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# DOES *TRYPANOSOMA EVANSI* HAVE THE *MAXICIRCLE* GENE, OR CAN *TRYPANOSOMA EQUIPERDUM* BE ISOLATED FROM BOVINES?

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**KEY WORDS**

*ITS1*
*ESAG6/7*
*kDNA* minicircle
*nad5-kDNA* maxicircle
Bovine
*Trypanosoma equiperdum*
*Trypanosoma evansi*

**ABSTRACT**

Identifying a trypanosome isolate is generally based on morphological observations and molecular identification of one of the genes, usually internal transcribed spacer 1 and 2 of ribosomal DNA (*ITS1* rDNA, *ITS2* rDNA), a variant surface glycoprotein of Rode *Trypanozoon* antigen type 1.2 (*VSG RoTat 1.2*), or expression site-associated genes (*ESAG*). However, this identification is insufficient because these genes cannot distinguish organisms in the subgenus *Trypanozoon* to the species level. A molecular approach using at least 5 sets of primers is needed, namely, ITS1, ESAG6/7, MINI, RoTat 1.2, and ND5, for stratified selection to obtain more targeted and conclusive results. Using this method to analyze isolates from Indonesia provided unexpected results: 9 isolates previously identified as *Trypanozoon* were found to have the *kDNA maxicircle* gene. Nine isolates of *Trypanosoma equiperdum* were identified for the first time in Indonesia, isolated from bovine (cattle and buffaloes). The identification of *T. equiperdum* in the 9 isolates was confirmed by analysis of the nucleotide sequence identity of the *nad5-kDNA maxicircle* gene.

Animal trypanosomiasis (AT) is widely reported in domestic animals in Africa, Asia, and South America (Nok, 2009). Typically, trypanosomiasis in animals is divided into 2 types, namely, tsetse-transmitted animal trypanosomiasis (TTAT), also known as African animal trypanosomiasis (AAT), and non-tsetse-transmitted animal trypanosomiasis (NTTAT), which generally occurs outside the African continent (Touratier, 2000; Yaro et al., 2016). The main cause of NTTAT in domestic animals is infection with *Trypanosoma evansi*, *Trypanosoma equiperdum*, and *Trypanosoma vivax* (Touratier, 2000). *Trypanosoma vivax* significantly impacts cattle production in South America, while *T. evansi* affects camels in Asia and horses, cattle, and domestic buffalo in South America, India, and Southeast Asia (Nok, 2009).

In Indonesia, AT has been declared a strategic disease of enormous economic importance. The most recent AT outbreak caused the death of more than 1,700 horses and buffaloes in East Nusa Tenggara province in 2010–2012 (Subekti and Yuniarto, 2020). In Indonesia, cases of AT in cattle, buffalo, and horses, often referred to as *surra*, are generally caused by *T. evansi*. This assumption is generally based on morphological identification, which is often considered insufficient. Specimens of 3 species in the subgenus *Trypanozoon*, namely, *Trypanosoma (Trypanozoon) brucei* (generally called *T. brucei*), *Trypanosoma (Trypanozoon) evansi* (generally called *T. evansi*), and *Trypanosoma (Trypanozoon) equiperdum* (generally called *T. equiperdum*), are very difficult to distinguish morphologically by either light or electron microscopy (Brun et al., 1998; Sánchez et al., 2015; Gizaw et al., 2017).

To determine if an isolate is *T. evansi*, researchers in Indonesia usually conduct molecular identification based on amplifying one of the following genes: *ITS1* rDNA, *ITS2* rDNA, *VSG RoTat 1.2*, or *ESAG* (Sawitri et al., 2015; Setiawan et al., 2021). Molecular identification based on these genes cannot distinguish each species in the subgenus *Trypanozoon* (Claes et al., 2005; Wen et al., 2016). Therefore, deciding whether the isolate is *T. evansi* is not possible using this approach.

Based on the current status of this field, molecular identification studies using polymerase chain reaction (PCR) and 5 primer

