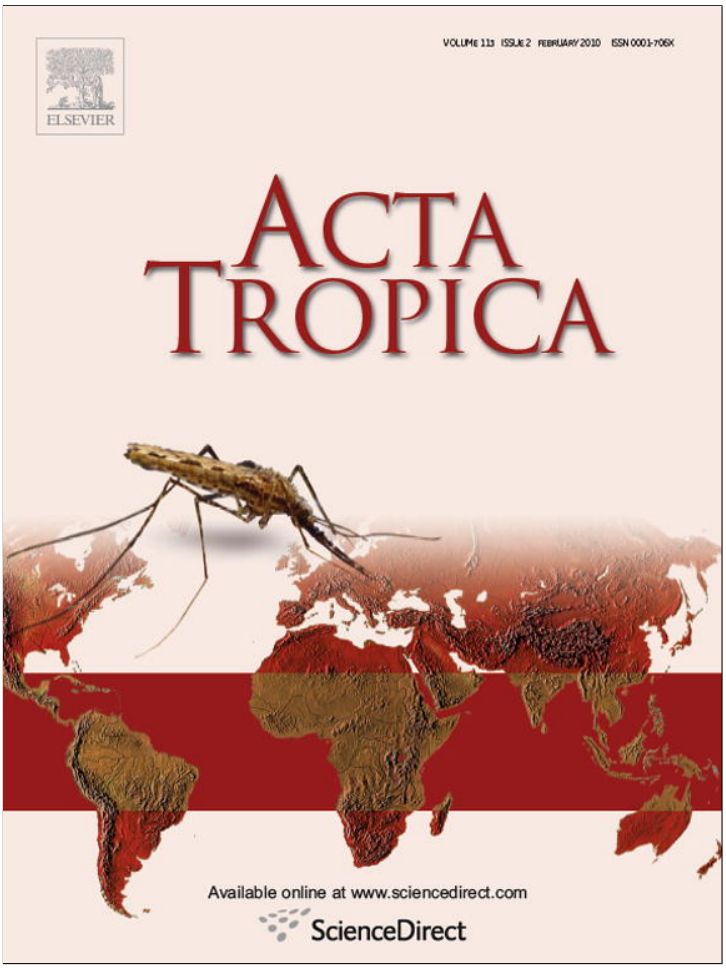

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Short communication

Amplified fragment length polymorphism (AFLP) analysis is useful for distinguishing *Leishmania* species of visceral and cutaneous forms

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ABSTRACT

The *Leishmania* strains belonging to cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) have been reported to possess close homology in genome profiles. To confirm this on genetic basis an attempt was made to differentiate *Leishmania major*; *Leishmania tropica* and *Leishmania donovani* genetically for the first time using amplified fragment length polymorphism (AFLP)—a high throughput DNA fingerprinting technique. The objective of this research work was to identify DNA markers of CL and VL. Ten combinations of selective primers detect a total of 1487 informative AFLP marker. Percentage of polymorphism was 45.12%. Three hundred and thirty-seven unique AFLP markers were also identified in three species of *Leishmania*. A clear distinction was revealed between *L. major* and *L. donovani*. It was inferred by AFLP analysis that a higher rate of polymorphisms occurred among *Leishmania* species which indicate the distinguished pattern of the disease cause by *Leishmania*, i.e. VL and CL. Analysis based on polymorphic AFLP markers revealed considerably high genetic variation among the genome of these species which was sufficient to distinguish between CL and VL.

1. Introduction

Trypanosomatid parasites of the genus *Leishmania* cause severe human diseases leishmaniasis that refers collectively a group of diseases with a wide spectrum of clinical manifestations ranging from self-healing cutaneous leishmaniasis (caused by *Leishmania major* and *Leishmania tropica*) to lethal visceral leishmaniasis (caused by *Leishmania donovani*) if untreated. *Leishmania* is an obligate intracellular protozoan parasite that is transmitted by sandflies as flagellated promastigote form. With the bite of the female vector the parasites are injected into the host and multiply in the phagolysosomes of macrophages into amastigotes. The mechanisms by which different *Leishmania* species cause different pathologies are largely unknown. Approximately 350 million people in 88 countries are estimated to be threatened by the disease (http://www.who.int/en/). The World Health Organization estimated that there are 12 million cases of all forms of leishmaniasis worldwide (Murray et al., 2005). More than 90% of the cutaneous leishmaniasis (CL) cases occur in Afghanistan, Saudi Arabia, Algeria, Brazil, Iran, Iraq, Syria and Sudan; while more than 90 percent of visceral leishmaniasis (VL) cases occur in India and Sudan (Singh and Sivakumar, 2003). Most of the drugs commonly used to treat different forms of leishmaniasis are toxic and have unacceptable side effects (Croft et al., 2006). Moreover, cases of drug resistant leishmaniasis are on the rise (Singh et al., 2003).

