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Evaluation of Fungal Diversity in High Altitude Soils in Different Temperature Conditions

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Abstract: Hot springs are like nature's spas, which include warm and humid aquatic habitats that serve as a sanctuary for a diverse range of microorganisms, including bacteria, archaea, viruses, and eukaryotes fungi. Among these, fungi are one of the most important microorganisms, carrying out essential functions that often go unnoticed but are crucial in accelerating biological processes. These organisms have adapted to survive under a wide range of thermal conditions. While thermophilic fungi can withstand the scorching heat of deserts and hyper-saline conditions, other variants like mesophilic and psychrophilic fungi prefer more moderate and colder temperatures, respectively. The study employs shotgun metagenomic sequencing to obtain a fine-grained taxonomic classification of lesser-known species and microbial eukaryotes. This study aims to explore the relationship between fungal diversity and temperature in three distinct thermal zones; thermophilic (hot spring), mesophilic (plain field), and psychrophilic (semi-frigid zone) zones. The findings of our study demonstrated that there is a notable and positive association between the diversity of fungi and temperature, suggesting that temperature is a key factor in moulding fungal communities. However, it is important to note that further research is necessary to elucidate the underlying causal mechanisms of this relationship. Overall, our study adds to the knowledge of how environmental factors influence microbial diversity and can aid in the development of strategies for the conservation and management of fungal communities. Fungi are essential for many industrial applications, and their role in causing illnesses is also significant, making this research valuable for both scientific and practical purposes.

Keywords: Metagenomic study; Fungal diversity; Thermophilic; Mesophilic; Psychrophilic.

Introduction

Microbial communities in natural and host-associated environments commonly harbour a mix of bacteria, archaea, viruses and microbial eukaryotes (Marcelino et al., 2020). Among these, bacteria can be easily identified at the species and strain levels (Scholz et al., 2016; Marcelino et al., 2020), however, it remains challenging to obtain a fine-grained taxonomic classification of lesser-known species and microbial eukaryotes (Nilsson et al., 2019). Eukaryotic microorganisms exhibit immense diversity, encompassing species with varying life cycles, morphological adaptations, and nutritional requirements. While viruses and bacteria are responsible for a larger number of diseases, certain microscopic eukaryotes, such as fungi, protists and algae, are associated with significant public health concerns. Fungi, in particular, play a crucial role in performing essential functions that are often invisible but vital for facilitating biological processes (El-Gendi et al., 2022).

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As eukaryotic organisms, fungi have a vital role in nutrient cycling and decomposition of organic matter across diverse ecosystems. Their remarkable adaptability allows them to flourish under various environmental conditions, including different temperature zones. Fungi occupy diverse ecological niches and make significant contributions to the well-being and sustainability of ecosystems in different regions.

Metagenomics is a powerful tool for studying the diversity and functionality of fungi in different thermal zones. In recent years, interest has aroused in exploring the fungal communities growing in extreme environments, such as hot springs, which can provide insights into the adaptations of fungi to high-temperature conditions. Some studies have used metagenomic approaches to investigate fungal communities in different thermal zones. In their investigation of soil fungal communities in a Mediterranean *Pinus pinaster* forest, Castaño et al. (2018) revealed a fascinating correlation between fluctuations in moisture and temperature levels and shifts in fungal community composition. This research uncovers the intricate relationships between environmental factors and the diversity of fungi in forest ecosystems. Moreover, the Global Fungi database provides a comprehensive collection of fungal communities from various habitats, including those in different thermal zones (Větrovský et al., 2020). This database can provide a useful reference for comparing the fungal communities in different thermal zones and can aid in the identification of potential fungal adaptations to extreme temperature conditions. These organisms evolved ways to flourish in a broad range of thermal conditions including deserts and hyper-saline conditions to meet their specific needs for growth (Salano et al., 2018). Fungus species *Aspergillus* and *Alternaria* were the most prevalent fungus in the hot spring water (Mehta and Satyanarayana, 2013; Wang and Pecoraro, 2021), whereas *Trichoderma* and *Cladosporium* dominated the sediments (Sterflinger et al., 2012). Thermophilic fungi are relatively less abundant compared to eubacteria and archaea.

In this study, our primary objective was to examine the fungal diversity within different thermal zones using shotgun metagenomics. We specifically focused on three zones: thermophilic, mesophilic, and psychrophilic. We hypothesised that each thermal zone would have unique fungal communities, with specific fungal taxa well-adapted to their respective environments.

Materials and Methods

Sampling Site Description and Sample Collection

Soil samples were collected from the two separate sampling zones; New Yumesamdong and Yumesamdong, North Sikkim, India. Each sampling zone has three distinct natural thermal zones such as thermophilic zones (hot spring) with temperature ranges of 55-65°C±2, mesophilic zones (having moderate temperature) with temperature ranges of 37-40°C±2, and psychrophilic zone (river; semi frigid zone) temperature ranges from 4-8°C ± 2 as listed in Table 1. The sampling site was unique as all three natural thermal zones are situated at very closest distance. The first sample was collected from where the hot spring originates and its temperature is around 60°C. As the water flows downwards the temperature of the hot spring and/or sampling site also decreased. The soil samples were collected at the mesophilic (37°C) and psychrophilic zone (8°C). The geographical coordinates and the elevation above mean sea level (AMSL) of the sampling sites were noted at the time of sampling with the help of a GPS meter- GPSMAP 78S (Garmin, India) (Das et al., 2022) and mapped through Q-GIS (Sehra et al., 2017) as depicted in Figure 1. Some physical parameters were also checked during the sample collection with help of thermometer and pH meter (Hi-Media). Twelve soil samples (approximately 100 gm.) were collected, i.e., two samples from each thermic zone at both sampling sites. All the samples were collected aseptically in sterile zip-lock bags and transported to the laboratory in ice-cooled condition for further experiments.

