

Genetic Characterization of Clinical *Acanthamoeba* Isolates from Japan using Nuclear and Mitochondrial Small Subunit Ribosomal RNA

Md Moshir Rahman¹, Kengi Yagita², Akira Kobayashi³, Yosaburo Oikawa⁴, Amjad I.A. Hussein⁵, Takahiro Matsumura¹ and Masaharu Tokoro^{1,*}

¹Department of Parasitology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Ishikawa, Japan; ²Department of Parasitology, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo, Japan; ³Department of Ophthalmology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Ishikawa, Japan; ⁴Department of Medical Zoology, Kanazawa Medical University, Kanazawa, Ishikawa, Japan; ⁵Department of Medical Laboratory Sciences, Faculty of Medicine and Health Sciences, An-Najah National University, Nablus, West-Bank, Palestine

Abstract: Because of an increased number of *Acanthamoeba* keratitis (AK) along with associated disease burdens, medical professionals have become more aware of this pathogen in recent years. In this study, by analyzing both the nuclear 18S small subunit ribosomal RNA (18S rRNA) and mitochondrial 16S rRNA gene loci, 27 clinical *Acanthamoeba* strains that caused AK in Japan were classified into 3 genotypes, T3 (3 strains), T4 (23 strains), and T5 (one strain). Most haplotypes were identical to the reference haplotypes reported from all over the world, and thus no specificity of the haplotype distribution in Japan was found. The T4 sub-genotype analysis using the 16S rRNA gene locus also revealed a clear sub-conformation within the T4 cluster, and lead to the recognition of a new sub-genotype T4i, in addition to the previously reported sub-genotypes T4a-T4h. Furthermore, 9 out of 23 strains in the T4 genotype were identified to a specific haplotype (AF479533), which seems to be a causal haplotype of AK. While heterozygous nuclear haplotypes were observed from 2 strains, the mitochondrial haplotypes were homozygous as T4 genotype in the both strains, and suggested a possibility of nuclear hybridization (mating reproduction) between different strains in *Acanthamoeba*. The nuclear 18S rRNA gene and mitochondrial 16S rRNA gene loci of *Acanthamoeba* spp. possess different unique characteristics usable for the genotyping analyses, and those specific features could contribute to the establishment of molecular taxonomy for the species complex of *Acanthamoeba*.

Key words: *Acanthamoeba*, keratitis, mixed sequence profile, 18S rRNA, 16S rRNA

INTRODUCTION

The genus *Acanthamoeba* has been isolated from various environmental samples such as soil [1], water [2], air [3], and also human nasal mucosa [4]. While, during the last few decades, this ubiquitous free-living amoeba [5] has become increasingly recognized as a causal agent of serious human diseases, such as vision-threatening *Acanthamoeba* keratitis (AK), life-threatening granulomatous amoebic encephalitis, and disseminated infections of other tissues [6].

Due to an increased number of *Acanthamoeba* infections along

with associated disease burdens, medical professionals have become more aware of this pathogen in recent years [7]. Since 1973 when the first case was reported in a contact lens wearer (CLW), AK has been reported from all over the world [8]. While the prevalence of AK was shown to vary from 1 per 10,000 to 1,000,000 CLW [7,9], the infection clearly appears to be dominant in CLW; on the other hand, the cases of AK in non-CLW are quite limited [10]. To date, the numbers of clinical cases worldwide have been increased as consequently gained the disease recognition [11]. Such a trend has also been observed in Japan, since the first case reported in 1988 [12].

The previous classification of *Acanthamoeba* spp., especially using morphology, caused various ambiguities and therefore has been revised several times [13]. In the early time, classification trials divided this species into 3 groups (I, II, and III) according to the cyst size and shape [14], which, however, was criticized by later studies showing numerous inconsistencies

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*Corresponding author (tokoro@med.kanazawa-u.ac.jp)

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between the morphological classification and previous species categories [13,15]. The current molecular classification divides *Acanthamoeba* spp. into 15 haplotypes (T1-T15), based on nucleotide sequence variations in the 18S rRNA gene [16]. While 2 additional genotypes, T16 and T17, have been recently reported [17,18], the number of these isolates is still limited. Therefore, far more reference information is required to confirm these novel clusters.

Among a total of 15 (or 17) genotypes, the majority of clinical and environmental isolates of amoeba belong to the T4 genotype [13,19]; however, phylogenetic reconstructions of the T4 sub-genotypes were problematic, due to low resolutions of the 18S rRNA gene [13]. On the other hand, the mitochondrial 16S rRNA gene locus seems to have some promising characteristics for the T4 sub-genotype analysis [20,21]. In addition, the 16S rRNA gene locus contains no intron and has more diversity than the 18S rRNA gene locus. However, the number of mitochondrial reference genes is still quite limited.

In this study, the genetic diversity of *Acanthamoeba* spp. isolated from keratitis patients was examined using both the nuclear and mitochondrial gene loci. Our results reveal the detailed diversity of T4 sub-genotypes.

