Genetic Characterization of Clinical *Acanthamoeba* Isolates Using Gene Loci of Nuclear and Mitochondrial Small Subunit Ribosomal RNA

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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, addition to the previously reported sub-genotypes T4a–T4h. Furthermore, the 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 the heterozygous nuclear haplotypes were observed from the 2 strains, the mitochondrial haplotypes were homozygous as T4 genotype in the both strains, and suggested that the 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 an 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 [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 trial divided this species into three groups (I, II, and III) according to the cyst size and shape [14], which, however, was criticized by later studies showing numerous inconsistencies 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 two 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 belong to the T4 genotype [13, 19]; however, phylogenetic reconstructions of the T4 sub-genotypes were problematic, due to the 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 culture medium, an amoeba saline containing 0.012% NaCl, 0.00035% KCl, 0.0003% $CaCl_2$, and 0.0004% $MgCl_2$, 7 H_2O 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 8000 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 DNA was preserved as an aqueous solution at −20°C until use.

18S rRNA Polymerase chain reaction (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 the previously published primers [19] on MyCycler™ (BioRad Laboratories, 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 trans-illuminator, Gel Doc™ EZ Imager, BIO RAD. 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 (1507–1533 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. While 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

124 profiles observed in the sequence data of JPH5 and JPH17, a sub-cloning strategy was
125 adopted to confirm the sequences. Those PCR amplicons were cloned into the EcoRV site of
126 pBluescript II SK(+) (Stratagene, California, USA) using the Mighty Cloning Reagent Set
127 (Blunt End) (Takara Bio Inc, Shiga, Japan). The recombinant plasmids were transformed into
128 *Escherichia coli* DH5a (Stratagene) and screened on Luria Broth (LB) agar plates
129 supplemented with 100 mg/L of ampicillin. The clones were picked up as *E. coli* DH5a
130 colonies on the plate and cultured overnight in the 2 ml LB supplemented with 100 mg/L of
131 ampicillin. From the *E. coli* pellet, plasmid purification was conducted using the QIAGEN®
132 Plasmid Mini Kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's
133 instructions, and their full-length sequences were confirmed using T3 and T7 primers. All
134 DNA sequences were assembled using the DNASIS-Mac v3.6 (Hitachi, Yokohama, Japan)
135 and confirmed in both directions.

136 **Sequence alignment and phylogenetic analysis:** All reference sequences of the 18S
137 rRNA and 16S rRNA genes of *Acanthamoeba* used in this study were obtained from the
138 DNA Data Bank of Japan (DDBJ) using the blastn algorithm (http://blast.ddbj.nig.ac.jp/top-
139 e.html). DNA sequence alignments were performed by ClustalW2 v2.1 on the European
140 Bioinformatics Institute (EBI) homepage (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The
141 phylogenetic reconstructions of neighbor-joining (NJ), maximum parsimony (MP) and the
142 maximum likelihood (ML) methods were conducted by MEGA5 [23] and used for its
143 comparative analysis. To construct NJ, MP and ML phylogenetic tree we used the same
144 options as bootstrap method with 1000 of replications, nucleotide substitution type, and
145 complete deletion of gaps/missing data treatment. The other and specific options we used in
146 NJ analysis: maximum composite likelihood model and uniform rates; in MP analysis: close-
147 neighbor-interchange (CNI) on random trees of MP search method and MP search level 1;
148 and in ML analysis: general time reversible model and nearest-neighbor-interchange (NNI)

of ML heuristic method. We also estimated the average evolutionary divergence of partial sequence of 18S rRNA (25 sequences including 2 sub-clone variations) and 16S rRNA (23 sequences) genes within the T4 genotype confirmed in this study by MEGA5 [23] with the options for the distance analysis preference: bootstrap method with 1000 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 approximate 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 two life cycle stages, an active trophozoite stage and a dormant cyst stage. After inoculation of the cyst forms, it took 2 to 12 days (average, 3.4 days) to detect trophozoites by microscopic examination. 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 PCR amplified from all the 27 samples. Of these amplicons, 25 sequences were confirmed by direct sequencing using the amplification primers, whereas the other two, JPH5 and JPH17, showed sequence heterogeneity. To separate mixed haplotypes from the amplicons of JPH5 and JPH17, we used the sub-cloning procedure described in “Materials and Methods”. The results confirmed the presence of two clones in each amplicon, designated as JPH5A and B

