Phylogenetic and Genetic Variation Analysis of ITS1 Gene of *Trypanosoma lewisi* in Wild Rats Using Polymerase Chain Reaction

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Abstract

Murine Trypanosomiasis is a disease caused by the blood protozoan *Trypanosoma lewisi* in rats, with the transmission process mediated by the flea species *Xenopsylla cheopis* and *Nosopsyllus fasciatus*. Cases of trypanosomiasis have been documented due to *Trypanosoma lewisi* infecting rats and humans in various countries. Diagnosis of *T. lewisi* is typically conducted using polymerase chain reaction (PCR), which amplifies target DNA using specific primers. One such target gene for detection is the Internal Transcribed Spacer-1 (ITS1). Subsequent sequencing of PCR products enables analysis of genetic variation employing parameters such as nucleotide composition, genetic distance, and phylogenetic analysis with MEGA software. Test results based on percent identity values indicated a 98.51% homology of blood samples with the Chinese strain of *T. lewisi* (FJ011094.1), demonstrating genetic variation. Phylogram reconstruction revealed that samples 18, 19, and 37 of *T. lewisi* exhibit very close intraspecies relationships with *T. lewisi* from NCBI genebank with genetic distance ranging from 0.007 to 0.01. While the closest interspecies relationship was found with *T. cruzi* (KT305857.1) with a genetic distance of (d = 0.61).

Keywords: ITS1, polymerase chain reaction, rats, *Trypanosoma lewisi*

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INTRODUCTION

Rats are the most common animals in the human urban environment (Yuliadi *et al*., 2016). According to Kristina *et al*. (2019), the negative impacts of rats are mainly in the agricultural, residential, and health domains. Within the health sector, rats possess the potential to serve as disease vectors or reservoirs, facilitating the transmission of zoonotic parasites to humans. These parasites include toxoplasmosis, echinococcosis, capillariasis, hymenolepiasis, trichinellosis, and trypanosomiasis (Mohd-Qawiem *et al*., 2022). Despite *T. lewisi* typically being deemed non-pathogenic in rats, under favorable conditions, it has been observed to cause disease in human hosts and other organisms (Shafiyyah *et al*., 2012). Trypanosomiasis, triggered by *T. lewisi*, is a blood protozoan within the Herpetosoma subgenus, primarily targeting blood plasma (Taylor *et al*., 2016). Rat fleas, including species such as *Xenopsylla cheopis* and *Nosopsyllus fasciatus*, serve as vectors for transmitting *T. lewisi* (Verma *et al*., 2011). *T. lewisi* has been detected in wild rats across various regions through multiple investigations. This includes findings among *Rattus* and *Bandicota species* in Thailand (Jittapalapong *et al*., 2008), wild rats in Kuala Lumpur, Malaysia (Shafiyyah *et al*., 2012), infected wild rats in Malang, East Java, Indonesia (Yesica *et al*., 2022), and black rats in Niger, West Africa (Dobigny *et al*., 2011).

In addition, it is reported that *T. lewisi* has infected humans in several countries. According to Sarataphan *et al*. (2007), there have been reports of *T. lewisi* infections in infants, including a 45-day-old Thai infant who had fever, anemia, coughing, and anorexia. According to Kaur *et al*. (2007), it was also observed in a two-month-old

infant in metropolitan Mumbai, India. The infection of a 2-month-old child in the Gambia demonstrated severe clinical signs, including generalized edema and a possible invasion of the central nervous system, as well as high and persistent parasitemia; the sequence of the SSU rDNA of the trypanosome in the child's blood was highly similar to that of a rodent-associated trypanosome also from the Gambia (Howie *et al*., 2006). Malang is one city with the greatest population density in East Java and is a well-known hotspot for the onset and spread of parasitic illnesses (Central Statistics Agency, 2023). Wulan and Widodo (2020) assert that the main factors contributing to the formation of slum settlements in Malang City include a scarcity of residential land, low community income, and insufficient public awareness regarding the importance of maintaining cleanliness in the vicinity. Given rats' affinity for unhygienic environments, there exists a significant risk of individuals contracting trypanosomiasis from *T. lewisi* (Yuliadi, 2016).

The polymerase chain reaction is a method for identifying and diagnosing *T. lewisi*. The PCR method offers distinct advantages over the blood smear test, primarily due to its capability to detect nucleic acid fragments with heightened sensitivity and specificity (Ehtisham *et al*., 2016; Purnama *et al*., 2021). Among the target genes utilized in the PCR approach for *T. lewisi* detection is the Internal Transcribed Spacer-1 (ITS1), localized within the nucleolus of *T. lewisi* cells (Pumhom *et al*., 2013). Amplification of the ITS1 gene involves the utilization of primer pairs tailored to complement the identified target DNA (Garibyan and Avashia, 2013). Notably, LEW1 stands out as one of the primers designed explicitly for ITS1 gene amplification, as indicated by Marc *et al*. (2011).

The resultant PCR data can be subjected to sequencing to explore the genetic variants of *T. lewisi*. Genetic variation analysis enables insights into molecular-level diversity in DNA, the evolutionary relationship between *T. lewisi* and other Trypanosoma species, the impact of mutations on evolution, and the occurrence of cross-species infections (Anantyarta, 2017). Key factors employed in genetic variation studies encompass phylogram construction, genetic distance measurement, single nucleotide polymorphism (SNP) analysis, and nucleotide composition assessment. The objective of this research is to diagnose and identify genetic variation in the species of *T. lewisi* that infects wild rats in Malang, utilizing four parameters: nucleotide composition, genetic distance, single nucleotide polymorphism (SNP), and phylogram analysis. By striving to mitigate the risk of emerging diseases, it is anticipated that this study would offer additional biomolecular insight into *T. lewisi*, contributing to preventive measures.

