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Source: Journal of Parasitology, 106(5) : 523-536

Published By: American Society of Parasitologists

URL: https://doi.org/10.1645/19-136

Journal of Parasitology 2020 106(5) 523–536

Published 8 September 2020

Contents and archives available through www.bioone.org or www.jstor.org

Journal of Parasitology

journal homepage: www.journalofparasitology.org

DOI: 10.1645/19-136

NATIVE AND INTRODUCED TRYPANOSOME PARASITES IN ENDEMIC AND INTRODUCED MURINE RODENTS OF SULAWESI

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KEY WORDS

Muridae
18S Ribosomal RNA (rRNA)
Protozoan Parasite
Metakinetoplastina
Parasite Biogeography
Indo-Australian Region

ABSTRACT

The Indonesian island of Sulawesi is a globally significant biodiversity hotspot with substantial undescribed biota, particularly blood-borne parasites of endemic wildlife. Documenting the blood parasites of Sulawesi's murine rodents is the first fundamental step towards the discovery of pathogens likely to be of concern for the health and conservation of Sulawesi's endemic murines. We screened liver samples from 441 specimens belonging to 20 different species of murine rodents from 2 mountain ranges on Sulawesi, using polymerase chin reaction (PCR) primers targeting the conserved *18S rDNA* region across the protozoan class Kinetoplastea. We detected infections in 156 specimens (10 host species) with a mean prevalence of 35.4% (95% confidence interval [CI] = 30.9–39.8%). Sequences from these samples identified 4 infections to the genus *Parabodo*, 1 to *Blechomonas*, and the remaining 151 to the genus *Trypanosoma*. Within *Trypanosoma*, we recovered 17 haplotypes nested within the *Trypanosoma theileri* clade infecting 117 specimens (8 host species) and 4 haplotypes nested within the *Trypanosoma lewisi* clade infecting 34 specimens (6 host species). Haplotypes within the *T. theileri* clade were related to regional Indo-Australian endemic trypanosomes, displayed geographic structuring but with evidence of long-term connectivity between mountains, and had substantial phylogenetic diversity. These results suggest *T. theileri* clade parasites are native to Sulawesi. Conversely, *T. lewisi* clade haplotypes were recovered from both endemic and introduced rodents, demonstrated complete geographic separation between clades, and had low genetic diversity. These results suggest that the *T. lewisi* clade parasites invaded Sulawesi recently and likely in 2 separate invasion events. Our results provide the first records of metakinetoplastids in Sulawesi's rodents and highlight the need for more extensive sampling for pathogens in this biodiversity hotspot.

Members of the genus *Trypanosoma* (Euglenozoa: Kinetoplastea) are protozoan parasites known to cause diseases in humans and wildlife. Trypanosome infections have been linked to declines and extinctions in native mammal populations, with disease outcomes ranging from subclinical effects to fever, anemia, weight loss, cachexia, and even death (Wyatt et al., 2008; Desquesnes et al., 2013; Cooper et al., 2017a, 2017b). By 1972, approximately 120 mammal-infecting trypanosome species had been described globally (Hoare, 1972; Thompson et al., 2014b). There are 4 major clades containing predominantly mammal-infecting trypanosomes—the *Trypanosoma brucei*, *Trypanosoma cruzi*, *Trypanosoma theileri*, and *Trypanosoma lewisi* clades (Hamilton et al., 2007). Rodent-infecting trypanosomes within these clades display significant variation in their geographic distributions, from locally endemic to cosmopolitan species (Pumhom et al., 2015). Additionally, trypanosomes rarely show the same level of host specificity often seen in other blood parasite groups (e.g., Haemosporidia), highlighting them as a particular concern for potential spillover from introduced host species into native host species (Sehgal, 2015). This is thought to have occurred on Christmas Island, where the introduction of an exotic trypanosome, *T. lewisi*, from introduced black rats (*Rattus rattus*) has been linked to the extinction of one of the island's endemic rodent species, Maclear's Rat (*Rattus macleari*) (Wyatt et al., 2008).

Trypanosoma lewisi is a well-known cosmopolitan species that has become widespread following human-assisted dispersal of infected commensal rodents (Milocco et al., 2013; Pumhom et al., 2014).

The genus *Trypanosoma* is phylogenetically positioned within Trypanosomatida, an order within the subclass Metakinetoplastina (Euglenozoa: Kinetoplastea) (Vickerman, 1978; Maslov et al., 2001; Moreira et al., 2004; Simpson et al., 2006). All species within Trypanosomatida are parasitic, some of which are monoxenous (i.e., only infect invertebrate hosts) such as members of the newly described genus *Blechomonas*, whereas others are dixenous (i.e., have a life cycle involving a vertebrate host and an invertebrate vector) such as members of the genus *Trypanosoma* (Votýpka et al., 2013; Yazaki et al., 2017). There are 3 other orders also recognized within Metakinetoplastina. Parabodonida and Neobodonida contain both free-living and parasitic species, and all species within Eubodonida are thought to be free-living (Moreira et al., 2004; Yazaki et al., 2017). In many wildlife populations, there is limited knowledge of the diversity, geographic distribution, and prevalence of species within Metakinetoplastina (Simpson et al., 2006; d'Avila-Levy et al., 2015). In order to identify potentially pathogenic parasites and to test hypotheses about links between host diversity and parasite prevalence/richness (such as the dilution effect hypothesis, which predicts that increased species diversity lowers the prevalence of diseases), increased surveillance of wildlife parasites is needed from species-rich host communities (Daszak et al., 2001; Clay et al., 2009; Searle et al., 2011; Pumhom et al., 2014; Salzer et al., 2016; Cooper et al., 2017a).

The tropical Indonesian island of Sulawesi is a globally significant biodiversity hotspot for terrestrial vertebrates that is also likely to support substantial undocumented parasite diversity (Groves, 2001; Carlson et al., 2017). Given Sulawesi's position between the Asian and Australian continental shelves, the island is likely to be relevant to the biogeography of trypanosomes distributed across the Indo-Australian region, such as the *T. theileri* clade (Stelbrink et al., 2012). These include *Trypanosoma cyclops* infecting Malaysian macaques and closely related but undescribed trypanosomes infecting wallabies and frogs from Australia and terrestrial leeches from Australia, New Guinea, and Sri Lanka (Weinman and Wiratmadja, 1969; Weinman, 1972; Hamilton et al., 2005; Cooper et al., 2017a). Currently, the only trypanosome species reported occurring on the island is *Trypanosoma evansi*, which has been recorded infecting livestock from the northern and southern peninsulas (Luckins, 1998). It is likely that the absence of trypanosome records from Sulawesi is due to sampling bias towards host taxa of economic importance rather than a lack of their presence, suggesting a need for more studies targeting Sulawesi's wildlife as a means to document trypanosomes in this important biogeographic region (Cooper et al., 2017a).

The endemic rodents of Sulawesi (Family Muridae) remain a particularly severely understudied group despite their high level of endemism (Groves, 2001; Kia et al., 2009; Desquesnes et al., 2016). To date, 48 endemic species of murid rodents have been recorded on the island (Rowe et al., 2016, 2019). Five recorded species of introduced rodents (*Rattus exulans*, *Rattus rattus* complex (Lineage IV), *Rattus norvegicus*, *Rattus argentiventer*, and *Mus musculus*) are encroaching on endemic species with the aid of anthropogenic habitat conversion and fragmentation (Whitten et al., 1987; Aplin et al., 2011). Several of these introduced rodent species are known reservoirs for the cosmopolitan trypanosome *T. lewisi*, including those found on neighboring landmasses of the Indo-Australia region, suggesting that this trypanosome species is also likely to be present in rodents on Sulawesi but has not yet been documented (Kartman, 1954; Anderson, 1990; Milocco et al., 2013; Pumhom et al., 2014; Alias et al., 2014; Thompson et al., 2014a).

