience by using MALDI-TOF compared with reference methods, only one single strain of *L. martiniquensis* (not identified with this nomenclature) was tested. Moreover, a strain of *L. martiniquensis* was reported by Pothirat et al. (2014), isolated from an authochtonous case of VL in Thailand. They suggested that several strains originally identified as *L.* 'siamensis' in Thailand might actually be *L. martiniquensis*.

A new genus *Porcisia* has proposed recently to accommodate the

Neotropical porcupine parasites originally described as *L. hertigi* and *L. deanei* (Espinosa et al., 2016; Barratt et al., 2017). However, due to the lack of in-depth genetic analysis, the number of subgenera constituting the *Paraleishmania* division is unstable, yet *L. herreri*, *L. equatorensis* and *L. colombiensis* as well as the former genus *Endotrypanum* are part of it.

Molecular-based typing was instrumental to reveal several cases of synonymy such as *L. tropica* (syn. *L. killicki*) (Schönian et al., 2001; Schwenkenbecher et al., 2006; Asato et al., 2009), *L. donovani* (syn. *L. archibaldi*) (Jamjoom et al., 2004; Kuhls et al., 2005, 2007), *L. infantum* (syn. *L. chagasi*) (Maurício et al., 2000; Kuhls et al., 2011), *L. mexicana* (syn. *L. pifanoi*) and *L. amazonensis* (syn. *L. garnhami*) (Schönian et al., 2010).

In the present study, we will focus on the 21 *Leishmania* species known to be pathogenic to humans (Akhoundi et al., 2016).

1.4. Hybrids pose a new threat

Expanding vector ranges driven by accelerating climate change provide not only more opportunities for human infections, but also increase chances of mixing between so far distinct species whose vectors now co-exist (Patz et al., 2000). In addition, higher human and livestock migration presents faster transmission of infected and asymptomatic cases to new non-endemic regions (Rijal et al., 2010). Likewise, susceptible people may move into and from endemic regions (WHO, 2008), elevating the chance of establishment increased infection and spread (Al-Salem et al., 2016). These trends are evidenced by the increasing frequency of leishmaniasis in people immune-compromised due to pre-existing infections (Cruz et al., 2006; Antinori et al., 2008).

There is copious evidence for the existence of natural hybridization between genetically discrete but also distant *Leishmania* species. These hybrids have been isolated from infected patients, thus reflecting real epidemiological patterns (Hamad et al., 2011). Furthermore, mating and genetic exchange has been demonstrated to occur in vectors experimentally infected by *L. major*, producing infective progeny (Akopyants et al., 2009), with no compelling evidence of the most parsimonious alternative (co-infection of diverse isolates in clinical samples).

Among Old World samples, hybridization between *L. donovani* and *L. aethiopica* (Odiwuor et al., 2011) and within *L. donovani* lineages has been found in Ethiopia using a combination of MLMT and MLST (Gelanew et al., 2014), and within Asian and African L. *tropica* using MLMT (Schwenkenbecher et al., 2006). Moreover MLMT and MLEE indicate the presence of *L. infantum* hybrids in Tunisia (Chargui et al., 2013). Using MLEE and DNA probes, inter-breeding has been found between *L. infantum* and *L. major* in Portugal (Ravel et al., 2006), and between *L. major* and *L. arabica* in Saudi Arabia (Evans, 1987; Kelly et al., 1991).

In South America, gene flow between species appears to be common (Noyes et al., 1996; Van der Auwera et al., 2013). Using MLMT, genetic mixing has been discovered between *L. lainsoni* and *L. naiffi* (Kuhls et al., 2013), and MLEE and DNA fingerprinting methods were instrumental to detect hybrids between *L. braziliensis* and *L. guyanensis* (Delgado et al., 1997), and between *L. braziliensis* and *L. panamensis* in Nicaragua (Darce et al., 1991; Belli et al., 1994). MLEE and RAPD typing (Dujardin et al., 1995), MLMT (Nolder et al., 2007) and DNA probes (Dujardin et al., 1993) detected hybrids between *L. braziliensis* and *L. peruviana* in Peru, some of which have been investigated subsequently in *in-vitro* experiments (Cortes et al., 2012). Later it was shown by MLST and *hsp*70 sequence analysis that *L. peruviana* separates as a unit within the *L. braziliensis* clusters (Van der Auwera et al., 2014), however the boundaries are still not completely resolved. Moreoever, both *L. panamensis* and *L. shawi* are nested within *L. guyanensis* (Boité et al., 2012), although the occurrence of hybrids does not require genetically close-related species (Ravel et al., 2006).

Many studies into the diversity of *Leishmania* spp. have used approaches such as MLEE that were insufficient for resolving their evolution and admixture. At present, the scale of the problem posed by novel hybrids is uncertain: few offspring are viable from *in vitro L. donovani* crosses (Sadlova et al., 2011), whereas higher rates are obtained for *L. major* (Inbar et al., 2013). It is unclear if the surviving reassortants have altered virulence or transmissibility, however *L. braziliensis/L. peruviana in vitro* hybrids are more resilient and have higher virulence than parental strains (Cortes et al., 2012). Moreover, a *L. infantum/L. major* hybrid had a higher transmission potential in the vector (Volf et al., 2007). Consequently, adopting technology providing significantly more information, such as genome sequencing will illuminate how outbreeding between genetically discrete strains can generate hybrid vigour.

1.5. Scope and aims

Leishmaniasis mainly affects low-income countries in tropical and sub-tropical regions, for which there is an urgent need for simple, versatile and cost-effective methods for the detection of pathogenic *Leishmania* species (De Ruiter et al., 2014). Consequently, tests should be considered in terms of their qualitative or quantitative nature, and their sensitivity and specificity at the level of genus, subgenus, species complex, species and population (Van der Auwera and Dujardin, 2015). It is crucial that major advances in our understanding of the *Leishmania* genome structure, gene activity, protein expression and metabolite changes are translated into better diagnostics.

A global map highlighting the geography of known endemic *Leishmania* species pathogenic to humans, drawn using World Health Organization information (WHO, 2016) and published literature (Fig. 2) shows that in more than half of endemic countries at least two or more *Leishmania* species are present, and are mostly sympatric. Distinguishing between sympatric species is of importance for medical purposes (*e.g.,* species-specific therapies) and fundamental to molecular epidemiology in endemic foci. Also, this map illustrates the extensive potential for hybridization between distinct species.

Although *Leishmania* species are frequently divided into those responsible for CL and VL, critical evaluation of available data reveals that this subdivision is ineffective for over a half of species, which have been so far associated with both. Additionally, the virulence of several zoonotic species (*e.g.*, *L. turanica*, *L. tarentolae*) is either unstudied, or ranges from slight pathogenicity to severe forms, apparently depending on a number of factors (Fig. 3) (Bordbar and Parvizi, 2014; Novo et al., 2015).

Herein, we present a complete list of methods and markers covering all commonly used assays and molecular DNA targets, as a basis for proposing a classification system for the suitability of these markers in examining the human-infecting *Leishmania* species. This assessment should be particularly considered in light of the goal to eliminate VL by 2020, and the necessity to track cases at the initial stages of infection with a low load of parasites (Medley et al., 2015).

2. Methods and markers to identify the *Leishmania* parasites

The key features of any detection assay for any infective agent including *Leishmania* are: i) sensitivity, ii) specificity, iii) speed, iv) accuracy, v) accessibility, vi) ease of use, and vii) applicability in the field. For species identification assays, additional factors should be considered, such as discriminatory power, versatility, cost-effectivness and ability to detect and quantify *Leishmania* spp. in

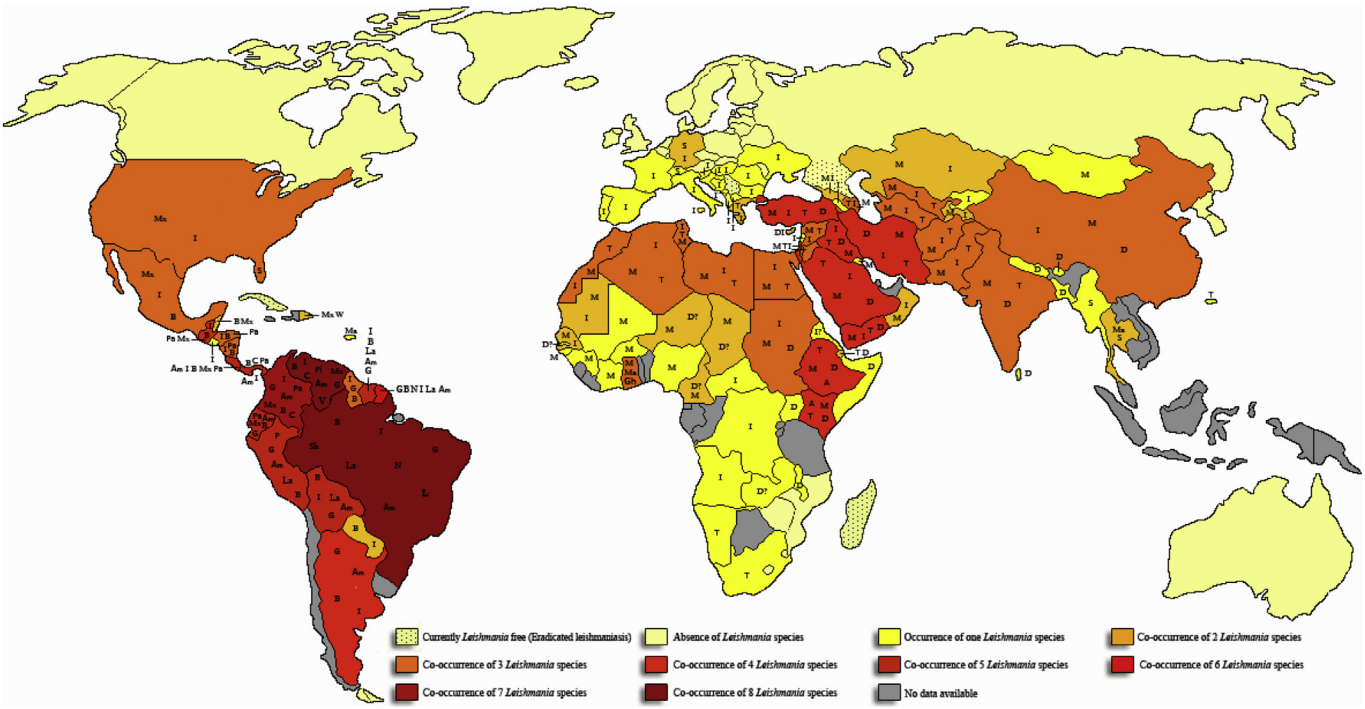

Fig. 2. Global distribution of 21 *Leishmania* species pathogenic for humans. The species name has been shown as abbreviation. A: *L. aethiopica*; Am: *L. amazonensis*; B: *L. braziliensis*; C: *L. colombiensis*; D: *L. donovani*; G: *L. guyanensis*; Gh: 'Ghana strain'; I: *L. infantum*; La: *L. lainsoni*; L: *L. lindenbergi*; M: *L. major*; Ma: *L. martiniquensis*; Mx: *L. mexicana*; N: *L. naiffi*; Pa: *L. panamensis*; P: *L. peruviana*; S: *L.* 'siamensis'; Sh: *L. shawi*; T: *L. tropica*; V: *L. venezuelensis* and W: *L. waltoni*. The species with question marks need to be confirmed by further genotyping studies.

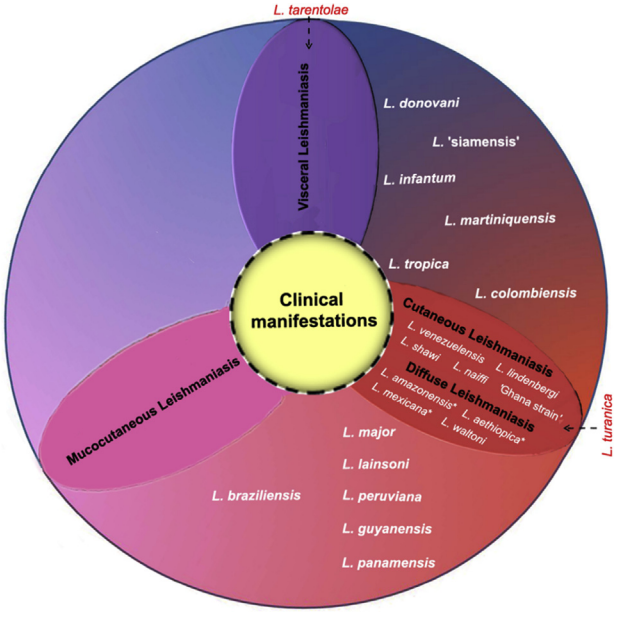

Fig. 3. Different *Leishmania* species causing various clinical manifestation of leishmaniasis (Among them, only the main clinical forms are shown in this figure). *: specific *Leishmania* species, the causative agents of diffuse leishmaniasis. The color range demonstrates the relative nearness of *Leishmania* species to each clinical manifestation based on the frequency of clinical disease cases (Dawit et al., 2013; Gradoni, 2013; Ready, 2014). The already known non-pathogenic species of *L. tarentolae* and *L. turanica*, reported recently to be pathogenic to human are shown as "outgroup".(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biological samples. Current methods for diagnosing leishmaniasis are mainly restricted to referral hospitals or research centers with well-equipped laboratory settings. Most widely used assays can only detect a single or a few *Leishmania* species and therefore are not suitable for areas with several sympatric species. Hence, there is a need for simplifying and adapting diagnostic assays in endemic areas. Different diagnostic methodologies and their power for detection, identification and quantification of *Leishmania* species, as well as their capacity for discrimination at different levels (genus, section, subgenus, species complex, species and population) are summarized in Table 1.

2.1. Detecting Leishmania in infections

2.1.1. Methods

Microscopic examination Lesion biopsy smears for CL and aspirates of the spleen, bone marrow and lymph nodes for VL are examined under the microscope following Giemsa-staining (Akhoundi et al., 2013). It is a rapid and cheap approach but invasive method (all tests based on biopsy material) which is not easy to perform. It gives only partial quantitative information on parasite load and does not allow discrimination between *Leishmania* species. In addition, sensitivity is highly dependent on the number and dispersion of parasites in the biopsy, as well as the sampling process and the technical skills of the personnel performing the tests. Nevertheless, in endemic areas, it is still a useful diagnostic approach for primary healthcare because of its cost-effectiveness and simplicity.

Isolation of *Leishmania* parasites in culture *In vitro* culture of *Leishmania* promastigotes from tissue aspirates, scrapings or biopsies is important for routine diagnostics. Unfortunately, the recovery of parasites in culture is rarely more than 70% efficient, even for strains that are easily maintained *in vitro*. It is a time-consuming

Table 1
Comparison of DNA-based methods, with other non DNA-based methods in detection, identification, discrimination and quantification of *Leishmania* species.