and JPH17A and B. In total, 29 sequences of the 18S rRNA gene from 27 isolates were confirmed. As the result of phylogenetic reconstruction, these 29 sequences and previous reference sequences formed three monophyletic clusters, T3, T4, and T5, with significant bootstrap values (100%, 81%, and 100%, respectively, by the NJ 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 number 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 (1520–1545 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 two 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 values (52/<50/<50%), the individual T4 sub-clusters 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, two previously proposed sub-clusters [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 follows: 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. lenticulata PD2S (AF479541). Overall, there was no dissimilarity between the genotyping results of 18S rRNA and 16S rRNA gene analyses.

Sub-cloning genotyping results: Among the 27 strains, only two, 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).

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. was 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. has reported T4 isolates in tap-water samples [25], and multiple clinical T4 cases and one 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 sub-cloned 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, the United Kingdom, and the United States. Therefore, these *Acanthamoeba* haplotypes might have been distributing around the world and maintaining individual haplotypes independently, despite their geographically dispersed conditions. A

246 robust feature of these haplotypes could be a confidential base for the molecular classification
247 of *Acanthamoeba* spp.

248 The results of 18S and 16S rRNA genotyping were found to match perfectly with each
249 other, and no contraindication depending on these loci was observed; however, the haplotype
250 diversity of AK-related strains was clearly different between the two analyses (Figs. 2, 3).
251 For example, while JPH3, 12, 17B, and 20 were identified as the same haplotype (U07403)
252 by the 18S rRNA gene analysis, 9 strains (JPH1, 3, 4, 8, 9, 12, 15, 20, and 26) were identified
253 as the same haplotype (AF479533) by the 16S rRNA gene analysis. Notably, JPH3, 12, and
254 20 were included in both analyses, but not other haplotypes. It is noteworthy that the
255 genotyping assessments were conducted using gene segments of partial 18S rRNA (517–570
256 bp) and partial 16S rRNA (1520–1540 bp). That is, even with the use of longer nucleotide
257 sequences, one third (9/27) of the strains were classified as one specific haplotype
258 (AF479533) by the 16S rRNA gene analysis. Although the differences was not statistically
259 significant, moreover the average evolutionary divergence of all T4 samples confirmed in this
260 study indicated higher value among 16S rRNA gene haplotypes (0.013 ± 0.003) than 18S
261 rRNA gene haplotypes (0.010 ± 0.003), may consistent with a previous notion of the
262 comparatively low divergence in the 18S rRNA gene locus [13, 20, 30-32]. While further
263 research is required before a conclusive hypothesis can be drawn, we speculate that a part of
264 T4 genotypes may be a dominant haplotype as a causal agent of AK and that the 16S rRNA
265 gene analysis is useful for the evaluation.

266 Since the sub-genotype classification of the T4 cluster seems to be useful for higher-
267 resolution molecular analyses [20-21, 32], 8 sub-clusters (T4a–T4h) have been proposed
268 [21]. In this study, the T4 sub-genotype analysis using the 16S rRNA gene locus reveals a
269 clear sub-conformation within the T4 genotype and also leads to the recognition of a new
270 sub-genotype, T4i, as the ninth sub-genotype in the T4 cluster (Fig. 3). We show that the two

newly identified sequences from JPH2 and JPH8 belong 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 sub-clusters and could be categorized into novel clusters as the number of the reference sequences increases in the future.

The differences were observed between nuclear and mitochondrial genetic characteristics; that is, the mixed haplotype profiles from two 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 one 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 one 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

296 clear evidence for the meiotic or sexual process in the *Acanthamoeba* life cycle, even though
297 the species apparently possesses a gene (Spo11) required for the meiotic recombination [34].
298 Whereas, the presence of heterozygous nuclear haplotypes in single strain, observed in this
299 study, suggesting a possibility of the genetic hybridization between different strains of
300 *Acanthamoeba*. Therefore, detailed evaluations of the presence of such mixed haplotypes in
301 the population of *Acanthamoeba* spp. are considered to be required for the precise molecular
302 taxonomy.

