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Diverse telomeres in trypanosomatids

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Research Article

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Abstract

Telomeres are the ends of linear eukaryotic chromosomes facilitating the resolution of the 'end replication and protection' problems, associated with linearity. At the nucleotide level, telomeres typically represent stretches of tandemly arranged telomeric repeats, which vary in length and sequence among different groups of organisms. Recently, a composition of the telomere-associated protein complex has been scrutinized in *Trypanosoma brucei*. In this work, we subjected proteins from that list to a more detailed bioinformatic analysis and delineated a core set of 20 conserved proteins putatively associated with telomeres in trypanosomatids. Out of these, two proteins (Ku70 and Ku80) are conspicuously missing in representatives of the genus *Blastocrithidia*, yet telomeres in these species do not appear to be affected. In this work, based on the analysis of a large set of trypanosomatids widely different in their phylogenetic position and life strategies, we demonstrated that telomeres of trypanosomatids are diverse in length, even within groups of closely related species. Our analysis showed that the expression of two proteins predicted to be associated with telomeres (those encoding telomerase and telomere-associated hypothetical protein orthologous to Tb927.6.4330) may directly affect and account for the differences in telomere length within the species of the *Leishmania mexicana* complex.

Introduction

Trypanosomatidae is a family of protozoan parasites possessing a single large mitochondrion, which encompasses a network of catenated circular DNA molecules, the so-called kinetoplast or kDNA (Maslov *et al.*, 2019). These species have been attracting research attention because of numerous unique or rare biochemical and molecular traits, such as *trans*-splicing and polycistronic transcription (Clayton, 2019; Michaeli, 2011), mitochondrial RNA editing (Aphasizheva *et al.*, 2020), presence of modified nucleotides (van Luenen *et al.*, 2012) and unusual organelles (Szöör *et al.*, 2014; Docampo, 2016), or a bizarre variation of the nuclear genetic code (Záhonová *et al.*, 2016). Most of these flagellates are monoxenous (with one host in their life cycle) parasites restricted to invertebrates (Maslov *et al.*, 2013), while members of the genera *Endotrypanum*, *Leishmania*, *Phytomonas*, *Porcisia* and *Trypanosoma* have switched to dixeny (two-host life cycle) and infect vertebrates or plants in addition to invertebrates (Lukeš *et al.*, 2018). It is established beyond a reasonable doubt that the dixenous species have evolved from the monoxenous ancestor(s) independently several times (Lukeš *et al.*, 2014). Notably, several *Leishmania* and *Trypanosoma* spp. are of medical importance, as they cause severe human diseases, and are fairly well-studied (Stuart *et al.*, 2008; Nussbaum *et al.*, 2010).

Telomeres typically represent repetitive physical ends of linear eukaryotic chromosomes, variable in length and sequence in different groups of organisms (Fulnečková *et al.*, 2013). Their main role is to protect chromosome ends from being recognized and processed as DNA double-strand breaks by the cellular repair machinery in order to prevent chromosomal end-to-end fusions (Pfeiffer and Lingner, 2013). Such shielding is provided by the telomere-associated protein complexes (Lewis and Wuttke, 2012) or specific complementary DNA structures, such as telomere loops (t-loops) facilitating the protection of chromosome ends (Tomáška *et al.*, 2019). It is generally assumed that telomeres undergo gradual shortening with each round of cell division because of incomplete lagging strand synthesis of linear DNA templates by DNA polymerases, known as the 'end replication problem' (Olovnikov, 1973; Greider, 1990; Hackett and Greider, 2002). In order to overcome this problem and, thus, prevent telomere shortening, cells engage a dedicated enzyme called telomerase (Greider and Blackburn, 1985).

Telomeres of kinetoplastids share many traits with those of other eukaryotes. They have the canonical sequence (5′-ttaggg-3′) found in vertebrates, end with a t-loop, are associated with capping protein complexes and maintained by telomerases (Muñoz-Jordán *et al.*, 2001; Conte and Cano, 2005; Fulnečková *et al.*, 2013). Similar to the situation in other eukaryotic pathogens, genes encoding trypanosomatid virulence factors are often located in the sub-telomeric regions and their expression may be co-regulated with telomeres (Chiurillo *et al.*, 1999;

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Table 1. Predicted telomere-associated protein complex composition in *T. brucei*

Protein ID	Annotation	Protein function	References
Tb927.2.6100	Hypothetical protein	Essential for cell growth and kinetoplast (k)DNA maintenance; kDNA was reduced in size or lost upon RNAi-mediated knock-down of the coding gene	Beck *et al.* (2013)
Tb927.3.1560	TRF-interacting factor 2, TIF-2	Interacts with the ttaggg binding factor (TRF), protecting it from degradation. Its transient depletion decreases level of TRF and increases frequency of variant surface glycoprotein (VSG) switching and sub-telomeric double-strand breaks (DSB)	Jehi *et al.* (2016), Jehi *et al.* (2014*b*)
Tb927.5.1700	Replication factor A 28 kDa subunit, RPA-2	Accumulates at DSB sites, where it forms RPA foci, stabilizing resected DNA and triggering cell cycle arrest, RAD51 accumulation and damage repair. The protein was shown to persist throughout the cell cycle in *T. brucei* and regulate metacyclogenesis in *T. cruzi*	Glover *et al.* (2019), Pavani *et al.* (2016)
Tb927.6.4330	hypothetical protein	Affects VSG allelic exclusion	Glover *et al.* (2016)
Tb927.9.10770	Polyadenylate-binding protein 2, PABP-2	An abundant mRNA binding protein involved in translation initiation and general mRNA metabolism	Kramer *et al.* (2013), Zoltner *et al.* (2018)
Tb927.9.15360	40S ribosomal protein S6	Regulates numerous cellular processes in eukaryotes	Ruvinsky and Meyuhas (2006)
Tb927.9.5020	HMG-box domain-containing protein	Generally, these small proteins bind DNA and regulate transcription, replication and DNA repair	Hock *et al.* (2007)
Tb927.9.8740	Double-stranded RNA Binding Domain protein 3, DRBD3	One of RNA-binding proteins (RBPs) that regulate abundance of the specific subset of mRNAs. Its depletion results in a growth arrest followed by the cell death	Estévez (2008)
Tb927.10.12850	ttaggg binding factor, TRF	Essential for telomere end protection. Its ablation caused drastic reduction of G overhangs and chromosome end fusions without affecting the overall telomere length. Expression of TRF with reduced DNA binding affinity leads to increased VSG switching	Jehi *et al.* (2014*a*), Li *et al.* (2005)
Tb927.10.2520	PrimPol-like protein 2, PPL-2	A translesion polymerase accumulating in G2 phase of trypanosome cell cycle and involved in postreplication tolerance of endogenous DNA damage. Its knock-down leads to the cell cycle arrest prior to mitosis in late S/G2 and activation of the DNA damage response	Rudd *et al.* (2013)
Tb927.10.6030	Proteasome Subunit Alpha type-1, PSA-1	A part of a eukaryotic proteasome 20S catalytic core complex. In parasites, proteasomes are involved in cell differentiation and replication	Paugam *et al.* (2003)
Tb927.10.6220	5′-3′ exoribonuclease D, XRND	A member of the XRN family of 5′-3′ exoribonucleases critical for ensuring the fidelity of cellular RNA turnover in eukaryotes. Its knock-down in *T. brucei* inhibited cell growth, but did not affect 5′ processing of several small RNAs	Li *et al.* (2006)
Tb927.11.370	Repressor Activator Protein 1, RAP-1	A telomeric protein recruited by TRF. Its depletion led to a de-repression of all VSGs in silent expression sites, without affecting telomere length, and resulted in the increased frequencies of the non-coding telomeric repeat-containing RNA (TERRA) and RNA:DNA hybrids and, subsequently, DSBs in telomeric and subtelomeric loci	Nanavaty *et al.* (2017), Yang *et al.* (2009)
Tb927.11.5550	DNA polymerase θ, Pol θ	A translesion DNA polymerase involved in the repair of DSBs *via* microhomology-mediated end joining. Its RNAi-mediated depletion resulted in reduced growth rate without a specific cell cycle arrest, accumulation of DNA damage and chromosome segregation defects, and substantial de-regulation of telomeric VSG genes. Orthologues in *T. cruzi* and *L. infantum* control DNA replication and resistance to oxidative damage	de Lima *et al.* (2019), Fernández-Orgiler *et al.* (2016), Leal *et al.* (2020)