Table 1: The physical parameters (temperature and pH) of the three natural thermal zones

	Sample Site	*Temperature*	*pH*
Site-I	Thermophilic zone	62°C	8.28
	Mesophilic zone	37°C	8.90
	Psychrophilic zone	4°C	9.00
Site-II	Thermophilic zone	55°C	8.6
	Mesophilic zone	37°C	9.16
	Psychrophilic zone	8°C	9.4

Shotgun Metagenomic Sequencing

All of the obtained soil samples were processed using the XpressDNA Soil Kit- MG20So-50 (MagGenome Pvt Ltd.) to extract total environmental DNA (eDNA). Using a NanoDrop 1000 UV-VIS Spectrophotometer (Thermo Scientific) the purity of the extracted DNA

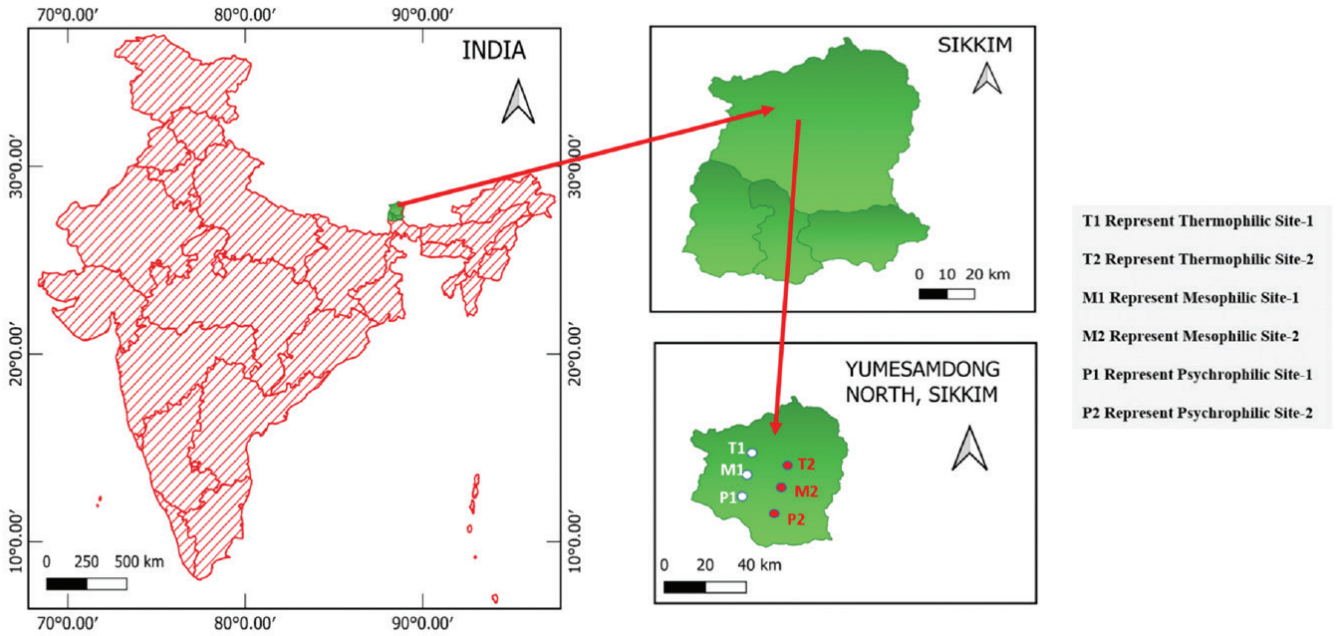


Figure 1: Geographic locations of the two sampling sites for the study.

was evaluated (Cheng et al., 2021). Conferring to the manufacturer's instructions, the eDNA samples underwent conventional paired-end DNASeq library construction utilising an Illumina 4000. Using 101bp paired-end module sequencing, samples were analysed.

Following the sequencing process, each library's reads were then processed for quality control by using the NGSQC tool set (Patel and Jain, 2012), resulting in high-quality (HQ) reads from the samples in Fastq format. A strict 80:30 criterion was applied to acquire high-quality filtered reads, and more than 80% of HQ bases with individual Phred scores greater than 30 were considered for assembling the genome. The sample's genome assemblies were created independently from one another using an HQ-filtered paired-end library.

Metagenomic Data Analysis

The raw sequence quality was thoroughly examined and assembled at the MG-RAST database (https://mg-rast.org). The fungal taxa were then determined using the assembled sequences. The MG-RAST database proved invaluable in annotating the fungal community's composition, function, and annotations (Keegan et al., 2016). Then, using the SILVA (Pruesse et al., 2007) and Refseq databases, taxonomic categorization of the representative Operational Taxonomic Units (OTUs) was completed (Pruitt et al., 2007). The readings were thoroughly categorized as a consequence of this approach, spanning several taxonomic levels, including kingdom, phylum, class, order, family, genus, and species. Sequences without a homologous pair of chromosomes were labelled as unidentified.

Statistical Analysis

The PAST4.0 tool was used to perform the Principal Component Analysis (PCA), and Graph Pad Prism was used to perform the regression and correlation analyses, t-tests, and ANOVA. Besides, the diversity indices (Shannon, Simpson's, chao1, and alpha diversity) were computed with the aid of the PAST tool (Chao et al., 2006). We employed the R software package iNEXT (iNterpolation and EXTrapolation) to plot a rarefaction curve at the species level using multiple soil samples (Rossi, 2011). Consequently, a heat map built by Clustvis was used to assess the relative fungal abundance at the genus level (Metsalu and Vilo, 2015).

