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Genotyping and physiological characteristics of *Acanthamoeba* isolated from beaches in Phuket Province, Thailand

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Abstract. *Boonman N, Wanna C, Chutrtong J, Chotpantarat S, Boonsilp S. 2022. Genotyping and physiological characteristics of* Acanthamoeba *isolated from beaches in Phuket Province, Thailand. Biodiversitas 23: 4377-4384.* *Acanthamoeba* is widely distributed in water, soil, dust and air. Beaches are popular tourist destinations for water-related activities while they can also be reservoirs for *Acanthamoeba* to humans. This study aimed to determine the genotyping of *Acanthamoeba* in the beach environment and characterized their pathogenicity potential. A total of 63 samples were collected from six beaches in Phuket Province, Thailand. Twenty-one samples of each source including wet sand, dry sand and seawater, were collected and cultivated on 2% non-nutrient agar coated with heat-killed *Escherichia coli*. *Acanthamoeba* was found in 100% (n=21) wet sand, 52.4% (n=11) dry sand and 14.3% (n=3) seawater. Sequence analysis of the GTSA.B1 region in 18S rRNA revealed that 83.9% of sequenced isolates belonged to the T5 genotype and 16.1% belonged to the T4 genotype. The T5 genotype was predominant, differing from other reports of *Acanthamoeba* prevalence in natural sources. *In vitro* thermal and salt-tolerance showed that all isolates had growth rates similar to clinical *Acanthamoeba* isolates. This research was undertaken to increase awareness of human eye and brain infections caused by *Acanthamoeba* on beaches. Further studies on these *Acanthamoeba* isolates are still required such as co-culture assay between the trophozoites and human cell lines to confirm their pathogenicity as well as their susceptibility to the chemotherapeutic agents.

Keywords: *Acanthamoeba*, beach, genotype, Thailand, thermo-tolerance, salt-tolerance

INTRODUCTION

The *Acanthamoeba* genus of free-living protozoa is found in diverse natural and man-made environments such as soil, freshwater, seawater, tap water, swimming pools, air-conditioning systems, contact lenses and lens cases (de Lacerda and Lira 2021), with two life cycle stages as active trophozoite or dormant cyst according to the surrounding conditions. *Acanthamoeba* is present in the trophozoite stage that feeds on bacteria, yeast or small organic particles by phagocytosis, and asexually reproduces by binary fission, whereas under harsh conditions such as food deprivation, high or low temperature and pH, and improper osmolarity or moisture, the trophozoite encysts to a cystic stage enclosed with double walls. The cyst survives by waiting for favorable conditions to exist as the trophozoite stage (Khan 2006). When *Acanthamoeba* in the environment enters the human body, it causes severe diseases including granulomatous amoebic encephalitis (GAE), a chronic central nervous system disease, or the dissemination of various organ systems resulting in death (Trabelsi et al. 2012; Kalra et al. 2020) and amoebic keratitis (AK) which causes blindness (Trabelsi et al. 2012; Szentmáry et al. 2019; Fanselow et al. 2021).

Currently, the *Acanthamoeba* genus is classified into 23 genotypes (T1-T23) based on the similarity of 18S ribosomal RNA gene (*Rns*) sequences (Putaporntip et al. 2021). The T4 genotype contains the largest number of *Acanthamoeba* strain from both environmental and clinical specimens (Fuerst et al. 2015; Maciver et al. 2013). Other genotypes including T1, T2, T3, T5, T6, T10, T11, T12, T15 and T18 were also associated with human infections (Qvarnstrom et al. 2013; Possamai et al. 2018). Historically, ASA.S1 or *Acanthamoeba*-specific amplimer S1 (500 bp) produced by JDP1 and JDP2, the genus-specific primers, was commonly used for *Acanthamoeba* identification. However, this was insufficient to distinguish closely related genotypes such as T3, T4 and T11. Therefore, GTSA.B1 or *Acanthamoeba* genotype-specific amplimer B1 (1,475 bp), containing eight variable regions, was used to differentiate and identify new genotypes (Schroeder et al. 2001; Shyrobokov et al. 2020). The pathogenicity of *Acanthamoeba* was not related to the genotype and may depend on many factors such as morphology, growth rate and adaptation to host environments (Khan et al. 2002). Thermo-tolerance and osmo-tolerance were the most acceptable techniques for differentiation between pathogenic and nonpathogenic *Acanthamoeba* (Khan et al. 2002; Paknejad et al. 2020).

Beaches are favored destinations for seawater recreation and promote the risk of *Acanthamoeba* infection. This study determined the presence of *Acanthamoeba* in dry sand, wet sand and seawater from the beach environment, genotyped and characterized the

pathogenicity potential of isolated strains using DNA sequencing and physico-chemical properties, respectively.