In this study, we used genetic sequencing to provide the first inventory of blood-borne parasites in the subclass Metakinetoplastina infecting murine rodents from Sulawesi. We predicted that native *Trypanosoma* species would show signatures of genetic diversity and population structure between isolated mountains, consistent with a long presence on Sulawesi (Gaither et al., 2013; Lymbery et al., 2014). In contrast, we predicted any introduced *Trypanosoma* species would show little genetic diversity or population structure among mountains reflecting a more recent arrival on Sulawesi (Dudaniec et al., 2008; Gaither et al., 2013; Lymbery et al., 2014). To explore the potential impacts of trypanosomes on rodent populations, we compared parasite prevalence of the native and introduced trypanosomes and among host species to determine if some species are at greater risk of infection, particularly threatened species.

MATERIALS AND METHODS

Study area, animal capture, and sample collection

We collected samples from rodents along elevational gradients on 2 mountains of South Sulawesi, Indonesia (Fig. 1). We obtained samples from Mount Latimojong in August 2016 at elevations between 700 and 2,600 m, with 95% of samples collected between 1,700 and 2,500 m. We obtained samples from Mount Bawakaraeng in October 2016 at elevations of 1,660–2,800 m. On both mountains, forests below 1,500 m have been cleared or are highly disturbed (Cannon et al., 2007; M. L. Winterhoff, pers. obs.). Forests above 1,500-m elevation remain largely intact with minimal disturbance from selective harvesting (Cannon et al., 2007; M. L. Winterhoff, pers. obs.). However, on Mount Bawakaraeng, forests were replaced by alpine grasslands above 2,700 m, with substantial disturbance from clearing for camping and accumulated rubbish occurring from 2,400 to 2,800 m elevation (M. L. Winterhoff, pers. obs.). These mountains were selected as part of an ongoing research program inventorying vertebrate and invertebrate species across Sulawesi. Meeting the objectives of the larger field project limited the sampling of sites at lower elevations. Rodents were collected by staff from the Indonesian Institute of Science using a combination of traps set in suitable locations on or near the ground.

We collected all blood and tissue samples from recently dead rodents (<18 hr). We collected liver samples into 70% ethanol. For future work, we also collected blood samples onto Whatman WB120210 FTA Micro Cards and/or Whatman Grade 3 Filter Paper (Sigma–Aldrich, Munich, Germany) as well as prepared blood smears on glass microscope slides. Additionally, voucher specimens and tissue samples collected as part of the larger project were lodged at Museums Victoria, Museum of Vertebrate Zoology, and Museum Zoologicum Bogoriense. Our sampling included a total of 441 specimens from 20 species of rodents (19 native, 1 invasive), with 192 samples representing 13 species of rodents collected from Mount Latimojong and 249 samples representing 9 species of rodents collected from Mount Bawakar-

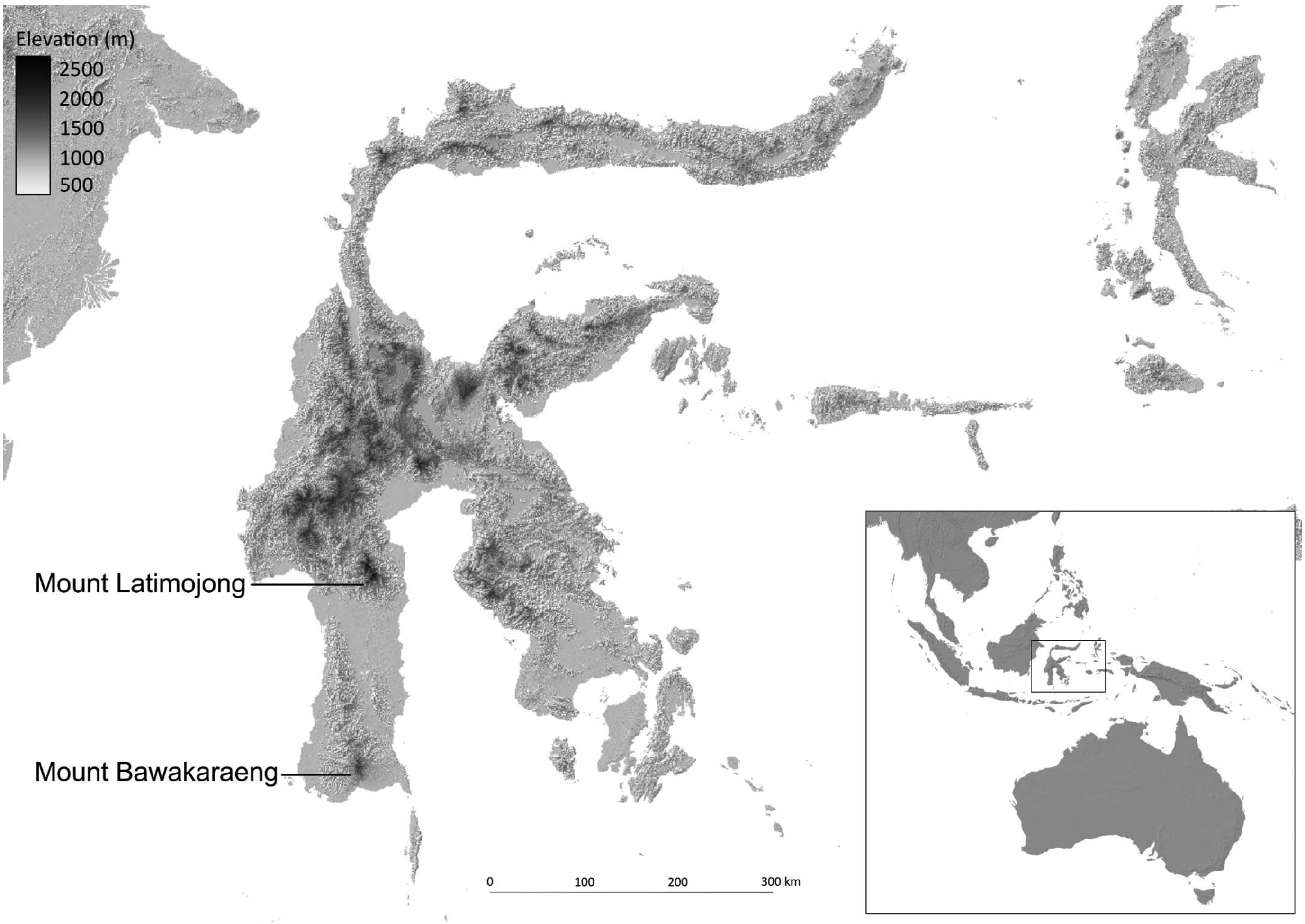

Figure 1. Location of field sites on Sulawesi, Indonesia: Mount Latimojong, Gamaru village, Belopa, South Sulawesi Province (3°25′9.0012″S, 120°5′40.8264″E); Mount Bawakaraeng, Gunung Perak village, Sinjai Regency, South Sulawesi Province, Indonesia (5°16′59.0808″S, 119°57′39.4596″E). Map made using QGIS 2.18.2 (2016).

aeng. Specimen sampling followed procedures approved under Museums Victoria Animal Ethics permit number MV AEC 15002.

Molecular detection and sequencing

We extracted genomic DNA from liver tissue (sample size [n] = 441) using a QIAextractor (DX reagents and plasticware), QIAGEN DNeasy blood and tissue kits (QIAGEN Inc., Valencia, California) or Wizard SV 96 Genomic DNA Purification Systems (Promega, Madison, Wisconsin) following standard manufacturers' guidelines. We screened DNA extractions for Metakinetoplastina using a 2-part nested set of primers targeting a ~906 base-pair (bp) fragment of the *18S* subunit of the rDNA following the PCR protocols previously described by McInnes et al. (2009, 2011; Table I). PCR products from the second reaction were screened by visualizing on a 2% agarose gel; we considered the presence of a band in the range of 900 bp a PCR-positive detection for Metakinetoplastina. For subsequent phylogenetic analysis, we purified any PCR-positive products using ExoSAP (USB Corporation, Cleveland, Ohio) and sequenced on an Applied Biosystems 3730 Automated DNA Sequencer (Applied Biosystems, Foster City, California). We edited individual sequences using Geneious v. 5.1.7 (Kearse et al., 2012).