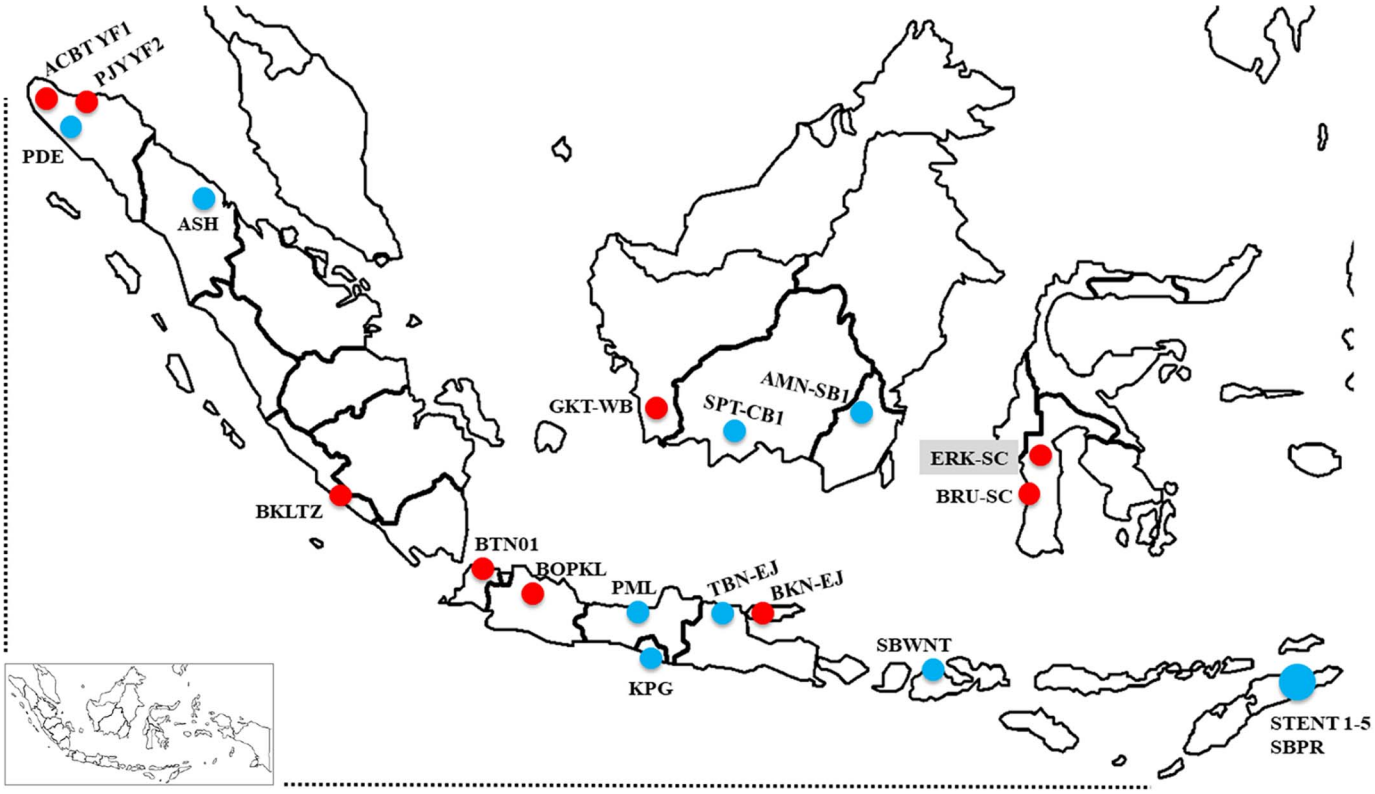


**Figure 1.** Map showing the origin of Indonesian isolates used in this study. The grey (red) dot was the origin of the isolate that was later identified as *Trypanosoma evansi*. The black (blue) dot was the origin of the isolate that was later identified as *Trypanosoma equiperdum*. Color version available online.

sets for specific genes, namely, ITS1, ESAG6/7, RoTat 1.2, MINI, and ND5, are needed. Here we use each primer set for sequential species identification to permit stratified selection so that molecular identification results provide greater specificity and certainty.

## MATERIALS AND METHODS

### Trypanosome isolates

Trypanosome isolates were collected from cattle and buffalo (bovines) in various regions in Indonesia (Fig. 1; Table I).

### Trypanosome propagation and purification

All trypanosome isolates were propagated intraperitoneally in DDY (Deutschland, Denken, and Yoken) mice. Trypanosomes were harvested when the parasite reached peak parasitemia (sixth or seventh day post-infection) with an estimated parasite density of approximately $10^8$–$10^9$ trypanosomes/ml. Parasite density was calculated after parasites were mixed (1:1) with 0.1% blood with 1% sodium dodecyl sulfate and then counted using a Neubauer-improved chamber (Subekti et al., 2013). All experiments using mice were conducted in accordance with and approved by the Experimental Animal Ethics Commission of the Indonesian Agricultural Research and Development Agency (approval number Balitbangtan/BB Litvet/Rd/06/2021).

Trypanosomes isolated from mouse blood were purified by anion exchange chromatography using diethylaminoethyl to separate them from blood cells (Subekti and Yuniarto, 2020). The anion exchange chromatography medium used in this study was Toyopearl 650M, DEAE-methacrylate polymer (Tosoh Bioscience, Philadelphia, Pennsylvania). The trypanosomes separated from blood cells were centrifuged in a collection tube at 3,361 *g* and 4 C for 15 min. The filtrate was washed with phosphate-buffered saline (pH 8) containing 1% glucose, and the process was repeated 3 times. Finally, supernatants were discarded, and the filtrate was resuspended with 1 ml $H_2O$ as purified trypanosomes and stored at −20 C until use.

### DNA extraction and PCR analysis

Genomic DNA extraction was performed on 200 µl of purified trypanosomes using DNAzol (Molecular Research Center Inc., Cincinnati, Ohio) according to the manufacturer's instructions. PCR was performed using an Applied Biosystems™ 2720 Thermal Cycler (Applied Biosystems, Waltham, Massachusetts). The amplification mixture contained 10 µl Bioline 5× My Taq reaction buffer (Meridian Life Science Inc., Memphis, Tennessee), 1 µl Bioline MyTaq HS DNA Polymerase (Meridian Life Science), 20 µM each primer, 1 µl DNA template (100 ng/µl), and ultrapure distilled water (Invitrogen, Waltham, Massachusetts) to 50 µl total reaction volume. The PCR cycle conditions are provided in Table II.

