Original Article

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Correlation of genetic diversity between hosts and parasites in *Entamoeba nuttalli* isolates from Tibetan and rhesus macaques in China

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Summary ***Entamoeba nuttalli* infection is prevalent in captive and wild macaques. Recent studies have suggested that genotypes of *E. nuttalli* isolates are correlated with the geographical distribution of host macaques. Correlation of amoebic genotypes with genetic diversity of host macaques was analyzed in present study. Sixty fresh stool samples were obtained from wild Tibetan macaques living in Mount Huang (HS) of the An-hui Province in China. PCR analysis revealed that the most prevalent *Entamoeba* species was *E. chattoni* (*E. polecki* ST2) (86.7%) followed by *E. nuttalli* (58.3%) and *E. coli* (25%). Six *E. nuttalli* HS isolates were successfully cultured. The tRNA-linked short tandem repeat (STR) loci and serine-rich protein gene of *E. nuttalli* isolates from four different regions of China (Mount Long-hu, Gui-yang, Mount E-mei, and HS, the former three isolates were obtained in previous studies) were studied and high numbers of polymorphisms were detected. When genetic diversity of different populations of *E. nuttalli* isolates was compared with geographical distance, an r^2 value of 0.919 was assigned by a Mantel test based on the tRNA-STR loci. In host macaques, the mtDNA HVS-I gene was also highly polymorphic in each of the genomes. Multiple regression analysis using *E. nuttalli* tRNA-STR loci genetic, macaque mtDNA HVS-I gene, and geographic distances showed an r^2 value of 0.943, indicating that a higher relevance was demonstrated when geographic and host gene factors were considered. Analysis of genetic factor of host would benefit for better understanding of the evolution of *E. nuttalli*.**

Keywords: Amoeba, host gene diversity, geographical distance

1. Introduction

Infection with *Entamoeba nuttalli*, a morphologically indistinguishable *Entamoeba* species from *E. histolytica*, is prevalent in captive and wild macaques (*1-7*). There are numerous wild macaque populations in China, in which the rhesus macaque is widely distributed, but the Tibetan macaque is unique to east central China. Our recent studies have detected *E. nuttalli* infection in wild rhesus and Tibetan macaques in China (*1,8*), indicating that these macaques are also natural hosts of *E. nuttalli*.

In addition to the epidemiology of *E. nuttalli* in non-human primates, genetic diversity of this amoeba also needs to be studied further. To date, several polymorphic markers for genotyping *E. histolytica* isolates have been well established. Of these, the serine-rich protein (SRP) gene was the most widely used genotyping marker for *Entamoeba* isolates (*4,9-15*). tRNA-linked short tandem repeats (STRs) were another effective polymorphic marker (*16*). Recent studies on *E. nuttalli* isolate genotypes have suggested that these

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are correlated with the geographical distribution of host macaques (*8,16*).

In addition to geographical factors, other aspects which could be impacting *E. nuttalli* genetic diversity remain unclear. Given that more information would be advantageous in studying factors contributing to *E. nuttalli* genetic polymorphism. In the present study, we aimed to detect *Entamoeba* spp. infection in another Tibetan macaque population living in Mount Huang (HS) of the An-hui Province in China. Genetic variation of the tRNA-linked STR loci and SRP gene of *E. nuttalli* isolates from Chinese macaques were analyzed.

2. Materials and Methods

2.1. *Sample collection*

Sixty fresh stool samples were obtained from wild Tibetan macaques living in Mount Huang of the An-hui Province in China in May 2016 (Figure 1).

2.2. *Stool examination and culture*

Stool samples, PCR-positive for *E. nuttalli*, were xenically cultured in modified Tanabe-Chiba medium (*17*). In addition, trophozoites of two *E. nuttalli* isolates from Mount Qian-ling (GY) of the Guizhou Province and six *E. nuttalli* isolates from Mount E-mei (EM) of the Si-chuan Province were axenically cultured in YIMDHA-S medium (*1,8*). Trophozoites from 16 *E. nuttalli* isolates from wild rhesus macaques in Mount Long-hu (LH) of the Guanxi Province were xenically cultured in modified Tanabe-Chiba medium. These isolates were used as reference strains.

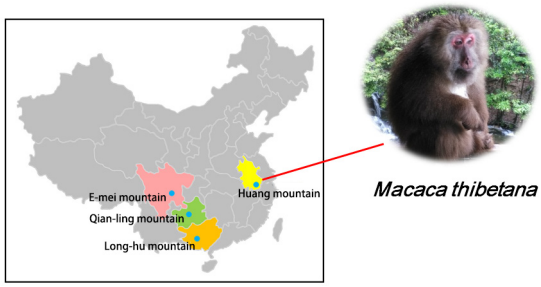

Figure 1. Geographic location of Mount Huang (HS), Mount E-mei (EM), Mount Qian-ling (GY), and Mount Long-hu (LH).

