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Protozoan ATP-binding cassette transporter based phylogenetic reconstruction and RNA secondary structure study

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Abstract
Protozoa are unicellular, mutualistic or parasitic responsible for several diseases of humans, animals and plants. Role of protozoa ATP-binding cassette (ABC) transporters is known as membrane proteins mediate the ATP-dependent transport of a wide variety of chemotherapeutic drugs away from their targets inside the parasites. The gene sequences of many protozoans have recently been available. The protozoan phylogeny has been always challenging relating to their evolutionary relationships. In the current study an attempt has been made to study the phylogenetic relationships among selected protozoan species based on ATP-binding cassette transporter gene along with their corresponding RNA secondary structures.

Keywords: protozoa, parasite, alignment, phylogeny, RNA secondary structure

Introduction
Protozoa are unicellular, eukaryotic, heterotrophic protists with motile behavior. They are microscopic organisms having membrane bound nucleus confined to moist or aquatic habitats, being ubiquitous in such environments worldwide. Protozoans can be flagellates, ciliates and amoebas possessing pseudopodia. Many species have close relationships with animals or plants. These relationships may be mutualistic, commensalistic or parasitic. In fact, some of the most important diseases of humans and animals are caused by parasitic protozoans. Klokouzas and coworkers (2003) [5] focused on the role of protozoan ABC transporters. Through the hydrolysis of ATP to generate energy, ABC transporters move a wide variety of substrates across membranes, including ions, sugars, amino acids, polypeptides, toxic metabolites, xenobiotics and even drugs and toxins. These membrane proteins mediate the ATP-dependent transport of a wide variety of chemotherapeutic drugs away from their targets inside the parasites. The genome sequences of many protozoans have recently been completed many are underway. The protozoan phylogeny has been always challenging. In the current study an attempt has been made to study the phylogenetic relationships among selected protozoan species based on ATP-binding cassette transporter gene along with their corresponding RNA secondary structures.

Materials and Methods
Retrieval of sequence and taxon sampling
The protozoan ATP-binding cassette transporter gene sequences were retrieved from NCBI-GenBank. A key word 'protozoa' in the search option with nucleotide option generated variety entries including the International Nucleotide Sequence Database Collaboration (INSDC), mRNA nucleotide sequence and reference sequences. The sequences (*Trypanosoma cruzi*, *Trypanosoma brucei and Entamoeba histolytica*) were sorted and edited using Bio Edit (Hall, 1999).

Multiple sequence alignment and phylogenetic analysis
The sequences were aligned using ClustalW (Thompson *et al*., 1994) [8]. For pair wise sequence alignment the gap opening penalty and gap extension penalty was 15 and 6.66 respectively. For multiple sequence alignment the gap opening penalty and gap extension penalty was 15 and 6.66 respectively. DNA weight matrix with transition weight of 0.5. The negative matrix was switched off and the delay divergent cut off percentage was kept at 30. The aligned file was exported to the local machine for phylogenetic analysis. Five different methods (maximum likelihood, neighbor joining, minimum evolution, UPGMA and maximum parsimony) were adopted to perform phylogenetic analysis. Phylogenetic trees were generated by five different methods (maximum likelihood, NJ, minimum evolution, UPGMA and MP) by MEGA 7 software (Kumar *et al*., 2016) [6]. All characters were equally weighted and unordered. Alignment gaps were treated as missing data.
The optimal trees with the sum of branch length were -7878.8389 for maximum likelihood, 32.52467491 for NJ, 32.52467491 for minimum evolution, 32.52923389 for UPGMA and 2559 for MP respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap was 500 replicates. The evolutionary distances were computed using the maximum composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The consistency index was (0.378421), the retention index is (0.445026), and the composite index is 0.168515 (0.168407) for all sites and parsimony-informative sites. The MP tree was

obtained using the Close-Neighbour-Inter-change algorithm. There were a total of 341 positions in the final dataset.

RNA secondary structure prediction

The consensus RNA secondary structures of different species were predicted using mfold web server (Zuker, 2003) [9]. It performed multiple alignments and generated consensus secondary structures using realistic energy models of RNAs. The structural variations are observed among different species. The no of bulge loops, hairpin loops and junction were observed. The generated. ct files of the secondary structures were further used with Varna (Darty *et al*, 2009) [1] to draw and annotate the secondary structures.

