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ORIGINAL PAPER

Molecular phylogeny of *Blastocystis* isolates from wild rodents captured in Indonesia and Japan

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Abstract

Blastocystis sp. is a common intestinal protist found worldwide in a variety of animals, including humans. Currently, 17 subtypes (STs) of *Blastocystis* isolates from mammalian and avian host species have been reported based on the small subunit ribosomal RNA gene (SSU rDNA). Among these, human *Blastocystis* were only identified among STs 1–9. Except ST9, all other STs comprised isolates from humans and other animal species. Entire sequence data of the SSU rDNA of nine *Blastocystis* isolates from laboratory rats or guinea pigs previously showed ST4, whereas *Blastocystis* isolates from wild rodents have not been addressed genetically. In this study, *Blastocystis* infection in wild rodents was surveyed in Indonesia and Japan, and 11 and 12 rodent *Blastocystis* parasites were obtained from *Rattus exulans* and *R. novercious*, respectively. All new *Blastocystis* isolates from wild rodents were identified as ST4 based on the SSU rDNA sequences. The best tree inferred with the entire sequences of the SSU rDNA of all ST4 isolates including 17 data registered in GenBank clearly showed monophyletic ST4A and ST4B clades. Although ST4 isolates from laboratory rats were separated into these two clades, all *Blastocystis* isolates from wild rodents in the present study were positioned into the clade ST4A and further separated into two sub-clusters within the clade ST4A according to the location of the host species. Considering the fact that laboratory rats were susceptible to both ST4A and ST4B, separation of the monophyletic sub-clusters of *Blastocystis* isolates from Indonesian Polynesian rats and Japanese brown rats may indicate the presence of geographical variations rather than a host-specific separation. In either way, the robust host preference to rodent species of ST4 *Blastocystis* was also confirmed.

Keywords *Blastocystis* · Rodents · Phylogeny · SSU rRNA gene · Subtype

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Introduction

Blastocystis is an anaerobic eukaryotic unicellular organism that infects the caecum and large intestine of a variety of animals including humans. Currently, *Blastocystis* species have been isolated from vertebrates including mammals, birds, reptiles, and amphibians, and rarely from invertebrates such as cockroaches (Yamada et al. 1987; Teow et al. 1992; Zaman et al. 1993; Quílez et al. 1995; Chen et al. 1997; Duda et al. 1998; Lee and Stenzel 1999; Abe et al. 2002; Yoshikawa et al. 2004a). Since these isolates are morphologically indistinguishable, genetic differences among *Blastocystis* isolates have been analyzed using various molecular techniques, including arbitrary primer polymerase chain reaction, restriction fragment length polymorphism, or sequencing of the partial or complete length of the small subunit ribosomal RNA gene (SSU rDNA) (Yoshikawa et al. 1996; Böhm-Gloning et al. 1997; Clark 1997; Snowden et al. 2000; Arisue et al. 2003;

Yoshikawa et al. 2004b). These studies revealed extensive genetic polymorphism among *Blastocystis* isolates, whereas genetically same or quite similar isolates exist within both humans and other animals (Yoshikawa et al. 2004c). Thus, a consensus terminology for *Blastocystis* isolates from avian and mammalian host species including humans has been proposed as *Blastocystis* sp. subtypes (STs) (Stensvold et al. 2007). This terminology was based on the phylogenetic interference among nine monophyletic clades inferred using SSU rDNA. All human *Blastocystis* isolates have been classified into these nine STs which comprise human and animal *Blastocystis* isolates except for ST9. Among ST1 to ST8, each ST comprises different animal species in addition to humans, and *Blastocystis* isolates from the same animal species also show different STs. Although the total number of ST4 *Blastocystis* isolates registered within GenBank is small, all complete sequence data for the SSU rDNA from rodent *Blastocystis* isolates in GenBank are limited to ST4. These rodent *Blastocystis* include two isolates from the USA, one isolate from France, three isolates from Singapore, and one isolate from Japan. These data are few and limit the evaluation of host specificity of *Blastocystis* ST4 in rodents. Therefore, the present study surveyed *Blastocystis* infection in wild rodents from Indonesia and Japan. The phylogenetic position of rodent *Blastocystis* isolates in this study was then inferred using the complete SSU rDNA sequences with other ST4 isolates from GenBank with ST8 used as an outgroup, because ST8 is the closest clade among nine STs (Allafilani et al. 2013; Yoshikawa et al. 2016a).