Our previous studies have established the genetic variation in sodium antimony gluconate (SAG) sensitive and resistant recent clinical isolates of *L. donovani* (Kumar et al., 2009) through AFLP—a DNA fingerprinting technique allowing the scanning of multiple loci across the whole genome for the presence of polymorphism (Vos et al., 1995). AFLP has good reliability (Gorni et al., 2004) and has been used widely for comparative genomic analysis of many organisms (Ross et al., 2003; Hill et al., 2004; Martinelli et al., 2005). Since, this is a relatively faster technique as compared to other fingerprinting techniques, we planned to assess the genomic differentiation among *Leishmania* genomes. The present research communication is a comparative genomic analysis through AFLP of three *Leishmania* species (*L. donovani*, *L. major* and *L. tropica*) that cause diverse human disease. The objectives of the present study were (1) to examine the usefulness of AFLPs in differentiating VL and CL, (2) to develop unique molecular markers against VL and CL and (3) to determine genetic relationships among these *Leishmania* species.

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Table 1
Leishmania species showing polymorphism on the basis of ten selective primer combinations.

Selective primer combinations	Number of all bands	Number of monomorphic bands	Number of polymorphic bands	Number of unique bands	Percentage of polymorphism
(1) MseI CAA/EcoRI ACA	137	105	32	3	23.35
MseI CAA/EcoRI ACG	30	21	9	7	30.00
MseI CAA/EcoRI AGC	61	27	34	22	55.73
(2) MseI CAC/EcoRI ACA	53	9	44	24	83.01
MseI CAC/EcoRI ACG	28	18	10	6	35.71
MseI CAC/EcoRI AGC	37	27	10	6	27.02
(3) MseI CAA/EcoRI ACT	54	12	42	22	77.77
MseI CAA/EcoRI AGG	70	42	28	14	40.00
MseI CAA/EcoRI AAC	13	9	4	4	30.76
(4) MseI CAC/EcoRI ACT	35	12	23	9	65.71
MseI CAC/EcoRI AAG	49	36	13	7	26.53
MseI CAC/EcoRI AAC	16	9	7	3	43.75
(5) MseI CAT/EcoRI ACA	40	12	28	20	70.00
MseI CAT/EcoRI ACG	26	12	14	12	53.84
MseI CAT/EcoRI AGC	22	3	19	9	86.36
(6) MseI CTC/EcoRI ACA	57	27	30	19	52.63
MseI CTC/EcoRI ACG	40	21	19	11	47.50
MseI CTC/EcoRI AGC	41	18	23	15	56.09
(7) MseI CAG/EcoRI ACA	65	36	29	17	44.61
MseI CAG/EcoRI ACG	41	27	14	8	34.14
MseI CAG/EcoRI AGC	64	36	28	10	43.75
(8) MseI CTA/EcoRI ACA	123	48	75	21	60.97
MseI CTA/EcoRI ACG	95	63	32	16	33.68
MseI CTA/EcoRI AGC	56	21	35	11	62.50
(9) MseI CTG/EcoRI ACA	52	27	25	13	48.07
MseI CTG/EcoRI ACG	62	42	20	10	32.25
MseI CTG/EcoRI AGC	38	30	8	8	21.05
(10) MseI CTT/EcoRI ACT	30	24	6	4	20.00
MseI CTT/EcoRI AAG	19	12	7	5	36.84
MseI CTT/EcoRI AAC	33	30	3	1	9.09
Total no. of bands	1487	816	671	337	

2. Materials and methods

2.1. Culture of Leishmania parasites

The six different strains of *L. donovani* – Dd8, 2001, 2039, 2041, 2087, 2093 – the visceral form (Dube et al., 2005; Samant et al., 2007; Kumar et al., 2009) and V121 and UR6 of *L. major* and L. *tropica*, respectively, of cutaneous form were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (Sigma, USA) at 25 °C as described previously (Dube et al., 2005).