MATERIALS AND METHODS

Isolates and culture condition

Twenty-seven cultured *Acanthamoeba* spp. isolates were used in this study. The samples JPH1 to JPH8 were reference isolates provided from the National Institutes of Infectious Diseases (Tokyo, Japan), originally isolated from AK patients all over Japan. While the samples JPH9 to JPH27 were collected from AK patients between 2006 and 2009, at Kanazawa Medical University and Kanazawa University Hospital, Ishikawa, Japan, and have been culturally maintained in our group. As the culture medium, an amoeba saline containing 0.012% NaCl, 0.00035% KCl, 0.0003% CaCl₂, and 0.0004% MgCl₂, 7 H₂O in 0.05mM Tris-HCl (pH 6.8) supplemented with inactivated *Escherichia coli* [22] was used, and maintained at 27°C.

DNA extraction

Cultured samples were centrifuged at 8,000 rpm for 5 min at 4°C. From the pellet fraction, the whole cell DNA was extracted using QuickGene DNA tissue kit S (FUJIFILM Corporation, Tokyo, Japan) according to the manufacturer's instructions, and concentrated by an ethanol precipitation method. The

Table 1. Primers used in this study

Primer ID	Sequences (5' to 3') and locations on genes ^a	Product size (bp)
For 18S rRNA gene		
YKF2	¹ CCTCCTTCTGGATTCCCGTTC ²¹	560
JDP2	⁵⁶⁰ TCTCACAAGCTGCTAGGGGAGTCA ⁵³⁷	
For 16S rRNA gene		
FP16	¹ TTGTATAACAATCGTTGGGTTTATT ²⁷	1533
RP16	¹⁵³³ GTCCAGCAGCAGGTTCCCTACCGCTA ¹⁵⁰⁷	

^aBase pair positions are according to *A. castellanii* Neff strain on 18S rRNA gene (U07416) [35] and on 16S rRNA gene (AF479560) [36].

DNA was preserved as an aqueous solution at -20°C until use.

18S rRNA PCR

A partial DNA fragment (537-560 bp) of the nuclear 18S rRNA gene of *Acanthamoeba* was amplified using a modified primer set (YKF2/JDF2, Table 1) based on previously published primers [19] on MyCycler™ (BioRad Laboratories, Hercules, California, USA). PCR amplifications were carried out in 20 µl reaction mixture as 1 × PrimeSTAR buffer containing a 1-2 µl of the extracted *Acanthamoeba* DNA template solution, 0.8 mM of each deoxynucleoside triphosphate (dNTP), 0.3 µM of primers, and 1 U of PrimeSTAR HS DNA polymerase (Takara Bio Inc., Shiga, Japan). The PCR cycling profile consisted of 98°C for 2 min, followed by 35 cycles of 98°C for 10 sec, an annealing temperature of 63°C for 5 sec, and 72°C for 40 sec, then a final extension of 72°C for 5 min. The PCR products were electrophoresed on 2% L03 agarose (TaKaRa) with ethidium bromide, and visualized on a UV transilluminator, Gel Doc™ EZ Imager (BioRad Laboratories). The target bands were then excised from the gel and purified using the Quantum Prep™ Freeze 'N Squeeze DNA Gel Extraction Spin Columns (BioRad Laboratories) according to the manufacturer's instructions.

16S rRNA PCR

A partial DNA fragment (1,507-1,533 bp) of the mitochondrial 16S rRNA gene of *Acanthamoeba* was amplified using a primer set with the reference of previously published primers (FP16/RP16, Table 1) [20]. The PCR conditions and following visualization and purification procedures of the amplicons were the same as used for 18S rRNA PCR. The PCR cycling profile consisted of 98°C for 2 min, followed by 30 cycles of 98°C for 10 sec, an annealing temperature of 62°C for 5 sec, and 72°C for 95 sec, then a final extension of 72°C for 5 min.

DNA sequencing

Generally, the purified PCR products were directly sequenced with amplification primers and/or appropriate sequencing primers in both directions on Applied Biosystems 3130 Genetic Analyzer by using the ABI Prism BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan). While, due to mixed-nucleotide profiles observed in the sequence data of JPH5 and JPH17, a subcloning strategy was adopted to confirm the sequences. Those PCR amplicons were cloned into the EcoRV site of pBluescript II SK(+) (Stratagene, City name, California, USA) using the Mighty Cloning Reagent Set (blunt-end) (Takara Bio Inc, Shiga, Japan). The recombinant plasmids were transformed into *Escherichia coli* DH5a (Stratagene) and screened on Luria Broth (LB) agar plates supplemented with 100 mg/L of ampicillin. The clones were picked up as *E. coli* DH5a colonies on the plate and cultured overnight in the 2 ml LB supplemented with 100 mg/L of ampicillin. From the *E. coli* pellet, plasmid purification was conducted using the QIAGEN[®] Plasmid Mini Kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions, and their full-length sequences were confirmed using T3 and T7 primers. All DNA sequences were assembled using the DNASIS-Mac v3.6 (Hitachi, Yokohama, Japan) and confirmed in both directions.

