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Novel motifs in amino acid permease genes from *Leishmania*

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Abstract

Eight amino acid permease genes from the protozoan parasite *Leishmania donovani* (AAPLDs) were cloned, sequenced, and shown to be expressed in promastigotes. Seven of these belong to the amino acid transporter-1 and one to the amino acid polyamino-choline superfamilies. Using these sequences as well as known and characterized amino acid permease genes from all kingdoms, a training set was established and used to search for motifs, using the MEME motif discovery tool. This study revealed two motifs that are specific to the genus *Leishmania*, four to the family trypanosomatidae, and a single motif that is common between trypanosomatidae and mammalian systems A1 and N. Interestingly, most of these motifs are clustered in two regions of 50–60 amino acids. Blast search analyses indicated a close relationship between the *L. donovani* and *Trypanosoma brucei* amino acid permeases. The results of this work describe the cloning of the first amino acid permease genes in parasitic protozoa and contribute to the understanding of amino acid permease evolution in these organisms. Furthermore, the identification of genus-specific motifs in these proteins might be useful to better understand parasite physiology within its hosts.

Keywords: Solute transport; Permease; Parasitic protozoa; *Leishmania*; Motif

In cells of unicellular as well as multicellular eukaryotes, amino acids play fundamental roles in numerous processes such as protein synthesis, energy and nitrogen metabolism, osmoregulation, hormone metabolism, nerve transmission, cell growth, nucleobase synthesis, and urea biosynthesis. Amino acid permeases (AAPs) are essential to these processes as they transport amino acids across membranes, thereby providing cells with the required precursors and help in maintaining cellular amino acid homeostasis.

Over the past two decades many AAP genes have been cloned, which enabled molecular characterization of transport systems in protists, plants, and mammals [1]. More than 20 different transport systems have been described for each kingdom. These have recently been grouped in five superfamilies, according to their function, number of membrane spanning regions, and protein domains. The families are amino acid polyamino-choline superfamily (APC), sodium dicarboxylate symporter superfamily (SDS), neurotransmitter superfamily (NTS), amino acid transporters with major facilitator superfamily (MFS), and amino acid transporter superfamily 1 (ATF-1) [1]. These superfamilies can be distinguished from one another by consensus sequences that characterize their specific domains (http://www.sanger.ac.uk/Software/Pfam/). However, motifs of AAP that are specific to taxonomic families and genera have not yet been described. Such sequences are important to improve classification and identification of intragenic regions that are likely to be significant for permease function [2,3].

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Protist AAP genes have been cloned and characterized only in yeast, none in protozoa [1]. Since this kingdom is the closest to prokaryotes, it confers diverse phylogenic relationships to both Pro and Eukaryotes. Here, we describe AAP genes from the parasitic protozoa *Leishmania donovani*.

Leishmania donovani are obligatory intracellular parasites that cause kala-azar, a fatal disease in humans [4]. These organisms cycle between phagolysosomes of mammalian macrophages (that act as the host) and alimentary tract of sand flies (that act as the vector) [5]. In the vector, they grow as flagellated extracellular promastigotes; in the host, they proliferate as aflagellated intracellular amastigotes. Promastigotes are introduced into the host during a vector's blood meal and are subsequently phagocytosed by macrophages, where they differentiate into amastigotes [6]. *Leishmanias*' route of development involves constant exposure to extreme environmental changes. Parasites' adaptation to different environments associates with the capacity to regulate their intracellular content, which includes selective uptake, metabolic utilization, and in some cases intracellular accumulation of substrates or osmolytes [7–9].

Promastigotes accommodate a large intracellular pool of free amino acids of approximately 300 mM [8]. Alanine is the most prominent intracellular amino acid (50–70 mM), while proline, that is transported at a very high level, is found in relatively low concentrations (3 mM) [10–12]. Other major amino acids in this pool are ornithine (34 mM), glutamate, and glycine (14 mM each). Promastigotes utilize this pool to maneuver amino acids across the plasma membrane in response to change in the osmolarity of the growth medium [13]. Although a high level of the internalized amino acid pool is typical to trypanosomatidae, its precise biochemical activity is not known. The major contributors to this pool are plasma membrane permeases that are responsible for amino acid uptake, and membrane potential created by H^+-pumps, which drives the uptake of most amino acids in this organism [8,9,14].

Despite the significance of AAPs in *Leishmania* none have yet been cloned and characterized at the molecular level. In the present work we describe the cloning of eight amino acid permease genes from *L. donovani*. Using computational tools, we identified and characterized *Leishmania* (genus) and trypanosomatidae (family) specific motifs within these genes as well as *Leishmania major* putative genes and looked for phylogenic relationships with other eukaryotes.

Materials and methods

Gene cloning. Primers from the 5′ and 3′ ends of eight putative amino acid permease genes from the *L. major* genome project were synthesized (Table 1). Polymerase chain reactions (PCRs) using *L. donovani* genomic DNA as template were carried out. Amplified DNAs were cloned into AT vector (pDrive, Qiagen, Hilden, Germany) and then sequenced three times each. The *L. donovani* gene names and accession numbers are summarized in Table 2.

Reverse transcriptase-polymerase chain reactions. Total RNA was prepared from promastigotes using Tri-Reagent (Sigma, St. Louis, USA) and was isolated using RNeasy kit (Qiagen, Hilden, Germany). Subsequently, cDNAs were made from isolated total RNA using reverse transcriptase (RT) reaction kit. Control genomic DNA was prepared from stationary phase promastigotes using the LiCl method [15]. PCR analyses were performed on cDNA and genomic DNA using primers from 5′ and 3′ ends for the entire gene or primers from intergenic regions.

Phylogenic trees. DNA sequences of 25 putative amino acid permease genes from *L. major* (http://www.GeneDB.org/) were aligned with the cloned *L. donovani* AAP sequences using Clustal-X (http://www-igbmc.u-strasbg.fr/BioInfo/). Based on the alignment, a NJ tree was constructed using Paup4.0 (http://paup.csit.fsu.edu/index.html) and bootstrapped with distance and parsimony algorithms independently and employing Jukes-cantor correction.

Multiple alignments of amino acid permease genes from the protozoan parasite *L. donovani* (AAPLD) protein sequences together with additional amino acid permease sequences from plant, human, and yeast were performed using Clustal-X (http://www-igbmc.u-strasbg.fr/BioInfo/). Subsequently, an unrooted phylogenic tree was constructed using Paup4.0 with distance and parsimony algorithms. In order to reduce redundancy, protein sequences with 80% or more identity were excluded from the data set.

Hydropathy analysis. Hydropathy analysis of the AAPLD proteins was performed using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) [16].

Motif elucidation. MEME motif search (http://meme.sdsc.edu/meme/website/meme.html) was employed on a training set that contained 80 non-redundant protein sequences (Table 3). The criteria by which the proteins were selected for the training are described in the results section. MEME was set to identify up to 100 motifs of 6 and 50 amino acids with zero or one occurrence per sequence, at p value of <0.0001.

Table 1
Sequences of oligonucleotide primers used to amplify the amino acid permease genes in *L. donovani*

Gene name	Source	Forward primer	Reverse primer	Product size (bp)
AAP1LD	LmjF31.1820	atgtcgaaccgatcagccga	tcaaacagtttcgtagatgg	1818
AAP2LD	LmjF31.0580	atgcaggataacggtgagta	ttacgagacgaaggagctct	1497
AAP3LD	LmjF31.0870c	atgagcaagcccagcaagtc	ctacacgaagaagctgtagt	1443
AAP8LD	LmjF35.5350	atgaaattttgggatcacgt	tcacatccgaatggcgatga	1236
AAP10LD	LmjF35.4410	atgtctcacgcttcacttct	ttacgtcccaacaagggcga	1554
AAP11LD	LmjF35.5360	atgcagcggacgccgctggt	tcaagcatgaagacgggcaa	1218
AAP13LD	LmjF33.1420	atggtcggctgcgcgatgat	tttacatgctgagtaggacg	1468
AAP15LD	LmjF27.1570	atgcagagcacccactcccc	tcagtggacctcgccgtaga	1443

The source for sequences was the *L. major* GeneDB database.