(*Continued*)

Table 1. (*Continued.*)

Protein ID	Annotation	Protein function	References
Tb927.11.9870	Telomere-associated protein 1, TelAP-1	Significantly upregulated in the bloodstream compared to the procyclic forms of *T. brucei*. Depletion of this protein mis-regulated developmental silencing of VSG silent expression sites	Reis *et al.* (2018)
Tb927.3.5030	Ku70 protein	Ku proteins play a central role in the 'classical' non-homologous end joining (NHEJ) pathway. In addition, they bind telomeres and facilitate recruitment of telomerase. Trypanosomatids mainly rely on other (not NHEJ) pathways for DNA repair, yet, with a few notable exceptions, they retained genes encoding Ku proteins in their genomes. Of note, ablation of Ku proteins resulted in rather ambiguous telomeric phenotypes in different organisms	Boulton and Jackson (1998), Chico *et al.* (2011), Janzen *et al.* (2004), Nenarokova *et al.* (2019), Riha and Shippen (2003)
Tb927.6.1760	Ku80 protein		
Tb927.11.10190	Telomerase reverse transcriptase	Comprised of two essential core subunits: the TElomerase RNA (TER) and TElomerase Reverse Transcriptase protein (TERT). Depletion of either component in *T. brucei* is associated with telomere shortening. Trypanosomes can maintain critically short telomeres using an alternative telomerase independent maintenance mechanism. Overexpression of this protein in *L. major* increased cell proliferation rate and resistance to oxidative stress	Campelo *et al.* (2015), Dreesen and Cross (2006), Dreesen *et al.* (2005), Sandhu *et al.* (2013)

Dobson *et al.*, 2006; Hovel-Miner *et al.*, 2012). Moreover, transposable elements are often found in association with telomeres (Pardue *et al.*, 1997; Rahnama *et al.*, 2020). In agreement with this, a sub-telomeric region of *Leptomonas pyrrhocoris* chromosome contains an integrated copy of an RNA-dependent RNA polymerase putatively originating from an RNA virus of the family *Tombusviridae* infecting this flagellate (Grybchuk *et al.*, 2018), and possibly contributing to the retrotransposon translocation within the trypanosomatid genome. Telomeric regions of kinetoplastid chromosomes also possess several features distinguishing them from their counterpart in most of the other eukaryotes. For example, the telomeres of *Trypanosoma brucei* increase in length (by approximately 10 bp per generation) until they reach an equilibrium (Bernards *et al.*, 1983; Pays *et al.*, 1983; Horn *et al.*, 2000). In trypanosomatids, a modified nucleobase, base J (*β*-D-gluco-pyranosyl-oxy-methyl-uracil) is involved in RNA polymerase II transcription termination and is preferentially localized to telomeres (Borst and van Leeuwen, 1997; Genest *et al.*, 2007; van Luenen *et al.*, 2012).

To the best of our knowledge, there has been very little systematic effort to analyse telomeres in trypanosomatids outside the medically relevant *Trypanosoma* and *Leishmania* spp. (Fu *et al.*, 1998; Fu and Barker, 1998*a*, 1998*b*; Chiurillo *et al.*, 1999, 2002; Muñoz-Jordán *et al.*, 2001; Janzen *et al.*, 2004; Conte and Cano, 2005; Genest and Borst, 2007). Therefore, we decided to do that for a wide range of trypanosomatids with a special emphasis on largely neglected parasites of insects, which are not pathogenic to humans. We selected more than 20 proteins from a set of recently defined putative trypanosomatid telomere-associated proteins (Reis *et al.*, 2018) for more detailed *in silico* analyses. For most of these proteins, some functional information is available [Table 1; the TriTrypDB (Aslett *et al.*, 2010) gene IDs are used throughout the text]. The predicted telomere-associated complex appears to be a cohort of proteins with widely variable functions, from ribosome and proteasome subunits to telomerase and even DNA repair proteins (Boulton and Jackson, 1998; Paugam *et al.*, 2003; Riha and Shippen, 2003; Janzen *et al.*, 2004; Dreesen *et al.*, 2005; Ruvinsky and Meyuhas, 2006; Chico *et al.*, 2011; Sandhu *et al.*, 2013; Nenarokova *et al.*, 2019). In this work, we analysed the evolutionary history of telomere-associated proteins in Kinetoplastea, performed a systematic analysis of telomere length variation among trypanosomatids on the dataset, which incorporates a wide range of understudied monoxenous members of the family Trypanosomatidae, and established a correlation between the level of transcription for several analysed telomere-associated proteins and the telomere length.

Materials and methods

In silico *analyses*

A putative set of telomere-associated proteins of *T. brucei brucei* TREU927 (Reis *et al.*, 2018) were used as queries for BLAST searches (Altschul *et al.*, 1990) against a dataset of annotated proteins of 64 trypanosomatids and the eubodonid *Bodo saltans*. First, BLASTp searches were performed with an *E*-value set to 1 and all the hits with an *E*-value not exceeding 10^{-15} were retained. If the respective sequence was not identified among annotated proteins, the searches were repeated with the tBLASTn algorithm against a database of genome sequences. In case no protein was identified in the genome, HMMER v.3.3 (Eddy, 2009), a more sensitive method for the identification of divergent homologues based on hidden Markov models was employed. Annotated proteins and assembled genome sequences were downloaded from the NCBI Genome (Sayers *et al.*, 2019) and TriTrypDB v. 45/46 (Aslett *et al.*, 2010) databases. The validity of the hits was confirmed using reciprocal BLAST searches against *T. brucei* proteins

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Table 2. Presence of genes putatively involved in telomere maintenance in kinetoplastids