Sequence alignment and phylogenetic analysis

All reference sequences of the 18S rRNA and 16S rRNA genes of *Acanthamoeba* used in this study were obtained from the DNA Data Bank of Japan (DDBJ) using the **blastn** (please confirm) algorithm (<http://blast.ddbj.nig.ac.jp/top-e.html>). DNA sequence alignments were performed by Clustal W2 v2.1 on the European Bioinformatics Institute (EBI) homepage (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The phylogenetic reconstructions of neighbor-joining (NJ), maximum parsimony (MP), and the maximum likelihood (ML) methods were conducted by MEGA5 [23] and used for its comparative analysis. To construct NJ, MP, and ML phylogenetic trees, we used the same options as bootstrap method with 1,000 of replications, nucleotide substitution type, and complete deletion of gaps/missing data treatment. The other and specific options we used were maximum composite likelihood model and uniform rates in NJ analysis, close-neighbor-interchange (CNI) on random trees of MP search method and MP search level 1 in MP analysis; and general time reversible model and nearest-neighbor-interchange (NNI) of ML heuristic method in ML analysis. We also estimated the average evolutionary divergence of partial

sequences of 18S rRNA gene (25 sequences including 2 subclone variations) and 16S rRNA gene (23 sequences) within the T4 genotype confirmed in this study by MEGA5 [23] with the options for the distance analysis preference; bootstrap method with 1,000 of replications from variance estimation method, nucleotide substitution type, maximum composite likelihood model, and complete deletion of gaps/missing data treatment. As the result of this option, the final sequence length used in the analysis were approximately 500 bp.

Nucleotide sequence accession numbers

All the newly identified partial sequences of the 18S rRNA and 16S rRNA genes of *Acanthamoeba* in the present study were deposited in the DDBJ/European Molecular Biology Laboratory (EMBL)/GenBank nucleotide sequence databases under accession numbers of AB741044-AB741047, AB795719, and AB795705-AB795718. respectively.

RESULTS

Microscopic examination

In all 27 cultured *Acanthamoeba* spp. samples, we observed 2 life cycle stages, an active trophozoite stage and a dormant cyst stage. After inoculation of the cyst forms, it took 2-12 days (average, 3.4 days) to detect trophozoites by microscopic examinations. The trophozoites, approximately 15-40 µm in diameter, were found to produce many spine-like processes, whereas the cysts, approximately 10-20 µm in diameter, typically had wrinkled double walls and were almost round in shape (Fig. 1).

18S rRNA gene analysis

The 18S rRNA gene segments (517-570 bp) were successfully amplified by PCR from all the 27 samples. Of these amplicons, 25 sequences were confirmed by direct sequencing using the amplification primers, whereas the other 2, JPH5 and JPH17, showed sequence heterogeneity. To separate mixed haplotypes from the amplicons of JPH5 and JPH17, we used the subcloning procedure described in "Materials and Methods". The results confirmed the presence of 2 clones in each amplicon, designated as JPH5 (A and B) and JPH17 (A and B). In total, 29 sequences of the 18S rRNA gene from 27 isolates were confirmed. As the results of phylogenetic reconstruction, these 29 sequences and previous reference sequences formed 3 monophyletic clusters, T3, T4, and T5, with significant bootstrap values (100%, 81%, and 100%, respectively, by the NJ

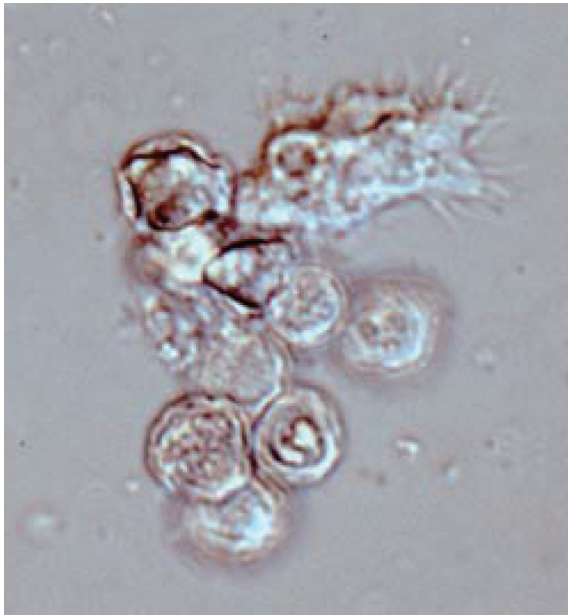


Fig. 1. Representative image of culture-isolated *Acanthamoeba* sp. from an AK case (JPH9). Nomarski interference contrast micrograph of cysts showing double-layered walls and a trophozoite showing spike-like pseudopodia (acanthopodia). Scale bar = 10 μ m.

method) (Fig. 2). In the T3 cluster, JPH6, 23, and 25 showed 100% identity to the reference sequences S81337, GQ397466, and GQ905499, respectively. In the T4 cluster, several groups also showed 100% identity to their respective reference sequences (accession no. in parenthesis): JPH10, 16, and 21 (U07410); JPH26 (AY703004); JPH3, 12, 15 17B, and 20 (U07403); JPH4 (U07413); JPH27 (AY148954); JPH11 (GU808328); JPH1, 9, and 17A (U07415); JPH5B and 22 (AY173000); JPH2 and 14 (GU936484); and JPH5A and 19 (U07408). As for the T5 cluster, only JPH7 was clustered with 98% homology to the reference sequence of *A. lenticulata* 25/1 (U94740).