MATERIALS AND METHODS

Sample collection

Samples were obtained from six popular beaches in Phuket Province, Thailand (Figure 1), including Nai Harn (7°46'26.8 "N, 98°18'23.7"E), Kata (7°48'51.1"N, 98°17'55.1"E), Karon (7°49'56.8"N, 98°17'40.1"E), Patong (7°53'16.3"N, 98°17'27.9"E), Kamala (7°57'13.6"N, 98°16'56.7"E) and Surin (7°58'28.3"N, 98°16'42.5"E), between December 2016 and February 2017 which was the high season period. Three sampling type were collected during low tide (Figure 2). Dry sand and wet sand were sampled from the surface to 10 cm depth at locations 5 m above the high-tide mark and halfway between the high-tide mark and the current water level, respectively. Seawater samples were collected at 5 m below the current water level. Each sampling distance was 500 meters apart. The number of samples collected depended on the length of each beach. Each sand sample was collected at 500 g and placed in a sterile plastic bag while each seawater sample was collected in a volume of 2L and placed in a sterile bottle. All samples were placed at room temperature and transported to the laboratory for subsequent analysis.

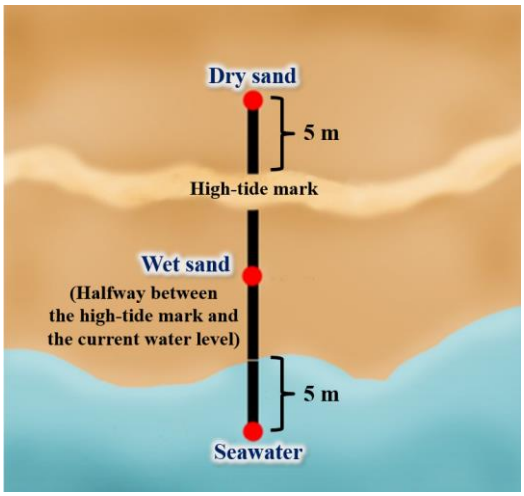

Figure 2. Sampling locations for dry sand, wet sand and seawater

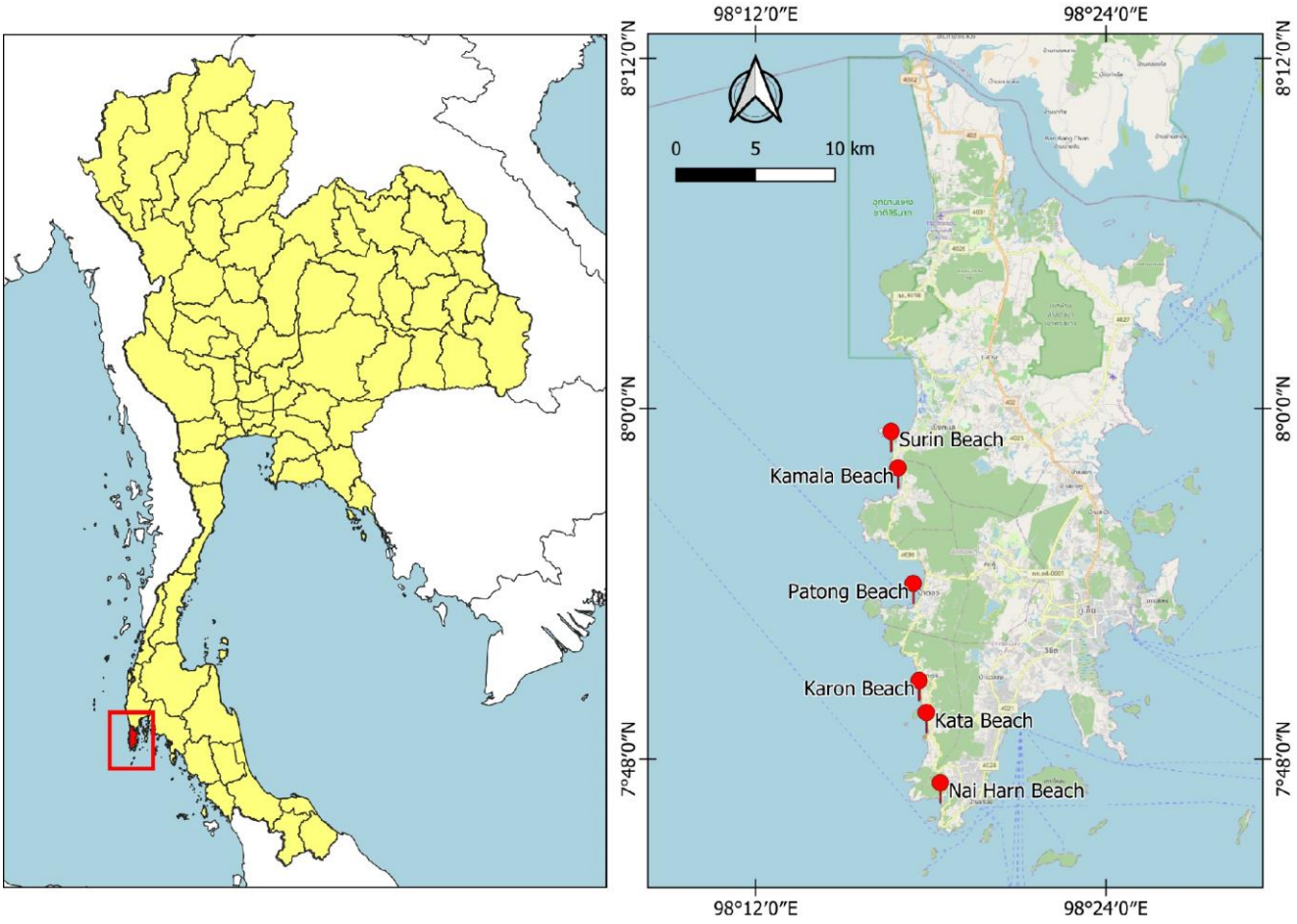

Figure 1. Map of six beaches in Phuket Province, Thailand depicting sample collection localities

Analysis of sample physico-chemical properties

Temperatures were measured at each sampling time. The thermometer was placed in the sand or seawater at 5 cm depth for at least 2 min and the measurement was repeated after 1 min. Values that varied at not more than 1°C were recorded.

For pH and salinity measurements, dry and wet sand was dried at 105°C for 24 h. Then, 40 g of each sample was mixed with 40 mL distilled water for 15 min and left for 1 min to settle. The pH of each sand mixture and seawater was measured using a pH meter, while the salinity was measured with an EC-meter (HI3512 Two Channels pH/ORP/ISE, EC/TDS/NaCl Resistivity Temperature Bench Meter, Romania).

The moisture percentages of dry and wet sand were determined by placing 30 g of each sample in a Petri dish, heating at 105°C for 24 h and then measuring the dry weight. The moisture percentage was calculated as follows:

$$\% \text{ Moisture} = [(30 - \text{dry weight})/30] \times 100$$

All experiments were performed in triplicate. The results were shown as mean ± S.D.

***Acanthamoeba* cultivation**

Each sand sample (≈ 0.5 g) was directly placed on the center of a 2% non-nutrient agar (NNA, consisting of agar 20 g; KCl 0.004 g; $CaCl_2$ 0.03 g; $MgSO_4 \bullet 7\ H_2O$ 0.01 g; distilled water to 1,000 mL; adjusted to pH 6.8-7.0 with 1 M HCl or 1 M Tris) plate coated with heat-killed *Escherichia coli* and incubated at 30°C. Seawater (500 mL) was filtered through a cellulose nitrate membrane with pore size 0.45 µm. Each filter was placed on a 2% NNA plate spread with heat-killed *E. coli* and incubated at 30°C for 3 days. The filter was then removed and the plate was further incubated. Each sand or water sample was cultivated in 3 plates and examined daily for the presence of *Acanthamoeba* trophozoites and cysts under an inverted light microscope (Nikon Eclipse TS100, Germany). A single cyst of each sample was picked up with an inoculating needle under the inverted light microscope and re-cultivated on a fresh NNA plate with heat-killed *E. coli*. The procedure was repeated until the pure isolate was established.

***Acanthamoeba* genotyping**

Acanthamoeba cysts from the NNA plates were harvested and washed with phosphate-buffered saline (pH 7.4). *Acanthamoeba* cysts (4×10^5 cells/mL) were centrifuged at 5,000 rpm, 4°C for 5 min. Two hundred microliters of 10% (w/v) Chelex solution in 0.1% Triton X-100 and 10 mM Tris buffer (pH 8.0) were added into the cyst suspension. The mixture was vortexed for 10 s then incubated at 95°C for 20 min and finally centrifuged at 10,000 rpm for 20 s. The supernatant containing genomic DNA was collected and used as the PCR template. PCR amplification of the GTSA.B1 amplicon was generated using genus-specific CRN5 primer (5'-CTGGTTGATCCTGCCAGTAG-3') and 1137 primer (5'-GTGCCCTTCCGTCAAT-3') (Schroeder et al. 2001). Thermal cycles were programmed to run a cycle of 7 min at 95°C, followed by 20 cycles of 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C and finished with 25 cycles of 1 min at 95°C and 2 min at 72°C. The amplicons (≈ 1,475 bp) were visualized by electrophoresis in 1% agarose with staining by ethidium bromide. The PCR product was purified and sequenced using six primers:

CRN5 primer (5′-CTGGTTGATCCTGCCAGTAG)
1137 primer (5′-GTGCCCTTCCGTCAAT)
373 primer (5'-TCAGGCTCCCTCTCCGGAATC-3')
570C primer (5'- GTAATTCCAGCTCCAATAGC -3')
892 primer (5'- CCAAGAATTTCACCTCTGAC -3')
892C primer (5'- GTCAGAGGTGAAATTCTTGG -3')

Multiple alignments were performed using ClustalW in BioEdit 7.0, blasted with known sequences from the NCBI database and submitted to GenBank. A phylogenetic tree was then constructed using maximum likelihood in MEGA6 with 1,000 replications (Tamura et al. 2013). Three clinical isolates, ALX and ANR from the AK patients and AGE from a GAE patient, were used as the control strains.

Thermo-tolerance and salt-tolerance

All isolates were determined for their thermo- and salt-tolerance properties as modified from Booton et al. (2004). Heat-killed *E. coli* was streaked in a straight line along the diameter of each 2% NNA plate. For thermo-tolerance, 5 µL of *Acanthamoeba* cysts (4×10^5 cells/mL) were inoculated at the end of the bacterial streak and incubated at 30°C, 34°C, 37°C and 42°C for 5 days. For salt-tolerance, 2% NNA was adjusted to 1%, 2% and 3% NaCl, then *Acanthamoeba* cysts were placed at the end of the *E. coli* streak and incubated at 30°C for 5 days. The experiments were performed in triplicate. The amoeba growth was indirectly determined by measuring the distance of trophozoite movement along the bacterial line. The percentage of trophozoite migration was calculated as follows:

$$\% \text{ Migration} = [\text{Migration distance} / \text{Total distance}] \times 100$$

Thermo- and salt-tolerance potentials were classified into five levels based on their percentage migration including-(0%), + (1-25%), ++ (26-50%), +++ (51-75%) and ++++ (76-100%). Thermo- and salt-tolerance properties were compared to the clinical isolates.

RESULTS AND DISCUSSION

Acanthamoeba detection

This research examined *Acanthamoeba* in dry sand (DS), wet sand (WS) and seawater (SW) collected from six beaches in Phuket Province, Thailand including Nai Harn (NH1-NH2), Kata (KT1-KT3), Karon (KR1-KR6), Patong (PT1-PT5), Kamala (KM1-KM3) and Surin (SR1-SR2). After cultivation, *Acanthamoeba* was found in 11 dry sand, 21 wet sand and 3 seawater samples as 52.4%, 100% and 14.3% of each sampling type, respectively (Table 1). Results indicated that *Acanthamoeba* could live in a variety of environments, concurring with Booton et al. (2004). They studied the prevalence of *Acanthamoeba* in beach samples collected from Fort Lauderdale Beach, Hollywood Beach and Hobe Beach, USA. *Acanthamoeba* was detected at 44% in dry sand and at 32% in wet sand, whereas the amoebae were not found in seawater samples.

Sample physico-chemical properties

The prevalence of *Acanthamoeba* in this study was higher than from the three beaches in Florida, USA as described above. This may be due to the different environment in terms of temperature, pH, moisture and salinity as major factors in the growth and survival of *Acanthamoeba.* Physico-chemical properties of dry sand samples revealed temperature between 31.00 and 34.00°C, pH 8.69-8.99, 0.02-0.54% salinity and 1.91-5.88% moisture. Wet sand samples exhibited temperature between 30.00 and 33.50°C, pH 8.48-9.51, 0.54-2.51% salinity and 7.15-31.00% moisture. For seawater samples, temperatures were between 31.30 and 31.90°C, pH 7.74-7.99 and 2.90-2.95% salinity (Table 1).

