Research paper

# Correlation between genotypes and geographic distribution of *Entamoeba nuttalli* isolates from wild long-tailed macaques in Central Thailand

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A B S T R A C T

*Entamoeba nuttalli* found in non-human primates is the phylogenetically closest species to *Entamoeba histolytica* and is potentially pathogenic. However, infection of wild long-tailed macaques (*Macaca fascicularis*) with *E. nuttalli* has not been found. In this study, the prevalence of *Entamoeba* infections in wild long-tailed macaques was examined in seven locations in six provinces of Thailand. The positive rate for *E. nuttalli* in 214 fecal samples was 43.9% using PCR, but no infection with *E. histolytica* or *Entamoeba dispar* was found, demonstrating that long-tailed macaque is one of the natural hosts for *E. nuttalli*. Twenty-four *E. nuttalli* isolates were successfully cultured and four of them were axenized. The sequences of the 18S ribosomal RNA genes of *E. nuttalli* from long-tailed macaques differed from those of *E. nuttalli* isolates from other species of wild macaques. Eleven types of sequences in serine-rich protein genes were identified in the 24 isolates and these were specific for each location in Thailand. By analysis of six tRNA-linked short tandem repeat loci, these isolates were divided into 14 types, and each type was also location-specific. Phylogenetic analysis revealed correlation between genotypes of the parasite and the geographic distribution of the host macaques. Genetic distance and geographic distance correlated significantly in a Mantel test, with r values of 0.888 based on the tRNA-linked short tandem repeat loci and 0.815 based on the serine-rich protein genes. These results suggest that genetic divergence and co-evolution of the parasite occurred during dispersion and colonization of the host macaque, and that genotypic analysis of the parasite may enable identification of the geographic localization of the host.

## 1. Introduction

*Entamoeba histolytica* is the causative agent of amebiasis, with an estimated 50 million cases of colitis and liver abscess resulting in 40,000 to 100,000 deaths annually (Haque et al., 2003; Stanley Jr., 2003). There are several *Entamoeba* species that are morphologically indistinguishable from *E. histolytica*. Of the amebas with quadrinucleated cysts, *Entamoeba dispar* is the most prevalent species, but is nonpathogenic; *Entamoeba moshkovskii* is primarily free-living; and *Entamoeba bangladeshi* is found in only a few countries (Ali et al., 2003; Heredia et al., 2012; Ngobeni et al., 2017; Royer et al., 2012).

It is important to understand the prevalence of *Entamoeba* infections in nonhuman primates for animal health and prevention of zoonosis. Recent studies have shown that infection with *Entamoeba nuttalli*, but

not *E. histolytica*, is prevalent in wild and captive macaques, in addition to *Entamoeba chattoni* (*Entamoeba polecki* ST2) and *Entamoeba coli* infections. *E. nuttalli* is the phylogenetically closest species to *E. histolytica* and also potentially pathogenic (Tachibana et al., 2007). Many *E. nuttalli* strains have been isolated by culture of feces from wild rhesus macaques (*Macaca mulatta*) in Nepal (Tachibana et al., 2013), wild and captive rhesus macaques in China (Feng et al., 2013), captive Japanese macaques (*Macaca fuscata*) in Japan (Tachibana et al., 2009), wild toque macaques (*Macaca sinica*) in Sri Lanka (Tachibana et al., 2016), and wild Tibetan macaques (*Macaca tibetana*) in China (Guan et al., 2016; Wei et al., 2018). Therefore, macaques seem to be a natural host for *E. nuttalli*.

*E. nuttalli* has also been isolated from a De Brazza's guenon in a zoo (Suzuki et al., 2008; 2007). In the zoo, fatal cases of liver abscess with

*E. nuttalli* have been observed in an Abyssinian colobus and a Geoffroy's spider monkey (Suzuki et al., 2008). Recently, an asymptomatic case of human infection with *E. nuttalli* occurred in a caretaker of nonhuman primates in a zoo (Levecke et al., 2015). Although the pathogenicity of *E. nuttalli* in human is still unknown, severe inflammatory reactions have been found in the formation of liver abscess in hamsters inoculated experimentally with *E. nuttalli* (Guan et al., 2018; Tachibana et al., 2009; 2007).

The long-tailed macaque (*Macaca fascicularis*), also known as a crab-eating macaque or cynomolgus monkey in laboratories, lives natively in Southeast Asia. Since long-tailed macaques are used as experimental animals for medical research, the macaques are exported from breeding colonies in the countries. Examination of 25 isolates of *Entamoeba* from long-tailed macaques imported to Japan identified only one isolate from China as *E. nuttalli*, while others were identified as *E. dispar* (Takano et al., 2005; 2007). Moreover, *E. nuttalli* infection has not been detected by screening of large breeding colonies of long-tailed macaques in China, despite *E. nuttalli* being prevalent in colonies of rhesus macaques in China (Feng et al., 2013; 2011). *E. nuttalli* was also not detected in a colony of long-tailed macaques in the Philippines, although *E. histolytica* and *E. dispar* infections were prevalent (Rivera et al., 2010). Therefore, it is important to know whether wild long-tailed macaques are also a natural host for *E. nuttalli*.

Here, we report the prevalence of *E. nuttalli* infections in wild long-tailed macaques in Central Thailand. More than twenty isolates of *E. nuttalli* were established by culture from the wild macaques and genotypes of the isolates were compared.

## 2. Materials and methods

### 2.1. Study area

Stool samples from wild long-tailed macaques were collected in seven locations in Central Thailand in February 2011 (Fig. 1). These areas were located in Phetchaburi, Prachuap Khiri Khan, Ratchaburi, Chonburi, Lopburi, and Saraburi Provinces. A total of 214 fresh stool samples were collected, carried to a laboratory with cooling by ice, and then subjected to direct microscopy and culture. Aliquots of each sample were preserved in 2% potassium dichromate solution.

