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Isolation and characterization of culturable thermophilic bacteria from hot springs in Benguet, Philippines

Socorro Martha Meg-ay V. Daupan[1] and Windell L. Rivera*[1,2]

[1]Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City, 1101, Philippines
[2]Natural Sciences Research Institute, University of the Philippines, Diliman, Quezon City, 1101, Philippines

Abstract—Despite the numerous geothermal environments in the Philippines, there is limited information on the composition of thermophilic bacteria within the country. This study is the first to carry out both culture and molecular-based methods to characterize thermophilic bacteria from hot springs in the province of Benguet in the Philippines. The xylan-degrading ability of each isolate was also investigated using the Congo red method. A total of 14 phenotypically-different isolates (7 from Badekbek mud spring and 7 from Dalupirip hot spring) were characterized. Phylogenetic analysis based on the nearly complete 16S rDNA sequences revealed that all the isolates obtained from Badekbek were affiliated with *Geobacillus*, whereas the isolates from Dalupirip clustered into 3 major linkages of bacterial phyla, Firmicutes (72%) consisting of the genera *Geobacillus* and *Anoxybacillus*; Deinococcus-Thermus (14%) consisting of the genus *Meiothermus*; and Bacteroidetes consisting of the genus *Thermonema* (14%). In addition, xylan-degrading ability was observed in all isolates from Badekbek and in 2 isolates from Dalupirip which showed high sequence similarity with *Geobacillus* spp. The results are also essential in understanding the roles of the physico-chemical properties of hot spring water as a driver of thermophilic bacterial compositions.

Keywords—16S rDNA sequencing, hot springs, Philippines, thermophilic bacteria

INTRODUCTION

The discovery of thermophilic bacteria capable of carrying out life processes in the boiling hot springs of Yellowstone National Park has become a foundation of developments in medicine and biotechnology. Since then, thermophiles have been isolated in geothermal features all over the world. Thermophilic bacteria are found in various geothermally-heated regions of the earth such as hot springs, deep sea hydrothermal vents and volcanic craters (Stetter et al. 1993). They can also live in fermenting materials that can produce heat such as compost piles and garbage landfills (Fujio and Kume 1991). The ability of thermophiles to proliferate at elevated temperatures is attributed to the thermally-stable macromolecules they possess (Zeikus et al., 1998). As a consequence of growth at high temperatures and unique macromolecular properties, thermophiles exhibit high metabolic rates, thereby generating greater end-products despite lower growth rates compared to mesophiles. They also provide physically and chemically stable enzymes that are of significant use to industries (Haki and Rakshit 2003). Thermophiles have provided an interesting and challenging platform for researchers since the time of their discovery. However, due to difficulties in isolation and maintenance of the pure culture, their diversity in thermal habitats remains to be explored (Kikani and Singh 2011).

*Corresponding Author
Email Address: wlrivera@science.upd.edu.ph
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The Philippines is geographically situated in the Pacific Ring of Fire and is subject to numerous active volcanoes. The country boasts of bountiful natural resources including hot springs that provide good conditions for thermophilic bacterial growth. However, there is limited knowledge on the thermophile community in Philippine hot springs. Hot springs in Los Baños, Laguna, Philippines have been shown to harbor unique thermophiles. Fluorescence in situ hybridization (FISH) analysis showed that this site is dominated by microbial community belonging to domain Archaea, of which 63% were Crenarchaeota and 8% were Euryarchaeota and 17% were bacteria and the remaining 12% were unidentified (Lantican et al. 2011). Two novel hyperthermophilic crenarchaeotes have also been discovered from the same hot spring and the proposed names for these isolates were *Caldivirga maquilingensis* and *Caldisphaera lagunensis* (Itoh et al. 1999, Itoh et al. 2003). Hongmei and colleagues, on the other hand, described thermophilic microbial mats from Laguna hot springs (Hongmei et al. 2005).

This present study therefore aimed to isolate and characterize thermophilic bacteria from two hot springs in Benguet, Philippines and to assess their phylogenetic relationships.