The PCR products were electrophoresed in 1.5% agarose gel mixed with 1st Base FloroSafe DNA stain (Axil Scientific Pte Ltd., Singapore, Singapore) using a runVIEW real-time gel visualization system (Cleaver Scientific Ltd., Warwickshire, U.K.). The PCR products were also sent to Bioneer Corp. (Daejeon,

**Table I.** Trypanosome isolates from Indonesia were included in the study.

| No. | Isolate | Natural host | Year | Region (district, province) | Geographic coordinates |
|---|---|---|---|---|---|
| 1 | AMN-SB1 | Buffalo | 2013 | Hulu Sungai Utara, South Kalimantan | 2°25′3.8″S, 115°14′57.9″E |
| 2 | KPG | Buffalo | 1985 | Kulon Progo, Yogyakarta | 7°51′26″S, 110°9′25″E |
| 3 | BOPKL | Buffalo | 1982 | Bogor, West Java | 6°35′40″S, 106°47′21″E |
| 4 | ACBT-YF1 | Cattle | 2015 | Aceh Besar, Aceh | 5°32′30″N, 95°19′60″E |
| 5 | PML | Buffalo | 1996 | Pemalang, Central Java | 6°53′30.9″S, 109°22′57.5″E |
| 6 | STENT1 | Buffalo | 2012 | Sumba Timur, East Nusa Tenggara | 9°39′24.1″S, 120°15′50.8″E |
| 7 | STENT5 | Buffalo | 2012 | Sumba Timur, East Nusa Tenggara | 9°39′24.1″S, 120°15′50.8″E |
| 8 | STENT3 | Buffalo | 2012 | Sumba Timur, East Nusa Tenggara | 9°39′24.1″S, 120°15′50.8″E |
| 9 | BTN01 | Buffalo | 2016 | Pandeglang, Banten | 6°18′30.2″S, 106°6′24.1″E |
| 10 | SPT-CB1 | Buffalo | 2013 | Kotawaringin Timur, Central Kalimantan | 2°28′22.4″S, 111°28′23.2″E |
| 11 | STENT2 | Buffalo | 2012 | Sumba Timur, East Nusa Tenggara | 9°39′24.1″S, 120°15′50.8″E |
| 12 | STENT4 | Buffalo | 2012 | Sumba Timur, East Nusa Tenggara | 9°39′24.1″S, 120°15′50.8″E |
| 13 | SBWNT | Buffalo | 1998 | Sumbawa Besar, West Nusa Tenggara | 8°29′35.4″S, 117°25′12.9″E |
| 14 | BKN-EJ | Madura cattle | 1998 | Bangkalan, East Java | 7°2′43.8″S, 112°44′6.4″E |
| 15 | TBN-EJ | Cattle | 2003 | Tuban, East Java | 6°53′51.4″S, 112°3′53.6″E |
| 16 | BKLTZ | Bali cattle | 2015 | Bengkulu Selatan, Bengkulu | 4°27′54.4″S, 102°54′13.9″E |
| 17 | PDE | Cattle | 1986 | Pidie, Aceh | 5°23′5.3″N, 95°57′39.2″E |
| 18 | ERK-SC | Bali cattle | 1986 | Enrekang, South Sulawesi | 3°33′′54.3″S, 119°46′12.9″E |
| 19 | PJY-YF2 | Cattle | 2015 | Pidie Jaya, Aceh | 4°59′17.8″N, 96°16′48.3″E |
| 20 | ASH | Buffalo | 1992 | Asahan, North Sumatra | 2°59′4.2″N, 99°36′56.9″E |
| 21 | SB-PR | Buffalo | 2012 | Sumba Timur, East Nusa Tenggara | 9°39′24.1″S, 120°15′50.8″E |
| 22 | BRU-SC | Bali cattle | 2016 | Barru, South Sulawesi | 4°24′43.2″S, 119°37′4.8″E |
| 23 | GKT-WB | Buffalo | 2011 | Ketapang, West Kalimantan | 1°50′19″S, 109°58′38.2″E |

Republic of Korea) for sequencing. Nucleotide sequences were compared for similarity with other trypanosome isolates using the basic local alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI). Dendrogram construction was based on trypanosome isolates from the NCBI database with very high similarity (typically above 90%) with Indonesian trypanosome sequences. The dendrogram was constructed with Mega 11 (Tamura et al., 2021) using the maximum parsimony and maximum likelihood method with Kimura-2 model and bootstrapping 100 replicates.

The 10 primer sets included in this study (Table II) were used in a successive workflow (Fig. 2). Trypanosome species identification was based on PCR amplification products using 5 primer sets used in PCR successively, namely, ITS1, ESAG6/7, MINI, RoTat 1.2, and ND5. The identification flowchart was constructed following the algorithm in Figure 2. The ITS1 primers were used for initial screening to identify trypanosome isolates as *Trypanozoon*, non-*Trypanozoon*, or both. The ESAG6/7 primers were used to ensure that the isolates could be definitively identified as *Trypanozoon*. MINI, RoTat 1.2, and ND5 primers were used as an eliminative step to distinguish between *T. brucei*, *T. evansi*, and *T. equiperdum*. All isolates that were positive by PCR using ND5 primers were subjected to additional tests using ND4/5, MaxiCyt1, and MURF2 Cox1.

## RESULTS

### Amplification results using 10 PCR primer sets

Positive PCR results were observed for all isolates detected using 3 primer sets, namely, ITS1, ESAG6/7, and RoTat 1.2. Negative PCR results were found in all isolates that had been detected using 2 primer sets, namely, ILO and EVAB. Negative PCR results obtained using the EVAB primers indicate that all Indonesian isolates studied were not *T. evansi* type B, while negative PCR using ILO primers proved that they were not *T. vivax*. *Trypanosoma evansi* type B can be distinguished based on the presence or absence of the *class B minicircle* gene. Amplification using the ND5 primers revealed 9 isolates were positive PCR and 14 negative PCR (Table III).