MATERIALS AND METHODS

Ethical Approval

The Universitas Brawijaya Animal Care Committee approved the animal ethics protocol under the designation 090-KEP-UB-2021.

Study Period and Location

This study was conducted in Malang, where wild rat traps were strategically placed across 8 locations: Kasin Market, Comboran Market, Blimbing Market, Dempo, Dieng Atas, Wagir, and Sukun. These locations were selected based on several criteria, including densely populated residential areas, an estimated population of approximately 20–30 families, the presence of slum surroundings, and frequent reports of wild rat sightings in the vicinity. The procedure for rats was conducted at the Laboratory of Anatomic Pathology, Faculty of Veterinary Medicine, Universitas Brawijaya. Euthanasia commenced with cleaning the injection site with alcohol-soaked cotton to prevent contamination. An anesthetic injection was administered at the caudal extremity using a dose five times higher than the standard anesthetic dose. Once the rat became unconscious, intracardiac blood collection was performed (AVMA: 2020 edition). Blood smear tests and Giemsa staining 10% were conducted at the Parasitology Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya. Biomolecular tests utilizing the polymerase chain reaction (PCR) technique were carried out at the

Animal Diseases Diagnostic (ADD) Laboratory of Veterinary Medicine. For safety precautions, researchers wore protective gear such as laboratory coats, double-layered gloves, and disposable N95 face masks to prevent infection transmission while handling the animals. Additionally, strict hand hygiene practices were adhered to, with hands washed thoroughly after any animal contact. Standard cleaning protocols were followed to maintain cleanliness in the post-mortem chamber, and all dissection kits and equipment were cleaned after each use. Carcasses were disposed of as clinical waste in yellow biohazard bags. The investigation spanned from September 2021 to March 2022.

Parasites Identification

Giemsa staining solution was applied to the glass slides after blood was smeared. According to Stuart *et al*. (2010), the procedure of Giemsa staining: air-dried samples were fixed for ten minutes in methanol. Dry by air until all of the methanol has evaporated. For 20 minutes, stain in a coplin jar with 10% Giemsa stain (diluted in tap water). Rinse the sample under tap water until the excess Giemsa stain is transparent. Dry it in the air and inspect it under a microscope. The prepared smear was examined with an oil immersion lens at 1000× magnification using an Olympus CX-23 (Olympus Corporation, Japan), and images were recorded using an OptiLab Advanced Plus camera (PT Miconos, Indonesia). *T. lewisi* possesses a long, modest posterior end and a kinetoplast in the sub-terminal area, according to Desquesnes *et al*. (2002). In addition, based on Yesica *et al*. (2022), *T. lewisi* exhibited a leaf-like morphology, a long, slightly tapering posterior end with an oval-shaped kinetoplast sub-terminally positioned, and an anterior nucleus. They have an elongated membrane and a free flagellum.

DNA Isolation

DNA was isolated from blood samples from rats with high *T. lewisi* infection (more than ten trypomastigotes in a single field of vision under the microscope). Then, 0.5 mL positive specimens were transferred into a microtube, and 200 µL of absolute ethanol was added to the blood samples' microtubes. Ethanol was employed to safeguard the parasite's DNA integrity until a molecular examination was carried out.

Subsequently, all positive blood samples were left to evaporate the ethanol over a period of three days at room temperature before DNA isolation. The tube was then supplemented with proteinase K, a broad-spectrum protease facilitating the breakdown of all cell proteins and the release of nucleic acids (Qamar *et al*., 2017). DNA isolation was conducted using a DeRiPROTM 3-In-1 Extraction Kit, which employs advanced technology for the simultaneous extraction of DNA, RNA, and proteins.

DNA Amplification

The *T. lewisi* DNA amplification procedure was performed using the PCR technique in a Bio-Rad® T100TM Thermal Cycler. Initially, a PCR sample comprising 6 µL of Thermo Scientific 2X Dream Taq Hot Start Green PCR Master Mix, 1 µL of forward primer for the ITS1 gene (LEW1S: 5'-ACC ACA CGC TCT CTTT CT-3'), 1 µL of reverse primer for the ITS1 gene (LEW1R: 5′-TGT ATG TGC GTG CTT GTT CA-3′), 1 µL of ddH2O or nuclease-free water (NFW), and 3 µL of DNA sample was prepared for amplification. Following 10 seconds of vortexing at 1800 rpm for homogenization, the sample was subjected to 35 cycles of denaturation, annealing, and elongation in the thermal cycler (Table 1).

Electrophoresis

The electrophoresis method serves to assess the quality of PCR products by leveraging the movement of charged molecules in an electric field, a principle known as the isoelectric point. Through electrophoresis, nucleic acids (DNA) and proteins are effectively separated. Various factors including the form and temperature of the molecules, their mass, as well as the viscosity and porosity of the medium, collectively influence their mobility during the electrophoresis process (Fuad *et al*., 2016). In this study, the quality of the PCR products was evaluated through electrophoresis using a 1.8% agarose gel

concentration. A 1.8% agarose gel concentration has been widely utilized in previous studies for the separation of DNA fragments with a base pair target of 220 bp (Marc *et al*., 2011).