Table 1. The physico-chemical properties of each *Acanthamoeba* positive sample

Sample	Temperature (°C)	pH	Salinity (%)	Moisture (%)
DSNH1	33.70 ± 0.49	8.86 ± 0.02	0.53 ± 0.01	5.88 ± 0.05
DSNH2	33.00 ± 0.21	8.80 ± 0.02	0.46 ± 0.05	5.22 ± 0.05
DSKT1	32.00 ± 0.28	8.81 ± 0.03	0.43 ± 0.05	4.33 ± 0.05
DSKT2	33.00 ± 0.00	8.92 ± 0.01	0.54 ± 0.01	5.48 ± 0.02
DSKT3	34.00 ± 0.07	8.77 ± 0.01	0.45 ± 0.01	4.54 ± 0.05
DSKR1	34.00 ± 0.14	8.71 ± 0.03	0.28 ± 0.01	2.98 ± 0.08
DSKR4	34.00 ± 0.21	8.69 ± 0.01	0.26 ± 0.01	2.55 ± 0.05
DSKM1	33.00 ± 0.00	8.99 ± 0.02	0.02 ± 0.00	3.80 ± 0.03
DSKM2	34.00 ± 0.07	8.97 ± 0.02	0.02 ± 0.00	2.03 ± 0.09
DSKM3	32.00 ± 0.28	8.84 ± 0.10	0.02 ± 0.00	3.63 ± 0.06
DSSR2	31.00 ± 0.14	8.86 ± 0.02	0.03 ± 0.00	1.91 ± 0.02
WSNH1	30.50 ± 0.42	9.22 ± 0.04	2.09 ± 0.02	19.51 ± 0.34
WSNH2	32.50 ± 0.49	9.22 ± 0.01	2.11 ± 0.09	23.44 ± 0.11
WSKT1	30.00 ± 0.07	8.71 ± 0.26	2.17 ± 0.13	29.95 ± 0.05
WSKT2	30.50 ± 0.21	9.11 ± 0.02	2.23 ± 0.07	31.00 ± 0.31
WSKT3	31.90 ± 0.28	9.19 ± 0.04	2.35 ± 0.01	26.57 ± 0.10
WSKR1	32.00 ± 0.00	9.48 ± 0.01	1.65 ± 0.01	17.53 ± 0.15
WSKR2	31.50 ± 0.07	9.51 ± 0.01	1.70 ± 0.10	19.04 ± 0.13
WSKR3	32.00 ± 0.14	9.42 ± 0.05	1.64 ± 0.06	17.27 ± 0.30
WSKR4	32.20 ± 0.21	9.22 ± 0.04	1.75 ± 0.07	14.58 ± 0.06
WSKR5	33.00 ± 0.00	8.86 ± 0.02	0.70 ± 0.03	7.15 ± 0.32
WSKR6	33.00 ± 0.07	9.29 ± 0.02	1.48 ± 0.05	19.18 ± 0.31
WSPT1	33.00 ± 0.00	9.10 ± 0.01	2.23 ± 0.06	25.36 ± 0.15
WSPT2	33.50 ± 0.14	9.27 ± 0.02	1.91 ± 0.06	22.81 ± 0.23
WSPT3	32.00 ± 0.00	9.30 ± 0.03	2.51 ± 0.18	26.12 ± 0.11
WSPT4	31.70 ± 0.35	9.31 ± 0.01	2.27 ± 0.06	25.33 ± 0.16
WSPT5	31.50 ± 0.28	9.30 ± 0.02	2.30 ± 0.08	25.70 ± 0.08
WSKM1	32.10 ± 0.49	8.97 ± 0.04	1.51 ± 0.01	17.75 ± 0.15
WSKM2	32.00 ± 0.00	8.95 ± 0.02	1.39 ± 0.07	18.16 ± 0.21
WSKM3	31.00 ± 0.42	8.48 ± 0.02	1.76 ± 0.04	25.76 ± 0.13
WSSR1	32.00 ± 0.21	8.74 ± 0.02	0.54 ± 0.03	7.23 ± 0.25
WSSR2	31.00 ± 0.00	9.04 ± 0.02	0.93 ± 0.03	12.91 ± 0.05
SWPT3	31.90 ± 0.00	7.99 ± 0.02	2.95 ± 0.04	ND
SWPT5	31.80 ± 0.07	7.74 ± 0.01	2.95 ± 0.00	ND
SWKM3	31.30 ± 0.07	7.95 ± 0.01	2.90 ± 0.02	ND

Note: Data are means ± standard deviation of three replication; ND represents did not determine