Methodology			*Leishmania* detection in clinical sample¥	*Leishmania* identification	*Leishmania* discrimination#¤						*Leishmania* quantification	Culture needed	Sensitivity	Specificity	Cost	References
					G	SL	SG	C	S	F						
DNA-based Methods	PCR-based Methods	PCR METHODS*	yes	yes	+	+	+	+	+		yes	no	XXX	XX	medium	Noyes et al., 1998
		RAPD	no	no						+	no	yes			medium	Tibayrenc et al., 1993
		AFLP	no	no						+	no	no	XX	XXX	medium	Kumar et al., 2010
		SNP	no	no	+	+	+	+	+	+	no	no	XXX	XXX	medium	Downing et al., 2012
		MLMT	no	no						+	no	yes	XXX	XXX	medium	Kuhls et al., 2007
		MLST	no	no	+	+	+	+	+	+	no	yes	XXX	XXX	medium	Ravel et al., 2006,
	POST-PCR Methods	OC-PCR	yes	yes	+	+	+	+	+		no	no	XXX	XX	medium	Basiye et al., 2010
		PCR-ELISA	yes	yes	+	+	+	+	+		no	no	XX	XXX	medium	De Doncker et al., 2005
		PCR-HRM	yes	yes	+	+	+	+	+	+	no	no	XX	XXX	medium	Nasereddin and Jaffe 2010
		PCR-RFLP	yes	yes	+	+	+	+	+		no	no	XX	XXX	medium	Dweik et al., 2007
		Gene sequencing	yes	yes	+	+	+	+	+	+	no	no	XXX	XXX	medium	Van Eys et al., 1992
	Non PCR-based Methods	PFGE	no	yes					+		no	yes	XX	XX	medium	Beverley, 1988
		NASBA	no	yes							no	no	XX	XXX	medium	Basiye et al., 2010
		LAMP	no	yes							no	no	XXX	XXX	medium	Ghasemian et al., 2014
Non DNA-based Methods	Parasitological Methods	Microscopic examination	yes	no	+						yes	no	XX	X	low	Bensoussan et al., 2006
		In vitro parasite culture	yes	no	+						no	yes	X	X	medium	Castilla et al., 1995
		Isolation in experimental animals	yes	no	+						no	no	X	XX	medium	Gupta, 2011; Loría-Cervera and Andrade-Narváez, 2014
		Dermal diagnostic tests	yes	no	+						no	no	XX	XXX	low	Sadeghian et al., 2013
		Xenodiagnosis	yes	no	+						no	no	XX	XXX	medium	Sadlova et al., 2015
	Serological Methods**	ELISA	yes	no	+						no	no	XX	XX	medium	Sundar and Rai 2002,
		IFAT	yes	yes	+						no	no	XXX	XX	medium	Figueiredo et al., 2010
		ICT	yes	yes	+						no	no	XX	XX	medium	da Silva et al., 2015
		DAT	yes	no	+						no	no	XX	XXX	medium	Adams et al., 2012
		CIE	yes	no	+						no	no	X	XX	medium	Mancianti & Meciani, 1988
		Western blot	yes	yes	+	+	+	+	+		no	no	XXX	XXX	medium	Gonçalves et al., 2002
	Protein based methods	MLEE	no	yes	+	+	+	+	+		no	yes			medium	Rioux et al., 1990
		MALDI-TOF	no	yes	+	+	+	+	+		no	yes			expensive	Lixia et al., 2012

*Including different specific PCR methods *e.g.* multiplex and nested PCR. **: based on the antibodies or antigens detection of *Leishmania* species. *Leishmania* detection ¥: capacity to detect *Leishmania* parasite at the genus level. *Leishmania* identification#: *Leishmania* identification refers to the ability to identify one *Leishmania* species whereas discrimination, refers to the capacity to identify all or several *Leishmania* species without "a priori". X: Grades of sensitivity and specificity are given by (X); X:low, XX: medium, XXX: high. ¤: Levels of discrimination amanable; G: Genus, SL: Section level, SG: Subgenus, C: Complex, S: Species and F: Foci.
Abbreviations RAPD (Random amplified polymorphic DNA); AFLP (Amplified fragment length polymorphism); SNP (single nucleotide polymorphism); MLMT (Multilocus microsatellite typing); MLST (Multilocus sequence typing); OC-PCR (Oligochromatography-PCR); PCR-ELISA (enzyme-linked immunosorbent assay); PCR-HRM (high-resolution melting); PCR-RFLP (restriction fragment length polymorphism); PFGE (pulsed-field gel electrophoresis); NASBA (nucleic acid sequence based amplification); LAMP (Loop-mediated isothermal amplification); ELISA (enzyme-linked immunosorbent assay); IFAT (indirect fluorescent antibody test); ICT (immuno-chromatography technology); DAT (direct agglutination test); CIE (Counterimmunoelectrophoresis); MLEE (Multilocus Enzyme Electrophoresis); MALDI-TOF (Matrix-assisted laser desorption ionization−time-of-flight).

and an expensive method that requires a sophisticated laboratory setup (Berman, 1997). On the other hand, parasite cultures are needed for a number of DNA-based and protein-based methods developed for discriminating between *Leishmania* species (Table 1).

Isolation of *Leishmania* parasites in experimental animals An alternative method of diagnosis is the inoculation of *Leishmania* cells isolated from biological materials into the footpad, nose or tail base of a susceptible BALB/c mouse or hamster. Histopathological evaluation of a biopsy can be informative, but it is rarely sufficient for a diagnosis (Loría-Cervera and Andrade-Narváez, 2014). This method is time-consuming, non-quantitative and not feasible without an animal facility.

Dermal diagnostic tests The *Leishmanin* skin test (LST), or Montenegro test, measures the delayed type hypersensitivity (DTH) reaction to an intradermal injection into the forearm of a suspension of killed *Leishmania* promastigotes. The comparison of the size of the induration with a control, represented by the injection of phenol-saline in the other forearm makes it possible to test for infection. It is a useful and important tool for leishmaniasis diagnosis because of its simplicity, sensitivity and specificity (Weigle et al., 1991). However, it does not allow for the identification of *Leishmania* species and does not discriminate between past and current infections.

Xenodiagnosis is conducted by exposing possibly infected tissues or lesions to a competent phlebotomine vector and the examination after feeding for the presence of *Leishmania* flagellates in the gut (Sadlova et al., 2015). It is relatively simple, with high specificity and reasonable sensitivity, but fails to discriminate between *Leishmania* species. Moreover, xenodiagnosis is time-consuming, non-quantitative and not feasible without animal/insectary facilities.

Conventional PCR enables the amplification of DNA or RNA through repetitive cycles *in vitro*. The description of several species-specific DNA fragments has made this method viable for leishmaniasis diagnosis since the early 1990s (Smyth et al., 1992). The PCR is superior to other parasitological methods such as microscopy or cultivation, particularly for samples with low parasite loads (Antinori et al., 2007). In addition to diagnosis, PCR has applications in quantification of the parasites and therefore also monitoring disease progression, predicting and controlling the outcome of anti-leishmanial therapy, defining parasite-specific features such as virulence or drug resistance and species discrimination (Schönian et al., 2011).

Oligochromatography-PCR (**OC-PCR**) provides a simple and rapid format for detection of PCR or nucleic acid sequence based amplification (NASBA) products (more below) visualized on a dipstick by hybridization with a gold-conjugated probe, allowing sequence-specific product detection. This detection format takes only 5–10 min and requires no equipment other than a water bath and a pipette. Internal controls for amplification and chromatographic migration are incorporated into the assay (Deborggraeve et al., 2008; Laurent et al., 2009). The method has high sensitivity, but does not allow for the discrimination of various *Leishmania* species (Mugasa et al., 2010; Saad et al., 2010).

Nucleic acid sequence based amplification (NASBA) is an isothermal, transcription-based amplification system designed for the detection of RNA targets. It usually works at an isothermic 41 °C, and has been widely used for the detection of *Leishmania* species because it gives results faster than PCR with more sensitivity, but is not suitable for quantifying and discriminating different *Leishmania* species (Van der Meide et al., 2005; Rodríguez-Lázaro et al., 2006; Saad et al., 2010; Mugasa et al., 2010).

2.1.2. Markers

Leishmania spp. and other trypanosomatids have unique genomic organization traits compared with eukaryotes, such as genes without introns, polycistrons and small chromosomes with high gene densities. Moreover, these flagellates possess a single mitochondrion called the kinetoplast, which contains a large network of kinetoplast DNA (kDNA) (Lukeš et al., 2002).

In the following chapter 2.1 all genetic markers are listed that are used for detection of *Leishmania* at genus level by PCR (presence of a PCR product) and other molecular methods. Depending on the targets and the respective PCR primers the assays can be genus-specific or even species specific (see also chapters 2.2 and 2.3).

2.1.2.1. Chromosomal DNA

2.1.2.1.1. Ribosomal DNA (rDNA). The ribosomal RNA (rRNA) genes are located mostly on chromosome 27, usually as multiple copies of tandem head-to-tail repeats of approximately 12.5 Kb (Yan et al., 1999) (Fig. 4). Among different components of these genes, the most variable ITS regions are ideal for species-typing (Schönian et al., 2001; Kuhls et al., 2005). Like most eukaryotes, large ribosomal subunit (LSU) is composed of 28S, 5.8S and 5S rRNAs, whereas the small subunit (SSU) contains 18S rRNA. The 18S, 5.8S and 28S rRNAs are encoded by a single transcription unit called the ribosomal cistron that is transcribed by RNA polymerase I to produce a large primary transcript (45S or pre-rRNA) (Torres-Machorro et al., 2010). The 5S rRNA gene is transcribed separately by RNA polymerase III. The processing of the transcripts results in the removal of the internal and external transcribed spacers (ITS and ETS, respectively), which are rapidly degraded. Therefore, the rRNA unit is composed of a transcribed region and an intergenic region (also called the intergenic spacer) consisting of a non-transcribed spacer (NTS) and an ETS (Sollner-Webb and Mougey, 1991). Repetitive sequences within the intergenic spacers or the NTS are subject to concerted evolution, because there is extensive sequence between the spacers within a species but little homology between the spacers of related species (Requena et al., 1997). In a single unit of repetition, the coding region is approximately 9 Kb (depending on the *Leishmania* species), whereas the NTS regions vary in length from 4 to 12 Kb. For complete list of primers targeting these regions see Supplementary Information: Table SI–1.

2.1.2.1.1.1. Ribosomal RNA transcription units

The ***18S rRNA*** is a structural RNA of the ribosomal SSU. The high conservation of this gene and its flanking regions make it suitable for reconstructing phylogenetic relationships.

The ***5.8S rRNA*** is a non-coding component of the ribosomal LSU, and is part of the 45S precursor that also contains the 18S and 28S rRNAs.

The ***28S rRNA*** acts as a ribozyme, catalysing peptide bond formation. *Leishmania* species are atypical in its composition, as they contain two large (24Sα and 24Sβ) and four small rRNA molecules (ϒ, ζ, δ, ε) (Soto et al., 2004).

Finally, the 120 nt-long ***5S rRNA*** is a structural and functional component of the ribosome's LSU (Schramm and Hernandez, 2002). The 5S rRNA gene is not usually linked to the ribosomal cistron (Paule and White, 2000). Due to its small size and thus low informational content, it is rarely used for molecular phylogenetics (Fig. 4, Supplementary Information: Table SI–1).

2.1.2.1.1.2. Internal transcribed spacer

The internal transcribed spacer (ITS) refers to the non-coding spacer DNA located between the SSU and LSU rRNAs (Fig. 4). The ***ITS1*** region ranges from 50 to 350 bp and is located between the 18S rRNA and 5.8S rRNA genes (Schönian et al., 2003). It has sufficiently high conservation to be a *Leishmania* PCR target but its polymorphism allow species typing, such as differentiating *L. aethiopica*, *L. tropica*, *L. major, L. turanica* and the *L. donovani* complex, *L. mexicana, L. amazonensis, L. guyanensis, L. braziliensis* (Cupolillo et al., 1995; Schönian et al., 2001; 2003; Mauricio et al.,

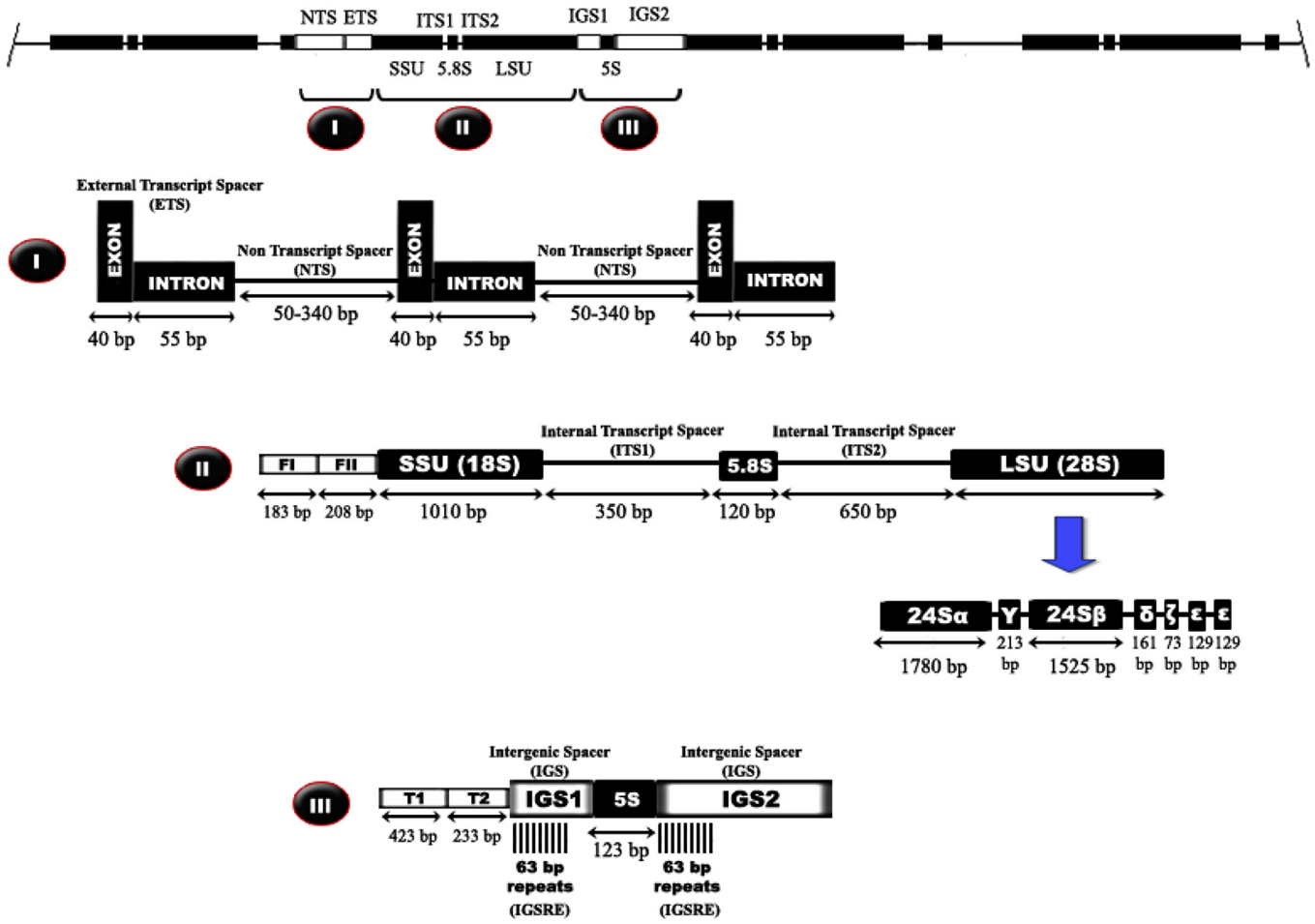

Fig. 4. Genetic map of rDNA genes (located in chromosome 27) with corresponding fragments length in *Leishmania* species

2004; Odiwuor et al., 2011). The ***ITS2*** region is of 50–650 bp-long and is located between the 5.8S rRNA and LSU rRNA genes. Amplification of *ITS2* with generic PCR primers revealed substantial differences between the Old and New World *Leishmania* spp. but also between species complexes and species of these subgenera (Schönian et al., 2003; Kuhls et al., 2005). These ITS (ITS1 & ITS2) regions were also considered by Cupolillo et al. (1995) as suitable targets for typing of the *Viannia* subgenus.