2.3. *Extraction of genomic DNA and PCR amplification*

PCR was used to detect various *Entamoeba* species. Genomic DNA from the 60 stool samples was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Germary) and then PCR with 35 cycles for amplification of partial 18S ribosomal RNA genes from *E. histolytica*, *E. dispar*, *E. nuttalli*, *E. coli*, and *E. chattoni* (*E. polecki* ST2) were performed as described previously (*2,3,18*). Genomic DNA was also extracted from cultured trophozoites using a DNeasy blood and tissue kit (Qiagen, Germany). Genomic DNA isolated from cultured trophozoites was used for amplification of amoeba genes and genomic DNA isolated from *E. nuttalli* positive stool samples were used for amplification of host genes. Complete 18S and 5.8S rRNA genes were amplified as described previously (*2*). The SRP gene and tRNA-linked STR fragments were amplified with Takara *Pyrobest* DNA polymerase from the genomic DNA of trophozoites using primers for five polymorphic STR loci (Locus D-A, N-K2, R-R, S-Q, and S^{TGA}-D) as described previously (*16,19*). The host macaque hypervariable segments I of mitochondrial DNA (mtDNA HVS-I) and partial MHC II DRB1 genes were amplified from the genomic DNA of stool samples using primers listed in Table 1. Briefly, the following PCR conditions were used: denaturation at 94°C for 15 s, annealing for 30 s, and extension at 72 C for 30 s (*Entamoeba* species diagnosis), 3 min (complete 18S and 5.8S rRNA genes), 45 s (five STR loci and SRP gene), or 1 min (mtDNA HVS-I and partial MHC II DRB1 genes). Annealing temperatures for amplifying mtDNA HVS-I and partial MHC II DRB1 genes are shown in Table 1. An initial 3-min denaturation step at 94°C and a final 7-min polymerization step at 72°C were also performed for all reactions.

2.4. *Sequencing and analysis of genes*

PCR products of the 2.4-kb rRNA gene, SRP gene, and five tRNA-linked STR fragments of *E. nuttalli* as well as those of mtDNA HVS-I and partial MHC II DRB1 genes of macaques were purified using an AxyPrep DNA Gel Extraction kit (Axygen, USA) and then subjected to direct sequencing by PCR primers using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The 2.4-kb rRNA gene were completely sequenced by primer walking. PCR products with multiple sequences were processed using a pMD®20-T Vector cloning kit (TaKaRa). More than

Table 1. Primers for macaque genes

Primer	Sequence (5′-3′)	Annealing temperature
Macaca HVS-I-F	CTGAATTGGAAGCGAACC	55°C
Macaca HVS-I-R	CCCGTGATCCATCGAGATGTCTT	
Macaca DRB1-F	CAACCTAAGGTGACTGTGTATC	50°C
Macaca DRB1-R	CACTCCATTCCACTGTGAGAG	

10 clones of each gene were sequenced. Reactions were run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA). Sequence data were analyzed using Bioedit 7.0.

2.5. *Genetic variation and population analyses*

Genetic distances were calculated using the SRP gene or five tRNA-STR loci using MEGA 5 software. Then, a Mantel test for isolation by distance was performed using IBD 1.5.2 software (*20*) with 10,000 randomizations.

A neighbor-joining (NJ) tree was constructed using the SRP gene or five of the tRNA-STR loci by MEGA 5 software.

Analysis and multiple alignments of macaque mtDNA HVS-I and partial MHC II DRB1 genes were performed using Clustal X; phylogenetic trees and genetic distances were constructed using the NJ method.

SPSS 20.0 was used for preparing the 3D spot figure. Haplotype and nucleotide diversities of these gene sequences between different groups were calculated using DnaSPv5 software as described previously (*21*).

3. Results

3.1. *Detection and isolation of Entamoeba spp. in stool samples*

We attepted to detected the five *Entamoeba* species by PCR from all 60 stool samples. *E. chattoni* (86.7%) was the most prevalent species, followed by *E. nuttalli* (58.3%) and *E. coli* (25%) (Figure 2). In addition, mixed infections were detected in more than half of the positive samples (65%). *E. histolytica* and *E. dispar* were not detected by PCR in these samples.