Table 2
L. donovani putative amino acid permease genes

Gene name	Accession number	ORF length (bp)	Predicted protein size (aa)	Copies in the genome	Membrane spanning (times)	Homology to *L. major*		
						Locus in *L. major* genome	Percentage nt identity (%)	Hit length
AAP1LD	AAN70991	1818	605	2	11	LmjF31.1820c	95	1811
AAP2LD	AAO88097	1497	498	1	11	LmjF31.0580	96	1497
AAP3LD	AAO88094	1443	480	2	11	LmjF31.0870c	96	1443
AAP8LD	AAO88098	1236	411	1	11	LmjF35.5350	93	1236
AAP10LD	AAO88095	1554	517	1	12	LmjF35.4410	96	1554
AAP11LD	AAO88096	1218	405	1	10	LmjF35.5360	96	1218
AAP13LD	AAR25622	1468	488	1	11	LmjF33.1420	96	1467
AAP15LD	AAR25623	1443	480	1	11	LmjF27.1570	96	1443

Membrane spanning domains were predicted using THHMM 2.0. Homology to *L. major* was calculated by GeneDB BLAST engine.

Pattern profiles. Motif sequences were aligned and pattern profiles were produced using Logos [17] (http://weblogo.berkeley.edu/logo.cgi). The several alignments were done using Clustal-X and were carried out as follows: For L1 and L2 we used *L. major* (LmjF.35.5350, LmjF35.5260, and LmjF33.1420) and *L. donovani* (AAP8LD, AAP11LD, and AAP13LD) sequences. For T1-4, *L. major* (LmjF.27.1580, LmjF31.0870c, LmjF31.0880c, LmjF31.1790c, LmjF31.1800c, and LmjF31.1820c), *L. donovani* (AAPLD1-3 and AAP15LD), *Trypanosoma cruzi* (PAT1-5 and 8), and *Trypanosoma brucei* sequences that appear in the training set in Table 3. For the N motif, system N and A1 proteins were added to the latter (NP_722518, NP_076294, NP_851604, NP_109599, NP_598847, NP_081328, and NP_277053).

BLAST validations. Regular expression profiles for PHI-BLAST analysis were constructed based on the identical amino acids within the T and N motifs. For example, in order to blast the N motif we entered the *Leishmania*-based consensus fvMLPLVIPKRVNSLRYVS AIGVTFMVYFVAVIVVHSAMNG along with the following regular expression in the PHI-BLAST command line: $XLPLX_8LX_3SX_6MX_2F$. For L motifs we used only the consensus sequences.

Results

Cloning amino acid permease genes from L. donovani

Using all amino acid permease pfam superfamily domains (http://www.sanger.ac.uk/Software/Pfam/), we searched the *L. major* genome and identified 16 open reading frames (ORF), all of which contained either pfam 01490 or 00324. These are included in the 25 amino acid permeases (AAPs) that have recently been annotated by the *Leishmania* Genome Network (http://www.ebi. ac.uk/parasites/leish.html). Phylogenic analysis of the 25 putative *L. major AAPs* suggested at least three major family branches (LmjFs in Fig. 1). Eight representatives of these families were selected for PCR amplification of the corresponding homologues in *L. donovani* genome (ldaaps in Fig. 1). PCR products that matched the corresponding *L. major* ORF size were recovered and the eight PCR products were cloned for further analyses (Table 2). The *L. donovani* genes exhibited 93–96% nucleotide and 97–99% amino acid identity to the corresponding genes in *L. major* (Table 2). ORF analyses suggested that these genes code for proteins of 405–605 amino acids long, reassembling their *L. major* homologues.

Hydropathy analysis using TMHMM 2.0 software showed that the cloned *AAPLDs* consist of 10–12 transmembrane spanning regions, similar to the pattern of the known amino acid permease superfamilies ATF-1 and APC (Fig. 2) [1]. Amino acid sequence comparison (Blastx) of the *L. donovani* putative AAPs (excluding *AAP10LD*) retrieved top hits of similarity to *T. brucei* putative amino acid transporters (e^{-95}–e^{-10} *E* value). In addition, significant hits contained exclusively system N and A1 mammalian amino acid transporters ($6e^{-18}$–0.001). Note that both systems N and A1 are members of the ATF-1 superfamily [1]. Blastx analysis of *AAP10LD* indicated top hit scores to *Arabidopsis thaliana* and *Oryza sativa* amino acid permeases, all of which belong to APC superfamily (228; e^{-58} or less).

In order to verify *AAPLD* expression, RT-PCR was performed on total RNA from *L. donovani* promastigotes using primers from either the entire ORF or from internal regions. As shown in Fig. 3, PCR amplified the same size DNA from both genomic and complementary DNAs, indicating that all of the above cloned *L. donovani* AAP genes are transcribed, e.g., they are all coding genes.

To further demonstrate that *AAPLDs* code for amino acid permeases, an additional phylogenic analysis was conducted, using the expressed AAPLD genes together with already known and characterized representatives of all superfamilies from yeast, plant, and mammalian. As illustrated in Fig. 4, *AAPLD 1*, *2*, *3*, *8*, *11*, *13*, and *15* are closely related to amino acid permeases of the ATF-1 superfamily. *AAP10LD* appears to be different from other AAPLD genes, as it relates to cationic transporters of the APC superfamily.

Cumulatively, expression data, domain similarity, hydropathy, blast, and phylogenic analyses indicated that all eight *AAPLDs* code for amino acid permeases.