2.1.2.1.1.3. Intergenic spacer region (IGS)

The IGS is a genomic element varying in size, number and sequence across *Leishmania* species, and isolates. The *Leishmania* IGS contains a 60–64 bp-long repeat element of 16–275 copies, causing length variations of 4–12 Kbp (Orlando et al., 2002). The size of the IGS region is roughly the same in *Sauroleishmania* and *Leishmania* species, demonstrating its conservation at the subgenus level. The IGS is less conserved than the rRNA genes and thus is more suitable to map the evolutionary relationships between closely related *Leishmania* species.

Mini-exon (or Spliced leader RNA; SL RNA) gene of kinetoplastid protists is present in 100–200 tandemly repeated copies per nuclear genome and has been used for *Leishmania* phylogenomics (Zelazny et al., 2005; Azmi et al., 2010). Each repeat consists of three major parts: i) a transcribed 39 bp-long exon (or mini-exon) that is highly conserved between positions 1–9 and 21–39 (Sturm et al. 2001); ii) a moderately conserved intron, ranging in size from 55 to 101 bp; and iii) a non-transcribed highly variable spacer (51–341 bp) used for *Leishmania* genotyping. Its variation in size allows preliminary discrimination between major Old and New World *Leishmania* complexes (Fernandes et al., 1994; Ramos et al., 1996; Harris et al., 1998; Marfurt et al., 2003). The 39 bp mini-exon can be used to quantify gene expression because it is present at the 5′ end of mRNAs (Haydock et al., 2015).

External transcribed spacer (ETS) is a RNA region that is closely related to the ITS and is situated outside the structural rRNA on a common precursor transcript (Fig. 4). The ~ 1 Kb–long ETS region of *Leishmania* species has a similar size among species: *L. amazonensis* (1 050 bp) (Uliana et al., 1996), *L. chagasi* (syn *L. infantum*) (1 060 bp) (Gay et al., 1996), *L. donovani* (1 020 bp) (Yan et al., 1999). Nevertheless, differences in the ETS sequences can distinguish *Sauroleishmania* from *Leishmania*.

Non transcribed spacer (NTS) is a 2 to 30 Kb-long intergenic rRNA spacer. It likely contains most of the regulatory elements needed for transcription (Sollner-Webb and Mougey, 1991).

2.1.2.1.2. Protein-coding genes. Chromosome structure differs among *Leishmania* between and within species. *L. major* is the optimal reference for studying chromosomal changes because its genome was assembled using long capillary sequence data. Members of the subgenera *L. (Leishmania)* possess 36 chromosomes (chr) with the exception of the *L. mexicana* complex, which has 34 due to fusion of the homologous *L. major* chr8 with chr29 as a single chr8 *in L. mexicana*, and another fusion of the homologous *L. major* chr20 with chr36 into *L. mexicana* chr29 (Rogers et al., 2011). The

subgenus *Viannia* has 35 chromosomes such that their long chr20 is orthologous to the homologous *L. major* chr20 plus chr36 (Britto et al., 1998). The subgenus *Sauroleishmania* has 38 chromosomes due to fission of those homologous to chr30 and chr36 in the *Leishmania* subgenus (Coughlan et al., 2017).

As expected for a single-cell obligatory parasite, the genomes of *Leishmania* are compact and streamlined due to gene loss: the *L. major* genome is 33 Mb compared with 40 Mb for free-living *Bodo saltans* (Jackson et al., 2016; Opperdoes et al., 2016). The highest number of genes has been recorded in *L. tarentolae* (8 530) followed by *L. major* (8 400), *L. infantum*/*L. donovani* (8 199), *L. braziliensis* (8 175), *L. mexicana* (8 106), *L. adleri* (7 959) and *L. panamensis* (7 748). However, these values tend to represent differences in genome assembly methods, sequence read type and length, and gene copy number resolution rather than true differences, with the exception of certain well-characterized gene families.

Some genes have been used as endogenous controls for asserting the expression level of target genes. These include several single or multiple-copy genes used for detection, identification, and phylogenetic analysis of *Leishmania* species such as alpha-tubulin and β-actin. A complete list of primers for the amplification of different protein-coding genes is given in Supplementary Information: Table SI–2. The chromosomal location of these genes and other MLMT and MLST markers is brought in Table 2. Moreover, several protein-based markers which are used for MLST genotyping (*i.g.* GOT, ASAT, GPI, NP1, NP2, ICD, ME, FH and etc.), we brought below just some of them which are commonly used for *Leishmania* identification and discrimination.

Major surface protease (MSP) is also called Gp63 or leishmanolysin, and is the most abundant surface glycoprotein. This 63 kDa molecule participates in the parasite's initial attachment to mammalian macrophages and likely contributes to their survival within the macrophage phagolysosomes. It has been identified as a virulence factor in several *Leishmania* species, and has been used as a species discriminatory tool (Supplementary Information: Table SI–2) (Victoir et al., 1998; Guerbouj et al., 2001; Mauricio et al., 1999, 2001, 2007).

Heat-shock proteins (HSPs) During stress, these highly conserved proteins maintain protein homeostasis by interacting with denatured proteins and inhibiting the formation of cytotoxic protein aggregates (Feder and Hofmann, 1999). Most HSPs exhibit chaperone activity and have multiple interaction targets to control their maturation, activation, translocation and degradation. Based on their molecular masses and primary structures, HSPs have been divided into the following families: HSP10, HSP40, HSP60, HSP70, HSP83, HSP90, HSP100, HSP110 and small HSPs (Feder and Hofmann, 1999). Small HSPs protect other proteins from aggregation and maintain cellular viability during stress. HSP20 and HSP23 are encoded by a single gene and are sometimes used for phylogenetic studies (Fraga et al., 2013).

The *hsp70* gene has been widely used for phylogenetic and taxonomic studies of *Leishmania* (Supplementary Information: Table SI–2, Fig. 5). Regions with *hsp70* homology have been found on chromosomes 26, 28, 30 and 35. These have been identified as suitable for PCR-RFLP diagnostics that does not require parasite culturing prior to amplification (Garcia et al., 2004, 2007; Montalvo et al., 2014), and so may become widely-used targets. The *hsp70* gene as well as other members of this group like *hsp20* and *hsp23* can differentiate *Leishmania* subgenera, such as *Leishmania*, *Viannia* and *Sauroleishmania*, and also species complexes *L. donovani*, *L. major*, *L. tropica*, *L. mexicana*, *L. braziliensis* and *L. guyanensis* as well as individual species (Fraga et al., 2013; Van der Auwera et al., 2013, 2014; Hombach et al., 2015). However, *hsp70* seems to be less discriminative for distinguishing *Viannia* species complexes (Fraga et al., 2010) because their geographic structure may have permitted more ancestral gene flow. Still, the resolution could be improved by simultaneous evaluation of other *hsp* loci such as *hsp20* (Fraga et al., 2013).

Variation at *hsp70* has been associated with the resistance to antimony in *L. donovani* (Vergnes et al., 2007), *L. tarentolae* and *L. infantum* (Brochu et al., 2004). Additionally, *hsp70* mutations were observed in a *L. donovani* focus where the total genetic diversity was minute (Downing et al., 2011). Consequently, *hsp70* has an extensive phylogenetic power, though a putative role in heavy metal metabolism could bias quantitative estimates of evolutionary distance.

Cysteine protease B (cpB) belongs to a large family of cysteine peptidases expressed by cysteine peptidase A, B and C (*cpa*, *cpb*, *cpc*). Various species–specific PCR assays targeting *cpb* genes and their non-coding intergenic sequences have been developed to discriminate different *Leishmania* species (Quispe Tintaya et al., 2004; Hide and Bañuls, 2008).

Hydrophilic acylated surface protein B (HASPB) belongs to a family of orthologous chr23 genes (including HASPA as well as HASPB) found in the Old and New World *Leishmania* species that was originally called the LmcDNA16 locus (Flinn and Smith, 1992). *Leishmania* HASPB is a lipoprotein, exported to the extracellular space via an unconventional secretory pathway present on the plasma membrane of metacyclic promastigotes and amastigotes. It is characterized by repetitive domains that show both inter- and intra-species polymorphism (McKean et al., 1997; Alce et al., 1999; Bhatia et al., 1999). HASPB is involved in metacyclogenesis and play role in promastigote localization within the sand fly vector (Sadlova et al., 2010). It is expressed only in vector metacyclic stages, correlating with the expression of metacyclic-specific LPG and providing the first definitive protein marker for this infective sand fly stage.

Catalytic subunit of DNA polymerase a (polA) The 60 kDa Pol α complex (or Pol α-DNA primase complex) consists of four subunits: the catalytic subunit POLA1, the regulatory subunit POLA2, and the small and the large primase subunits PRIM1 and PRIM2, respectively. The catalytic subunit POLA1 has been used for *Leishmania* species discrimination (Croan et al., 1997; Noyes et al., 2002; Tsokana et al., 2014).

N-acetylglucosamine-1-phosphate transferase (NAGT) is a transmembrane protein of the endoplasmic reticulum, whose enzymatic action catalyses the first step of *N*-glycosylation pathway. It is expressed from a single-copy gene that is highly conserved and functionally indispensable and was used for species discrimination and phylogenetic analyses (Waki et al., 2007).

Small hydrophilic ER-associated protein SHERPs are expressed in metacyclic promastigotes in culture and the insect vector. Their product is a 6.2 kDa peripheral membrane protein that localizes to the outer mitochondrial membrane (Knuepfer et al., 2001), yet the function of this unusual small protein is still unknown (Sadlova et al., 2010).

Mitogen-activated protein kinase (MAPK) cascades are evolutionary conserved three-kinase modules that play a key role in the regulation of cellular functions in response to a variety of extracellular stimuli. They are composed of several members, including MAPK2, MAPK3, MAPK4, MAPK5 and MAPK7. MAPK genes that become activated in response to a wide variety of physiological and stress-related stimuli and are believed to have the same role in *Leishmania* species (Brumlik et al., 2011; Ashutosh et al., 2012).

The ***A2 gene*** family at chr22 comprises the 5′A2 rel, 3′A2 rel, internal A2 rel and A2 genes (Zhang and Matlashewski, 2001;

Table 2
Chromosomal location of different molecular markers of multilocus microsatellite typing (MLMT), multilocus sequence typing (MLST) and protein coding genes.

Chromosome	MLMT	MLST	Protein coding genes
1	Li22-35, Lm2TG, Lm4TA, Li21-34, 36GTG, 39GTG, 45GTG, 1GACA, LiBTG, Li71-5/2, LiBTA	hypothetical protein (LinJ.01.0010)	heat shock protein 70 (LmxM.01.0640, LmjF.01.0640, LinJ.01.0660, LbrM.01.0790), GP63 (LinJ.10.0490), Poly A (LmjF.01.0320, LmxM.01.0320, LinJ.01.0330, LdBPK_010330, LbrM.01.0360), DNA-damage inducible 1 protein (LbrM.01.0030, LmjF.01.0610, LmxM.01.0610, LinJ.01.0630, LdBPK_010630)
2	GA2, GT4, 2079372-72-448, 2079372-119-374		
3	LIST7006, 27GTG, 1 GC, ARP, M233	Inorganic pyrophosphatase (AC125735), Elongation initiation factor 2 alpha (LdBPK_030960, LmxM.03.0980)	
4	LIST7004, LIST7007, ST436, 2079447-148-646, 2079447-193-429	Spermidine synthase 1 (LinJ.04.0570, LdBPK_040570, LmjF.04.0580, LmxM.04.0580, LbrM.04.0630)	
5	28AT, G09, M202, M207, M402		
6	LIST7022	Dihydrofolate reductase-thymidylate synthase (LbrM.06.0830, LmjF.06.0860, LmxM.06.0860, LinJ.06.0890, LdBPK_060890)	Histone H4 (LinJ.06.0010, LmxM.06.0010)
7	M166		Cysteine protease b (LmxM.07.0550, LdBPK_070600)
8	DPB1, DPB2, Rossi 1		Poly A (LmjF.08.1010, LmxM.08_29.2600), Beta tubulin (LbrM.08.1040, LbrM.08.1040, LmjF.08.1230, LmxM.08.1230, LinJ.08.1280, LinJ.08.1280)
9			Elongation Factor-1 gamma (LinJ.09.1020), Histone H2B (LmxM.09.1340, LinJ.09.1410)
10	CSg55, LIST7025, LIST7031, LIST7032, 2079619-25-438, 2079619-166-373, Ibh3	Isocitrate dehydrogenase (LinJ10.0200, LmjF10.0290), Zinc binding dehydrogenase like protein (LmjF.10.0560, LmxM.10.0560, LmjF.10.0570, LmxM.10.0570, LdBPK_100610, LinJ.10.0610, LdBPK_100620, LinJ.10.0620, LbrM.10.0690, LbrM.10.0700), Gp63 (LmxM.10.0405, LmjF.10.0470, LinJ.10.0490, LdBPK_100520, LbrM.10.1630)	GP63 (LmxM.10.0405, LmjF.10.0470, LdBPK_100520, LbrM.10.1630), Histone H3 (LmxM.10.0870, LinJ.10.0920)
11	AC16R, AC16, 3 (Lmj), 21 (Lmj)	Stearic acid desaturase (LbrM.11.0330), hypothetical protein (LinJ.11.0280)	
12	LIST7034, GA3, 19 (Lmj), 26 (Lmj, Ld), LIST7002, M196, Li71-7, CS19	Alanine aminotransferase (LinJ.12.0580, LbrM.12.0630), Glucose-6-phosphate isomerase (LinJ.12.0490, LbrM.12.0490, LdBPK-120490, LmjF.12.0530, LmxM.12.0530), Translation initiation factor alpha (LinJ.12.0001, LmjF.12.0010, LdBPK_120010, LmxM.12.0010, LbrM.12.0020)	
13	LIST7026, 2079709-131-590, 2079709-205-450, LIST7001, M149, M155		
14	LIST7010, LIST7011, Rossi2, ISA136, 2079734-89-538, 2079734-196-471	Enolase (LinJ.14.1240, LbrM.14.1330), Nucleoside hydrolase-like protein (LdBPK_140130, LmxM.14.0130), Stearic acid desaturase (LmjF.14.0510, LmxM.14.0510, LinJ.14.0520, LdBPK_140520)	Poly A (LmjF.14.1180, LmxM.14.1180, LdBPK_141260, LinJ.14.1260, LbrM.14.1350)
15	2079764-93-547, LRC, 2079764-207-434, 8 (Lmj, Ld), 11 (Lmj, Ld)		Histone H4 (LinJ.15.0010, LmxM.15.0010)
16	B6F, GA1, GA3, GA6, GTG1, GTG3, Li45-24, Li72-14		Histone H3 (LmxM.16.0570)
17	LIST7030, M140		Elongation Factor-1 alpha (LmxM.17.0080, LmxM.17.0083, LinJ.17.0110), Histone H2B (LinJ.17.1320)
18	CSg46, 10F, 22 (Lmj, Li)	Aconitase (LinJ.18.0510, LbrM.18.0570), nucleoside hydrolase 1 or nucleoside phosphorylase 1 (LDBPK_181570), nucleoside hydrolase 2 or nucleoside phosphorylase 2 (LDBPK_181570)	
19	AC01R, AC01, CS20, LIST7003, LIST7009, LIST7012, 2079862-64-549, 2079862-235-376, CS20		Poly A (LinJ.19.1450, LbrM.19.1680), Histone H2B (LinJ.19.0030)
20	CSg48, AC52, M292	Malate dehydrogenase mitochondrial (LbrM.20.0010)	Elongation Factor-1 beta (LbrM.20.0770), LPG2 (LbrM.20.2700)
21	CSg53, 11H, 71AT, LIST7008, LIST7013, LIST7037, Mix9, HG	Hypoxanthine-guanine phosphoribosyltransferase (LinJ.21.0980, LbrM.21.0990), Phosphoglucomutase (LbrM.21.0700, LinJ.21.0700), Hypoxanthine-guanine phosphoribosyl transferase (AL596287)	Beta tubulin (LmjF.21.1860, LmxM.21.1860, LbrM.21.2150, LbrM.21.2150, LinJ.21.2240, LinJ.21.2240), Histone H2A (LinJ.21.1160), Histone H4 (LmxM.21.0015, LinJ.21.0020, LmxM.21.0915)
22		cytb5R-Ch22II (LinJ.22.0590)	A2 protein (LinJ.22.0670, LmxM.22.0691)
23	LIST7035	Pteridine reductase 1 (LmjF.23.0270, LmxM.23.0270, LbrM.23.0300, LdBPK_230310, LinJ.23.0310)	Small hydrophilic ER-associated protein (LmxM.23.1050, LmjF.23.1086, LinJ.23.1210), Hydrophilic acylated surface protein B (LinJ.23.1220), Poly A (LbrM.23.0650, LinJ.23.0660)