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

307

308 **ACKNOWLEDGEMENT**

309 We would like to dedicate our work with our deepest empathy in the memories of Mr.
310 Yoshizaki Kentaro who passed away before the completion of this study. This research was
311 partly supported by Grants-in-Aid for Scientific Research from Ministry of the Environment,
312 Japan. We would like to thank Editage for providing editorial assistance.

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FIGURE LEGENDS

Figure 1. Representative image of culture-isolated *Acanthamoeba* spp. 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 μm.

Figure 2. NJ tree reconstructed with the 18S rDNA sequences of *Acanthamoeba*.

The evolutionary history was inferred using the neighbor-joining method as described in "Materials and Methods". Isolates from *Acanthamoeba* keratitis are shown in boldface text, mixed infection with underlining, 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 (1000 replicates) are shown next to the branches. The evolutionary distances are shown in the units of the number of base substitutions per site.

Figure 3. **NJ tree reconstructed with the 16S rDNA sequences of *Acanthamoeba*.**

Isolates from *Acanthamoeba* keratitis are shown in boldface text, mixed infection 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 (1000 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.

Table 1. Primers used in this study

Primer ID	Sequences (5′ to 3′) and locations on genes*	Product Size (bp)
For 18S rRNA gene		
YKF2	^{1}CCTCCTTCTGGATTCCCGTTC^{21}	560
JDP2	^{560}TCTCACAAGCTGCTAGGGGAGTCA^{537}	
For 16S rRNA gene		
FP16	^{1}TTGTATAAACAATCGTTGGGTTTTATT^{27}	1533
RP16	^{1533}GTCCAGCAGCAGGTTCCCCTACCGCTA^{1507}	

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

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 sub-cloning procedure described in "Materials and Methods". JPH5 and JPH17 samples, which were consisted of two sub-genotypes: JPH5 (JPH5A and JPH5B) and JPH17 (JPH17A and JPH17B).

Table 3. Sequence heterogeneities observed of 2 genotypes in 18S rRNA nucleotide sequences by using genotype T4 isolated from a single strain.

From the two strains, JPH5 and JPH17, each strain consists of 2 sub-genotypes: (A) JPH5A (U07408) and JPH5B (AY173000), and (B) JPH17A (U07415) and JPH17B (U07403) were identified according to the reference accession number in parenthesis. Only the substituted positions are showing with each nucleotide position number. Hyphen indicates an insertion/deletion mutation.

(A) JPH5

Nucleotide positions*	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

*Nucleotide positions are shown according to the reference sequence (U07408).

(B) JPH17

Nucleotide positions*	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

*Nucleotide positions are shown according to the reference sequence (U07415).

501 **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_PCN18c	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]

Isolate name	Accession No.	Genotype	Isolation/Place of origin	Reference
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

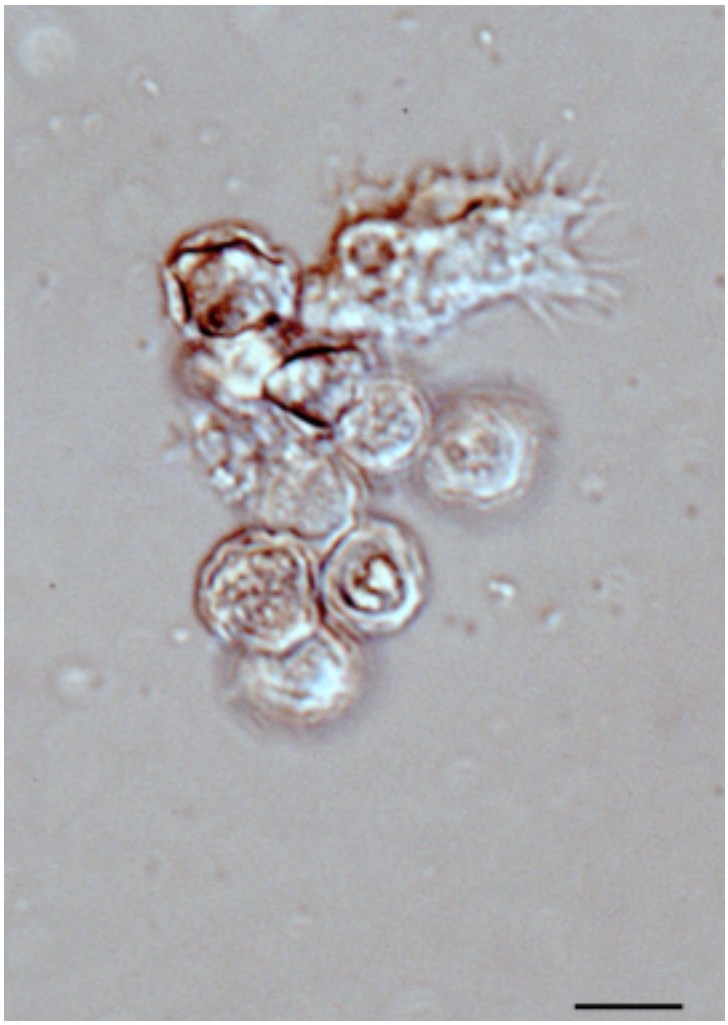
518 **Table 5**: 16S rRNA gene sequence used in this study