Results

Phylum Level Diversity

All of the soil samples that were gathered contained members of the fungal phyla Ascomycota and Basidiomycota. With 36.83 % of Ascomycota and 8.52 % of Basidiomycota at the phylum level diversity, the thermophilic zone had the largest percentage of fungus. The fungal community of the mesophilic zone contained 31.86 % Ascomycota and 7.46 % Basidiomycota. In comparison, the Ascomycota and Basidiomycota

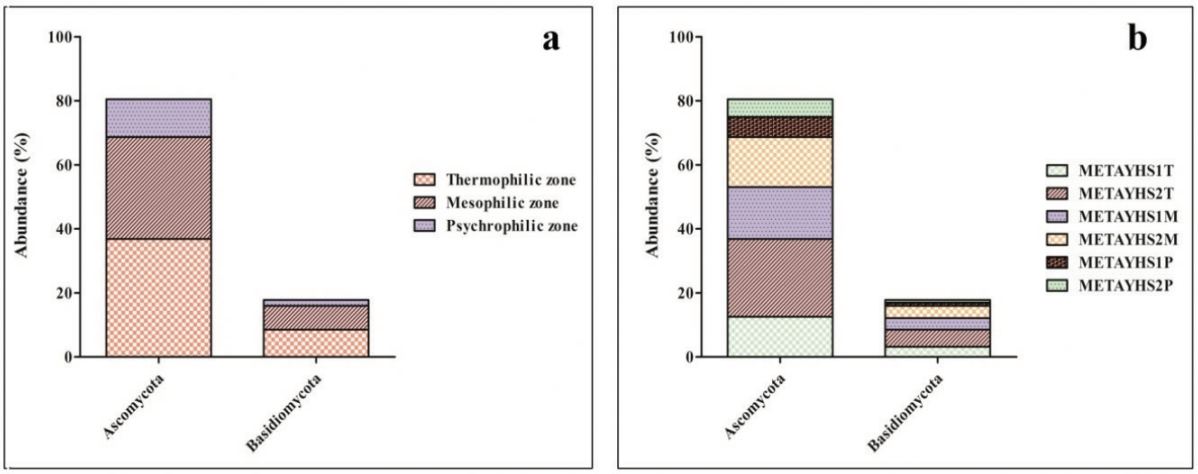


Figure 2: The phylum-level diversity of the dominant fungi in (a) different thermal zones and (b) at distinct samples.

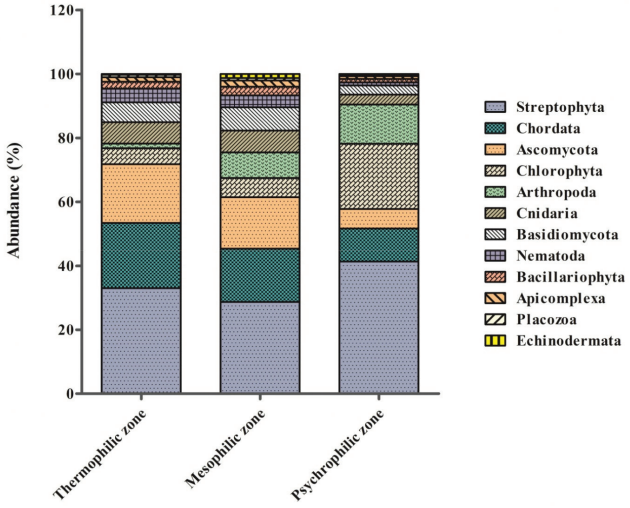


Figure 3: Abundance of distinct phyla found in various thermal zones.

populations in the psychrophilic zone were only 11.77% and 1.84%, respectively as depicted in Figure 2a**.** The samples that were gathered also contain a small number of other fungi as depicted in Figure 2b. Interestingly, we observed that as the temperature drops from thermophilic to psychrophilic via mesophilic zones, the abundance of fungi also declines as depicted in Figure 3, which suggests that temperature plays an important role in diversity of fungi.

Genus Level Diversity

The phylum-level diversity results, which demonstrate that fungi predominate the thermophilic zone followed by the mesophilic and psychrophilic zones, are supported by the genus-level diversity findings. We observed that in all three natural thermal zones (thermophilic, mesophilic, and psychrophilic) the most prevalent genera were *Ricinus*, *Hydra*, *Homo*, and *Neosartorya* as seen in Figure 4**.** *Neosartorya* (13.10 %), *Aspergillus* (10.82 %), *Malassezia* (5.70 %), *Gibberella* (5.50 %), and *Penicillium* (5.12 %) were the most prevalent fungal genera in the thermophilic zone. The most diverse genera in the mesophilic zone were *Gibberella* (8.14 %), *Neosartorya* (7.48 %), *Schizosaccharomyces* (6.71 %), *Aspergillus* (6.60 %), and *Ustilago* (5.39 %). Whereas the dominant genera in the psychrophilic zone were *Aspergillus* (8.08 %), *Gibberella* (7.24 %), *Magnaporthe* (7.07 %), *Neosartorya* (6.40 %), and *Yarrowia* (5.39 %), as seen in Figure 5.

Species Level Diversity

In the thermophilic zone, *Neosartorya fumigata* (10.62 %), *Malassezia globosa* (5.70 %), *Gibberella zeae* (5.50 %), *Talaromyces stipitatus* (4.74 %), and *Ustilago maydis* (4.55 %) were the dominant species. In the mesophilic zone, *Gibberella zeae* (8.14 %) was the most dominant species followed by *Neosartorya fumigata* and *Schizosaccharomyces pombe* (5.72 %), *Ustilago maydis* (5.39 %), and *Magnaporthe oryzae* (4.51 %). In contrast, psychrophilic zone had *Gibberella zeae* (8.08 %), *Neosartorya fumigata* (6.39 %), *Schizosaccharomyces pombe* (6.40 %), *Neurospora crassa* (5.39 %), *Yarrowia lipolytica* (4.54 %), and *Ustilago maydis* (4.21 %) were the most dominant species as shown in Figure 6.