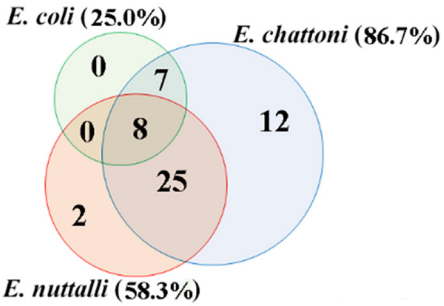

Figure 2. Detailed compositions of *Entamoeba* infections in Tibetan macaques of Mount Huang. The Venn diagram shows quantities of co-infection of *E. nuttalli*, *E. polecki* ST2, and *E. coli* in the stool samples obtained from wild Tibetan macaques of Mount Huang. Each circle represents one *Entamoeba* species. Numbers in an area overlapped by two or three circles indicate the quantity of samples of coinfections, accordingly.

Stool samples were xenically cultured in modified Tanabe-Chiba medium. Six isolates were successfully cultured and designated as follows: HS24, HS25, HS31, HS42, HS48, and HS59.

3.2. *rRNA gene analysis*

The 2.4-kb region containing 18S and 5.8S rRNA genes was amplified by PCR and directly sequenced. Nucleotide sequences of the Mount Huang samples indicated that the isolates were of *E. nuttalli*. Moreover, the nucleotide sequence of the rRNA gene of the isolates obtained from Tibetan macaques in Mount Huang was identical to that the rRNA gene of *E. nuttalli* isolates from wild Tibetan macaques in Mount E-mei and wild rhesus macaques in Mount Qian-ling and Mount Long-hu. The 2.4-kb region containing 18S and 5.8S rRNA genes of these Chinese *E. nuttalli* isolates were completely identical and these isolates exhibited three nucleotide differences compared with the reference strain P19-061405 (AB282657), which was obtained from a wild rhesus macaque in Kathmandu in Nepal (*1,2*).

3.3. *SRP gene and tRNA-linked STR loci typing*

Given that differentiation of STR types are indispensable for high-resolution genotyping of amoebic isolates, we analyzed the SRP gene and tRNA-linked STRs of Chinese *E. nuttalli* isolates collected from two *Macaca* species at diverse geographic locations (Table 2). Six isolates from the Tibetan macaques collected from Mount Huang shared identical STR sequence types for the SRP gene and five tRNA-linked STRs loci (Figure 3A to 3E). The newly identified sequences were deposited in the DDBJ/EMBL/GenBank database (LC379241-LC379247).

3.4. *NJ tree of E. nuttalli isolates*

Table 2. Characteristics of *E. nuttalli* isolates using SRP and tRNA-STR markers

Isolate	No. of isolates	Host macaque species	Location of collection	Genotypes of SRP gene	Genotypes of tRNA-STRs				
					N-K2	S-Q	S^{TGA}-D	R-R	D-A
EM	6	*M. thibetana*	E-mei mountain	Cn1SRP	Cn1NK	Cn1SQ	Cn1SD	Cn1RR	Cn1DA
GY	2	*M. mulatta*	Qian-ling mountain	Cn2SRP	Cn2NK	Cn2SQ	Cn2SD	Cn2RR	Cn2DA
				Cn3SRP	Cn2NK	Cn3SQ	Cn2SD	Cn2RR	Cn3DA
LH	16	*M. mulatta*	Long-hu mountain	Cn4SRP	Cn3NK	Cn4SQ	Cn3SD	Cn3RR	Cn5DA
				Cn5SRP					
HS	6	*M. thibetana*	Huang mountain	Cn6SRP	Cn4NK	Cn5SQ	Cn4SD	Cn4RR	Cn6DA

