Genetic diversity analysis of Chinese *Leishmania* isolates and development of *L. donovani* complex specific markers by RAPD

Dongmei Yuan
Sichuan University West China School of Basic Medical and Forensic Medicine
Hanxiao Qin
Sichuan University West China School of Basic Medical and Forensic Medicine
Dali Chen
Sichuan University West China School of Basic Medical and Forensic Medicine
Jianping Chen (jpchen007@163.com)

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Abstract

Background: Leishmaniasis is one of the most neglected tropical diseases in the world and is still endemic in some underdeveloped regions including western of China. The phylogeny and classification of Chinese *Leishmania* has not yet been clarified, especially within *Leishmania* (*L.*) *donovani* complex, although phylogenetic analyses based on a series of gene markers were carried out. More different analytic method and data still be needed. Random amplified polymorphic DNA (RAPD) technology could identify slight intraspecific differences sensitively, and it always be a powerful tool to seek the species-specific markers. This work aimed to identify Chinese *Leishmania* isolates from diverse geographic regions at genomic level. Meanwhile, the specific markers of *L. donovani* complex were also developed by RAPD.

Methods: The RAPD was applied on 14 Chinese *Leishmania* isolates from diverse geographic regions and 3 WHO reference strains. The polymorphic sites of amplification were transformed into data matrix, based on which genetic similarity were calculated and UPGMA dendrogram were constructed to analyze genetic diversity of these *Leishmania* isolates. Meanwhile, the specific amplification loci of *L. donovani* complex were TA-cloned, sequenced and converted into sequence characterized amplified regions (SCAR) markers, which were validated preliminarily in available 17 *Leishmania* strains in this study and analyzed by bioinformatics.

Results: The cluster analyses showed that the three *Leishmania* sp. isolates SC10H2, SD and GL clustered together and apart from others, and the strains of *L. donovani* complex clearly divided into two clades and the three isolates Cy, WenChuan and 801 formed a subclade. Three specific SCAR markers of *L. donovani* complex, i.e. 1-AD17, 2-A816 and 3-O13, were successfully obtained and validated on available 17 *Leishmania* strains in this study. Through bioinformatics analyses, Marker 1-AD17 may have more specificity on PCR detection of VL and Marker 3-O13 has the potential of encoding protein.

Conclusions: The RAPD result verified that the undescribed *Leishmania* species causing visceral Leishmaniasis (VL) in China was a unique clade distinguished from *L. donovani*, and revealed that there was genetic differentiation among Chinese *L. donovani*. The development of *L. donovani* specific markers may provide the foundation for developing new specific diagnostic markers of VL and the research of specific gene function.

Background

Leishmaniasis is a tropical disease caused by obligate intracellular protozoa genus *Leishmania* and transmits through bite of genus *Phlebotomus*, which threaten 350 million people over 98 countries, mainly in developing countries [1]. There are four main forms of Leishmaniasis according to different clinical syndromes, i.e., visceral Leishmaniasis (VL, kala-azar), post-kala-azar dermal leishmaniasis (PKDL), cutaneous Leishmaniasis (CL) and mucocutaneous Leishmaniasis (MCL), which caused by different species of *Leishmania*. There are more than 64 species of genus *Leishmania* consist of

subgenera *Euleishmania*, *Paraleishmania* and *Sauroleishmania* [2], among which 20 species have been considered human-infectious [1].