Materials and methods

Ethical considerations

Ethical review was performed and approval for this study in Indonesia was granted by the Ethics Committee of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. On the other hand, wild rodents captured in Nishinomiya city and Tennoji city were considered as pest control under the control of the Environmental Hygiene Division in Nishinomiya city and Osaka Municipal Tennoji Zoological Gardens, respectively.

Sources and isolation of *Blastocystis* from wild rodents

Polynesian rats (*Rattus exulans*) were captured using mouse traps at resident houses from 2009 to 2012 at the Wainyapu area, Southwest Sumba District, East Nusa Tenggara Province, Indonesia as described previously (Yoshikawa et al. 2016b). Wild brown rodents (*Rattus novercious*) were captured using traditional wire-gagged rat traps set at two different locations in Nishinomiya city, Hyogo Prefecture, and in Tennoji city, Osaka Prefecture. The former traps were set in sewers, whereas the latter ones were set within the Osaka Municipal Tennoji Zoological Gardens from 2012 to 2014. The captured wild rats were euthanized and the caecal contents of the rodents were cultured in a liquid medium (Yoshikawa et al. 2004a) in 2.0 ml microtubes or in 15 ml culture tubes at 37 °C. After 3 to 4 days of cultures, the sediments were observed by standard light microscopy and examined for the presence of *Blastocystis*. When typical vacuolar or granular forms of *Blastocystis* were observed, they were subcultured once into a new medium. The *Blastocystis* suspensions were then centrifuged at 3000×*g* for 1 min and the total DNA of the pellets was extracted using DNAzol® reagent according to the manufacturer's protocol (Invitrogen™ Life Technologies, CA, USA). In total, 11 and 12 *Blastocystis* isolates were obtained from 67 wild rodents in Indonesia and 48 rodents in Japan, respectively. The concentration of the extracted DNA was adjusted to 5 μg/ml based on values from a Qubit® 2.0 fluorometer (Invitrogen™ Life Technologies, CA, USA) and preserved at − 20 °C until use.

PCR amplification and sequencing of SSU rDNA

The entire SSU rDNA sequence was amplified as fragments using the following primer pairs of the SR primer series: 1F/618R, 539F/1149R, and 1089F/1R constructed from the conserved regions of SSU rDNA sequences (Table 1) (Yoshikawa and Iwamasa 2016). PCR was performed using high-fidelity polymerase, KOD-Plus-Ver. 2 (TOYOBO Co. Ltd., Osaka, Japan). The PCR profile consisted of an initial denature at 94 °C for 3 min, followed by 35 cycles at 98 °C for 10 s, 54 °C for 30 s, and 68 °C for 45 s, with a final extension at 68 °C for 7 min. After purification of the PCR products, the amplicons were subjected to dA-addition using an A-attachment mix included in a kit of Mighty TA-cloning Reagent Set for PrimeSTAR® (TaKaRa Bio Inc., Shiga, Japan). The modified amplicons were then cloned using pMD20-T vector and *Escherichia coli* JM109 (TOYOBO

Table 1 Primer sequences used in this study

Primer[a]	Sequence
1F	5′-GCTTATCTGGTTGATCCTGCCAGT-3′
539F	5′-AAGTCTGGTGCCAGCAGCC-3′
1089F	5′-GAGTATGGTCGCAAGGCTGAA-3′
618R	5′-CAACTACGAGCTTTTTAACT GCAAC-3′
1149R	5′-CTCCACTCCTGGTGGTGCC-3′
1R	5′-TTGATCCTTCCGCAGGTTCACCTA-3′