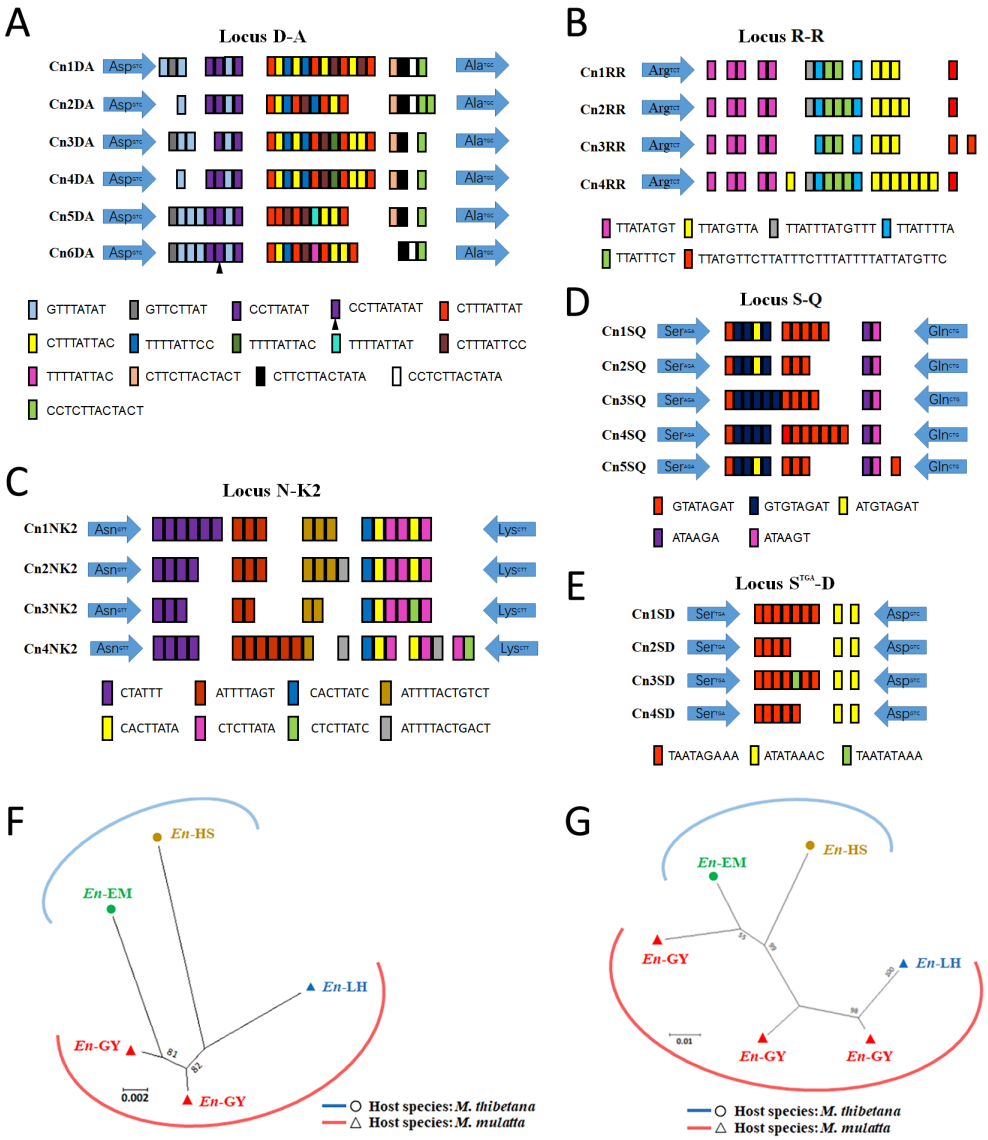

Figure 3. Schematic representation of tRNA-linked short tandem repeat types of each loci based on the nucleotide sequence and phylogenetic tree constructed using the NJ method of *E. nuttalli* isolates. Schematic representation of Locus D-A **(A)**, R-R **(B)**, N-K2 **(C)**, S-Q **(D)**, and STGA-D **(E)**. tRNA genes and STRs are depicted as arrows and rectangles, respectively. Diagrams and sequence types are based on the study of Ali *et al*. (*34*). **(F)** NJ tree based on the tRNA-STR loci of *Entamoeba* species. **(G)** NJ tree based on the SRP gene of *Entamoeba* species

In the distance-based NJ tree obtained using the tRNA-STR loci (Figure 3F), *E. nuttalli* isolates were presented into five paraphyletic branches. HS, EM, and LH isolates were categorized into an independent branch and GY isolates were categorized into two branches. In the distance-based NJ tree obtained using the SRP gene (Figure 3G), *E. nuttalli* isolates were represented by six paraphyletic branches. HS and EM isolates were categorized into an independent branch, whereas GY and LH isolates were categorized within two branches.

3.5. *Effect of geographical distance on genetic diversity*

The genetic diversity of different populations was compared with respect to geographical distance to estimate the importance of the geographical pattern for genetic relationships among the four *E. nuttalli* populations. Chinese *E. nuttalli* isolates were collected from four neighboring provinces (Figure 1). Results of the Mantel test gave an r^2 value of 0.919 ($P < 0.05$, for 10,000 randomizations) based on the tRNA-STR loci and an r^2 value of 0.88 ($P < 0.05$, for 10,000 randomizations) based on the SRP gene. These results indicated that a significant isolation by the distance effect was found in the populations based on the tRNA-STR loci.

