Identification and Distribution of Trichoderma Species in the Four Regions of Papua New Guinea

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Abstract: *Trichoderma*, a soil-dwelling fungus, is a reliable biocontrol agent that combats crop diseases, enhances plant resistance to environmental stresses, improves nutrient uptake efficiency, and promotes plant growth. Studies were conducted in the Papua New Guinea University of Technology (PNGUoT) laboratory using soil samples collected from four regions across 16 provinces in PNG. The objectives were identifying Trichoderma species, determining their phylogenetic relationships, and analyzing their population distribution. Twenty isolates were morphologically identified as *Trichoderma,* and further categorized into five species, i.e., *T. harzianum*, *T.virens*, *T. hamatum*, *T. lixii,* and *T. asperellum*, through molecular characterization. The phylogeny analysis generated an outgroup and two clades. *Trichoderma lixii* from Chimbu Province was an outgroup compared to the other four species. The first clade showed that *T. harzianum* (SHP) is closely related to *T. virens* (Manus) with a bootstrap value of 100%. The second clade showed that *T. harzianum* (Sandaun) is closely related to *T. asperellum* (East Sepik); *T. harzianum* (Central) with *T. harzianum* (NCD) and *T. harzianum* (Morobe); *T. virens* (Madang) with *T.virens* (New Ireland); and *T. asperellum* (Madang) with *T. hamatum* (ENB), respectively. *Trichoderma harzianum* was the dominant species. The highest population of *Trichoderma* was found in New Ireland Province with 15 882 CFU (*A. manihot*), while the lowest of 47 CFU (*I. batatas*) was in Western Highlands Province. The findings from this study can contribute towards the potential development of a *Trichoderma* species into a biological control against soil-borne pathogens in PNG.

Keywords: Prevalence, morphology, molecular identification, phylogeny.

1. Introduction

Trichoderma, a soil-inhabiting fungi, is a formidable weapon against many plant pathogenic fungi and nematodes. Its production of volatile and non-volatile metabolites allows it to effectively counter these threats (Kumar & Ashraf, 2017). But, its significance goes beyond disease suppression. *Trichoderma* also serves as a plant growth promoter, promising improved crop yields and agricultural productivity [1,2].

Trichoderma species are widely distributed in various soil types, habitats, and climatic zones. They can adapt to various habitats, including a tropical rainforest's diverse habitat and a biotechnological fermenter's environments. The diversified metabolic capacity and aggressive competitive character may be the basis of their prevalence in soil [2, 3]. Apart from a few research focusing on specific ecological niches, most studies on the diversity of *Trichoderma* have been undertaken in Asia, Europe, and America [4, 5].

The growth, distribution, and population of *Trichoderma* are affected by various factors. It prefers mesophilic temperatures (25-35°C), a wide range of pH, and moist conditions, and it does not tolerate water stress conditions [6].

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Despite the overwhelming importance of *Trichoderma* spp, few systematic and detailed studies have been conducted in Papua New Guinea. Ban (2015) demonstrated that *Trichoderma* species effectively controlled key soil-borne pathogens affecting vegetable crops in Papua New Guinea [7].

Hence, the study was conducted to isolate, identify, and determine the biodiversity and phylogenetic relationship of the *Trichoderma* isolates from the four different regions of Papua New Guinea.

2. Materials and Methods

2.1 Study Location

The study was conducted at the Papua New Guinea University of Technology (PNGUoT) Biotechnology Centre (UBC) and PNGUoT Analytical Services Laboratory (UASL). In addition, Macrogen Inc., South Korea, performed DNA fingerprinting to identify specific *Trichoderma* species.

2.2 Isolation of *Trichoderma* Species

Trichoderma was isolated from soil samples collected in 2020 and 2021 from 16 provinces covering four regions of PNG. These include the Highlands region, the Momase region, the Southern region, and the New Guinea Islands region with varying climatic conditions (Table 1). Composite samples were prepared from soils from different cropping areas in the various provinces. The soil moisture and pH of the soils were recorded on a subsample using the protocol by Robertson & Simmons at the UASL [8]. The samples were sealed in plastic bags and refrigerated at 4^{o}C in the UBC for *Trichoderma* isolation.