Results showed that *Acanthamoeba* content in all wet sand samples was substantially greater than in dry sand and seawater samples. This may be because the wet sand received moisture from the seawater tide and was not directly exposed to sunlight like the dry sand; therefore, temperature and moisture were more appropriate for *Acanthamoeba* growth and survival. Conversely, *Acanthamoeba* was found in a few seawater samples. Many factors in seawater, including sunlight, airwaves and cold and warm water flows, made the temperature unstable and unsuitable for *Acanthamoeba* growth. The amoebae do not directly live in the seawater but establish themselves in the wet sand. Sometimes they are flushed into the seawater by the water current. Microbial factors also affected the growth of *Acanthamoeba* in the environment. Bonilla et al. (2007) surveyed fecal coliform bacteria in sand and seawater samples. They found that dry sand and wet sand contained more bacterial contamination than seawater at up to 30-460 times and 2-23 times, respectively. These bacteria served as a food source for *Acanthamoeba*. These data supported our results that the prevalence of *Acanthamoeba* was higher in sand than in seawater. Although dry sand provided more abundant food, the growth of *Acanthamoeba* depended more on the physico-chemical properties of the sand than the number of food bacteria. Halliday and Gast (2011) reported that bacteria can attach to sand particles longer than to seawater. Therefore, *Acanthamoeba* attached preferentially to the bacterial biofilm that protected them from being destroyed and lived longer in the sand. Moreover, Mohd Hussain et al. (2022) determined a significant correlation between total coliforms and the presence of *Acanthamoeba* which confirmed the higher *Acanthamoeba* prevalence in areas with more abundant bacteria.

Acanthamoeba genotyping

A total of 35 *Acanthamoeba* isolates from the beach samples were further cultivated to obtain the pure isolates. However, only 31 pure cultures were successfully established because four samples, including DSKM3, WSNH1, WSKM2 and SWPT5 were contaminated with fungi and could not be maintained (Table 2). Genotypes of these 31 pure isolates were determined using three clinical isolates, ALX, ANR and AGE, as the positive control. The PCR product was approximately 1,475 bp and specific to the genotype of *Acanthamoeba* since it covered eight variable sub-regions that were sufficient to distinguish the genotypes. Each sequence has been deposited in GenBank database under accession numbers between OP218959 and OP218992. A phylogenetic tree (Figure 3) was obtained using maximum likelihood in MEGA6 with 1,000 replications. Sequence analysis revealed that the T5 genotype was the most prevalent in 83.9% of the sequenced isolates obtained from 10 dry sand (DSNH1, DSNH2, DSKT1, DSKT2, DSKT3, DSKR1, DSKR4, DSKM1, DSKM2 and DSSR2), 14 wet sand (WSNH2, WSKT3, WSKR1, WSKR3, WSKR4, WSKR5, WSKR6, WSPT1, WSPT2, WSPT5, WSKM1, WSKM3, WSSR1 and WSSR2), and 2 seawater (SWPT3 and SWKM3) samples. The rest of the sequences belonged to T4 genotypes as 16.1% of sequenced isolates originating from 5 wet sand samples (WSKT1, WSKT2, WSKR2, WSPT3 and WSPT4) (Figure 3 and Table 2). Our results demonstrated that both T4 and T5 genotypes were widely distributed in beach environments, with T5 predominant.

Our results were remarkably different from Booton et al. (2004). They determined that out of 20 *Acanthamoeba* isolates from dry and wet sand samples in Florida, USA only one belonged to the T5 genotype, with the others classified into T4. This may be explained because beach temperatures in Thailand are higher than in the USA. In addition, Mahmoudi and Karanis (2020) reported the presence of T2 and T4 genotypes in the Caspian Sea, Iran, with T2 predominant. These findings indicated the different predominant genotypes in the beach samples of different countries. *Acanthamoeba* in the T5 genotype has been reported to grow better at high temperatures than the T4 genotype (Booton et al. 2004), caused by the expression of specific genes that adapt to the environment. Genotypes of *Acanthamoeba* isolated from natural sources with high temperatures such as hot springs should be examined. The predominant genotype should be T5, as found in this study. Sudjaroen et al. (2017) determined the characteristics of mineral water from natural hot springs in Ranong, Thailand. Although fecal coliform bacteria were not found, the water contained nutrients and minerals sufficient for *Acanthamoeba* survival.

Thermo-tolerance and salt-tolerance

Thermo-tolerance and osmotolerance assays are the easiest and acceptable techniques for determining *Acanthamoeba* pathogenicity. Khan et al. (2002) reported that pathogenic *Acanthamoeba* grew well at 37°C in medium with 1 M mannitol, while the non-pathogenic *Acanthamoeba* grew slowly or not at all. In this study, *Acanthamoeba* isolated from different sources were cultivated and incubated at 30°C, 34°C, 37°C and 42°C for 5 days. Migration rates of the trophozoites along the bacterial line were evaluated by grading ranges from -, +, ++, +++ and ++++, as shown in Table 2. Results indicated that *Acanthamoeba* isolated from beach samples had the same growth characteristics as *Acanthamoeba* isolated from patients and grew well at 34°C and 37°C as the average temperature in the human eyes and body, respectively. Therefore, all *Acanthamoeba* isolates in this study could cause diseases in humans. However, the growth of *Acanthamoeba* in this research did not depend on genotype since all isolates did not grow at 42°C. Booton et al. (2004) reported that two T5 genotypes isolated from soil samples grew at 37°C and 40°C but did not grow at 42°C, whereas one T5 genotype isolated from a dry sand sample grew at 37°C, 40°C and 42°C. On the other hand, all T4 genotypes only grew at 37°C. This finding supported that the T5 genotype can grow at higher temperatures than the T4 genotype but some isolates cannot grow at 42°C.