MATERIALS AND METHODS

Sampling Sites

Benguet was selected because of its unique temperate climate compared to other provinces of the country (National Statistical Coordination Board 2005). Out of its 13 municipalities, hot springs are found in five of them. Two hot springs were

randomly selected for the isolation of thermophilic bacteria (Figure 1). These hot springs include Badekbek mud spring in Bokod and Dalupirip hot spring in Itogon. The temperature and pH of the hot springs were measured during sampling and the turbidity was visually assessed. The selected hot springs were found to vary greatly in terms of their physico-chemical properties. Badekbek mud spring had a temperature range of 78-80°C and Dalupirip hot spring had lower temperature range of 45-48°C. In terms of pH, the former had lower pH ranging from 3-4 and the latter had nearly neutral pH of 7-8.

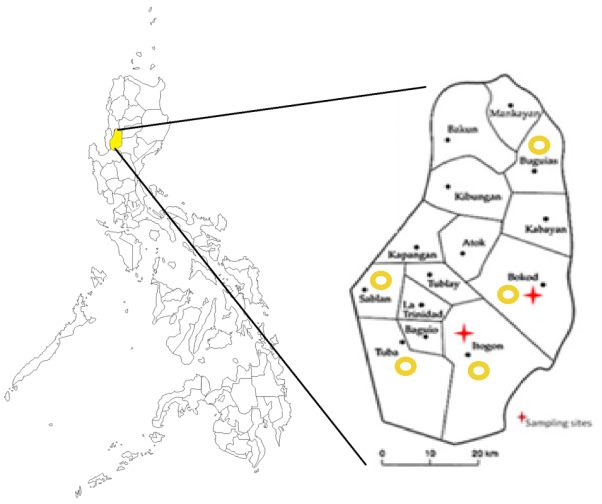

Figure 1. Map showing the geographical location of the sampling sites in Benguet, Philippines (marked with red star) and municipalities with hot springs (marked with yellow circle).

Sample Collection

Water samples were collected in triplicates at different points from the sampling sites using sterile thermal flasks (Ledbetter et al. 2007). All samples were immediately transported to the laboratory and directly inoculated onto solidified Thermus medium (ATCC medium 697) plates. This medium has the following composition (in micrograms per liter of deionized water): $CaSO_4 \cdot 2H_2O$, 60; $MgSO_4 \cdot 7H_2O$, 100; NaCl, 1000; KNO_3, 103; $NaNO_3$, 689; Na_2HPO_4, 111; $FeCl_3$, 2.8; $MnSO_4 \cdot H_2O$, 22; $ZnSO_4 \cdot 7H_2O$, 5; H_3BO_3, 5; $CuSO_4$, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.3; $CoCl_2 \cdot 2H_2O$, 0.5; and EDTA, 6 (Brock and Freeze 1969). Yeast extract (0.08%) and peptone (0.05%) were added and the medium was solidified with Phytagel (Sigma) at 1% final concentration. $MgSO_4$ (0.5%) was also added to make the solidifying agent heat stable. Concentrated H_2SO_4 (0.5ml) was added to dissolve the salts and the pH of the medium was adjusted to 7.0. All plates were then incubated for a maximum of 3 days at 60°C.

Purification and preservation of the isolates

Colonies were selected from each plate and were subjected to streaking on solidified Thermus medium at least three times. Single colony was picked and re-streaked on fresh solid media to obtain pure cultures. The colonies were observed under the microscope after several streaking to check for purity by assessing the homogeneity of cell morphology. Pure isolates were grown at 60°C for 24 hours and were suspended in Thermus broth containing 15% glycerol and stored at -70°C until use.

Phenotypic characterization

The morphological and biochemical characterizations of the isolates were done according to the methods of Elnasser et al. (2007) and Narayan et al. (2008) using 18 to 24-hour old cultures. These include examination of cultural characteristics, Gram staining, motility test, Kovac's oxidase and catalase test.

Screening for xylan-degrading isolates

Pure isolates were tested for xylan-degrading ability because xylanases offer a wide range of industrial and environmental significance since these enzymes are being used to replace chlorinated compounds in bleaching of wood for paper production (Kaur et al. 2010). The screening was done by inoculating a loopful of each isolate to Thermus medium containing 0.5% xylan. After incubation of the plates for 48 hours, 0.1% Congo red solution was poured onto the plates. The plates were incubated for 30 minutes at 65°C and washed with 1M NaCl solution. Clear zones surrounding the colonies on the red background dyed with Congo red solution is indicative of positive activity for xylanase since Congo red binds only to carbohydrate polymers (Cordeiro et al. 2002; Teather and Wood 1982).