Table 1: Information regarding the soil samples from four regions of PNG.

Regions	Provinces	Sample number per province	Districts	Average temperature (^{o}C)	Average rainfall (mm)	Cropping site
Highlands	Eastern Highlands (EHP)	2	Goroka Unggai-Bena	17.9	2097	Sweet Potato (*Ipomoea batatas*) & Taro (*Colocasia esculenta*)
	Chimbu	1	Sinasina Yongomul	16.9	378.5	Sweet Potato (*I. batatas*)
	Jiwaka	1	Banz	15-22.8	243.8	Sweet Potato (*I. batatas*)
	Western Highlands (WHP)	1	Hagen Central	17.5	335.1	Sweet Potato (*I. batatas*)
	Southern Highlands (SHP)	2	Ialibu Pangia Mendi	19.5	376.8	Sweet Potato (*I. batatas*) & Peanut (*Arachis hypogaea*)
	Hela	1	Dauli	20	630.0	Sweet Potato (*I. batatas*)
Momase	Morobe	2	Markham Lae	23.6	6797	Banana (*Musa acuminata*) & Taro (*C. esculenta*)
	Madang	2	South-Coast Usino Bundi	25.5	3665	Cocoa (*Theobroma cacao*) & Sugarcane (*Saccharum officinarum*)
	East Sepik	1	Ambunti-Drekikia	24.9	366.3	Cocoa (*T. cacao*)
	Sandaun	1	Vanimo- Green River	26.2	262.8	Bokchoy (*Brassica rapa*) & Aibika (*A. manihot*)
Southern	NCD	1	NCD	25.8	1526	Peanut (*A. hypogaea*)

	Central	1	Kairuku-Hiri	28.0	237.5	Banana (*M. acuminata*)
NGI	East New Britain (ENB)	1	Kokopo	25.8	2413	Cocoa (T. *cacao*)
	West New Britain (WNB)	1	Kandarian-Glouster	29.0	432.75	Oil Palm (*Elaeis guineensis*)
	Manus	1	Los Negros LLG	29.0	436.02	Mixed Cropping: (Coconut (*Cocos nucifera*), Cassava (*Manihot esculenta*))
	New Ireland	1	Kavieng	27.0	287.8	Aibika (*A. manihot*)

Trichoderma was isolated from the soil samples using the dilution plate method [9] using *Trichoderma* Selective Media (Lincoln University TSM-LU). Each sample had three replicates. The colonies were purified through a single spore culture, transferred to PDA slants, and stored in the refrigerator.

2.3 Enumeration of *Trichoderma* in Soil

When visible colonies appeared on the TSM plates, Colony Forming Units (CFU) were counted based on color and compaction on the TSM plates. The population per gram of soil was calculated using the following formula [9]:

Total population per gram of soil = Mean number of colonies per plate X *dilution factor*

2.4 Morphological Identification

Trichoderma was identified morphologically by observing the appearance, color, and growth rate of the colony and a microscopic view of the hyphal branching structures and conidia shape. *Trichoderma* isolates were morphologically identified according to Ranasingh et al. [10].

2.5 Molecular Identification

A pure culture of *Trichoderma* was used for DNA extraction. *Trichoderma* tissues were scraped from 5-day-old cultures using sterile scalpel blades on PDA plates. Twenty milligrams of the sample were disrupted using a sterile mortar and pestle under liquid nitrogen. The total DNA of *Trichoderma* was isolated as per the protocol by DNeasy Plant Mini Kit (Qiagen) [7].

The DNA of the *Trichoderma* isolates identified morphologically [10] were amplified using Polymerase Chain reaction (PCR) in the automated Eppendorf Mastercycler. Bidirectional generic primers, ITS1('5'-TCCGGTAGGTGAACCTGCGG-'3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'3'), were used (Ban, 2015).