The sequences of PCR products were compared to sequences in the NCBI-BLAST database to determine sequence similarity. The sequence similarities of 23 Indonesian isolates demonstrated >92% similarity with *T. brucei*, *T. evansi*, and *T. equiperdum* based on the *ESAG6/7* gene sequence. The sequences from the 23 Indonesian isolates also demonstrated >96% similarity with *T. evansi* and *T. equiperdum* based on the *gRNA-kDNA minicircle* gene sequence (Table IV), and none of these isolates had a similarity with *T. brucei*. In contrast, only 7 of 23 Indonesian isolates showed >94% sequence similarity, and 2 isolates showed sequence similarities of about >84% compared with *T. equiperdum* and *T. brucei* based on the sequence of the *nad5-kDNA maxicircle* gene; none of the 9 Indonesian isolates had sequence similarity with *T. evansi* (Table IV).

### Phylogenetic tree analysis based on *kDNA minicircle* and *maxicircle* genes

The results of the phylogenetic tree analysis using both maximum likelihood and maximum parsimony indicated that the 9 Indonesian isolates formed sister relationships with DNA sequences from *T. equiperdum* (Fig. 3). The phylogenetic grouping is consistent with the PCR diagnostic algorithm, which also identified these 9 isolates as *T. equiperdum*.

## DISCUSSION

The classification of *T. equiperdum* remains controversial (Claes et al., 2005; Li et al., 2006; Wen et al., 2016). The controversy is partially due to the difficulty in distinguishing trypanosome species within the subgenus *Trypanozoon*. Isolates of *T. evansi* and *T. equiperdum* have also historically been morphologically

**Table II.** The nucleotide sequence of the 10 primer set and their amplification program.

| Code | Set primer (nucleotide sequences) | Approximate fragment size amplified | References | PCR program used in this study |
|---|---|---|---|---|
| ITS1 | Forward: CCGGAAGTTCACCGATATTG<br>Reverse: TTGCTGCGTTCTTCAACGAA | 480 bp | Njiru et al. (2005) | 5 min at 94 C, 35 cycles: [1 min at 94 C, 1 min at 58 C, 90 sec at 72 C] and 5 min at 72 C |
| ESAG6/7 | Forward: ACATTCCAGCAGGAGTTGGAG<br>Reverse: CACGTGAATCCTCAATTTTGT | 237 bp | Holland et al. (2001) | 1 min at 94 C, 35 cycles: [1 min at 94 C, 2 min at 55 C, 2 min at 72 C] and 10 min at 72 C |
| MINI | Forward: CAACGACAAAGAGTCAGT<br>Reverse: ACGTGTTTTGTGTATGGT | 357 bp | Artama et al. (1992) | 1 min at 94 C, 35 cycles: [1 min at 94 C, 2 min at 55 C, 2 min at 72 C] and 10 min at 72 C |
| ND5 | Forward: TGGGTTTATATCAGGTTCATTTATG<br>Reverse: CCCTAATAATCTCATCCGCAGTACG | 395 bp | Li et al. (2007) | 5 min at 95 C, 35 cycles: [1 min at 94 C, 1 min at 55 C, 2 min at 72 C] and 10 min at 72 C |
| RoTat 1.2 | Forward: GCGGGGTGTTTAAAGCAATA<br>Reverse: ATTAGTGCTGCGTGTGTTCG | 205 bp | Claes et al. (2004), Recka et al. (2020) | 4 min at 94 C, 35 cycles: [1 min at 94 C, 1 min at 59 C, 1 min at 72 C] and 5 min at 72 C |
| ND4/5 | Forward: GTGTTTTATTAGCAAGTATTGTGC<br>Reverse: CCCTAATAATCTCATCCGCAGTACG | 1515 bp | Lai et al. (2008), Suganuma et al. (2016) | 5 min at 94 C, 35 cycles: [1 min at 94 C, 1 min at 47 C, 1 min at 72 C] and 5 min at 72 C |
| MaxiCyt1 | Forward: AGC GGA GAA AAA AGA AAG GGT<br>Reverse: TCA CAA AAT GCA TCA GAA CTC A | 599 bp | Sánchez et al. (2015) | 5 min at 94 C, 35 cycles: [1 min at 94 C, 1 min at 49 C, 1 min at 72 C] and 5 min at 72 C |
| MURF2 Cox1 | Forward: AGT AGA AGT TGT TAT ATA TTG ATG CC<br>Reverse: TAA GTG GGT TTT TGA CTG AAG AG | 1551 bp | Lai et al. (2008), Suganuma et al. (2016) | 3 min at 95 C, 35 cycles: [1 min at 95 C, 1 min at 46 C, 2 min at 72 C] and 3 min at 72 C |
| ILO | Forward: CAGCTCGCCGAAGGCCACTTGGCTGGG<br>Reverse: TCGCTACCACAGTCGCAATCGTCGTCTCAAGG | 400 bp | Gonzales et al. (2006), Mekata et al. (2009) | 3 min at 94 C, 30 cycles: [1 min at 94 C, 2 min at 55 C, 2 min at 72 C] and 5 min at 72 C |
| EVAB | Forward: CACAGTCCGAGAGATAGAG<br>Reverse: CTGTACTCTACATCTACCTC | 436 bp | Njiru et al. (2006), Birhanu et al. (2016) | 5 min at 95 C, 30 cycles: [1 min at 94 C, 1 min at 60 C, 1 min at 72 C] and 10 min at 72 C |

indistinguishable (Li et al., 2006; Sánchez et al., 2016; Wen et al., 2016; Gizaw et al., 2017). This challenge has led to the consideration of molecular identification methods. The *nad5-kDNA maxicircle* gene is the identification key to distinguishing *T. evansi* from *T. equiperdum* (Li et al., 2007; Wen et al., 2016), while the *gRNA-kDNA minicircle* (Artama et al., 1992) and *VSG RoTat 1.2* (Claes et al., 2004) can be used to distinguish *T. brucei* from other species within the subgenus *Trypanozoon*. Combining these 3 primer sets in a consecutive testing strategy guided by the appropriate algorithm will permit isolates of each species in the subgenus *Trypanozoon* to be distinguished.