Visceral leishmaniasis, which is acute and fatal if left untreated, is the main form of leishmaniasis that prevailed in China. Although Chinese VL has been effectively restricted since 1950s, it still presents localized and sporadic outbreaks now, mostly in Xinjiang Uygur Autonomous Region, Sichuan and Gansu province [3]. What' more, China is still one of 14 VL high-burden countries [4]. Over the past few decades, epidemiological characteristics, kinetoplast and chromosomal DNA have been applied successively to Chinese VL typing [5,6,7]. In recent years, through a series of gene markers, such as internal transcribed spacer 1 (ITS1), cytochrome oxidase II (COX II), cytochrome *b* (cyt *b*) and HSP70 were applied to establish phylogenic trees, *L. donovani* complex (include *L. donovani* and *L. infantum*), *Leishmania gerbilli*, *Leishmania tropica* and *Leishmania turanica* has been identified, along with an undescribed *Leishmania* species which has clustered with Lizard *Leishmania* [8-11]. However, the species classification and pathogen identification of Chinese leishmaniasis is far from illuminated, especially within *L. donovani* complex. Our previous phylogenetic analyses on HSP70 presented clear relationship within *L. donovani* complex [11]. It inferred that *L. infantum*, which is one of the causative agents of VL, is primarily distributed in western mountainous areas and plain of northwestern China, including Sichuan, Gansu, and Xinjiang provinces. While the identification of isolate MHOM/CN/80/801 from VL patients in Kashi of Xinjiang, was different from the study of ITS1 sequences [12]. Moreover, the analysis of HSP70 also concluded that *L. donovani* is the pathogen of CL in Karamay of Xinjian and *Phlebotomus major wui* is the vector, which challenged the previous determination that the *L. infantum* is the pathogen of CL in Karamay based on gene hybridization and animal inoculation [13, 14]. Thus, application of more diverse analytical methods and more classified data would be needed to add our understanding of genetic relationship of Chinese *Leishmania* isolates.

Rapid species identification is essential to early diagnosis of Leishmaniasis and conduce to accurate treatments. Multilocus enzyme electrophoresis (MLEE) is still the golden standard in identification of *Leishmania* species [15], but it is rarely used now because of its time-consuming procedure. DNA markers have been widely applied for phylogenic research of *Leishmania* [16]. The phylogenetic trees of these markers provided a lot of evidence for the taxonomy of main *Leishmania* complex, while the relatively slow evolutionary rate of these genes were insufficient to solve the species relationship within complex [17]. Furthermore, the discrimination capability is diverse among markers and which in turn makes identification of species and subspecies sometimes inconsistent [18]. The Random Amplification Polymorphic DNA (RAPD) is a technique which could make polymorphic analysis for unknown genomes on the basis of PCR [19, 20]. This technique is easy and sensitive, which always be applied to species identification [21], the correlation analysis of population differentiation with geographical origins on *Leishmania* [22]. Also, RAPD has its advantage on the taxonomy at subgeneric level [23]and species level [24]. Meanwhile, through the selection of DNA markers among differentially amplified bands, the specific genetic markers of one species can be developed to species identification or assist diagnosis by making a probe. So it proved to be an effective method to obtain genetic markers to develop relevant *Leishmania* DNA assays [25, 26].

In this study, the RAPD was applied on Chinese *Leishmania* isolates from diverse geographic regions, which could help us more thoroughly understand the genetic difference of these isolates combining with our previous phylogenic analyses. Meanwhile, the specific amplified bands of *L. donovani* complex were screened for converting of *L. donovani* complex specific sequence characterized amplified regions (SCAR) markers, and these SCAR markers were validated preliminarily in available 17 *Leishmania* strains in this study and analyzed by bioinformatics, which may provide the foundation for the research of specific gene function and developing new specific diagnostic markers of VL.

Methods

Leishmania strains and DNA extraction

Fourteen Chinese *Leishmania* isolates and three WHO reference strains were used in this study and listed in Table 1 (see Additional file 1). The 14 Chinese *Leishmania* isolates were collected from the plain, hill, and desert foci of China. These parasites were cultured in Medium 199 supplemented with 15% heat-inactivated fetal bovine serum, 100 U/mL penicillin (Sigma) and 100 μg/mL streptomycin (Sigma) at 26℃. The promastigotes were collected at logarithmic phase and centrifuged at 3300×g for 10min. Total DNA was extracted using a commercially available DNA extraction kit (TianGen Cell DNA Kit). The concentrations of 17 DNA samples were detected (Thermo Scientific™ NanoDrop™ One) and adjusted to the same level before the following RAPD amplification.

RAPD-PCR

Twenty decamer primers were selected according to previous studies [27, 28]and commercially synthesized (Invitrogen). All primers were prepared as 10 μM (10 pmol/μl) working solutions. These primers firstly were screened through three independent RAPD amplifications on the same *L. donovani* isolate DNA, then 10 primers that presenting polymorphic, reproducible and clear amplification profile were selected finally (Table 2). RAPD amplification was performed in 50μl reactions containing 0.8μM primer, 10ng genomic DNA sample, 25μl 2×TaqMater Mix (Tsingke, China) and PCR grade distilled water. The PCR procedure were described as follows: initial denaturation at 94℃ for 5min followed by 45 cycles of 94℃ for 1min, 36℃ for 1min, 72℃ for 2min, and a final extension at 72℃ for 8min. The PCR products were separated using 1.5% agarose gel electrophoresis. Each PCR and electrophoresis were taken three times under the same protocol and operator to assure reproducibility.