### 2.2. Stool examination and culture

Stool samples were examined microscopically with iodine staining. Samples with quadrinucleated cysts of *Entamoeba* in microscopy were suspended in water for > 18 h to prevent growth of *Blastocystis* spp. trophozoites, and then cultured in modified Tanabe-Chiba medium at 37 °C (Tachibana et al., 2007). Grown trophozoites of at least one isolate from each area were treated with a cocktail of antibiotics and then cultured monoxenically with *Crithidia fasciculata* in YIMDHA-S medium supplemented with 15% adult bovine serum at 37 °C (Kobayashi et al., 2005). Finally, some of the isolates were cultured axenically in the medium and an isolate was subjected to cloning by limiting dilution, followed by microscopy examination.

### 2.3. Extraction of DNA and PCR

Extraction of DNA from preserved stool samples was performed as previously described (Tachibana et al., 2007). Briefly, the samples in potassium dichromate solution were mixed with diethyl ether and centrifuged at 400*g* for 2 min. Sediments were washed twice with phosphate-buffered saline and then genomic DNA was isolated using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Genomic DNA was also isolated from cultured trophozoites. PCR amplification of partial 18S rRNA genes from *E. nuttalli*, *E. histolytica*, *E. dispar*, *Entamoeba coli* and *Entamoeba chattoni* was performed using species-specific primers (Tachibana et al., 2009; 2013; 2007; Verweij et al., 2001).
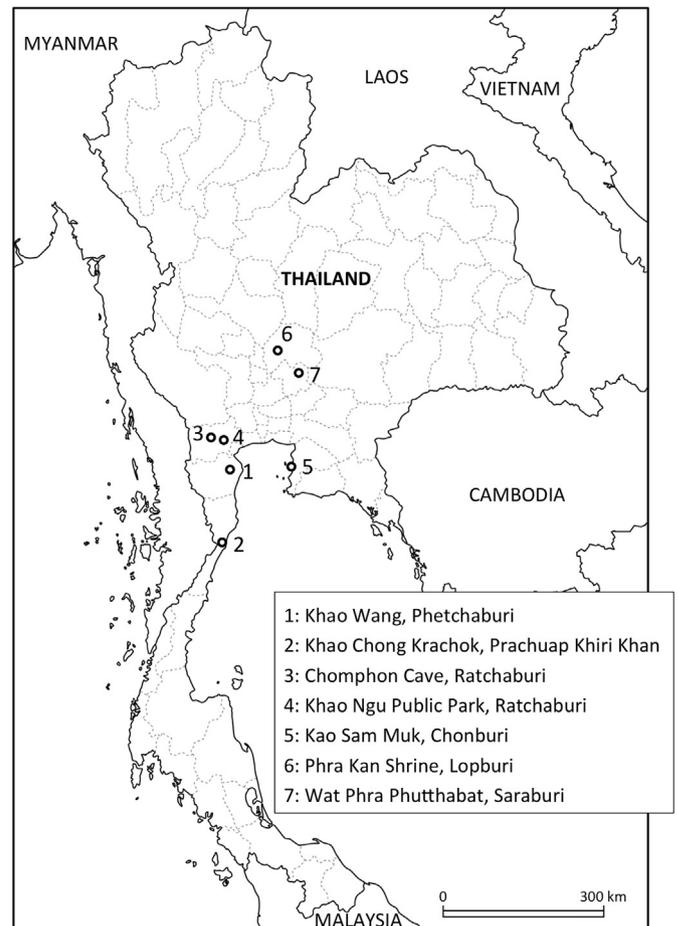


**Fig. 1.** Map of the study sites in Central Thailand. Stool samples were collected in seven locations of six provinces.

Amplification of an approximately 2.4 kb region containing 18S and 5.8S rRNA genes was performed (Tachibana et al., 2007). The serine-rich protein (SRP) gene was amplified essentially as reported (Ghosh et al., 2000). Amplification of tRNA-linked short tandem repeats (STR) of six loci (locus A-L, D-A, N-K2, R-R, S-Q and $S^{TGA}$-D) was also performed (Ali et al., 2005).

### 2.4. Sequencing

PCR products of 2.4 kb rRNA genes, SRP genes and tRNA-linked STR fragments were purified using a QIAquick PCR purification kit (Qiagen) or QIAEX II gel extraction kit (Qiagen), and then directly sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA). Reactions were run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

### 2.5. Phylogenetic analysis and alignment

Genetic distances were calculated using the SRP gene or tRNA-linked STR loci using MEGA 5 software. A Mantel test for analysis of isolation by distance was performed using IBD 1.5.2 software with 10,000 randomizations (Jensen et al., 2005). Exact *P* values for data in the Mantel test were calculated by Pearson correlation coefficient analysis using SPSS 20.0. A neighbor-joining (NJ) tree was constructed using the SRP gene or tRNA-linked STR loci using MEGA 5 software.

**Fig. 2.** A long-tailed macaque in Phetchaburi. The macaque would take food from human hands.



**Fig. 3.** Venn diagram of co-infections among *E. nuttalli*, *E. chattoni* and *E. coli* in 214 fecal samples from wild long-tailed macaques detected by PCR. Each ring represents one *Entamoeba* species, and the numbers of positives samples and percentages are shown.