Phylogenetic analyses

We used BLAST to identify sequences to putative metakinetoplastids genera and confirmed that sequences were nested within genera using phylogenetic analyses. For *Trypanosoma*, we identified sequences to major clades and conducted separate phylogenetic analyses on each of these clades. From our edited sequences, we identified unique haplotypes using ALTER (Glez-Peña et al., 2010). We searched each unique haplotype against the NCBI GenBank database using the nucleotide BLAST tool to identify sequences to genus and retained all sequences that shared 97–100% similarity to the Sulawesi haplotypes (GenBank accession numbers in supplementary material, Table S1; Altschul et al., 1990). We also included published sequences representing 13 genera that are phylogenetically dispersed across Metakinetoplastina and representatives of each major clade within *Trypanosoma* following Hamilton et al. (2005) and Yazaki et al. (2017). We aligned sequences using MUSCLE (Edgar, 2004) and

Table I. Trypanosome-universal primers, polymerase chain reaction (PCR) conditions and sequencing procedures for the *18S rDNA* gene targeted in this study.

			Cycle conditions: temperature (degrees Celsius)/time (seconds)*			
Primer	PCR	Forward (F) and reverse (R) PCR primers	Denaturation	Annealing	Extension	Band size (base pairs [bp])
SLF	Outer	F: GCT TGT TTC AAG GAC TTA GC	94/30	52/30	72/140	~1,500 bp
S762R		R: GAC TTT TGC TTC CTC TAA TG				
S823F	Inner	F: CGA ACA ACT GCC CTA TCA GC	94/30	52/30	72/140	~906 bp
S662R		R: GAC TAC AAT GGT CTC TAA TC				

* All reactions included an initial denaturation period of 5 min at 95 C, repeated for 35 cycles, and a final extension period of 7 min at 72 C. PCR conditions follow those as previously described in McInnes et al. (2009, 2011).

manually edited alignments in Geneious. DNA sequences are available in GenBank under accessions MN383196–MN383322.

We conducted phylogenetic analyses on 3 alignments. Analysis of the first alignment was to infer phylogenetic relationships across all samples within the subclass Metakinetoplastina and to confirm identifications from BLAST sequence similarity. Once we were able to infer the placement of the Sulawesi samples within the broader Metakinetoplastina, we then conducted 2 additional phylogenetic analyses on separate alignments for the 2 major *Trypanosoma* clades we detected in our initial BLAST analysis to infer within-clade phylogenetic relationships. For all 3 analyses, we used jModelTest to select the best-fitting substitution model (Darriba et al., 2012). jModelTest estimated GTR + G + I to be the optimal model of substitution for the broader Metakinetoplastina alignment. However, the use of the invariant parameter (I) in RAxML is not recommended and consequently was only used in the Bayesian analyses (Stamatakis et al., 2008). jModelTest selected K80 to be the optimal model of substitution for the separate *Trypanosoma* clades alignments. We estimated phylogenetic relationships for each of our 3 data sets using both Bayesian and maximum-likelihood approaches. We performed Bayesian phylogenetic analyses using MrBayes 3.2 (Ronquist and Huelsenbeck, 2003; Huelsenbeck and Ronquist, 2005) via the online portal CIPRES (Miller et al., 2010). Analyses ran for 10 million generations, sampling every 1,000 trees. We discarded the first 25% of trees as burn-in and produced a majority-rule consensus tree from the remaining trees. We examined convergence and adequacy of effective sample sizes in Tracer v1.7 (Rambaut et al., 2018). We performed maximum-likelihood analyses using RAxML (Stamatakis et al., 2008) with 1,000 bootstrap pseudoreplicates.

Population genetic analyses

For each *Trypanosoma* clade identified, we used Arlequin v3.5 (Excoffier and Lischer, 2010) to calculate standard genetic diversity indices (haplotype number, number of polymorphic sites, and θ_{π}). To calculate genetic distances we used a Tamura model, which best reflects the substitution model used in our phylogenetic analyses (Tamura and Nei, 1993). In Arlequin, we also calculated population structure (analysis of molecular variance [AMOVA], F_{st}, and population pairwise differences) between (and within) mountains for each *Trypanosoma* clade. We also constructed haplotype networks within each clade to explore genetic relationships within and between mountains using *pegus* (Paradis, 2010) in R version 3.1.3 (R Development Core Team, 2015).

Statistical analyses of prevalence

For each *Trypanosoma* clade identified, we conducted statistical analyses to determine if infections were randomly distributed between sexes, host species, and between mountains. To determine if one sex was more prone to parasite infections than the other, we compared prevalence between males and females using chi-squared tests. To test if some host species were more susceptible to infections, we compared each infected host species to the respective *Trypanosoma* clade's average parasite prevalence across all infected rodent species using chi-squared tests (χ^2) and Fisher's exact tests (when the category sample size was <5). Finally, to determine if the prevalence of infection was related to host species richness, we compared parasite prevalence between Mount Bawakaraeng (n = 9 rodent species) and Mount Latimojong (n = 13 rodent species). Statistical analyses were conducted in R version 3.1.3 (R Development Core Team, 2015). When relevant, we present our results with 95% confidence intervals (95% CI) or ±1 standard deviation of the mean (SD).

RESULTS

Molecular detection and sequencing

Of the 441 Sulawesi rodents we screened for the presence of *Trypanosoma*, we had a PCR-positive detection in 35.4% (n = 156) of individuals, representing infections in 50% (n = 10) of sampled species (Table II; Suppl. Data, Table S2). Our BLAST search revealed that 4 sequences matched closely to those from the genus *Parabodo* (99%), 1 to *Blechomonas* (98%), and 151 to *Trypanosoma* (97–100%; see Table S2). Phylogenetic analyses of our Metakinetoplastina alignment, which contained 74 sequences from across Metakinetoplastina (24 unique sequences from this study), supported that our samples were nested within each of these genera. These analyses strongly supported the placement of 2 Sulawesi haplotypes within the genus *Parabodo* and another within the genus *Blechomonas* ([MLBS] ≥70%, Bayesian posterior probability [BPP] ≥ 0.95; Fig. 2). *Parabodo* was recovered from *Rattus mollicomulus* (n = 2), *Bunomys penitus* (n = 1), and *Maxomys musschenbroekii* (n = 1). *Blechomonas* sp. was recovered from *R. mollicomulus* (n = 1). There was strong support for the placement of 4 Sulawesi haplotypes (H1 to H4) representing 34 samples into the *T. lewisi* clade (MLBS = 95%, BPP = 1; Fig. 2). The remaining 17 haplotypes (H5 to H21) representing 117 samples were placed within the *T. theileri* clade with strong support from both maximum likelihood and Bayesian analyses (MLBS = 100%, BPP = 1; Fig. 2).