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]

Isolate name	Accession No.	Genotype	Isolation/Place of origin	Reference
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

533 Figures

534 Fig.1



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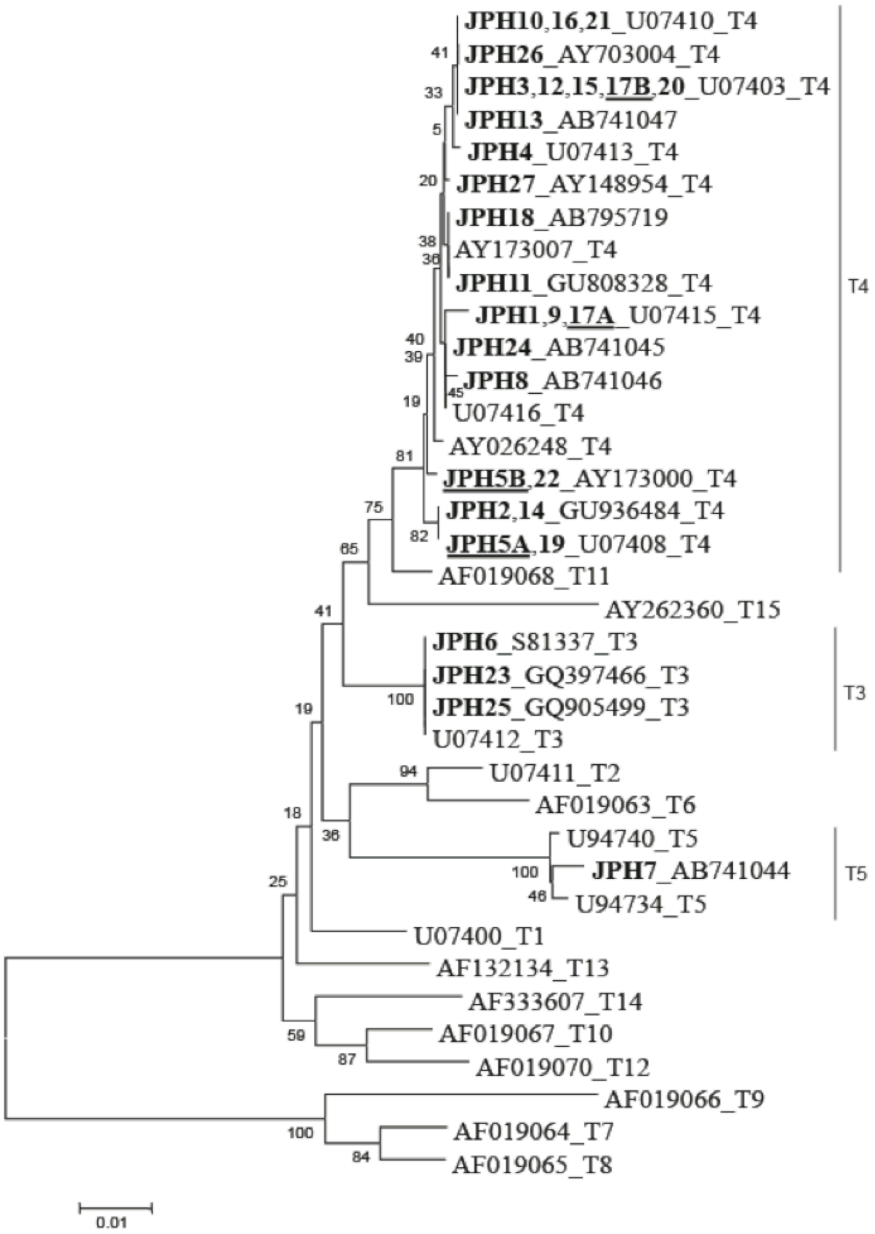
537 Fig. 2