16S rRNA gene analysis

The 16S rRNA gene segments (1,520-1,545 bp) were also successfully PCR amplified from all the 27 samples, and subsequently, all the sequences were confirmed by direct sequencing. Unlike the 18S rRNA gene analysis results, no sequence heterogeneity was observed in any of the strains analyzed. The reconstructed neighbor-joining tree further showed that these 27 sequences of the 16S rRNA gene and previous reference sequences formed 2 monophyletic clusters, T3 and T5, with significant bootstrap values (100/99/100% and 100/99/100%, respectively, by NJ/MP/ML methods) (Fig. 3). Although the whole T4 cluster was not statistically supported by the bootstrap val-

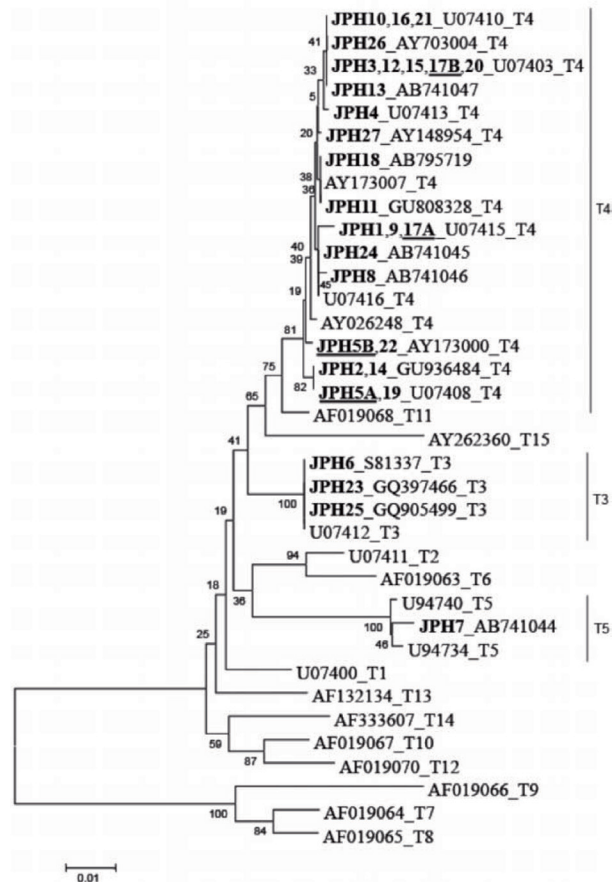


Fig. 2. Neighbor-joining (NJ) tree reconstructed with the 18S rDNA sequences of *Acanthamoeba*. The evolutionary history was inferred using the NJ method as described in “Materials and Methods”. Isolates from *Acanthamoeba* keratitis are shown in boldface text, mixed infections with underlines, and with new or reference accession numbers. All reference sequences are shown with the accession numbers and the genotypes. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap value (1,000 replicates) are shown next to the branches. The evolutionary distances are shown in the units of the number of base substitutions per site.

ues (52/<50/<50%), the individual T4 subclusters were significantly supported as follows: T4a (96/91/98%, excluding the reference AF479553), T4b (99/98/99%, excluding the reference AF479507), T4c (100/99/100%), T4d (100/99/100%), T4e (100/99/100%), T4g (99/74/99%), and T4i (100/99/100%). The bootstrap values were not calculated for T4f and T4h, 2 previously proposed subclusters [21], since none of the 27 samples analyzed in this study was clustered with the reference sequences. Based on the phylogenetic reconstruction results, all the 27 sequences were classified as specific genotypes as fol-