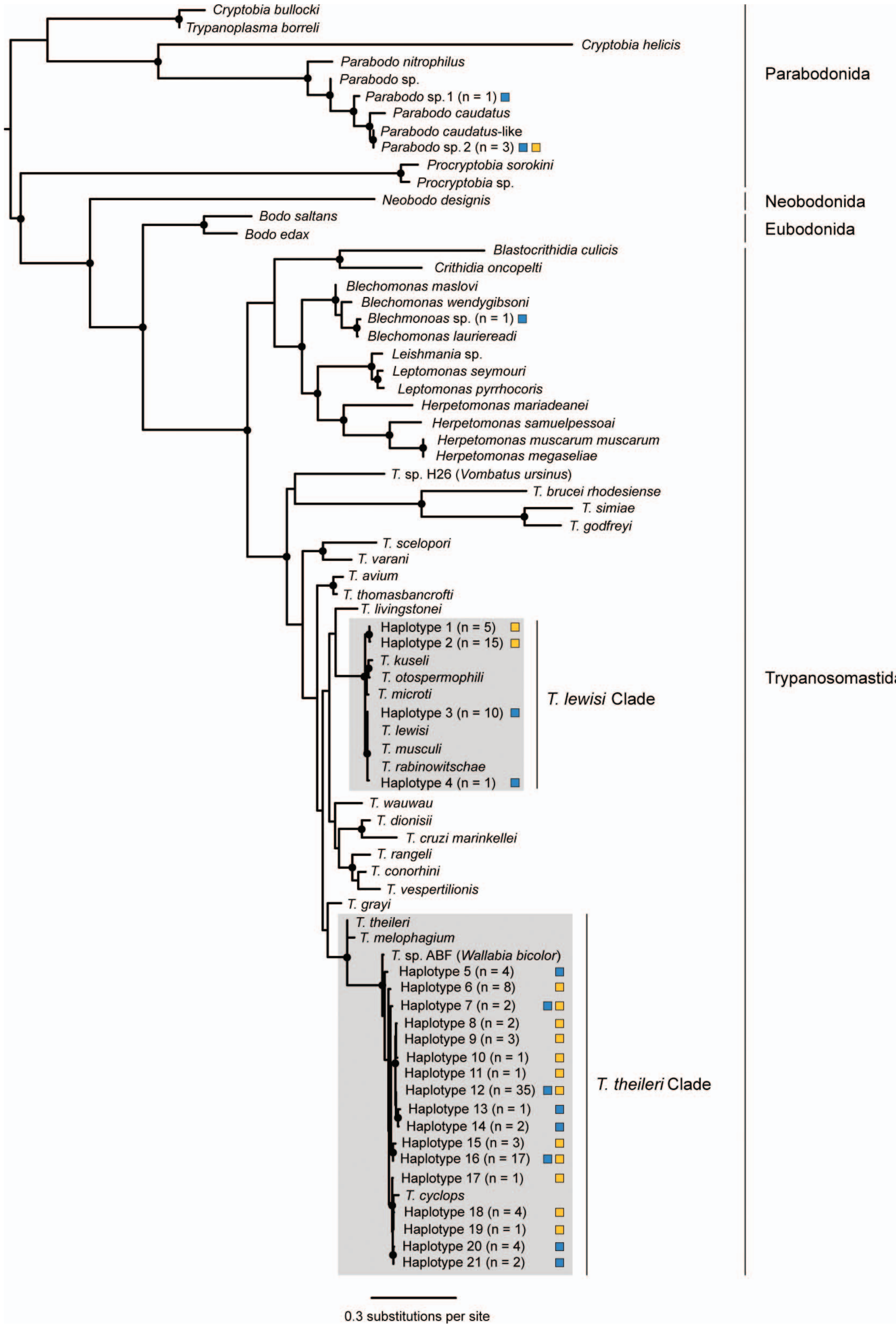

Figure 2. Phylogenetic relationships inferred using maximum likelihood analysis based on the Metakinetoplastina alignment of partial *18S rDNA* gene sequences, including 24 Sulawesi haplotypes and 50 GenBank sequences. The phylogenetic tree is midpoint rooted. Nodes supported by ≥70% maximum likelihood bootstrap values and ≥95% posterior probabilities are marked with black circles. Samples sequenced in this study are represented by haplotype number with sample sizes in brackets. Samples from GenBank are represented by metakinetoplastid species name with host species in brackets, if indicated. Colors of squares indicate haplotypes recovered from Mount Bawakaraeng (■, blue) and from Mount Latimojong (■, yellow). Color version available online.

Table II. Prevalence of *Trypanosoma* via polymerase chain reaction (PCR) screening in endemic (n = 19) and introduced (n = 1) murine species from Mount Latimojong and Mount Bawakaraeng in South Sulawesi.

Murine species	Locality	No. sampled	n (% prevalence, 95% confidence interval [CI]) *Trypanosoma lewisi*	n (% prevalence, 95% confidence interval [CI]) *Trypanosoma theileri*
Bunomys andrewsi	Bawakaraeng	2	0	0
*Bunomys coelestis**	Bawakaraeng	70	4 (5.7%, 0.28–11.2)	17 (24.3%, 14.2–34.4)
*Bunomys penitus**	Latimojong	82	15 (18.3%, 9.9–26.7)	42 (53.7%, 42.9–64.5)
*Bunomys torajae**	Latimojong	66	8 (12.1%, 4.2–20.0)	44 (66.7%, 55.3–78.0)
*Eropeplus canus**	Latimojong	2	0	0
Lenomys meyeri	Bawakaraeng	2	0	0
Margaretamys elegans	Latimojong	1	0	0
Margaretamys sp. nov.	Bawakaraeng	1	0	0
Maxomys dollmani	Latimojong	1	0	0
Maxomys musschenbroekii	Bawakaraeng	31	0	4 (8.5%, 05–16.5)
	Latimojong	16	0	0
*Paruromys dominator**	Bawakaraeng	36	0	6 (16.7%, 4.5–28.8)
	Latimojong	9	0	1 (11.1%, −9.4 to 31.6)
Paucidentomys vermidax	Latimojong	1	0	1 (100%, 100–100)
Rattus bontanus	Bawakaraeng	33	1 (3.0%, −2.8 to 8.9)	1 (3.0%, −2.8 to 8.9)
Rattus exulans†	Bawakaraeng	19	2 (10.5%, −3.3 to 24.3)	0
Rattus facetus	Latimojong	4	0	1 (25%, −17.4 to 67.4)
Rattus hoffmanni	Latimojong	3	0	0
*Rattus mollicomulus**	Bawakaraeng	54	4 (7.4%, 0.4–14.4)	0
Sommeromys macrorhinos	Latimojong	3	0	0
Taeromys callitrichus	Latimojong	2	0	0
Tateomys rhinogradoides	Latimojong	3	0	0
20 species	Two localities	441	34 (7.7%, 5.2–10.2)	117 (26.5%, 22.4–30.7)

* No. sampled includes positive PCR reactions that failed at sequencing and were excluded from analyses *B. coelestis* (n = 2), *B. penitus* (n = 4), *B. torajae* (n = 3), *E. canus* (n = 1), *P. dominator* (n = 1), *R. mollicomulus* (n = 1).
† Introduced species.

Phylogenetic analyses

The second phylogenetic analysis aimed to determine the relationships of Sulawesi haplotypes within the *T. theileri* clade. The *T. theileri* clade alignment contained 17 haplotypes isolated from 117 specimens (8 species) of rodents generated from this study and sequences from 8 trypanosome species representing all species within the *T. theileri* clade available from GenBank (Tables II, S1). The *T. theileri* clade alignment contained 846 nucleotide positions (53 parsimony informative sites) with a minimum of 764 bp per sequence. Although the *T. theileri* clade-specific phylogenetic analysis offered little support for many recent relationships, there was strong support for the placement of the Sulawesi haplotypes (H5–H21), *T. cyclops*, and *Trypanosoma* sp. TL.AQ.22 (isolated from an Australian leech, *Philaemon* sp.) in a clade containing undescribed trypanosomes infecting Australian leeches and wallabies (*Trypanosoma* sp. ABF, *Trypanosoma* sp. TL.AV.43, and *Trypanosoma* sp. TL.AQ.45) (MLBS $\geq$70, BPP $\geq$0.95; Fig. 3). We did not recover any haplotypes that shared 100% similarity to *T. theileri,* an ungulate-infecting trypanosome species.

The *T. lewisi* clade alignment contained 4 novel haplotypes isolated from 34 specimens (6 species) of rodents from this study as well as sequences from 3 representative trypanosome species within the *T. lewisi* clade obtained from GenBank (Tables II, S1). The *T. lewisi* clade alignment contained 808 nucleotide positions (7 parsimony-informative sites) with a minimum of 745 bp per sequence. jModelTest selected K80 to be the optimal model of substitution. The *T. lewisi* clade haplotypes had distinct geographic separation, with each mountain hosting a distinct clade containing 2 haplotypes each (Fig. 3). This reciprocal monophyly among mountains was strongly supported by both maximum likelihood and Bayesian analyses (MLBS = 100%, BPP = 1; Fig. 3). Additionally, we recovered sequences from the introduced rodent, *Rattus exulans*, that were genetically identical to sequences recovered from endemic rodents that had overlapping distributions with *Rattus exulans* on Mount Bawakaraeng (Fig. 3). This haplotype (H3) also shared 100% similarity to *T. lewisi* clade parasites infecting the Chinese white-bellied rat, *Niviventer confucianus,* a species that is not recorded from Sulawesi.