Table 2. The nucleotide sequences of 10 primers and amplified results of *Leishmania* isolates.

Primers	Nucleotide Sequence 5'-3'	Total Bands
OP-AD17	GGCAAACCCT	13
OP-AY5	TCGCTGCGTT	12
OP-AY8	AGGCTTCCCT	18
OP-O13	GTCAGAGTCC	10
OP-U3	CTATGCCGAC	11
AB1-13	TTCCCCCGCT	12
AB1-09	TGGGGGACTC	12
AB1-04	GGACTGGAGT	7
3301[35]	TCGTAGCCAA	12
A816	GTGACGTAGG	14
Total		121

Phenetic analysis of RAPD results

The bands of all polymorphic RAPD gel were marked as “0” for absence and “1” for presence. The relative intensity among all bands were regardless. The 0/1 data matrix was formed in Microsoft Excel 2013 and analyzed using Numerical Taxonomy and Multivariate Analysis System (NTSYS) [29]. The similarity module was used for calculating similarity matrix. The SAHN function of the clustering module was employed for clustering analysis and the phenetic dendrogram was output under the unweighted pair-group method with arithmetic means (UPGMA).

Cloning, sequencing and verification of SCAR markers

The *L. donovani* and *L. infantum* strain-specific bands that stably reproduced in one gel electrophoresis were considered as potential SCAR markers of *L. donovani* complex to extract. These gel blocks were purified and then cloned into pGM-T vector (Tiangen, China) overnight. Recombined products were mixed with DH5α competent E. coli and then screened by blue-white selection. The white colonies were picked up and identified by colony PCR, then the positive samples were proliferated in liquid LB medium and collected for DNA sequencing (Tsingke, China). For the obtained SCAR markers sequences, specific primer pairs were designed using primer 5.0, then PCR was performed on now available 17 *Leishmania* strains to verified these markers’ specificity for *L. donovani* complex in this study. In the PCR conditions, annealing temperature were tested by gradient according to different primers of each SCAR marker.

Bioinformatics analysis of SCAR markers

The obtained SCAR markers sequences were submitted to online BLAST for homologous analyses on NCBI, and the base component was analyzed using Lasergene EditSeq. The open reading frames (ORFs) were predicted and located using NCBI-ORF Finder. To further determine whether the ORFs contained in the sequences have potential possibility to express protein, the promotor binding sites were analyzed and

predicted online using Promotor Scan. Using Lasergene, the ORFs that have potential protein expression were translated and the components of these presumed proteins were analyzed. The secondary structures were predicted by loading Chou-Fasman method. The hydrophobic regions were calculated using Kyte-Doolittle method. The antigenic determinants were analyzed through JamesonWolf method and the surface probability was assessed using Emini method.

Results

RAPD analysis of 17 Leishmania strains

Through RAPD-PCR on the 17 *Leishmania* strains, a total of 121 RAPD bands were observed, of which 120 bands were polymorphic (99.17%). An average of 12.1 bands were amplified by each primer, and segments ranged from 200 to 3000 bp. According to the gel photograph, there are differences among 17 *Leishmania* isolates. Fig. 1 showed one gel photo as examples of polymorphism. While same species tend to form similar bands model. The genetic similarity of 17 *Leishmania* strains ranged from 0.4393 to 1.0000 with an average of 0.6758 (Table 3, see Additional file 2), which indicated a considerable genetic differentiation among these isolates. The UPGMA dendrogram established basing on the similarity matrix was showed as Fig. 2. The isolates SC10H2, SD and GL clustered into Clade I with a highly average similarity index of 0.9715, which had a distant genetic relationship with others. Other strains formed Clade II, which further consist of Clade A, B and C. The isolate EJNI-154 clustered with *L. gerbilli* WHO reference strain MRHO/CN/60/GERBILLI and formed as Clade C. The strains that identified as *L. donovani* complex before did not cluster as one clade but two clades, Clade A and B, instead. The isolates Cy, WenChuan and 801 clustered with *L. donovani* reference strain DD8 as Clade A, whose average similarity index is 0.9439. The other *L. donovani* complex strains clustered as Clade B, in which there still existed genetic differences. Within Clade B, the similarity index of SC6 from Sichuan province with others is 0.9019, which significantly lower than the average index of Clade B (0.9537), so it was separated as an independent clade.