Table 3
Training set of permease sequences for MEME assessment

Accession number	Gene name	Name of organism	Pfam
Superfamily: amino acid polyamine-choline (APC)			
NP_193844	AAT-1	*Arabidopsis thaliana*	0.0324
NP_010851	CAN-1	*Saccharomyces cerevisiae*	0.0324
NP_036376	LAT-2	*Homo sapiens*	0.0324
NP_000332	rBAT (SLC3A1)	*Homo sapiens*	0.0324
NP_003973	y+LAT-1	*Homo sapiens*	0.0324
NP_003036	CAT-1	*Homo sapiens*	0.0324
NP_446178	asc-1	*Rattus norvegicus*	0.0324
NP_009625	Tat1p	*Saccharomyces cerevisiae*	0.0324
NP_014993	Put4p	*Saccharomyces cerevisiae*	0.0324
NP_010071	GABA specific	*Saccharomyces cerevisiae*	0.0324
NP_010444	ssy1 receptor	*Saccharomyces cerevisiae*	0.0083
Superfamily: amino acid transporters-1 (ATF-1)			
NP_109599	A1	*Homo sapiens*	0.1490
NP_196484	aap2	*Arabidopsis thaliana*	0.1490
NP_565019	lht1	*Arabidopsis thaliana*	0.1490
NP_568848	Auxin transporter	*Arabidopsis thaliana*	0.1490
NP_191133	ProT2	*Arabidopsis thaliana*	0.1490
NP_076294	Nat-1	*Mus musculus*	0.1490
NP_598847	Nat-2	*Mus musculus*	0.1490
NP_277053	N2	*Homo sapiens*	0.1490
Superfamily: neurotransmitter (NTS)			
NP_003035	Betaine/GABA transporter	*Homo sapiens*	0.0209
Superfamily: sodium-dicarboxylate transporters (SDS)			
NP_033227	ASCT1	*Mus musculus*	0.0375
NP_035523	GLT-1	*Mus musculus*	0.0375
NP_683740	GLAST	*Mus musculus*	0.0375
Superfamily: amino acid transporters within the major facilitator (MFS)			
NP_064705	Bnip	*Homo sapiens*	0.0083
NP_032193	Iglur glut receptor	*Mus musculus*	0.0083
NP_061063	T-type amino acid transporter-1	*Homo sapiens*	0.0083
Prokaryotes			
AAC44538Bottom of Form	Proline betaine transporter	*Escherichia coli*	0.0083
AAB40774	Phenylalanine-specific permease	*Escherichia coli*	0.0324
NP_388122	Similar to histidine permease	*Bacillus subtilis*	0.0324
NP_755241	Serine transporter	*Escherichia coli*	0.3222
Plasmodium falciparum			
NP_703952	Amino acid transporter, putative	*Plasmodium falciparum*	0.1490
Trypanosoma brucei			
CAC86544	AATP1	*Trypanosoma brucei*	0.1490
CAC86545	AATP3	*Trypanosoma brucei*	0.1490
CAC86546	AATP5	*Trypanosoma brucei*	0.1490
CAC86547	AATP6	*Trypanosoma brucei*	0.1490
CAC86548	AATP7	*Trypanosoma brucei*	0.1490
CAC86549	AATP8	*Trypanosoma brucei*	0.1490
CAC86550	AATP9	*Trypanosoma brucei*	0.1490
CAC86551	AATP10	*Trypanosoma brucei*	0.1490
CAC86552	AATP11	*Trypanosoma brucei*	0.1490
Leishmania donovani			
AAN70991	AAP1LD	*Leishmania donovani*	0.1490
AAO88097	AAP2LD	*Leishmania donovani*	0.1490
AAO88094	AAP3LD	*Leishmania donovani*	0.1490
AAO88098	AAP8LD	*Leishmania donovani*	0.1490
AAO88095	AAP10LD	*Leishmania donovani*	0.0324
AAO88096	AAP11LD	*Leishmania donovani*	0.1490
AAR25623	AAP13LD	*Leishmania donovani*	0.1490
AAR25622	AAP15LD	*Leishmania donovani*	0.1490

Table 3 (*continued*)

Accession number	Gene name	Name of organism	Pfam
Leishmania major			
	LmjF2.0440	*Leishmania major*	0.1490
	LmjF11.0520	*Leishmania major*	0.0324
	LmjF14.0320	*Leishmania major*	0.0324
	LmjF22.0230	*Leishmania major*	0.1490
	LmjF31.350	*Leishmania major*	0.1490
	LmjF27.0680	*Leishmania major*	0.1490
	LmjF36.6830	*Leishmania major*	0.1490
	LmjF36.0420	*Leishmania major*	0.1490
Non-amino acid permeases			
XP340640	ABC transporter, putative	*Trypanosoma brucei*	0.0005
NP187917	ABC-transporter	*Arabidopsis thaliana*	0.0005
NP011636	Ammonium transporter	*Saccharomyces cerevisiae*	0.0909
NP035519	Na–K–Cl cotransport protein	*Mus musculus*	0.0324
NP859435	Putative choline transporter	*Leishmania major*	0.1553
NP036119	Fatty acid transport protein	*Mus musculus*	0.0501
CAD21449	Probable biopterin transporter	*Trypanosoma brucei*	0.3092
B48442	Membrane transport protein	*Leishmania donovani*	0.0083
AAB70519	Hexose transporter	*Schizosaccharomyces pombe*	0.0083
CAC32260	Possible monocarboxylate transporter	*Leishmania major*	0.0008
AAF74264	Inosine-guanosine nucleoside transporter	*Leishmania donovani*	0.1733
NP701001	UDP-galactose transporters	*Plasmodium falciparum*	0.4142
NP472970	Monosaccharide transporter, putative	*Plasmodium falciparum*	0.4142
AAF04490	Nucleoside transporter 2	*Trypanosoma brucei*	0.1733
NP594801	UDP-GlcNAc transporter	*Canis familiaris*	0.4142
NP012323	Opt1p oligopeptide transporter	*Schizosaccharomyces pombe*	0.3169
NP172592	Sugar transporter	*Arabidopsis thaliana*	0.0083
NP013583	Phosphate transporter	*Saccharomyces cerevisiae*	0.0083
NP703633	Sugar transporter, putative	*Plasmodium falciparum*	nf
NP594801	Vitamin H transporter	*Schizosaccharomyces pombe*	0.2133
NP565165	Sulfate transporter	*Arabidopsis thaliana*	0.0916
NP011847	Urea transporter	*Saccharomyces cerevisiae*	0.0474
NP031498	Aquaporin-1	*Mus musculus*	0.0230
NP733768	Sodium-coupled citrate transporter	*Rattus norvegicus*	0.0939

Accession numbers, names and source organisms are shown together with their Pfam domains. The term "transporter" refers to permease with proofed function.

Identification of Leishmania-specific motifs

To date, information on amino acid permease domains enable functional classification within superfamilies, but cannot relate motifs to organisms. In the following set of experiments we used both *L. major* and *L. donovani* AAP sequences to identify motif elements that are conserved specifically within *Leishmania*.

A training set with maximal variability and reliability was constructed, which included a non-redundant set of AAP protein sequences (Table 3). This set included protein sequences of: eight *L. donovani* AAP (AAPLDs) that were cloned in this work; eight *L. major* putative AAPs of low similarity to AAPLDs; 26 fully characterized amino acid transporters from fungi, plants, and mammalians according to the known superfamilies; nine amino acid permeases from *T. brucei* (NCBI); one putative amino acid permease from *Plasmodium falciparum* (NCBI); and four amino acid permeases from Prokaryotes [18,19]. As a control, 24 non-amino acid solute permeases from various organisms were added to the training set.

Variability was achieved by avoiding redundancy of highly homologous (>80%) proteins. Reliability was attained by selecting proteins according to the following priorities: permeases with proved transport activity; that were manually sequenced and annotated and lastly, automatically annotated. The plant, mammalian, yeast, and bacterial proteins listed in Table 3 are highly reliable as they have been functionally characterized. The *P. falciparum* and *T. brucei* genes were manually annotated and the *L. major* genes were automatically annotated. Hence, this training set enables segregation and identification of consistent *Leishmania*-specific motifs when searched by a motif discovery tool.

We used MEME as a motif discovery tool, requesting up to 100 motifs with zero or one occurrence per sequence at p values <0.0001. The analysis revealed 57 such motifs, indicating that the maximal number of possible motifs was obtained.

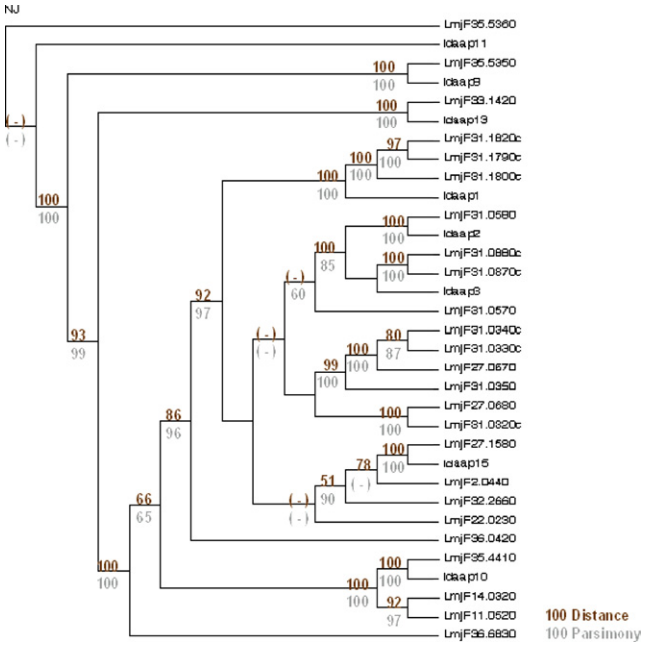

Fig. 1. Neighbor joining tree of *Leishmania* amino acid permease genes. A phylogenetic tree was constructed, using Paup 4.0 sequences of 25 putative *L. major* amino acid permease genes and eight *L. donovani* AAP genes. Numbers above branches indicate bootstrap distance values while numbers below branches indicate bootstrap parsimony values.