DNA Sequencing

First Base Laboratory managed the sequencing process, while PT. Genetic Science Indonesia facilitated sample delivery. The transmitted PCR product was in the form of an unpurified amplicon, packaged by inserting it into a single tube. Each tube was labeled with a sample number code, and the label was securely sealed with parafilm. Subsequently, the tubes were placed on a foam board, which was further covered in plastic to ensure dryness. The Sanger DNA sequencing technique, performed using the BigDye® Terminator v3.1 cycle sequencing kit, yields high-quality sequences for relatively large DNA segments, up to approximately 900 base pairs. This method involves the DNA polymerase enzyme, which catalyzes the annealing of amplified DNA to an oligonucleotide primer and extends the resulting product, incorporating either deoxynucleotide triphosphates (dNTPs: dATP, dGTP, dCTP, and dTTP) or dideoxynucleotide triphosphates (ddNTPs: ddATP, ddGTP, ddCTP, and ddTTP) (Crossly *et al*., 2020). Arham (2015) explains that the Sanger sequencing process necessitates a DNA template and specific primers. Its fundamental principle involves a synthetic approach to generating new DNA molecules using deoxynucleoside triphosphates (dNTP) as the primary constituents and terminating the synthesis process at specific bases using dideoxynucleoside triphosphates (ddNTP).

Statistical Analysis

The genetic variation and phylogenetic analysis of *T. lewisi* are elucidated through DNA sequence data, wherein comparisons are drawn between DNA sequences derived from *T. lewisi* specimens collected from diverse countries and other Trypanosoma species sourced from the NCBI GeneBank database. Four factors are employed in this comparative analysis: phylogram construction, genetic distance measurement, single nucleotide polymorphism (SNP) analysis, and assessment of nucleotide composition. Utilizing MEGA version 11.0 software, a phylogenetic tree or phylogram was constructed using the bootstrapped Neighbor-Joining (NJ) technique. Bootstrap confidence intervals for phylogenetic trees were calculated based on 1000 repetitions. The results generated by the MEGA program will comprise a matrix depicting genetic distance similarities among nucleotide bases (Tang *et al*., 2012).

RESULTS AND DISCUSSION

The ITS1 gene was amplified in five blood samples (D17, D18, D19, D29, and D37) from rats exhibiting high *T. lewisi* infection, defined as having more than ten trypomastigotes observed in a single field of vision under microscope examination. *T. lewisi* exhibited a leaf-like morphology, characterized by a long and slightly tapering posterior end, an oval-shaped kinetoplast sub-terminally positioned, and a nucleus located in the anterior region. *T. lewisi* has an elongated membrane and a free flagellum (Figure 1). This finding was aligned with study of Yesica *et al*. (2022), the structure of *T. lewisi* isolated from wild rats in malang city was resembled a leaf, with its nucleus situated in the anterior section and a long, slightly curved posterior end featuring an oval-shaped kinetoplast sub-terminally, an undulating membrane, and a free flagellum are notable features of this species. This finding was also supported by study of Hoare (1972) and Desquesnes *et al*. (2022), *T. lewisi* is distinguished by its elongated posterior end, a subterminal oval kinetoplast, a nucleus located in the anterior region of the body, and a flagellum with a free portion.

Based on the electrophoresis data, all five amplified samples exhibited visible DNA bands. Notably, blood sample D19, labeled as L19 in the electrophoresis code, displayed the brightest DNA band, whereas blood sample D17, identified by the L17 electrophoresis code, showed a fainter DNA band. Furthermore, the electrophoresis data indicated that the amplified DNA samples all exhibited base pair sizes consistent with the target, specifically 220 bp. Among the five DNA

samples analyzed, blood samples D18, D19, and D37 demonstrated the highest PCR product quality. In contrast, blood samples D17 and D29 were deemed less suitable due to their less intense DNA bands. This diminished band intensity may be attributed to factors such as high annealing temperature or incorrect utilization of DNA fractions in the amplicon creation process.

Table 1. PCR procedures, including temperature settings, cycle repetitions, and duration

Number of cycle repetitions	Process	Temperature	Duration
I (1 cycle)	Pre-denaturation	94°C	5 minutes
	Denaturation	94°C	30 seconds
II (35 cycles)	Annealing	56.2°C	45 seconds
	Elongation	72°C	1 minute
III (1 cycle)	Post-elongation	72°C	5 minutes

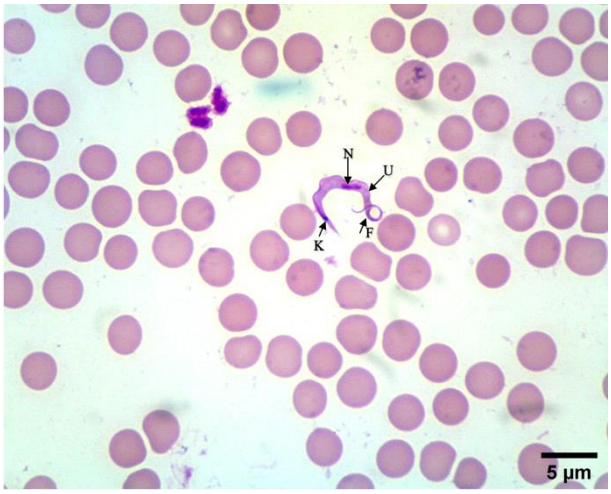

Figure 1. The results of microscopic studies conducted at 1000× magnification on blood smear preparations stained with Giemsa staining revealed distinct morphological features of Trypomastigote. These included an anteriorly positioned nucleus, an oval sub-terminal kinetoplast, a single free flagellum, and a wavy membrane extending from the anterior to the posterior. Key identifiers observed during this analysis were kinetoplast (K), nucleus (N), and undulating membrane (U), and flagellum (F).