Cloning, sequencing and verification of SCAR markers

A total of four fragments that only appeared in all *L. donovani* complex strains were successfully T-cloned and sequenced. The obtained fragments sequences were named partly after their primers, i.e. 1-AD17, 2-A816, 3-O13 and 4-09. For evaluating the species-specificity, an 18 base pair primer was designed for each potential marker respectively. By PCR amplification on 17 strains in this study, three of the four markers manifested strict species-specific single band at their respective corresponding loci and were tentatively converted to the potential SCAR markers of *L. donovani* complex (Fig. 3). The primers and annealing temperature of the three SCAR markers are showed in Table 4. The DNA sequences are showed in Table S1 (see Additional file 4).

Table 4. The converted SCAR markers of *L. donovani* complex in this study.

SCAR marker	Length	Primer sequences 5'-3'	Annealing temp (°C)
1-AD17	636	F: TGGCAAACCCTGTATGAGGAAAACGT R: TTGGCAAACCCTCATAGGTTGC	61
2-A816	416	F: GTGACGTAGGCATGCCAGCAAGGTGG R: GTGACGTAGGGGTGGGGATGAAGAGG	63
3-O13	1210	F: GTCAGAGTCCTCGCGGGGTATTC R: GTCAGAGTCCAGTAGATAGGATCGATGCG	65

Bioinformatics analysis of SCAR markers

All the three markers were undergone BLAST on NCBI, the query coverage was 99% and the identity percent were greater than 98% with *L. donovani/infantum* reference sequences. Besides, the E-valves were close to '0'. All these indicated that the three markers sequences had a very highly homology with *L. donovani/infantum* reference sequences. According to distribution of Blast hits of SCAR markers, excepted the first two *L. donovani/infantum* reference sequences, the marker 1-AD17 had a query coverage less than 90% and had no matching primer binding sites with other *Leishmania* species sequences (Fig. 4a). While marker 2-A816 and 3-O13 had more matching primer binding sites with other *Leishmania* species sequences excepted *L. donovani/infantum* reference sequences (Fig 4b, c). Therefore, the primers of marker 1-AD17 has a greater specificity for amplification of *L. donovani* complex.

The results of sequence components, chromosomal assignment, ORF and promotor prediction for the three markers are listed in Table 5 (see Additional file 3). There are 4 to 7 ORFs in these markers, in which only 3-O13 has two potential promotors, located on 611-816 bp of sense strand and 1056-806 of antisense strand. The distance between predicted promotor sites and ORFs implied that ORF-4 has the potential of encoding proteins. The gene sequence was then translated into protein and analyzed by Lasergene. The protein sequence of ORF-4 contains 7 strong basic amino acids, 6 strong acid amino acids, 19 hydrophobic residues and 10 polar residues, and the putative isoelectric point is 8.835. The structure predication of the ORF-4 protein sequence by Lasergene Protean was showed in Fig. 5. The results showed that a clear structure of one hydrophilic β turns region (19-36 residues) was flanked by two hydrophobic α helices (1-18 and 37-50 residues), which implied that the α helices might located in interior of the protein and β turns might on the surface. It was confirmed by the surface probability analysis (Emini method). In addition, the β region has a higher antigen index. Furthermore, there is no homologous protein according to BLAST on GenBank.

Discussion

Generally, DNA marker is now the most widely used method in identification and classification of *Leishmania*, since it is both effective and efficient. While different evolutionary rate of diverse gene markers may lead to different classification result. Thus, to help us understand interspecific relationship more comprehensively, the usage of more dissimilar identification method would be necessary. RAPD has been widely used in genetic maps construction, breeding lines identification and gene markers screening, as has been in the realm of genetic evolution of parasites such as trypanosome, schistosome and trichinella spiralis [30-32]. As we all know, RAPD has its innate drawbacks in stability and repeatability of bands due to the highly random hybrid sites with template DNA. Therefore, to gain stable bands and repeatable results of RAPD, we followed the PCR amplified condition whose stability and repeatability has been already reported [27, 28]and had validated the results before formal experiments. Meanwhile, the usage of commercial Taq DNA Mix from the same batch replaced the addition of dNTP, Mg^{2+} and Taq DNA polymerase one by one also improved the reaction stability in this study. Theoretically, the larger the number of polymorphic sites of RAPD, the more reliable the genetic relationship can be concluded. In this study, 10 of 20 random primers has produced 121 polymorphic bands, with the proportion of polymorphic bands was more than 99%, which could reflect genetic diversity among these isolates credibly.