Table 2 (*continued*)

Chromosome	MLMT	MLST	Protein coding genes
24	7 (Lmj), 28 (Lmj), LIST7002, Li71-7, CS19	Malic enzyme (LinJ24.0440, LmxM.24.0761, LmjF.24.0770, LinJ.24.0780, LbrM.24.0780, LdBPK_240780), Aspartate Aminotransferase (LinJ.24.0370, LbrM.24.0370, LmjF.24.0370, LdBPK_240370, LmxM.24.0370)	Poly A (LinJ.24.0110)
25	CSg59, Li23-41, LIST7033		Poly A (LinJ.25.0080, LmjF.25.0080, LdBPK_250080, LmxM.25.0080), Elongation Factor-1alpha (LinJ.25.0130), Histone H4 (LmxM.25.2450, LinJ.25.2560)
26	LIST7027, LIST7036, LIST7038, 20 (Lmj), 17 (Lmj), 27 (Ld), M147, 21 (Lmj), E11		Adenine phosphoribosyltransferase (LinJ.26.0120, LbrM.26.0130, LmjF.26.0140)
27	6F, ITSbraz, ITS1, M329		
28	B3H, GACA1, Li71-19	Leishmania-activated C kinase (LmxM.28.2740, LdBPK_282970), heat shock protein 70 (LmjF.28.2770, LmxM.28.2780, LinJ.28.2950, LdBPK_282950, LbrM.28.2970), hypothetical protein (LinJ.28.0190)	heat shock protein 70 (LmxM.28.2770, LmjF.28.2780, LinJ.28.2950, LdBPK_282960, LinJ.28.2960, LbrM.28.2980, LinJ.28.3060)
29	CSg47, M80, M81	Fumarate hydratase (LmjF29.1960), Mitogen-activated Protein Kinase (AJ243188)	heat shock protein 70 (LmjF.29.1240, LbrM.29.1320, LdBPK_291330, LinJ.29.1330), Poly A (LinJ.29.2710, LdBPK_292710), Apical Membrane Antigen 1 (LmxM.29.1420)
30	LIST7024, LIST7029	RNA polymerase II largest sub-unit (LmxM.30.2610)	Apical Membrane Antigen 1 (LmjF.30.1410, LinJ.30.1470, LdBPK_301470, LbrM.30.1520), RNA polymerase II largest sub-unit (LmxM.30.2610)
31	LIST7039, LIST7040, Li46-67, Li71-33, Li72-20, 23 (Lmj, Ld, Li), 13 (Lmj), CAK, 5 (Lmj, Ld), 6 (Lmj)	RNA polymerase II largest sub-unit (LmjF.31.2610, LinJ.31.2680, LdBPK_312680), Hypothetical protein (Lmj.31.0280, LbrM.31.0280)	GP63 (LbrM.31.2250), RNA polymerase II largest sub-unit (LmjF.31.2610, LinJ.31.2680, LdBPK_312680), Thiol dependent reductase 1 (LbrM.31.0550), Histone H4 (LinJ.31.3320)
32	Li71–42, GA09, GA10, 1 (Lmj), 4 (Lmx), 15 (Ld)	Mannose phosphate isomerase (LmjF32.1580, LinJ32.1600)	Mitogen-activated protein kinase (LmxM.32.1380), heat shock protein 20 (LmjF.32.2260, LdBPK_322410, LinJ.32.2410, LbrM.32.2490), Beta tubulin (LmxM.32.0792),hypothetical protein (LinJ32.1600), Mannose Phosphate Isomerase genes (LmjF.32.1580)
33	11C, LIST7023, M269, 4 (Ld)		Mitogen-activated protein kinase (LmjF.33.1380, LinJ.33.1470, LbrM.33.1650), Beta tubulin (LmjF.33.0794, LinJ.33.0860, LbrM.33.0950, LbrM.33.0950), Macrophage migration inhibitory factor (LmjF.33.1740, LinJ.33.1840), Thiol dependent reductase 1 (LmjF.33.0240, LinJ.33.0260), Lipophosphoglycans2 (LmxM.33.3120), Elongation Factor-1 beta (LmxM.33.0820)
34	TubCA, 18 (Lmj, Ld, Li), 16 (Lmj)	Aspartate Aminotransferase (LmxM.34.0820), heat shock protein 70 (LbrM.34.4680), Glucose-6-phosphate dehydrogenase (LbrM.34.3250, LmxM.34.3340, CT005271), Adenylate kinase 2 (AF156853), Malate dehydrogenase mitochondrial (LmjF.34.0160, LinJ.34.0170, LdBPK_340170), hypothetical protein (LinJ.34.0550)	heat shock protein 70 (LbrM.34.4680), A2 protein (LmxM.34.3020), Poly A (LmxM.34.4130), Elongation Factor-1 beta (LinJ.34.0870), Kinetoplastid membrane protein-11 (LbrM.34.2140, LmxM.34.2210), Lipophosphoglycans2 (LmjF.34.3120, LinJ.34.4290)
35	Li72-17/2, 7 GN, M75, M78, LIST7005, 4GTG, 2080398-188-553, 2080398-243-507, EMI	heat shock protein 70 (LmjF.35.4710, LinJ.35.4780, LdBPK_354780), Phosphomannomutase (LbrM.35.2180), 6-Phosphogluconate dehydrogenase (LmjF.35.3340, LinJ.35.3390, LdBPK_353390), Aspartate Aminotransferase (LmjF.35.0820, LinJ.35.0840, LdBPK_350840)	heat shock protein70 (LmjF.35.4710, LinJ.35.4780, LdBPK_354780), A2 protein (LbrM.35.2200, LdBPK_353070), Poly A (LinJ.35.2390, LmjF.35.4130), *N*-acetylglucosamine-1-phosphate transferase (LbrM.35.4430), Histone H4 (LmjF.35.0015, LbrM.35.0050, LinJ.35.1320), Kinetoplastid membrane protein-11 (LmjF.35.2210, LinJ.35.2250, LdBPK_352260)
36	Li41-56, LBA, 1CA, LIST7021, LIST7028, 2080492_169_582, 2080492_192_516, 2080455_268_439, 2080455_230_522, 2080483_178_461, 2080476-49-610, 2080476-227-539, 2080483-70-595	Phosphomannomutase (LinJ.36.2070), hypothetical protein (LinJ.36.1190, LinJ.36.0350)	A2 protein (LmjF.36.1980, LmxM.36.1980, LinJ.36.2090, LdBPK_362090, LdBPK_354200), Poly A (LinJ.36.6630), Elongation Factor-2 alpha (LmxM.36.0180, LinJ.36.0190), *N*-acetylglucosamine-1-phosphate transferase (LmjF.36.4180, LinJ.36.4390), Histone H4 (LinJ.36.0020, LmxM.36.0020)

Different copies of a gene with their systematic ID have given in the brackets.

Zhang et al., 2003). It is the only gene family known to be responsible for visceralization and has a species-specific copy number in *Leishmania*. The A2 proteins are abundant amastigote-specific virulence factors (ranging from 42 to 100 kDa) required for survival within the mammalian host. The A2 proteins are similar to the *Leishmania* stage specific S antigen.

Elongation Factor-1α (EF-1α) Elongation factors are used for protein synthesis and include elongation factors 1-alpha, 1-beta, 1-gamma and elongation factor 2. As a highly-conserved ubiquitous protein EF-1α may be suitable for phylogenetic inference between species complexes (Momen and Cupolillo, 2000). However, genes encoding EF-1α are members of a large gene family that undergo

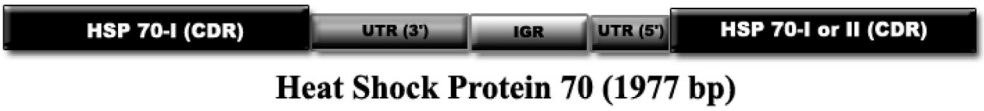

Fig. 5. Hsp70 gene used for diagnosis and typing *Leishmania* species

extensive changes in copy number in *Leishmania* species and also during short-term drug-related stress. EF-1α orthologs were previously reported as amplified in *L. infantum* JPCM5 (7–12 copies) and in *L. major* Friedlin (7–21 copies) (Rogers et al., 2011). One L. *donovani* EF-1α homolog (LdBPK_354220, also known as a HSP70 subfamily B suppressor 1 or HBS1) had a reduced copy number due to a single CNV spanning of 674 Kb during miltefosine-resistance induction (Shaw et al., 2016).

The ***Macrophage migration inhibitory factor (MIF)*** plays an important role in the regulation of macrophage function in host defence through the suppression of the anti-inflammatory effects of glucocorticoids. It has rarely been used for *Leishmania* identification (Weiser et al., 1989) (Supplementary Information: Table SI–2).

Glucose-6-phosphate dehydrogenase (G6PD) is one of the enzymes used to identify *Leishmania* by multilocus enzyme electrophoresis (MLEE) and MLST analysis (Zemanová et al., 2007; Boité et al., 2012). The polymorphic pattern revealed by partial characterization of the G6PD gene by PCR generated eight isoforms of this enzyme that can be used as molecular markers for different *Leishmania* species (Cupolillo et al., 1994; Castilho et al., 2003).

6-Phosphogluconate dehydrogenase (6PGDH) is a key enzyme of the oxidative pathway involved in the generation of NADPH and ribulose 5-phosphate. The gene encodes a 52 kDa protein. It has been frequently used in MLEE and MLST analyses for typing of Old and New World *Leishmania* species (Zemanová et al., 2007; Boité et al., 2012).

Histones are major components of the nucleosome and play an important role during gene transcription. They are classified as core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5). Their molecular masses vary from 14 (H2A and H2B) to 21 kDa (H1) (Khorasanizadeh, 2004). Some histone genes have been reported as potent candidates for *Leishmania* typing (Supplementary Information: Table SI–2) (Lukeš and Maslov, 2000).

Tubulins constitute a family of protein-coding genes that includes alpha, beta, gamma, zeta and epsilon tubulin. The highly conserved alpha and beta-tubulins form α/β-tubulin heterodimers that are fundamental components of the eukaryotic cytoskeleton, cell division machinery, intracellular transport, and ciliary and flagellar motility (McKean et al., 2001). Tubulin synthesis correlates with morphological changes during the promastigote-to-amastigote transition, where promastigotes synthesizing more tubulin than amastigotes (Fong and Chang, 1981). Among different members of the tubulin family, the α- and β-tubulin genes are widely used for *Leishmania* typing because they are present in multiple tandem copies, forming separate clusters of α- and β-tubulin genes, and are dispersed throughout the genome (Landfear et al., 1983; Jackson et al., 2006).

Mannose phosphate isomerase genes (MPI) encode proteins facilitating the interconversion of fructose 6-phosphate (F6P) and mannose-6-phosphate (M6P), applied in MLEE analysis. They have also been extensively used for examining variation within the species complexes using MLST (Zemanová et al., 2007; Boité et al., 2012). MPI was found to be the only reliable enzyme marker that can distinguish between *L. peruviana* and *L. braziliensis*, two genetically closely related members of the subgenus *Viannia* (Arana et al., 1990). It plays an important role in parasite infection in macrophages and BALB/c mice (Garami and Ilg, 2001).

Lipophosphoglycans (LPG) The surface of the promastigote stages is coated with a variety of interrelated glycoconjugates including LPG and is considered to be a critical factor for parasite survival in both the insect vector and the mammalian host. It is commonly modified by the addition of carbohydrate side chains that vary significantly across life cycle stages and species. Consequently, genes encoding enzymes involved in LPG side chain modification exhibit high variability among species, with several loci located in subtelomeric regions (Llanes et al., 2015).

RNA polymerase II largest subunit (RPOIILS) RNA polymerase (RNAP) II is a multi-subunit enzyme responsible for the transcription of proteins that forms part of a large multifunctional 'holoenzyme' involved in splicing, polyadenylation and DNA damage repair (Hampsey and Reinberg, 1999; Holstege and Young, 1999). The largest subunit (RPOIILS) carries a carboxy-terminal domain formed by long repeats of multiple 7 amino acids. It has been used for evolutionary studies of the genus *Leishmania* (Croan et al., 1997; Noyes et al., 2002).

2.1.2.2. Non-chromosomal DNA

2.1.2.2.1. Kinetoplast DNA (mitochondrial DNA). All Kinetoplastid flagellates possess a single mitochondrial genome known as the kinetoplast DNA (kDNA), which consists of several thousand circular DNA molecules linked together in a concatenated network (Lukeš et al., 2002).

It is a mass of circular DNA that consists of thousands of minicircles (~1 Kb each) and several dozen maxicircles (~23 Kb each). Maxicircles encode genes homologous to those present in the mitochondrial DNA of other eukaryotes. Minicircles make up approximately 95% of kDNA and encode small RNA molecules termed guide RNAs, which provide information for RNA-editing of the maxicircle–encoded transcripts (Stuart et al., 2005). kDNA is traditionally the most frequently used target for detection and typing of *Leishmania* because of its multicopy nature and though high sensitivity (Jara et al., 2013). Sequence diversity between the minicircle and maxicircle kDNA is know to be variable in nature.

2.1.2.2.1.1. kDNA maxicircle

This kDNA component encodes subunits of the mitochondrial respiratory chain complexes, one ribosomal subunit and two unusually small (12S and 9S) rRNAs. In contrast to most mitochondrial DNAs, the kDNA does not encode tRNAs (Alfonzo et al., 1997; Lukeš et al., 2005).

The maxicircle consists of a protein- and rRNA-coding conserved region and a non-transcribed variable region (VR). The VR is composed of a variety of repeated sequences with unknown function, and has a similar structure in all *Leishmania* species, with long GC-rich repeats interspersed by short AT-rich repeats. The maxicircle encodes three unassigned open reading frames, MURF1, MURF2, and MURF4, which likely encode proteins (Fig. 6). Maxicircle genes possess varying levels of sequence conservation, ranging from the highly conserved cytochrome oxidase subunit I (COI), to the least-conserved COIII, another subunit of the same complex. Maxicircle kDNA differences among *Leishmania* species are almost exclusively at the VR. The structure of the *Leishmania* maxicircles (Fig. 6) and list of primers used for the amplification of their genes is in Supplementary Information: Table SI–3.