2.2. Genomic DNA isolation

Genomic DNA of *L. donovani* strain – Dd8 (WHO reference strain), *L. major* – V121 and *L. tropica* – UR6 was isolated as per protocol described previously (Kelly, 1993). Briefly, logarithmic phase promastigotes were disrupted in NET lysis buffer (50 mM NaCl, 50 mM EDTA, 1% SDS, 50 mM Tris–HCl, pH 8.0), and then incubated overnight with proteinase K (100 mg/ml, Sigma–Aldrich) at 37 °C. DNA was purified further by phenol–chloroform extraction and ethanol precipitation.

2.3. AFLP reactions

AFLP was performed as per protocol described by Vos et al. (1995). Briefly, 500 ng genomic DNA was digested using two restriction endonucleases, EcoRI and Tru9I (an isoschizomer of MseI) for 4 h at 37 °C. Adapter ligation was performed in a 50 μL reaction mixtures containing EcoRI and MseI adapter primers, T4 DNA ligase and digested template DNA at 16 °C over night. After ligation, the pre selective PCR amplification reaction was performed using template DNA, a pair of primers based on the sequences of the EcoRI and MseI adapters, including one additional selective nucleotide at the 3′end of the MseI primer (MseI + C) and the EcoRI primer (EcoRI + A). The preamplified PCR products was diluted 20-fold with T10E0.1 buffer and selective PCR amplifications were carried out using ten different MseI and EcoRI primer combinations (Applied Biosystems, USA). The selective PCR products were loaded on a 5% denaturing polyacrylamide gel using an ABI PRISM® 377 DNA sequencer. AFLP reactions were carried out in triplicate using the amplification modules and the guidelines supplied by Applied Biosystems, USA. As for reference DNA we have used DNA of Dd8 strain of *L. donovani* which is a WHO reference strain as a specific control.

2.4. AFLP data analysis

Analysis of AFLP output was performed using the ABI GENESCAN Analysis Software (Applied Biosystems, USA) which automatically detects the DNA fragments. Different DNA fragments amplified with each primer were treated as discrete characters and numbered sequentially. For diversity, analysis bands were scored as present (1) or absent (0) to form a raw data matrix. Matrix of similarity was then obtained using Jaccard similarity coefficient (Jaccard, 1908) by SPSS 10.0.1 software. The average similarity matrix was used to generate a tree for cluster analyses by Unweighted Pair Group Method with Arithmetic average (UPGMA) method using NTSys v2.1. Principal component analysis was carried out with the help

of SPSS software. Support values for the degree of confidence at the nodes of the dendrogram were analyzed by BOOTSTRAP analysis (Felsenstein, 1985) using FREETREE software (Pavlicek et al., 1999).

3. Results

3.1. AFLP analysis

A total of 1487 informative AFLP markers were generated using ten selective primer combinations (Table 1). Out of 1487 AFLP bands, number of polymorphic ones identified were 816 and that of monomorphic 671. On the basis of all the ten primer combinations used the percentage of polymorphism among *Leishmania* species was calculated to be 45.12.

3.2. Dendrogram and phylogeny

The phylogenetic tree was obtained by using FREETREE software. The results distinctly separated *L. donovani* from *L. major* and *L. tropica* which clearly indicated towards cutaneous and visceralization dichotomy (data not shown). Here we have also compared this result with our previous data of SAG sensitive and resistant clinical isolates of *L. donovani* (Kumar et al., 2009). The first cluster

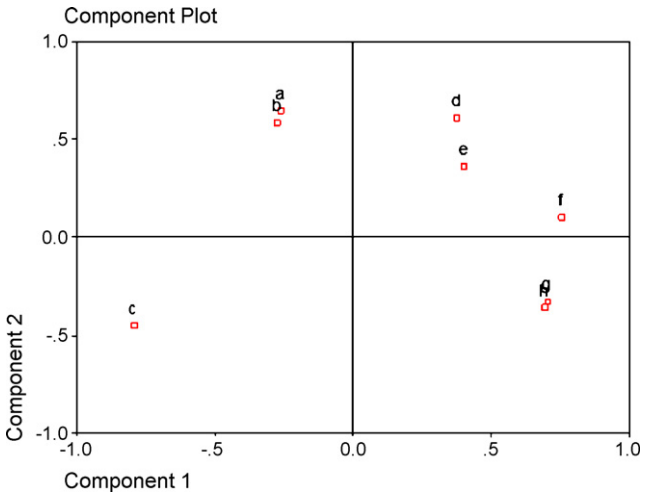

Fig. 1. Principal component plot based on the AFLP data of six clinical isolates of *L. donovani L. major* and *L. tropica* (a: Dd8, b: 2001, c: 2039, d: 2041, e: 2087, f: 2093, g: Lm, h: Lt).