Results and Discussion

T. cruzi is a species of parasitic euglenoids. Amongst the protozoa, the trypanosomes characteristically bore tissue in another organism and feed on blood (primarily) and also lymph. *T. brucei* is a species of parasitic kinetoplastid belonging to the genus *Trypanosoma*. The parasite is the cause of a vector-borne disease of vertebrate animals, including humans, carried by genera of tsetse fly in sub-Saharan Africa. In humans *T. brucei* causes African trypanosomiasis, or sleeping sickness. In animals it causes animal trypanosomiasis. *E. histolytica* is an anaerobic parasitic amoebozoa, part of the genus *Entamoeba* predominantly infecting humans and other primates causing amoebiasis. The phylogenetic analysis showed *T. brucei* sharing a clade with *E. histolytica*.

The phylogenetic tree (Fig. 1) showed evolutionary relationships among the three species.

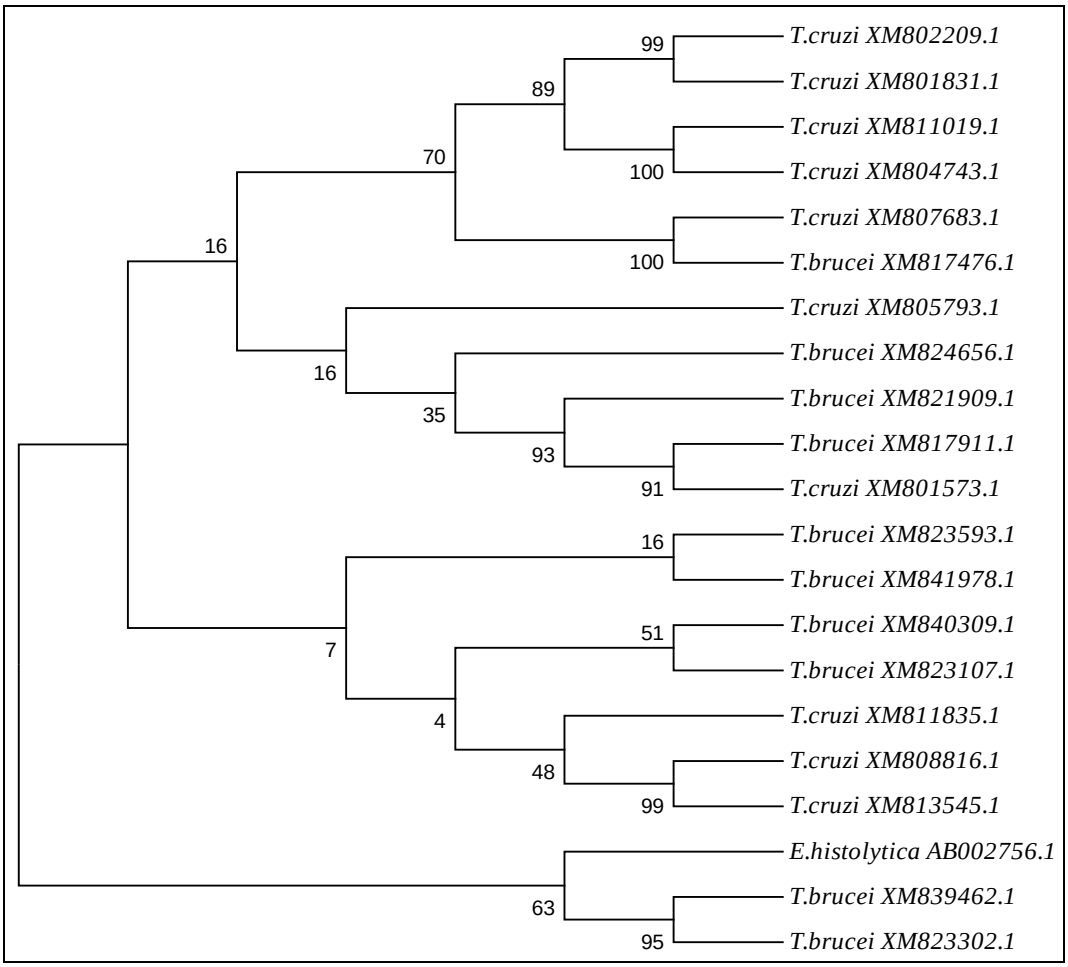

Fig 1: Phylogenetic tree showing evolutionary relationships inferred using maximum likelihood method. The numbers indicate GenBank accession numbers.