Statistical analyses of *Trypanosoma* prevalence

We detected *Trypanosoma* in 151 of 441 (34.2%) of our samples (Table II). *Trypanosoma theileri* clade haplotypes were recovered from 117 individuals representing 8 host species and had a significantly higher mean prevalence of 26.5% (22.4–30.7%) compared to *T. lewisi*, which was detected in only 34 individuals from 6 species for a mean prevalence of 7.7% (5.2 – 10.2%; χ^2 = 11.9, degrees of freedom [df] = 1, *P* value [*P*] = 0.006; Tables I, II). The host species infected by *T. theileri* clade haplotypes included 3 species of *Bunomys* (*Bunomys coelestis*, *Bunomys penitus*, and *Bunomys torajae*), 2 species of native *Rattus* (*Rattus bontanus*, *Rattus facetus*), *Maxomys musschenbroekii*, *Paruromys dominator*, and *Paucidentomys vermidax* (Table II). The host species infected by *T. lewisi* clade haplotypes were similar but not identical and included 3 species of *Bunomys* (*B. coelestis*, *B. penitus*, *B. torajae*), 2 species of native *Rattus* (*R. bontanus* and *R. mollicomulus*), and

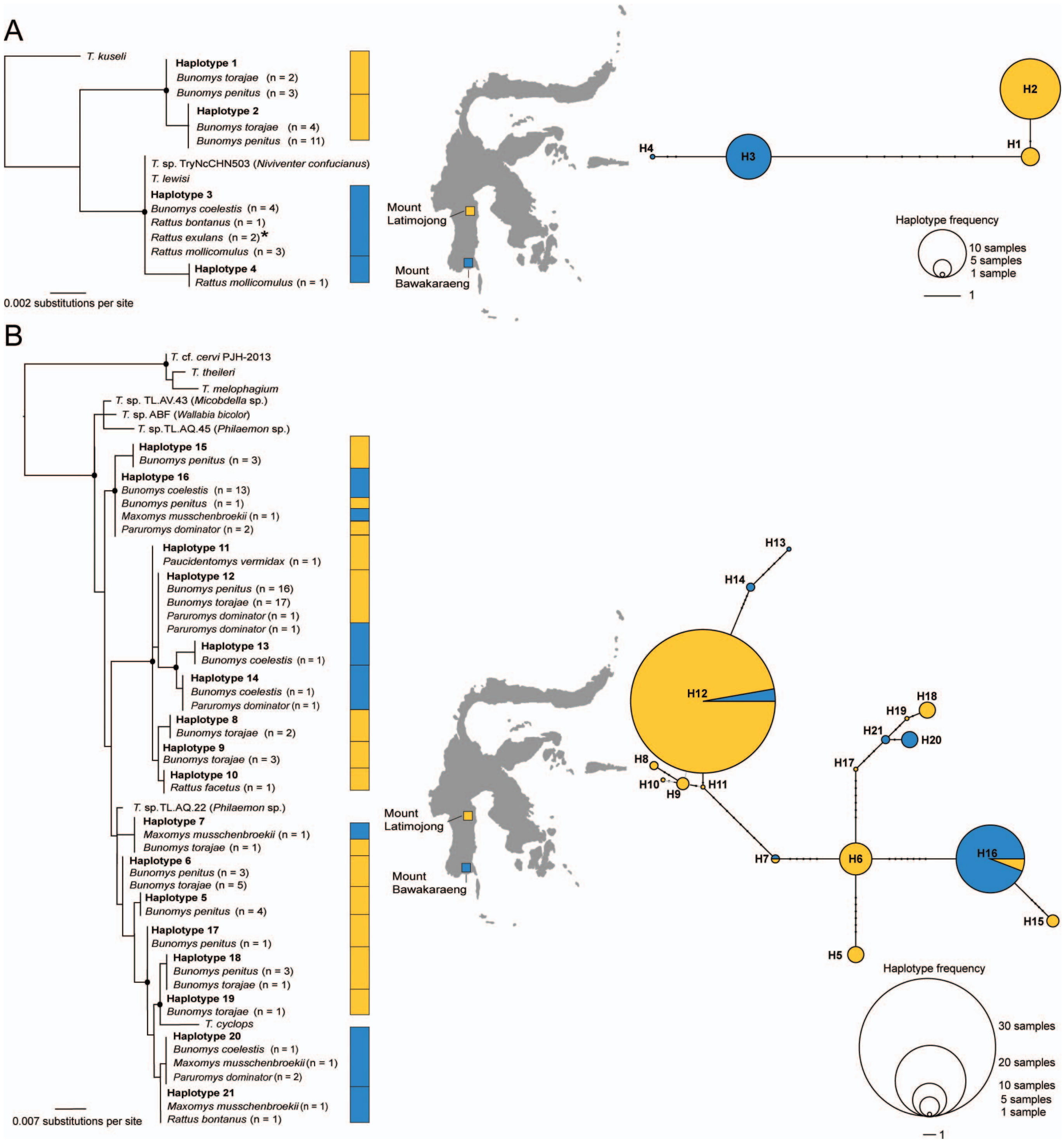

Figure 3. Phylogenetic relationships inferred using maximum likelihood analyses based on (**A**) the *Trypanosoma lewisi* clade alignment and (**B**) the *Trypanosoma theileri* clade alignment of *18S rDNA* fragments, with corresponding haplotype networks. Nodes supported by >70% maximum likelihood bootstrap values and ≥95% posterior probabilities are marked with black circles. Samples sequenced in this study are represented by haplotype number in bold. Below each haplotype are the species names of the rodent hosts they infected, with sample sizes in brackets. Colors of bars indicate samples recovered from Mount Bawakaraeng (■, blue) and from Mount Latimojong (■, yellow). Samples from GenBank are represented by *Trypanosoma* species name with host species in brackets, if reported. Haplotype networks of partial *18S rDNA* fragments depict the number of mutations between haplotypes, with pie charts showing haplotype frequencies separated by mountain (Mount Bawakaraeng = ●, blue, Mount Latimojong = ●, yellow). Dots along links in network represent mutational steps between haplotypes. The introduced host species screened in this study, *Rattus exulans*, is denoted by an asterisk (*). Color version available online.

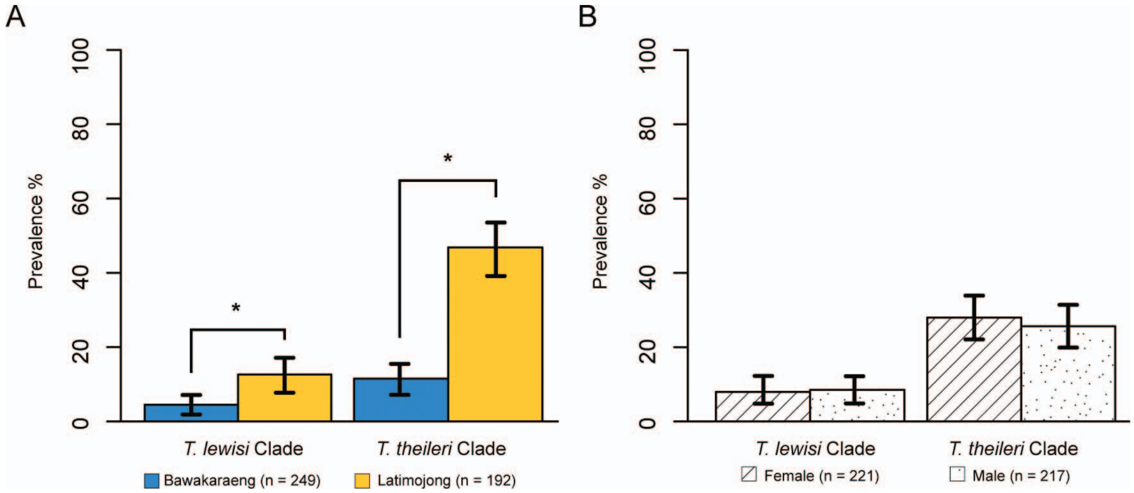

Figure 4. Percent prevalence of *Trypanosoma* spp. from *Trypanosoma lewisi* and *Trypanosoma theileri* clades in murines from Sulawesi, by PCR; (**A**) prevalence across field sites, and (**B**) prevalence within each sex. Confidence intervals (95%) are represented by error bars. Statistical significance ($P < 0.05$) is represented by an asterisk (*). Color version available online.