Table 2
Unique AFLP marker bands (size in bp) specific for Ld, Lm and Lt, i.e. for VL and CL.

Selective primer combinations	Ld (Dd8)	Lm (V121)	Lt (UR6)
(1) MseI CAA/EcoRI ACA		57,83,182	
MseI CAA/EcoRI ACG	92, 239, 241, 262, 347	360	135
MseI CAA/EcoRI AGC	56, 58, 60, 62, 82, 91, 124, 152, 153, 184, 215, 222, 245, 263, 271, 272, 284, 342, 346		50, 145, 239
(2) MseI CAC/EcoRI ACA	59, 83, 91, 111, 245, 255, 265, 292, 309, 340, 367, 379	293	64, 108, 114, 139, 176, 184, 252, 268, 269, 279, 349
MseI CAC/EcoRI ACG	143, 371	66, 174	95, 98
MseI CAC/EcoRI AGC	83, 143, 347	102, 215, 218	
(3) MseI CAA/EcoRI ACT	134, 182, 196, 248, 269, 274, 319	183, 186, 202, 218, 222, 233	87, 112, 119, 164, 263, 270, 320, 337, 342
MseI CAA/EcoRI AGG	98, 203, 245, 319	57, 211, 327	73, 76, 87, 110, 141, 186, 322
MseI CAA/EcoRI AAC	110		83, 91, 92
(4) MseI CAC/EcoRI ACT	183, 251, 296, 347	157, 215	112, 166, 202
MseI CAC/EcoRI AAG	95, 173, 260		88, 186, 263, 264
MseI CAC/EcoRI AAC			50, 91, 92
(5) MseI CAT/EcoRI ACA	115, 123, 125, 186, 202, 251	117	57, 118, 161, 164, 171, 172, 185, 223, 250, 255, 258, 335, 340
MseI CAT/EcoRI ACG	92, 94, 253, 260, 318, 319, 320, 321	90	93, 172, 208
MseI CAT/EcoRI AGC	51, 90, 299, 321, 326, 334, 345		89, 215
(6) MseI CTC/EcoRI ACA	165, 377	210, 211, 212, 214, 217, 221, 248, 315	61, 132, 133, 134, 157, 250, 255, 340, 343
MseI CTC/EcoRI ACG	87, 98, 185	78, 207, 320	61, 65, 205, 212, 232
MseI CTC/EcoRI AGC	91, 92, 190, 260	217	59, 60, 61, 62, 108, 125, 143, 153, 172, 191
(7) MseI CAG/EcoRI ACA	58, 137, 171, 172, 223, 242		84, 95, 119, 182, 202, 245, 263, 294, 314, 329, 340
MseI CAG/EcoRI ACG	172	62, 344	64, 66, 69, 264, 320
MseI CAG/EcoRI AGC	51, 62, 125, 197, 212, 246, 274		111, 184, 270, 275, 285
(8) MseI CTA/EcoRI ACA	62, 65, 68, 80, 83, 88, 101, 154, 175, 233, 346	96, 176, 202, 240	117, 204, 208, 213, 209, 379
MseI CTA/EcoRI ACG	63, 95, 112, 162, 193, 199, 264, 306, 308, 316	68, 263, 368	62, 205, 375
MseI CTA/EcoRI AGC	199, 263, 304, 305, 378	119, 144, 176	209, 215, 307
(9) MseI CTG/EcoRI ACA	89, 95, 119, 179, 180, 216, 217	83, 183, 190	213, 215
MseI CTG/EcoRI ACG	69, 106, 165, 166, 180, 197, 207, 378	212	204
MseI CTG/EcoRI AGC	112, 199, 308, 369, 378	179, 218	243
(10) MseI CTT/EcoRI ACT	178, 208, 209		179
MseI CTT/EcoRI AAG	62, 189, 205, 209, 375		
MseI CTT/EcoRI AAC	89		

from the top constitutes of different strain of *L. donovani*. They were grouped together because all are the causative agent of VL. The second cluster constitutes of *L. major* and *L. tropica* sharing 90% similarity (because both cause CL) was observed to be completely out grouped from *L. donovani*, showing cutaneous behavior of it.