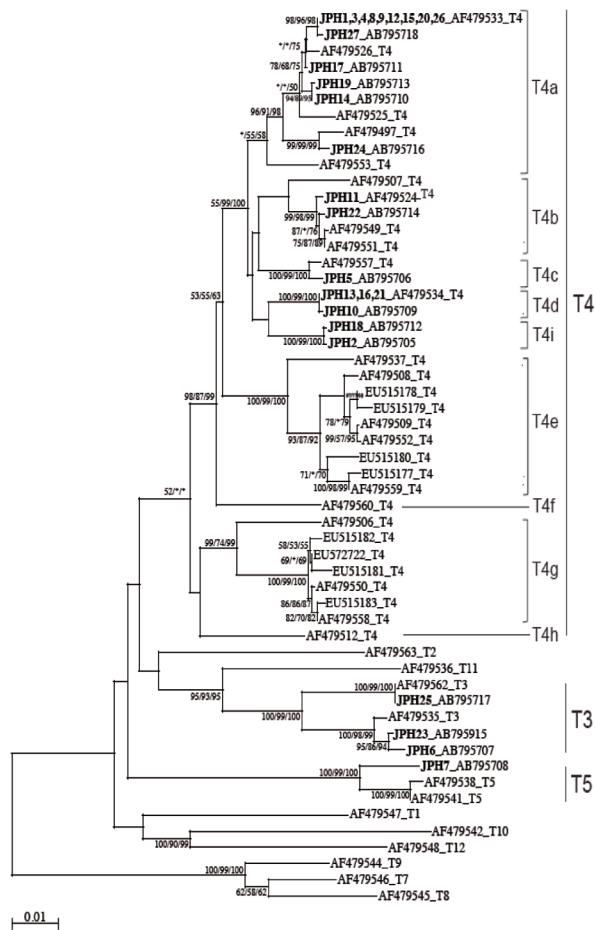


Fig. 3. NJ tree reconstructed with the 16S rDNA sequences of *Acanthamoeba*. Isolates from *Acanthamoeba keratitis* are shown in boldface text, mixed infections with underlining, and with new or reference accession numbers. All reference sequences are shown with the accession numbers and the genotypes. Representative NJ tree with bootstrap value (1,000 replicates) for NJ, MP, and ML methods, conducted as described in “Materials and Methods”, are shown. The analysis involved 55 nucleotide sequences. An asterisk indicates a value of less than 50% or if a position of the node is differ according to each analysis method. The evolutionary distances are shown in the units of the number of base substitutions per site.

lows: T3 cluster, JPH6 (AB795707), JPH23 (AB795915), and JPH25 (AB795717); T4a cluster, JPH1, 3, 4, 8, 9, 12, 15, 20, and 26 (AF479533), JPH27 (AB795718), JPH17 (AB795711), JPH19 (AB795713), JPH14 (AB795710), and JPH24 (AB795716); T4b cluster, JPH11 (AF479524) and JPH22 (AB795714); T4c cluster, JPH5 (AB795706); T4d cluster, JPH13, 16, and 21 (AF479534), and JPH10 (AB795709); and T4i cluster, JPH18 (AB795712) and JPH2 (AB795705). In the T5 cluster, only JPH7 was clustered with 97% homology to the reference sequence of *A. len-*

Table 2. Comparison of genotyping results assessed in this study

Sample name	Target loci for genotyping	
	18S rRNA gene	16S rRNA gene
JPH1	T4 (U07415)	T4 (AF479533)
JPH2	T4 (GU936484)	T4 (AB795705)
JPH3	T4 (U07403)	T4 (AF479533)
JPH4	T4 (U07413)	T4 (AF479533)
JPH5A*	T4 (U07408)	T4 (AB795706)
JPH5B*	T4 (AY173000)	
JPH6	T3 (S81337)	T3 (AB795707)
JPH7	T5 (AB741044)	T5 (AB795708)
JPH8	T4 (AB741046)	T4 (AF479533)
JPH9	T4 (U07415)	T4 (AF479533)
JPH10	T4 (U07410)	T4 (AB795709)
JPH11	T4 (GU808328)	T4 (AF479524)
JPH12	T4 (U07403)	T4 (AF479533)
JPH13	T4 (AB741047)	T4 (AF479534)
JPH14	T4 (GU936484)	T4 (AB795710)
JPH15	T4 (U07403)	T4 (AF479533)
JPH16	T4 (U07410)	T4 (AF479534)
JPH17A*	T4 (U07415)	T4 (AB795711)
JPH17B*	T4 (U07403)	
JPH18	T4 (AB795719)	T4 (AB795712)
JPH19	T4 (U07408)	T4 (AB795713)
JPH20	T4 (U07403)	T4 (AF479533)
JPH21	T4 (U07410)	T4 (AF479534)
JPH22	T4 (AY173000)	T4 (AB795714)
JPH23	T3 (GQ397466)	T3 (AB795715)
JPH24	T4 (AB741045)	T4 (AB795716)
JPH25	T3 (GQ905499)	T3 (AB795717)
JPH26	T4 (AY703004)	T4 (AF479533)
JPH27	T4 (AY148954)	T4 (AB795718)

*Mixed haplotype profiles of T4 sub-genotypes observed in 18S rRNA gene were analyzed using the subcloning procedure described in “Materials and Methods”. JPH5 and JPH17 samples, which were consisted of 2 sub-genotypes: JPH5 (JPH5A and JPH5B) and JPH17 (JPH17A and JPH17B).

ticulata PD2S (AF479541). Overall, there was no dissimilarity between the genotyping results of 18S rRNA and 16S rRNA gene analyses.

Subcloning of genotyping results

Among the 27 strains, only 2, JPH5 and JPH17, showed mixed sequencing profiles (sequence heterozygosity at some nucleotide positions) of their 18S rRNA gene sequences. Specifically, among 7 clones isolated from JPH5, 4 and 3 clones were identified as JPH5A (U07408) and JPH5B (AY173000), respectively, whereas among 7 clones isolated from JPH17, 3 and 4 clones were identified as JPH17A (U07415) and JPH17B (U07403), respectively (Table 2; Fig. 2).