## 3. Results

### 3.1. Detection of Entamoeba species by PCR

Of the 214 stool samples from long-tailed macaques, 34 were collected in Phetchaburi, 32 in Prachuap Khiri Khun, 48 in Ratchaburi, 41 in Chonburi, 37 in Lopburi, and 22 in Saraburi Provinces (Figs. 1, 2). Almost all of the samples were formed, and none were watery or bloody. In direct microscopy, uninucleated *Entamoeba chattoni* cysts were highly prevalent, and *Entamoeba coli* cysts and quadrinucleated *Entamoeba histolytica/E. dispar/E. nuttalli* cysts were also prevalent. In addition, cysts of *Iodamoeba buetschlii* were frequently found. By PCR analysis of the stool samples, the quadrinucleated *Entamoeba* cyst was confirmed to be *E. nuttalli*, but not *E. histolytica* or *E. dispar*, in all of the locations (Table 1). The positive rates for *E. nuttalli* were 71% in Chonburi, 50% in Prachuap Khiri Khan and Saraburi, 48% in Phetchaburi, 29% in Ratchaburi, and 22% in Lopburi Province. The positive rates in PCR for *E. chattoni* and *E. coli* from the six provinces were 65% to 100% and 38% to 68%, respectively. The rates of co-infections among the three *Entamoeba* species are shown as a Venn diagram in Fig. 3. Co-infection was recorded in 138 samples (64.5%), and 64 samples (29.9%) were positive for all three *Entamoeba* species. Of 94 *E. nuttalli* positive samples, all except two were co-infected with *E. chattoni*.

### 3.2. Isolation of E. nuttalli

A total of 142 stool samples containing *Entamoeba* cysts based on direct microscopy were cultured xenically in modified Tanabe-Chiba medium, and 24 isolates of *E. nuttalli* were obtained (Table 2). Of these, six isolates from five provinces were cultured in YIMDHA-S medium with *Crithidia fasciculata*; four isolates, TFP6, TFC145, TFL205 and
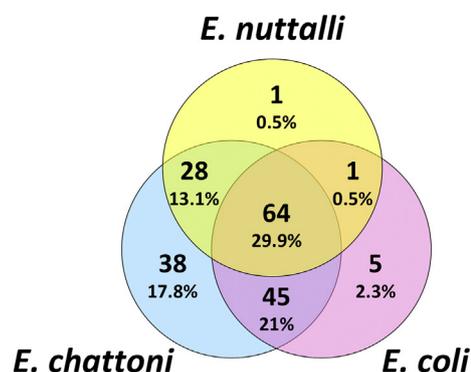
**Table 2**
Characteristics of *E. nuttalli* isolates from seven locations in Central Thailand.

| Name of isolate | Province of isolation (Numbers on map) | Final culture condition | 18S rDNA | Type of serine-rich protein gene (size of amplicon in bp[a]) |
|---|---|---|---|---|
| TFP6 | Phetchaburi (1) | Axenic | Mfas-type | I (552) |
| TFPK43 | Prachuap Khiri Khan (2) | Xenic | ND | II (540) |
| TFR74 | Ratchaburi (3) | Monoxenic | Mfas-type | V (528) |
| TFR79 | Ratchaburi (3) | Xenic | ND | V (528) |
| TFR101 | Ratchaburi (4) | Xenic | ND | VI (528) |
| TFC115 | Chonburi (5) | Xenic | ND | III (534), IX (498) |
| TFC120 | Chonburi (5) | Xenic | ND | III (534) |
| TFC121 | Chonburi (5) | Xenic | ND | IV (534) |
| TFC122 | Chonburi (5) | Xenic | ND | IV (534) |
| TFC126 | Chonburi (5) | Monoxenic | Mfas-type | III (534) |
| TFC128 | Chonburi (5) | Xenic | ND | III (534), XI (426) |
| TFC137 | Chonburi (5) | Xenic | ND | IV (534) |
| TFC138 | Chonburi (5) | Xenic | ND | III (534), IX (498) |
| TFC142 | Chonburi (5) | Xenic | ND | III (534), IX (498) |
| TFC145 | Chonburi (5) | Axenic | Mfas-type | III (534), IX (498) |
| TFC148 | Chonburi (5) | Xenic | ND | III (534) |
| TFC149 | Chonburi (5) | Xenic | ND | III (534) |
| TFC155 | Chonburi (5) | Xenic | ND | IV (534) |
| TFL197 | Lopburi (6) | Xenic | ND | X (477) |
| TFL203 | Lopburi (6) | Xenic | ND | X (477) |
| TFL205 | Lopburi (6) | Axenic, cloned | Mfas-type | X (477) |
| TFL206 | Lopburi (6) | Xenic | ND | X (477) |
| TFS228 | Saraburi (7) | Axenic | Mfas-type | VII (528) |
| TFS240 | Saraburi (7) | Xenic | ND | VIII (516) |

[a] Primer regions are excluded; Mfas-type, identical with EHMfas1 strain; ND, not determined.

