Research Article

Phylogenetic Diversity of Allspice (*Pimenta dioica*) Collections from Tanzania Using Chloroplast (cp) *rbcL* Gene

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ABSTRACT

This study investigates the phylogenetic relationships among diverse collections of Allspice (*Pimenta dioica*) sourced from Tanzania. The chloroplast (cp) *ribulose-1, 5-bisphosphate carboxylase* (rbcL) gene is employed as a Deoxyribonucleic Acid (DNA) barcode for this purpose. Allspice holds significance due to its economic, medicinal, and cultural applications. The phylogenetics study will help to identify the evolutionary relationships and genetic diversity within the *Myrtaceae* family, aiding in accurate identification and understanding its evolutionary history. The molecular phylogeny involved deoxyribonucleic acid extraction, polymerase chain reaction amplification, and rbcL gene sequencing. Cetyltrimethylammonium Bromide (CTAB) method is employed for genomic DNA extraction, while the rbcL gene is amplified using specific primers. Results reveal an average amplicon size of 560bp, BLASTN search exhibited over 96% similarity to seventeen *Myrtaceae* family members, including *Eucalyptus torquata* (NC_022401), Eucalyptus spathulata (NC_022400), *Eucalyptus torquata* (KC180794), *Eucalyptus spathulata* (KC180793), *Syzygium polyanthum* (OQ355361), Syzygium aromaticum (ON920513), Luma apiculata (KX162972), Eugenia aggregata (OP650216), *Eugenia Selloi* (MN095411), *Myrcianthes pungens* (MN095409), *Campomanesia xanthocarpa* (KY392760), *Acca sellowiana* (KX289887), *Syzygium samarangense* (NC_060657), *Lophomyrtus bullata* (MW214669), *Lenwebbia prominens* (MW214668), *Lenwebbia lasioclade* (MW214667), and *Syzygium nervosum* (NC_053907). The phylogenetic tree portrayed Allspice rbcL gene's proximity to *Myrtaceae* family members. This study demonstrates substantial genetic diversity within Tanzanian Allspice collections and among *Myrtaceae* family constituents. Furthermore, it establishes a basis for future research on Allspice's evolutionary history and population genetics in Tanzania.

Key Words: DNA barcoding; Genetic analyses; *Myrtaceae* family; Primers; Sequence alignment

Introduction

Myrtaceae is a highly diverse family of plants that includes over 130 genera and 4000 species that are distributed around the world [1]. One such species, Allspice (*Pimenta dioica*) grow up to 20 meters tall and has sweet-smelling leaves that measures between 9 centimeters and 20 centimeters [2]. Allspice thrives in semitropical lowland regions with an average temperature of 15°C to 32°C, 600 meters above sea level, and an average rainfall of 1,500 mm to 2,500mm annually [3]. This species is indigenous to Southern Mexico and Central America, and was introduced to Tanzania by Arab traders in the 19th century, and it quickly became popular in the country's coastal areas [4]. Allspice has spread too many countries due to the trade of spices, as well as seeds dispersal by birds [5]. Allspice is found in various countries around the world, and in Africa, the tree has been reported in 27 countries [1].

The tree produces a fragrant fruit that measures between 4 mm -8 mm in size, which is used as an ingredient in the food industry [6]. Allspice is highly valued economically, as it is used in the manufacture of spices in the food industry (65%-70%), pimento oil production (20-25%), and for domestic purpose (5% - 10 %) [7]. Allspice is known for its pesticidal properties, and it has been used as an insecticide for plant protection in various pests such as *Reticulitermes speratus*, *Acanthoscelides obtectus*, and *Sitophilus zeamais* [8, 9]. Oils derived from this has antifungal, antibacterial, and antinematicidal properties [10-12]. Additionally, the use of Allspice in traditional medicine has been prevalent for its therapeutic properties. The powder extracted from its fruit is known to be effective in treating several health problems, including menstrual discomfort, inflammation, stomach aches, and

Received: 08-July-2023, Manuscript No. ijocs-23-111136; **Editor assigned:** 09-July-2023, PreQC No. ijocs-23-111136 (PQ); **Reviewed:** 16-July-2023, QC No. ijocs-23-111136 (Q); **Revised:** 19-July-2023, Manuscript No. ijocs-23-111136 (R); **Published:** 28-July-2023, DOI: 10.37532/1753-0431.2023.17(8).322

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muscle pain [13]. Therefore, it is imperative to accurately identify it to guarantee its safe and effective use. Also, there is a need to create awareness among local communities and promote proper identification and utilization of Allspice.