3.6. *Analysis of host genetic variation*

The macaque mtDNA HVS-I and partial MHC II DRB1 genes were highly polymorphic in each of the genomes. In the distance-based NJ tree obtained using the mtDNA HVS-I gene (Figure 4A), different macaque populations were represented by six paraphyletic branches. HS and

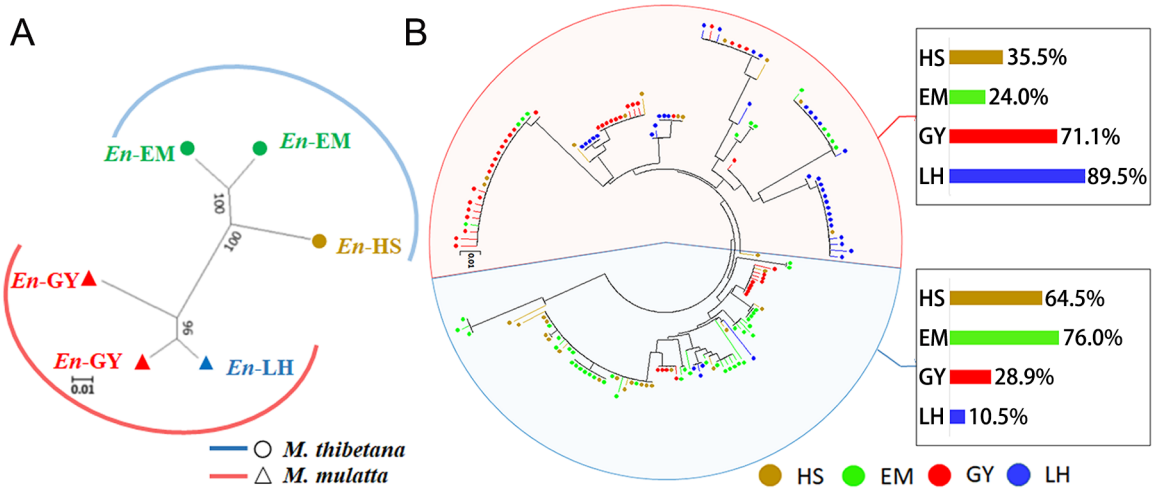

Figure 4. Phylogenetic tree constructed using the NJ method for host macaques. (A) NJ tree based on the mtDNA HVS-I gene of host macaques. **(B)** NJ tree based on the partial MHC II DRB1 gene of host macaques. Pie chart displaying the percentage of each population in the upper and lower clusters.

LH macaques were categorized into an independent branch, whereas EM and GY macaques were categorized into two branches. In the distance-based NJ tree obtained using the partial MHC II DRB1 gene (Figure 4B), different macaque populations were represented by two large clusters. The upper semi-cluster contained 89.5%, 71.1%, 35.5%, and 24% alleles of LH, GY, HS, and EM macaques, respectively, and the lower half cluster contained 76%, 64.5%, 28.9%, and 10.5% alleles of EM, HS, GY, and LH macaques, respectively. The results indicated that most alleles of LH and GY macaques were located in the upper semi-cluster and that most of the alleles of the EM and HS macaques were located in the lower half cluster. The results suggested that HVS-I gene could distinguish the four regions of macaques better than MHC II DRB1 gene. The newly identified sequences were deposited in the DDBJ/EMBL/GenBank database (LC379248- LC379253).

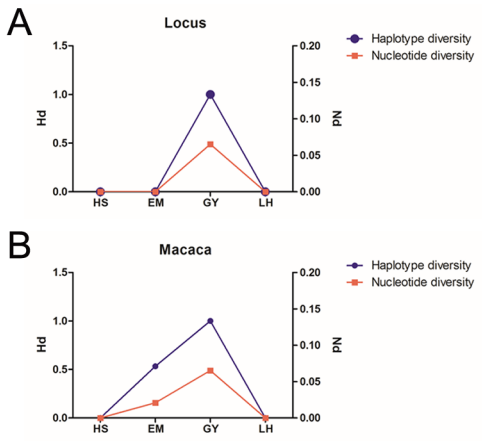

Figure 5. Gene diversity of *E. nuttalli* isolates and the macaque. (A) Haplotype and nucleotide diversities of the tRNA-STR loci of *E. nuttalli* isolates. **(B)** Haplotype and nucleotide diversities of mtDNA HVS-I gene of macaques.

3.7. *Effect of macaque gene diversity on E. nuttalli genetic diversity*

Quantifiable data revealed both high haplotype (1.0) and nucleotide diversities (0.065) of tRNA-STR loci sequences from GY isolates (Figure 5A). The mtDNA HVS-I gene of macaques infected with GY isolates also indicated high haplotype (1.0) and nucleotide diversities (0.06) (Figure 5B). GY macaques and *E. nuttalli* GY isolates both had the highest genetic diversity among the four Chinese populations. The haplotype diversity and nucleotide diversity of the MHC II DRB1 gene were also calculated. But the results suggested that the MHC II DRB1 gene was too polymorphism to distinguish difference among the four regions of macaques in the present study.