The consensus secondary structures of different species were observed in terms of bulge loops, hairpin loops and junctions were observed. Variations were observed with little similarities in case of species which are present in a single clade. The consensus RNA secondary structures computed by mfold web server were given below (Fig: 2), the annotated secondary structures generated by Verna were depicted (Fig: 3).

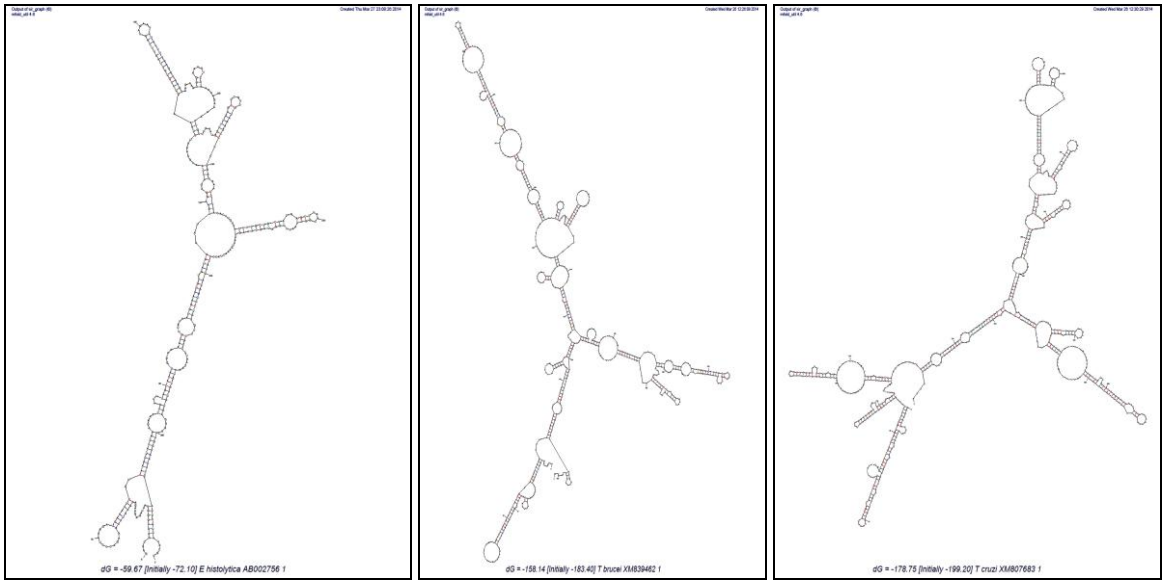

Fig 2: Consensus RNA secondary structures of abc transporter mitochondrial RNA genes of *E. histolytica, T. brucei* and *T. cruzi* generated by mfold.