The complexity of plant diversity is shaped by various biotic and abiotic factors [14]. Environmental factors, including climate, topography, resource availability, and disturbance like wildfires, flooding, deforestation, and agriculture, play a critical role in determining species composition and shaping evolutionary processes [15]. Two of the most important climatic factors, temperature and rainfall, are strong predictors of plant diversity. Ecosystems with average temperatures above the thresholds of 25°C - 30°C (77°F - 86°F) for temperate and boreal areas and 28°C - 32°C (82°F - 90°F) for tropical regions exhibit lower plant diversity. Conversely, low temperature stress on plant diversity is usually observed at temperatures around -10°C to -15°C (14°F to 5°F) in temperate and boreal ecosystems and between 5°C and 10°C (41°F to 50°F) in tropical ecosystems [16]. Plant diversity is highest at intermediate rainfall levels (1000 mm per year -1500 mm per year) and declines when rainfall falls below 500 mm or rises above 1500 mm in tropical forests, and below 400 mm per year in grasslands [17]. Also, edaphic factors, such as soil type and nutrient availability, also influence plant diversity [18]. On the other hand, genetic variation, mutation, and genetic drift are important mechanisms that drive evolution and shape genetic diversity within populations [19].

However, Allspice has been misidentified as *Pimenta racemosa* and other similar trees in *Myrtaceae* family. This confusion is primarily due to the striking similarities in their morphological characteristics, which can make it difficult to distinguish between the two [20]. DNA barcoding is a useful method for identifying and classifying plant species, especially when morphological features are insufficient or ambiguous [21]. It utilizes a specific genetic marker, such as the chloroplast (cp) rbcL gene, which encodes the large subunit of ribulose bisphosphate carboxylase [22]. This gene has average length of 1400 bp, and is commonly used as a DNA barcode in plants due to its high variability between species, highly conserved, experiencing low levels of mutations and its presence in nearly all plants [23]. Therefore, *cprbcL* gene is a potentially tool that can be used in the study of evolutionary, intraspecies diversity, and phylogenetic variations among species.

This study aims to investigate the phylogenetic relationship between various collection of Allspice from different locations in Tanzania and other related members of the *Myrtaceae* family using *cprbcL* gene, and the outcomes of this research are anticipated to offer valuable understandings into the evolutionary relationships among species and the interspecies diversity within the *Myrtaceae* family.

Materials and Methods

Plant material

The study sampled a total of fifty nine (59) collections of Allspice from various locations in Tanzania. These samples were collected from different areas: Kizimbani – Zanzibar (23), Kizugu botanical garden (25), Zigi Amani forest (9), and World Vegetable Center (AVRDC) – Arusha (2). Fresh leaves were collected from the field and then subjected to a drying process using silica gels. All samples were kept in storage at a temperature of -80 °C until they were processed. Provide more detailed information about collections and locations where the samples were collectedas shown in Figure 1.

DNA extraction

The CTAB procedure was used to extract genomic DNA. Leaves were dried overnight at a temperature of 70°C. Then, CTAB buffer was warmed at 65°C for 15 minutes. After that, pieces of dried leaves were ground into a fine powder using Geno grinding machine (2010 Geno/Grinder®) with two steel grinding media at 1200rpm for 40 seconds, two times. 1ml of warm CTAB buffer was added to the eppendorf tube containing the fine powder and then warmed at 65°C for 30 minutes, while shaking the tube in the interval of 10 minutes. Next, centrifugation was done at 13,000rpm for 15 minutes at room temperature, and the supernatant was transferred to a new eppendorf tube. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added and centrifuged again for 15 minutes. The upper layer was collected, and 0.7 of the total volume of cold isopropanol was added and stored at -20°C for 1 hour, then centrifuged at 13,000