For osmotolerance assays, we slightly modified to determine salt-tolerance since *Acanthamoeba* in this study naturally lived in environments containing NaCl. All isolates were cultivated on 2% NNA with 0%, 1%, 2% and 3% NaCl. After incubation at 37°C for 5 days, the

migration rate was recorded in the same manner by the thermo-tolerance assay. Results showed that all environmental isolates grew in 0%, 1% and 2% NaCl, similar to the clinical isolates (Table 2). Average salinity in the human body is between 0.85 and 0.90% NaCl; therefore, all beach *Acanthamoeba* isolates in this research had the potential to infect humans. Seven isolates did not grow in 3% NaCl. By contrast to thermo-tolerance, salt-tolerance did not depend on the genotype.

Both T4 and T5 genotypes involve in AK and GAE infections (Walochnik et al. 2015). Interestingly, Boonman et al. (2012) found that the T5 genotype was more resistant to treatment drugs than the T4 genotype. Therefore, providing information about the dangers of *Acanthamoeba* to tourists may reduce the risk of blindness or death caused by this silent pathogen lying in wait on the beaches.

Table 2. The genotype, thermo-tolerance, and salt-tolerance of each *Acanthamoeba* isolate

Sample	Genotype	Thermo-tolerance				Salt-tolerance			
		30°C	34°C	37°C	42°C	0% NaCl	1% NaCl	2% NaCl	3% NaCl
ALX	T5	+++	++++	++++	-	+++	++	++	+
ANR	T4	+++	++++	++++	-	+++	++	++	+
AGE	T4	+++	++++	++	-	+++	+	+	+
DSNH1	T5	++++	++++	++++	-	++++	++	+	+
DSNH2	T5	+++	++++	++++	-	+++	++	++	+
DSKT1	T5	+++	++++	++++	-	+++	++	++	+
DSKT2	T5	+++	++++	+++	-	+++	++	++	+
DSKT3	T5	+++	++++	++++	-	+++	++	++	+
DSKR1	T5	++++	++++	++++	-	++++	++	++	-
DSKR4	T5	++++	+++	+++	-	++++	++	+	-
DSKM1	T5	++++	++++	++++	-	++++	++	+	+
DSKM2	T5	+++	++++	++++	-	+++	++	+	+
DSKM3	ND	ND	ND	ND	ND	ND	ND	ND	ND
DSSR2	T5	++++	++++	++++	-	++++	++	+	-
WSNH1	ND	ND	ND	ND	ND	ND	ND	ND	ND
WSNH2	T5	++++	++++	++++	-	++++	+++	+	+
WSKT1	T4	+++	++++	+++	-	+++	++	++	+
WSKT2	T4	++	++++	++++	-	++	++	++	-
WSKT3	T5	+++	++++	++++	-	+++	++	+	+
WSKR1	T5	+++	++++	++++	-	+++	++	++	+
WSKR2	T4	++	+++	++	-	++	++	++	+
WSKR3	T5	+++	++++	++++	-	+++	++	++	-
WSKR4	T5	+++	++++	++++	-	+++	++	++	+
WSKR5	T5	++	++++	++++	-	++	++	++	+
WSKR6	T5	+++	++++	++++	-	+++	++	++	+
WSPT1	T5	+++	++++	++++	-	+++	++	++	+
WSPT2	T5	++++	++++	++++	-	++++	++	++	+
WSPT3	T4	+++	++++	++++	-	+++	++	++	+
WSPT4	T4	+++	++++	++++	-	+++	++	+	+
WSPT5	T5	+++	++++	++++	-	+++	++	++	+
WSKM1	T5	+++	++++	++++	-	+++	++	+	+
WSKM2	ND	ND	ND	ND	ND	ND	ND	ND	ND
WSKM3	T5	++++	++++	++++	-	++++	++	++	-
WSSR1	T5	++++	+++	++++	-	++++	++	+	+
WSSR2	T5	++++	++++	++++	-	++++	++	++	-
SWPT3	T5	++++	++++	++++	-	++++	++	+	+
SWPT5	ND	ND	ND	ND	ND	ND	ND	ND	ND
SWKM3	T5	++++	++++	++++	-	++++	++	++	+