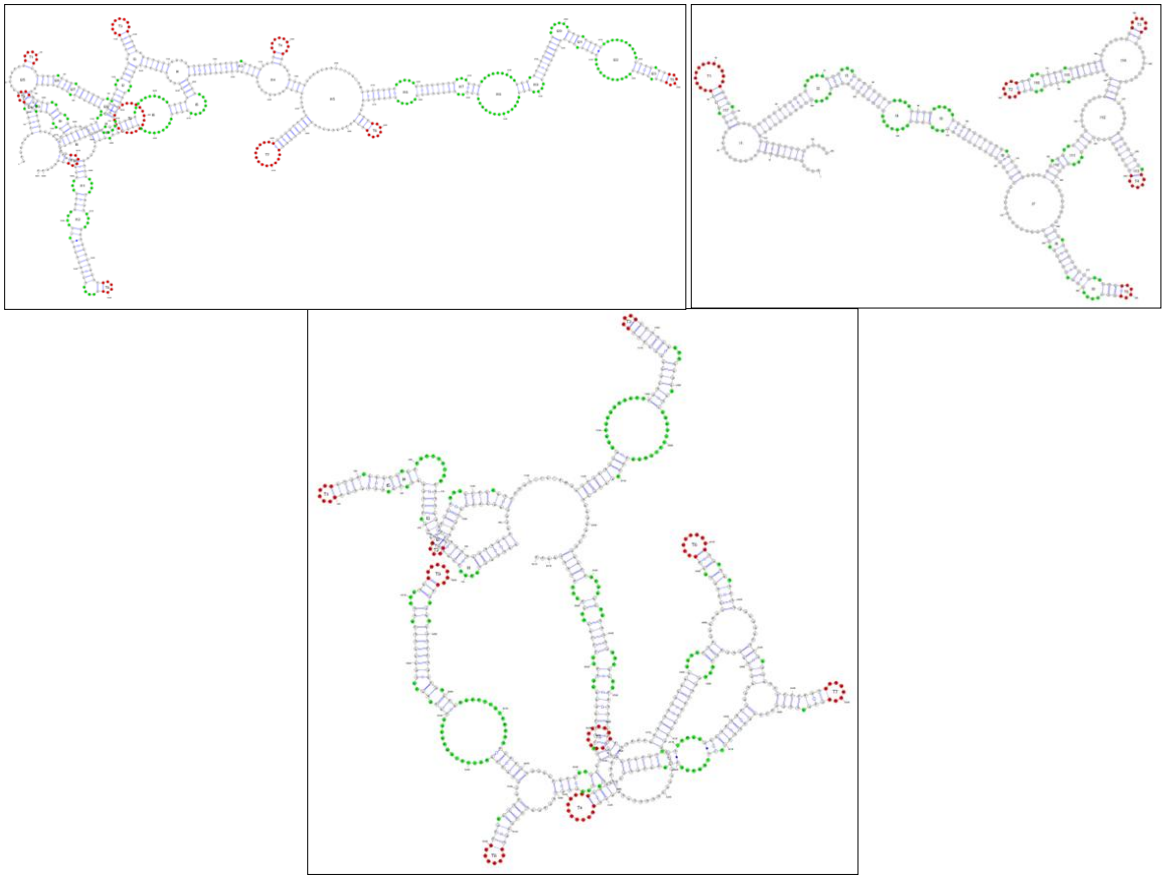

Fig 3: Consensus RNA secondary structures of abc transporter genes of *E. histolytica, T. brucei and T. cruzi* generated by Varna.

RNA secondary structure elements (bulge loop, hairpin loop and junctions) of the three species were generated and analyzed. In the consensus RNA secondary structures, *T. brucei* and *T. cruzi* have somewhat same number of bulge loops, hairpin loops and junctions were found. But in case of *E. histolytica*, less number of bulge loops, more hairpin loops and junctions were noticed. ABC transporters play important roles in protozoans by participating in physiological and pathological processes. In order to modulate the function of ABC transporters, detailed knowledge regarding their structure and dynamics is necessary. RNA secondary structures of ABC genes and corresponding proteins indicate three major conformations, a nucleotide-bound "bottom-closed" state with the two nucleotide binding domains (NBDs)

tightly closed, and two nucleotide-free conformations, the "bottom-closed" and the "bottom-open", which differ in the extent of separation of the NBDs (Gyimesi *et al*., 2011) [2].

P-glycoprotein is an ABC drug pump. A common feature of ABC proteins is that they are organized into two wings. Each wing contains a transmembrane domain (TMD) and a nucleotide-binding domain (NBD). Drug substrates and ATP bind at the interface between the TMDs and NBDs, respectively. Drug transport involves ATP-dependent conformational changes between inward-(open, NBDs far apart) and outward-facing (closed, NBDs close together) conformations. P-gps crystallized in the presence of detergent show an open structure. Human P-gp is inactive in detergent but basal ATPase activity is restored upon addition of lipids. The lipids might cause closure of the wings to bring the NBDs close together to allow ATP hydrolysis (Loo and Clarke, 2016). The spread of drug resistance has been a major obstacle to the control of protozoan parasitic infections. The mechanisms underlying drug resistance seem to be complex and genetically controlled. The recent literature on multiple drug resistance against antibiotics has documented ATP-binding cassette protein, as an important determinant of resistance. In the Protozoan genomes, there are several ABC transporters some of which could be putative drug transporting proteins. In order to understand the molecular mechanisms underlying drug resistance, characterization and evolutionary significance of these transporters is essential (Kavishe *et al*., 2009).

Conclusion

The phylogenetic tree showed similar species remain cluster together with little alterations. This may be attributed to adaptive radiation or mutations. The secondary structures shared high degree of similarities among the related species supporting the classical taxonomy. The protozoan ATP-binding cassette transporters in evolutionary related species may show similar pattern of drug resistance.

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