In this study, the isolates SC10H2, SD and GL, which were identified as *L.* (*SauroLeishmania*) sp. previously [11], had a lower genetic similarity with other strains and firstly clustered as Clade I. This result from the view of genome adds to evidence that in China there exist an undescribed *Leishmania* species and it is a distinct branch which has low homology with Chinese *L. donovani* strains [9, 33, 34]. Particularly, this RAPD result also demonstrated the discrimination and differentiation in the relationship among subspecies of *L. donovani* complex. Three isolates Cy, WenChuan and 801, which isolated from Gansu, Sichuan and Xinjiang respectively, were clustered together and separated from other *L. donovani* isolates. This result confirmed that the genetic differentiation really existed in Chinese *L. donovani*. Combining with the previous study [11, 35], it can be concluded that Cy, WenChuan and 801 should be identified as *L. infantum* and *L. infantum* is the causative agent of canine leishmaniasis (CanL) in Sichuan and Gansu. Accordingly, it could be inferred that the VL in Sichuan, Gansu and Xinjiang were caused by *L. infantum*. This conclusion is also in accord with the previous report [5]. From the UPGMA tree, the isolates KXG-918, KXG-927 were identified as *L. donovani* in this study, which confirmed again that *L. donovani* was the pathogen of CL in Karamay of Xinjiang. Generally, CL were not the mainly prevalent types in China and the majority were imported. It still needs to carry out extensive investigation for indigenous CL in China to obtain solid conclusion about the causative agent. Besides, the dendrogram of UPGMA showed that *L. donovani* reference strain DD8 did not cluster with KXG-XU, KXG-LIU, 9044, KXG-65 and SC6 that identified as *L. donovani* previously. This result indicated there were differences between these Chinese *L. donovani* strains and *L. donovani* reference strain from India on genomic level, which was inconsistent with the phylogenetic analysis results of gene markers [8, 34]. For this, as a gene marker only contains partial information of genome and the selective pressure is various among different genes, intraspecific genetic differentiation probably could not be reflected fully. On the other hand, great genetic variation is generally generated between species or genera in RAPD amplification, so that using an individual represents a species may cause deviation of phylogenic results. Consequently, the

divergence in this study need to be further verified by enlarging the sample size or combining with other methods. In addition, the cluster dendrogram showed that the clade B divided into two small branches: one is Sichuan isolate SC6 from hill foci, another included Shandong isolate 9044 from plain foci and other five Xinjiang isolates from desert foci. It revealed that there were still differences among VL isolates from hill, plain and desert in China, which supported the previous report [36]. Although RAPD technology has been waned gradually, while it is very sensitive to identify slight intraspecific differences, which is suitable for differentiation of sibling species.

For the species-specific segments of RAPD, further bioinformatics analysis is benefit for genetic information mining and the development of specific genetic markers. In this study, although three *L. donovani* complex species-specific DNA markers were obtained and have been preliminarily verified. While, according to their distribution of Blast hits in NCBI, only the primers of marker 1-AD17 has a greater specificity for amplification of *L. donovani* complex. We considered that the differences of primer binding sites or annealing sites of amplification was the cause for generation of differential DNA fragments of diverse species in RAPD, which was speculated in previous report [25]. Thus, the SCAR marker 1-AD17 have the potentiality to be developed into a rapid diagnosis marker of kala-azar. Admittedly, the *L. donovani* complex specific DNA marker in this study still have certain limits because the species of *Leishmania* are multifarious and less genomic information is available. Therefore, more parasite samples and patient specimens would be needed to test the specificity.

Through bioinformatic analysis, the three markers were all located in large chromosomes instead of kinetoplast, which was similar with some other reports [28, 37, 38]. It may be related to that multicopy genes are found preferentially on disomic chromosomes [39], which would increase the probabilities of random primers binding to them. As genome sequences of different *Leishmania* species are highly conserved [39], the amplification loci of RAPD frequently located in variable regions. In this study, although there were 4 to 7 ORFs in the three markers, in which only 3-O13 has two potential promotors and its ORF-4 has the potential of encoding proteins. The following protein prediction analysis showed that the hypothetical protein had higher antigenic index and surface probability. Nevertheless, all these need further experiments to be verified.