	Tb927.2.6100: hypothetical protein	*Tb927.3.1560:* TRF-interacting factor 2	*Tb927.3.5030:* KU70 protein	*Tb927.3.5150:* exonuclease, putative	*Tb927.5.1700:* replication factor A 28 kDa subunit	*Tb927.6.1760:* KU80 protein	*Tb927.6.4330:* telomere-associated protein	*Tb927.9.10770:* polyadenylate-binding protein 2	*Tb927.9.15360:* 40S ribosomal protein S6	*Tb927.9.3930:* hypothetical protein	*Tb927.9.4000:* hypothetical protein	*Tb927.9.5020:* HMG-box domain-containing protein
Crithidia bombi 08.076			+	+	+	+	+	+	+			+
Crithidia expoeki BJ08.175			+	+	+	+	+	+	+			+
Crithidia fasciculata Cf-Cl			+	+	+	+	+	+	+			+
Leptomonas pyrrhocoris H10			+	+	+	+	+	+	+			+
Leptomonas seymouri ATCC30220			+	+	+	+	+	+	+			+
Lotmaria passim SF			+	+	+	+	+	+	+			+
Endotrypanum monterogeii ATCC30507			+	+	+	+	+	+	+			+
Endotrypanum monterogeii LV88			+	+	+	+	+	+	+			+
Porcisia deanei TCC258			+	+	+	+	+	+	+			+
Porcisia hertigi TCC260			+	+	+	+	+	+	+			+
Leishmania (M.) enriettii LEM3045			+	+	+	+	+	+	+			+
Leishmania (M.) macropodum LV756			+	+	+	+	+	+	+			+
Leishmania (M.) martiniquensis LEM2494			+	+	+	+	+	+	+			+
Leishmania (S.) adleri HO174			+	+	+	+	+	+	+			+
Leishmania (S.) tarentolae ParrotTarII			+	+	+	+	+	+	+			+
Leishmania (L.) aethiopica L147			+	+	+	+	+	+	+			+
Leishmania (L.) tropica L590			+	+	+	+	+	+	+			+
Leishmania (L.) arabica LEM1108			+	+	+	+	+	+	+			+
			+	+	+	+	+	+	+			+

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Table 2. (*Continued.*)

	Tb927.2.6100: hypothetical protein	*Tb927.3.1560:* TRF-interacting factor 2	*Tb927.3.5030:* KU70 protein	*Tb927.3.5150:* exonuclease, putative	*Tb927.5.1700:* replication factor A 28 kDa subunit	*Tb927.6.1760:* KU80 protein	*Tb927.6.4330:* telomere-associated protein	*Tb927.9.10770:* polyadenylate-binding protein 2	*Tb927.9.15360:* 40S ribosomal protein S6	*Tb927.9.3930:* hypothetical protein	*Tb927.9.4000:* hypothetical protein	*Tb927.9.5020:* HMG-box domain-containing protein
Leishmania (L.) turanica LEM423												
Leishmania (L.) gerbilli LEM452			+	+	+	+	+	+	+			+
Leishmania (L.) major Friedlin			+	+	+	+	+	+	+			+
Leishmania (L.) major LV39			+	+	+	+	+	+	+			+
Leishmania (L.) major SD75			+	+	+	+	+	+	+			+
Leishmania (L.) donovani BPK282A1			+	+	+	+	+	+	+			+
Leishmania (L.) infantum JPCM5			+	+	+	+	+	+	+			+
Leishmania (L.) amazonensis M2269			+	+	+	+	+	+	*			+
Leishmania (L.) mexicana M379			+	+	+	+	+	+	+			+
Leishmania (V.) braziliensis M2903			+	+	+	+	+	+	+			+
Leishmania (V.) braziliensis M2904			+	+	+	+	+	+	+			+
Leishmania (V.) peruviana PAB-4377			+	+	+	+	+	+	+			+
Leishmania (V.) panamensis L13			+	+	+	+	+	+	+			+
Novymonas esmeraldas E262AT			+	+	+	+	+	+	+			+
Blastocrithidia sp. p57				+	+		+	+	+			+
Vickermania ingenoplastis CP21			+	+	+	+	+	+	+			+
Phytomonas francai TCC064			+	+	+	+	+	+	+			+
Phytomonas serpens 9T			+	+	+	+	+	+	+			+
Phytomonas sp. HART1			+	+	+	+	+	+				+

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Table 2. (*Continued.*)

	Tb927.2.6100: hypothetical protein	*Tb927.3.1560:* TRF-interacting factor 2	*Tb927.3.5030:* KU70 protein	*Tb927.3.5150:* exonuclease, putative	*Tb927.5.1700:* replication factor A 28 kDa subunit	*Tb927.6.1760:* KU80 protein	*Tb927.6.4330:* telomere-associated protein	*Tb927.9.10770:* polyadenylate-binding protein 2	*Tb927.9.15360:* 40S ribosomal protein S6	*Tb927.9.3930:* hypothetical protein	*Tb927.9.4000:* hypothetical protein	*Tb927.9.5020:* HMG-box domain-containing protein
Phytomonas sp. EM1			+	+	+	+	+	+	+			+
Wallacemonas collosoma ATCC30261			+	+	+	+	+	+	+			+
Wallacemonas rigidus Sld			+	+	+	+	+	+	+			+
Wallacemonas sp. MBr04			+	+	+	+	+	+	+			+
Wallacemonas sp. 195SL			+	+	+	+	+	+	+			+
Wallacemonas sp. Trypx			+	+	+	+	+	+	+			+
Wallacemonas sp. Wsd			+	+	+	+	+	+	+			+
Angomonas deanei TCC036E			+	+	+	+	+	+	+			+
Angomonas desouzai TCC079E			+	+	+	+	+	+	+			+
Strigomonas culicis TCC012E			+	+	+	+	+	+	+			+
Strigomonas galati TCC219			+	+	+	+	+	+	+			+
Strigomonas oncopelti TCC290E			+	+	+	+	+	+	+			+
Blechomonas ayalai B08-376		+	+	+	+	+	+	+	+			+
Trypanosoma brucei gambiense DAL972	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma brucei brucei Lister 427	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma evansi STIB_805	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma equiperdum OVI_V2	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma congolense IL3000	+	+	+	+	+	+	+	+	+			+
Trypanosoma vivax Y486	+	+	+	+	+	+	+	+	+			+

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Table 2. (*Continued.*)

	Tb927.2.6100: hypothetical protein	*Tb927.3.1560:* TRF-interacting factor 2	*Tb927.3.5030:* KU70 protein	*Tb927.3.5150:* exonuclease, putative	*Tb927.5.1700:* replication factor A 28 kDa subunit	*Tb927.6.1760:* KU80 protein	*Tb927.6.4330:* telomere-associated protein	*Tb927.9.10770:* polyadenylate-binding protein 2	*Tb927.9.15360:* 40S ribosomal protein S6	*Tb927.9.3930:* hypothetical protein	*Tb927.9.4000:* hypothetical protein	*Tb927.9.5020:* HMG-box domain-containing protein
Trypanosoma cruzi CL-EL	+	+	+	+			+	+	+			+
Trypanosoma cruzi CL-Br NEL	+	+	+	+	+	+	+	+	+			+
Trypanosoma cruzi marinkellei B7	+	+	+	+	+	+	+	+	+			+
Trypanosoma rangeli SC58	+	+	+	+	+	+	+	+				+
Trypanosoma grayi ANR4	+	+	+	+	+	+	+	+	+			+
Trypanosoma theileri Edinburgh	+	+	+	+	+	+	+	+	+			+
Paratrypanosoma confusum CUL13		+	+	+	+	+	+	+	+			+
Bodo saltans Lake_Konstanz			+	+	+	+	+	+	+			