Multiple regression analysis using *E. nuttalli* tRNA-STR loci genetic, macaque gene, and geographic distances indicated an r^2 value of 0.943 ($P < 0.05$, 10,000 randomizations). The results indicated that a higher relevance was shown when both geographic and host gene factors were considered.

4. Discussion

Recent studies have demonstrated high polymorphism among *E. nuttalli* SRP gene and tRNA-linked STR from Nepalese, Japanese, and Chinese isolates (*1-3,6,22*). The present study also demonstrated the presence of multiple genotypes in *E. nuttalli* isolates from different macaque populations. However, factors affecting polymorphism of *E. nuttalli* genes remained largely unknown. The current study attempted to provide insights into this amoeba gene polymorphism.

It has been demonstrated that genetic diversity of Chinese *E. nuttalli* isolates is associated with geographic distance based on the tRNA-STR loci (*8*). The importance of the geographic factor was proved by investigation of other *E. nuttalli* isolates in Nepal (*16*). In present study, a Mantel test indicated similar results when

the Mount Huang population was included. Geographic factors were regarded as important contributors that could explain polymorphism of *E. nuttalli* genes. However, there were exceptions, as GY isolates included in present study demonstrated separate genetic features in the NJ tree. In the distance-based NJ tree, differences in genetic distance among *E. nuttalli* HS, EM, GY, and LH isolates were demonstrated. *E. nuttalli* genes from four macaque populations could be divided into five branches, with only GY isolates being attributed to two different branches. Genetic diversity in *E. nuttalli* GY isolates was quite prominent even if the amoeba was isolated from macaques in a very close geographical range. Therefore, we could not exclude the possibility that other factors may also affect genetic polymorphism of *E. nuttalli* isolates.

Host genetic diversity or pathogen-host co-evolution have been considered as critical factors affecting pathogen genetic diversity in a number of studies (23-28). Accordingly, the host macaque species and host genetic diversity could be important factors affecting *E. nuttalli* genetic diversity. Both rhesus and Tibetan macaques were included in present study, and all the samples were examined by DNA sequence analysis to verify that they were of *Macaca* species origin. Furthermore, the hypervariable region of host mtDNA and MHC II DRB1 genes were first used to determine the possibility that the host species was correlated with the genetic diversity of the amoeba. The results indicated that mtDNA HVS-I gene was compatible for distinguishing between the four Chinese macaque populations. GY macaques contained genetic features of two clusters in the NJ tree. The high haplotype and nucleotide diversity of GY macaques was also the highest among all populations. Results were similar to the *E. nuttalli* genetic diversity of GY isolates. This may indicate that host genetic diversity could affect parasite genetic diversity and explain *E. nuttalli* genetic diversity of GY isolates.

The host factor was probably non-negligible in the study on *E. nuttalli*. Host specificity has already been proved to be an important subject in the epidemiology of *Entamoeba* (*29,30*). In present study, a further analysis including amoebic genetic distance, geographic distance, and host genetic distance was performed. The result gave an r^2 value of 0.943, which was higher than that obtained with the Mantel test (0.919). This higher r^2 value may have demonstrated a more reasonable fitting method to analyze *E. nuttalli* genetic features. The genetic variation of the tRNA-linked STR loci of *E. nuttalli* isolates from Chinese macaques may be partially attributable to both host geographic locations and host genetic variation.

Genetic differences between the two different macaque species included in present study could not be ignored; however, the Tibetan macaque, a unique non-human primate in China, was identified as a closely related species to the rhesus macaque (*31,32*). A more in-depth study involving the same species of macaque to evaluate the relationship between host genetic variation and *E. nuttalli* genetic variation would be more powerful.

Both the SRP gene and the tRNA-linked STR genotyping systems have been commonly used to distinguish *E. histolytica* genotypes (*10,19,33-36*). In the five tRNA-STR loci distance-based NJ tree, HS isolates were attributed to a slightly further branch to distinguish it from the *E. nuttalli* EM and GY isolates. However, data based on the SRP gene showed slightly different results. *E. nuttalli* HS isolates were attributed to a branch closer to the EM isolates than to the GY isolates. These results suggest that genetic evolution of SRP gene has a little differences with tRNA-STR loci. Furthermore, the tRNA-STR loci had more relativity than the SRP gene when used in the Mantel test. In summary, analysis in present study was focused on data of the tRNA-STR loci of Chinese *E. nuttalli* isolates. In comparison with single-genes analysis, polygenic analysis would be better for obtaining more credible results.