DNA extraction

Cells from each of the 18 to 24-hour old cultures were harvested by centrifugation for 2 minutes at 10,000 rpm. The pellet was washed with 1X PBS; after which, it was resuspended in 10µl sterile distilled water. A 200µl aliquot of 5% Chelex was then added and the mixture was mixed vigorously. The mixture was incubated for 20 to 30 minutes in water bath at 56°C and spun at high speed using a vortex. The product was incubated for 8 minutes in a boiling water bath and spun for 2 to 3 minutes at 13,000 rpm. The supernatant was transferred to a new tube and stored at -20°C until use. The nucleic acid concentration of the crude DNA was estimated using NanoDrop 2000 (Thermo Scientific).

PCR amplification

Amplification of the 16S rRNA gene was conducted using a pair of universal primers, 27F/1492R (Lau et al. 2009). All PCR reactions were carried out under the following conditions: 4 minutes at 94°C, 30 cycles of 1 minute at 92°C, 1 minute at 45°C, 1 minute at 72°C; followed by 10 minutes at 72°C. PCR products were purified using Expin™ PCR SV Purification Kit according to the manufacturer's instructions. The PCR products were separated by gel electrophoresis at 100V for 25 minutes on 1X TAE buffer (Tris: Acetic acid: EDTA) and analyzed by staining with ethidium bromide under UV light. The purified PCR products were sent to Macrogen, Inc., South Korea for sequencing.

Phylogenetic analyses

The sequences consisting of 1,400-1,500 nucleotides were determined and assembled using MEGA 5.05 (Tamura et al. 2011). The sequences were then compared with those available in GenBank using BLAST search (http://www.ncbi.nlm.gov/blast/). All sequences were aligned using Clustal W algorithm in BioEdit 7.0.5.3 (Hall, 1999). A total of 24 sequences were consolidated. The optimal model for DNA substitution was determined using Bayesian Information Criterion (BIC) as selection strategy in jModelTest (Posada 2008). The site saturation test (Xia test) in DAMBE (http://dambe.bio.uottawa.ca/dambe.asp) was also performed to check for extreme substitution saturation that could be present in the sequence data (Xia et al. 2003). If saturation was observed in the sequence data, the succeeding phylogenetic analysis was discarded.

Phylogenetic trees were constructed from the aligned dataset using the neighbor-joining (NJ) (Saitou and Nei 1987) and maximum-likelihood (ML) (Cavalli-Sforza and Edwards 1967; Felsenstein 1981) methods with 1,000 bootstrap resamplings. A total of 1,510 unambiguously aligned nucleotide sites were used for the phylogenies using the nearly complete 16S rRNA dataset. NJ and ML trees were generated using PAUP* 4.0 (Swofford 2002) and PhyML 3.0 (Guindon 2010) programs, respectively.

Nucleotide sequence accession numbers

All DNA sequences were deposited in GenBank, under accession numbers KC252975-KC252981 for Badekbek isolates and KC252983-KC252989 for Dalupirip isolates.

RESULTS

Phenotypically different colonies that appeared after incubation on Thermus medium at 60°C from each hot spring site were selected for purification and characterization. A total of 14 thermophilic bacterial isolates (7 from each site) were obtained. It was noted that the isolates were either Gram-negative or Gram-variable. All the isolates had circular colony form and the cells were rod-shaped of several different morphologies from slender, long rods (thread-like) to small, nearly rounded rods. Several pigmented colonies were also obtained. Motility varied among the isolates, of which 79% were found to be motile (Table 1).

TABLE 1. Phenotypic characteristics of the 14 thermophilic bacterial isolates from Badekbek (Ba 1-7) and Dalupirip (Da 1-7) hot springs in Benguet, Philippines.

Isolate	Cultural characteristics			Gram-stain	Cell shape	Catalase activity	Oxidase activity	Motility	Xylanolytic activity
	Color	Form	Size (mm)						
Ba1	light yellow	circular	0.5-1	–	rods (in pair, forming v-shape)	–	–	+	+
Ba2	light yellow	circular	0.25-0.5	–	rods (in pair, forming v-shape)	–	–	+	+
Ba3	light yellow	circular	0.25-0.5	V	rods (short and long)	–	–	+	+
Ba4	light yellow	circular	0.25-0.5	V	rods (short and long)	–	+	+	+
Ba5	light yellow	circular	0.25-0.5	–	short rods	–	–	–	+
Ba6	light yellow	circular	0.25-0.5	V	rods (short and long)	–	+	+	+
Ba7	light yellow	circular	0.25-0.5	V	rods (short and long)	–	–	+	+
Da1	yellow	circular	0.5-0.75	–	long rods (thread-like)	+	–	–	–
Da 2	white	circular	0.5-0.75	–	Rods	+	–	+	+
Da 3	white	circular	1-1.5	–	rods in pairs	+	+	+	+
Da 4	white	circular	0.5-1	–	small rods	–	–	–	–
Da 5	pink	circular	0.5-0.75	–	rods	+	–	+	–
Da 6	white	circular	0.5-0.75	–	small rods	+	–	+	–
Da 7	white	circular	1-1.25	–	small rods	–	–	+	–