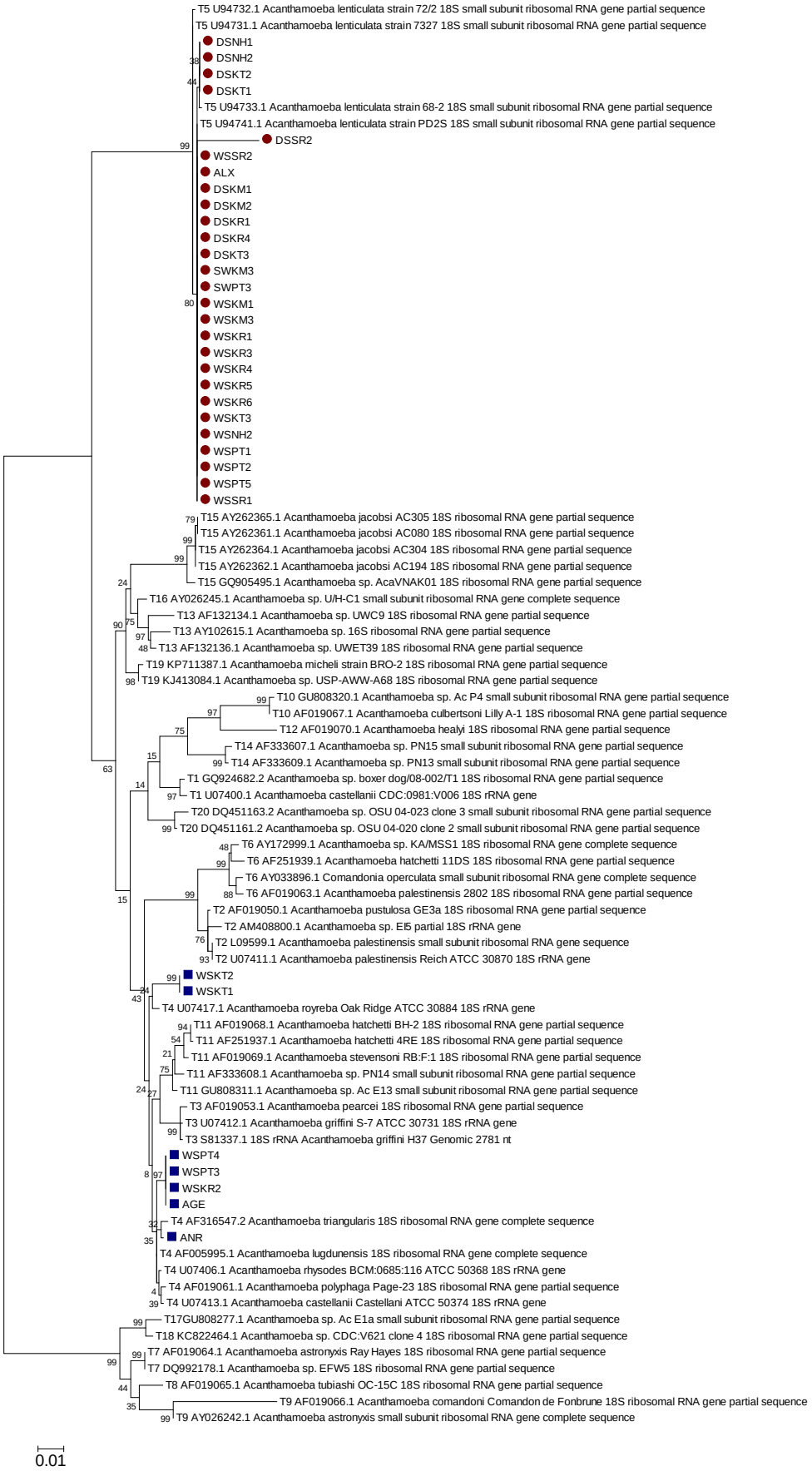

Figure 3. Phylogenetic tree of *Acanthamoeba* isolated from beaches in Phuket Province, Thailand constructed by Maximum likelihood with 1,000 replications

In conclusion, this research examined *Acanthamoeba* in sand and seawater samples collected from 6 popular beaches in Phuket Province, Thailand during the high season. The amoebae were recovered mainly from the wet sand, followed by the dry sand and the seawater samples, respectively. This may be due to the temperature, pH, moisture and salinity in the wet sand provide a more favorable environment for *Acanthamoeba* than the others. Both T4 and T5 genotypes were widely distributed in this investigation, with T5 predominant. All isolates revealed their pathogenic potential based on thermo-tolerance and salt-tolerance properties. However, further study on their ability to produce a cytopathic effect on human cell monolayer and their sensitivity to the chemotherapeutic agents are still required to obtain more valuable information.

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