In order to select and classify from the resultant list motifs that are specific to *Leishmania*, the following filters were applied:

1. Sequences that either do not appear in *Leishmania* AAPs or are highly redundant through the entire training set were omitted. This excluded 38 motifs from the list.
2. Motifs that appear within *Leishmania* sequences related to the APC superfamily, such as AAP10LD, were found inconsistent due to their little representation among the training set. Thus, three additional motifs were excluded.
3. In order to exclude false positives that might be misrecognized as *Leishmania*-specific motifs, an additional training set that contained 27 sequences from the pfam 01490 seed, and *AAPLD1*, *2*, *3*, *8*, *11*, *13*, and *15* was created and analyzed by MEME. In this way we may identify motifs that overlap individual sequences from the Pfam seed that cannot be considered as specific to *Leishmania*. In this way, eight additional pfam 01490 overlapping motifs were identified and excluded from the list.

The result of this selection left us with seven motifs, which were sorted into three groups: two *Leishmania*-specific motifs (L1 and L2, Fig. 5A); four motifs that are conserved among *Trypanosoma* and *Leishmania* (Trypanosomatidae-specific; T1–T4; Fig. 5B). Unexpectedly, a Trypanosomatidae-motif was found to be conserved within mammalian genes from the systems N and A1, exclusively (N motif, Fig. 5B). L1 and L2 are present in AAP8LD, AAP11LDm and AAP13LD while T1-4 and N appear in AAP1LD, AAP2LD, AAP3LD, and AAP15LD.

Fig. 6 summarizes the pattern profiles of each motif. The L motif pattern was constructed by multiple alignments of the AAP8LD, AAP11LD, and AAP13LD protein sequences together with the corresponding putative amino acid permease sequences from *L. major*. While summarizing the results of this work, the *T. cruzi* genome annotations have been published in GenBank. This database included 12 putative AAP genes that were

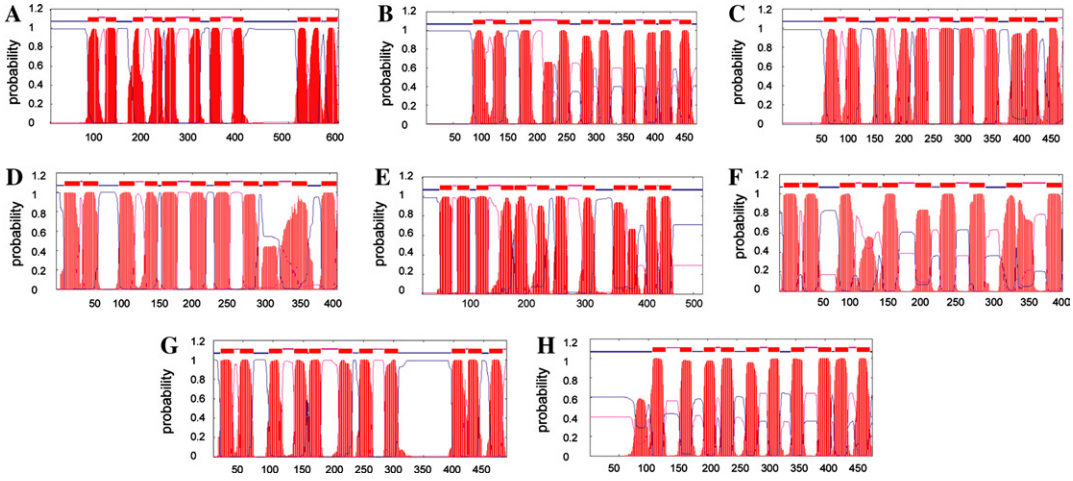

Fig. 2. Hydropathy analyses of the AAPLDs. Protein sequences from (A) AAP1LD, (B) AAP2LD, (C) AAP3LD, (D) AAP8LD, (E) AAP10LD, (F) AAP11LD, (G) AAP13LD, and (H) AAP15LD were analyzed by TMHMM 2.0 in order to identify transmembrane domains. Hydrophobic transmembrane domains are showed on the graph by columns; the dark lines correspond to intracellular domains and the light lines to extracellular domains.

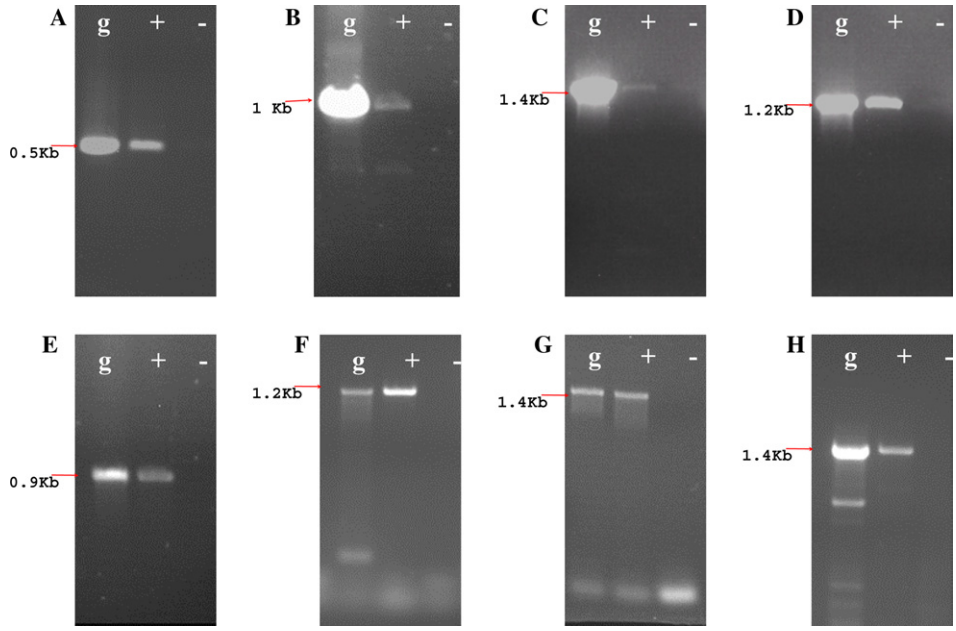

Fig. 3. mRNA expression of AAPLDs. RT-PCRs were performed on total RNA that was isolated from promastigotes in the presence (+) or absence (−) of reverse transcriptase. A control PCR was performed for each gene using genomic DNA as template (g). (A) AAP1LD, (B) AAP2LD, (C) AAP3LD, (D) AAP8LD, (E) AAP10LD, (F) AAP11LD, (G) AAP13LD, and (H) AAP15LD.