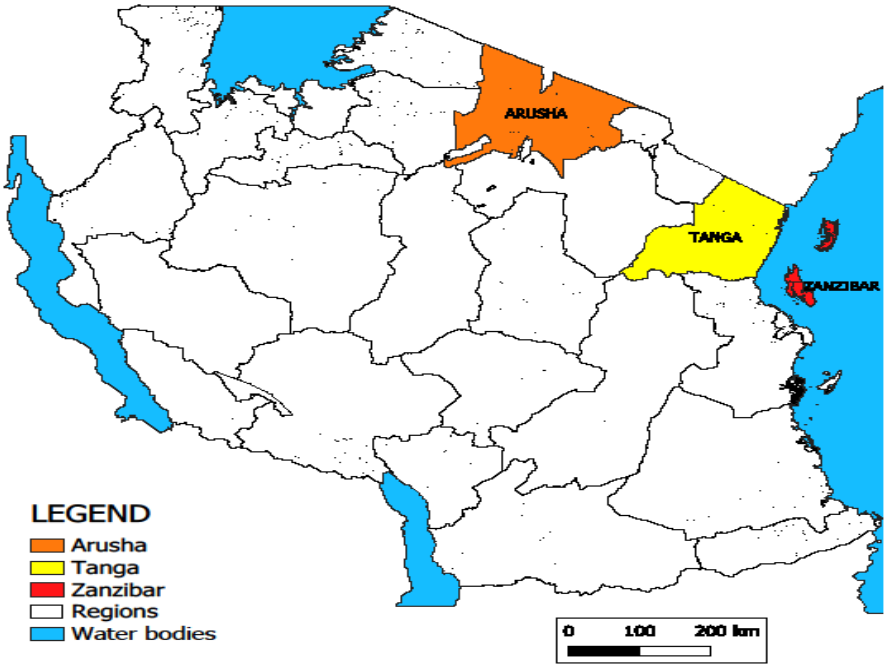

Figure 1: Map of Tanzania showing the locations where allspice samples were collected, particularly in Arusha, Tanga, and Zanzibar.

rpm for 30 minutes. The pellet was collected, washed with 500µl of ethanol, and centrifuged again at 13,000 rpm for 10 minutes. The pellet was air-dried for 45 minutes and suspended in nuclease-free water, which was then treated with *RNase* (10 mg/mL) and incubated at 37°C for 1 hour. Finally, the DNA concentration was measured and purity was determined by agarose electrophoresis and estimating the ratio of absorbance at 260 nm to that at 280 nm (A260/A280), respectively. The spectrophotometer (Metter Toledo UV/VIS) was used to measure the purity of DNA that was isolated. The quality assessment was performed by utilizing agarose gel electrophoresis with a 1.5% concentration (Bio-Rad's Mini-sub cell GT electrophoresis system). Subsequently, the gels were observed via a UV transilluminator manufactured by Invitrogen under which the image was taken using a Gel documentation system.

■ PCR amplification and sequencing of *cprbcL* gene

The Allspice *cprbcL* gene marker from cpDNA was used in this study. Primer pairs used in PCR amplification were P610 as forward primer 5'-TGTCACCACAAACAGAGACTAAAGC-3' and P609 as reverse primer 5'-GTAAAAT-CAAGTCCACCRCG-3'. The PCR program was as follows: denaturation at 94°C for five minutes was followed by 35 cycles at 94°C for 30 seconds, annealing at 52°C for 30 seconds, an extension of 72°C for 1 minute and final extension of 72°C for 10 minutes. Each reaction contained 10.5µl of water, 12.5µl *Taq DNA Polymerase* 2x Master Mix RED (Ampliqon A/S, Stenhuggervej 22, Denmark), 0.5µl of each primer and 1.0µl of DNA template were added to make a final volume of 25µl. The PCR products of the *cprbcL* gene were visualized by running them on a 1.5% agarose gel with ethidium bromide staining. The single band of PCR amplicon were purified using a column-based DNA purification kit. Then after, the purified PCR product was sent to Inqaba Biotech Pretoria, South Africa for Sanger sequencing using Sanger's di-deoxy sequencing method on an ABI prism 3700 DNA analyzer. The resulted *cprbcL* sequence obtained from Allspice was submitted to the NCBI database and the accession numbers obtained range from OP985342 to OP9885400.