All the isolates from Badekbek mud spring and 2 more isolates from Dalupirip hot spring showed xylan-degrading ability as indicated by the cleared zones that formed on xylan plates following the Congo red method. Isolate Da3 exhibited the largest clearing zones. The other isolates had clearing zones with diameter ranging from 0.8mm to 1.5mm (Figure 2).

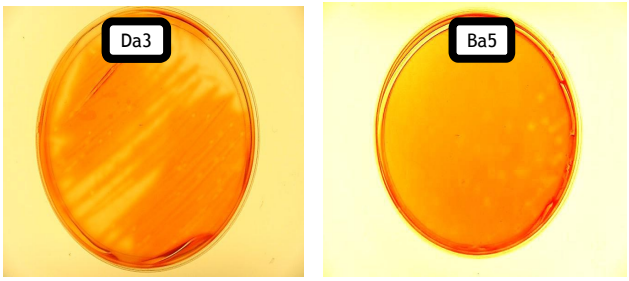

Figure 2. Thermus plates with 0.5% xylan showing clearing zones following the Congo red method. (a) Large clearing zones from plate inoculated with Da3 (previously assigned with code I4-b) (b) small rounded clearing zones from plate inoculated with Ba5 (previously Ba5-a).

BLAST searches based on the nearly complete 16S rDNA sequences of the 14 isolates showed that there was a strong similarity (> 98%) between the test isolates and representative strains of *Geobacillus*, *Thermonema*, *Meiothermus* and *Anoxybacillus*. Isolates fom Badekbek had high sequence similarity with either *G. thermoparaffinivorans* or *G. thermoleovorans*. In contrast, isolates from Dalupirip hot spring showed high sequence homology to the aforementioned genera (Table 2).

TABLE 2. Identity of the 14 thermophilic bacterial isolates based on BLAST searches.

Code (accession number)	Identity based on BLAST searches	Max Identity (%)	GenBank Accession No.	E-value (Query Coverage %)
Ba1(KC252975)	*Geobacillus thermoparaffinivorans* it-12	100	EU214615	0.0 (98)
Ba2(KC252976)	*Geobacillu sthermoparaffinivorans* it-12	99.9	EU214615	0.0 (99)
Ba3(KC252977)	*Geobacillus thermoparaffinivorans* it-12	99.8	EU214615	0.0 (99)
Ba4(KC252978)	*Geobacillus thermoparaffinivorans* it-12	99.7	EU214615	0.0 (99)
Ba5(KC252979)	*Geobacillus thermoleovorans*CCB	99.9	CP003125	0.0 (100)
Ba6(KC252980)	*Geobacillus thermoparaffinivorans* it-12	99.9	EU214615	0.0 (99)
Ba7(KC252981)	*Geobacillus thermoparaffinivorans* it-12	100	EU214615	0.0 (99)
Da1(KC252983)	*Thermonema rossianum* strain SC-1	99.4	Y08957.1	0.0 (96)
Da2(KC252984)	*Geobacillus kaustophilus* HTA426	99.8	BA000043	0.0 (99)
Da3(KC252985)	*Geobacillus stearothermophilus* P4	99.4	JF713055	0.0 (99)
Da4(KC252986)	*Anoxybacillus flavithermus* clone LK4	99.8	EU816689	0.0 (98)
Da5(KC252987)	*Meiothermus* sp. SK3-2	99.9	GU129930	0.0 (94)
Da6(KC252988)	*Geobacillus stearothermophilus*	99.0	AY491497	0.0 (99)
Da7(KC252989)	*Geobacillus stearothermophilus*	98.9	AY491497	0.0 (98)