[a] *F*, forward; *R*, reverse

Co. Ltd., Osaka, Japan) following the manufacturer's instruction of the above kit. In this study, at least five clones were randomly chosen from each sample and the insertion size was confirmed by electrophoresis. Plasmid DNA was purified using a FastGene™ Plasmid Mini Kit (Nippon Genetics Co. Ltd., Tokyo, Japan). Sequencing of the plasmid DNA was performed using a BigDye® Terminator v3.1. Cycle Sequencing Kit (Life Technologies Japan, Tokyo, Japan) on an ABI 3130 sequencer. Both strands were sequenced and ambiguous nucleotides were confirmed by additional sequences from different clones. Sequence data for 23 new rodent isolates were deposited in GenBank (MH127488-MH127500).

Phylogenetic analysis

The sequences obtained in the present study along with all other complete sequences of ST4 *Blastocystis* isolates registered in GenBank and the outgroup ST8 isolates were used for phylogenetic analysis (Table 2). ST8 was previously confirmed as the closest clade to ST4 (Allafilani et al. 2013; Yoshikawa et al. 2016a) (Table 2). Sequence alignment was conducted using the alignment tool MUSCLE implemented in MEGA6 (Tamura et al. 2013). The alignment was edited manually to remove regions of ambiguity. Subsequently, 1683 substituted positions in these sequences were applied to the final phylogenetic analyses using the neighbor-joining (NJ) and maximum likelihood (ML) algorithms implemented in MEGA6. On the ML algorithm, conducted based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985), the best model was selected by ModelTest, implemented in MEGA6. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining (NJ) method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete gamma distribution was used to model the evolutionary rate differences among sites [8 categories (+G, parameter = 0.1415)]. The best tree with the highest log likelihood was presented along with the bootstrap proportions (BP, 1000 replicates), whereas less than 50% BP was not shown. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 5% site coverage were eliminated. That is, fewer than 95% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Results and discussion

Currently, up to 17 STs of *Blastocystis* organisms have been identified from homoiotherm (warm blood) animals including

Table 2 SSU rDNA sequences of *Blastocystis* ST4 and ST8 used for phylogenetic analysis

ST	Isolate/ strain	Host	Country isolated	Accession no. in GenBank
4	HG00-10	Human	Germany	AY244619
4	HG00-12	Human	Germany	AY244620
4	HJ01-7	Human	Japan	AY244621
4	DMP02-212	Human	Denmark	JN682513
4	AS1	Monkey	Colombia	KF002511
4	AS2	Monkey	Colombia	KF002512
4	MKJ04-19	Kangaroo	Japan	EU427516
4	MKJ04-20	Kangaroo	Japan	EU427517
4	WR1	Wistar rat	Singapore	AY590113
4	WR2	Wistar rat	Singapore	AY590114
4	S1	Sprague-Dawley rat	Singapore	AY590111
4	RN94-9[a]	Brown Norway rat	Japan	AB071000
				AB091251
4	No name[a]	Rat	France	AY135407
				AY135408
4	NIH	Guinea pig	USA	U51152
4	No name	Guinea pig	USA	U26177
8	MJ99-132	Lemur	Japan	AB107970
8	BJ99-319	Great argus	Japan	AB107970
8	Dm15	Opossum	Colombia	KF002524

[a] Two clone sequences are reported from an isolate in GenBank

mammalian and avian hosts, while human *Blastocystis* organisms are only limited to STs 1–9 (Stensvold et al. 2007; Alafillani et al. 2013a). Among these STs, multiple genetically distinct STs are commonly found from the same animal species. In rodent, however, all seven isolates from laboratory rats (*Rattus norvegicus*) and guinea pigs (*Cavia porcellus*) in worldwide only showed ST4. It is not clear whether this is due to a small number of the isolates or whether the rodent infects only the ST4. In this study, therefore, we investigated *Blastocystis* organisms from wild rodents in Indonesia and Japan, respectively.