Rarefaction Curve and Principal Component Analysis (PCA)

A rarefaction curve is an intriguing tool for analysing species richness across varying levels of diversity. This allowed us to determine the species richness of

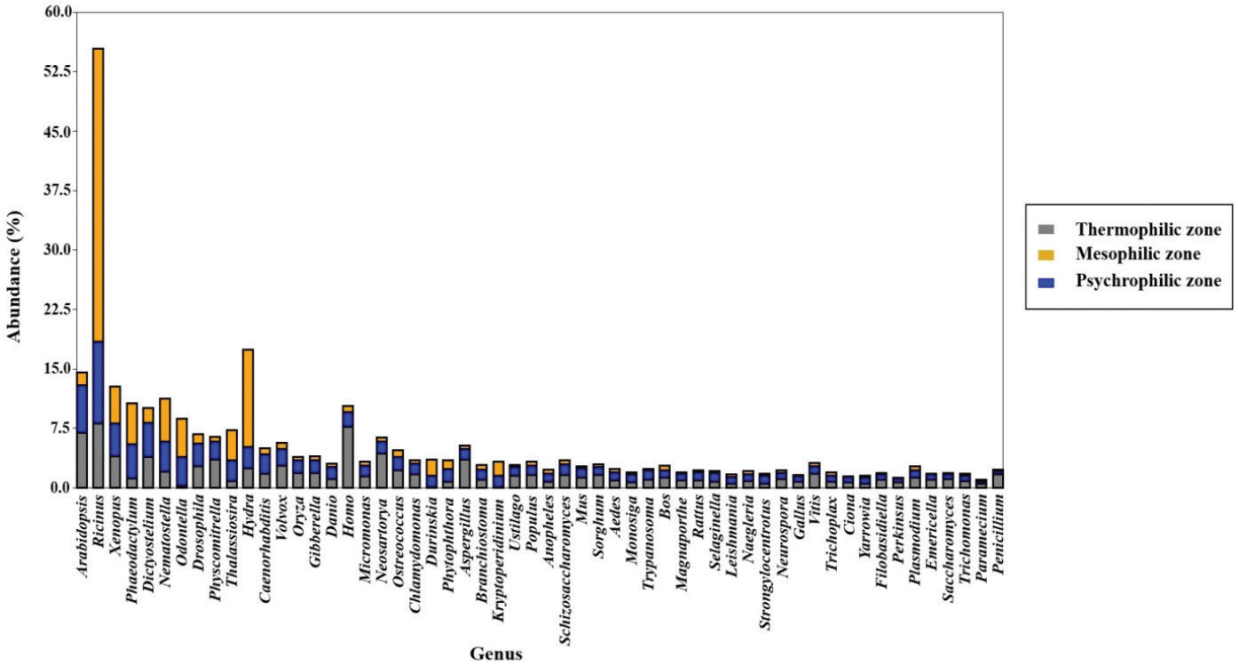


Figure 4: Abundance of the genus-level diversity in different thermal zones.