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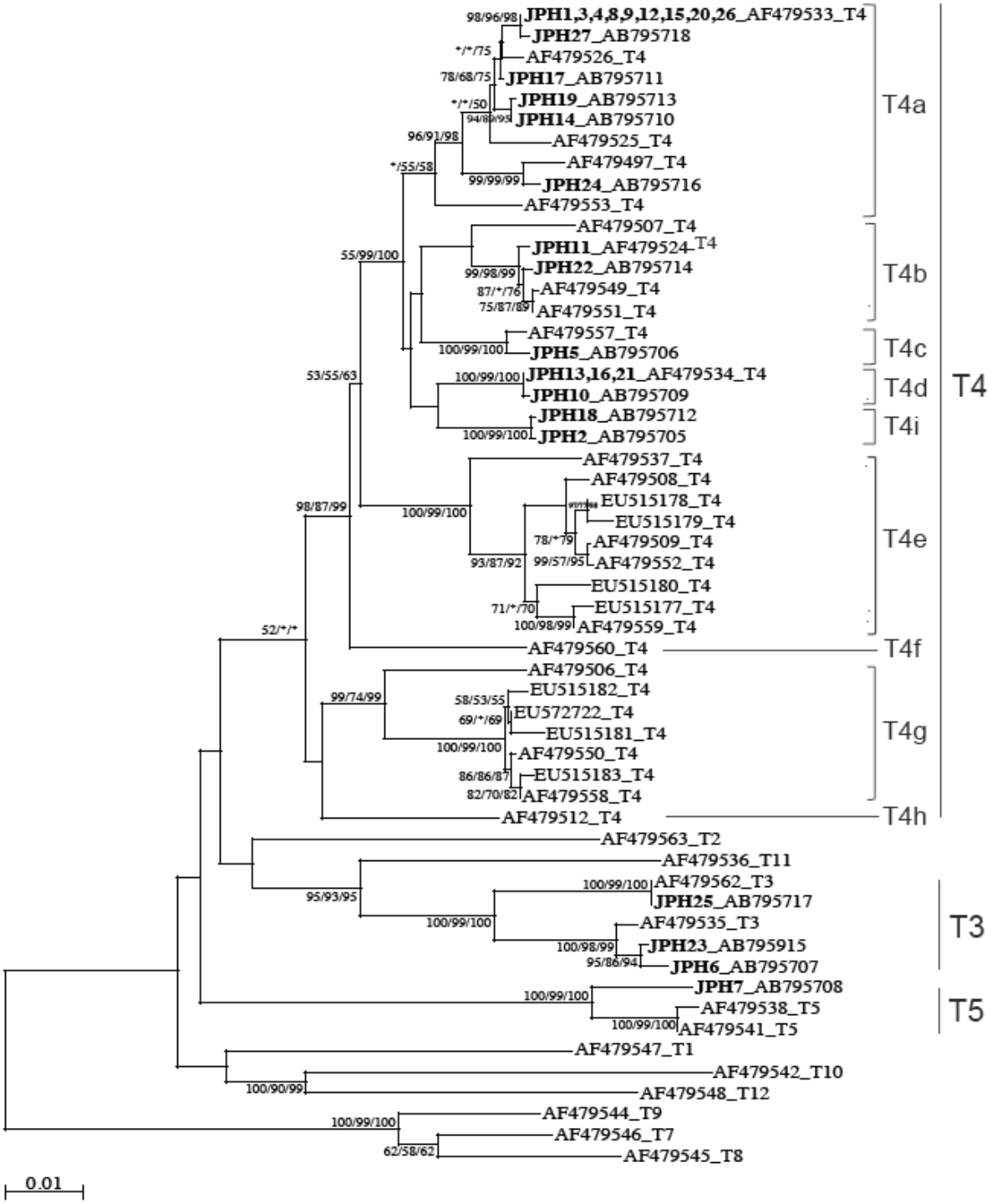
541 Fig. 3



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