The un-purified PCR products were loaded onto electrophoresis gel for DNA detection, according to Ban [7]. A Gel Doc EZ Imager was used to view bands and was saved on the computer.

Sixteen isolates of un-purified PCR products of the *Trichoderma i*solates that showed morphological characteristics for *Trichoderma* and bands on the electrophoresis gel were sent to Macrogen Inc (South Korea) for sequencing to identify the isolates to species. The processed sequences were edited using the DNA subway platform. The Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) was used to match and confirm *Trichoderma* species [7].

The nucleotide sequences sent from Macrogen Inc (South Korea) were used to construct a phylogeny tree using the DNA subway platform (https://dnasubway.cyverse.org/). The nucleotide sequences were submitted, trimmed, and paired, and the phylogeny tree was constructed from the identified species using the platform [7].

3. Results

3.1 Morphological Characterization

Table 2 presents the morphological characteristics of twenty isolates of *Trichoderma* collected from sixteen provinces of PNG. The table includes observations of the fungal growth on the PDA medium and microscopic observations of the colony characteristics, conidiospores, and conidia.

Table 2: Morphological characteristics and species prediction of 20 *Trichoderma* isolates from 16 provinces of PNG.

Isolate ID	Province	Morphological characters	Specie prediction
D6 D11 D10	EHP (Goroka) Chimbu NCD	Colony: Whitish mycelium, grew densely and cushion-like. Conidiospores: in compact turfs. Side branches are short and thick. Phialides: crowded, short, and plump. Phialospore: Hyaline, small. No discoloration of media.	*T. polysporum*
D3 D4 D24 D22 D9	EHP (Unggai-Bena) WHP SHP (Ialibu-Pangia) Madang (South-coast) ENB	Colonies: yellowish, dull to dark green. Long and slender. Thick, cushion-like. Conidiospore: branches long and slender. No sterile hyphae. Phialides: not crowded. Phialospore: Rough Discoloration of media.	*T. viride*
D13 D16 D12 D1 D5	Jiwaka SHP (Mendi) Hela Morobe (Markham) Sandaun	Colonies: Yellowish, Dull dark green. Velvety, Hard on the media surface. Conidiospore: in a compact tuft, side branches are long and slender. Phialide: not crowded Phialospore: smooth, elliptical, simple branching. No discoloration of media.	*T. longibrachiatum*
D2 D15 D17 D8	Morobe (Lae) East Sepik WNB New Ireland	Colonies: Whitish green, long and thick, often sterile hyphae. The texture is velvety. Conidiospores: in compact turfs, side branches are short and thick. Phialides: crowded, short, and plump Phialospore: short ovoid. No discoloration of media.	*T. harzianum*
D14 D7 D23	Madang (Usino-Bundi) Manus Central	Colonies: Whitish green. Often sterile hyphae. Thick and cushion-like. Conidiospores: in compact tufts, side branches are short and thick. Phialides: crowded, short, and plump Phialospore: Oblong, angular No discoloration of media	*T. koningii*

The morphological characteristics of *Trichoderma* isolates, i.e., D1, D5, D12, D13, and D16 resembled *T. longibrachiatum. Trichoderma isolates* D7, D14, and D23 resembled *T. koningii.* Isolates D2, D8, D15, and D17 resembled *T. harzianum*. Isolates D3, D4, D9, D22 and D24 resembled *T. viride* and isolates D6, D10 and D11 resembled *T. polysporum. Trichoderma longibrachiatum* and *T. viride were* found to be the dominant species.