Conclusions

Our results verified that the undescribed *Leishmania* species causing visceral Leishmaniasis (VL) in China was a unique clade distinguished from *L. donovani*, and revealed that there was genetic differentiation among Chinese *L. donovani* isolates at the genome level. Three *L. donovani* complex species-specific DNA markers in available 17 *Leishmania* strains were developed and analyzed preliminarily through Blast and bioinformatics, which may provide the foundation for developing new specific diagnostic markers of VL and the research of specific gene function. Nevertheless, collection of more strains from different origins and patient specimens would be necessary to obtain more accurate intra-specific classification of Chinese *L. donovani* and effective verification of these specific SCAR markers.

Abbreviations

RAPD: Random amplified polymorphic DNA; SCAR: sequence characterized amplified regions; VL: visceral Leishmaniasis; CanL: canine leishmaniasis; cyt *b*: cytochrome *b*; HSP70: heat shock protein 70; NTSYS: Numerical Taxonomy and Multivariate Analysis System; UPGMA: unweighted pair-group method with arithmetic means.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YD carried out the RAPD studies and drafted the manuscript, QH performed the statistical analysis and edited the manuscript, CD participated in discussions about the analysis, CJ conceived and coordinated the study. All authors have read and approved the final manuscript.

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Figures

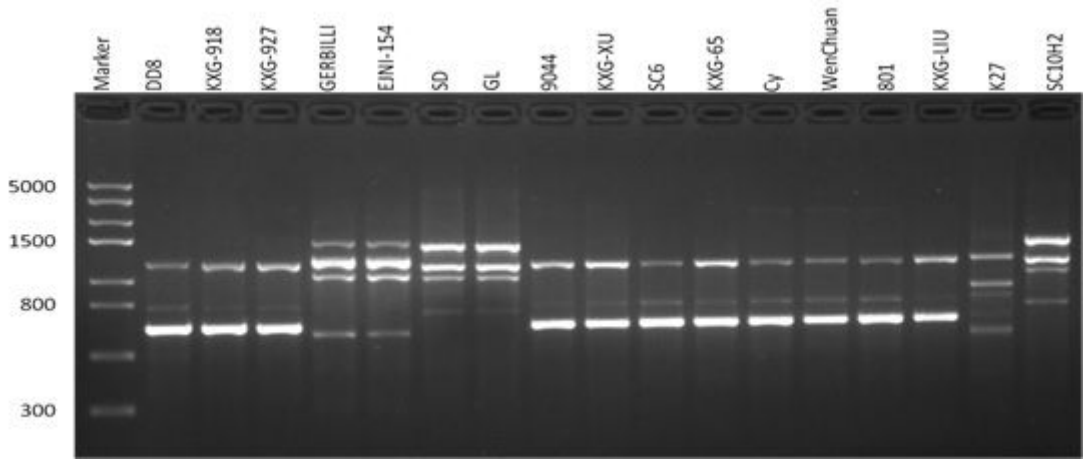

Figure 1

The RAPD profiles of 17 Leishmania strains with primer OP-AD17.