Table 2. The nucleotide composition of the ITS1 rDNA nucleolar region was found to be modified in *T. lewisi* 18, 19, and 37, compared to sequences of *T. brucei*, *T. cruzi*, *T. evansi*, and additional *T. lewisi* strains sourced from NCBI GeneBank, following alignment using the Clustal W method

Protozoan	Nucleotide Composition					
	T(U)	C	A	G	A+T	C+G
(MK132184.1) *T. brucei* Japan	37	13.33	30	19.66	67	32.99
(KT305857.1) *T. cruzi* Brazil	32.68	17.72	25.08	24.51	57.76	42.23
(LC521916.1) *T. evansi* Phillippines	35.17	15.86	30.11	18.85	65.28	34.71
(MK005296.1) *T. lewisi* Brazil	31.08	18.31	28.91	21.68	59.99	39.99
(HQ437158.1) *T. lewisi* France	29.31	18.70	28.95	23.02	58.26	41.72
(GU252222.1) *T. lewisi* Brazil	31.08	18.55	29.16	21.20	60.24	39.75
(FJ011094.1) *T. lewisi* China	30.24	18.70	27.79	23.25	58.03	41.95
(EU599639.1) *T. lewisi* China	30.24	18.70	27.79	23.25	58.03	41.95
(DQ345394.1) *T. lewisi* Thailand	30.17	18.21	28.75	22.85	58.92	41.06
T. lewisi 18	37.31	13.43	23.88	25.37	61.19	38.80
T. lewisi 19	37.31	13.43	23.88	25.37	61.19	38.80
T. lewisi 37	37.31	13.43	23.88	25.37	61.19	38.80
Average	32.02	17.58	27.76	22.61	60.56	39.43

Table 3. The findings of the intraspecies and interspecies genetic distance analysis conducted on *T. lewisi* 18, 19, and 37, in comparison to sequences from *T. brucei*, *T. evansi*, *T. cruzi*, and other *T. lewisi* strains obtained from NCBI GeneBank, are based on ITS1 rDNA sequence analysis

Protozoan	Genetic Distance											
	MK	**LC**	**KT**	**HQ**	**GU**	**MK**	**FJ**	**EU**	**DQ**	**18**	**19**	**37**
(MK132184.1) *Trypanosoma brucei* Japan												
(LC521916.1) *Trypanosoma evansi* Phillippines	0.03											
(KT305857.1) *Trypanosoma cruzi* Brazil	1.24	1.01										
(HQ437158.1) *Trypanosoma lewisi* France	1.16	1.16	0.9									
(GU252222.1) *Trypanosoma lewisi* Brazil	1.12	1.10	0.78	0.02								
(MK005296.1) *Trypanosoma lewisi* Brazil	1.15	1.15	0.77	0.004	0.01							
(FJ011094.1) *Trypanosoma lewisi* China	1.13	0.98	0.88	0.01	0.01	0.002						
(EU599639.1) *Trypanosoma lewisi* China	1.12	1.07	0.91	0.02	0.007	0.01	0.01					
(DQ345394.1) *Trypanosoma lewisi* Thailand	1.15	1.09	0.88	0.02	0.02	0.009	0.01	0.002				
T. lewisi 18	1.14	1.14	0.61	0.01	0.01	0.007	0.01	0.007	0.007			
T. lewisi 19	1.14	1.14	0.61	0.01	0.01	0.007	0.01	0.007	0.007	0.0		
T. lewisi 37	1.14	1.14	0.61	0.01	0.01	0.007	0.01	0.007	0.007	0.0	0.0	0.0

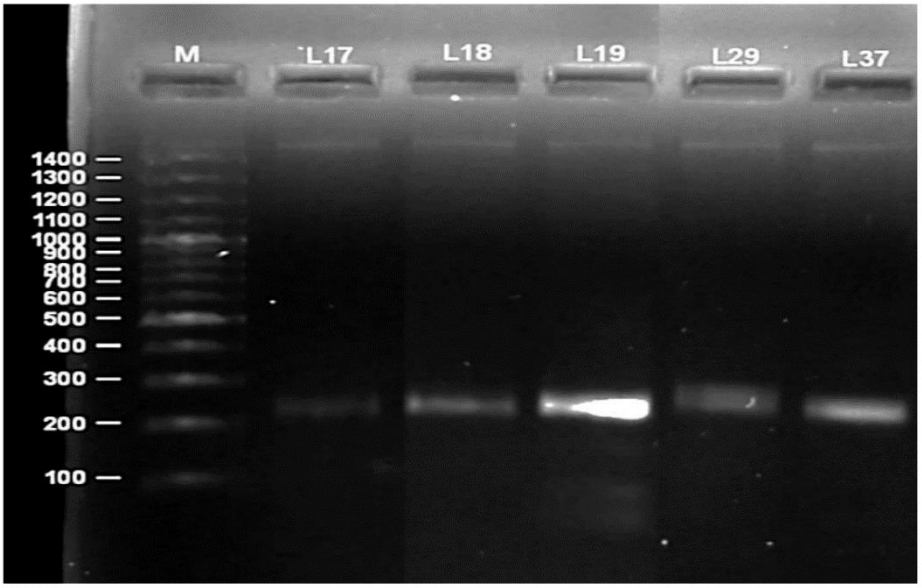

Figure 2. The outcomes of 1.8% agarose gel electrophoresis conducted on blood samples from wild rats following amplification of the ITS1 gene are as follows: Marker M, along with blood samples labeled as follows: L17 (blood sample 17, designated as D17), L18 (blood sample 18, designated as D18), L19 (blood sample 19, designated as D19), L29 (blood sample 29, designated as D29), and L37 (blood sample 37, designated as D37).