3.2 Identification of *Trichoderma* Using Molecular Techniques

The sixteen *Trichoderma* isolates were amplified, and the results are shown in Figure 1. From the agarose gel electrophoresis, detection under ethidium bromide showed 12 isolates. D2, D3, D5, D7, D8, D10, D11, D12, D14, D15, D2 and D23 produced expected amplified products of ≈ 600 bps. Isolates D1, D4, D9, and D16 revealed some morphological similarities, such as those of Trichoderma, but they did not show any bands on the gel. Unpurified PCR products were sent to Macrogen Inc. (South Korea) for sequencing, and the results are shown in Table 3. From the similarity indices from Genbank, twelve isolates were confirmed to be *Trichoderma,* in which isolates D2, D5, D10, D16, and D23 were identified as *T. harzianum* in which D2 and D10 closely resembled *T. harzianum* strain CEN859 from Brazil, D5 matched *T. harzianum* strain NECC21358 from China, D16 matched *T. harzianum* strain TBR-12 from India and D23 matched *T. harzianum* strain CEN732 from Brazil. Isolates D7, D8, and D22 were identified as *T. virens* strains and matched strain Tvien 3 from China, *T. virens* G1D9 from China, and *T. virens* strain SVPRT-Tvir01 from India, respectively. Isolate D9 was identified as *T. hamatum* strain resembling *T. hamatum* strain JAHLH2 from Iraq; isolate D11 was *T. lixii* and closely resembled isolate *T. lixii* Thar-10 from India. Isolate D14 and D15 were identified as *T. asperellum* strains and resembled *T. asperellum* strain AMUTA-2 from India, and D15 resembled strain T203 from Israel. *T. harzianum* was the dominant species identified based on molecular characterization. Nucleic acid sequence analysis showed a range of 85-100% similarity and an average nucleotide length of 600-700 bps for the twelve isolates.