The most important genetic elements of maxicircles are briefly described below:

Mitoribosomal 9S and 12S RNAs of trypanosomatids are the shortest mitochondrial rRNAs known and are highly conserved among these flagellates (Fig. 6, Supplementary Information: Table SI–3).

Cytochrome oxidase (CO) I, II and III form the three subunits of the terminal enzymatic complex of the respiratory chain. COI gene is unusual in its organization because it is encoded on the opposite strand of the maxicircle compared to other genes. The less conserved COII gene has been used for examining variation within the *L. donovani* complex (Ibrahim and Barker, 2001). However, it has been used for studies targeting phylogenetic relationships of *Leishmania* species (Cao et al., 2011).

MURFs The function of MURFs genes remains unknown. Due to their variation and species-specific editing pattens (Verner et al., 2015), they are unsuitable for diagnostic purposes.

The same applies for subunits of the NADH dehydrogenase (ND) complex that catalyses the oxidation of NADH by ubiquinone. The ND1 gene is not believed to be involved in catalysis. The trypanosomatid ND complex is reduced, and under cultivation conditions is non-essential (Verner et al., 2015).

A single mitochondrial − encoded subunit of the cytochrome reductase complex and other Cytochrome b (Cyt b), Cytochromes have been occasionally used for *Leishmania* discrimination (Luyo-Acero et al., 2004; Kato et al., 2007; Yang et al., 2013), due to their high sequence conservation (Asato et al., 2009).

The ***Divergent region (DR)*** was initially described as a variable and presumably non-coding maxicircle segment (Maslov et al., 1984). It is composed of various repeat arrays that differ between *Leishmania* species. DR sequences include two types of long GC-rich repeats alternating with clusters of short AT-rich repeats, ranging in length from 10 Kb in L. *amazonensis* to 12 Kb in L. *major* (Flegontov et al., 2006).

Out of the several hundreds of guide RNA (gRNA) genes only a few are encoded on the maxicircle. These small RNAs play a role in the precise insertion and deletion of uridines in 12 out of 18 of maxicircle transcripts (Estévez and Simpson, 1999; Lukeš et al., 2005).

The sizes and positions of five intergenic (IG) regions (12S–9S, 9S-ND8, ND7-COIII, COIII-CyB and RPS12-ND5) in the *Leishmania* maxicircles are shown in Fig. 6.

2.1.2.2.1.2. kDNA minicircle

The kDNA minicircles have species-specific sizes ranging from approximately 0.8 to 1.0 Kb. Since these small DNA circles are present in thousands of copies per cell, they are ideal targets for highly sensitive detection of *Leishmania* from biological samples with low parasite numbers (Ceccarelli et al., 2014). Minicircles constitute >95% of the total kDNA mass, and their typically heterogenous sequences (there are about 10 different sequences classes and the number of minicircles in each class is variable) may lose diversity during continuous culturing (Barker, 1987; Lukeš et al., 2002). Each minicircle is composed of one to four 150–200 bp conserved regions containing the origins of replication for both strands, and a variable region encoding usually a single gRNA gene (Sturm and Simpson, 1991; Stuart et al., 2005). A map of the *Leishmania* minicircle is shown in Fig. 7, and a complete list of primers for the amplification of different minicircle regions is shown in Supplementary Information: Table SI–4.

The ***conserved minicircle*** region contains conserved sequence blocks (CSB–I, CSB-II and CSB-III) that are variable in length throughout the genus *Leishmania* (Ray, 1989). Hence, they are effective targets for PCR amplification of all minicircle classes. The conserved region can discriminate *Leishmania* only at sub-generic level (Lachaud et al., 2002). The ***variable region*** of minicircles is approximately 150 bp long, and can be used for accurate discrimination between *Leishmania* species and between strains (Smyth et al., 1992; Piarroux et al., 1993; Minodier et al., 1997; Morales et al., 2001; Salotra et al., 2001; Lachaud et al., 2002; Gangneux et al., 2003; Jirku et al., 2006).

In addition, primers entitled “Anonymous markers” are listed in Supplementary Information: Table SI–5. These primers have been used for the detection and identification of *Leishmania*, but their targets are currently not known.

2.2. Distinguishing between species complexes

2.2.1. Methods

None of the parasitological or non-PCR based methods outlined above are effective in distinguishing between *Leishmania* species complexes. Most PCR-assays based on the above described targets are genus-specific proving simply the presence or absence of a PCR-product (detection of the parasite); some can be used for parasite load quantification in clinical samples (Verma et al., 2010).

PCR Identification and discrimination of *Leishmania* species complexes and species can be done through different technical approaches. Amplified fragments from samples can be examined by electrophoresis for length differences, restriction site variation using restriction enzymes, sequencing or HRM curve analysis. However, the variability of many of the previously listed targets is too low that makes other methods as RFLP or sequencing useless and only some of these targets are ideal for differentiation and typing below the genus level. Another approach is the use of species-complex or species specific primers (*e.g.*, in multiplex PCR, nested PCR).

Nested and semi-nested PCR are non-quantitative versions of PCR intended to reduce non-specific binding. They involve two sets of primers used in two successive runs. The second set of primers amplifies a secondary target within the first product. This ensures that the product from the second PCR has little contamination from primer dimers, hairpins, and alternative primer targets (Akhavan et al., 2010). It can be used to discriminate at the species with the appropriate primers.

Multiplex PCR refers to the simultaneous amplification of several different DNA targets. It involves performing several separate PCR reactions together in one experiment. It uses multiple primer sets within a single PCR mixture to produce amplicons of varying sizes of specific DNA targets. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction tubes, and amplicon sizes should be sufficiently distinct to be visualized via gel electrophoresis. This method has been used for leishmaniasis diagnosis using different type of markers, such as multicopy SL RNAs (Harris et al., 1998; Jorquera et al., 2005) and kDNA minicircles (Pita-Pereira et al., 2008).

Real-time PCR (quantitative PCR) enables the reliable measurement of DNA products generated by monitoring the amplification of the target DNA molecule during each PCR cycle. It can be used quantitatively (quantitative real-time PCR) or semi-quantitatively, and these methods are important for certain leishmaniasis treatments (Paiva-Cavalcanti et al., 2010). Different internal controls (genes such as GAPDH, β-microglobulin, β-actin, 18S rRNA) are most often used as indicators of optimal nucleic acid extraction, quality of samples, quality of PCR, as well as PCR inhibition (Mortarino et al., 2004). Absolute quantification needs also the use of a DNA standard.

Sequencing The first-generation DNA sequencing technology was developed by Sanger et al. (1977) based on the selective incorporation of chain-terminating dideoxynulceotides and the first automatic sequencing machine (Applied Biosystems 370A) was produced in 1987. It is rapidly improving in quality, read length,

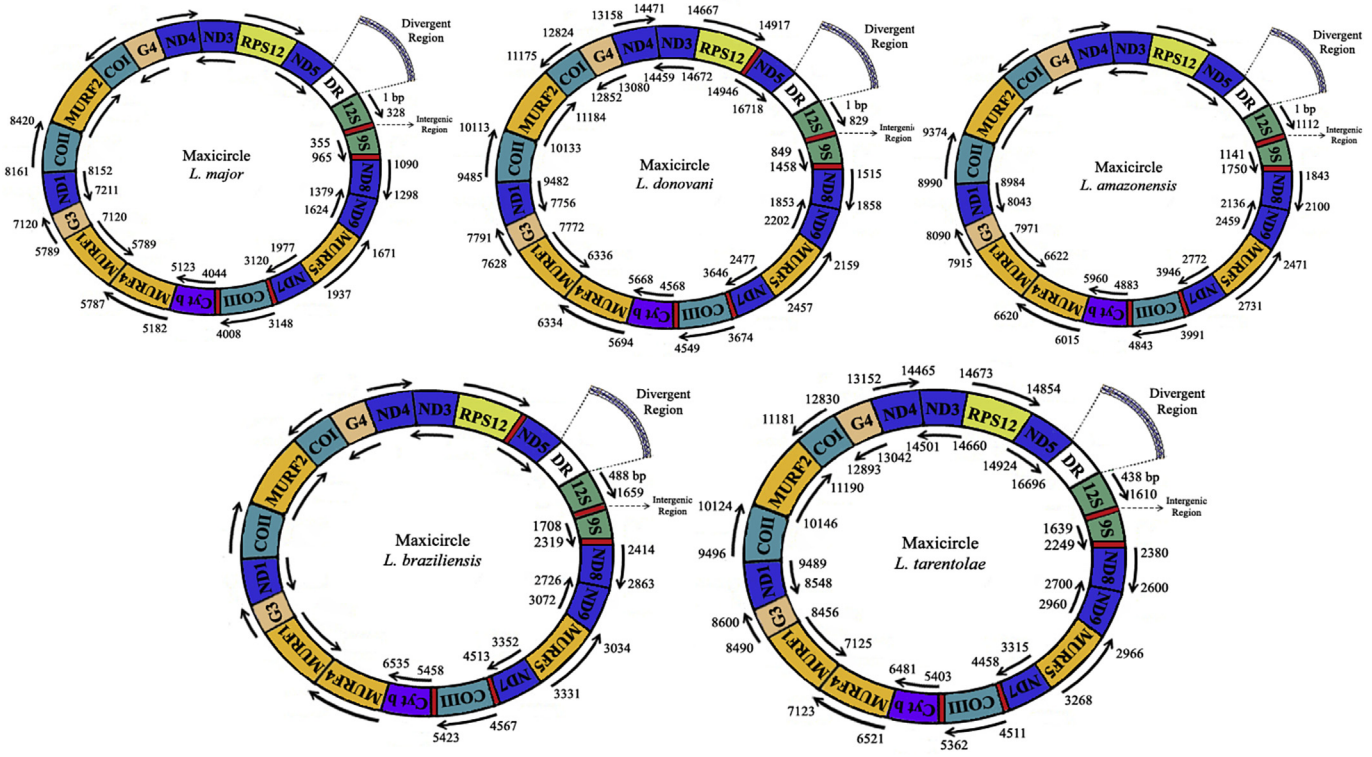

Fig. 6. kDNA maxicircle and its component genes and fragments lengths in 5 *Leishmania* species of *L. major*, *L. donovani*, *L. amazonensis*, *L. braziliensis* and *L. tarentolae*.

Fig. 7. kDNA minicircle of *Leishmania* sp. and its component fragments. CSB: Conserved Sequence Block.

speed and cost and it is widely used for the identification of *Leishmania* species (complexes) and phylogenetic studies. **High-throughput sequencing** has become essential in studies on genomics, epigenomics, and transcriptomics. It is therefore becoming more widely used to identify and discriminate different species of parasites including *Leishmania* (Cantacessi et al., 2015).

Multilocus sequence typing (MLST) is a method where multiple unlinked housekeeping genes (typically 4 to 6) are (simultaneously) amplified using PCR and subsequently analyzed by sequencing (Zemanová et al., 2007; Mauricio et al., 2006; Boité et al., 2012). MLST has extensive power to differentiate samples where the total number of distinct biomarkers is large. A complete list of MLST markers and their characteristics is given in the Supplementary Information: Table SI–6.

Randomly amplified polymorphic DNA (RAPD) is based on the PCR amplification of DNA using only one short primer that is arbitrarily defined and thus can be used for any organism without prior knowledge of the target sequences. RAPD can be used alone or in conjunction with other techniques to investigate genetic inter- and intra-specific diversity within *Leishmania* species and complexes (Toledo et al., 2002; Zemanová et al., 2004; Botilde et al., 2006). Nevertheless, its use is restricted due to its need for highly pure *Leishmania* DNA and precise standardisation of PCR conditions to guarantee specificity. It is also difficult to standardise and interpret the results of assays with poor reproducibility (Schönian et al., 1996; Eisenberger and Jaffe, 1999). A complete list of RAPD markers and their characteristics is in Supplementary Information: Table SI–7.

Amplified fragment length polymorphism (AFLP) is a PCR-based tool used in DNA fingerprinting of *Leishmania* species. It is based on the selective PCR amplification of restriction fragments of a total digest of genomic DNA, linked to an oligonucleotide adapter (Vos et al., 1995). The amplified fragments are separated and visualized on polyacrylamide gels, and scored as presence/absence polymorphisms. It is a powerful and high-throughput tool for marker identification and to investigate genetic variation in strains or closely related *Leishmania* species (Kumar et al., 2010) such as in the *L. braziliensis* complex (Odiwuor et al., 2011).

Multilocus microsatellite typing (MLMT) is based on the amplification of microsatellites (also called simple sequence repeats SSR or short tandem repeats – STR), which are tandem repeats of a simple 1–6 nucleotide motif. Microsatellites are present in coding (rarely) and non-coding regions allover the genome and they mutate at rates 5–6 orders of magnitude higher than the bulk

DNA. Microsatellites are neutral and co-dominant markers. Microsatellite length variation results from gain or loss of repeats, which can be detected after PCR amplification using specific primers flanking the locus. The sizes of the PCR fragments are determined by metaphor agarose gel or polyacrylamid gel electrophoresis or automated fragment analysis (use of florescence labelled primers). MLMT generally uses a set of 10–20 unlinked microsatellite loci and assumes a step-wise mutation model. Microsatellites have high discriminatory power within species complexes (Kuhls et al., 2007; Schönian et al., 2010). The primers are usually species or species-complex specific (as for the complexes *L. donovani, L. tropica, L. major*) and cannot be applied for comparison of species belonging to different species complexes. Regions flanking the microsatellites are not strongly conserved between related species of *Leishmania* and repeats polymorphic in one species can be absent or non-informative in other species (Jamjoom et al., 2004; Schwenkenbecher et al., 2006; Schönian et al., 2010). There are some acceptations of primers working also for different species complexes, *e.g.,* a standard set of primers developed for the subgenus *Viannia* (Oddone et al., 2009). In many studies standard sets of microsatellite primers specific for a certain species or species complex are used in order to achieve comparable results between different laboratories and to use a given data base for already studied strains. A complete list of MLMT markers and their characteristics is in Supplementary Information: Table SI–8.

PCR-ELISA is an immunological method to detect and quantify the PCR products directly after immobilization of biotinylated DNA on a microplate using three steps: amplification, immobilization, and detection. The gene of interest is amplified by PCR in the presence of digoxigenin-11-dUTP (DIG-dUTP). DIG-labelled PCR products bind to specific oligonucleotide probes labelled with biotin at their 5′ ends so that they can be immobilized on the microplate and their nucleic acids can be detected. PCR-ELISA is more sensitive than conventional PCR, has shorter analysis times and lower detection limits. Combined with its semi-quantitative nature, this method may be a powerful detection tool in medical assays, but not for discrimination between species (De Doncker et al., 2005a,b; Sue et al., 2014).

PCR-HRM measures changes in the fluorescence intensity of a DNA-intercalating dye during dissociation of double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA). It can detect dsDNA alternatives and discriminate different *Leishmania* species based on their composition, length, GC content and strand complementarity (Nasereddin and Jaffe, 2010; Hernández et al., 2014).

PCR-RFLP is a method that detects variation between the patterns of DNA fragments produced by restriction enzyme digestion visualized using gel electrophoresis. These RFLPs are caused by alternative nucleotides at the restriction sites, and were often used as markers on genetic linkage maps. Length variation and difference in the numbers and patterns of the fragments can be used for *Leishmania* species differentiation (Marfurt et al., 2003; Akhoundi et al., 2013), particularly when applied to ITS1 (Schönian et al., 2003) and mini-exon (Minodier et al., 1997) regions or the *hsp70* (Montalvo et al., 2012).