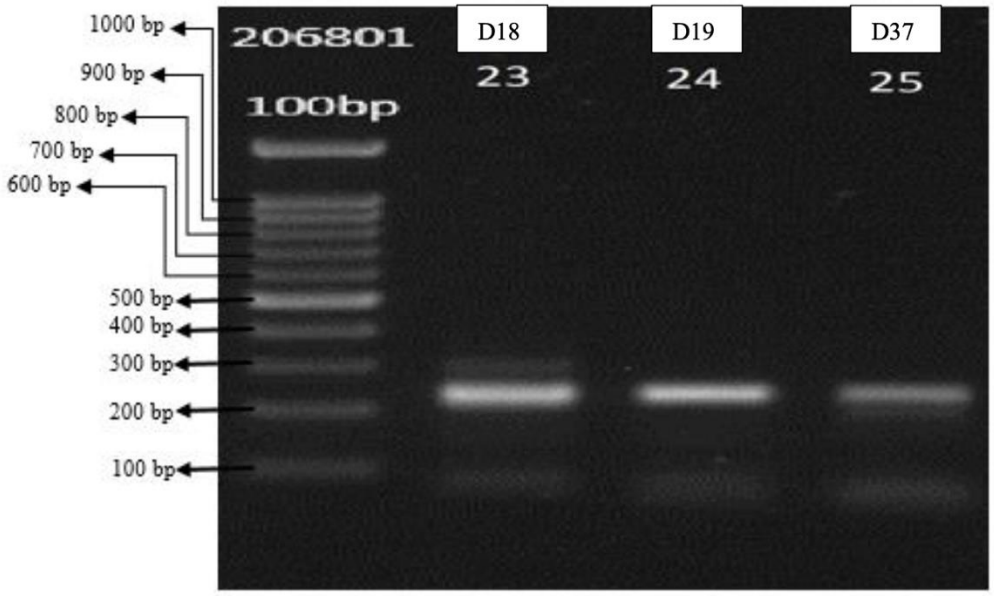

Figure 3. The electrophoresis results of PCR products for samples D18, D19, and D37 were observed using a 1% agarose solution. All three samples displayed distinct DNA bands at 220 bp, characterized by a single, thick, and intense appearance, consistent with the target base pair size.

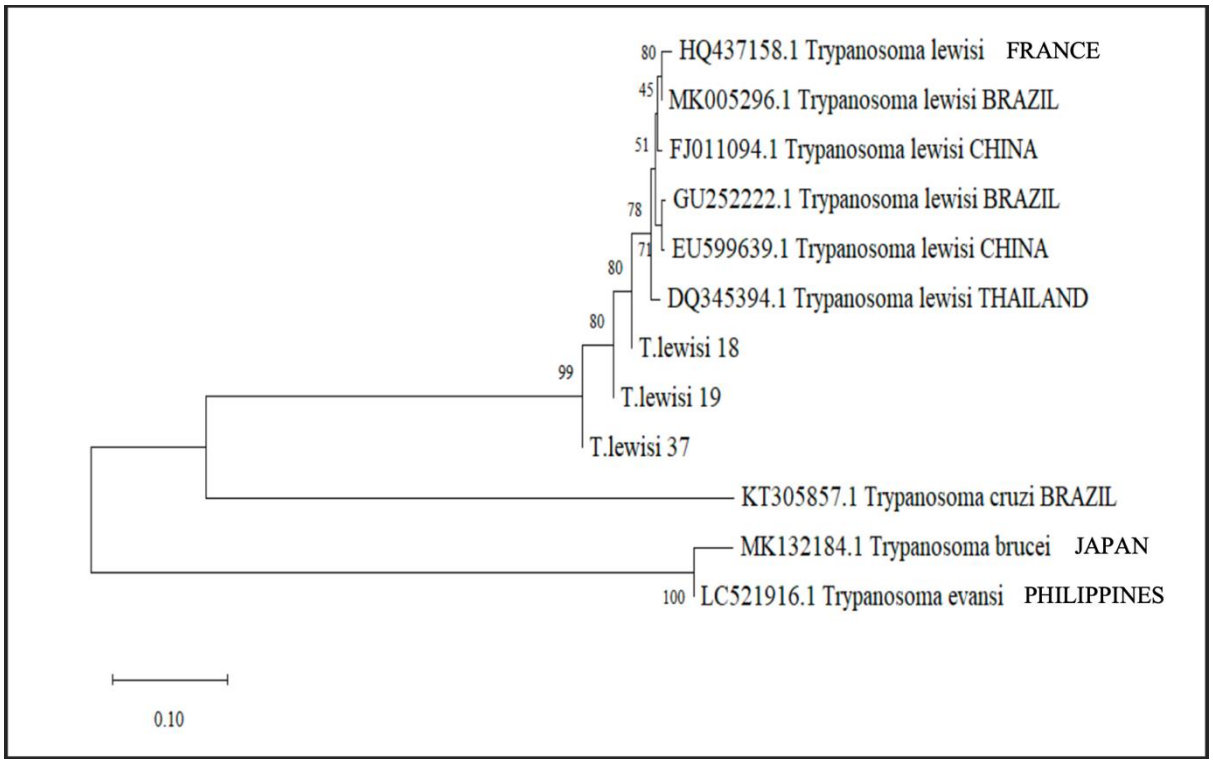

Figure 4. The reconstruction of the original phylogenetic tress using sequences from *T. lewisi,* 18, 19, and 37, alongside sequences obtained from *T. Brucei*, *T. Evansi,* and *T. Cruzi* sourced from NCBI Genebank.