In conclusion, the current study demonstrated high positive rates of *E. nuttalli* infection in Chinese macaques. These data showed that different macaque species in China were susceptible to *E. nuttalli* infection. In addition, *E. coli* and *E. chattoni* infections were commonly detected in these macaques. Present study also demonstrated that *E. nuttalli* HS isolates had unique genetic differences from other Chinese isolates based on tRNA-linked STR loci and the SRP gene. Additionally, analysis of genetic factor of host would benefit for better understanding of the evolution of *E. nuttalli*.

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References

1. Feng M, Cai J, Min X, Fu Y, Xu Q, Tachibana H, Cheng X. Prevalence and genetic diversity of *Entamoeba* species infecting macaques in southwest China. Parasitol Res. 2013; 112:1529-1536.
2. Tachibana H, Yanagi T, Pandey K, Cheng X, Kobayashi S, Sherchand J, Kanbara H. An *Entamoeba* sp. strain isolated from rhesus monkey is virulent but genetically different from *Entamoeba histolytica*. Mol Biochem Parasitol. 2007; 153:107-114.
3. Tachibana H, Yanagi T, Akatsuka A, Kobayashi S, Kanbara H, Tsutsumi V. Isolation and characterization of a potentially virulent species *Entamoeba nuttalli* from captive Japanese macaques. Parasitology. 2009; 136:1169-1177.
4. Tachibana H, Yanagi T, Lama C, Pandey K, Feng M, Kobayashi S, Sherchand J. Prevalence of *Entamoeba nuttalli* infection in wild rhesus macaques in Nepal and characterization of the parasite isolates. Parasitol Int. 2013; 62:230-235.
5. Tachibana H, Yanagi T, Feng M, Bandara K, Kobayashi