The aligned sequences for the nearly complete 16S rRNA had a length of 1,510 bp. Site saturation test (Xia test) revealed little saturation in the sequences. The optimal models of DNA substitution for the 16S rRNA as determined by jModelTest (Posada 2008) using Bayesian Information Criterion (BIC) was TRN +G. The phylogenetic relationships of the 14 thermophilic bacterial isolates and closely-related species were determined using the neighbor-joining (NJ) (Saitou and Nei 1987) and maximum-likelihood (ML) (Cavalli-Sforza and Edwards 1967; Felsenstein 1981) methods. The branches that corresponded to partitions reproduced below 50% bootstrap replicates were collapsed. The generated dendrogram revealed 3 clades supported by high bootstrap values (Figure 3). These clades are represented by 3 major lineages, namely: Firmicutes (72%) consisting of the genera *Geobacillus* and *Anoxybacillus*; Deinococcus-Thermus (14%) consisting of the genus *Meiothermus*; and Bacteroidetes consisting of the genus *Thermonema* (14%). This third clade was observed to form distinct lineage from the first two clades. The xylan-degrading isolates clustered within the clade of Firmicutes and the brightly-pigmented isolates that did not exhibit xylan-degrading ability formed separate clades. Interestingly, 2 isolates (Da 6 and Da 7) that did not exhibit xylan-degrading ability formed a separate cluster within the first clade. It is also noted that isolates from Badekbek mud spring did not exhibit great diversity and all isolates fell under the first clade showing their close association with *G. thermoparaffinivorans*. The dendrogram also shows clustering of a member of the genus *Bacillus* to *Geobacillus* spp.

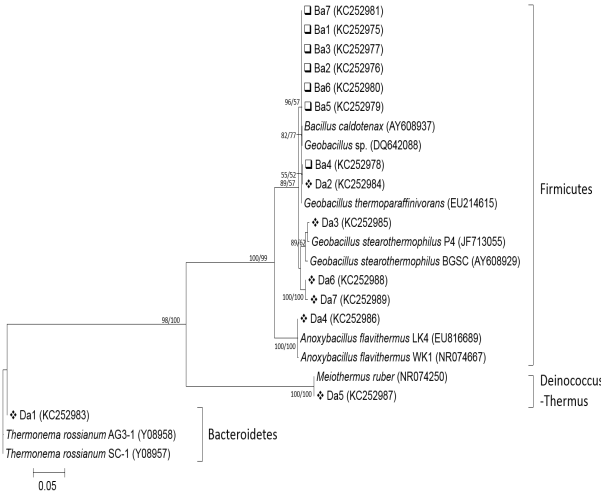

Figure 3. Maximum-likelihood tree of the 14 isolates and their closest relatives based on the nearly complete 16S rRNA gene (1,510 bp) using TRN+G as model of DNA substitution. The two values at the nodes represent the bootstrap values from ML and NJ. Only bootstrap values above 50% are shown at the nodes (based on 1000 bootstrap resamplings). Scale bar indicates 5 nucleotide changes for every 1,000 nucleotides. Bullets indicate xylan-degrading isolates.

DISCUSSION

This study is the first to describe thermophilic bacterial compositions in Benguet hot springs in the Philippines using both culture-based methods and molecular techniques. Non-culturable bacteria may play significant roles in nutrient cycling and can be identified by molecular techniques such as denaturing gradient gel electrophoresis (DGGE) and FISH. Despite potential significant ecological roles, non-culturable bacteria will be of little significance for industrial applications. Among the 14 isolates, 79% were found to be closely-related to the genus *Geobacillus* based on BLAST searches. Representatives of this genus have been found in both natural and man-made habitats throughout the world, indicating that these groups of thermally-adapted bacteria are widespread in various habitats and may have significant impact on soil and water geochemistry (Rahman et al. 2007). The growth temperature for *Geobacillus* ranges from 35 to 78°C and the major fatty acids are iso-branched saturated fatty acids (iso-15:0, iso-16:0 and iso 17:0) (Rahman et al. 2004). Members of the genus *Geobacillus* have rod-shaped cells, occurring singly or in short chains; colonies may show variable shape and may exhibit pigmentation (Nazina et al. 2001). All isolates from Badekbek mud spring had light yellow pigmentation and were found to be affiliated with *Geobacillus*. Although most species of *Geobacillus* are Gram-positive, their cell wall structure using Gram stain may vary between positive or negative (Nazina et al. 2001). Members of the genus *Geobacillus* have been previously shown to produce xylanases. *G. stearothermophilus* T-6, for instance, produces two selective family 10 xylanases that complete the degradation of xylan (Solomon et al. 2007). Research has focused mainly on two of families of xylanase containing glycoside hydrolase and these are families 10 and 11. Family 10 xylanases have been isolated from various thermophilic and hyperthermophilic microorganisms including species of *Thermotoga, Caldocellulosiruptor, Rhodothermus,* and *Bacillus*. The other enzymes with xylanase activity that belong to other families are also studied, albeit to a lesser extent. Most of the xylanases that are of bacterial origin have optimum activity at approximately 40 to 60°C and a number of extremophilic xylanases have been described due to the industrial demand for such enzymes that can operate under process conditions (Collins et al. 2005). In the present study, only 2 isolates with high sequence similarity to *Geobacillus* did not exhibit xylan-degrading ability.