Loop-mediated isothermal amplification (LAMP) uses four different primers specifically designed to recognize six distinct regions at a target locus, such that amplification and detection of the dsDNA target can be completed in a single step at isothermal temperature (Notomi et al., 2000). The DNA polymerase used has strand displacement activity, so that there is no need for heat (to denature dsDNA to ssDNA for primer annealing and amplicon elongation). Each initial ssDNA product provides additional template for a chain-reaction using second inner and outer primers via a stem-loop intermediate structure (Fu et al., 2011). One inner primer then binds this loop section to allow production of a copy of the template ssDNA fragment, permitting the amplification process to swiftly amplify the target to a high copy number. This can be detected by eye as a white precipitate or as a yellow-green solution after the addition of SYBR green dye. This sensitive, simple, rapid and cost-effective method does not require special reagents or sophisticated equipment and may even be combined with a reverse-transcription step to allow the detection of RNA. LAMP can be more sensitive than conventional PCR and has been used for detection of *Leishmania* species (Khan et al., 2012; Ghasemian et al., 2014; Sriworarat et al., 2015). Its main limitations are the requirement of a non-extreme GC content, the risk of secondary DNA structures, and the limited suitable temperature range. In addition, the four primers have numerous design requirements, such as there should be no homology with host DNA, the 5′ ends of the outer primers should be 120 to 180 bases apart, the 5' ends of the inner primers should be 0 to 20 bases apart, and the loop regions should be 40 to 60 bases long.

Protein-based methods include **multilocus enzyme electrophoresis (MLEE),** which examines enzymes with similar or identical specificities, but different structures termed isoenzymes (Rioux et al., 1990). Isoenzymes are produced by multiples genes, are non-redundant, and are important for metabolic processes in various cellular compartments. Using a defined set of isoenzymes, a characteristic species- or strain-specific “mobility pattern” can be established. However, MLEE does not discriminate among populations due to homoplasy, low marker number or target gene heterozygosity causing more than one mobility pattern (Jamjoom et al., 2004). On the other hand, this method was found effective in detecting major inter- and within-complex hybridization events (see sections C2 and C3, respectively). MLEE is not recommended due to lack of discriminative power within some species and populations (*e.g.*, *L. infantum* MON-1), laborious parasite culturing, and requirement of specialised equipment (Schönian et al., 2010).

Matrix-assisted laser desorption ionization–time-of-flight (**MALDI-TOF**) mass spectrometry (MS) is a powerful tool for *Leishmania* species identification (Mouri et al., 2014). After ionization of the sample in a specific acidic solution, MALDI-TOF MS uses laser beams from (expensive) spectrometers to evaporate the sample towards the sensor, where the “time of flight” depends on the molecular weight of the ionized molecules. This protein spectral fingerprint of an isolate can be compared with a reference spectral database. This method is suitable only for cultured parasites; therefore it does not work with clinical samples.

2.2.2. Markers

MLST have allowed an efficient discrimination between *L. braziliensis, L. guyanensis, L. naiffi* and *L. lainsoni,* as well as within species complexes using four housekeeping genes: glucose-6-phosphate dehydrogenase (*g6pd*), 6-phosphogluconate dehydrogenase (*6pgd*), mannose phosphate isomerase (*mpi*) and isocitrate dehydrogenase (*icd*) (Boité et al., 2012). Similarly, MLST of seven genes was effective for examining diversity within complexes and species (El Baidouri et al., 2013). These genes encoded an elongation initiation factor 2 alpha subunit, spermidine synthase 1, a Zinc-binding dehydrogenase protein, a translation initiation factor alpha subunit, a nucleoside hydrolase protein, a hypothetical protein, and the largest subunit of RNAP II. Indeed, MLST of these genes (4677 concatenated bp in total), the SL RNA locus (176–397 bp) and *hsp70* (1245 bp) provided better resolution of inter-complex and intra-species variation than the rDNA ITS1 locus (257–302 bp), 7SL RNA gene (184–187 bp), or MLEE (Van der Auwera et al., 2014). Beside mentioned targets, one of important targets, used for *Leishmania* identification is based on characterization of the *Leishmania* telomeres named LCTAS (*Leishmania* conserved telomere-associated sequence) (Cantacessi

et al., 2015).

An assessment of several members of the genus *Leishmania* using PCR amplifications of different chromosomaland and mitochondrial markers showed that among them, ITS1 and ITS2 regions, Mini-exon/Spliced Leader (rDNA), *gp63*, *hsp70*, cpb, POLA, G6PD, 6PGDH, MPI, Histones, RPOIILS, NAGT, A2, EF-1α (proein coding gene), *cytb, COII* (kDNA maxicircle) and kDNA minicircle are informative and have a reasonable power to differentiate between species complexes (Harris et al., 1998; Momen and Cupolillo, 2000; Noyes et al., 2002; Marfurt et al., 2003; Schönian et al., 2003; Zhang et al., 2003; Kuhls et al., 2005; Mauricio et al., 2007; Waki et al., 2007; Hide and Bañuls, 2008; Cao et al., 2011; Leelayoova et al., 2013; Fraga et al., 2013; Yang et al., 2013) (Table 3).

A complete list of different markers and primers including chromosomal markers, MLST, RAPD and MLMT used for distinguishing between species complexes are summarized in Supplementary Information: Tables SI-1, 2, 6, 7 and 8.

2.3. Identifying species within complexes

2.3.1. Methods

Of the above methods, **MLST**, **MLMT** and **PCR-RFLP** are effective for examining variation within species complexes. AFLP has been used to examine *L. peruviana* (Dujardin et al., 1998) and central and South American L. *braziliensis* (Odiwuor et al., 2011), but may have over-estimated intraspecific diversity in comparison to MLMT-based studies.

MLMT has been effective for scrutinising subspecies diversity within species complexes. Paradigms for this include *L. donovani* lineages from Ethiopia Gelanew et al., 2014), *L. donovani* (*infantum*) in Tunisia (Chargui et al., 2013), France (Hide et al., 2013), Turkey (Gouzelou et al., 2012), *L. donovani* from across the Old World (Downing et al., 2012), and *L. infantum* from South America (Kuhls et al., 2007, 2011). MLMT- and MLST-based studies of *L. chagasi* in South America (Kuhls et al., 2007) and *L. archibaldi* in East Africa (El Baidouri et al., 2013), identified the former as *L. infantum,* and the latter as an indistinct entity within the *L. donovani* complex. MLMT has served as a powerful tool for the analysis of other *Leishmania* species, such as Asian and African L. *tropica* (Schwenkenbecher et al., 2006) and Iranian L. *major* (Tashakori et al., 2011). It has also been used to investigate *L. lainsoni* and *L. naiffi* (Kuhls et al., 2013), *L. braziliensis* and *L. peruviana* (Nolder et al., 2007) and *L. braziliensis* and *L. guyanensis* (Rougeron et al., 2010).

2.3.2. Markers

Combined MLST of seven genes listed above and the SL RNA locus was far superior in its discriminant power than the use of single genes, illustrating the importance of a large number of biomarkers (Boité et al., 2012; Van der Auwera et al., 2014). Deeper resolution has been provided by MLST-style analysis of 49 genes across 215,644 genome-wide SNPs (Harkins et al., 2016). An additional advantage of the multi-locus approach is that reconstructing the genealogical history of a species requires vertically inherited biomarkers from many neutrally evolving DNA sites, so the variation at a single gene such as *hsp70* reflects one ancestral signal, whereas independent regions indicate other patterns. If many regions are targeted in parallel, phylogenetic inconsistencies can be identified by independent evaluations of gene genealogies to differentiate loci with neutral vs non-neutral patterns. Moreover, among all markers listed in section B1, it appears that only *ITS1* region, *Mini-exon/Spliced Leader* (rDNA), *gp63, hsp70, cpb, POLA, G6PD, 6PGDH, MPI, Histones, RPOIILS, NAGT, A2, EF-1α* (proein coding gene), *cytb, COII* (kDNA maxicircle) and kDNA minicircle have the capacity to differentiate within complexes (Harris et al., 1998; Momen and Cupolillo, 2000; Noyes et al., 2002; Marfurt et al., 2003; Schönian et al., 2003; Zhang et al., 2003; Kuhls et al., 2005; Mauricio et al., 2007; Waki et al., 2007; Hide and Bañuls, 2008; Cao et al., 2011; Leelayoova et al., 2013; Fraga et al., 2013; Yang et al., 2013) (Table 3).

2.4. Variation within foci and populations

2.4.1. Methods

A number of studies have examined genetic variation within or between infection foci or species categories. MLST of a large number of genes could in principle be effective (Boité et al., 2012), but, this was supported by genome-wide Indian L. *donovani* diversity (Downing et al., 2011). Differences between L. *braziliensis* foci from Bolivia and Peru sampled at two time intervals (Rougeron et al., 2009), the diversity of *L. major, L. tropica, L. donovani* and *L. infantum* in the Old World (Bulle et al., 2002; Ochsenreither et al., 2006; Schwenkenbecher et al., 2006; Kuhls et al., 2007; Al-Jawabreh et al., 2008) and in Tunisia sampled in years 1991–1992 and 2008–2012 (Harrabi et al., 2015), could be effectively assessed using MLMT. Nevertheless, for certain foci the homogeneity of the marker alleles has limited inferences about population structure (Downing et al., 2012), so MLMT would appear more suited for inter- and intra-complex discrimination (Schönian et al., 2010).

2.4.2. Markers

A **single nucleotide polymorphism (SNP)** is a DNA position with more than one allele (typically two). These are distinct from indels (insertion-deletions) where a single base is added or removed, causing a change in total sequence length. Assuming disomy with a known reference allele, a SNP can either be homozygous if both bases are changed to the alternative allele, or heterozygous if only one is changed. SNPs at protein-coding regions either alter the corresponding amino acid for the particular codon (a nonsynonymous SNP) or encode the same amino acid (a synonymous SNP). Purifying selection reduces change at coding regions so diversity is higher at non-coding regions, and correspondingly reduced for nonsynonymous changes (Downing et al., 2011). Screening with a large number of SNPs permits high discriminatory power, as evidenced by their use in typing different species, *e.g.*, *L. donovani* and *L. braziliensis* (Downing et al., 2011; Vanaerschot et al., 2012; Zhang et al., 2014; Imamura et al., 2016). A complete list of SNP markers and their characteristics is shown in Supplementary Information: Table SI–9.

SNP genotyping is a powerful method for discriminating at any level: an appropriate design can yield information at the level of subgenus, species complex, species and within populations (Mardis, 2011). SNP genotyping is typically completed in a high-throughput manner across extensive specimen panels and for a large number of biomarkers, providing this amplification approach with power superior to MLST, MLMT or other approaches, as shown in *L. donovani* (Downing et al., 2012) and other species (Tokarska et al., 2009). This approach requires known SNPs to create an array that can be assessed simultaneously using specific primers, and so is more effective for investigating single foci and populations because higher genetic differentiation is associated with more structural changes that would eliminate effective amplification. Prior selection of the SNP panel can bias results due to rare alleles (Clark et al., 2005), non-neutral evolution (Rosenberg et al., 2003) or a lack of local conservation at the target. Moreover, SNP genotyping can only assess point mutations, and thus cannot decipher alterations in chromosome copy number, episome or other structural variants. SNP genotyping is also limited by the target's GC content, variation within homologs, and the proximity of markers because of SNP-specific amplification.

In addition to above mentioned approaches, kDNA minicircle,

Table 3
Characteristics of DNA genes [or loci] used for Leishmania genus detection and their applicability for within- [or intra-] genus discrimination and parasite quantification.

DNA target			DNA locus	Number of copies	Gene conservation	Detection	Identification	Discrimination level*					Quantification**
				Single/Multiple	Conserved/Variable			SL	SG	C	S	F	
CHROMOSOMAL DNA	RIBOSOMAL DNA (rDNA)		I8S	M	C	Y	N						Y
			5.8S	M	C	Y	N						N
			28S (24Sα)	M	C	Y	N						N
			28S (24Sβ)	M	C	Y	N						N
			28S (Ɛ)	M	C	Y	N						Y
			28S (Ƴ)	M	C	Y	N						N
			28S (ζ)	M	C	Y	N						N
			28S (δ)	M	C	Y	N						N
			5S	M	C	Y	N						N
			ITS1	M	C	Y	Y	+	+	+	+		Y
			ITS2	M	C	Y	Y	+	+	+	+		N
			IGS	M	C	Y	N						N
			Mini-exon (Spliced Leader)	M	C/V	Y	Y	+	+	+	+	+	Y
			ETS	M	C	Y	N						N
			NTS	M	C	Y	N						N
	PROTEIN CODING GENES		MSP or gp63	M	C	Y	Y	+	+	+	+	+	N
			hsp20, 23, 70	S	C	Y	Y	+	+	+	+	+	N
			cpb	M	C	Y	Y	+	+	+	+		Y
			HASPB	S	C	Y	N						N
			POLA	S	C	Y	Y	+	+	+	+		N
			NAGT	S	C	Y	Y	+	+	+	+		N
			SHERP	S	C	Y	N						N
			MAP kinase	S	C	Y	N						N
			A2	S (CL)/M (VL)	C	Y	Y	+	+	+	+		N
			EF	S	C	Y	Y	+	+	+	+		N
			MIF	S	C	Y	N						N
			G6PD	S	C	Y	Y	+	+	+	+		Y
			6PGDH	S	C	Y	Y	+	+	+	+		N
			Histone	M	C	Y	N						N
			Tubulin	M	C	Y	N						Y
			MPI	S	C	Y	Y	+	+	+	+		N
			LPG	S	V	Y	N						N
			RPOIILS	S	C	Y	Y	+	+	+	+		N
NON CHROMOSOMAL DNA	KINETOPLAST DNA (MITOCHONDRIAL DNA)	Maxicircle	12S	M	C	Y	N						N
			9S	M	C	Y	N						N
			COI, COII, COIII	M	C	Y	Y	+	+	+	+		N
			MURF1, MURF2, MURF4, MURF5	M	C	Y	N						N
			ND1, ND3, ND4, ND7, ND8 and ND9	M	C	Y	N						N
			CYT B	M	C	Y	Y	+	+	+	+		Y
			RPS12	M	C	Y	N						N
			G3, G4	M	C	Y	N						N
			DR maxicircle	M	V	Y	N						N
			gRNA maxicircle	M	C	Y	N						N
			IG maxicircle	M	C	Y	N						N
		Minicircle	CSB-1, CSB-2, CSB-3	M	C	Y	Y	+	+	+	+	+	Y
			Variable region	M	V	Y	Y						Y
			gRNA minicircle	M	C	Y	N						N

C: Conserved, M: Multiple, N: No, S: Single, V: Variable, Y: Yes. *: Different levels of discrimination; SL: Section level, SG: Subgenus, C: Complex, S: Species and F: Foci. **: It is based on the results published in the literature.

hsp70, *gp63* and Mini-exon/Spliced Leader markers are used to discriminate at subspecies or strain level (Harris et al., 1998; Marfurt et al., 2003; Gangneux et al., 2003; Jirku et al., 2006; Mauricio et al., 2007; Fraga et al., 2013) (Table 3).

The complete list of MLST, MLMT, SNP markers used for distinguishing variation within foci and populations are in Supplementary Information: Tables SI-6, 8 and 9.