1 species of introduced *Rattus* (*R. exulans*; Table II). We never recovered trypanosomes from 10 of our sampled host species including *Bunomys andrewsi* (n = 2), *Eropeplus canus* (n = 2), *Lenomys meyeri* (n = 2), *Margaretamys elegans* (n = 1), *Margaretamys* sp. nov. (n = 1), *Maxomys dollmani* (n = 1), *Rattus hoffmanni* (n = 3), *Sommeromys macrorhinos* (n = 3), *Taeromys callitrichus* (n = 2), and *Tateomys rhinogradoides* (n = 3).

For *T. theileri* clade infections, we observed significant variation in prevalence among host species and between mountains, but not between sexes (Figs. 4, 5). The prevalences of *T. theileri* clade infections in *B. penitus* and *B. torajae* were significantly higher than the average *T. theileri* clade prevalence of all infected host species at 36.8% (*B. penitus*, mean parasite prevalence = 51.2%, n = 82; $\chi^2 = 15.6$, df = 1, $P < 0.001$; *B. torajae*, 66.7%, n = 66; $\chi^2 = 31.0$, df = 1, $P < 0.001$ respectively). For *P. dominator*, *M. musschenbroekii*, and *R. bontanus*, *T. theileri* clade prevalence was significantly lower than the average *T. theileri* clade prevalence (*P. dominator*, 15.6%, n = 45, $\chi^2 = 37.0$, df = 1, $P < 0.001$; *M. musschenbroekii*, 8.5%, n = 47, $\chi^2 = 50.3$, df = 1, $P < 0.001$; *R. bontanus*, 3.0%, n = 33, $\chi^2 = 43.4$, df = 1, $P < 0.001$). *Trypanosoma theileri* clade prevalence in *B. coelestis* was not significantly different from the average *T. theileri* clade prevalence (24.3%, n = 70, $\chi^2 = 2.8$, df = 1). Although for *Paucidentomys vermidax* and *R. facetus* *T. theileri* clade prevalence was also not significantly different from the average, we lacked power to detect differences with our sample sizes (*P. vermidax*, 100%, n = 1, Fisher's exact test, $P = 0.37$; *R. facetus*, 25%, n = 4, Fisher's exact test, $P = 0.15$). Between mountains, *T. theileri* clade prevalence was significantly higher on Mount Latimojong (46.4%, 95% CI = 39.3–53.4%) than on Mount Bawakaraeng (11.2%; 95% CI = 7.3–15.1%, $\chi^2 = 80.7$, df = 1, $P < 0.001$, Fig. 4a). There was no significant difference in prevalence between males and females ($\chi^2 = 0.22$, df = 1, $P = 0.64$, Fig. 4b).

As with the *T. theileri* clade samples, *T. lewisi* clade infections on Sulawesi displayed significant variation in prevalence among species and between mountains, but not between sexes (Figs. 4, 5). The prevalences of *T. lewisi* clade infections in *B. penitus* and *B. torajae* were significantly higher than the average *T. lewisi* clade prevalence of all infected host species at 9.5% (*B. penitus*, 18.3%, n = 82, $\chi^2 = 10.8$, df = 1, $P = 0.001$ and *B. torajae*, 12.1%, n = 66; $\chi^2 = 6.1$, df = 1, $P = 0.01$ respectively). For *R. bontanus*, *T. lewisi* clade prevalence was significantly lower than the average *T. lewisi* clade prevalence (3.0%, n = 33, $\chi^2 = 96.8$, df = 1, $P < 0.001$). *Rattus exulans*, *R. mollicomulus*, and *B. coelestis* were not significantly different from the average *T. lewisi* clade prevalence (*R. exulans*, 10.5%, n = 19, Fisher's exact test, $P = 0.75$; *R. mollicomulus*, 7.4%, n = 54, Fisher's exact test, $P = 0.19$; *B. coelestis*, 5.7%, n = 70, Fisher's exact test, $P = 0.18$, respectively). There was a significantly higher ($\chi^2 = 22.5$, df = 1, $P < 0.001$) prevalence on Mount Latimojong (12.0%; 95% CI = 7.4–16.6%) compared to Mount Bawakaraeng (4.4%; 95% CI = 1.8–7.0%, Fig. 4a). There were no significant differences in prevalence between males and females ($\chi^2 = 0.09$, df = 1, $P = 0.76$, Fig. 4b).

Population genetic analyses of *Trypanosoma*

For samples within the *T. theileri* clade, our combined analyses found significant population structure between mountains but with substantial variation within mountains. We identified 47 polymorphic sites within the 17 *T. theileri* clade haplotypes with a nucleotide diversity (θ_π) of 9.05 (±4.66 SD). We detected significant population structure between mountains in the *T. theileri* clade haplotypes but with most variation explained by difference among individuals within mountains ($F_{st} = 0.31$; $P < 0.0001$; Table S3). Although the average number of pairwise differences between mountains (11.29) was higher than within mountains, both mountains contained substantial differences among individuals (Mount Bawakaraeng = 8.38, Mount Latimojong = 7.30). Population structure between mountains was also supported by the *T. theileri* haplotype network where we recovered 10 haplotypes exclusively on Mount Latimojong and 4 exclusively on Mount Bawakaraeng. However, 3 haplotypes were shared between mountains including the 2 haplotypes with the highest frequencies (Haplotype 12, n = 35 and Haplotype 16, n = 17).

In contrast to the *T. theileri* clade samples, our combined analyses suggested that haplotypes in the *T. lewisi* clade were