■ DNA sequence alignment and phylogenetic analyses

Geneious Prime version 2023.0.1 (www.geneious.com) was used to assess the quality of the nucleotide sequences, which allowed for obtaining consensus arrangements of nucleotides. The study of the evolution of Allspice involved constructing a phylogenetic tree with major species from the *Myrtaceae* family. To perform this analysis, a BLASTn search (http://www.ncbi.nlm.nih.gov) was conducted to compare the query sequence with the subject sequence

available in the NCBI database. For each event, six to ten closely related sequences were selected and multiple sequence alignment was performed using MUSCLE 5.1, which is integrated into Geneious Prime software. In order to examine the differences among the sequences, a distance matrix was created, and using the dissimilarities expressed in the matrix, a phylogenetic tree was constructed using Geneious Prime software. To assess the structure of the phylogenetic tree, the bootstrap method was employed with 1000 replicates for all nodes. *Eucalyptus behriana* (MW446388) was used as an out group to position the root of tree. Bootstrap analysis was used to re-evaluate the resulting rooted tree topologies using 1,000 resampling of the data.

Results

■ PCR amplification and sequence analysis

The electrophoresis of the Allspice *cprbcL* gene amplified by PCR was successful on a 1.5% agarose gel. In the agarose gel, the first well shows ladder DNA and the remaining wells indicate amplified *cprbcL* gene product. In this case, the gel was able to separate the amplified *cprbcL* gene product from other DNA fragments. The thick and single band present in the gel confirms that the amplification was successful and that the size of the amplified *cprbcL* gene was 560 bp. The nucleotide content of the Allspice *cprbcL* gene was analyzed using Geneious Prime software version 2023.0.1. The nucleotide statistics for all 59 sequences were calculated, and it was determined that the amplified *cprbcL* gene contained a total of 33,064 nucleotides with a mean molecular weight of 173kDa for ssDNA and 346.234kDa for dsDNA. The nucleotide composition of the amplified Allspice *cprbcL* gene was analyzed, and it was found to consist of 9,381 bases of adenine (A), 7,149 bases of cytosine (C), 7,420 bases of guanine (G), and 9,114 bases of thymine (T). These bases correspond to 28.4%, 21.6%, 22.4%, and 27.6% of the gene, respectively with a GC content of 44% as given in Table 1.

■ Multiple sequence alignment and Phylogenetic tree analyses

The BLASTn searches conducted in this study yielded significant results, with a high degree of similarity ranging from 96% – 100% and E-value of 0.0 between the query sequence and subject sequences obtained from GenBank. Specifically, the 59 sequences of Allspice *cprbcL* genes were found to be similar to 17 species within the *Myrtaceae* family, as shown in Results of the study indicate that the Allspice isolate with accession number TZnK2_OP985343 showed 100% similarity with six members of the *Myrtaceae* family, including *Eucalyptus torquata* (NC_022401), *Eucalyptus spathulata* (NC_022400), *Eucalyptus torquata* (KC180794), Eucalyptus spathulata (KC180793), Syzygium polyanthum (OQ355361), and *Syzygium aromaticum* (ON920513). Additionally, three isolates from Kizimbani – Zanzibar and Kizugu botanical garden exhibited genetic similarity ranging from 96.77% to 96.59% with 11 members of the *Myrtaceae* family, including *Luma apiculata* (KX162972), *Eugenia aggregata* (OP650216), *Eugenia Selloi* (MN095411), *Myrcianthes pungens* (MN095409), Campomanesia xanthocarpa (KY392760), *Acca sellowiana* (KX289887), *Syzygium samarangense* (NC_060657), *Lophomyrtus bullata* (MW214669), *Lenwebbia prominens* (MW214668), *Lenwebbia lasioclade* (MW214667), and *Syzygium nervosum* (NC_053907). Furthermore, twenty-three Allspice isolates exhibited 100% to 99.82% similarity with the 11 members of the *Myrtaceae* family, while 22 isolates showed 98.94% to 98.76% similarity to the same group of 11 members. Additionally, five other isolates exhibited 98.92% to 98.75% and 98.91% to 98.73% similarity to the same members of the *Myrtaceae* family. The isolates was collected from various locations, including Kizimbani – Zanzibar, Kizugu botanical garden, Amani Zigi Forest, and World vegetable center – Arusha.