### Identifying species within the subgenus *Trypanozoon*

The *ITS1* rDNA gene has been widely applied to identify and differentiate several trypanosome species based on their amplicon size (Njiru et al., 2005; Gaithuma et al., 2019). Therefore, the ITS1 primers are eminently suitable for screening in the early identification of trypanosome isolates. In this study, ITS1-PCR amplicons from Indonesian isolates were uniform in size, approximately 480 bp, so we concluded that they belonged to 1 of the 3 species of the subgenus *Trypanozoon*: *T. brucei*, *T. evansi*, or *T. equiperdum* (Njiru et al., 2005; Salim et al., 2011).

Twenty-three Indonesian isolates that we identified as *Trypanozoon* were also confirmed by amplification using the ESAG6/7 primers, reportedly specific for *Trypanozoon* (Holland et al., 2001). These results were also supported by the results of *ESAG6* gene sequencing, which were similar to 3 species of subgenus *Trypanozoon*. The sequences of the 23 Indonesian isolates demonstrated 95.06–98.33%, 95.04–97.91%, and 92.18–94.56% similarity to *T. brucei*, *T. evansi*, and *T. equiperdum*, respectively. These results generally confirm that all Indonesian isolates were members of the subgenus *Trypanozoon*.

### *Trypanosoma brucei* was not identified in this study

The MINI primers were specifically designed by Artama et al. (1992) to distinguish between *T. evansi* and *T. brucei*. As the *gRNA-kDNA minicircle* gene was amplified in all isolates using the MINI primers, none of the isolates were of the species *T. brucei*. However, the MINI primers have not been tested to detect *T. equiperdum*; therefore, its ability to distinguish *T. evansi* from *T. equiperdum* is unknown. Sequence analysis of the PCR products showed that all Indonesian isolates had *gRNA-kDNA minicircle* sequence similarities with *T. evansi* and *T. equiperdum* (Table III). It would seem from the results here that the MINI primers alone cannot distinguish *T. evansi* from *T. equiperdum*.

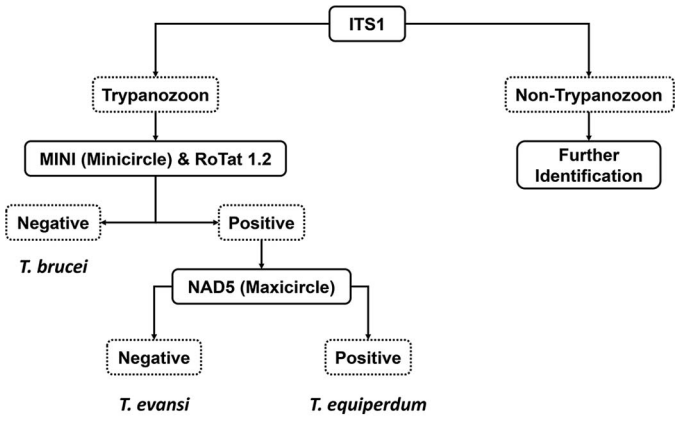


**Figure 2.** The algorithm used in this study for the identification and differentiation of *Trypanosoma* species within the subgenus *Trypanozoon*. ITS1 primers: amplify the internal transcribed spacer 1 gene region of ribosomal deoxyribonucleic acid (*ITS1* rDNA); ESAG6/7 primers: amplify the expression site-associated genes (*ESAG*) region 6 and 7; MINI primers: amplify the guide RNA of the kinetoplast DNA (*gRNA-kDNA*) *minicircle*; RoTat 1.2 primers: amplify the DNA region lacking similarity to other known variant surface glycoprotein (*VSG*) genes; ND5 primers: amplify the NADH-dehydrogenase subunits 5 (*nad5*)-*kDNA maxicircle* gene.

Therefore, all the Indonesian isolates could be classified as *T. evansi* or *T. equiperdum*.

The RoTat 1.2 primers are known to amplify the VSG of the Rode Trypanozoon Antigen (RoTat) type 1.2 gene region of *T. evansi* and *T. equiperdum*. Claes et al. (2004) demonstrated that the RoTat 1.2 primers could provide PCR-positive results for isolates of *T. evansi* and *T. equiperdum*. This evidence provides additional support that all Indonesian isolates were likely *T. evansi* or *T. equiperdum*.

## Distinguishing *T. equiperdum* and *T. evansi* based on molecular identification

Isolates of *T. evansi* and *T. equiperdum* can be distinguished consistently by PCR using ND5 primers that amplify the *nad5-kDNA maxicircle* gene (Li et al., 2006, 2007; Suganuma et al., 2016; Büscher et al., 2019). Negative PCR results using the ND5 primers in isolates of *T. evansi* are due to the absence of the *kDNA maxicircle* gene (Sánchez et al., 2016; Gizaw et al., 2017). Therefore, the 9 Indonesian isolates amplified using the ND5 primers were considered *T. equiperdum*, while the 14 isolates which were not amplified using the ND5 primers were determined to be *T. evansi*.