**Table 1**
PCR detection of *Entamoeba* spp. in fecal samples of wild long-tailed macaques in Central Thailand.

| Location, Province (Numbers on map) | No. of samples examined | No. of positives (%) | | | | |
|---|---|---|---|---|---|---|
| | | E. nuttalli | E. histolytica | E. dispar | E. chattoni | E. coli |
| Khao Wang, Phetchaburi (1) | 34 | 16 (47.1) | 0 (0) | 0 (0) | 29 (85.3) | 16 (47.1) |
| Khao Chong Ka Jok, Prachuap Khiri Khan (2) | 32 | 16 (50) | 0 (0) | 0 (0) | 28 (87.5) | 21 (65.6) |
| Jom Pon Cave, Ratchaburi (3) | 24 | 6 (25) | 0 (0) | 0 (0) | 19 (79.2) | 16 (66.7) |
| Khao Ngu, Ratchaburi (4) | 24 | 8 (33.3) | 0 (0) | 0 (0) | 16 (66.7) | 11 (45.8) |
| Khao Sam Muk, Chonburi (5) | 41 | 29 (70.7) | 0 (0) | 0 (0) | 41 (100) | 22 (53.7) |
| Phra Kan Shrine, Lopburi (6) | 37 | 8 (21.6) | 0 (0) | 0 (0) | 24 (64.9) | 14 (37.8) |
| Wat Phra Phutthachai, Saraburi (7) | 22 | 11 (50) | 0 (0) | 0 (0) | 18 (81.8) | 15 (68.2) |
| Total | 214 | 94 (43.9) | 0 (0) | 0 (0) | 175 (81.8) | 115 (53.7) |
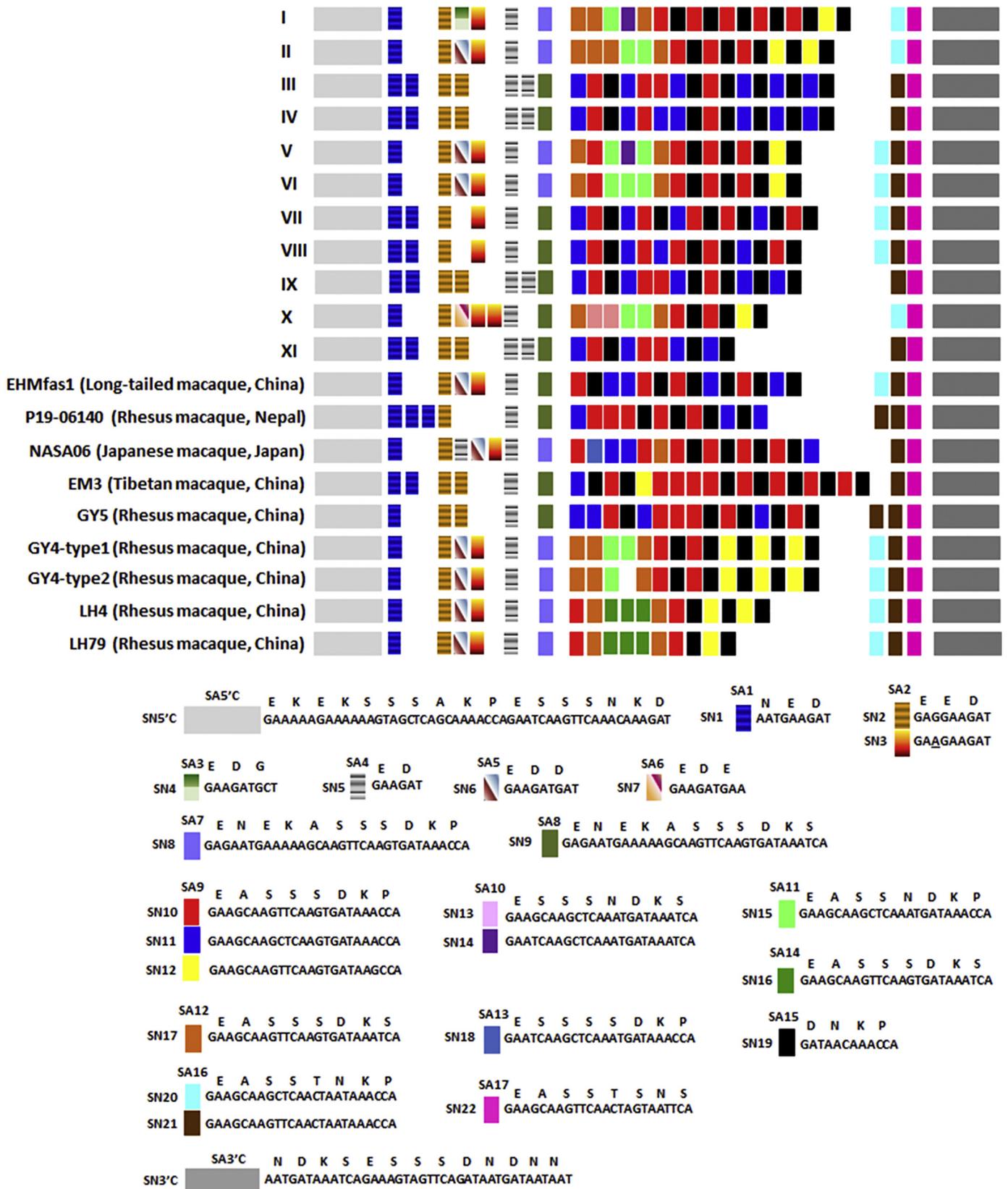
**Fig. 4.** Schematic representation of 11 genotypes in the repeat region of serine-rich protein genes of *E. nuttalli* isolated from long-tailed macaques in Central Thailand. Data for *E. nuttalli* isolates from Nepal, Japan and China in our previous studies were added to the analysis. Each unit component with a unique nucleotide sequence is shown as a rectangle and given a number for the SN series. Each deduced amino acid sequence is also shown and given a number for the SA series.

TFS228, were axenized; and TFL205 was further cloned.

### 3.3. Analysis of rRNA gene

The approximately 2.4 kb region including 18S and 5.8S rRNA genes was sequenced in two monoxenic and four axenic isolates of *E. nuttalli*. No differences in nucleotide sequences were found among the six isolates. The sequence including internal transcribed spacer 1 and 2 regions was identical with those of the EHMfas1 strain (AB197936) isolated from a captive long-tailed macaque exported from China to Japan as an experimental animal, and the NASA06 strain (AB485592) isolated from a captive Japanese macaque.