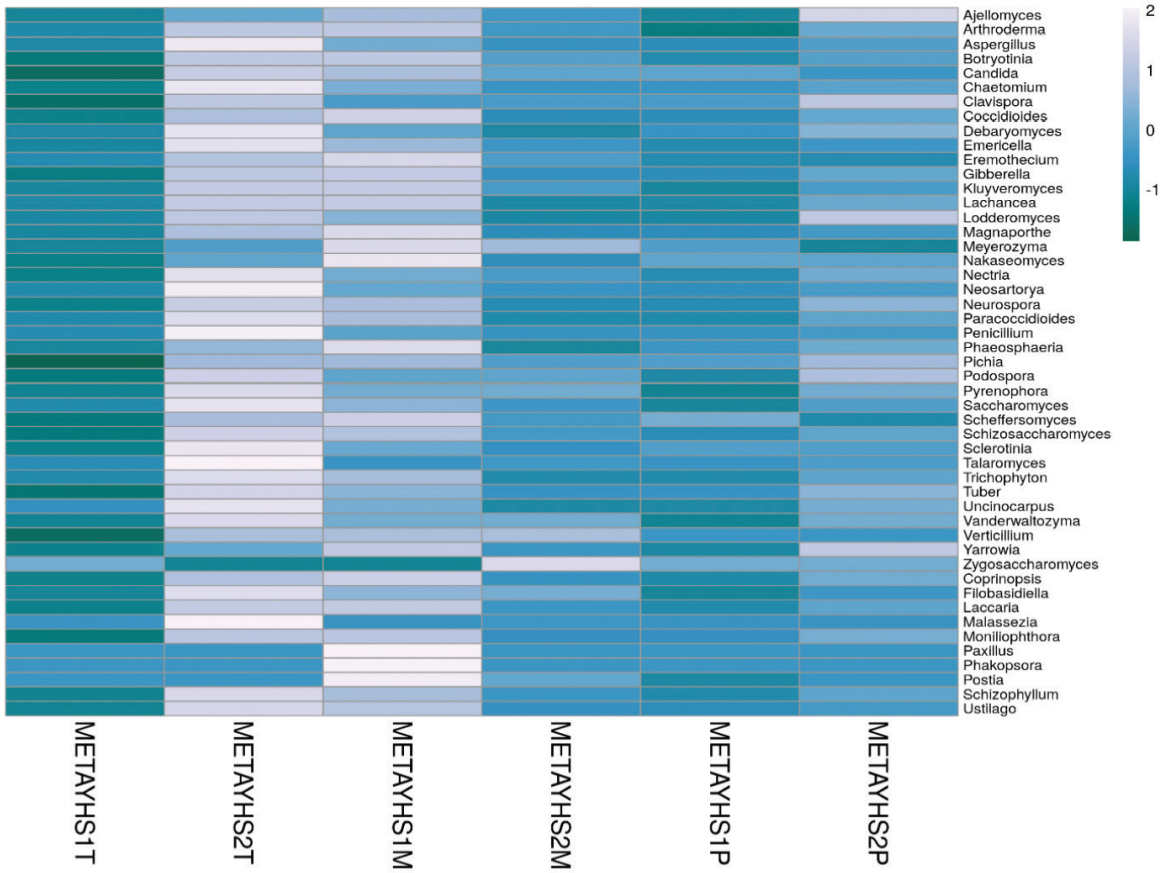


Figure 5: Heat map depicting the genus-level diversity.

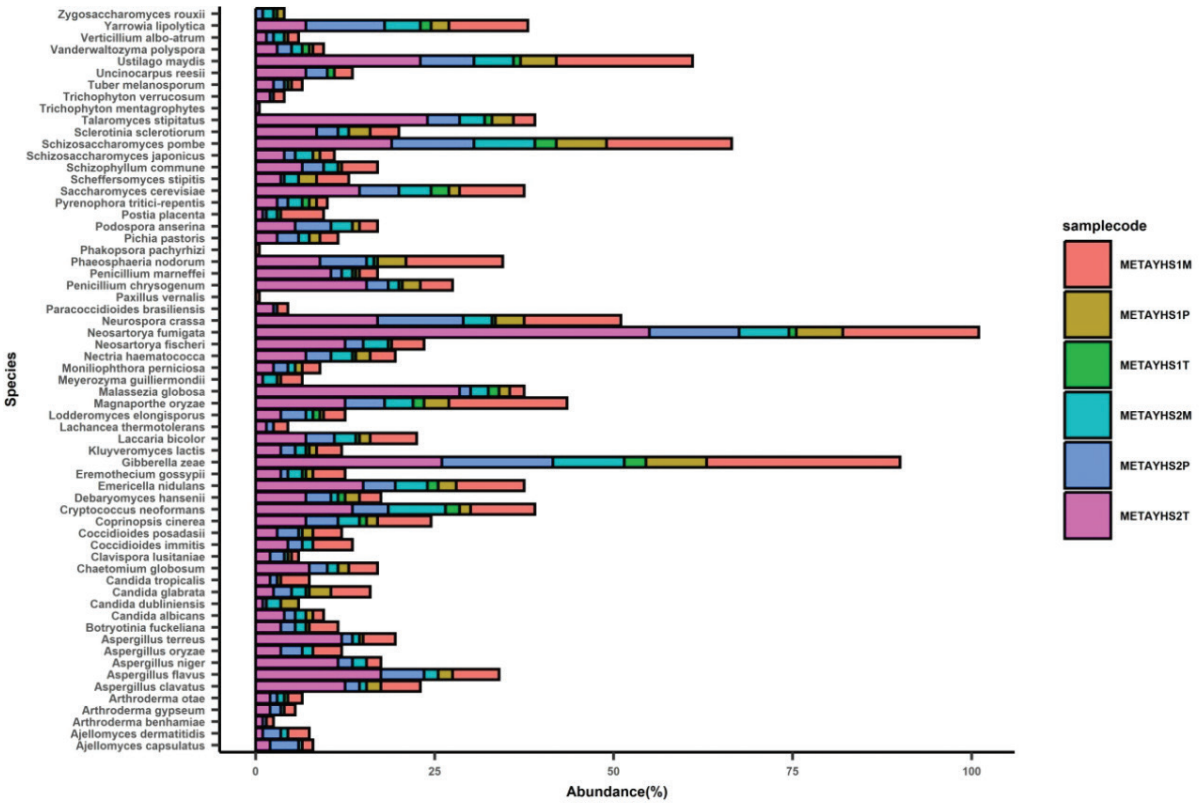


Figure 6: Species-level fungal diversity of samples from different thermal zones used in the study.

each individual and compare the results between two different sampling sites (Rossi, 2011). At sampling site-I, the mesophilic zone (METAYHS1M) displayed the greatest projection and population, followed by the psychrophilic zone (METAYHS1P) and the thermophilic zone (METAYHS1T). Conversely, at sampling site II, the thermophilic zone (METAYHS2T) showed the highest projection and population, followed by the psychrophilic zone (METAYHS2P) and the mesophilic zone (METAYHS2M) as shown in Figure 7. Interestingly, when we compared the thermal zones across both sampling sites, the thermophilic zone exhibited the greatest diversity as shown in Supplementary Figure S1a,b. These findings suggest that the optimal conditions for maximal fungal growth and community structure are found in thermophilic zones.