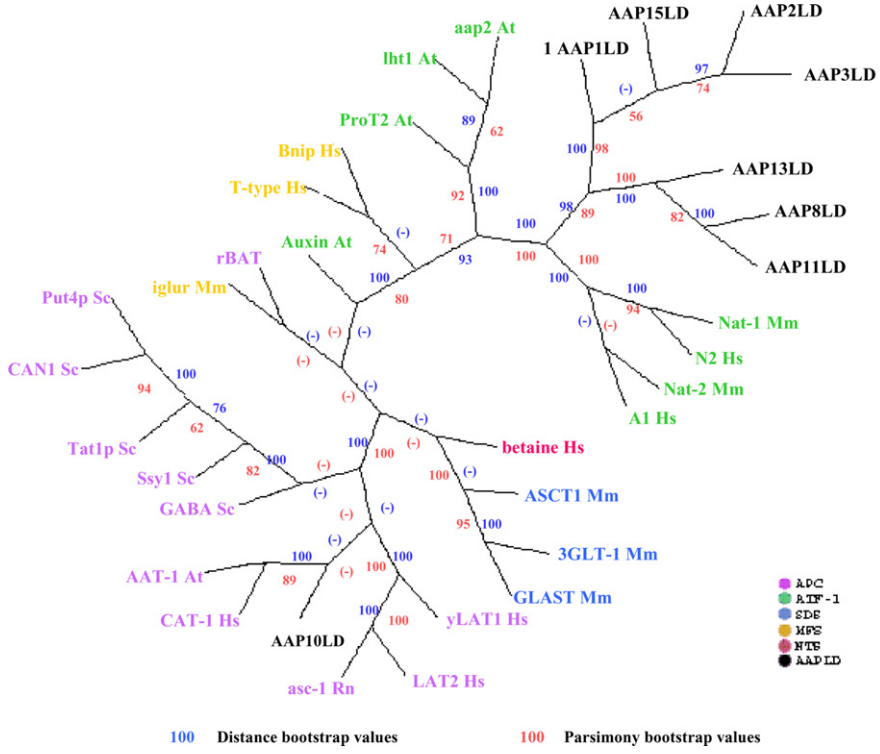

Fig. 4. AAPLDs are members of the ATF-1 and APC amino acid permease superfamilies. Phylogenic analysis of AAPLD with representatives of all amino acid permease superfamilies was carried out, using Paup 4.0. Bootstrap distance values are shown in blue, parsimony bootstrap values are in red. ATF-1 (green), APC (pink), SDS (blue), NTS (red), and MFS (yellow) superfamilies and AAPLDs (black). Accession number of each gene is listed in Table 3.

added to the pattern analysis list. T motif profiles were constructed by multiple alignments of the AAP1LD, AAP2LD, AAP3LD, and AAP15LD protein sequences together with the corresponding putative amino acid permease sequences from *L. major*, *T. brucei*, and *T. cruzi*. N motif profiles were constructed by conducting a

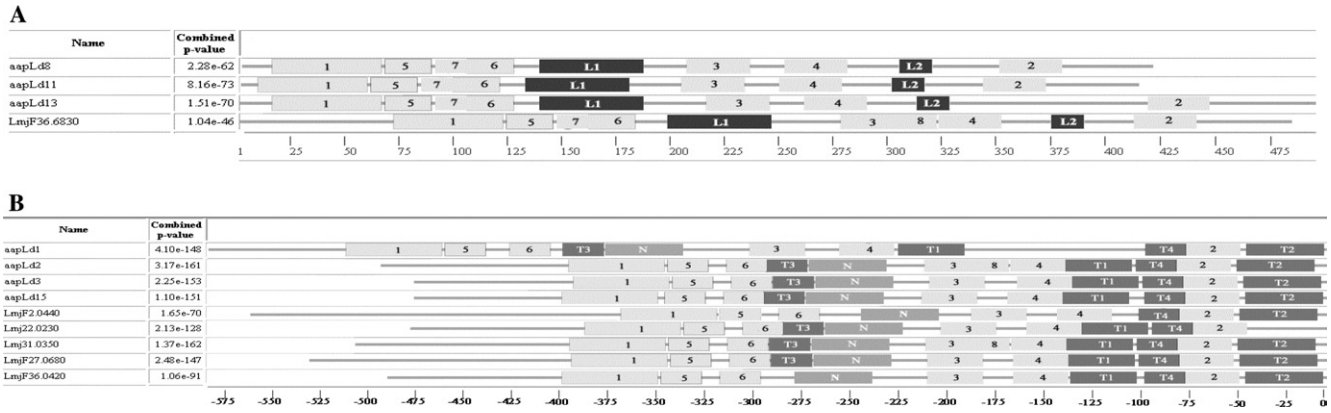

Fig. 5. Motif localization within AAPLD proteins. (A) *Leishmania*-specific motifs (L1, L2) appear in AAPLDs 8, 11, and 13 while (B) Trypanosomatidae-specific motifs (T1–T4) and mammalian-trypanosomatidae motif (N) appear in AAPLDs 1–3 and 15. The light gray boxes numbered 1-8 correspond to motifs conserved within the pfam 01490 domain. Black boxes indicate L1-2 and T1-4 motifs and a dark gray box represents the N motif. Note that in (B) the sequences are aligned to the C-terminus and labeled with negative numbers.

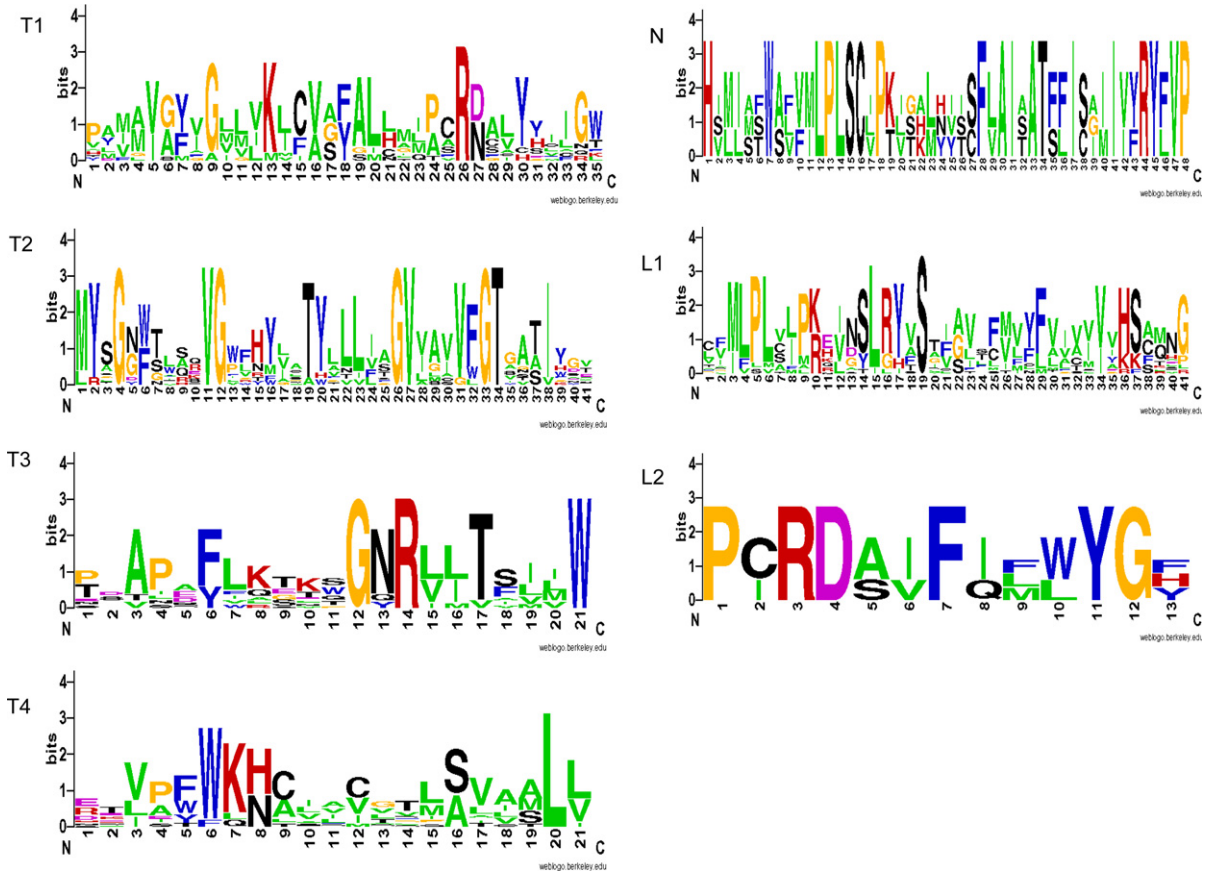

Fig. 6. Pattern profiles of the motifs.