3. Markers available for diagnosis

The ability to distinguish between *Leishmania* species is a prerequisite for accurate diagnosis of the disease, and has an important consequence for establishing the correct treatment and implementing control measures. This is especially true in areas where several *Leishmania* species co-exist (Fig. 2).

3.1. Classification scheme

A wide range of molecular tools and markers have been exploited to address key epidemiological and clinical questions related to leishmaniasis. The choice of the most suitable ones depends on the goal of the study. Common characteristics include: i) stability during *in vitro* or *in vivo* passages, ii) reproducibility of analyses between laboratories, iii) comparability of methods, iv) direct testing on clinical samples and v) simplicity and cost-effectiveness.

While all of the above approaches provide a multitude of valid characters for diagnosing leishmaniasis, ideally they should differentiate *Leishmania* species, establish parasite load in mammalian hosts and insect vectors, and monitor the efficacy of drug treatment (Shaw et al., 2015). They should also identify sample origins, association with clinical pathologies, relationship with new non-human reservoirs, and new phlebotomine and/or arthropod vectors. In addition, discrimination within foci is required in order to assess population structure, geographic differentiation, spread of new mutations, transmission patterns between cases, intra-host evolution, and the origin of relapses due to recrudescence or *Leishmania* re-infection (Mathis and Deplazes, 1995; Bhattacharyya et al., 1996).

Some factors are essential for validation of suitable PCR-based methods: i) the target sequence (size of expected amplicon, genetic stability, copy number in the genome, genus- and species-specificity); ii) methodological efficacy (specificity, sensitivity, precision, accuracy and reproducibility); and iii) clinical-diagnostic sensitivity and specificity (testing clinical samples, patients with similar symptoms but different etiology).

Appropriate choice of target genes and primers is essential. It is worth noting that for the investigations of *Leishmania*, there are substantial differences between multi- or single-copy gene-based studies in terms of the sensitivity and efficiency (Bossolasco et al., 2003; Talmi-Frank et al., 2010). Multi-copy genes are often preferred because they enhance the sensitivity of the detection and are advantageous for detection, but may produce off-target amplification. Additionally, variation in the target gene copy number between and within species (Weirather et al., 2011) can be challenging and confounding for quantification using standard curves. Thus, multi-copy genes such as kDNA markers are preferable for investigations that focuse on *Leishmania* detection, whereas single-copy genes are more suitable for phylogenetic studies. In the same way, studies aiming to discriminate between *Leishmania* species are more effective with highly polymorphic genes, such as some mitochondrial protein-coding genes. The characteristics of different genes applied for detection, identification and quantification of *Leishmania* species, as well as their capacity for discrimination at the levels of genus, section, subgenus, species and population level are summarized in Table 3.

To carry out an exhaustive analysis of DNA-based molecular markers used for the detection and identification of all *Leishmania* species known to infect humans, we screened the available literature and compiled a list of all known markers and primers, including kDNA, rDNA, protein-coding genes, RAPD, MLMT, MLST, and SNPs, as well as anonymous markers and probes. This includes more than 1200 primer pairs with their characteristics: primer name, sequence, probe, amplified fragment length, corresponding gene length, TM, GC richness, PCR program, chromosome location, dye label, repeat array, allele range, number of alleles, mono/polymorphism, single/multiple copy, conservation/variability of a given gene, and reference to the genome assemblies for 21 *Leishmania* species along with their WHO IDs. Molecular markers and primers suitable for detection and typing have been categorized as follows:

i) The sets of markers used specifically for *Leishmania* typing such as MLST, RAPD, MLMT and SNP were clustered separately, according to species (Supplementary Information: Tables SI-6, 7, 8 and 9).
ii) The kDNA, rDNA and protein-coding genes were separated based on their components, and subsequently categorized into two sections: a) markers for detection (with the same amplified fragment length in all species) and b) markers for identification (underlined with different amplified fragment lengths for individual species). In each section, the markers were subdivided again into two sub-sections according to their use in the Old World and New World (or both). (Supplementary Information: Tables SI-1, 2, 3, 4 and 5). The proposed molecular markers used for discrimination at different hierarchical levels of genus, section, subgenus, species and population are listed in Table 3. A complete list of species with published or assembled genomes is in Table 4.

Descriptive statistics on the different molecular markers and primers used for leishmaniasis diagnosis and molecular *Leishmania* identification are given in Figs. 8 and 9. In these figures, the difference in use of various types of genes and their interfragments as well as their application to identify different *Leishmania* species (based on the literature) are shown in details.

3.2. Inter-complex hybridization

Genetic exchange between *Leishmania* species has most frequently been detected for those with higher synteny and fewer differences in gene complement, such as the hybridization of *L. major* with *L. infantum* in the sandfly *Lutzomyia longipalpis* (Romano et al., 2014). Assuming that mixing between distinct species complexes is rare, resolving the hybrids arising from known species is not challenging because large numbers of mutations will have accumulated with independent patterns of genetic drift over a substantial period.

In an ideal system of mating between two euploid samples at Hardy-Weinberg equilibrium and no additional mating or parasexuality (Rougeron et al., 2015), mutations detected in the progeny with respect to either parent (or ancestral lineage) should be heterozygous. These alleles will appear similar to both ancestral lineages, so a large number of biomarkers would be required to detect hybrids (Schönian et al., 2010). This is illustrated by MLST approaches that use four housekeeping genes (*g6pd*, *6pgd*, *mpi*, *icd*) on a collection of 96 isolates of *L. braziliensis*, *L. guyanensis*, *L. naiffi* and *L. lainsoni* that clearly discriminate both among and within species

Table 4
Leishmania species with published or assembled genomes arranged according to taxonomic group.

Subgenus	Complex	(Sub)species	WHO ID (if genome-sequenced)	Publication, or Consortium and NCBI Assembly/SRA ID
Leishmania	*major*	*arabica*	MPSA/SA/1983/JISH220	Kinetoplastid Genomes Consortium, GCA_000410695, see also SRP021540
		gerbilli	MRHO/CN/1960/GERBILLI	Kinetoplastid Genomes Consortium, GCA_000443025, see also SRP021543
		major	MHOM/IL/1981/Friedlin	Ivens et al., 2005
		major	MHOM/SN/1974/SD	Kinetoplastid Genomes Consortium, GCA_000250755
		turanica	MMEL/SU/1979/MEL	Kinetoplastid Genomes Consortium, GCA_000441995, see also SRP021547
	tropica	*aethiopica*	MHOM/ET/1972/L100	Kinetoplastid Genomes Consortium, GCA_000444285
		tropica	MHOM/IL/1990/LRC-L590	Kinetoplastid Genomes Consortium, GCA_000410715 see also SRP015935
	donovani	*donovani*	MHOM/NP/2003/BPK282/0cl4	Downing et al., 2011
		infantum	MCAN/ES/1998/LLM-87	Peacock et al., 2007
	mexicana	*amazonensis*	MHOM/BR/1973/M2269	Real et al., 2013
		mexicana	MHOM/GT/2001/U1103cl25	Rogers et al., 2011
Sauroleishmania	*tarentolae*	*adleri*	MARV/ET/1975/HO174	Coughlan et al., 2017
		tarentolae	RTAR/DZ/1939/Parrot-TarII	Raymond et al., 2012
Viannia	*braziliensis*	*braziliensis*	MHOM/BR/1975/M2903	Kinetoplastid Genomes Consortium, GCA_000340355
		braziliensis	MHOM/BR/1975/M2904	Peacock et al., 2007
		naiffi	MCAN/CO/1986/CL223	Coughlan et al., 2017, ERX180449
		panamensis/guyanensis	MHOM/PA/1994/PSC-1	Llanes et al., 2015
		panamensis/guyanensis	MHOM/COL/1981/L13	Kinetoplastid Genomes Consortium, GCA_000340495
		guyanensis	MCAN/CO/1985/CL085	Coughlan et al., 2017, ERX180458
Mundinia	*martiniquensis*		MHOM/MQ/1992/MAR1	Kinetoplastid Genomes Consortium, GCA_000409445
	enriettii		MCAV/BR/1995/CUR3	Kinetoplastid Genomes Consortium, GCA_000410755

complexes (Boité et al., 2012). Similar approaches have been insightful for the Old World samples, where hybridization has been found between *L. donovani* and *L. aethiopica* in Ethiopia (Odiwuor et al., 2011). MLEE is adequate for detecting inter-complex hybridization, as indicated by the detection of the *L. infantum/L. major* hybrids isolated in Portugal (Ravel et al., 2006) and also those of *L. major/L. arabica* from Saudi Arabia (Evans, 1987; Kelly et al., 1991), but it has far less resolution power when compared to MLST.

3.3. Within-complex hybridization

Mating within a species complex can be assessed using similar approaches to those for inter-complex events, again based on the assumption that they are rare. Previous work demonstrates that a lack of recent gene flow in the *L. donovani* complex was sufficient to identify specimens from Turkey with mixed ancestry: one parent was related to *L. infantum,* and the genetic origin of the other was enigmatic (Rogers et al., 2014). Although MLEE is not recommended for assessing within-complex variation (Schönian et al., 2010; Van der Auwera et al., 2014), it has shown mixing between *L. braziliensis* and *L. panamensis* (Belli et al., 1994), *L. braziliensis* with *L. peruviana* (in combination with RAPD data) (Dujardin et al., 1995) or with *L. guyanensis* (including *L. panamensis*). These patterns are supported by MLMT of *L. braziliensis* and *L. peruviana* (Nolder et al., 2007).

3.4. Applicability to mating within subspecies

Instances where gene flow is a continuous or frequent requires different approaches based on a large sample size and numerous biomarkers to resolve multiple events of genetic exchange (Rougeron et al., 2015). Methods for inferring evolution over long periods of time are underpowered for evaluating change during shorter intervals, and *vice versa*. The assumption of independent genetic drift between samples no longer applies and thus only minorities of biomarkers are informative. As a result, only multilocus screening of large numbers of biomarkers has sufficient power to detect and classify unusual or mixed infections (Rougeron et al., 2009). This is illustrated by admixture between *L. donovani* lineages (*L. donovani* and *L. infantum*) in Turkey and Cyprus originally detected with PCR-RFLP (Svobodová et al., 2009) and MLMT (Gouzelou et al., 2013), subsequently verified and quantified by genome-sequencing that allowed the inference of the two genetic backgrounds of the original cross (Rogers et al., 2014). This approach is essential for accuracy because natural genetic diversity within individual foci may be low, yielding potential hybrids with few detectable variable loci (Rougeron et al., 2009; Imamura et al., 2016). Screening with extended panels of variation facilitates tracing the evolution and epidemiology underlying clinical infections, differentiating transmission patterns between hosts, pinpointing origins of novel variants, discovering mutations associated with drug tolerance, distinguishing between relapse and reinfection, and inferring the original evolutionary sources of new cases.

4. High-throughput screening and an update of available genomes

Systems-wide high-throughput genomic, transcriptomic, proteomic and metabolomic screening represents an opportunity to more comprehensively integrate taxonomic, evolutionary and clinical results (Cantacessi et al., 2015) and can provide insights into gene regulation (Haydock et al., 2015). Although inter-species differences exist in chromosomal and gene structure, it is possible to resolve this at an elementary level on the basis of homology, inference of ancestral states and phylogenetics. More advanced approaches based on DNA read-mapping can deduce changes in aneuploidy, a universal property of all these species (Mannaert et al., 2012). Similarly, inference of proteins expressed in the host and vector can make use of high-throughput genome data to improve life cycle stage specificity of protein-based diagnostics (Nirujogi et al., 2014).

Our understanding of the genomes of *Leishmania* spp. is improving (Table 4). The genomes of *L. major* MHOM/IL/1981/Friedlin (Ivens et al., 2005), *L. infantum* MCAN/ES/1998/LLM-87 (Peacock et al., 2007), *L. donovani* MHOM/NP/2003/BPK282/0cl4

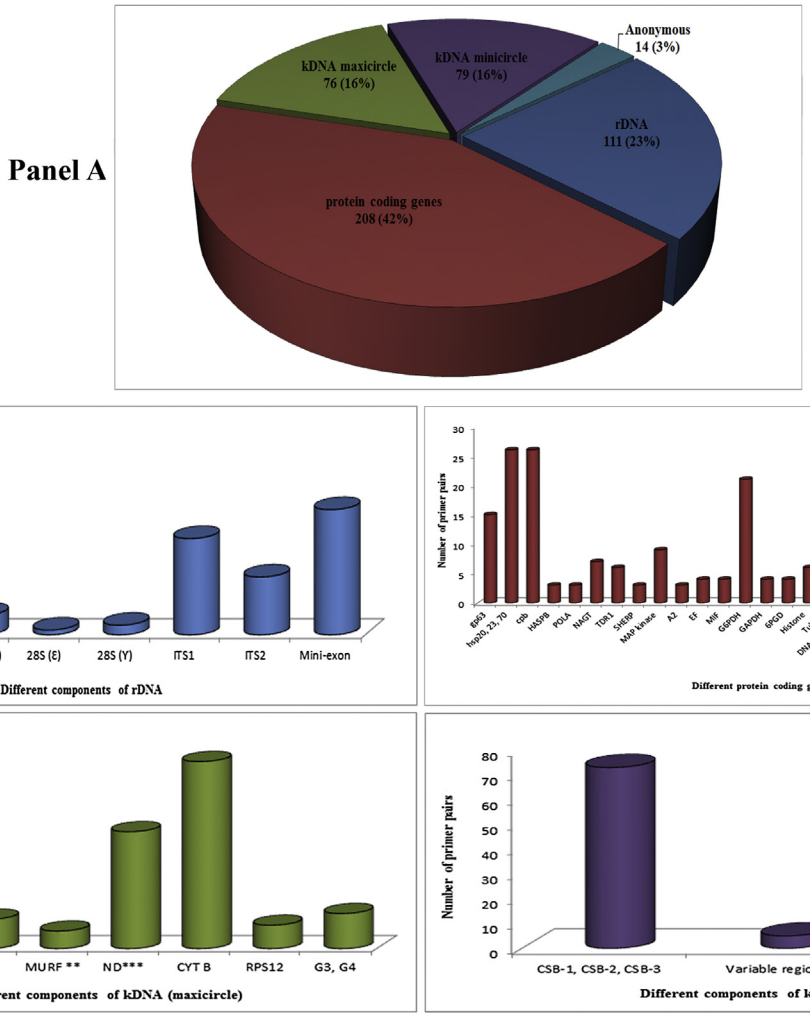

Fig. 8. Panel A. Graphic representation showing the proportion of the various molecular markers (or genes) currently identified for molecular diagnosis or typing, Panel B. Number of primer pairs for each type of marker (or gene)-and/or their components-used for detection and identification of 21 human *Leishmania* species. *: COI, COII, COIII; **: MURF1, MURF4; ***:ND1, ND3, ND4, ND5, ND7, ND8 and ND9.