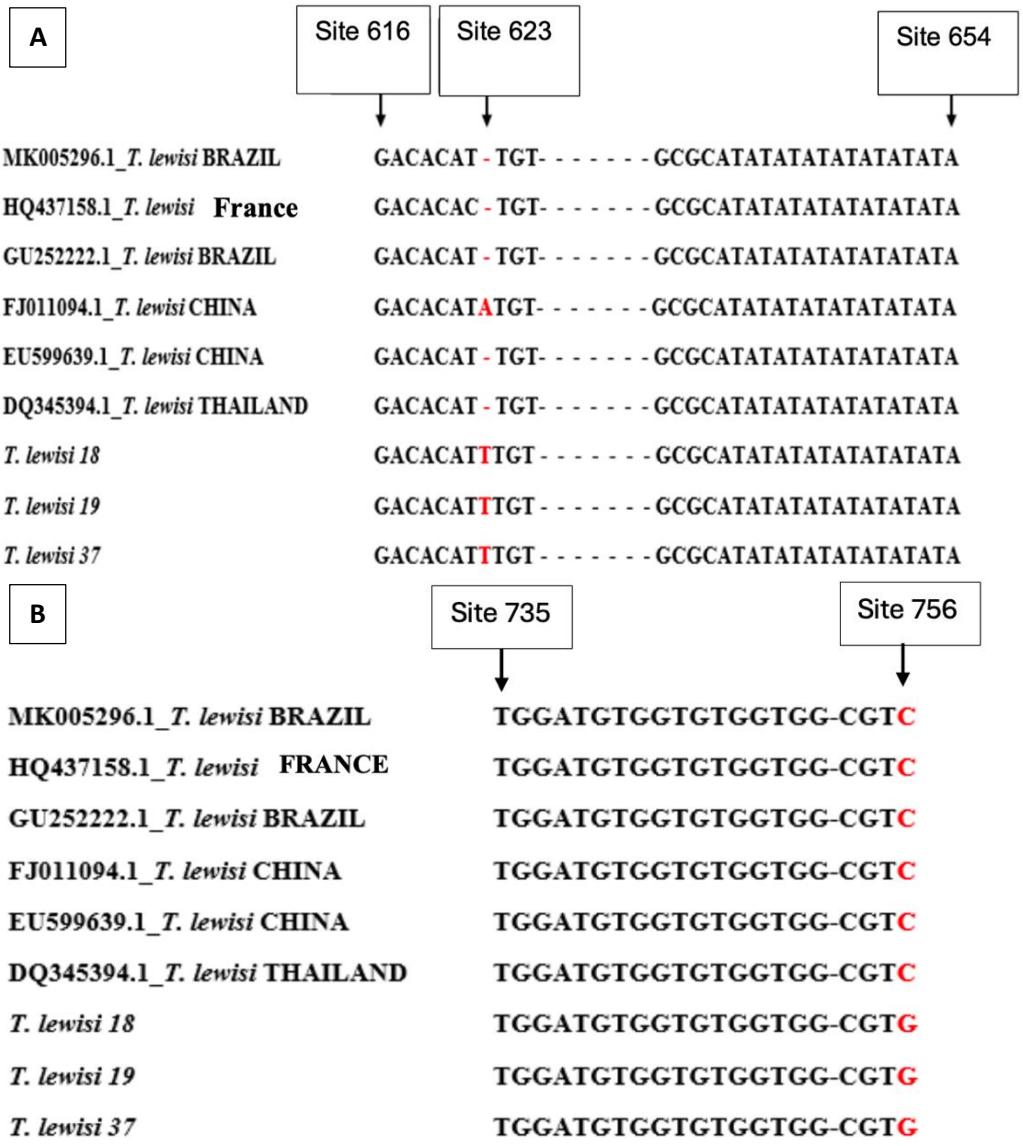

Figure 5. *T. lewisi* sequences retrieved from NCBI GeneBank were aligned with single nucleotide polymorphisms (SNPs) identified in *T. lewisi* 18, 19, and 37. This comparison revealed the presence of an SNP at position 623 (A) and 756 (B), visually represented by characters highlighted in red, denoting the SNP occurrence.

According to Figure 2 and 3, depicting the electrophoresis result, three amplicons (D18, D19, D37) exhibiting prominent DNA bands were selected for sequencing. These three amplicon samples displayed optimal DNA band characteristics: single-shaped, thick, brilliant, and aligned with the target base pair size. Sequencing findings were presented in Fasta data and chromatogram graph format. Due to its versatility and widespread adoption within the bioinformatics community, the Fasta format was chosen. It is well-suited for diverse bioinformatics tasks, facilitating sequence data storage, retrieval, and manipulation.

Chromatogram graphs are employed for their ability to visually depict sequencing data, simplifying interpretation and assessment for researchers. They enable precise base calling and quality evaluation of sequencing reactions, ensuring the reliability of the acquired sequence data. A high-quality chromatogram graph is characterized by evenly spaced single peaks without overlap (Makarova *et al*., 2013). When processing the received Fasta data, one of the considerations is the chromatogram graph, which is aligned through editing to arrange different nucleotide bases (Crossly *et al*., 2020). The sequencing results for samples D18, D19, and

D37 were obtained after editing using the MEGA version 11.0 tool and BLAST on NCBI GeneBank. The percentage identity was determined to be 98.51%, indicating homology with the Chinese-originating *T. lewisi* species, with accession number FJ011094.1. Although the target base length for three DNA samples in this study was 220 bp, only 134 bp of nucleotide bases were acquired from alignment and editing procedures. This phenomenon arises from the alignment and editing procedures, which involve comparing forward and reverse sequencing outcomes of DNA samples—specifically, D18, D19, and D37—utilizing the Clustal W algorithm. Editing encompasses excising regions with substantial gaps and sequences from both forward and reverse directions that exhibit relatively poor quality (such as missing nucleotide bases or unreadable portions), as indicated by Crossly *et al*. (2020).