multiple alignment analysis of the AAP1LD, AAP2LD, AAP3LD, and AAP15LD protein sequences together with the corresponding putative amino acid permease sequences from *L. major*, *T. brucei*, *T. cruzi*, and various mammalian N and A1 permeases. Interestingly, although the training set for motif elucidation did not include the *T. cruzi* AAP sequences, PAT1-5 and 8 contained motifs T1-4 and N. This observation further supports the accuracy of the motifs.

L, T, and N motifs co-localize in clusters

The two L motifs appear to be position-specific in AAPLD 8, 11, and 13 (Fig. 5A). Hydropathy analysis

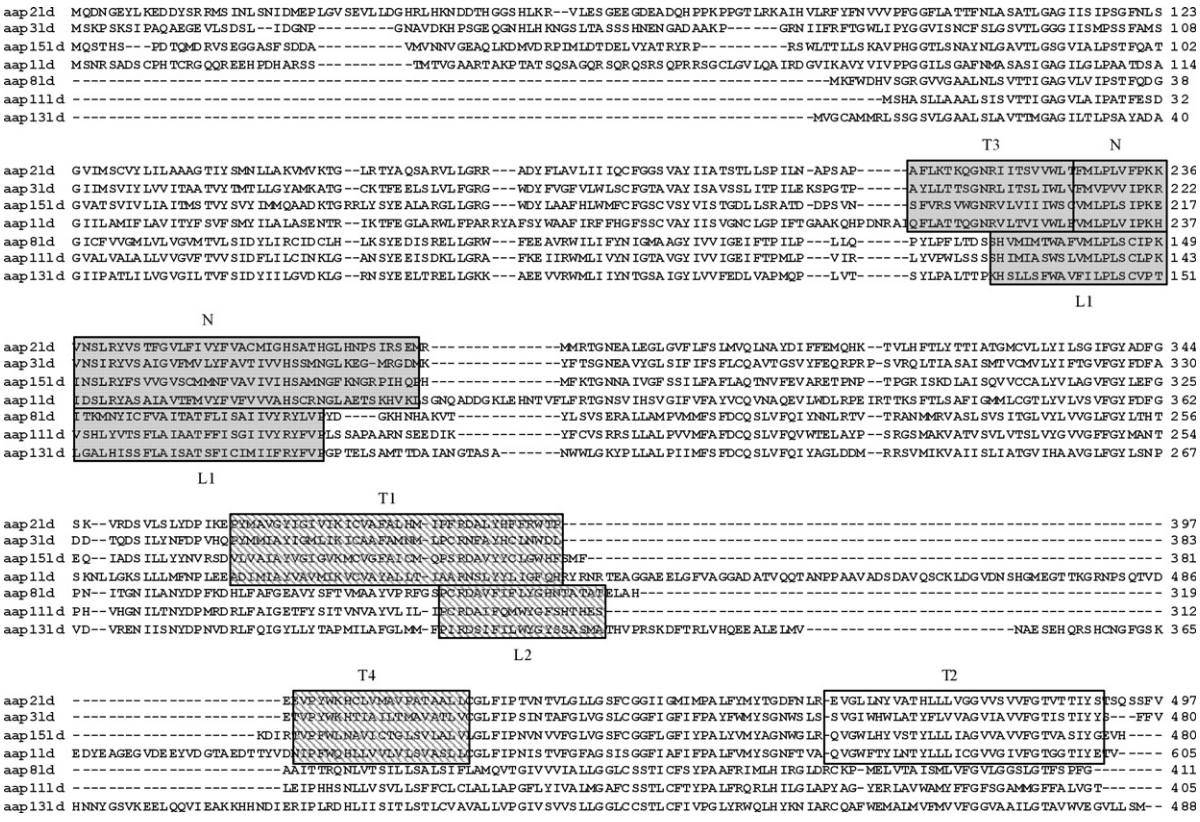

Fig. 7. Multiple alignment of AAPLD amino acid sequences. AAPLDs 1, 2, 3, 8, 11, 13, and 15 were aligned by Clustal-x. Cluster 1 (gray boxes) contains T3 and N in AAPLDs 1–3 and 15, L1 in AAPLDs 8, 11, and 13. Cluster 2 (dashed boxes) contains T1 and T4 in AAPLDs 1–3 and 15 and L2 in AAPLDs 8, 11, 13. In AAP1LD, T1 and T4 are separated by a region of ~100 hydrophilic amino acids. The C-terminus (white box) contains the motif T2 specifically on AAPLDs 1–3 and 15.

of these genes indicated that L1 is localized to transmembrane helixes four and five, which are separated by a very short hydrophilic stretch (between 2 and 5 amino acids). L2 is localized to the eighth transmembrane domain.

Motifs T and N co-localize when AAPLDs 1, 2, 3, and 15 are aligned to their C-termini (Fig. 5B). Hydropathy analysis of these genes indicated co-localization of N and T3 with transmembrane domains seven and eight, respectively, starting from the C-terminus. T1 and T4 co-localize between the third and the fourth C-justified transmembrane region, and T2 is located at the very C terminal membrane spanning domain. Motifs T1 and T4 might be seen as a single conserved region in most genes. However, in AAP1LD they are separated by an extracellular hydrophilic stretch of ~100 amino acids (Fig. 5A). We assume that this is the reason MEME considered them as two separate motifs.

Multiple alignment of AAPLDs 1, 2, 3, 8, 11, 13, and 15 amino acid sequences indicated that L1 and N co-localized in their corresponding proteins. Likewise, L2 coincided with T1 (Fig. 7). Three amino acids in each co-localized pair, the LPL trimer in N and L1 and the PXRD tetramer in T1 and L2, are likely to be the co-localization anchor as the other amino acid compositions are different between L and T/N motifs (Fig. 7, underline). This indicates that most of the motifs appear in two clusters of homology, which we named cluster 1 and cluster 2 (Fig. 7, gray and dashed rectangles, respectively).

The conservation pattern of the T2 motif is especially interesting due to its symmetry ($GX_6VGX_7TX_6GVX_5GT$) and localization in the C-terminus of the protein.

The next step was to validate the results by probing the motif consensus sequences in BLASTp, using *Leishmania*-based consensuses from each motif and a PHI-BLAST regular expression profile. Only L motifs were probed without an expression profile and retrieved high scores ($3e^{-17}$–$4e^{-4}$) exclusively for AAPLD 8, 11 and 13 (Appendix); T motifs retrieved high scores only for trypanosomatidae (Appendix). N motif BLAST analysis resulted in a mixture of trypanosomatidae and mammalian N and A1 system protein sequences (Appendix).

Discussion

Eight amino acid permease genes were cloned, sequenced, and shown to be expressed in *L. donovani*

promastigotes. Seven of these belong to the ATF-1 and one to APC superfamilies, respectively [1]. Since these permeases are highly identical (>90%) to the putative *L. major* AAPs and since they represent the different family branches within *Leishmania* (Fig. 1), we predict that the *L. major* permeases also belong to these superfamilies.