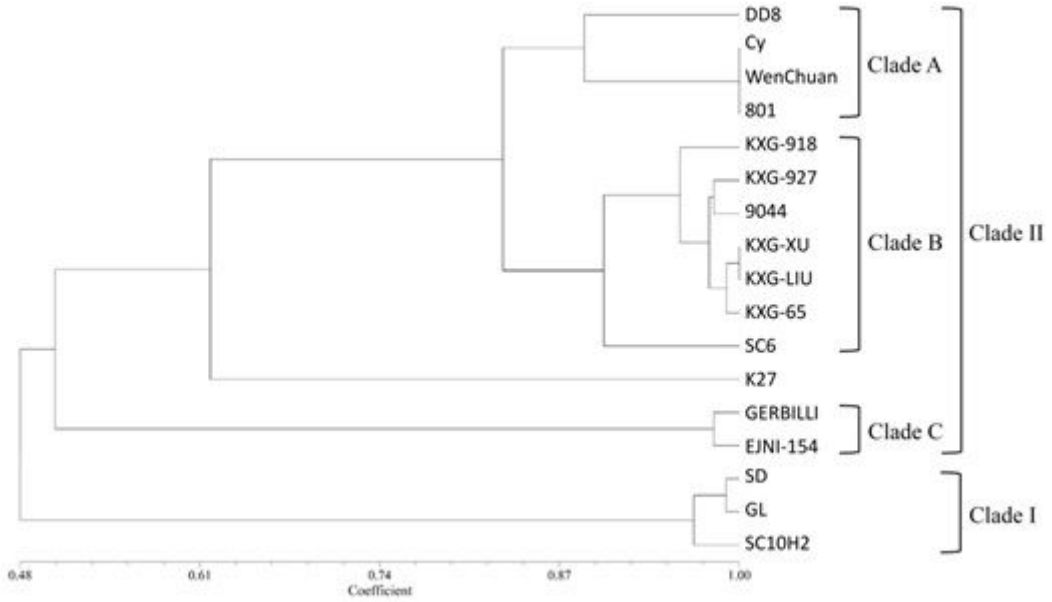

Figure 2

The UPGMA tree of 17 Leishmania strains clustering based on genetic similarity.

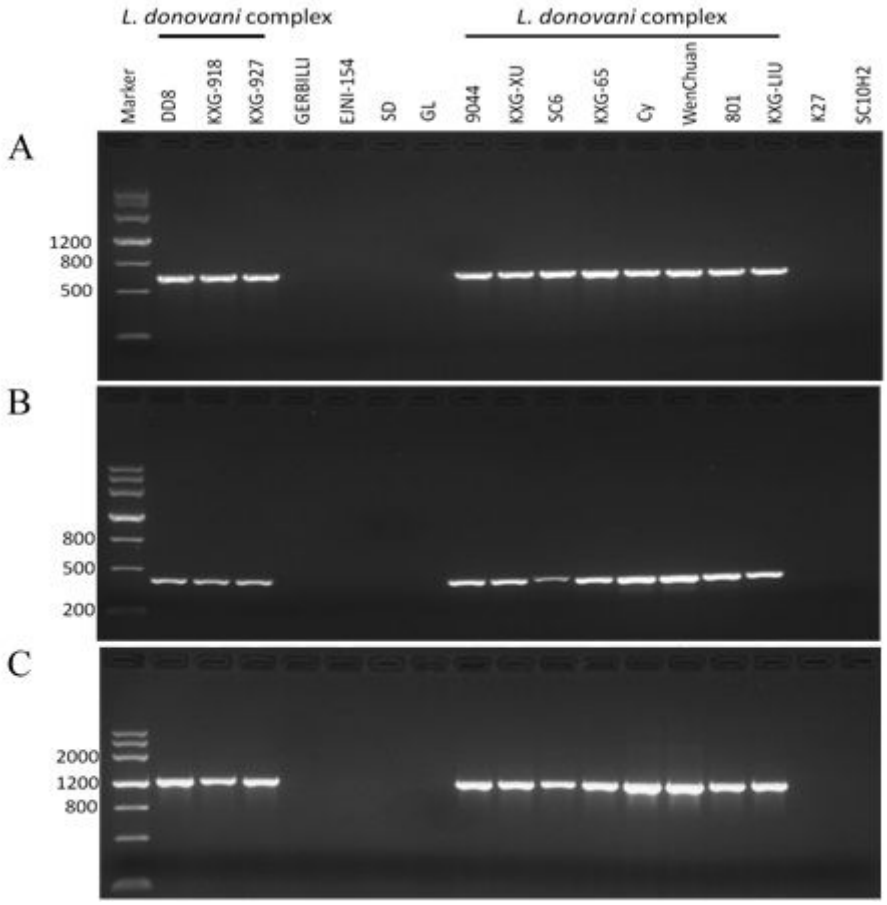

Figure 3

Species-specific amplification of the three SCAR markers to L. donovani complex. A: 1-AD17; B: 2-A816; C: 3-O13.