	Tb927.9.8740: double RNA binding domain protein 3	*Tb927.10.12850:* ttaggg binding factor	*Tb927.10.2200:* hypothetical protein	*Tb927.10.2520:* PrimPol-like protein 2	*Tb927.10.4220:* hypothetical protein	*Tb927.10.6030:* proteasome subunit alpha type-1	*Tb927.10.6220:* 5′-3′ exoribonuclease D	*Tb927.11.10190:* telomerase reverse transcriptase	*Tb927.11.16120:* hypothetical protein	*Tb927.11.370:* repressor activator protein 1	*Tb927.11.5550:* DNA polymerase theta	*Tb927.11.9870:* telomere-associated protein 1
Crithidia bombi 08.076	+	+	+	+	+	+	+	+	+	+	+	+
Crithidia expoeki BJ08.175	+	+	+	+	+	+	+	+	+	+	+	+
Crithidia fasciculata Cf-Cl	+	+	+	+	+	+	+	+	+	+	+	+
Leptomonas pyrrhocoris H10	+	+	+	+	+	+	+	+	+	+	+	+
Leptomonas seymouri ATCC30220	+	+	+	+	+	+	+	+	+	+	+	+
Lotmaria passim SF	+	+	+	+	+	+	+	+	+	+	+	+
Endotrypanum monterogeii ATCC30507	+	+	+	+	+	+	+	+	+	+	+	+
Endotrypanum monterogeii LV88	+	+	+	+	+	+	+	+	+	+	+	+
Porcisia deanei TCC258	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+

(*Continued*)

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Table 2. (*Continued.*)

	Tb927.9.8740: double RNA binding domain protein 3	*Tb927.10.12850:* ttaggg binding factor	*Tb927.10.2200:* hypothetical protein	*Tb927.10.2520:* PrimPol-like protein 2	*Tb927.10.4220:* hypothetical protein	*Tb927.10.6030:* proteasome subunit alpha type-1	*Tb927.10.6220:* 5′-3′ exoribonuclease D	*Tb927.11.10190:* telomerase reverse transcriptase	*Tb927.11.16120:* hypothetical protein	*Tb927.11.370:* repressor activator protein 1	*Tb927.11.5550:* DNA polymerase theta	*Tb927.11.9870:* telomere-associated protein 1
Porcisia hertigi TCC260												
Leishmania (M.) enriettii LEM3045	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (M.) macropodum LV756	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (M.) martiniquensis LEM2494	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (S.) adleri HO174	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (S.) tarentolae ParrotTarII	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) aethiopica L147	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) tropica L590	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) arabica LEM1108	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) turanica LEM423	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) gerbilli LEM452	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) major Friedlin	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) major LV39	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) major SD75	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) donovani BPK282A1	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) infantum JPCM5	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) amazonensis M2269	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (L.) mexicana M379	+	+	+	+	+	+	+	+	+	+	+	+

(*Continued*)

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Table 2. (*Continued.*)

	Tb927.9.8740: double RNA binding domain protein 3	*Tb927.10.12850:* ttaggg binding factor	*Tb927.10.2200:* hypothetical protein	*Tb927.10.2520:* PrimPol-like protein 2	*Tb927.10.4220:* hypothetical protein	*Tb927.10.6030:* proteasome subunit alpha type-1	*Tb927.10.6220:* 5′-3′ exoribonuclease D	*Tb927.11.10190:* telomerase reverse transcriptase	*Tb927.11.16120:* hypothetical protein	*Tb927.11.370:* repressor activator protein 1	*Tb927.11.5550:* DNA polymerase theta	*Tb927.11.9870:* telomere-associated protein 1
Leishmania (V.) braziliensis M2903	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (V.) braziliensis M2904	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (V.) peruviana PAB-4377	+	+	+	+	+	+	+	+	+	+	+	+
Leishmania (V.) panamensis L13	+	+	+	+	+	+	+	+	+	+	+	+
Novymonas esmeraldas E262AT	+	+	+	+	+	+	+	+	+	+	+	+
Blastocrithidia sp. p57	+	+	+	+	+	+	+	+	+	+	+	
Vickermania ingenoplastis CP21	+	+	+	+	+	+	+	+	+	+	+	
Phytomonas francai TCC064	+	+	+	+	+	+	+	+	+	+	+	
Phytomonas serpens 9T	+	+	+	+	+	+	+	+	+	+	+	+
Phytomonas sp. HART1	+	+	+	+	+	+	+	+	+	+	+	
Phytomonas sp. EM1	+	+	+	+	+	+	+	+	+	+	+	+
Wallacemonas collosoma ATCC30261	+	+	+	+	+	+	+	+	+	+	+	+
Wallacemonas rigidus Sld	+	+	+	+	+	+	+	+	+	+	+	+
Wallacemonas sp. MBr04	+	+	+	+	+	+	+	+	+	+	+	+
Wallacemonas sp. 195SL	+	+	+	+	+	+	+	+	+	+	+	+
Wallacemonas sp. Trypx	+	+	+	+	+	+	+	+	+	+	+	+
Wallacemonas sp. Wsd	+	+	+	+	+	+	+	+	+	+	+	+
Angomonas deanei TCC036E	+	+	+	+	+	+	+	+	+	+	+	+
Angomonas desouzai TCC079E	+	+	+	+	+	+	+	+	+	+	+	+

(*Continued*)

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Table 2. (*Continued.*)

	Tb927.9.8740: double RNA binding domain protein 3	*Tb927.10.12850:* ttaggg binding factor	*Tb927.10.2200:* hypothetical protein	*Tb927.10.2520:* PrimPol-like protein 2	*Tb927.10.4220:* hypothetical protein	*Tb927.10.6030:* proteasome subunit alpha type-1	*Tb927.10.6220:* 5′-3′ exoribonuclease D	*Tb927.11.10190:* telomerase reverse transcriptase	*Tb927.11.16120:* hypothetical protein	*Tb927.11.370:* repressor activator protein 1	*Tb927.11.5550:* DNA polymerase theta	*Tb927.11.9870:* telomere-associated protein 1
Strigomonas culicis TCC012E	+	+	+	+	+	+	+	+	+	+	+	+
Strigomonas galati TCC219	+	+	+	+	+	+	+		+	+	+	+
Strigomonas oncopelti TCC290E	+	+	+	+	+	+	+	+	+	+	+	+
Blechomonas ayalai B08-376	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma brucei gambiense DAL972	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma brucei brucei Lister 427	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma evansi STIB_805	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma equiperdum OVI_V2	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma congolense IL3000	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma vivax Y486	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma cruzi CL-EL	+	+		+	+	+	+	+	+	+	+	+
Trypanosoma cruzi CL-Br NEL	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma cruzi marinkellei B7	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma rangeli SC58	+	+		+	+	+	+	+	+		+	+
Trypanosoma grayi ANR4	+	+	+	+	+	+	+	+	+	+	+	+
Trypanosoma theileri Edinburgh	+	+	+	+	+	+	+	+	+	+	+	+
Paratrypanosoma confusum CUL13	+	+	+	+	+	+	+	+	+	+	+	+
Bodo saltans Lake_Konstanz	+	+	+	+	+	+	+	+	+		+	+

Species analysed by Southern blotting are shaded.
+, identified; empty, not identified; *, identified in strain UA301.