This study revealed two motifs that are specific to the genus *Leishmania* (L motifs), four to the family trypanosomatidae (T motifs), and a single motif that is common between trypanosomatidae and mammalian A1 and N permeases (N motif) [20,21]. These localized to membrane spanning domains and are excluded from regions that are highly similar to pfam 01490 seed.

The most significant observation of this work is that the motifs are clustered in two regions of 50–60 amino acids. A multiple alignment of the protein sequences indicated that cluster 1 contains T3 and N motifs (AAPLD1-3 and 15), which co-localize with L1 (AAPLD8, 11, and 13). Cluster 2 contains T1 and T4 (AAPLD1-3 and 15), which co-localize with L2 (AAPLD8, 11 and 13). The anchors that determine the co-localizations are the highly conserved residues "W" and "LPL" in cluster 1 and "PXRD" in cluster 2. These clusters may indicate regions that are significant for permease function in *Leishmania*.

The C-terminus region of the *Leishmania* AAP genes contains the T2 motif that shows a symmetric conservation pattern ($GX_6VGX_7TX_6GVX_5GT$). The existence of a motif in this region suggests that T2 might play a regulatory role in permease activity. A previous work on GLT-1 glutamate transporter indicated a residue of 43 amino acids at the C-terminus that binds protein kinase C and thereby regulates transport activity [22].

BLAST search indicated a close relationship between *L. donovani* and *T. brucei* amino acid permeases. While working on this paper, 12 amino acid permeases from the *T. cruzi* were annotated (PAT1-12). BLAST analysis and regular expression profiles indicated that T and N motifs are also present in the *T. cruzi* AAPs (PAT1–5 and 8) further validating the conservation of the motifs.

Furthermore, our phylogenic analysis showed that AAPLD genes that belong to ATF-1 superfamily are more related to mammalian systems N and A1 than to plant AAPs. In addition, N and A1 share with *Leishmania* AAPs the following features: (1) They span the membrane 9–11 times, (2) both are cation-dependent, similar to the physiologically characterized transport systems from *L. donovani* [12] (Shaked-Mishan et al. unpublished data), (3) as in *Leishmania*, osmoregulation is activated by amino acid fluxes, which is mediated by system A1 in mammalian cells [23,24], and (4) both system N and A1 genes are expressed in the liver, which is the main target tissue of *L. donovani* [4].

Similarity between *Leishmania* and mammalians genes is in contrast with a recent study on metabolic enzymes that indicated a close relationship to corresponding genes in plants. They suggested gene transfer to be the mechanism responsible for the observed similarity [25]. The resemblance between AAPLD and mammalian AAP genes cannot be explained as the product of conservation of an ancestral precursor. If that was the case, we should see representatives from other animal species sharing this similarity. Thus, it is more likely to explain the specific similarity between AAPLD and N/A1 in the basis of parasite–host relationships. Since N and A1 proteins are expressed in specific organs such as the liver and given that this organ is the main target of *L. donovani*, it is likely that proteins that are both expressed in the mammalian liver and the parasite had evolved similarly due to the same evolutionary forces and environmental conditions or gene transfer. However, the existence of genera and family specific motifs suggests that the evolution of these proteins is also affected by additional factors that are unique to the parasite.

Except for AAP10LD, the *L. donovani* amino acid permeases described in this work contain 11 transmembrane sequences (TMS). Although this is not unusual for members of the ATF-1 superfamily, the relatively large number of permeases with an odd number of TMS raises questions on the evolution of these proteins. In a series of bioinformatic analyses, Saier [26] recently proposed that gene duplication and fusion lead to the formation of 10–14 TMS permeases. He suggested that an odd number of TMS was developed by either fusion between genes that code for permeases with even and odd TMS, or following gene duplication, a single TMS was lost from the C-termini of these proteins during evolutionary history [27]. Hence, it will be interesting to assess this hypothesis in relation to *Leishmania* evolution and host–parasite interactions.

The results of this paper describe the cloning of the first amino acid permease genes in parasitic protozoa. Phylogenic analyses contribute to the understanding of amino acid permease evolution. Furthermore, the identification of genus-specific motifs in these proteins might be useful to better understand parasite physiology within its hosts. Lastly, these motifs might improve molecular diagnosis of leishmaniasis.

Acknowledgments

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Appendix

Validation of the motifs by BLASTp

L1

		Score	E-value
☑	gb|AAO88096.1| amino acid permease AAP11LD [Leishmania donovani]	89	3e-17
☑	gb|AAO88098.1| amino acid permease AAP8LD [Leishmania donovani]	68	4e-11
☑	gb|AAR25623.1| amino acid permease AAP13LD [Leishmania donovani]	65	2e-10
☐	gb|AAH39731.1| PRAME protein [Homo sapiens]	31	4.9
☐	ref|NP_006106.1| preferentially expressed antigen in melanoma; m...	31	5.1
☐	gb|EAA40403.1| GLP_43_3422_2040 [Giardia lamblia ATCC 50803]	31	5.2
☐	gb|AAH31853.1| BC031853 protein [Mus musculus]	31	6.8
☐	ref|NP_766346.1| hypothetical protein D430050E18 [Mus musculus] ...	30	7.1

L2

☑	gb|AAO88096.1| amino acid permease AAP11LD [Leishmania donovani]	59	2e-08
☑	gb|AAR25623.1| amino acid permease AAP13LD [Leishmania donovani]	55	5e-07
☑	gb|AAO88098.1| amino acid permease AAP8LD [Leishmania donovani]	45	4e-04
☐	ref|NP_611578.1| CG30394-PB [Drosophila melanogaster] >gi|246569...	33	2.0

T1

		Score	E-value
☑	gi|13751810|emb|CAC37212.1| possible amino acid transporter [Lei...	61	6e-09
☑	gi|29650766|gb|AAO88094.1| amino acid permease AAP3LD [Leishmani...	59	2e-08
☑	gi|13751797|emb|CAC37199.1| possible amino acid transporter [Lei...	55	3e-07
☑	gi|29650899|gb|AAO88097.1| amino acid permease AAP2LD [Leishmani...	55	4e-07
☑	gi|25005075|gb|AAN70991.1| amino acid permease [Leishmania donov...	45	3e-04
☑	gi|38607492|gb|AAR25622.1| amino acid permease AAP15LD [Leishman...	43	0.001
☐	gi|22003080|emb|CAC86552.1| amino acid transporter AATP11 [Trypa...	38	0.049
☐	gi|22003076|emb|CAC86550.1| amino acid transporter AATP9 [Trypan...	35	0.32
☐	gi|22003070|emb|CAC86547.1| amino acid transporter AATP6 [Trypan...	35	0.36
☐	gi|22003066|emb|CAC86545.1| amino acid transporter AATP3 [Trypan...	35	0.37
☐	gi|22003064|emb|CAC86544.1| amino acid transporter AATP1 [Trypan...	35	0.42
☐	gi|13751838|emb|CAC37240.1| probable transporter [Leishmania major]	33	1.1
☐	gi|22003068|emb|CAC86546.1| amino acid transporter AATP5 [Trypan...	32	2.4