### 3.4. Analysis of the SRP gene

SRP genes were analyzed in all 24 *E. nuttalli* isolates. Isolates from each location had different gene sequences (Table 2, Fig. 4). In total, 11 genotypes were identified in 24 isolates and none was identical to those of the EHMfas1 strain from long-tailed macaques, P19-061405 from rhesus macaque, NASA06 strain from Japanese macaque, GY and LH strains from rhesus macaque, and the EM3 strain from Tibetan macaque (Fig. 4). Genotypes I, II, V, VI and X from Phetchaburi, Prachuap Khiri Khan, Ratchaburi and Lopburi Provinces contained similar types of unit components (shown as rectangles), whereas components of genotypes III, IV, VII, VIII, IX and XI from Chonburi and Saraburi Provinces were similar. Unique nucleotide sequence components SN13 and SN14 were found in type X and types I and V, respectively. These two components were first observed in *E. nuttalli* isolates.

### 3.5. Analysis of tRNA-linked STR loci

We also analyzed sequences of tRNA-linked STR loci of the Thailand *E. nuttalli* isolates. The 24 isolates from seven locations showed 8 variations in locus D-A, 8 variations in locus N-K2, 8 variations in locus R-

R, 5 variations in locus $S^{TGA}$-D, 7 variations in locus S-Q, and 10 variations in locus A-L sequence types (Table 3, Fig. 5). These patterns had a highly significant difference in their distributions. By combination of sequences from six loci, the 24 isolates were divided into 14 patterns (Table 2). In addition, there were obvious differences between the Thailand isolates and Chinese isolates in compositions of locus D-A and locus N-K2 (Fig. 5A,B).

### 3.6. Phylogenetic tree analysis

In the distance-based NJ tree obtained using the SRP gene, *E. nuttalli* isolates formed two paraphyletic branches (Fig. 6A). Phetchaburi, Prachuap Khiri Khan, Ratchaburi and Lopburi isolates were categorized into one branch, and Chonburi and Saraburi isolates were categorized in another branch. In the distance-based NJ tree obtained using the tRNA-STR loci, the Thailand *E. nuttalli* isolates formed 8 paraphyletic branches (Fig. 6B). Isolates from six locations were categorized into independent branches, and Chonburi isolates were categorized into two branches. Essentially, the tree mirrored localization of isolation sites on the map shown in Fig. 1. The newly identified sequences have been deposited in the DDBJ/EMBL/GenBank database (LC436927-LC436983).
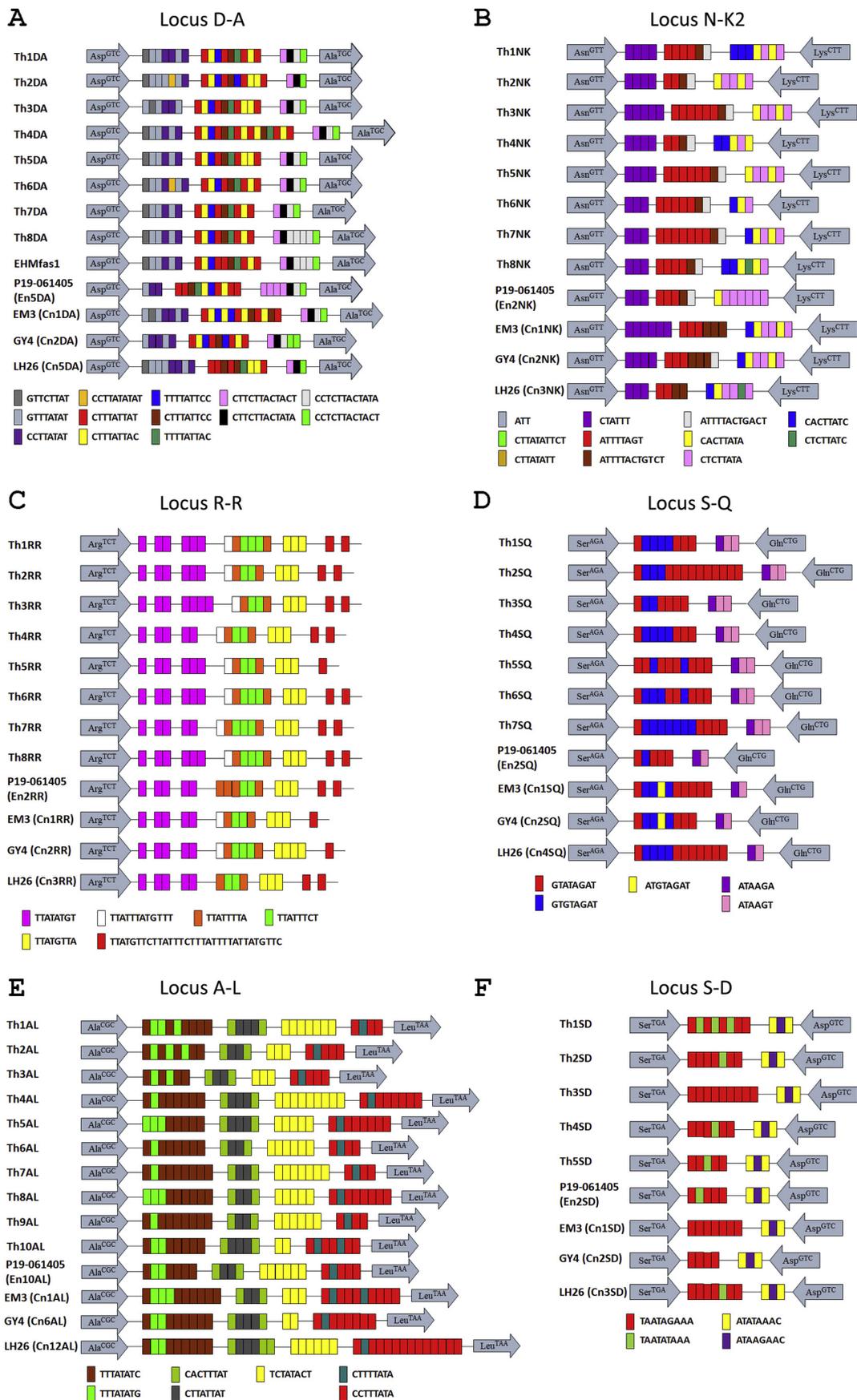
### 3.7. Effect of geographical distance on genetic diversity