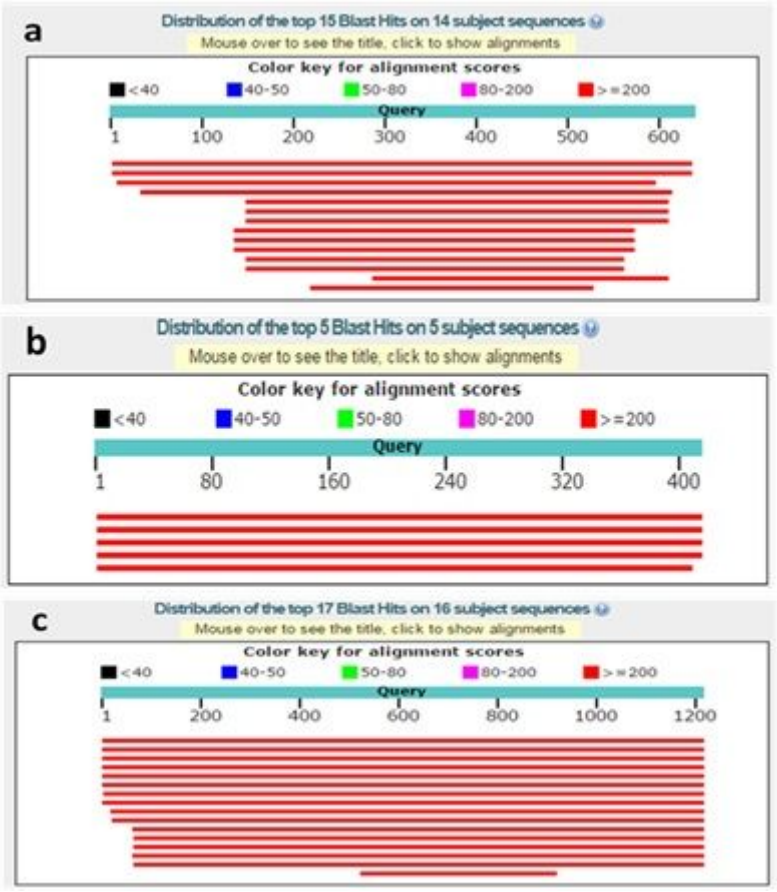

Figure 4

Distribution of Blast hits of SCAR markers. a. 1-AD17; b. 2-A816; c. 3-O13.

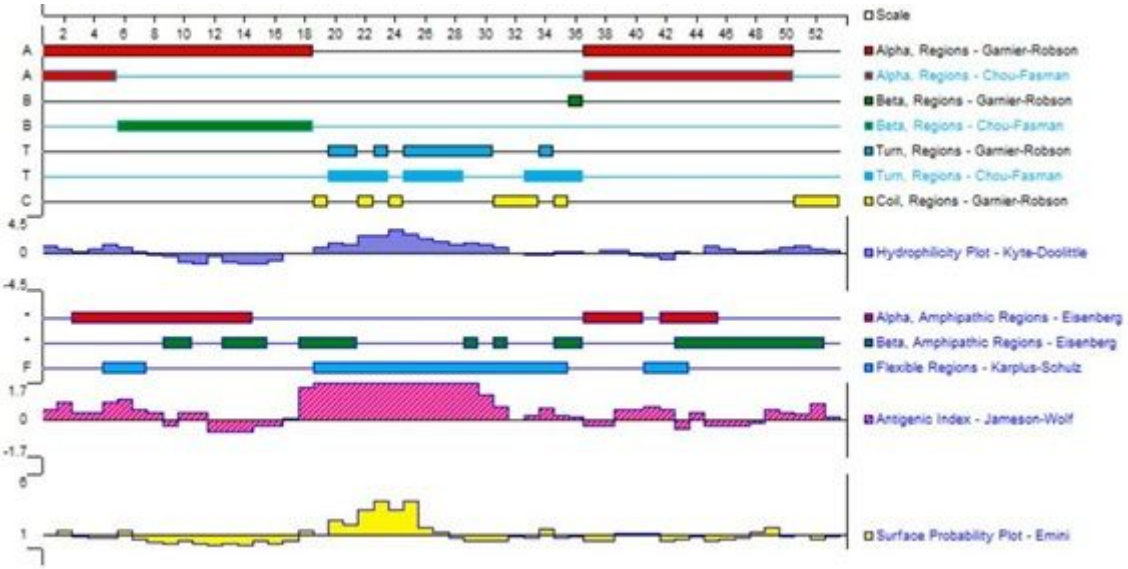

Figure 5

Protein predictive analytics of ORF-4 of Marker 3-O13 by Lasergene Protean.

Supplementary Files

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