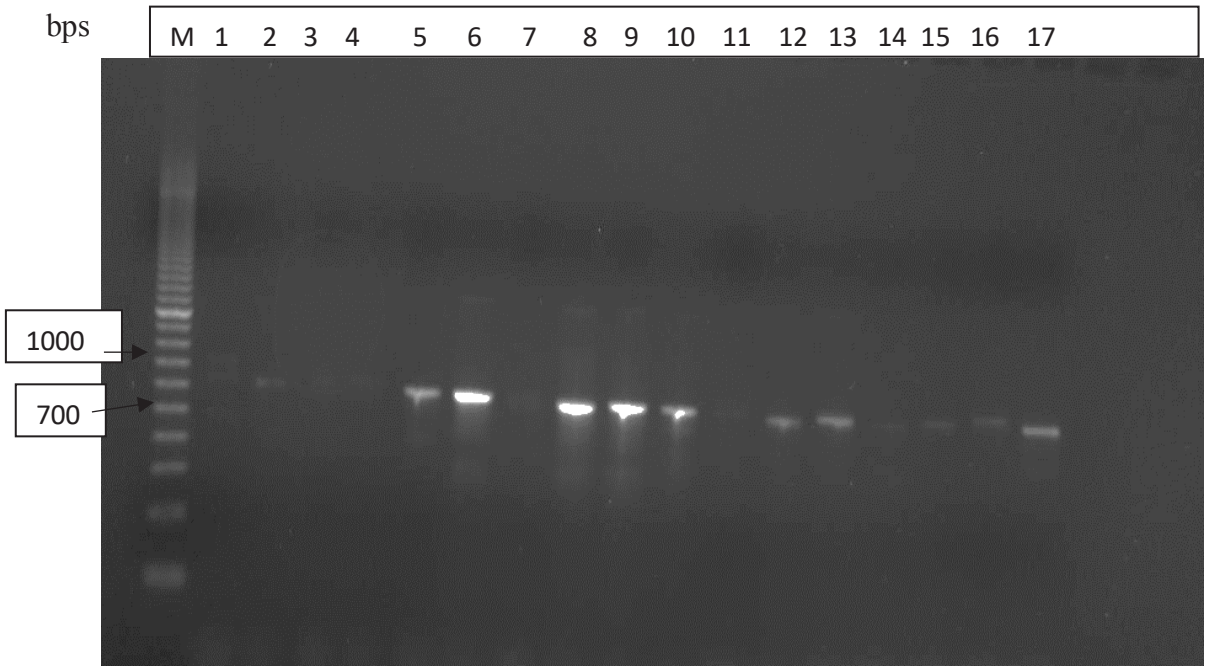

Fig 1: Amplified products of 16 *Trichoderma* isolates using primers ITS 1 and ITS 4. The DNA molecular marker is denoted as Lane M; Lane 1 is negative control (water); Lanes 2,5, 6, 8, 9, 10, 12, 13, 14, 15, 16, and 17 are isolates D2, D5, D7, D9, D10, D11, D15, D14, D16, D22, D23 and D24, respectively. Lanes 3, 4, 7, and 11 are isolates D3, D4, D8, and D12 confirmed *Trichoderma* species but appeared negative under electrophoresis.

Table 3: Molecular identifications of *Trichoderma* isolates by sequence analysis using NCBI Database.

Trichoderma isolates	*Trichoderma* isolates ID	Sequence nucleotide size	Sequence homology (%)	Matched *Trichoderma* species
Morobe	D2	1401	98.1	*T. harzianum*
Sandaun	D5	1 320	99.5	*T. harzianum*
Manus	D7	1 454	99.7	*T. virens*
New Ireland	D8	1 459	99.8	*T. virens*
ENB	D9	1 494	99.1	*T. hamatum*
NCD	D10	1 367	98.4	*T. harzianum*
Chimbu	D11	1 387	100	*T. lixii*
Madang 1	D14	1359	99.8	*T. asperellum*
East Sepik	D15	743	88.9	*T. asperellum*
SHP 1	D16	591	90.0	*T. harzianum*
Madang 2	D22	1 321	99.7	*T. virens*
Central	D23	1376	98.3	*T. harzianum*

3.3 Phylogeny Studies

The neighboring joining analysis generated a consistent phylogeny tree with two clades and one outgroup. For this study, *T. lixii* from Chimbu province was outgrouped from the other species in the same genus. This is because it has a nucleotide sequence that differs from the other clade members, occupying a base position on the tree. The two clades are apart from outgrouped species. The first clade showed that *T. harzianum* (SHP) is closely related to *T. virens from* (Manus) at a bootstrap value of 100 %. The second clade had two sub-branches in which *T. harzianum* (Sandaun) is closely related to *T. asperellum* (East Sepik) at a bootstrap value of 76% in the first sub-branch. The second sub-branch had two sub-branches at 100% bootstrap value. The first sub-branch indicated that *T. harzianum* from central and NCD are closely related (53% bootstrap value) and related to *T. harzianum* from Morobe (78% bootstrap value). The second sub-branch showed that *T. virens* from Madang and New Ireland are closely related (60% bootstrap value), *T. asperellum* (Madang) and *T. hamatum* (ENB) are closely related (100% bootstrap value), and all four isolates are related to each other (Figure 2).