In this study, 11 and 12 complete sequences of *Blastocystis* SSU rDNA were obtained from wild rodents of the Japanese *R. novercious* (brown rats) and Indonesian *R. exulans* (Polynesian rats), respectively. Phylogenetic analyses using these 23 new sequences with 17 references of ST4 revealed the presence of two monophyletic clades (ST4A and ST4B) within ST4 (Fig. 1). Both ST4A and ST4B clades were statistically supported by the bootstrap values of NJ/ML as 87/99% and 100/100%, respectively. All the new wild rodent *Blastocystis* isolates confirmed in this study were clustered into clade ST4A with several reference ST4 sequences. On the other hand, the other 11 ST4 reference sequences were clustered into another monophyletic clade, ST4B (Fig. 1). Interestingly, the *Blastocystis* isolates from wild Polynesian rats captured in Indonesia and the ones from wild brown rats captured in Japan were addressed into each independent subcluster. Although it was not statistically supported, the

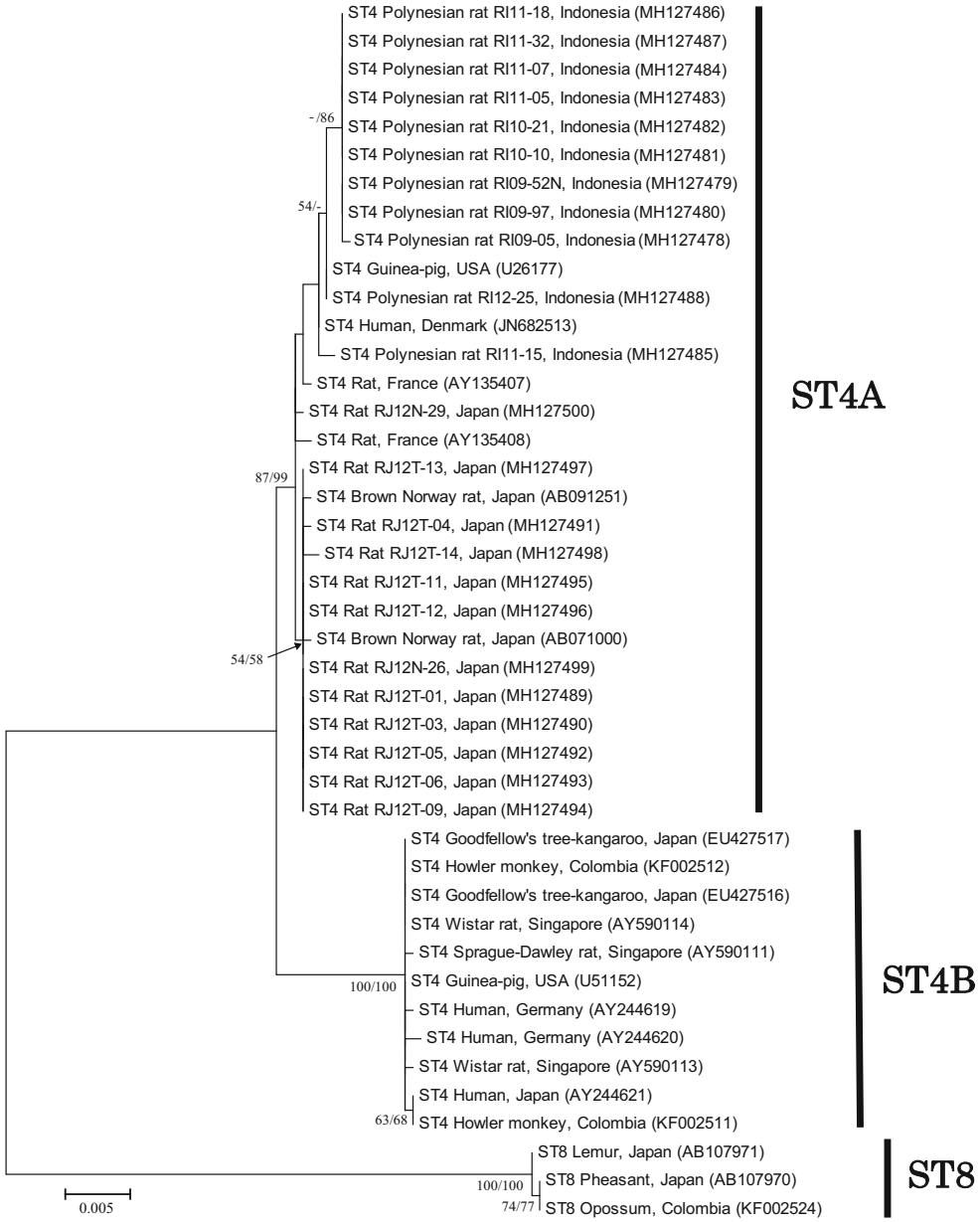

Fig. 1 Phylogenetic tree inferred using the complete SSU rDNA sequences from 23 newly isolated rodent isolates and sequence data for 15 ST4 registered in GenBank with ST8 isolates as an outgroup. The best tree of ML analysis with the Hasegawa-Kishino-Yano model is shown with bootstrap proportion (BP) values for the ML and NJ methods. The tree shows two distinct monophyletic ST4 clades (A and B) with ST8 as the outgroup. All 23 newly isolated rodent isolates from Indonesia and Japan were positioned within ST4A. Interestingly, 11 wild rodent isolates from Polynesian rats (*Rattus exulans*) in Indonesia were positioned separately from the 12 wild rodent isolates from brown rats (*Ruttus novercious*) in Japan. BP values less than 50% and for the internal branch seen in each monophyletic clade are not shown. Names of the host species and countries of origin are given with the accession numbers in parentheses. The newly isolated rodent isolates in this study are shown with the name of the host species, followed by the isolates, the country of origin, and the accession numbers in parentheses (MH127488-MH127500). The horizontal length of each branch is proportional to the estimated number of substitutions