Table 3. 18S rRNA sequence heterogeneities observed in two isolates of genotype T4

(A) JPH5

Nucleotide positions ^a	890	891	902	893	894	900	901	906	908	1295	1319	1320	1324	1325	1326	1327	1344
JPH5A	T	G	C	G	G	C	A	C	T	-	C	C	A	C	G	G	T
JPH5B	-	-	-	A	T	G	C	G	-	T	-	-	-	-	-	-	C

^aNucleotide positions are shown according to the reference sequence (U07408). Sequence heterogeneities were observed in JPH5, and the subcloning procedure could confirm 2 sub-genotypes, JPH5A (U07408) and JPH5B (AY173000). Only the substituted nucleotides are shown with position numbers. Hyphen indicates an insertion/deletion mutation.

(B) JPH17

Nucleotide positions ^a	874	888	889	894	895	897	909	1283	1302	1303	1304	1305	1312	1313	1314	1315	1318	1325
JPH17A	A	A	-	-	T	C	T	T	T	C	G	G	C	C	G	G	T	A
JPH17B	G	G	T	A	C	G	C	C	G	G	T	C	G	G	C	C	C	G

^aNucleotide positions are shown according to the reference sequence (U07415). Sequence heterogeneities were observed in JPH17, and the subcloning procedure could confirm 2 sub-genotypes, JPH17A (U07415) and JPH17B (U07403). Only the substituted nucleotides are shown with position numbers. Hyphen indicates an insertion/deletion mutation.

Estimation of average evolutionary divergence of T4 genotypes

The numbers of base substitutions per site from averaging over all sequence pairs of the 18S rRNA gene and 16S rRNA gene were analyzed as described in "Materials and Methods". The confirmed average evolutionary divergences and standard errors of 18S rRNA gene and 16S rRNA gene were 0.010 ± 0.003 and 0.013 ± 0.003 , respectively.

DISCUSSION

The increased risk of AK has been widely recognized worldwide, as well as in Japan. A recent survey conducted by the Japan Contact Lens Society and the Japanese Association for Ocular Infection in 224 facilities all over Japan from April 2007 to March 2009 revealed the high prevalence of AK in Japan. Specifically, among 350 patients who were diagnosed with contact lens-associated microbial keratitis, *Acanthamoeba* spp. were identified in 85 (24.3%) cases [24]. To date, the data regarding the distribution of various genotypes in Japan are still quite limited. Edagawa et al. [25] reported T4 isolates in tap-water samples, and multiple clinical T4 cases and 1 T11 case [26-27] have been reported so far.

In this study, 27 *Acanthamoeba* strains that caused AK in Japan were classified into 3 genotypes, T3 (3 strains), T4 (23 strains), and T5 (one strain) (Table 2; Figs. 2, 3), consistent with previous findings that both T3 and T4 genotypes were prevalent among AK patients around the world (Tables 4, 5).

On the other hand, the T5 genotype was mostly detected in the environment [13,19,21,25], and the reports of the T5 genotype in AK patients [28,29] or human nasal mucosa [4,13] were very rare.

It seems interestingly, most of the haplotypes identified in this study showed 100% identity to the reference sequences available in the database. In the 18S rRNA gene analysis, only 5 were newly recognized haplotypes, and the rest of 22 haplotypes (including subcloned ones) had 13 homologous references; on the other hand, in the 16S rRNA gene analysis, 14 were newly identified, and the rest of 13 haplotypes had 3 references (Tables 4, 5). The places where the references were originally isolated include many areas all over the world, such as Africa, Argentina, France, Germany, India, Israel, Japan, Korea, Pakistan, Slovakia, Thailand, UK, and USA. Therefore, these *Acanthamoeba* haplotypes might have been distributing around the world and maintaining individual haplotypes independently, despite their geographically dispersed conditions. A robust feature of these haplotypes could be a confidential base for the molecular classification of *Acanthamoeba* spp.

The results of 18S and 16S rRNA genotyping were found to match perfectly with each other, and no contraindication depending on these loci was observed; however, the haplotype diversity of AK-related strains was clearly different between the 2 analyses (Figs. 2, 3). For example, while JPH3, 12, 17B, and 20 were identified as the same haplotype (U07403) by the 18S rRNA gene analysis, 9 strains (JPH1, 3, 4, 8, 9, 12, 15, 20, and 26) were identified as the same haplotype (AF479533) by the