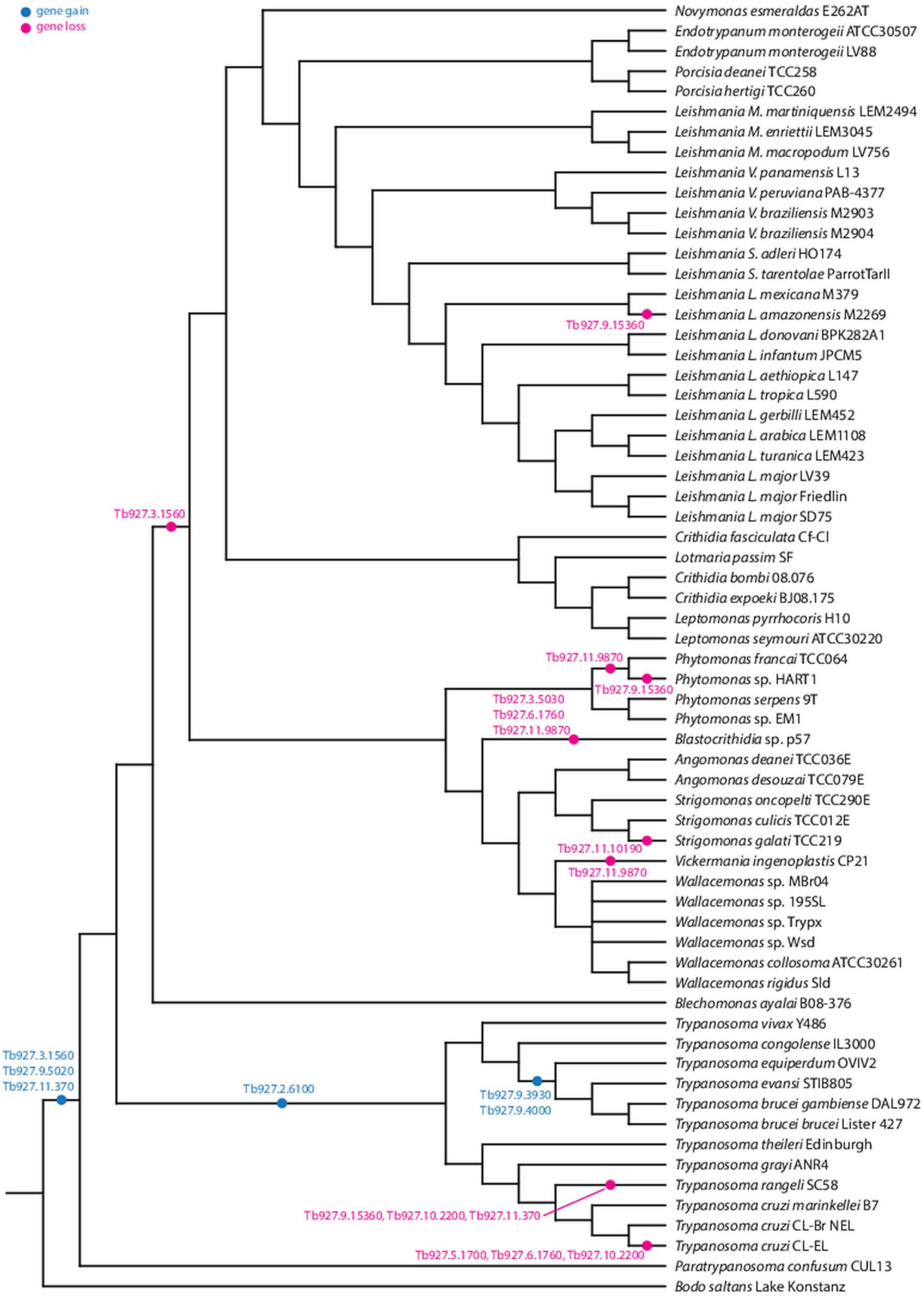


Fig. 1. Gains and losses of genes encoding putative telomere-associated proteins in kinetoplastids.

and alignments including the query and all identified proteins, if necessary. The resulting gene presence/absence table and a cladogram manually written in a Newick format based on recent publications (Butenko *et al.*, 2019; Kostygov *et al.*, 2014, 2020; Kostygov and Yurchenko, 2017; Lukeš *et al.*, 2018; Frolov *et al.*, 2019; Kato *et al.*, 2019) were used for Dollo parsimony analysis in the Count software (Csűrös, 2010) and results were visualized in a graphical editor.

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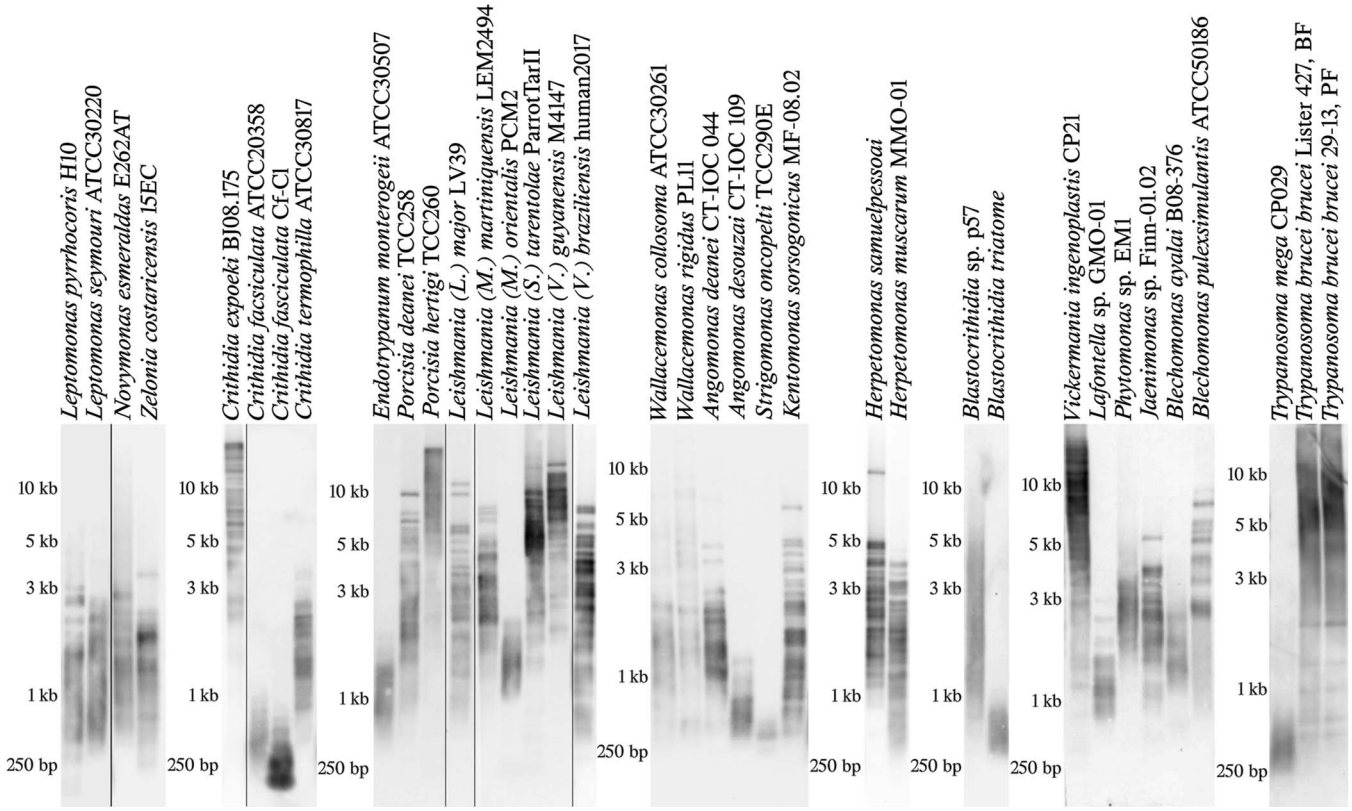