Utilising a statistical method known as Principal Component Analysis (PCA), we were able to reduce the dimensionality of our data while still retaining a majority of the variance present in the dataset. We applied PCA to analyse fungal diversity at the genus level and found that the thermophilic zone in both sampling locations exhibited similar results. Conversely, the samples from the mesophilic and psychrophilic

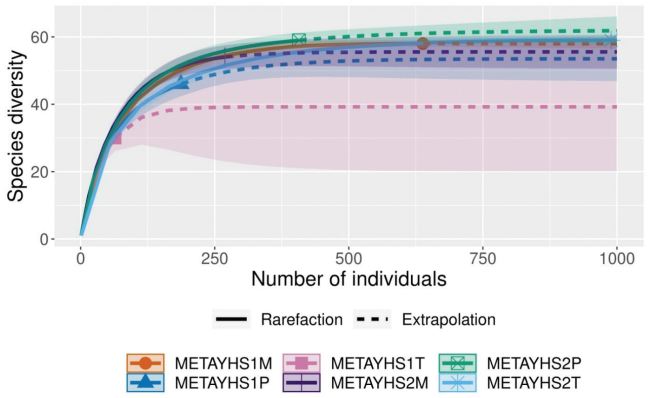


Figure 7: Rarefaction curve demonstrating species richness.

zones displayed similar results compared to the thermophilic zone as shown in Figure 8. Therefore, it can be deduced that the fungal diversity in the thermophilic zone differs significantly from that of the mesophilic and psychrophilic zones. This difference may be attributed to the thermophilic zone's higher temperature in comparison to the other two zones. It is plausible that temperature plays a crucial role in regulating fungal diversity in these thermal zones.

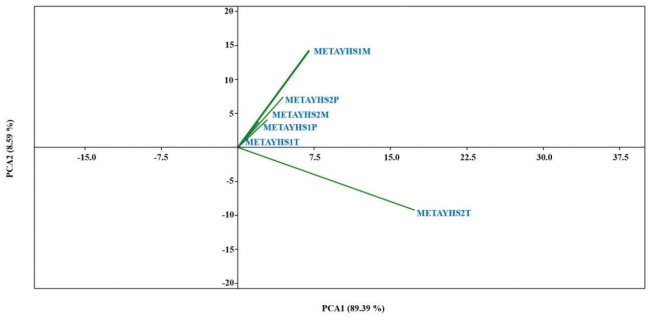
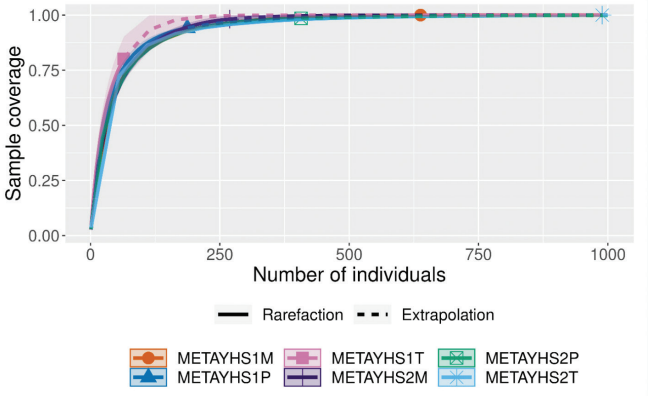
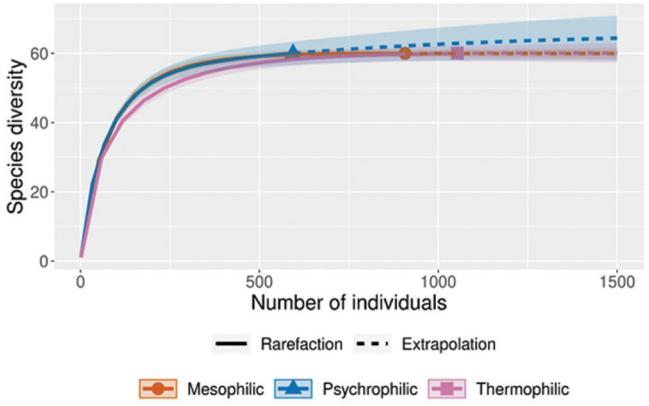


Figure 8: Principal Component Analysis (PCA) to analyse fungal diversity at the genus levels.



Supplementary Figure S1a: Displays a rarefaction curve plotting the relationship between sample coverage and the number of individuals.



Supplementary Figure S1b: Displays a rarefaction curve plotting the relationship between species diversity and the number of individuals.

Discussion

Fungi have a wide range of temperature tolerances, and their response to temperature varies depending on the species and their ecological niche (Robinson, 2001). Some fungi are adapted to extreme temperatures, such as those found in thermal vents, while others have more moderate temperature ranges. Fungal growth and metabolism are largely dependent on temperature, with most species having an optimal temperature range for growth and reproduction. At lower temperatures, fungal growth and metabolism slow down, and they may enter a dormant state (Mensah-Attipoe and Toyinbo, 2019). This is why food preservation techniques such as refrigeration can effectively prevent fungal growth and thereby increase the shelf life to a certain extent. On the other hand, fungal growth and metabolism may increase at higher temperatures, but if the temperature becomes too high (Maheshwari et al., 2000), it can lead to cell damage and death. Some fungi are capable of responding to changes in temperature by adjusting their gene expression or physiological processes. For example, when exposed to cold temperatures, some fungi produce cold-shock proteins that protect their cell membranes from damage, whereas few fungi produce heat-shock proteins when exposed to high temperatures thus protecting the cells from heat stress (Bakar et al., 2020).

Two phyla of fungi, Ascomycota and Basidiomycota, were identified in all soil samples collected from the different thermal zones. Ascomycota is a type of fungi characterised by the presence of asci and ascospores, while Basidiomycota is a fungal phylum distinguished by hyphae with septate structures and spores produced on a basidium. Ascomycota and Basidiomycota, the dynamic duo of the fungal world, are critical to the health of soil ecosystems (Frąc et al., 2018). Their ability to break down organic matter into simpler compounds facilitates the carbon cycle. These two phyla have distinct specialties: Ascomycota is nature's plant material decomposer, while Basidiomycota has a knack for breaking down lignin, the tough polymer found in woody material (Purohit et al., 2018). They also engage in mutually beneficial relationships with plants, a symbiosis with far-reaching ecological implications. The fact that they are ubiquitous in all soil samples is a testament to their tenacity and ability to thrive across diverse thermal zones.