A total of 17 sequences from different species of *Myrtaceae* family were selected for the analysis, and multiple sequence alignment was performed using the Geneious prime software. show the phylogenetic parameters obtained from sequences alignment.

Table 1: Location and sample identity of Allspice collections from various areas in Tanzania.

Location	Sample Identity	Longitude	Latitude	Altitude (masl)	No. of collections
Kizimbani – Zanzibar	TZnK	39°12'90"E	6°5'34" S	119	23
	TAR	36°41'15"E	3°23'19"S	1400	2
	TTKB	38°39'52"E	5°6'49"S	2289	25
	TTZF	38°38'59"E	5°3'49"S	2289	9
Total					**59**

The degree of genetic divergence between sequences ranges from 0.01 – 0.04 at gene level. The results presented in of this study indicate that a total of 24 isolates exhibited a patristic distance of 0.01 to 17 members of the *Myrtaceae* family, which represents the minimum level compared to isolate 32, which showed an intermediate level of 0.02. However, three Allspice isolates showed a significantly higher level of 0.04, which is the maximum level observed. However, the results indicate that the *Myrtaceae* family members have a relatively low level of genetic diversity, as evidenced by the small range of patristic distances observed. In contrast, the Allspice isolates exhibited a higher level of genetic diversity, with some isolates showing a significantly higher patristic distance.

The phylogenetic tree shows one main clade that contains four subclades. The first subclade is composed of Eucalyptus torquata (KC180794), Eucalyptus spathulata (NC_022400), Syzygium aromaticum (ON920513), and Eucalyptus spathulata (KC180793), which are very closely to Allspice isolate with accession numbers TZnK2_OP985343 and TTKB5_OP985371 from the Kizimbani – Zanzibar and Kizugu botanical gardens respectively. Additionally, Syzygium polyanthum (OQ355361) was closely related to Allspice isolates with accession numbers TTZF4_OP985395, TTZF3_OP985394, and TTZF6_OP985397 from Amani Zigi Forest, TZnK8_OP985349, TZnK9_OP985350, and TZnK11_OP985352 from Kizimbani – Zanzibar. Luma apiculata (KX162972) and Eugenia aggregata (OP650216) clustered with Allspice isolates with accession numbers TZnK12_OP985353 and TZnK14_OP985355