Fig. 2. Southern blotting analysis of telomere repeats in selected species of Trypanosomatidae. Marker sizes are indicated on the left. The vertical lines denote a composite image from the same blot. DNA integrity controls are presented in Supplementary Fig. 1 (left and middle panels).

Trypanosomatid isolates and cultivation

Cultures of *Crithidia expoeki* (BJ08.175), *C. fasciculata* (Cf-Cl), *C. fasciculata* (ATCC20358), *C. termophilla* (ATCC30817), *L. pyrrhocoris* (H10), *L. seymouri* (ATCC30220), *Novymonas esmeraldas* (E262AT), *Strigomonas oncopelti* (TCC290E) and *Zelonia costaricensis* (15EC) were grown in BHI medium (Oxoid/Thermo Fisher Scientific, Basingstoke, UK) supplemented with 2 μg mL^{-1} Hemin (Sigma-Aldrich, St. Louis, USA) and 50 units mL^{-1} of Penicillin/Streptomycin (BioSera, Nuaillé, France) at 23°C. Cultures of *Endotrypanum monterogeii* (ATCC30507), *Herpetomonas samuelpessoai*, *Leishmania* (*Leishmania*) *major* (LV39), *L.* (*L.*) *amazonensis* (Josefa, LV78, LV79 and PH8), *L.* (*L.*) *mexicana* (M379), *L.* (*Mundinia*) *martiniquensis* (LEM2494), *L.* (*M.*) *orientalis* (PCM2), *L.* (*Sauroleishmania*) *tarentolae* (ParrotTarII), *L.* (*Viannia*) *braziliensis* (human2017), *L.* (*V.*) *guyanensis* (M4147), *Phytomonas* sp. (EM1), *Porcisia deanei* (TCC258) and *P. hertigi* (TCC260) were grown in M199 medium supplemented with 2 μg mL^{-1} Biopterin, 2 μg mL^{-1} Hemin (all Sigma-Aldrich), 25 mM HEPES (Lonza, Basel, Switzerland), 50 units mL^{-1} of Penicillin/Streptomycin (BioSera) and 10% fetal bovine serum (BioWest, Nuaillé, France) at 23°C. Cultures of *Angomonas deanei* (CT-IOC 044), *A. desouzai* (CT-IOC 109), *Blastocrithidia* sp. (p57), *Blastocrithidia triatomae*, *Blechomonas ayalai* (B08-376), *Blechomonas pulexsimulantis* (ATCC50186), *Herpetomonas muscarum* (MMO-01), *Jaenimonas* sp. (Finn-01.02), *Kentomonas sorsogonicus* (MF-08.02), *Lafontella* sp. (GMO-01), *Vickermania ingenoplastis* (CP21), *Wallacemonas collosoma* (ATCC30261) and *W. rigidus* (PL11) were maintained in SDM medium (BioWest) supplemented with 10% fetal bovine serum (BioWest) and 50 units mL^{-1} of Penicillin/Streptomycin (BioSera) at 23°C. In the cases of *Lafontella* and *Endotrypanum*, cultures were grown in a bi-phasic medium, overlaying blood agar. All species were validated by amplifying and sequencing the 18S rRNA gene as described previously (Kostygov *et al.*, 2014).

Quantification of transcription level of genes encoding telomeric proteins using RT-qPCR

RNA was isolated and transcript levels of the telomeric proteins were assessed by RT-qPCR as described previously (Záhonová *et al.*, 2014; Kraeva *et al.*, 2019). Sequences of the specific primers for *L. mexicana*/*amazonensis* orthologues of *T. brucei* genes are listed in Supplementary Table 1. Expression values were normalized to those of 18S rRNA.

Southern blotting

The previously established terminal restriction fragment analysis of telomere lengths protocol was followed (Janzen *et al.*, 2004). In brief, total genomic DNA from the log-phase grown cells was isolated and digested with *Alu*I, *Hinf*I and *Rsa*I overnight. Restriction fragments were separated in 0.75% agarose gel, transferred to a ZetaProbe blotting membrane (Bio-Rad, Hercules, USA), probed with the DIG-labelled telomeric probe $[CCCTAA]_{x25}$ in the DIG Easy Hyb buffer (Roche Diagnostics, Indianapolis, USA), and visualized with the DIG Luminescent Detection Kit (Roche Diagnostics). The probe was labelled by the Dioxigenin NT Labeling Kit (Jena Bioscience GmbH, Jena, Germany). Statistics of the telomere lengths were obtained with an online tool WALTER (Web-based Analyser of the Length of TElomeRes) (Lyčka *et al.*, 2021). For the loading and integrity control in the *L. mexicana* complex analysis, DNAs were processed as above, and the membrane was probed against a fragment of a gene encoding telomerase (*LmxM.36.3930*) (Supplementary Table 1).