## *Trypanosoma evansi* or *Trypanosoma equiperdum* isolated from bovines?

The classification of *T. equiperdum* is still a matter of debate, and the results of this study indicate 2 possible hypotheses. The first hypothesis, based on the proposal that *T. equiperdum* only naturally infects horses, indicates that 9 Indonesian trypanosome isolates should be considered to be *T. evansi*, which possesses a *kDNA maxicircle* gene or before losing all its *maxicircle* gene (maxicircle remnants). This possibility refutes the well-established evidence that *T. evansi* generally lacks the *kDNA maxicircle* gene.

**Table III.** Molecular identification using 10 primer sets on 23 Indonesian isolates.*

| No. | Isolate | ITS1 | ESAG6/7 | ILO | MINI | RoTat 1.2 | EVAB | ND5 | ND4/5 | MaxiCyt1 | MURF2 Cox1 | *Trypanosoma* species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | AMN-SB1 | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 2 | KPG | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 3 | BOPKL | + | + | − | + | + | − | + | − | − | − | *T. equiperdum* |
| 4 | ACBT-YF1 | + | + | − | + | + | − | + | − | − | − | *T. equiperdum* |
| 5 | PML | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 6 | STENT1 | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 7 | STENT5 | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 8 | STENT3 | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 9 | BTN01 | + | + | − | + | + | − | + | − | − | − | *T. equiperdum* |
| 10 | SPT-CB1 | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 11 | STENT2 | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 12 | STENT4 | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 13 | SBWNT | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 14 | BKN-EJ | + | + | − | + | + | − | + | − | − | − | *T. equiperdum* |
| 15 | TBN-EJ | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 16 | BKLTZ | + | + | − | + | + | − | + | − | − | − | *T. equiperdum* |
| 17 | PDE | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 18 | ERK-SC | + | + | − | + | + | − | + | − | + | − | *T. equiperdum* |
| 19 | PJY-YF2 | + | + | − | + | + | − | + | + | + | − | *T. equiperdum* |
| 20 | ASH | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 21 | SB-PR | + | + | − | + | + | − | − | nd | nd | nd | *T. evansi* |
| 22 | BRU-SC | + | + | − | + | + | − | + | + | + | − | *T. equiperdum* |
| 23 | GKT-WB | + | + | − | + | + | − | + | + | + | − | *T. equiperdum* |

* ITS1 primers: amplify the internal transcribed spacer 1 gene region of ribosomal deoxyribonucleic acid (*ITS1* rDNA); ESAG6/7 primers: amplify the expression site-associated genes (*ESAG*) region 6 and 7; ILO primers: designed to amplify specific genes of *Trypanosoma vivax*; MINI primers: amplify the kinetoplast DNA (*kDNA*) *minicircle*; RoTat 1.2 primers: amplify the DNA region lacking similarity to other known variant surface glycoprotein (*VSG*) gene; EVAB primers: amplify the class B minicircle gene of *T. evansi*; ND5 primers: amplify the NADH-dehydrogenase subunit 5 (*nad5*)-*kDNA maxicircle* gene; ND4/5 primers: amplify the NADH-dehydrogenase subunits 4 and 5 of the kDNA maxicircle gene; MaxiCyt1 primers: amplify the Cytochrome b region of the *kDNA maxicircle* gene; MURF2 Cox1 primers: amplify the maxicircle unknown reading frame 2 and cytochrome oxidase subunit 1 of the *kDNA maxicircle* gene; + = PCR positive/amplified; − = PCR negative/not amplified; nd = not done.

**Table IV.** Sequence similarity of Indonesian isolates based on *gRNA-kDNA minicircle* and *nad5-kDNA maxicircle* genes.*

| No. | Isolate | *gRNA-kDNA minicircle* gene | | *nad5-kDNA maxicircle* gene | |
|---|---|---|---|---|---|
| | | *Trypanosoma evansi* Similarity (accession no.) | *Trypanosoma equiperdum* Similarity (accession no.) | *Trypanosoma equiperdum* Similarity (accession no.) | *Trypanosoma brucei* Similarity (accession no.) |
| 1 | AMN-SB1 | 97.76% (M57462) | 96.90% (EU155058) | – | – |
| 2 | KPG | 99.17% (M81594) | 99.09% (EU155058) | – | – |
| 3 | BOPKL | 99.72% (M81594) | 99.70% (EU155058) | 99.44% (DQ401131) | 98.89% (M94286) |
| 4 | ACBT YF1 | 97.71% (M81594) | 97.45% (EU155058) | 94.81% (U03738) | 95.21% (M94286) |
| 5 | PML | 96.99% (M57462) | 96.07% (EU155058) | – | – |
| 6 | STENT1 | 97.26% (M81594) | 96.90% (M14763) | – | – |
| 7 | STENT5 | 97.26% (M81594) | 96.90% (M14763) | – | – |
| 8 | STENT3 | 97.26% (M81594) | 96.90% (M14763) | – | – |
| 9 | BTN01 | 96.98% (M81594) | 96.67% (EU155058) | 98.23% (U03738) | 98.23% (M94286) |
| 10 | SPT-CB1 | 96.91% (M81594) | 96.84% (M14763) | – | – |
| 11 | STENT2 | 96.98% (M81594) | 97.13% (M14763) | – | – |
| 12 | STENT4 | 96.71% (M81594) | 96.34% (M14763) | – | – |
| 13 | SBWNT | 99.18% (M81594) | 99.09% (EU155058) | – | – |
| 14 | BKN-EJ | 96.16% (M81594) | 95.77% (EU155058) | 98.24% (U03738) | 98.49% (M94286) |
| 15 | TBN-EJ | 96.71% (M81594) | 96.59% (M14763) | – | – |
| 16 | BKLTZ | 97.49% (M81594) | 97.26% (EU155058) | 97.74% (U03738) | 97.74% (M94286) |
| 17 | PDE | 99.44% (M81594) | 99.39% (EU155058) | - | – |
| 18 | ERK-SC | 96.70% (M81594) | 96.36% (EU155058) | 98.45% (U03738) | 98.70% (M94286) |
| 19 | PJY YF2 | 97.76% (M81594) | 97.53% (EU155058) | 98.47% (DQ401131) | 97.46% (M94286) |
| 20 | ASH | 96.17% (M81594) | 96.03% (M14763) | – | – |
| 21 | SB-PR | 96.98% (M81594) | 96.88% (M14763) | – | – |
| 22 | BRU-SC | 96.23% (M81594) | 96.23% (EU155058) | 89.66% (DQ401131) | 88.73% (M94286) |
| 23 | GKT-WB | 99.35% (M81594) | 98.70% (M14763) | 84.43% (U03738) | 84.08% (M94286) |