The nucleotide composition of the nucleolar region of ITS1 rDNA exhibited modifications in *T. lewisi* strains 18, 19, and 37 compared to sequences from *T. brucei, T. cruzi, T. evansi,* and additional *T. lewisi* strains obtained from NCBI GeneBank. This comparison was conducted through alignment using the Clustal W method. The nucleotide composition data is presented in Table 2, where thymine (T) predominates at 37.31%, representing the primary nucleotide base in the ITS1 region of *T. lewisi* samples 18, 19, and 37, as depicted in the table. Conversely, cytosine (C) is the least abundant nucleotide base, comprising only 13.43% of the sequence. Variations in nucleotide composition are invaluable for understanding genetic diversity, evolutionary history, and population structure within species. They provide insights into the mechanisms underlying genetic variation and its role in driving adaptability and species divergence. Lin *et al.* (2015) highlighted variations in nucleotide composition as indicative of significant genetic differentiation. Such variation entails differences in the types and frequencies of nucleotides (adenine, thymine, cytosine, and guanine) in DNA sequences across different individuals or populations. This variation serves as a key indicator of genetic differentiation, reflecting the extent of genetic divergence between populations or individuals within a species. Analyzing the nucleotide composition of T. lewisi in this study, data suggest a discernible pattern of genetic diversity among Trypanosoma species from the Malang region of Indonesia and several other regions worldwide.

Furthermore, in each sequence retrieved from NCBI GeneBank for *T. lewisi, T. evansi, T. cruzi,* and *T. brucei*, the nucleotide base thymine (T) is predominant. The adenine-thymine (AT) combination represents a prevalent arrangement, constituting 61.19% of the nucleotide base pairs in the sequences of *T. lewisi 18, T. lewisi 19, and T. lewisi 37*. This consistent prevalence of nucleotide base pairs is observed throughout the sequences sourced from NCBI GeneBank for *T. lewisi, T. evansi, T. cruzi*, and *T. brucei*. This observation aligns with Marc *et al*. (2011), where in the ITS1 gene region, AT base pairs (adenine and thymine) predominate among the nucleotides. Specifically, the AT nucleotide base pair (adenine-thymine) tends to dominate unstable DNA sequences and is prone to repeated mutations due to weak base pairing interactions between AT and GC. Unlike GC base pairs, which possess three hydrogen bonds, AT base pairs only form two bonds. In AT base pairs, only two hydrogen bonds are present, whereas GC base pairs consist of three bond (Marc *et al*., 2011). Due to its composition, the ITS1 gene region undergoes frequent mutations based on base pair composition, making it an excellent choice for amplification to detect genetic differences in the blood protozoan *Trypanosoma lewisi* (Smarda *et al*., 2014).

The results of intraspecies and interspecies genetic distance analyses conducted using MEGA version 11.0 software, employing the maximum likelihood composite model method are presented in Table 3. Examination of this table reveals significant interspecies genetic distances between samples of *T. lewisi* 18, 19, and 37, and *T. brucei, T. evansi*, and *T. cruzi* retrieved from NCBI GeneBank, with respective values of d = 1.14, d = 1.14, and d = 0.61. Notably, *T. lewisi* exhibits the closest intraspecies genetic distance to *T. cruzi*

compared to other *Trypanosoma species* from NCBI GeneBank. Furthermore, when comparing several *T. lewisi* strains from NCBI GeneBank with *T. brucei*, *T. evansi*, and *T. cruzi*, considerable genetic distances between species are observed, with average values of d = 1.13, d = 1.09, and d = 0.85, respectively. In addition, the intraspecies genetic distance between *T. lewisi* 18, 19, and 37, and six other *T. lewisi* strains from NCBI GeneBank demonstrates relatively close genetic proximity, ranging from d = 0.007 to 0.01. Specifically, *T. lewisi* sequences (NCBI GeneBank) exhibiting the closest genetic distance to samples 18, 19, and 37 were identified as *T. lewisi* (MK005296.1), *T. lewisi* (EU599639.1), and *T. lewisi* (DQ345394.1), with a genetic distance value of d = 0.007. According to Wirdateti *et al*. (2016), the obtained genetic distance reflects the degree of relationship between species. A lower genetic distance value indicates a closer relationship between individuals, while a higher value indicates a more distant relationship. Generally, the intraspecies genetic distance value is lower than the interspecies genetic distance value.