Isolate Da4 was found to be affiliated with *Anoxybacillus*. Members of the genus *Anoxybacillus* are predominantly aerotolerant anaerobe although aerobic growth has been observed in some species (Pikuta et al. 2003).

One isolate (Da5) was found to be affiliated with *Meiothermus ruber*. In natural environments, representative strains of this genus are exclusively found in thermal limnetic systems, predominantly in terrestrial hot springs (Zhang et al. 2010). This isolate showed similar phenotypic properties with *Meiothermus* sp. SK3-2 having circular colony form with smooth margin and glistening surface with

pink pigmentation (Goh et al. 2011). Although this isolate was not able to degrade xylan, its pigmentation is noteworthy. Pigments from bacteria are being exploited for the production of natural dyes since bacteria produce better yields as opposed to dye extraction from eukaryotes. Natural dyes are preferred over synthetic ones since the former exhibit better biodegradability (Ahmad et al. 2012).

Another isolate (Da1) showed high sequence similarity with *Thermonema rossianum*, a bacterium that has been found to be polyextremophile being both thermophilic and halophilic. This isolate exhibited yellow pigmentation and filamentous cellular morphology typical of *T. rossianum* (Tenreiro et al. 1997). The yellow pigment is probably carotenoid.

The clustering of the genus *Geobacillus* with *Bacillus caldotenax* as seen in Figure. 3 may suggest the need for further reclassification of certain members of the genus *Bacillus*. The genus *Bacillus* is a diverse group of bacteria that has progressively been subdivided into the novel genera *Brevibacillus, Paenibacillus, Salibacillus*, and most recently, *Geobacillus* based on separate phylogenetic groupings derived from the 16S rRNA gene sequence information (Nazina et al. 2001). This also supports the conclusion of stating that 16S rDNA analysis alone may be insufficient to distinguish between some closely-related species possibly because of the existence of multiple 16S rRNA operons and the occurrence of recombination within the strain (Meintanis 2008; Vandamme et al. 1996). A more reliable approach to discriminate thermophilic bacteria even at strain levels would be the use of other genomic fingerprint method, like REP-PCR.

The constructed phylogenetic tree also shows that culturable thermophilic bacterial community in Dalupirip hot spring is more diverse in comparison to that of Badekbek, which is sulfur, acidic mud spring. This implies the importance of physico-chemical properties of hot spring water as a driver of thermophilic bacterial compositions.

CONCLUSION

This study has identified the thermophilic bacterial compositions of hot springs in Benguet and revealed the industrial significance of the isolates, particularly in xylan degradation. The sites were found to be dominated by species of *Geobacillus*. Members of *Meiothermus*, *Thermonema*, and *Anoxybacillus* were also isolated in Dalupirip hot spring. Dalupirip hot spring showed greater diversity of bacteria because of the difference in physico-chemical characteristics of the location. It had a lower temperature range of 45-48°C that can support more bacteria. The higher temperature range in Badekdek hot spring supports the existence of the genus *Geobacillus,* which have been previously shown to produce xylanases. The clustering of *Bacillus caldotenax* with *Geobacillus* spp. in the generated dendrogram suggests the need for further analysis and possibly reclassification of certain species of *Bacillus* to *Geobacillus*. The results also imply the need to develop medium for culturing higher number of thermophilic bacteria. This work paves the way for a comparative diversity studies for thermophilic bacteria in other hot springs of the Philippine archipelago that have different climatic regimes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

SMMVD and WLR conceptualized this study. The experiments were conducted by SMMVD. SMMVD and WLR prepared the manuscript.

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