haplotypes determined from Indonesian rodents were clustered with guinea pig (USA), human (Denmark), and rat (France) isolates, and this sub-cluster was addressed as a sister clade of the Japanese rodent sub-cluster including a part of Japanese isolates (11 haplotypes of this study and 2 reference haplotypes isolated from *R. novercious*), which was supported by the bootstrap values of NJ/ML as 54/58%, within the ST4A cluster.

Considering the fact that laboratory rats were susceptible to both ST4A and ST4B (Fig. 1), separation of the monophyletic sub-clusters of *Blastocystis* from wild Indonesian *R. exulans* and Japanese *R. novercious* may indicate the presence of geographical variations rather than a host-specific separation. Although no geographical concordance with ST4A and ST4B has been observed thus far, the results of this study suggest that the evolutionary origin of such sub-clusters can be elucidated by verifying genetic traces of geographical separation by further sample collection.

Regarding those host specificities of ST4 strains, it seems difficult to determine why the wild rodents were only infected with ST4 *Blastocystis*. The susceptibilities of human ST4 isolates to laboratory rats and chickens were shown by the experimental infection trials (Iguchi et al. 2007). However, there is no epidemiological report of ST4 infection in avian species. Moreover, in our previous epidemiological survey in a local Indonesian community where wild Polynesian rats were captured, no ST4 infections in resident people and domestic pigs and chickens were observed (Yoshikawa et al. 2016b). Although more data is apparently required to confirm the host specificity issue of ST4, the ST4 strains appear to be dominantly maintained in wild rodents, and the potent infections of ST4 to other host species such as human, monkey, and kangaroo are considered to be the accidental ones.

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Compliance with ethical standards

Ethical review was performed and approval for this study in Indonesia was granted by the Ethics Committee of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. The capture of wild rodents in Japan was conducted in accordance with the guideline of Protection and Control of Wild Birds and Mammals and Hunting Management Law.

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