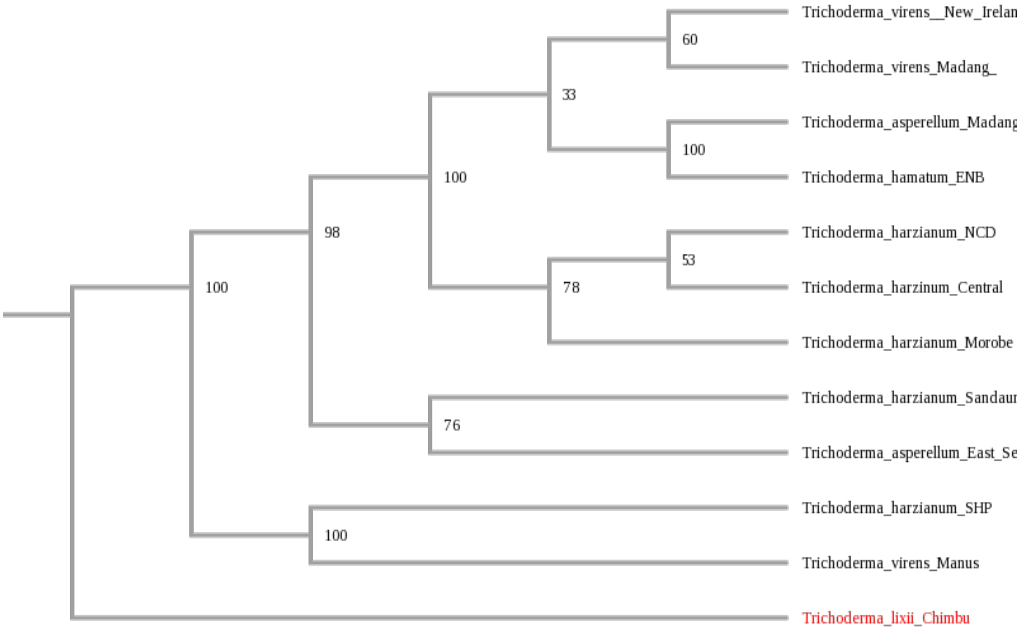

Fig 2: Phylogenic tree from Neighbouring Joining (NJ) of the 12 taxa of *Trichoderma* isolates of 700 base pairs aligned sequences of ITS 1 and ITS 4 primers.

3.4 Soil Characteristics and Population Distribution of Trichoderma Isolates

Table 4: Soil pH, moisture, and *Trichoderma* population from different cropping areas.

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Province	Soil pH (potentiometry)	Soil moisture (% w/w)	*Trichoderma* population g-^{1} of dry soil (CFU)
EHP (Goroka)	5.55	11.3	372
EHP (Bena)	6.37	5.51	95
Chimbu	7.70	9.33	414
Jiwaka	6.45	6.82	585
WHP	5.64	14.0	47
SHP	5.77	13.2	426
Hela	7.84	19.6	871
Morobe (Mutzing)	6.74	11.0	1607
Morobe (PNGUoT Farm)	5.68	7.40	2181
Madang (Ramu)	5.82	6.69	514
Madang (South-Coast)	7.15	16.5	4790
East Sepik	8.09	12.7	2222
Sandaun	8.09	4.68	4,474
ENB	7.62	8.38	589
West New Britain	5.83	7.97	575
New Ireland	8.05	25.7	15,882
Manus	7.87	22.4	1,231
Central	5.33	15.0	365
NCD	7.86	7.82	1,307

The soil pH, moisture content, and the average population of *Trichoderma* recorded from the different provinces under various cropping areas are presented in Table 4. The highest population of *Trichoderma* was recorded from New Ireland province with 15 882 CFU under *A. manihot* soil with a pH of 8.05 and soil moisture of 25.7%ww, followed by Madang (South-Coast) province (4 790 CFU, pH 7.15, 16.5% ww) under *T. cacao* soil, and Sandaun province (4 474 CFU, pH 8.09, 4.68%ww) under *B. rapa* and *A. manihot*. The lowest population of *Trichoderma* was recorded from Western Highlands province with 47 CFU under *I. batatas* soil at a pH of 5.33 and moisture content of 14.0%ww. The population per gram of dry soil for the Highlands Region (Eastern Highlands, Chimbu, Jiwaka, Western Highlands, and Southern Highlands) and some provinces in the coastal regions (Madang (Usino-Bundi), East New Britain, West New Britain, and Central) were lower than that of the soil samples collected from other provinces in the coastal regions (New Ireland, Manus, NCD, Sandaun, East Sepik, Madang (South-Coast) and Morobe) of Papua New Guinea. The total population for soil pH below 7 is 6,767 g^{-1} of dry soil, while the total population for soil above 7 is 31,780 g^{-1} of dry soil.