The highest fungal diversity was observed in the thermophilic zone, with 36.83% Ascomycota and 8.52% Basidiomycota. A recent metagenomic study conducted by Das et al. in 2021 also revealed a similar pattern in two hot springs located in North Sikkim, India, where diverse microbiomes were present. The habitats in these hot springs were dominated by Ascomycota genera (Das et al., 2021).

Fungal species that dominate in high-temperature environments, like thermophilic habitats, have unique

adaptations that enable them to survive (Newsham et al., 2016). They possess heat-resistant enzymes and proteins, efficient nutrient uptake mechanisms, and protective systems against temperature-induced cellular damage. They have stable enzymes that efficiently break down complex organic matter for nutrition. Also, they have protective mechanisms like heat shock proteins that maintain proper protein folding and prevent cellular harm. In high-temperature environments, competition from other organisms is often reduced, giving thermophilic fungi a competitive advantage. Species may exhibit varying degrees of competitive ability. Some species possess traits or adaptations that give them a competitive advantage over others, allowing them to outcompete and displace competing species. As a result, species with higher competitive abilities tend to occupy and dominate specific niches or habitats, limiting the presence and distribution of other species within the areas (Lyu and Alexander, 2022).

The extreme conditions limit the growth of other microorganisms, allowing thermophilic fungi to dominate ecological niches with fewer competitors for resources. High temperatures can inhibit the growth of heat-sensitive microbial species like bacteria and other fungi. This further creates a favourable environment for thermophilic fungi to thrive and become the dominant organisms in these hot environments.

In a study conducted by Liu et al. in 2018, the fungal assemblages dwelling in the Rehai hot springs were categorized into 5 phyla and 67 orders. As expected, the Ascomycota phylum reigned supreme, with Basidiomycota following closely behind. However, the Chytridiomycota, Glomeromycota, and Zygomycota groups made up only a meagre fraction of the total communities (Liu et al., 2018). This trend was not isolated to Liu et al.'s research, as similar findings were reported in the works of Mouchacca (1997), Morgenstern et al. (2012), and Powell et al. (2012), all of which showcased Ascomycota and Basidiomycota as the most prevalent phyla (Mouchacca, 1997; Morgenstern et al., 2012; Powell et al., 2012). A recent investigation by Salano et al. (2017) in Kenya also noted that Ascomycota and Basidiomycota dominated the fungal communities in hot springs, pointing to the possibility that these two groups harbour genuine thermophilic communities that can withstand high temperatures (Salano et al., 2017). The ubiquitous presence of Ascomycota and Basidiomycota in thermal habitats indeed suggests that they could be considered heat-tolerant or even thermophilic.

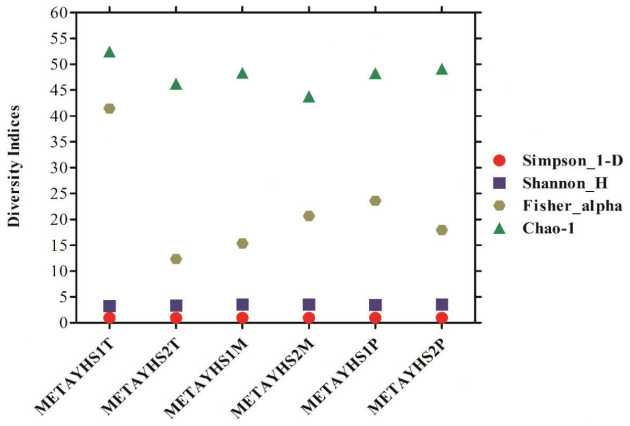
Fungal diversity in the three thermal zones was also observed at the genus level, and the most prevalent and diverse genera were identified. *Ricinus*, *Hydra*, *Homo*, and *Neosartorya* were the most prevalent genera across all sampling zones. In the thermophilic zone, *Neosartorya*, *Aspergillus*, *Malassezia*, *Gibberella*, and *Penicillium* were the most prevalent genera. In the mesophilic zone, *Gibberella*, *Neosartorya*, *Schizosaccharomyces*, *Aspergillus*, and *Ustilago* were the most diverse. In the psychrophilic zone, *Aspergillus*, *Gibberella*, *Magnaporthe*, *Neosartorya*, and *Yarrowia* were the dominant genera.

Our findings from the metagenomic data emphasising fungal genera suggested variation between the three thermal zones which is somewhat equivalent to previous research where a relationship between temperature and variations in the make-up of microbial communities was observed. Hot springs are a never-ending plethora for a diverse range of fungal communities, with certain genera taking center stage. Kambura et al. (2016) and Salano et al. (2017) revealed that *Penicillium*, *Cladosporium*, *Aspergillus*, *Fusarium*, *Aureobasidium*, and *Debaryomyces* were among the most predominant genera in these habitats (Kambura et al., 2016; Salano et al., 2017). Meanwhile, Li et al. (2012) detected the presence of *Penicillium*, *Aspergillus*, *Davidiella*, and *Fusarium* in geothermal environments located in the Tibetan Plateau (Li et al., 2012). These findings illuminate the fascinating and diverse world of fungal life in extreme environments, where certain genera seem to thrive and dominate. The presence of *Aspergillus, Gibberella,* and *Neosartorya* in all three thermal zones suggests that these fungal genera may be adapted to a wide range of thermal conditions. Such widespread presence across different ecosystems highlights the versatility and resilience of these fungal genera and underscores their ability to adapt to diverse conditions.