T2

☑	gi|38607492|gb|AAR25622.1| amino acid permease AAP15LD [Leishman...	85	1e-16
☑	gi|13751810|emb|CAC37212.1| possible amino acid transporter [Lei...	72	1e-12
☑	gi|29650766|gb|AAO88094.1| amino acid permease AAP3LD [Leishmani...	68	2e-11
☑	gi|13751797|emb|CAC37199.1| possible amino acid transporter [Lei...	65	2e-10
☑	gi|29650899|gb|AAO88097.1| amino acid permease AAP2LD [Leishmani...	59	7e-09
☑	gi|25005075|gb|AAN70991.1| amino acid permease [Leishmania donov...	55	1e-07
☑	gi|13751838|emb|CAC37240.1| probable transporter [Leishmania major]	54	3e-07
☑	gi|22003070|emb|CAC86547.1| amino acid transporter AATP6 [Trypan...	52	1e-06
☑	gi|22003076|emb|CAC86550.1| amino acid transporter AATP9 [Trypan...	49	8e-06
☑	gi|22003078|emb|CAC86551.1| amino acid transporter AATP10 [Trypa...	48	2e-05
☑	gi|22003074|emb|CAC86549.1| amino acid transporter AATP8 [Trypan...	48	2e-05
☑	gi|22003072|emb|CAC86548.1| amino acid transporter AATP7 [Trypan...	48	2e-05
☑	gi|22003068|emb|CAC86546.1| amino acid transporter AATP5 [Trypan...	47	3e-05
☑	gi|22003080|emb|CAC86552.1| amino acid transporter AATP11 [Trypa...	45	1e-04
☑	gi|22003064|emb|CAC86544.1| amino acid transporter AATP1 [Trypan...	44	2e-04
☑	gi|22003066|emb|CAC86545.1| amino acid transporter AATP3 [Trypan...	44	3e-04

(*continued on next page*)

Appendix (*continued*)

T3

☑	qb|AA088097.1|	amino acid permease AAP2LD [Leishmania donovani]	75	3e-13
☑	emb|CAC37199.1|	possible amino acid transporter [Leishmania major]	73	1e-12
☑	emb|CAC37212.1|	possible amino acid transporter [Leishmania major]	55	3e-07
☑	qb|AA088094.1|	amino acid permease AAP3LD [Leishmania donovani]	55	4e-07
☑	emb|CAC86544.1|	amino acid transporter AATP1 [Trypanosoma brucei...	52	2e-06
☑	emb|CAC86550.1|	amino acid transporter AATP9 [Trypanosoma brucei...	51	4e-06
☑	emb|CAC86545.1|	amino acid transporter AATP3 [Trypanosoma brucei...	51	5e-06
☑	emb|CAC86549.1|	amino acid transporter AATP8 [Trypanosoma brucei...	50	7e-06
☑	emb|CAC86548.1|	amino acid transporter AATP7 [Trypanosoma brucei...	50	1e-05
☑	emb|CAC86551.1|	amino acid transporter AATP10 [Trypanosoma bruce...	50	1e-05
☑	emb|CAC86546.1|	amino acid transporter AATP5 [Trypanosoma brucei...	47	1e-04
☑	qb|AAN70991.1|	amino acid permease [Leishmania donovani]	45	2e-04
☑	qb|AAR25622.1|	amino acid permease AAP15LD [Leishmania donovani]	44	8e-04
☐	emb|CAC86552.1|	amino acid transporter AATP11 [Trypanosoma bruce...	37	0.063
☐	emb|CAC86547.1|	amino acid transporter AATP6 [Trypanosoma brucei...	37	0.098
☐	ref|ZP_00058117.1|	COG0839: NADH:ubiquinone oxidoreductase subun...	32	3.9

T4

☑	qb|AA088097.1|	amino acid permease AAP2LD [Leishmania donovani]	78	4e-14
☑	emb|CAC37199.1|	possible amino acid transporter [Leishmania major]	76	2e-13
☑	emb|CAC86550.1|	amino acid transporter AATP9 [Trypanosoma brucei...	51	5e-06
☑	emb|CAC86545.1|	amino acid transporter AATP3 [Trypanosoma brucei...	51	6e-06
☑	emb|CAC37212.1|	possible amino acid transporter [Leishmania major]	50	9e-06
☑	emb|CAC86544.1|	amino acid transporter AATP1 [Trypanosoma brucei...	50	1e-05
☑	qb|AAR25622.1|	amino acid permease AAP15LD [Leishmania donovani]	49	2e-05
☑	qb|AA088094.1|	amino acid permease AAP3LD [Leishmania donovani]	49	3e-05
☑	emb|CAC86547.1|	amino acid transporter AATP6 [Trypanosoma brucei...	44	0.001
☐	emb|CAC86549.1|	amino acid transporter AATP8 [Trypanosoma brucei...	43	0.001
☐	emb|CAC86546.1|	amino acid transporter AATP5 [Trypanosoma brucei...	42	0.002
☐	emb|CAC86548.1|	amino acid transporter AATP7 [Trypanosoma brucei...	41	0.005
☐	emb|CAC86551.1|	amino acid transporter AATP10 [Trypanosoma bruce...	40	0.013
☐	emb|CAC37240.1|	probable transporter [Leishmania major]	39	0.022
☐	qb|AAN70991.1|	amino acid permease [Leishmania donovani]	35	0.30
☐	emb|CAC86552.1|	amino acid transporter AATP11 [Trypanosoma bruce...	32	2.0

N

☑	gi|38607492|qb|AAR25622.1|	amino acid permease AAP15LD [Leishman...	22	1e-05
☑	gi|25005075|qb|AAN70991.1|	amino acid permease [Leishmania donov...	17	4e-04
☑	gi|22003068|emb|CAC86546.1|	amino acid transporter AATP5 [Trypan...	17	5e-04
☐	gi|31543737|ref|NP_081328.2|	solute carrier family 38, member 4;...	6	0.87
☐	gi|18543357|ref|NP_570104.1|	amino acid transport system A3 [Rat...	6	0.87
☐	gi|18482385|ref|NP_060488.2|	solute carrier family 38, member 4;...	6	0.87
☐	gi|18369791|dbj|BAB84091.1|	system A amino acid transporter 3 [M...	6	0.87
☐	gi|7022083|dbj|BAA91481.1|	unnamed protein product [Homo sapiens]	6	0.87
☐	gi|7023123|dbj|BAA91846.1|	unnamed protein product [Homo sapiens]	5	2.1
☐	gi|21361602|ref|NP_061849.2|	solute carrier family 38, member 2;...	5	2.1
☐	gi|15216171|emb|CAC51434.1|	putative 40-9-1 protein [Homo sapiens]	5	2.1

Appendix (*continued*)

gi|22760478|dbj|BAC11215.1|	unnamed protein product [Homo sapiens]	4	2.6
gi|22760410|dbj|BAC11186.1|	unnamed protein product [Homo sapiens]	4	2.6
gi|31543733|ref|NP_598847.2|	solute carrier family 38, member 1 ...	4	2.6
gi|21361929|ref|NP_109599.2|	amino acid transporter system A1 [H...	4	2.6
gi|26338786|dbj|BAC33064.1|	unnamed protein product [Mus musculus]	4	2.6
gi|20301960|ref|NP_620187.1|	solute carrier family 38, member 1;...	4	2.6
gi|14042788|dbj|BAB55394.1|	unnamed protein product [Homo sapiens]	4	2.6
gi|26325174|dbj|BAC26341.1|	unnamed protein product [Mus musculu...	4	2.6
gi|13124897|gb|AAG43433.2|	amino acid transporter Nat-2 [Mus mus...	4	2.6
gi|11359856|pir||JC7328	amino acid transporter A1 - human >gi|11...	4	2.6
gi|22760727|dbj|BAC11310.1|	unnamed protein product [Homo sapiens]	4	2.6
gi|8677401|gb|AAF75589.2|	system A transporter isoform 2 [Rattus...	4	4.0
gi|26327471|dbj|BAC27479.1|	unnamed protein product [Mus musculus]	4	4.0
gi|31543735|ref|NP_780330.2|	solute carrier family 38, member 2 ...	4	4.0
gi|37360348|dbj|BAC98152.1|	mKIAA1382 protein [Mus musculus]	4	4.0

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