4. Discussion

In the present study, the population of the *Trichoderma* isolates varied for each province and the cropping sites. The highest *Trichoderma* population was recorded from New Ireland province under the Aibika cropping area, while the sweet potato cropping area from the Western Highlands had the lowest population. In addition, the diversity and abundance of the *Trichoderma* strains varied with cropping type and climatic conditions of the respective provinces from which the soil samples were collected.

The growth and distribution of *Trichoderma* are affected by biotic and abiotic factors in the soil. Abiotic factors, such as temperature, moisture, nutrients, and pH, significantly affect the growth, sporulation, and effectiveness of *Trichoderma* against phytopathogens [11]. Two critical factors affecting occurrences of *Trichoderma* are the soil temperature and the pH. This explains the higher population in the Coastal regions than in the Highlands regions of PNG, as the temperature ranges between 20-35°C on the coast. This confirms the research findings of Singh et al. [12] and Srivastava et al. [11] that *Trichoderma* strains prefer optimum temperatures ranging from 25-30°C to produce sufficient biomass. The pH is another critical determinant of the occurrence of *Trichoderma*. Bitton and Boylan reported that the development of *Trichoderma* is more efficient in acidic than alkaline soils [14]. Studies conducted by several researchers revealed that *Trichoderma* species showed optimum growth and biomass production at different pH values ranging from 4 to 7 [13]. Other studies showed that *Trichoderma* strains could grow at high pH values between 9 and 11 [15]. The different pH preferences of *Trichoderma* strains for proliferation explain the variations in the population with the respective soil pH for each province.

The type of plant cultivated in an area determines the growth and distribution of *Trichoderma* [16]. *Trichoderma* proliferates better under plants with widespread and shallow rooting systems than those deeply rooted perennial crops like coffee and tea trees. In addition, studies by Ban [7] showed that cropping type determined the population distribution of *Trichoderma* in the rhizospheres. The different plants also affect soil nutrients as they add to the litter formation [3, 17], confirming the high population of *Trichoderma* in aibika, taro, peanut, and vegetables and the low population in cocoa and oil palm soil in this study. Sweet potato soil in WHP had a low *Trichoderma* population due to continuous intensive cultivation as it is a staple crop in the region. According to Okoth et al. [16], continuous cultivation of the same crop results in the depletion or altering of soil nutrients that may affect the occurrence and population of *Trichoderma*.

In the present study, five species, i.e., *T. harzianum, T. koningii, T. viride, T. polysporum,* and *T. longibrachiatum*, were tentatively identified morphologically. However, morphological species identification of *Trichoderma* was unreliable due to the lack of similarity in the morphological characteristics and the increasing number of cryptic species of *Trichoderma,* resulting in incorrect identification [18]. This observation was confirmed by the results from the molecular identification performed in the current study; *T. harzianum*, *T. virens, T. hamatum, T. asperellum, and T. lixii* were identified.

The phylogeny analysis showed the genetic relativeness of the twelve identified species. Phylogenetic trees show the degree of relationship between taxa. On a phylogenetic tree, nodes closer to the ends of the tree connect terminal taxa that are more closely related, and the nodes closer to the base of the tree connect terminal taxa that are distantly related [19]. For example, *T. lixii* from Chimbu Province was out-grouped from the other species in the same genus. This is because it has a nucleotide sequence that differs from the other clade members, occupying a base position on the tree [20, 21]. Clade one showed that *T. harzianum* from SHP is closely related to *T. virens* from Manus. The second clade showed that *T. harzianum* from Sandaun is closely related to *T. asperellum from* East Sepik, *T. harzianum* from Central, and NCD are closely related and associated with *T. harzianum* from Morobe. *T. virens* from Madang and New Ireland are closely related*, and T. asperellum* from Madang and *T. hamatum* from ENB are closely related. All four species are related to each other under the same sub-branch.

The phylogenetic analysis conducted in this study reveals the relationships between these species and their distribution within the country. Identifying *Trichoderma* species across the four regions of Papua New Guinea provides a foundation for future research to develop biocontrol agents and growth-promoting supplements for farmers.

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