The genetic diversity of isolates from different locations were compared with respect to geographical distance to estimate the importance of the geographical pattern for genetic relationships among the Thailand *E. nuttalli* isolates. In analysis of 24 *E. nuttalli* isolates collected from seven locations ranging from 20 km to 286 km apart, a Mantel test (10,000 randomizations) gave r values of 0.815 ($P < 0.001$ in Pearson correlation coefficient analysis) based on the SRP gene (Fig. 7A) and 0.888 ($P < 0.001$) based on tRNA-linked STR loci (Fig. 7B). These results indicate significant isolation by distance for *E. nuttalli* isolates from Central Thailand based on the SRP gene and tRNA-linked STR loci.

### 4. Discussion

This is the first study to show that *E. nuttalli* infection is prevalent in wild long-tailed macaques. The prevalence of *E. nuttalli* in Central Thailand, 43.9%, was comparable with those in wild rhesus macaques in Kathmandu, Nepal (50.9%) and Tibetan macaques in Mount Huang, China (58.3%), and higher than those of toque macaques in Sri Lanka (18.5%) and Tibetan macaques in Mount *E*-Mei, China (16.9%) (Guan et al., 2016; Tachibana et al., 2016; 2013; Wei et al., 2018). Since no infection with *E. histolytica* or *E. dispar* was found in the macaques, long-tailed macaque is thought to be a natural host for *E. nuttalli*. The wild long-tailed macaques infected with *E. nuttalli* did not show diarrhea or bloody stools, similarly to other macaques, which suggests that *E. nuttalli* infections must be commensal for the macaques. The most prevalent *Entamoeba* species in wild long-tailed macaques was *E. chattoni*, followed by *E. coli*. This trend of high prevalence of these two nonpathogenic species is the same as that in other macaques, including captive macaques (Feng et al., 2013; Guan et al., 2016; Tachibana et al., 2013). In contrast to wild long-tailed macaques, a high positive rate of *E. dispar* infection in captive long-tailed macaques in China has been reported (Feng et al., 2013; 2011; Takano et al., 2005). High positive rates of *E. dispar* infection are also present in captive macaques and chimpanzees in Japan (Tachibana et al., 2000; 2001). Therefore, susceptibility to *E. dispar* infection must be high in macaques and other nonhuman primates, but its prevalence in wild macaques is lower.

Sequences of the 18S rRNA gene in *E. nuttalli* isolates from the wild long-tailed macaques were identical with the EHMfas1 strain (AB197936) from a long-tailed macaque exported from China (Takano et al., 2007). Since no wild long-tailed macaques are distributed in

**Table 3**
Genotypes of six tRNA-STR loci of *E. nuttalli* isolates from seven locations in Central Thailand.

| Isolate | Locus | | | | | |
|---|---|---|---|---|---|---|
| | N-K2 | R-R | $S^{TGA}$-D | S-Q | D-A | A-L |
| TFP6 | Th2NK | Th2RR | Th2SD | Th2SQ | Th2DA | Th2AL |
| TFPK43 | Th3NK | Th2RR | Th3SD | Th3SQ | Th1DA | Th2AL |
| TFR74 | Th4NK | Th3RR | Th3SD | Th3SQ | Th3DA | Th3AL |
| TFR79 | Th4NK | Th3RR | Th3SD | Th3SQ | Th3DA | Th3AL |
| TFR101 | Th5NK | Th4RR | Th3SD | Th3SQ | Th3DA | Th3AL |
| TFC115 | Th1NK | Th1RR | Th1SD | Th4SQ | Th1DA | Th4AL |
| TFC120 | Th1NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th1AL |
| TFC121 | Th1NK | Th5RR | Th4SD | Th1SQ | Th4DA | Th4AL |
| TFC122 | Th1NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th1AL |
| TFC126 | Th1NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th4AL |
| TFC128 | Th2NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th4AL |
| TFC137 | Th1NK | Th1RR | Th4SD | Th1SQ | Th1DA | Th1AL |
| TFC138 | Th1NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th1AL |
| TFC142 | Th1NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th1AL |
| TFC145 | Th1NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th1AL |
| TFC148 | Th1NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th1AL |
| TFC149 | Th1NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th1AL |
| TFC155 | Th1NK | Th1RR | Th1SD | Th1SQ | Th1DA | Th1AL |
| TFL197 | Th6NK | Th6RR | Th5SD | Th5SQ | Th5DA | Th5AL |
| TFL203 | Th7NK | Th6RR | Th5SD | Th6SQ | Th6DA | Th6AL, Th7AL |
| TFL205 | Th7NK | Th6RR, Th7RR | Th1SD, Th5SD | Th6SQ | Th6DA, Th7DA | Th5AL, Th8AL, Th9AL |
| TFL206 | Th7NK | Th6RR | Th1SD | Th5SQ | Th5DA | Th7AL, Th9AL |
| TFS228 | Th8NK | Th8RR | Th2SD | Th7SQ | Th8DA | Th10AL |
| TFS240 | Th8NK | Th8RR | Th2SD | Th7SQ | Th8DA | Th10AL |