* Accession number is the highest sequence similarity only.

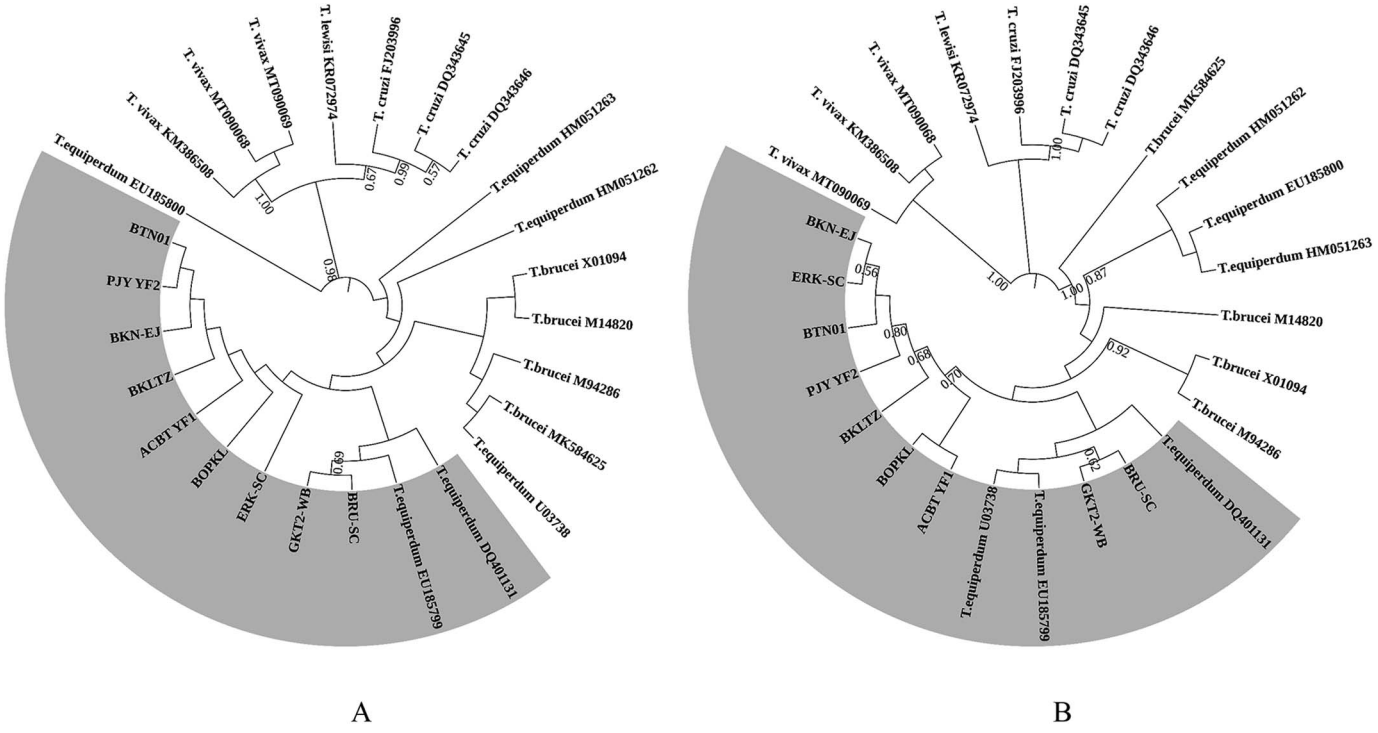


**Figure 3.** Dendrograms of *Trypanosoma equiperdum* from Indonesia based on the sequence of the *nd5-kDNA maxicircle* gene and constructed using the Maximum Likelihood (**A**) and Maximum Parsimony (**B**) methods. The numbers in the phylogenetic tree show the bootstrap value. All Indonesian isolates are in gray.

This hypothesis is not supported by sufficient scientific evidence at this time.