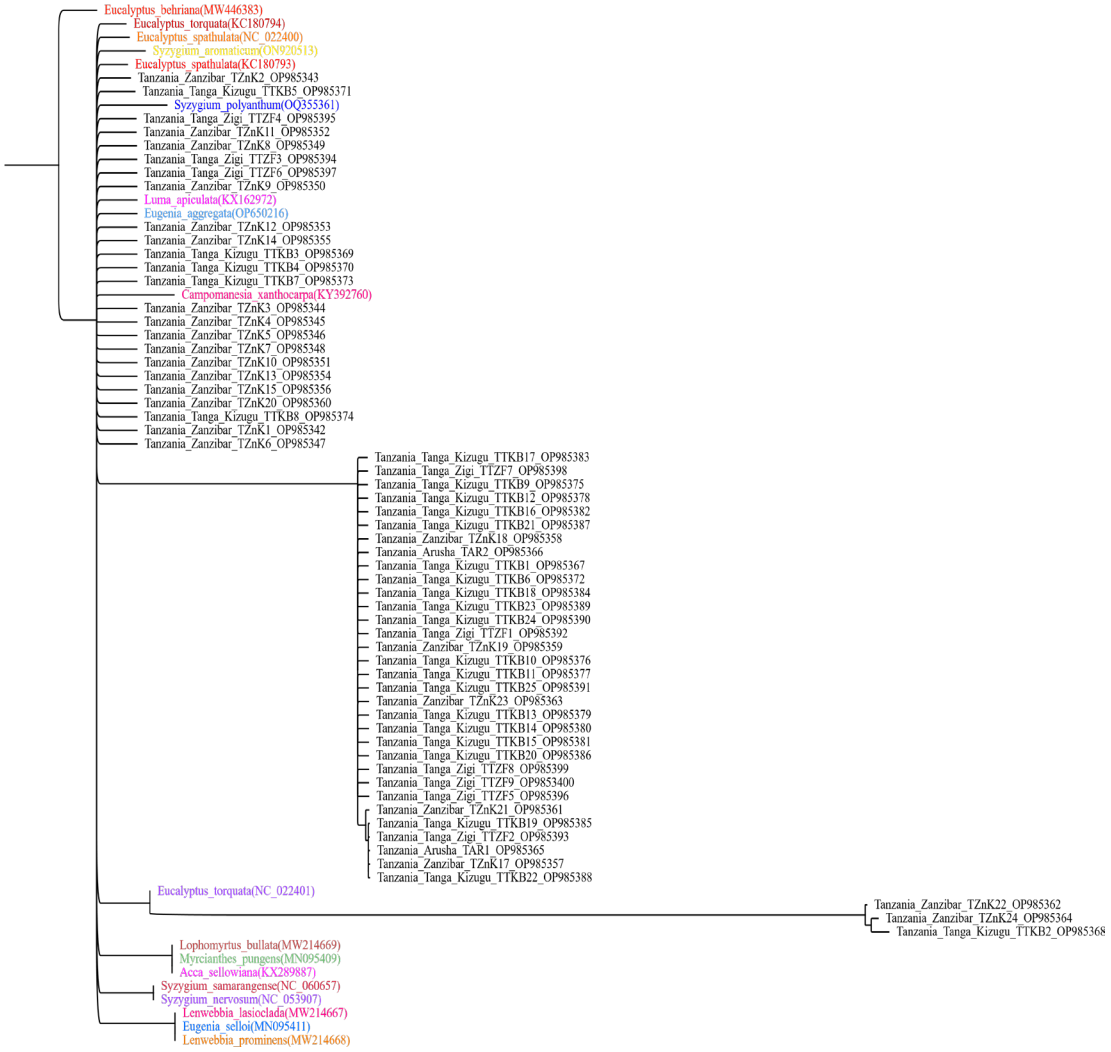

Figure 2: Phylogenetic relationship of cprbcL gene of Allspice from Tanzania. The inference tree was constructed through Geneious Prime 2023.0.1 using Tamura-Nei, maximum likelihood as a statistical method and neighbor-joining method. Eucalyptus behriana (MW446383) was used as an outgroup to position the root of the tree.

from Kizimbani – Zanzibar, TTKB3_OP985369, TTKB4_OP985370, and TTKB7_OP985373 from Kizugu botanical garden. Campomanesia xanthocarpa (KY392760) was closely related to isolate with accession numbers TZnK1_OP985342, TZnK6_OP985347, TZnK3_OP985344 to TZnK15_OP985356, and TZnK20_OP985360 from Kizimbani – Zanzibar and TTKB8_OP985374 from Kizugu botanical garden.

The Allspice isolate gene sequences with accession numbers TTKB1_OP985367, TTKB6_OP985372, TTKB9_OP985375 to TTKB25_OP985391 from the Kizugu botanical garden, TAR1_OP985365 and TAR2_OP985366 from World vegetable center – Arusha, TZnK17_OP985357, TZnK18_OP985358, TZnK19_OP985359, TZnK21_OP985361, and TZnK23_OP985363 from Kizimbani –Zanzibar, as well as TTZF1_OP985392, TTZF2_OP985393, TTZF5_OP985396, TTZF7_OP985398, TTZF8_OP985399, and TTZF9_OP9853400 from Amani Zigi Forest, were found to be closely related to one another within the second subclade.