**Fig. 5.** Schematic representation of genotypes in tRNA-linked short tandem repeats of six loci of *E. nuttalli* isolated from long-tailed macaques in Central Thailand. tRNA genes and STRs are depicted as arrows and rectangles, respectively, while non-tRNA and non-STR regions are shown as lines. Data for *E. nuttalli* isolates from Nepal and China in our previous studies were added to the analysis. (A) Locus D-A; (B) Locus N-K2; (C) Locus R-R; (D) Locus S-Q; (E) Locus A-L; (F) Locus S$^{TGA}$-D.
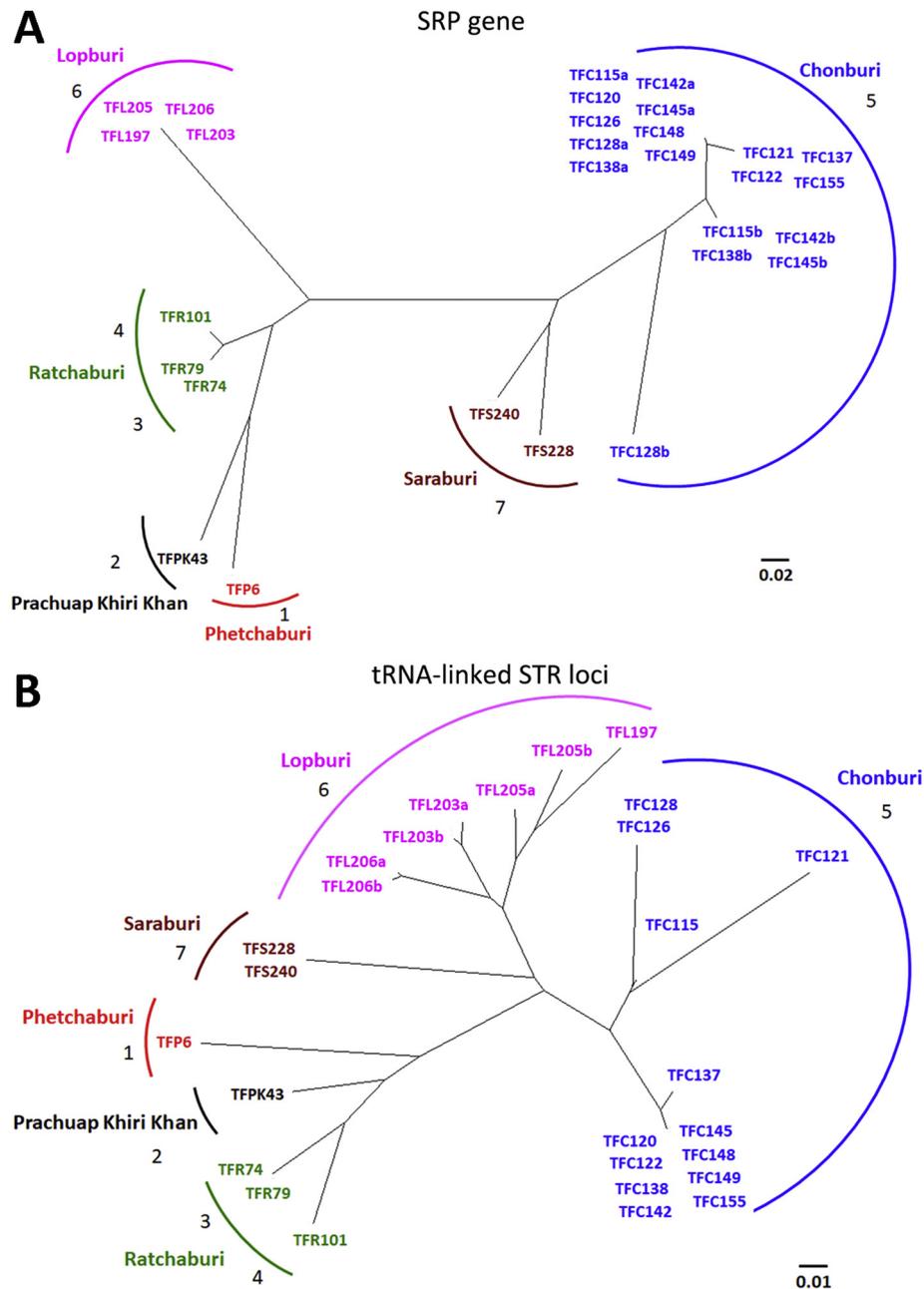
**Fig. 6.** Phylogenetic tree constructed using the NJ method based on serine-rich protein genes (A) and six tRNA-linked STR loci (B) of *E. nuttalli* isolates from Thailand. Numbers 1 to 7 refer to the locations on the map in Fig. 1.

China, the species of macaque in breeding colonies might be derived from neighboring countries such as Vietnam, Laos, Cambodia and Myanmar (Feng et al., 2011). The 18S rRNA gene sequence in long-tailed macaques showed two nucleotide differences in comparison with that from rhesus macaques (AB282657) and may be unique in long-tailed macaques (Tachibana et al., 2007). However, the 18S rRNA sequence from the Thailand isolates was also identical with the NASA06 strain isolated from a captive Japanese macaque (Tachibana et al., 2009). By tracking the origin of the captive Japanese macaques, it became clear that these macaques were derived from a zoo in Japan. Therefore, we cannot exclude the possibility that the NASA06 strain was derived from other species of nonhuman primates, including long-tailed macaque. Studies on *Entamoeba* infections in wild Japanese macaques are in progress to clarify this situation. Transmission of *Entamoeba* spp. to other host species in zoos and facilities is probable (Levecke et al., 2015; Suzuki et al., 2008; 2007).