Another argument concludes that *T. evansi* has been misclassified as *T. equiperdum* and is thus positive for PCR amplification using the RoTat 1.2 primers (Claes et al., 2004). This argument was not accepted because *T. equiperdum*, negative by PCR using the RoTat 1.2 primers, was identified in only 2 of 9 (22%) strains (BoTat 1.1 and OVI). The conclusion proposed by Claes et al. (2004) indirectly implies that a substantial degree of misclassification (78%) of *T. equiperdum* has occurred. This misclassification is certainly very surprising regarding *Trypanozoon* identification. However, other studies showed that BoTat 1.1, OVI, and other *T. equiperdum* strains were positive for PCR amplification using the ND5 primers, while all *T. evansi* isolates were consistently PCR negative (Li et al., 2006). This evidence leads to 2 possible conclusions. The first possibility is that *T. equiperdum* was always detected in the PCR amplification of the *kDNA maxicircle*, while *T. evansi* was not amplified. The second one, the study conducted by Claes et al. (2004), proves that PCR using the RoTat 1.2 primers can amplify the *VSG* gene of *T. evansi* and most of *T. equiperdum*. Thus the RoTat 1.2 primers should be inferred not only for *T. evansi* but also for most of *T. equiperdum*. This conclusion implies that *T. equiperdum* had been examined in previous studies as a result of earlier misclassifications is an incorrect opinion. Thus, the argument proposed by Claes et al. (2004) cannot be used as evidence to support the first hypothesis.

The second hypothesis is that 9 Indonesian isolates are of the *T. equiperdum* even though they were isolated from bovines. This result represents the first isolation of a *T. equiperdum* from a nonequine. Isolates of this species are considered natural and exclusive equine parasites (Brun et al., 1998; Li et al., 2007; Sánchez et al., 2015). However, the claim that *T. equiperdum* occurs naturally in the equine does not necessarily exclude its parasitization of other mammals and the possibility to isolate it from other mammals. It could be interpreted that naturally, *T. equiperdum* causes severe disease (known as dourine) only in equines, but infection may be asymptomatic or mild in other mammals.

This hypothesis was supported by several direct and indirect lines of evidence. Researchers have experimentally infected dogs (Krumbhaar, 1918) and sheep (Gimenez and Bello, 2019) with *T. equiperdum* isolates. Artificial infection with *T. equiperdum* isolates in sheep has been shown to cause chronic disease with leading parasitemia up to 55 days post-infection (Gimenez and Bello, 2019). This provided evidence that *T. equiperdum* may be able to infect mammals other than equines naturally, although not acutely or with severe symptoms.

Other researchers have also reported that *T. equiperdum* can also be found and isolated from the horse bloodstream such as *T. equiperdum* Dodola 713 and 834 strains (Hagos et al., 2010). They have also successfully performed artificial transmission using *T. equiperdum* Dodola 713 and 834 strains in horses intravenously (Hagos et al., 2010). Some researchers have also reported that *T. equiperdum* in the bloodstream is generally short-lasting and has mild parasitemia (Pascucci et al., 2013; Suganuma et al., 2016). Overall this cannot be ignored for its role as a bridge for transmission to other mammals through blood-sucking vectors. However, *T. equiperdum* transmission through mechanical vectors such as blood-sucking flies cannot be completely ruled out (Suganuma et al., 2016). Taken together, this evidence suggests that natural infection by *T. equiperdum* is very likely to occur in bovines and perhaps in other mammals.

Other evidence also strengthens the second hypothesis that the 9 Indonesian isolates were *T. equiperdum*. So far, no reports have been found indicating the discovery of *T. evansi* that has a remnant *kDNA maxicircle* gene. On the other hand, it has been known that *T. evansi* lacks *kDNA maxicircle* whereas *T. equiperdum* and *T. brucei* still have it (Lun et al., 2010; Sánchez et al., 2016; Gizaw et al., 2017). At this point, it becomes the main basic principle to be followed for further molecular identification to differentiate the species within the subgenus *Trypanozoon*.

The 6 Indonesian isolates had high sequence similarity with *T. equiperdum* ATCC 30019 (accession no. U03738), and the other 3 isolates had sequence similarity with *T. equiperdum* STIB 818 (accession no. DQ401131). Both *T. equiperdum* ATCC 30019 and STIB 818 have been reported to be positive only when amplified using the ND5 primers as used in this study. In contrast, *T. evansi* showed PCR-negative results with these primers (Li et al., 2006; Lai et al., 2008). Although ATCC 30019 and STIB 818 were PCR negative when using other primers to amplify several loci in the *kDNA maxicircle*, they remain identified and well accepted as *T. equiperdum* (Li et al., 2006; Lai et al., 2008). Another study on a Mongolian isolate, namely, IVM-t1, was also identified as *T. equiperdum* although only ND4/5 primers can amplify *kDNA maxicircle* (Suganuma et al., 2016). In general, although only 1 primer was successful in amplifying a particular locus within the *kDNA maxicircle*, this suggests a possible identification of *T. equiperdum*.

Other evidence came from the result of phylogenetic tree analysis using maximum likelihood and maximum parsimony, which places the 9 Indonesian isolates that have been identified as having *kDNA maxicircle* falling into 1 cluster with *T. equiperdum*, namely, ATCC 30019 and STIB 818 strains (Fig. 3). This fact strongly supports that the 9 isolates were *T. equiperdum*, especially for the 4 isolates that also showed positive PCR using another 2-primer set, namely, MaxiCyt1 and ND4/5 (Table III). This evidence confirms that the 4 isolates (ERK-SC, PJY YF2, BRU-SC, and GKT-WB) were definitively *T. equiperdum* that had been isolated from bovine. Considering all the evidence above, this discussion tends to strengthen the conclusion that leads to the 9 Indonesian isolates that were positive PCR for the *nad5-kDNA maxicircle* were *T. equiperdum* although isolated from bovines.

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