S, Cheng X, Hirayama K, Rajapakse R. Isolation and molecular characterization of *Entamoeba nuttalli* strains showing novel isoenzyme patterns from wild toque macaques in Sri Lanka. J Eukaryot Microbiol. 2016; 63:171-180.
6. Takano J, Tachibana H, Kato M, Narita T, Yanagi T, Yasutomi Y, Fujimoto K. DNA characterization of simian *Entamoeba histolytica*-like strains to differentiate them from *Entamoeba histolytica*. Parasitol Res. 2009; 105:929-937.
7. Vlčková K, Kreisinger J, Pafčo B, Čížková D, Tagg N, Hehl A, Modrý D. Diversity of *Entamoeba* spp. in African great apes and humans: An insight from Illumina MiSeq high-throughput sequencing. Int J Parasitol. 2018; S0020-7519:30055-9.
8. Guan Y, Feng M, Cai J, Min X, Zhou X, Xu Q, Tan N, Cheng X, Tachibana H. Comparative analysis of genotypic diversity in *Entamoeba nuttalli* isolates from Tibetan macaques and rhesus macaques in China. Infect Genet Evol. 2016; 38:126-131.
9. Feng M, Yang B, Yang L, Fu Y, Zhuang Y, Liang L, Xu Q, Cheng X, Tachibana H. High prevalence of *Entamoeba* infections in captive long-tailed macaques in China. Parasitol Res. 2011; 109:1093-1097.
10. Feng M, Cai J, Yang B, Fu Y, Min X, Tachibana H, Cheng X. Unique short tandem repeat nucleotide sequences in *Entamoeba histolytica* isolates from China. Parasitol Res. 2012; 111:1137-1142.
11. Fu Y, Nagakura K, Cheng X, Tachibana H. Comparison of serine-rich protein genes of *Entamoeba histolytica* isolates obtained from institutions for the mentally retarded in Kanagawa and Shizuoka Prefectures, Japan. Parasitol Res. 2010; 107:999-1002.
12. Haghighi A, Kobayashi S, Takeuchi T, Masuda G, Nozaki T. Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. J Clin Microbiol. 2002; 40:4081-4090.
13. Haghighi A, Kobayashi S, Takeuchi T, Thammapalerd N, Nozaki T. Geographic diversity among genotypes of *Entamoeba histolytica* field isolates. J Clin Microbiol. 2003; 41:3748-3756.
14. Haghighi A, Rasti S, Nazemalhosseini Mojarad E, Kazemi B, Bandehpour M, Nochi Z, Hooshyar H, Rezaian M. *Entamoeba dispar*: Genetic diversity of Iranian isolates based on serine-rich *Entamoeba dispar* protein gene. Pak J Biol Sci. 2008; 11:2613-2618.
15. Zaki M, Meelu P, Sun W, Clark C. Simultaneous differentiation and typing of *Entamoeba histolytica* and *Entamoeba dispar*. J Clin Microbiol. 2002; 40:1271-1276.
16. Feng M, Komiyama T, Yanagi T, Cheng X, Sherchand J, Tachibana H. Correlation between genotypes of tRNA-linked short tandem repeats in *Entamoeba nuttalli* isolates and the geographical distribution of host rhesus macaques. Parasitol Res. 2014; 113:367-374.
17. Yoshimura M, Ebukuro M. A modified Tanabe-Chiba medium for detection of *Entamoeba histolytica*. Jikken Dobutsu. 1988; 37:321-324. (in Japanese)
18. Verweij J, Polderman A, Clark C. Genetic variation among human isolates of uninucleated cyst-producing *Entamoeba* species. J Clin Microbiol. 2001; 39:1644-1646.
19. Ali I, Zaki M, Clark C. Use of PCR amplification of tRNA gene-linked short tandem repeats for genotyping *Entamoeba histolytica*. J Clin Microbiol. 2005; 43:5842-5847.
20. Jensen J, Bohonak A, Kelley S. Isolation by distance, web service. BMC Genet. 2005; 6:13.
21. Liu J, Feng M, Wang X, Fu Y, Ma C, Cheng X. Unique *Trichomonas vaginalis* gene sequences identified in multinational regions of Northwest China. Biosci Trends. 2017; 11:303-307.
22. Dong H, Li J, Qi M, Wang R, Yu F, Jian F, Ning C, Zhang L. Prevalence, molecular epidemiology, and zoonotic potential of *Entamoeba* spp. in nonhuman primates in China. Infect Genet Evol. 2017; 54:216-220.
23. Brites D, Gagneux S. The nature and evolution of genomic diversity in the *Mycobacterium tuberculosis* complex. Adv Exp Med Biol. 2017; 1019:1-26.
24. Cacciò S, Lalle M, Svärd S. Host specificity in the *Giardia duodenalis* species complex. Infect Genet Evol. 2017; S1567-1348:30418-5.
25. Kabamba E, Tuan V, Yamaoka Y. Genetic populations and virulence factors of *Helicobacter pylori*. Infect Genet Evol. 2018; 60:109-116.
26. Latinne A, Bezé F, Delhaes L, Pottier M, Gantois N, Nguyen J, Blasdell K, Dei-Cas E, Morand S, Chabé M. Genetic diversity and evolution of *Pneumocystis* fungi infecting wild Southeast Asian murid rodents. Parasitology. 2017; 9:1-16.
27. Le Bailly M, Maicher C, Dufour B. Archaeological occurrences and historical review of the human amoeba, *Entamoeba histolytica*, over the past 6000 years. Infect Genet Evol. 2016; 42:34-40.
28. Scarborough J, Paul J, Spencer J. Evolution of the ability to modulate host chemokine networks via gene duplication in human cytomegalovirus (HCMV). Infect Genet Evol. 2017; 51:46-53.
29. Wilson I, Weedall G, Hall N. Host-Parasite interactions in *Entamoeba histolytica* and *Entamoeba dispar*: What have we learned from their genomes? Parasite Immunol. 2012; 34:90-99.
30. Ximenez C, Moran P, Rojas L, Valadez A, Gomez A. Reassessment of the epidemiology of amebiasis: State of the art. Infect Genet Evol. 2009; 9:1023-1032.
31. Li Q, Zhang Y. Phylogenetic relationships of the macaques (Cercopithecidae: Macaca), inferred from mitochondrial DNA sequences. Biochem Genet. 2005; 43:375-386.
32. Yan X, Li A, Zeng L, Cao Y, He J, Lv L, Sui L, Ye H, Fan J, Cui X, Sun Z. Identification of MHC class I sequences in four species of Macaca of China. Immunogenetics. 2013; 65:851-859.
33. Ali I, Clark C, Petri W. Molecular epidemiology of amoebiasis. Infect Genet Evol. 2008; 8:698-707.
34. Ali I, Haque R, Alam F, Kabir M, Siddique A, Petri W. Evidence for a link between locus R-R sequence type and outcome of infection with *Entamoeba histolytica*. Clin Microbiol Infect. 2012; 18:E235-237.
35. Ali I, Mondal U, Roy S, Haque R, Petri W, Clark C. Evidence for a link between parasite genotype and outcome of infection with *Entamoeba histolytica*. J Clin Microbiol. 2007; 45:285-289.
36. Jaiswal V, Ghoshal U, Mittal B, Dhole T, Ghoshal U. Association between allelic variation due to short tandem repeats in tRNA gene of *Entamoeba histolytica* and clinical phenotypes of amoebiasis. Acta Trop. 2014; 133:1-7.

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