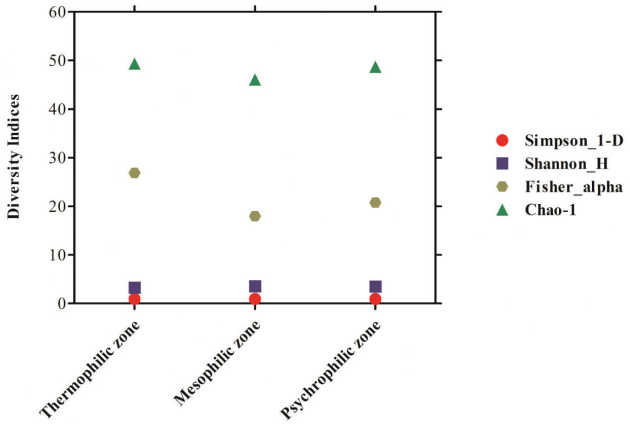
In the present study, it was observed that the dominant fungal species varied significantly among temperature zones. *Gibberella zeae* and *Neosartorya fumigata* were found to be the most dominant species in both the mesophilic and psychrophilic zones, but in the thermophilic zone, *Neosartorya fumigata* was the most dominant with a much higher percentage than in the other zones. This finding is consistent with other research articles that have reported on the distribution of fungal species across temperature zones. Some taxa were more dominant in cooler temperatures, while others were more dominant in warmer temperatures. Similarly, Liu et al. (2019) investigated fungal

diversity in different temperature zones in soil from a subalpine coniferous forest in China and found that the fungal community composition and diversity varied significantly among different temperature zones. These studies suggest that temperature is a critical factor that shapes the distribution and abundance of fungal species in different environments, and different species have different temperature preferences and adaptations. Understanding the factors that drive fungal community dynamics across various temperature zones is crucial in predicting the impacts of climate change on fungal diversity and ecosystem functions.

This study implies that the species richness and fungal diversity across various settings can be analysed using the rarefaction curve and principal component analysis. The findings revealed that the fungal diversity and species richness were highest in the thermophilic zone as shown in Supplementary Figure S2a,b, indicating that temperature may be a key factor in controlling fungal diversity in thermal zones. The interplay between microbial communities and their environmental surroundings has long been a subject of fascination for researchers, with temperature and pH levels emerging as key factors shaping these communities (Rousk et al., 2010; Zhang et al., 2016; Liu et al., 2018). Our study reinforces this notion, with a significant correlation observed between temperature and the fungal community. This underscores the delicate balance between organisms and their surroundings, and the need to understand the intricacies of these relationships to fully appreciate the diversity of life on our planet. The results also point to the potential for PCA to aid in dimensionality reduction while keeping the bulk of the variation found in the dataset, enabling a more specialized examination of fungal diversity at the genus level. Altogether, our findings offer insightful information on the variables affecting fungal diversity in various habitats, which may have significant implications for understanding the ecology and evolution of fungi.



Supplementary Figure S2a: Presents diversity Indices for various samples.



Supplementary Figure S2b: Presents diversity indices at distinct thermal zone.

Conclusions

Fungal diversity in hot springs reveals new insights into their adaptability and variety in extreme environments. These habitats offer unique niches for fungi, allowing them to thrive in inhospitable conditions. Metagenomics identified diverse fungal species in each thermal zone, shedding light on their distribution and their relationship with their environments. Dominant species varied across zones: *Neosartorya fumigata*, *Malassezia globosa*, *Gibberella zeae*, *Talaromyces stipitatus*, and *Ustilago maydis* in the thermophilic zone; *Gibberella zeae*, *Neosartorya fumigata*, *Schizosaccharomyces pombe*, *Ustilago maydis*, and *Magnaporthe oryzae* in the mesophilic zone; and *Gibberella zeae*, *Neosartorya fumigata*, *Schizosaccharomyces pombe*, *Neurospora crassa*, *Yarrowia lipolytica*, and *Ustilago maydis* in the psychrophilic zone. Rising temperatures correlated with increased fungal diversity, highlighting temperature's influence. Studying fungal diversity in thermal zones enhances our understanding of survival mechanisms and the potential for new drugs and biotechnologies. This research unlocks new frontiers in our understanding of the natural world and its applications.

Data Availability

To get the Bio-Project, Bio-Sample, and Sequence Read Archive (SRA) accession numbers, raw metagenomic data has been submitted to National Center for Biotechnology Information (NCBI). Table 2 includes all accession numbers.

Table 2: The metagenomic sequences submitted to National Center for Biotechnology Information (NCBI)

Sn.	*Sample Code*	*Bio Project*	*Bio Sample*	*SRA*
1	META03THERMH2	PRJNA889353	SAMN31237803	SRR21858985
2	META04THERMH2	PRJNA889693	SAMN31249784	SRR21870253
3	META07THERMH3	PRJNA889714	SAMN31250946	SRR21870628
4	META08THERMH3	PRJNA889719	SAMN31251116	SRR21871181
5	META01MESOH2	PRJNA890034	SAMN31265957	SRR21887601
6	META02MESOH2	PRJNA890150	SAMN31266807	SRR21890346
7	META09MESOH3	PRJNA890169	SAMN31269251	SRR21890547
8	META10MESOH3	PRJNA890178	SAMN31269369	SRR21890604
9	META05PSYH2	PRJNA890418	SAMN31277341	SRR21901715
10	META06PSYH2	PRJNA890425	SAMN31278271	SRR21902168
11	META11PSYH3	PRJNA890440	SAMN31278452	SRR21902415
12	META12PSYH3	PRJNA890557	SAMN31280924	SRR21903161

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