In the third subclade *Eucalyptus torquata* (NC_022401) showed a closely relationship with Allspice isolate with accession numbers TZnK22_OP985362, TZnK24_OP985364 from Kizimbani – Zanzibar and TTKB2_OP985368 from Kizugu botanical garden as shown in Figures 2, 3.

The fourth subclade was consisting only of the following Myrtaceae species *Acca sellowiana* (KX289887), *Syzygium samarangense* (NC_060657), *Eugenia Selloi* (MN095411), *Myrcianthes pungens* (MN095409), *Lophomyrtus bullata* (MW214669), *Lenwebbia prominens* (MW214668), *Lenwebbia lasioclade* (MW214667), and *Syzygium nervosum* (NC_053907).

Discussion

Studying the phylogenetic diversity of plants is crucial to understand its evolutionary history, identifying genetic variations and traits, and developing effective conservation. In this study, the amplification of the Allspice *cprbcL* gene was observed at a length of 560bp. The yield and appearance of the DNA band were attributed to the gel's capacity to sort DNA molecules by size and the primer's efficacy in amplifying the conserved region of the Allspice *cprbcL* gene, as reported previously. The nucleotides statistics of *cprbcL* gene was found to have GC contents of 44%, however, there is a difference in GC content between genes within a genome and between genomes of one species and another. It has been reported that GC levels in plants range from 28.81% to 42.14% . Therefore, in this study GC levels were found to be 44% which is a relatively high level and indicate the thermal stability of the *cprbcL* gene in Allspice.

BLASTn search as presented in shows a high level of similarity between the query and subject sequence, with a range of 96% to 100%. Additionally, the number of identical bases was 539, representing 94.90% of the total bases, with a pairwise identity between sequences of 99.20%. The mean alignment was 553, and the standard deviation was 26.2. The patristic distance ranged from 0.01 to 0.04. These findings suggest that the query and subject sequences are highly similar, indicating a possible evolutionary relationship between them. The high level of similarity also implies that the sequences may be functionally related. These results are consistent with previous studies that have used BLASTn searches to identify sequence similarities between organisms and genes. For example, a study that compared

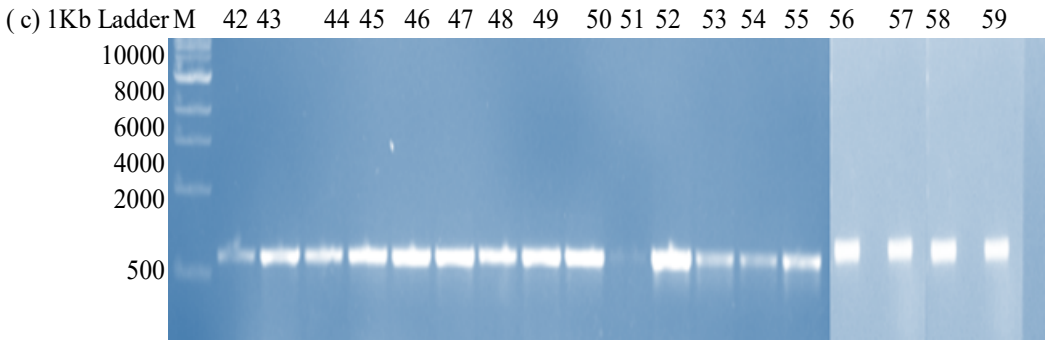

Figure 3: PCR amplified products for the cprbcL gene by Allspice collections.

the genetic information of loblolly pine and Arabidopsis thaliana, it was found that there was a higher level of apparent homology between their expressed genes from wood-forming tissues.