The SRP gene and tRNA-linked STR loci genotyping system was used to distinguish *E. histolytica* genotypes in previous studies (Ali et al., 2008; 2012; 2007; 2005; Feng et al., 2012; Jaiswal et al., 2014). The present study demonstrated the presence of 11 genotypes of SRP gene and 5 to 10 types in each of six tRNA-linked STR loci in 24 *E. nuttalli* isolates from Central Thailand. In the distance-based NJ based on tRNA-linked STR loci, isolates from seven locations clearly belonged to different clusters, whereas these were divided into two clusters based on SRP gene data. These results illustrate that tRNA-linked STR loci are more effective in distinguishing *E. nuttalli* isolates, compared to SRP genes. This might be due to the higher number of nucleotides in the analysis using tRNA-linked STR loci compared to that using SRP genes. Furthermore, using a combination of SRP gene and tRNA-linked STR loci sequences, the 24 isolates could be divided into 17 genotypes, demonstrating that fingerprinting analysis of the parasite may be possible in the area.
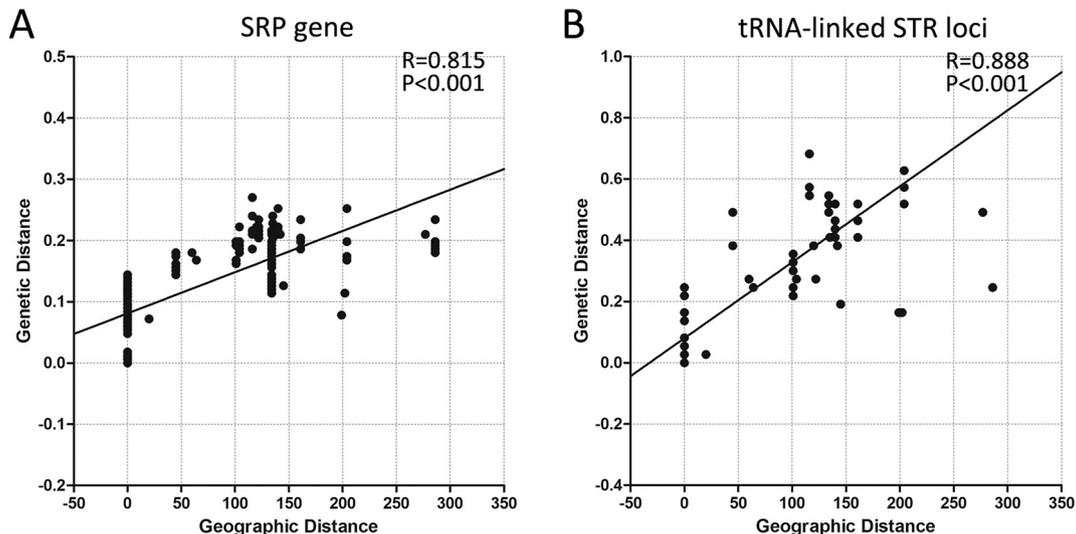
**Fig. 7.** Scatter plots of genetic distance vs. geographic distance comparisons by Mantel test. R values are from the Mantel test and *P* values were calculated using Pearson correlation coefficient analysis. (A) SRP gene. (B) tRNA-linked STR loci.

Distribution of different genotypes in several geographical locations has been observed in rhesus macaques in Nepal and China and in Tibetan macaques in China (Feng et al., 2014; Guan et al., 2016; Tachibana et al., 2013; Wei et al., 2018). The present study based on data from seven locations revealed that the genotypes of *E. nuttalli* isolates correlated phylogenetically with the geographical distribution of host macaques. The r values of 0.888 based on tRNA-STR loci and 0.815 based on SRP genes both indicated that geographic factors are important contributors that could explain the polymorphism of *E. nuttalli* genes. *E. nuttalli* isolates from two close locations in Ratchaburi showed separate genetic features in the NJ tree based on tRNA-STR loci. This observation also suggests that tRNA-linked STR loci are a better marker than SRP genes. Genetic diversity of Nepali isolates and Chinese GY isolates of *E. nuttalli* was also quite prominent (Feng et al., 2014; Guan et al., 2016). Therefore, genotypes of *E. nuttalli* isolates may be polymorphic even if the ameba was isolated from macaques in a close geographical range. Genetic analysis of host macaques was not performed in this study, but it is well known that phylogenetic differences are present in long-tailed macaques (Bunlungsup et al., 2017a; 2017b; Klegarth et al., 2017; Tosi and Coke, 2007).

There are many long-tailed macaques and other species of macaques in Thailand. Some live closely with human communities and food can be given by hand, as shown in Fig. 2. We have recently detected *E. chattoni*, but not *E. nuttalli*, in feces of residents in Kathmandu, Nepal, where rhesus macaques live nearby (Feng et al., 2018). Further screening of *Entamoeba* infections in human and non-human primates, especially in macaques, should be performed because a case of human infection with *E. nuttalli* in a caretaker in zoo has been reported and *E. nuttalli* is potentially pathogenic (Guan et al., 2018; Levecke et al., 2015; Tachibana et al., 2009; 2007).

In conclusion, *E. nuttalli* infection is prevalent even in wild long-tailed macaques in Central Thailand, which shows that this species of macaque is a natural host of the ameba. Divergence and co-evolution of the parasite occurred during dispersion and colonization of the host macaque, and genotypic analysis of the parasite may enable identification of the geographic localization of the host.

## Conflict of interest

All authors declare no conflicts of interest.

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