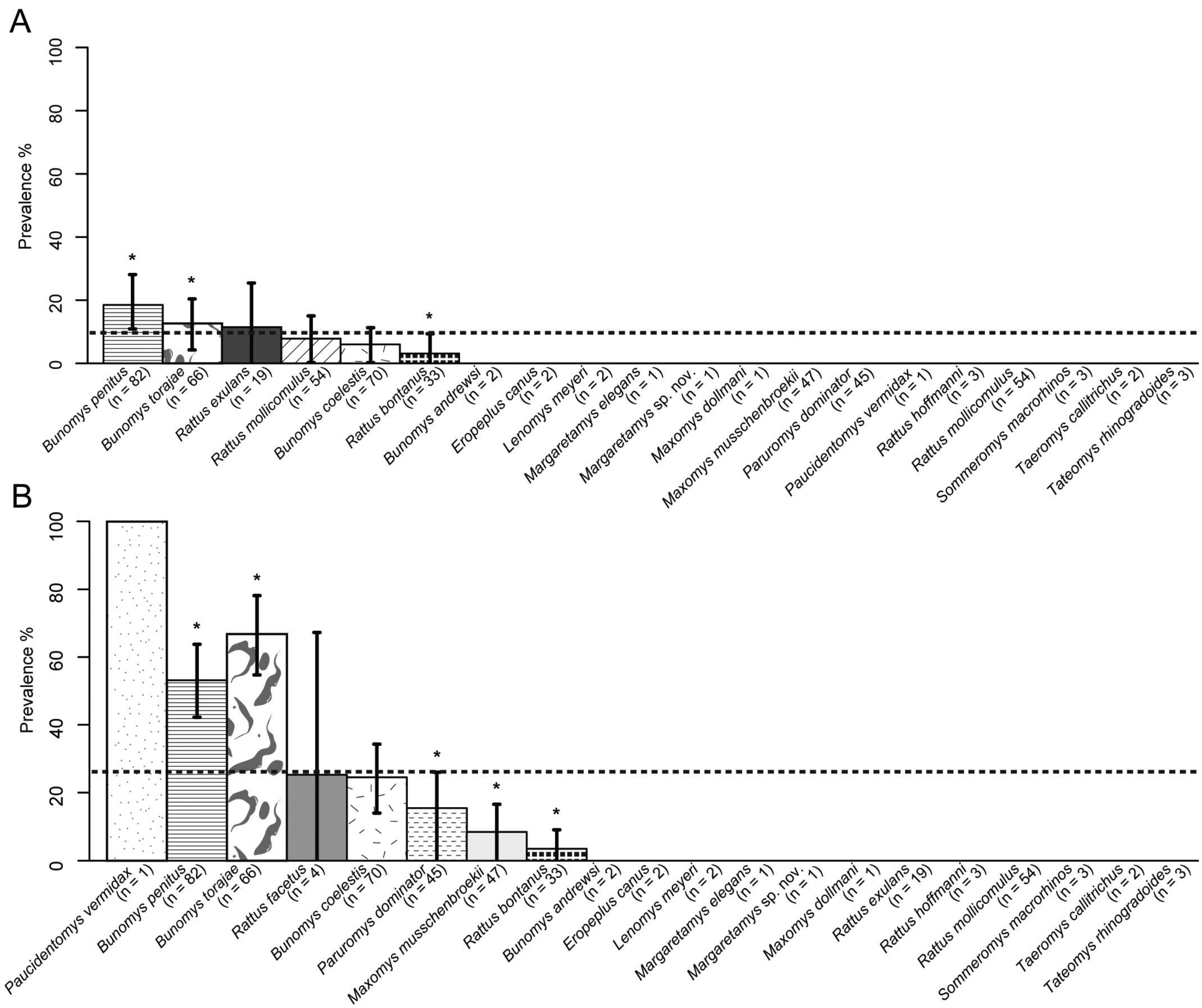

Figure 5. Prevalence of (**A**) *Trypanosoma lewisi* clade and (**B**) *Trypanosoma theileri* clade *Trypanosoma* spp. per murine species from Sulawesi. Confidence intervals (95%) are represented by error bars. Average prevalence across all infected species is represented by a dashed line. Species with a prevalence significantly different ($P < 0.05$) from the average parasite prevalence is represented by an asterisk (*).

completely structured between mountains with almost no genetic diversity within each of the mountains. We identified 15 polymorphic sites within the 4 *T. lewisi* clade haplotypes with a nucleotide diversity (θ_π) of 4.01 (±2.29 SD). Each of the 4 haplotypes was restricted to 1 mountain with 2 haplotypes per mountain ($F_{st} = 0.95$, $P < 0.0001$; Table S4). This population structure was evident from the average number of pairwise differences between mountains (8.04) compared to the average number of pairwise differences within mountains (Mount Bawakaraeng = 0.37, Mount Latimojong = 0.40, Fig. 3). The distinct geographic separation of haplotypes was also supported by the *T. lewisi* haplotype network. Seven mutational steps separated Haplotype 1 on Mount Latimojong from Haplotype 3 on Mount Bawakaraeng, whereas within mountains, haplotypes were only 1 or 2 mutational steps apart (Fig. 3).

DISCUSSION

This is the first study to survey Sulawesi's murine rodents systematically for blood-borne parasites in the subclass Metakinetoplastina. With samples from 2 mountains, Mount Bawakaraeng and Mount Latimojong, we recorded 3 genera of Metakinetoplastina in murine rodents from Sulawesi. Our detection of *Parabodo*, a genus currently thought to be free-living (Lee et al., 2005; Tikhonenkov et al., 2012; Suyanto, 2016) suggests that the genus may include parasitic species. As *Parabodo*-like flagellates are substantially understudied, *Parabodo* may contain undescribed species that parasitize vertebrates (Lukes et al., 2014). This study adds to an increasing body of literature detecting metakinetoplastids, that were considered to be free-living, in the tissues of vertebrates (Yuan et al., 2012; Dario et al., 2017). Similarly, species within the genus *Blechomonas* have previously only been isolated from insect hosts (i.e., are monoxenous parasites), with the exception of *Blechomonas pulexsimulantis* (formerly *Leptomonas pulexsimulantis*) recorded opportunistically infecting an HIV-positive patient in Brazil (Pacheco et al., 1998; Votýpka et al., 2013; Lukes et al., 2014). However, we have not confirmed infection in our 1 rodent sample testing positive for *Blechomonas*, which may reflect noninfective transfer of *Blechomonas* DNA from an infected biting insect. Our sampling also produced the first records of trypanosomes infecting rodents of Sulawesi, including 2 major *Trypanosoma* clades; the *T. lewisi* clade containing a globally distributed invasive parasite, and the *T. theileri* clade containing trypanosomes endemic to Australia, Malaysia, and Sri Lanka (see Hamilton et al., 2005; Pumhom et al., 2014). Because the *18S rDNA* gene alone is not sufficient to resolve haplotypes to species, the haplotypes recovered in this study can only be identified to parasite clades and thus may represent multiple trypanosome species within these clades.

Our phylogenetic and population genetic analyses suggest that the parasites we recorded from the *T. theileri* clade display characteristics indicative of them being species that are native to Sulawesi. Within the *T. theileri* clade our samples were most closely related to *T. cyclops* and related species which have been recorded from Malaysia, Sri Lanka, Australia, and New Guinea (Weinman and Wiratmadja, 1969; Weinman, 1972; Hamilton et al., 2005; Cooper et al., 2017a). The phylogenetic placement of our Sulawesi trypanosomes, within a clade containing Australian and Asian trypanosomes, suggests that parasites within this clade are native to the Indo-Australian region. Our phylogeny also nested the Malaysian trypanosome, *T. cyclops*, within our Sulawesi samples suggesting that Sulawesi harbors substantial diversity in this Indo-Australian clade (Hamilton et al., 2005). Sulawesi's parasites within the *T. theileri* clade also displayed high haplotype and sequence diversity with 47 polymorphic sites identified within 17 haplotypes. Additionally, they displayed high average nucleotide pairwise differences both between and within mountains. Although there was significant geographic structuring of haplotype frequencies between mountains, there was also evidence for some long-term connectivity between mountains for these parasites. For example, there was strong support for at least 3 clades that included haplotypes from each mountain (Fig. 3). Furthermore, 3 haplotypes that were shared across both mountains were present in a host species with broad geographic distributions that span both mountains.

In contrast to the *T. theileri* clade parasites, the *T. lewisi* clade parasites we recorded in rodents from these 2 mountains on Sulawesi display characteristics indicative of an introduced parasite species. *Trypanosoma lewisi* clade parasites are cosmopolitan trypanosomes recorded from all continents excluding Antarctica. They are known to be carried by introduced rodent species on neighboring landmasses in the Indo-Australia region (Jittapalapong et al., 2008; Pumhom et al., 2014, 2015; Desquesnes et al., 2016). We recovered an identical sequence to *T. lewisi* from an introduced host species, *Rattus exulans*, a known reservoir for *T. lewisi* clade parasites with recent records of spillover into native rodent species elsewhere (Milocco et al., 2013; Pumhom et al., 2014). The *T. lewisi* clade parasites displayed low haplotype and sequence diversity with 15 polymorphic sites identified within 4 haplotypes. Additionally, they displayed low average nucleotide pairwise differences recorded both between and within mountains. Similar to patterns reported from introduced parasites in other island systems (Dudaniec et al., 2008; Gaither et al., 2013), these results suggest that parasites in the *T. lewisi* clade on Sulawesi were recently introduced to the island. Furthermore, there were no haplotypes shared between mountains (thus $F_{st} = 0.95$), suggesting that the *T. lewisi* clade haplotypes had no connectivity between mountains. The 2 haplotypes on each mountain also formed reciprocally monophyletic clades, further demonstrating that there was complete isolation between mountains (Fig. 3). This isolation suggests that there were 2 independent introductions of *T. lewisi* clade parasites into these 2 areas of Sulawesi. Though *R. exulans* were only recovered from Mount Bawakaraeng and no introduced species was collected from Mount Latimojong, introduced rodent species have been recorded across Sulawesi including Mount Latimojong that may be potential reservoirs for *T. lewisi* (Whitten et al., 1987).