Table 4. 18S rRNA gene sequences used in this study

Isolate name	Accession No.	Genotype	Isolation/place of origin	Reference
V006	U07400	T1	GAE, Brain, Georgia, USA	[32]
Reich	U07411	T2	Soil, Israel	[32]
H37	S81337	T3	Keratitis, UK	[37]
S-7	U07412	T3	Shallow beach, New London, CT, USA	[32]
AcaVN04	GQ397466	T3	Air conditioner scrape, Slovakia	[38]
AcaVNAK05	GQ905499	T3	Keratitis, Slovakia	[38]
V042	U07403	T4	Keratitis, Illinois, USA	[32]
Castellani	U07413	T4	Yeast culture, London, UK	[32]
Neff	U07416	T4	Soil, USA	[32]
JAC/S2	U07415	T4	Soil, Japan	[32]
U/Oft1	AY026248	T4	Brazil	Direct submission
M3	GU936484	T4	Cooling towers water, France	[39]
82-12-324	U07408	T4	Keratitis, Houston, TX, USA	[32]
KA/MSS8-1	AY173000	T4	Marine sediment, Korea	Direct submission
88-2-37	U07410	T4	Keratitis, Houston, TX, USA	[32]
KA/MSG15	AY173007	T4	Marine sediment, Korea	Direct submission
V390	AY703004	T4	Skin, Atlanta, GA, USA	[40]
KA/E5	AY148954	T4	Keratitis, Korea	Direct submission
Ac_PCNI8c	GU808328	T4	Keratitis, Thailand	[18]
407-3a	U94734	T5	Acid waste dump, Atlantic Ocean, USA	[13]
25/1	U94740	T5	Nasal mucosa, Germany	[13]
2802	AF019063	T6	Swimming pool, France	[13]
Ray & Hayes	AF019064	T7	Lab water, Washington, USA	[13]
OC-15C	AF019065	T8	Freshwater, Maryland, USA	[13]
Comandon & de Fonbrune	AF019066	T9	Soil, France	[13]
Lilly A-1	AF019067	T10	Human cell culture, Indiana, USA	[13]
BH-2	AF019068	T11	Brackish water, Maryland, USA	[13]
V013	AF019070	T12	GAE, brain, Barbados, BWI	[13]
UWC9	AF132134	T13	Keratitis, MN, USA	[41]
PN15	AF333607	T14	Human cell culture, Pakistan	[42]
AC005	AY262360	T15	Marine source, USA	[43]
JPH7	AB741044	T5	Keratitis, Kanazawa, Japan	This study
JPH8	AB741046	T4	Keratitis, Kanazawa, Japan	This study
JPH13	AB741047	T4	Keratitis, Kanazawa, Japan	This study
JPH18	AB795719	T4	Keratitis, Kanazawa, Japan	This study
JPH24	AB741045	T4	Keratitis, Kanazawa, Japan	This study

16S rRNA gene analysis. Notably, JPH3, 12, and 20 were included in both analyses, but not other haplotypes. It is noteworthy that the genotyping assessments were conducted using gene segments of partial 18S rRNA (517-570 bp) and partial 16S rRNA (1,520-1,540 bp). That is, even with the use of longer nucleotide sequences, one third (9/27) of the strains were classified as 1 specific haplotype (AF479533) by the 16S rRNA gene analysis. The differences were statistically not significant; moreover, the average evolutionary divergence of all T4 samples confirmed in this study indicated higher values among 16S rRNA gene haplotypes (0.013 ± 0.003) than 18S rRNA gene haplotypes (0.010 ± 0.003), which may be consistent with a

previous notion of the comparatively low divergence in the 18S rRNA gene locus [13,20,30-32]. While further research is required before a conclusive hypothesis can be drawn, we speculate that a part of T4 genotypes may be a dominant haplotype as a causal agent of AK and that the 16S rRNA gene analysis is useful for the evaluation.

Since the sub-genotype classification of the T4 cluster seems to be useful for higher-resolution molecular analyses [20-21,32], 8 subclusters (T4a-T4h) have been proposed [21]. In this study, the T4 sub-genotype analysis using the 16S rRNA gene locus revealed a clear sub-conformation within the T4 genotype and also leads to the recognition of a new sub-genotype, T4i, as the