The patristic distance in shows a range of 0.01 to 0.04 which indicates that the query and subject sequences are relatively closely related, with a low degree of divergence between them. This may suggest a recent common ancestor or a relatively short evolutionary distance between the sequenced. This finding is in line with previous studies that have shown that patristic distance is a useful metric for measuring the evolutionary distance between DNA sequences.

The phylogenetic tree presented in depicts a common ancestor and cluster of plant species that are closely related to Allspice, as evidenced by their *cprbcL* genes. The tree represents a shared genetic origin for the plant species within a subclade, indicating their evolutionary relatedness and the diversification of their genetic material over time. The identified plant species are derived from various geographic regions, including Australia, China, the United Kingdom, Brazil, and New Zealand.

The first subclade in the phylogenetic tree identifies a group of plant species that show less genetic heterogeneity between members of the *Myrtaceae* family and the Allspice isolate from Tanzania. The observation of a close evolutionary relationship between Allspice and members of the *Myrtaceae* family is a significant finding, as it suggests that these plant species share a common ancestry and have likely undergone similar evolutionary processes. This is consistent with previous studies that have investigated the evolutionary relationships between different plant taxa based on molecular information . A study discovered that the plant families *Boraginaceae* and *Convolvulaceae* exhibit a close relationship based on genetic data, in accordance with their comparable floral characteristics and habitat preferences . The closeness among Allspice and other *Myrtaceae* family members also implies potential ecological relationships among them. This is supported by studies that have shown that closely related plant species tend to share ecological niches and adaptive traits. A research conducted on oak species in California revealed that leaf characteristics linked with resistance to drought and efficient use of resources are analogous among closely related species. The identification of plant species from different geographical regions and their placement on the phylogenetic tree has significant implications for plant taxonomy, ecology, and conservation .

However, the second subclade of the phylogenetic tree in indicate a lower level of genetic diversity within the Allspice population from Tanzania. This finding is consistent with previous studies that have shown that small, isolated populations are more susceptible to genetic drift and inbreeding, which can reduce genetic diversity .

The third subclade in the phylogenetic tree of Allspice and related species reveals valuable insights into the evolution and genetic variation of these plant species. Specifically, the genetic diversity observed between Allspice isolates from Kizimbani – Zanzibar with accession numbers TZnK22_OP985362 and TZnK24_OP985364, and from Kizugu botanical garden with accession number TTKB2_OP985368. And *Eucalyptus torquata* (NC_022401) suggests that these species have diverged significantly over time. The level of genetic diversity observed in this subclade aligns with findings from prior research that have shown that genetic diversity can be influenced by various factors such as geographic distance, environmental conditions, and reproductive isolation. A study on Eucalyptus species in southeastern Australia revealed high genetic diversity, likely due to geographic isolation and adaptation to diverse environmental conditions. Another study on the genetic diversity of Allspice in Jamaica found that the population structure was influenced by the geographic distribution of the species and the type of soil in which it grows. Conservation strategies such as habitat protection and restoration can help preserve the genetic diversity of these plant species and maintain their ecological relationships for future generations.

The phylogenetic tree reveals that there is substantial genetic diversity among the population of Allspice from Tanzania. This finding is supported by the presence of distinct subclades that share a common ancestor but display significant genetic variations. The existence of these subclades provides evidence that Allspice population in Tanzania has undergone genetic differentiation, which may have occurred due to various factors such as geographic isolation, founder effects, or natural selection. In a recent study on a rare plant species known as *Silene tatarica*, it was discovered that the species exhibited considerable genetic differentiation as a result of both geographic isolation and founder effects . Another study on the genetic diversity

of *Pinus koraiensis* populations in China also found evidence of genetic differentiation within populations, which was attributed to natural selection and genetic drift.

Conclusion

The main objective of this study is to investigate the phylogenetic diversity present within Allspice (*Pimenta dioica*) collections originating from Tanzania. The phylogenetic analysis of the *cprbcL* gene revealed genetic diversity within Allspice population found in Tanzania, and had a strong evolutionary relationship with other species in the *Myrtaceae* family. Also, the obtained results provide new insights to understand the biology and ecology of Allspice and aid in conservation efforts.

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