According to Xia *et al*. (2019), a phylogram, also referred to as a phylogenetic tree, is a branching diagram illustrating the evolutionary relationships between individuals based on gene sequences. The phylogram obtained illustrates the intraspecies relationship among research samples *T. lewisi* 18, 19, and 37, along with *T. lewisi* strains sourced from NCBI GeneBank (accession numbers: MK005296.1, HQ427158.1, GU252222.1, FJ011094.1, EU599639.1, and DQ345394.1), as well as interspecies relationships with *T. brucei* (ID: OK422213.1), *T. cruzi* (KT305857.1), and *T. evansi* (LC521916.1) also retrieved from NCBI GeneBank (Figure 4). Based on the phylogram, it is evident that the reconstructed phylogram segregates into three main clades. Notably, the samples tested—*T. lewisi* 18, 19, and 37—are clustered within the same clade as the six *T. lewisi* strains from NCBI GeneBank. This observation indicates a very close intraspecies relationship between *T. lewisi* 18, 19, and 37 and those from NCBI GeneBank, as determined by genetic distance. According to Saleky and Merly (2021), clade formation within a group of individuals is predicated on genetic distance; individuals with close genetic proximity form a distinct clade, whereas those with greater genetic divergence form separate ones. The second clade in the phylogram comprises exclusively *T. cruzi*, suggesting a relatively distant genetic relationship between *T. cruzi* and *T. evansi* as well as *T. brucei*, but a closer proximity to *T. lewisi* 18, 19, and 37.

Single nucleotide polymorphism (SNP) represents a prevalent form of DNA variation serving as a genetic marker, manifesting as a single nucleotide alteration within the genome sequence (Xia *et al*., 2019). According to Karki *et al*. (2018), Single Nucleotide Polymorphism (SNP) refers to a DNA sequence variation occurring in a population with a frequency of 1% or more. In the research sample sequences, *T. lewisi* 18, 19, and 37, two distinct single nucleotide variations were identified in comparison to the six *T. lewisi* sequences referenced from NCBI GeneBank. Utilizing the Clustal W method, the specific nucleotide variances were aligned with the corresponding positions in *T. lewisi* sequences retrieved from NCBI GeneBank, pinpointing discrepancies at the 623rd and 756th positions (Figure 5). At the 623rd site, the nucleotide is exclusive to *T. lewisi* 18, 19, 37, and *T. lewisi*, referenced from NCBI GeneBank with accession number FJ011094.1. Discrepancies exist in the nucleotides recorded in the *T. lewisi* sequence (ID: FJ011094.1) compared to the findings of *T. lewisi* 18, 19, and 37 research. Specifically, at site 623, the nucleotides in the sequencing results of *T. lewisi* 18, 19, and 37 are Thymine (T), whereas the nucleotide listed in the *T. lewisi* sequence (ID: FJ011094.1) is Adenine (A). At the 756th site, the nucleotide listed in the *T. lewisi* sequence sourced from NCBI GeneBank with accession numbers MK005296.1, HQ427158.1, GU252222.1, FJ011094.1, EU599639.1, and DQ345394.1 is Cytosine (C), whereas the nucleotide in the sample sequence from *T. lewisi* research 18, 19, and 37 is Guanine (G). SNPs are non-functional point mutations that can manifest in coding sequence areas, introns, or intergenic regions.

These variations serve as genetic markers in populations, facilitating the study of predispositions to certain traits in individuals (Xia *et al*., 2019). In this study, it was discovered that DNA markers at sites 623 and 756 of T. lewisi 18, 19, and 37 differed from those of T. lewisi retrieved from NCBI GeneBank data.

Based on the bootstrap values, it is evident that samples *T. lewisi* 18, 19, and 37 exhibit relatively robust bootstrap values at 80, 80, and 99, respectively. Notably, *T. lewisi* 37 demonstrates a higher bootstrap value compared to *T. lewisi* 18 and *T. lewisi* 19, although the bootstrap values of both *T. lewisi* 18 and *T. lewisi* 19 remain sufficiently reliable. As noted by De Moraes Russo (2018), low bootstrap values may arise due to insufficient characters supporting a node or alignment errors resulting from the diversity and ambiguity of the aligned sequences. The application of bootstrapping in phylogenetic tree construction aims to assess the internal consistency of molecular datasets supporting the same clade through the analysis of altered alignments. Bootstrap values exceeding 95 are deemed significant, while those surpassing 70 are considered reliable (Kelley, 2018; Sukoco *et al*., 2023). Anafarida (2020) suggests the likelihood of alterations in the composition of a small clade falls within the bootstrap value range of 70–100, whereas changes in the composition of a large clade typically occur with bootstrap values < 70. Furthermore, higher bootstrap values and parallel lines observed between individuals signify a closer kinship relationship among them. that the likelihood of compositional changes within a substantial clade is indicated by a bootstrap value < 70, whereas for a minor clade, the likelihood ranges from 70 to 100.

CONCLUSION

The sequencing analysis of samples D18, D19, and D37 revealed a 98.51% similarity to *Trypanosoma lewisi* species originating from China, with accession number (FJ011094.1). In the ITS1 gene sequence regions of *T. lewisi* 18, 19, and 37, thymine (T) bases predominate, comprising 37.31% of the sequence. Additionally, two Single Nucleotide Polymorphisms (SNPs) were identified at the 623rd and 756th sites. Based on phylogram reconstruction, a close intraspecies relationship was observed with a genetic distance (d = 0.007–0.01), while the closest interspecies relationship was found with *T. cruzi* (KT305857.1) with a genetic distance of (d = 0.61). This study serves as a pilot investigation into the genetic variation of *T. lewisi* in Indonesia, particularly in the city of Malang. Further research is warranted to explore the genetic diversity of *T. lewisi* in other cities around Malang and across Indonesia, to gather more comprehensive data regarding its characteristics.

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AUTHORS' CONTRIBUTIONS

RY: Conceptualization, Validation, Investigation, Fieldwork, Writing - Original Draft, Supervision, Funding acquisition. ABH: Methodology, Software, Data Curation, Formal analysis. YO: Methodology, Validation, Data Curation, advice on PCR process. SK: Advice on parasitology field, Review & Editing, Project administration. GJGP: Investigation, Writing - Original Draft, Visualization, fieldwork. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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