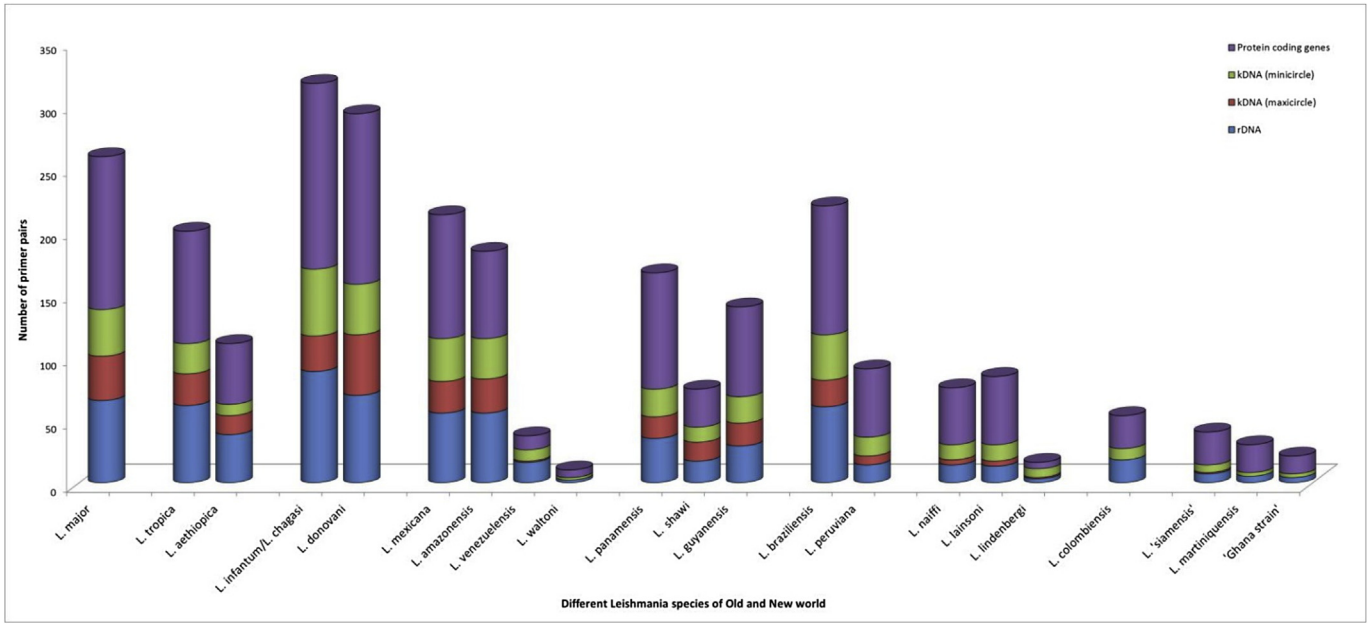

Fig. 9. Number of primer pairs of different molecular markers (genes), used for detection and identification of 21 *Leishmania* species obligatory pathogenic in humans.

(Downing et al., 2011), *L. mexicana* MHOM/GT/2001/U1103cl25 (Rogers et al., 2011) and *L. amazonesis* MHOM/BR/1973/M2269 (Real et al., 2013) have been assembled using a combination of long and short DNA read sequencing. Genomes of L. *tropica* MHOM/IL/1990/LRC-L590 (Kinetoplastid Genomes Consortium, KGC) and *L. aethiopica* MHOM/ET/1972/L100 (KGC) will represent a distinct complex once released. Assemblies of the relatives of *L. major* (Harkins et al., 2016), include *L. arabica* (the *L. major* complex) LEM1108 aka MPSA/SA/1983/JISH220 (KGC), *L. turanica* LEM423 aka MMEL/SU/1979/MEL (KGC), *L. gerbilli* LEM452 aka MRHO/CN/1960/GERBILLI (KGC), and SD-75.1 aka MHOM/SN/1974/SD (KGC). Other studies have assessed variation within three of these complexes using short read reference mapping of *L. major* LV39 aka MRHO/SU/1959/P and *L. mexicana* MNYC/BZ/1962/M379 (Rogers et al., 2011) and numerous *L. donovani* isolates (Imamura et al., 2016). Alignment-based approaches have been used for *L. pifanoi* MHOM/VE/1960/Ltrod, *L. tropica* MHOM/SU/60/LRC-L39 and *L. aethiopica* MHOM/ET/1972/L100 (Harkins et al., 2016).

For the subgenus *Viannia,* the genomes of only *L. braziliensis* MHOM/BR/1975/M2904 (Peacock et al., 2007) and *L. panamensis/guyanensis* MHOM/PA/1994/PSC-1 (Llanes et al., 2015) have been published, although this small group will be enlarged by the genomes of *L. guyanensis* MCAN/CO/1985/CL085 and the related *L. naiffi* MCAN/CO/1986/CL223 (Coughlan et al., 2017). The genomes of *L. braziliensis* MHOM/BR/75/M2903 (KGC) and *L. panamensis* MHOM/COL/1981/L13 (KGC) will be informative but are not yet available. Comparative power within the *L. braziliensis* complex has been improved by sequence data of *L. peruviana* LEM1537 aka MHOM/PE/1984/LC39 and PAB-4 377 (Valdivia et al., 2015), and by comparative alignments of *L. lainsoni* MHOM/BR/1981/M6426, *L. guyanesis* MHOM/BR/75/M4147, *L. naiffi* MDAS/BR/70/M5533, *L. panamensis/guyanensis* MHOM/PA/1974/WR120, and *L. shawi/guyanensis* MCEB/BR/1984/M8408 (Harkins et al., 2016).

For the subgenus *Sauroleishmania*, the first genome sequenced was for *L. tarentolae* RTAR/DZ/1939/Parrot-TarII (Raymond et al., 2012) to which a second typed as *L. adleri* MARV/ET/1975/HO174 (Coughlan et al., 2017) was added. This pair has been compared with *L. adleri* RLAT/KE/1957/SKINK-7 (Harkins et al., 2016), highlighting the additional chromosomes present in certain *Sauroleishmania* isolates.

Genomes related to the subgenus *Mundinia* are *L. martiniquensis* LEM2494 aka MHOM/MQ/1992/MAR1 (Mouri et al., 2014), *L. enriettii* LEM3045 aka MCAV/BR/1995/CUR3 (KGC), *L. enriettii* MCAV/BR/1945/L88 (Harkins et al., 2016), *L.* 'siamensis' and *L. macropodum* (Barratt et al., 2017) isolated from a kangaroo in Australia (Rose et al., 2004).

Also related but distinct is a variety of *Paraleishmania* species likely including the assembly of *Endotrypanum monterogeii* LV88 (KGC, GCA_000333855), which may be more related to the *Paraleishmania* than *Euleishmania,* as determined on the basis of the data available for *Endotrypanum schaudinni* MCHO/BR/80/M6159, and genus *Porcisia* including *L. deanei* MCOE/BR/1978/M5088 and *L. hertigi* MCOE/PA/2000/M4051 (Harkins et al., 2016). *L. colombiensis, L. equatorensis* and *L. herreri* remain unexamined.

The next most closely related genomes are *Crithidia fasciculata* C1 (KGC, GCA_000331325), and then *Leptomonas seymouri* (Kraeva et al., 2015) and *Leptomonas pyrrhocoris* (Flegontov et al., 2016). Additional relevant outgroup genomes for comparison primarily belong to the more distantly related *Trypanosoma brucei* TREU927 (Berriman et al., 2005), Lister 427 (Becker et al., 2004), *T. gambiense* DAL 972 (Jackson et al., 2010), *T. grayi* ANR4 (Kelly et al., 2014), *T. evansi* strain STIB 805 (Carnes et al., 2015), *T. congolense* IL3000 (Jackson et al., 2012), *T. vivax* Y486 (Jackson et al., 2013), and *T. cruzi* strains Tula cl2 (Hamilton et al., 2011), B7 (Franzén et al., 2012), Dm28c (Grisard et al., 2014), Sylvio X10/1 (TcI) (Franzén et al., 2011), Esmeraldo (TcII), non-Esmeraldo (TcIII) and CL Brenner (TcVI) (El-Sayed et al., 2005; Weatherly et al., 2009). Furthermore, for comparative analyses with more distantly related flagellates *Paratrypanosoma confusum* (Flegontov et al., 2013) is an obvious choice.

5. Available molecular approaches and perspectives

A crucial point in the laboratory diagnosis is etiological agent detection and species identification. Despite the great progress in diagnostic techniques, there is a gap between the scientific advances and the diagnostics process of *Leishmania* infections in the endemic areas. Thus, the real challenge for the researchers and clinicians has concentrated to fill this distance. Historically, the diagnosis of leishmaniasis has been confirmed by isolating, visualizing, and culturing the parasite from infected tissue. Routine diagnosis was usually according to the clinical and epidemiological parameters but definitive diagnosis was based on the parasitological observation of *Leishmania* in direct smear, culture or animal inoculation. After long time use of classical parasitological methods, the advent of the PCR method provided a powerful diagnostic approach for application of molecular biology techniques in parasite identification. Various molecular approaches mainly based on DNA-based techniques have been developed for detection and identification of parasites. Simultaneously, a number of species-specific probes have been developed for *Leishmania* identification in many different samples.

Applying molecular applications depends mainly on several criteria including the goal of study, availability of methods in-use, sensibility/specificity, technical skills requirement as well as the expense. In the context of leishmaniasis diagnosis, several goals are crucial. These objectives can be classified in two main categories: i) Diagnosis (*e.g.*, detection, identification, discrimination and quantification) and ii) Typing (*e.g.*, fingerprinting). Therefore, based on each goal, the appropriate molecular methods and markers are needed.

Several detection systems based on PCR methodology have been developed and are actually available for leishmaniasis diagnosis. Of these laboratory methods, PCR is still considered suitable for diagnosis. Among different molecular markers, repetitive sequences such as kinetoplast minicircle DNA, ribosomal RNA genes, miniexon-derived RNA genes or genomic repeats are considered as the best targets with maximum sensitivity for PCR (Schallig and Oskam, 2002). A descriptive diagram for diagnosis of leishmaniasis is proposed in Fig. 10.

Despite major advances in diagnostic techniques during last decade, there is no specific gold standard for detection and diagnosis of *Leishmania* infections. At present, there is no single method for diagnosing all pathogenic *Leishmania* species, nor for use in the field in endemic regions. The chosen method often depends upon the application. Due to the lack of this standardisation, the main goal was the development of a reliable diagnosis approach negating these defects.

Beside these molecular techniques, the complete sequencing of several *Leishmania* genomes has opened a new era in leishmaniasis diagnosis (Van der Auwera et al., 2014). These genomes permit the investigation of many additional biomarkers for diagnosis, which will ultimately yield more effective diagnostics. Several studies have examined different *Leishmania* species with high-throughput technologies. Ongoing whole-genome sequencing and SNP analysis as well as further analysis by MLST and MLMT will contribute to further improvement of the identification of different *Leishmania* species.

Despite the introduction of novel techniques and

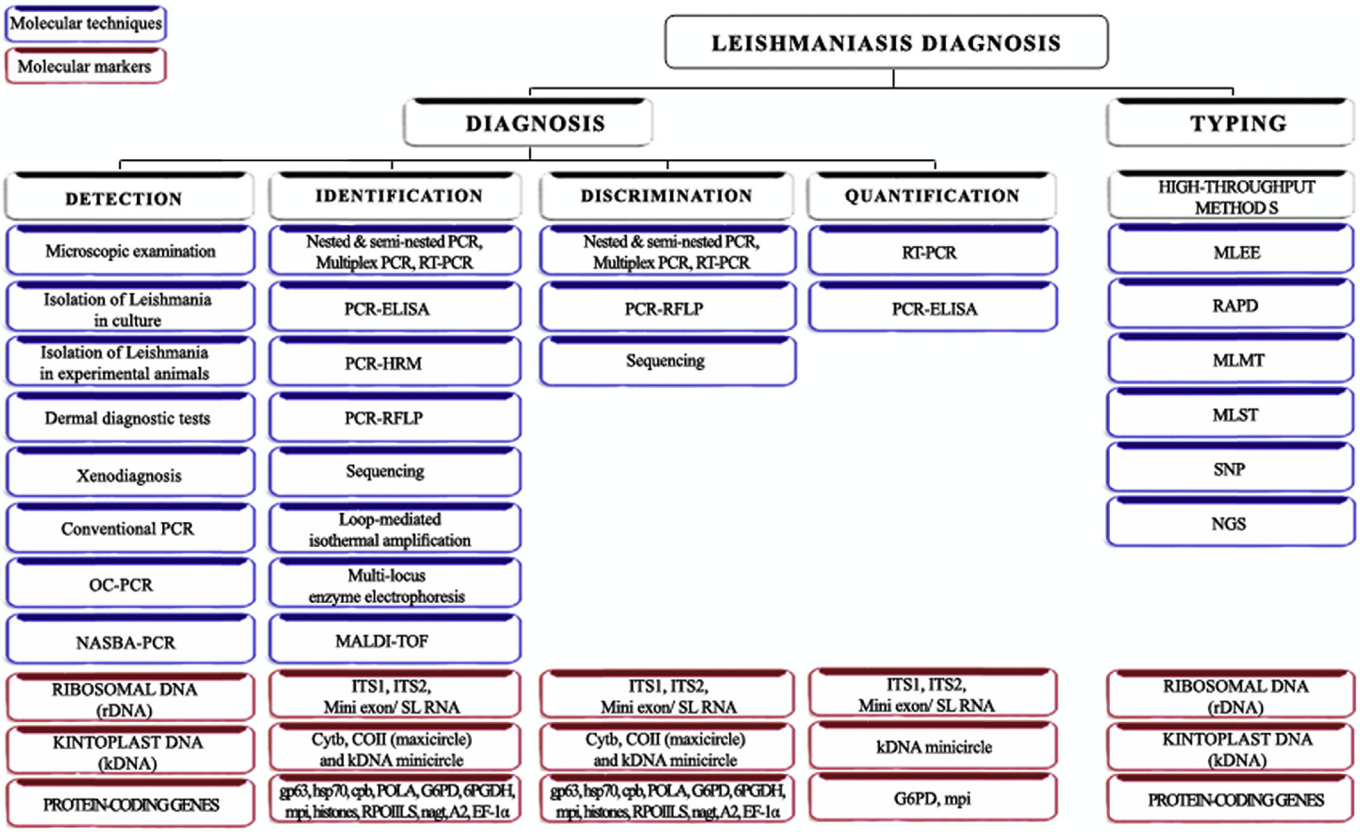

Fig. 10. Molecular markers and tools proposed for different goals of leishmaniasis diagnosis.

improvements to existing ones, there is still a serious need for development of more precise, rapid, cheap, sensitive and specific diagnostic tests which can be used not only in health centers but also in endemic regions. In addition, it is necessary to simplify the laboratory diagnostic tests interpreted easily with high sensitivity and specificity. Therefore, more effort will be required to improve molecular methodologies for diagnosing leishmaniasis.

6. Conclusion

The detection and identification of *Leishmania* species is essential for accurate, rapid and sensitive diagnosis of leishmaniasis, which has a major influence on effective treatment and control measures (Medley et al., 2015). Therefore, knowledge about different methodologies and their targets is crucial. Our goal was to propose the update classification of *Leishmania* species and their synonymies. Our global map highlights the geography of known endemic *Leishmania* species pathogenic to humans. We presented a complete list of molecular techniques currently in use, and to compare and discuss their advantages and limitations. Moreover, we have assembled a comprehensive list of DNA-based molecular markers and categorized them according to their application in detection, identification, discrimination and quantification of *Leishmania* parasites. Our next objective was to sketch chromosomal locations of diagnostic genes. We have compiled a list of over 1200 primer pairs with their characteristics, and suggest optimal primers and probes for detection and typing of the 21 known pathogenic species. Finally, we highlight the sensitivity and specificity of different markers to detect parasites at different taxonomic levels (genus, subgenus, species complex, species and foci). This information will improve diagnosis of leishmaniasis in hospitals, clinical health centers and research institutes. Furthermore, the presented resource can be applied to study the epidemiology and geographic ranges of sand fly vectors and animal reservoirs of *Leishmania* spp.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mam.2016.11.012.

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