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Table 3. Telomere lengths (weighted median, minimum–maximum) in selected species of Trypanosomatidae

	Median (min–max) of telomere length, bp
Crithidia expoeki BJ08.175	4,271 (1619–33 446)
C. fasciculata ATCC20358	506 (252–1047)
C. fasciculata Cf-Cl	368 (252–983)
C. termophilla ATCC30817	1374 (512–3655)
Leptomonas pyrrhocoris H10	874 (328–4859)
L. seymouri ATCC30220	875 (386–2941)
Endotrypanum monterogeii ATCC30507	916 (450–2182)
Porcisia deanei TCC258	1875 (705–10 469)
P. hertigi TCC260	4992 (1305–27 381)
Leishmania (*Mundinia*) *martiniquensis* LEM2494	2519 (1009–8231)
L. (*M.*) *orientalis* PCM2	1488 (1033–2327)
L. (*Sauroleishmania*) *tarentolae* ParrotTarII	3938 (1263–27 381)
L. (*Leishmania*) *major* LV39	1842 (573–13 381)
L. (*L.*) *amazonensis* LV78	435 (247–779)
L. (*L.*) *amazonensis* LV79	3363 (252–34 459)
L. (*L.*) *amazonensis* PH8	362 (253–1260)
L. (*L.*) *amazonensis* Josefa	443 (253–2481)
L. (*L.*) *mexicana* M379	393 (271–840)
L. (*L.*) *mexicana* M379 ΔKu80	630 (248–3463)
L. (*L.*) *mexicana* M379 ΔKu70	521 (248–3421)
L. (*Viannia*) *braziliensis* human2017	1911 (587–7865)
L. (*V.*) *guyanensis* M4147	5105 (1782–27 381)
Novymonas esmeraldas E262AT	1238 (535–15 198)
Zelonia costaricensis 15EC	937 (264–3818)
Blastocrithidia sp. p57	1500 (478–10 944)
B. triatomae	614 (390–1011)
Vickermania ingenoplastis CP21	4078 (815–33 440)
Herpetomonas muscarum MMO-01	1282 (388–4675)
H. samuelpessoai	1989 (862–32 804)
Lafontella sp. GMO-01	1169 (708–3080)
Phytomonas sp. EM1	2554 (1507–4906)
Jaenimonas sp. Finn-01.02	2112 (879–5679)
Walacemonas collosoma ATCC30261	1290 (466–7332)
W. rigidus PL11	1253 (408–9550)
Angomonas deanei CT-IOC 044	1245 (466–3948)
A. desouzai CT-IOC 109	588 (294–1334)
Strigomonas oncopelti TCC290E	420 (282–600)
Kentomonas sorsogonicus MF-08.02	1017 (384–6178)
Blechomonas ayalai B08-376	1693 (1097–3152)
Ble. pulexsimulantis ATCC50186	1972 (687–8889)
Trypanosoma brucei brucei Lister 427 (BF)	3422 (474–24 711)
T. b. brucei Lister 427 29-13 (PF)	3108 (470–24 281)
T. mega CP029	414 (252–715)

Results and discussion

The core set of proteins putatively involved in telomere maintenance in kinetoplastids is conserved

To study the phylogenetic distribution of proteins predicted to be involved in telomere maintenance (Reis *et al.*, 2018), we analysed the presence/absence of the corresponding 24 genes in the available genomes of trypanosomatids and their close eubodonid relative, *B. saltans* (Table 2). Most of the studied proteins (20 of 24) are well conserved and we consider them as a core set putatively involved in telomere maintenance in kinetoplastids. It is worth noting that the telomere association and function in telomere maintenance has already been confirmed for some of these proteins, while some others have not been functionally characterized yet. Thus, the composition of the core set of proteins involved in telomere maintenance, as defined previously (Reis *et al.*, 2018) and discussed herein, should be taken with caution. Despite the fact that most of the respective genes are conserved across Kinetoplastea (Fig. 1, Table 2) and, thus appear to be present in the kinetoplastid common ancestor, we came across several interesting exceptions that are discussed in detail below.

A set of three proteins (orthologues of *T. brucei* Tb927.3.1560, Tb927.9.5020 and Tb927.11.370) was acquired by the common ancestor of trypanosomatids upon the separation from bodonids (Fig. 1). One of them, Tb927.3.1560 [TIF-2, an orthologue of mammalian TINF2 (Jehi *et al.*, 2014*a*; 2014*b*)] was suggested to be essential, as it is involved in shelterin (a protein complex implicated in telomere protection) assembly and telomerase-mediated telomere length maintenance in other organisms (Walne *et al.*, 2008; Frank *et al.*, 2015). Yet, it is not present in bodonids and was secondarily lost in all other trypanosomatids outside of the genera *Paratrypanosoma*, *Trypanosoma* and *Blechomonas* (Fig. 1), raising a question of how do they cope with its absence or whether they replaced it with a functional analogue? Tb927.2.6100 is *Trypanosoma*-specific, confirming previous report (Beck *et al.*, 2013). Surprisingly, this protein was shown to be specifically associated with kDNA, so its role in telomere maintenance, if any, remains to be elucidated by functional genetics approaches. Two proteins (orthologues of *T. brucei* Tb927.9.3930 and Tb927.9.4000) are present only in four species of the *T. brucei* group and may determine specific traits of these parasites.

An orthologue of Tb927.11.9870 (TelAP-1) is present in most species, but it is conspicuously absent from the representatives of two monoxenous groups (*Blastocrithidia* and *Vickermania* spp.) and most *Phytomonas* spp., plant pathogens with streamlined genomes (Porcel *et al.*, 2014). While we cannot rule out a possibility that the protein is divergent beyond recognition by available bioinformatics tools, there may exist another component fulfilling the role of TelAP-1 in these species.

Of special attention is the absence of Tb927.3.5030 (Ku70) and Tb927.3.5030 (Ku80) orthologues in *Blastocrithidia* sp., which has a non-canonical nuclear genetic code with all three stop codons reassigned to encode amino acids (Záhonová *et al.*, 2016). It has been recently proposed that such an absence may lead to the accumulation of numerous insertions in many protein-coding genes of these organisms (Nenarokova *et al.*, 2019).

Trypanosomatid telomeres are variable in length

We performed a systematic screen of the telomere length across Trypanosomatidae by Southern blotting (Fig. 2, Table 3). Our analysis revealed that monoxenous Leishmaniinae (Kostygov and Yurchenko, 2017) of the genera *Leptomonas*, *Novymonas* and *Zelonia* have fairly short telomeres (weighted medians