As *T. lewisi* is an introduced parasite known to cause disease in naïve host species (Wyatt et al., 2008), further research into its potential health impacts on Sulawesi's endemic rodents is required. In a naïve host, the prevalence of introduced parasites can be much higher than native parasites (Darji et al., 1992; Guerrero et al., 1997; Dudaniec et al., 2008; Wyatt et al., 2008). Specifically, for *T. lewisi*, its introduction into Uganda led to a prevalence 3 and a half times higher than native trypanosome species (Salzer et al., 2016). However, in our study on Sulawesi, native trypanosomes in the *T. theileri* clade had a prevalence 3 and a half times higher than introduced trypanosomes in the *T. lewisi* clade (26.8% and 7.7%). This suggests that the native rodent hosts are resistant to the introduced trypanosome and infections by native trypanosomes are more common. Another possibility is that the introduced parasites are more pathogenic (Morner et al., 2002), allowing rodent hosts to tolerate higher infection rates of the native trypanosomes compared to the introduced trypanosomes. However, we lack any data on the pathogenicity of trypanosomes in Sulawesi rodents to test this. Finally, this study was unable to detect mixed infections within individuals, and PCR amplification bias towards parasites within the *T. theileri* clade could have resulted in a higher observed prevalence than is occurring (see Smith et al., 2005; Paparini et al., 2011; Botero et al., 2013).

Despite an inability to detect mixed infections within an individual, there is evidence that 4 rodent host species were infected with haplotypes from both trypanosome clades within the same mountain. Both *T. lewisi* and *T. theileri* clade haplotypes were recovered from *Bunomys penitus*, *B. coelestis*, and *B. torajae* on Mount Latimojong, and *Rattus bontanus* on Mount Bawakaraeng. Laboratory rats with *T. lewisi* infections are more susceptible to infections by other parasites due to trypanosome-elicited immunosuppression (Darji et al., 1992; Guerrero et al., 1997). This potentiated pathogenicity also has been observed in wild marsupials (woylies, *Bettongia penicillata*, and koalas, *Phascolarctos cinereus*) with mixed trypanosome infections (Botero et al., 2013). Further research is required to determine if co-infections of native and introduced trypanosomes are occurring in Sulawesi's rodents, what the health impacts of these infections are, and whether or not co-infections are potentiating pathogenicity in otherwise nonpathogenic trypanosomes (Botero et al., 2013).

Our results show that the infection rates for *T. theileri* and *T. lewisi* clade haplotypes were not randomly distributed among rodent host species but were randomly distributed across sexes. Two host species in the genus *Bunomys* (*B. penitus* and *B. torajae*) supported infection rates that were significantly higher than the average across all rodents sampled for both trypanosome clades. Other species within *Bunomys* did not share this elevated infection rate, including the critically endangered *B. coelestis*. Four host species had infection rates that were not significantly different from the average, whereas 3 host species had significantly lower rates of infection than the average. For most species, our power to detect differences was high (>0.80) except for *Rattus facetus* (n = 4), *R. exulans* (n = 19), and *Paucidentomys vermidax* (n = 1). Trypanosome infections were not detected in 10 rodent species; however, these species had low sample sizes (n < 3) suggesting that there was a low probability of detection (Table I) (see also Salzer et al., 2016). Future research is needed to determine if *B. penitus* and *B. torajae* are more susceptible to trypanosome infections and what factors may be influencing this high parasite prevalence.

The dilution-effect hypothesis predicts that increased species diversity lowers the prevalence of diseases because of lower host densities, lower rates of transmission, or higher mortality of infected hosts (Clay et al., 2009; Searle et al., 2011). Our results indicate that Mount Latimojong, which supported a higher diversity of murine rodent species (n = 14), had a significantly higher prevalence of trypanosome parasites compared to Mount Bawakaraeng which supports a lower diversity of murine rodents (n = 9) (Keesing et al., 2006; Johnson and Thieltges, 2010; Brugat et al., 2014). As such, our study adds to the increasing body of literature suggesting that the dilution effect hypothesis is not consistently supported and may only apply to specific circumstances (Randolph and Dobson, 2012). Our results are instead more consistent with the amplification effect hypothesis, where disease prevalence increases with increasing species diversity due to higher encounter rates between hosts and thus higher rates of transmission, or increased availability of secondary hosts (Clay et al., 2009; Randolph and Dobson, 2012; Young et al., 2017). Even though fewer species were infected with trypanosomes on Mount Latimojong (5 host species) compared to Mount Bawakaraeng (6 host species), the noninfected rodent species could act as amplification agents (Clay et al., 2009). Amplification agents can increase rates of transmission through increasing interactions between rodents of susceptible species by altering their behavior and microhabitat use (Clay et al., 2009). These trends remain speculative, as this study has not addressed key mechanisms that can influence parasite prevalence (e.g., host–parasite–vector dynamics, host population ecology, and behaviors, vector dynamics, seasonality, interannual variability, pathogenicity, etc.; Clay et al., 2009; Young et al., 2017). However, our baseline data suggest that regions within Sulawesi with high host diversity are more likely to have a higher parasite prevalence than regions with lower host diversity (Clay et al., 2009; Searle et al., 2011).

The findings of this study provide important initial data on the prevalence and genetic diversity of previously undescribed metakinetoplastids in Sulawesi rodents. Although there has recently been a greater interest in screening understudied regions for trypanosomes, this study highlights the need for a more extensive sampling of wildlife in remote biodiversity hotspots, not only for trypanosomes, but also other parasites (DiEuliis et al., 2016; Cooper et al., 2017a; Dario et al., 2017; Dunnum et al., 2017). Additionally, there is a critical need for targeted assessment of the health impacts of trypanosome infection in Sulawesi's endemic rodents, particularly in threatened species such as *B. coelestis* and those displaying high infection rates, such as *B. torajae* and *B. penitus*.

ACKNOWLEDGMENTS

The authors assert all applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All specimen collection was conducted in accordance with permits from the Indonesian Ministry of Research Technology and Higher Education (RISTEK) and with the authorization of the Indonesian Ministry of Environment and Forestry (BKSDA). We thank the State Ministry of Research and Technology (RISTEK) and the Ministry of Forestry, Republic of Indonesia, for providing permits to carry out fieldwork. We are indebted to the Research Center for Biology, Indonesian Institute of Sciences (RCB-LIPI), and the Museum Zoologicum Bogoriense (MZB, Cibinong, Indonesia) for providing staff and support. Thank you to Sarah Nielsen and Molly Watchorn for assistance in the collection of blood smears in the field. We thank Jimmy A. McGuire, Rauri Bowie, Lydia Smith, Bryan Bach, and Cynthia Wang of the Museum of Vertebrate Zoology (MVZ), and Kelly Kroft of the American Museum of Natural History (AMNH) for their invaluable advice and assistance with laboratory work. Thank you to Kath Handasyde for insightful comments and edits of this manuscript. Funding was provided by the United States National Science Foundation (DEB-1457654), National Geographic Society (WW-160R-17), The Mohamed bin Zayed Species Conservation Fund (172516185), the American Society of Mammalogists (Grants-in-Aid of Research to M. L. Winterhoff), and Museums Victoria (1854 Student Scholarship to M. L. Winterhoff).

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