3.3. Component plot analysis

The principal component plots generated from AFLP data using SPSS software have been shown in Fig. 1. On the basis of principal component analysis, all *Leishmania* species were placed in four quadrants. In the first quadrant, there was occurrence of three species of *L. donovani*: 2041, 2087, and 2093. In the second one Dd8 & 2001 and in third one 2039 strain of *L. donovani* were placed but interestingly again, *L. major* and *L. tropica* were placed in fourth quadrant which was totally distinct from all other strains of *L. donovani*.

3.4. Genetic polymorphism

Fingerprinting of *L. donovani*, *L. major* and *L. tropica* using ten combinations of EcoRI/MseI primers revealed a total number of 671 (45.12%) unambiguous polymorphic AFLP fragments (Table 1). Results confirmed that AFLP was capable of detecting high polymorphism. The primer combination MseI CAT/EcoRI AGC revealed the highest percentage of polymorphic fragments (86.36%), while the lowest percentage (9.09%) was generated by the primer combination of MseI CTT/EcoRI AAC (Table 1).

3.5. AFLP markers specific for VL and CL

Total 337 unique molecular markers were detected through AFLP analysis for *Leishmania* species which has been listed in Table 2. Maximum number of unique bands (19) was observed in *L. donovani* using primer combination of MseI CAA/ECoRI AGC, (8) in *L. major* with primer combination MseI CTC/EcoRI ACA and (13) in *L. tropica* using primer combination MseI CAT/EcoRI ACA.

4. Discussion

Our previous works have indicated that AFLP is a powerful and high throughput tool for genetic variation and marker identification in case of Leishmaniasis (Kumar et al., 2009). In this communication we further exploited this technique for comparative genomic study between VL and CL. Since, the way in which different human-infective species of the *Leishmania* genus cause diverse clinical disease is poorly understood, the identification of AFLP marker differentially distributed in parasite genome should facilitate timely experimental verification of their role in disease development. Documentation of genetic relatedness of *Leishmania* species has been a significant impediment to advances in studying these pathogens. Since, prior sequence knowledge is not necessary to conduct AFLP experiments; hence, this method is particularly applicable for comparative analysis of parasite genomes (Masiga and Turner, 2004). AFLP accessed multiple independent sites within the genome and allowed a better definition of the phylogenetic relatedness of different species (Vos et al., 1995). We have taken here ten combinations of primers in our study that yielded 20–40 DNA fragments after the PCR, within the size range of 100–400 bp for more accuracy, reliability, and distinguish genetic relations in our result. It is believed that by choosing the six combinations of primers, it is possible to explain more than 80% of the expected relatedness (Chen et al., 2004). The results, obtained from a large number of primer combinations, were utilized to check the robustness of the findings, estimation of phylogeny that clearly establish that the polymorphism revealed by AFLP is not only abundant but also statistically reliable (Ellis et al., 1997).

From the AFLP analysis a clear cut large genetic distance and divergence has been observed that separates *L. major* and *L. tropica* from *L. donovani* species which was evident from the component plot and distance analysis. *L. donovani* is evolutionary divergent from *L. major* which suggests about its visceralization nature. Data from AFLP studies support that species causing cutaneous and visceral disease have diverged primarily due to mutations, insertions, deletions, and/or other genetic rearrangements. However, it has been shown by (Samaras and Spithill, 1987) that DNA sequences are rearranged during speciation in *Leishmania*. Parasite genome plasticity has been hypothesized to play a driving role in parasite speciation. In addition, the unexpected identification of few unique markers that is species specific might be significant and provides a comprehensive and manageable resource to target efforts in identifying parasite factors that influence infection. Conversely, factors that are unique to the each *Leishmania* species may be used to develop the diagnostic markers against CL and VL.

Future studies may also include molecular cloning and recovery of that unique AFLP bands for identification of disease specific markers of VL and CL. The genomic fingerprinting data of *Leishmania* obtained from AFLP study serves as a basic framework in order to obtain more detailed information on the genome and markers linked to genes responsible for visceralization and cutanization mechanism. The Unique AFLP markers may throw the light on genes involved in VL and CL progression and also the mechanism involving their habitat priorities for skin and visceral organs of human. An appreciation of the genomic variability between the three *Leishmania* species, *L. major*, *L. tropica* and *L. donovani* is an important aspect of our understanding in the evolution of the different genomes within the same genus. Further, this preliminary exercise of AFLP fingerprinting could be further extended to a broad survey of populations of different geographic regions.

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