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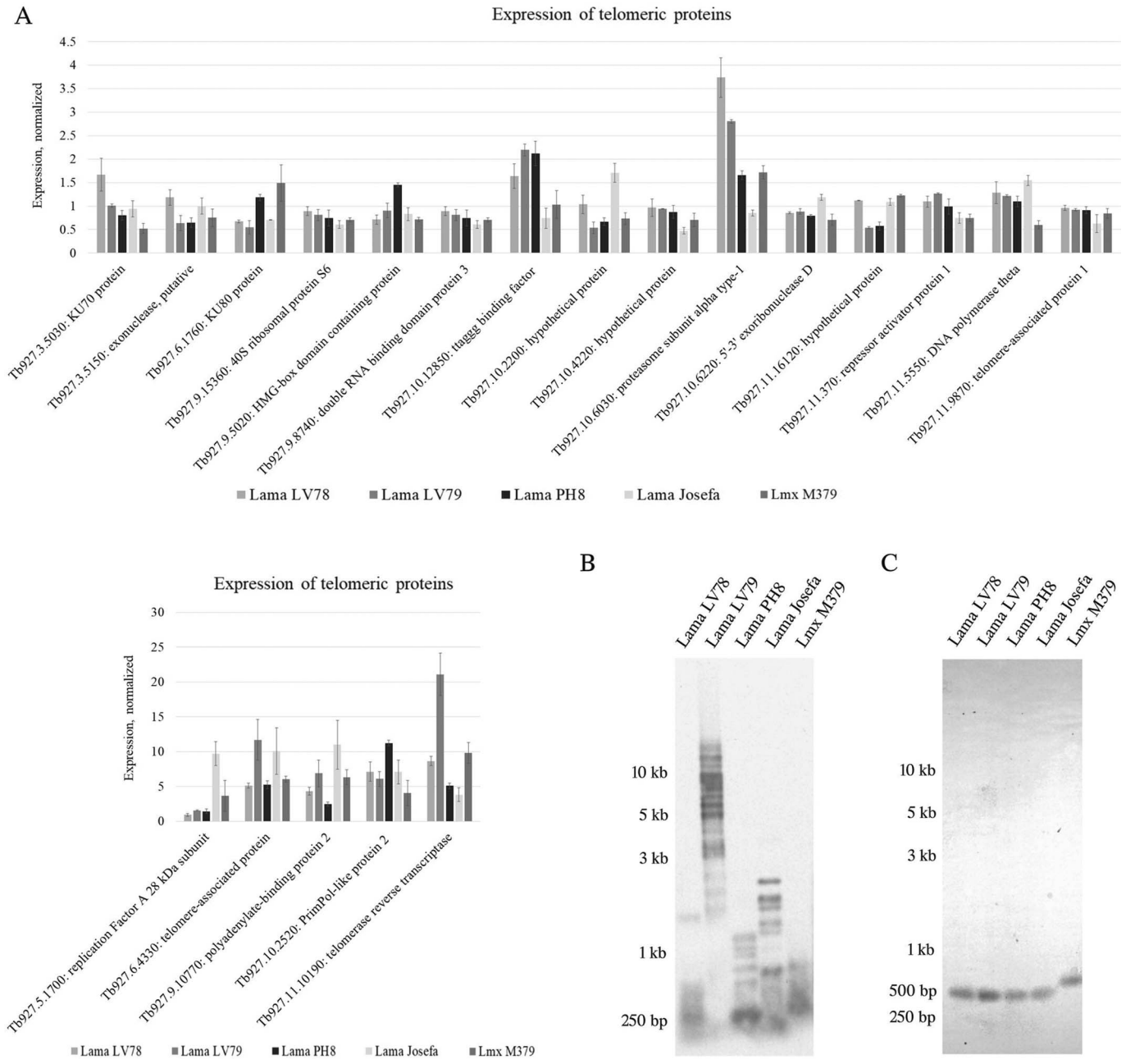


Fig. 3. Transcript levels of telomere-associated proteins and telomere lengths in the species of *L. mexicana* complex. (A) Quantitative RT-PCR analysis of the core set of proteins implicated in telomere maintenance. Gene expression is presented as normalized means and standard deviations of three replicates. Data are presented in two graphs to account for differences in expression values. (B, C) Southern blotting analysis of telomere repeats (B) and telomerase-encoding gene (C, used as an additional DNA integrity control) in *L. amazonensis* LV78, LV79, PH8, Josefa and *L. mexicana* M379. Marker sizes are indicated on the left. DNA integrity controls are presented in Supplementary Fig. 1 (right panel).

900–1200 bp; hereafter only rounded weighted median data are compared in the text, see Table 3 for minimum and maximum values), while telomeres in analysed *Crithidia* spp. ranged from 400 bp in *C. fasciculata* Cf-C1 to 4300 bp in *C. expoeki*. These numbers correlate well with previous reports on telomere length in the selected representatives of the genera *Crithidia*, *Leishmania* and *Trypanosoma* (Genest *et al.*, 2007). Of note, the repertoire of genes implicated in telomere maintenance is identical in these flagellates (Table 2), so these differences can be explained by either the presence of other proteins involved in this process, or (more likely) differences in gene expression. Telomeres of *Blechomonas*, *Herpetomonas*, *Jaenimonas* and *Wallacemonas* spp. are 1300–2100 bp long. The endosymbiont-containing Strigomonadinae [*Angomonas*, *Kentomonas* and *Strigomonas* spp. (Votýpka *et al.*, 2014)] differ in telomere length, with *S. oncopelti* bearing the shortest chromosome ends of ~400 bp.

Representatives of three genera (*Blastocrithidia*, *Leishmania* and *Trypanosoma*) deserved special attention. Uniquely among trypanosomatids, *Blastocrithidia* spp. lack Ku proteins (Nenarokova *et al.*, 2019), yet their telomeres are of similar length to telomeres of other trypanosomatids (600 and 1500 bp in *B. triatomae* and *Blastocrithidia* sp., respectively), arguing that either Ku proteins are dispensable for the telomere length maintenance in these species, or their loss can be compensated by other factors. Telomere sizes vary in different *Trypanosoma* spp. represented by short telomeres in *T. mega* (400 bp) and substantially longer telomeres in *T. brucei* Lister 427 (3100–3400 bp). In contrast to the previous report (Dreesen and Cross, 2008), we did not document differences in telomeres' length between the procyclic and

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bloodstream stages of *T. brucei*. However, both strains in our analysis have the same origin (Lister 427), while the abovementioned study compared Lister 427 and TREU927 strains. Similar to the cases discussed above, despite possessing the same repertoire of telomere-bound proteins, the distribution of telomere sizes in the *Leishmania–Porcisia–Endotrypanum* clade (Espinosa *et al.*, 2018) is wide, exemplified by two extreme cases of *P. hertigi* (5000 bp) and *L. mexicana* (400 bp, Fig. 2). Variable telomere length in *Leishmania* spp. (and possibly other Leishmaniinae) may be explained by the presence of a stress-sensitive telomere-proximal replication activity outside S phase of the cell cycle in these species (Damasceno *et al.*, 2020, 2021).

RNA level of telomerase and several telomere-associated proteins correlates with telomere length in the species of L. mexicana *complex*

We analysed telomere length and expression of the core set of proteins putatively involved in telomere maintenance in closely related species forming the *L. mexicana* complex (Eresh *et al.*, 1994). Similar to the cases discussed above, telomeres in *L. mexicana* and four isolates of *L. amazonensis* greatly differed in length from ~400 bp in *L. mexicana* M379 to ~3400 bp in *L. amazonensis* LV79 (Fig. 3, Table 3, Supplementary Fig. 1). Such a wide range of telomere lengths correlated well with the expression of the *Leishmania* spp. telomerase (orthologue of Tb927.11.10190) and a telomere-associated hypothetical protein (orthologue of Tb927.6.4330). The higher expression of these proteins correlated with longer telomeres. The specific roles of these and other proteins remain to be further elucidated by functional studies.

Conclusions

The genome analysis has allowed us to identify a core set of 20 conserved proteins predicted to be responsible for telomere maintenance in trypanosomatids. Several proteins, previously identified in *T. brucei* pull-downs, are trypanosome-specific. Out of 20 proteins conserved in Trypanosomatidae, two (Ku70 and Ku80) are conspicuously missing in *Blastocrithidia* spp., yet telomeres in these species do not appear to be affected by their loss. We documented that telomeres of trypanosomatids are diverse in length, even within groups of closely related species. One such group is a complex of species, related to *L. mexicana*. Our analysis demonstrated that the expression of several telomere-associated proteins correlates with the documented differences in telomere length within species of the *L. mexicana* complex, which is indicative of a potential role these proteins may play in the telomere length maintenance.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021000378.

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Conflict of interest. None.

Ethical standards. Not applicable.

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