Table 5. 16S rRNA gene sequence used in this study

Isolate name	Accession No.	Genotype	Isolation/Place of origin	Reference
CDC V006	AF479547	T1	GAE, Brain, Georgia, USA	[21]
Reich	AF479563	T2	Soil, Israel	[21]
Panola Mtn.	AF479535	T3	Soil, Georgia, USA	[21]
S-7	AF479562	T3	Beach bottom, Connecticut, USA	[21]
Ma	AF479533	T4	Keratitis, New York, USA	[21]
JAC E2	AF479497	T4	Keratitis, Japan	[21]
Neff	AF479560	T4	Soil, California, USA	[21]
CDC V014	AF479550	T4	Keratitis, India	[21]
AA2	EU515178	T4	Soil, Morocco	[44]
1652	EU515180	T4	Soil, Mauritania	[21]
SAWL 93/1	AF479512	T4	Keratitis, South Africa	[21]
AA1	EU515179	T4	Soil, France	[44]
CCAP, 1501-3D	AF479537	T4	Keratitis, UK	[21]
CDC V029	AF479526	T4	Keratitis, Massachusetts, USA	[21]
CEI 73-01-16	AF479557	T4	Keratitis, Texas, USA	[21]
CEI 85-6116	AF479553	T4	Keratitis, Texas, USA	[21]
Singh	EU515177	T4	Soil, UK	[21]
Oak Ridge	AF479559	T4	Human tissue culture	[21]
SH621	EU515183	T4	Human feces, France	Direct submission
CEI 88-2-27	AF479558	T4	Keratitis, Texas, USA	[21]
CDC V125	AF479524	T4	Keratitis, California, USA	[21]
CDC V383	AF479534	T4	Keratitis, Argentina	[21]
CDC V168	AF479525	T4	Skin infection, USA	[21]
KA/E9	EU515181	T4	Keratitis, Korea	Direct submission
KA/E17	EU572722	T4	Keratitis, Korea	Direct submission
KA/E23	EU515182	T4	Keratitis, Korea	Direct submission
LVPEI 402/97	AF479506	T4	Keratitis, India	[21]
LVPEI 773/96	AF479507	T4	Keratitis, India	[21]
LVPEI 1035/99	AF479508	T4	Keratitis, India	[21]
LVPEI 98/00	AF479509	T4	Keratitis, India	[21]
LVPEI 1060/96	AF479549	T4	Keratitis, India	[21]
LVPEI 1002/99	AF479551	T4	Keratitis, India	[21]
LVPEI 749/98	AF479552	T4	Keratitis, India	[21]
SAWS 87/1	AF479538	T5	Sewage sludge, South Africa	[21]
PD2S	AF479541	T5	Swimming pool, France	[21]
Ray & Hayes	AF479546	T7	Lab water, Washington, USA	[21]
NMFS OC-15C	AF479545	T8	Freshwater, Maryland, USA	[21]
AIP	AF479544	T9	Soil, France	[21]
CDC 409	AF479542	T10	Horse brain, USA	[21]
OHSU M001	AF479536	T11	Keratitis, Oregon, USA	[21]
CDC V013	AF479548	T12	GAE, brain, British West Indies	[21]
JPH2	AB795705	T4	Keratitis, Kanazawa, Japan	This study
JPH5	AB795706	T4	Keratitis, Kanazawa, Japan	This study
JPH6	AB795707	T3	Keratitis, Kanazawa, Japan	This study
JPH7	AB795708	T5	Keratitis, Kanazawa, Japan	This study
JPH10	AB795709	T4	Keratitis, Kanazawa, Japan	This study
JPH14	AB795710	T4	Keratitis, Kanazawa, Japan	This study
JPH17	AB795711	T4	Keratitis, Kanazawa, Japan	This study
JPH18	AB795712	T4	Keratitis, Kanazawa, Japan	This study
JPH19	AB795713	T4	Keratitis, Kanazawa, Japan	This study
JPH22	AB795714	T4	Keratitis, Kanazawa, Japan	This study
JPH23	AB795715	T3	Keratitis, Kanazawa, Japan	This study
JPH24	AB795716	T4	Keratitis, Kanazawa, Japan	This study
JPH25	AB795717	T3	Keratitis, Kanazawa, Japan	This study
JPH27	AB795718	T4	Keratitis, Kanazawa, Japan	This study

ninth sub-genotype in the T4 cluster (Fig. 3). We showed that the 2 newly identified sequences from JPH2 and JPH8 belonged to T4i, with statistically significant bootstrap values. Nevertheless, the number of genetic references for the 16S rRNA gene locus does not seem to be sufficient for completing the precise identification of all sub-genotypes. In the phylogenetic reconstruction (Fig. 3), some haplotypes, for example, AF479553 in T4a and AF479507 in T4b, were positioned as out-groups of the main cluster. These out-group haplotypes might be members of yet-to-be identified subclusters and could be categorized into novel clusters as the number of the reference sequences increases in the future.

Differences were observed between nuclear and mitochondrial genetic characteristics; that is, the mixed haplotype profiles from 2 individual strains, JPH5 and JPH17, were detected only in the nuclear gene analysis, but not in the mitochondrial gene analysis (Table 3; Figs. 2, 3). The formation of JPH5A/5B in the JPH5 strain and JPH17A/17B in the JPH17 strain seemed to be stable. To evaluate the contamination risk with other strains, we tried 1 trophozoite PCR after years of cultivation and got the same results for both strains (data not shown). The contamination might also be ruled out by the result of the 16S rRNA gene analysis, which showed only a single genotype for each strain.

Taken these results together, these strains are considered to possess heterozygous nuclear 18S rRNA genes and homozygous mitochondrial 16S rRNA genes. The polyploid genome conformation of *Acanthamoeba* spp. has been suggested [33]; therefore, an accumulation of allelic sequence heterogeneity in polyploid genomes within a single cell might be a possible explanation for the mixed haplotype profile. However, such a possibility may be ruled out in our case, since all haplotypes identified in the sub-cloning analysis had their respective homologous haplotypes as individual strains. Specifically, JPH5A, 5B, 17A, and 17B were found to be identical to previous reported reference strains 82-12-324 (U07408), KA/MSS8-1 (AY173000), JAC/S2 (U07415), and V042 (U07403), respectively. Currently, there is no clear evidence for the meiotic or sexual process in the *Acanthamoeba* life cycle, even though the species apparently possesses a gene (Spo11) required for the meiotic recombination [34]. Whereas, the presence of heterozygous nuclear haplotypes in single strain, observed in this study, suggesting a possibility of the genetic hybridization between different strains of *Acanthamoeba*. Therefore, detailed evaluations of the presence of such mixed haplotypes in the

population of *Acanthamoeba* spp. are considered to be required for the precise molecular taxonomy.

The nuclear 18S rRNA gene and mitochondrial 16S rRNA gene loci of *Acanthamoeba* spp. possess different and unique characteristics usable for the genotyping analyses, and those specific features could contribute to the establishment of molecular taxonomy for the species complex of *Acanthamoeba*.

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