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Phylogenetic Analysis of *Acacia nilotica* and *Coffea arabica* Using Protein Sequences from the Chloroplast RBCL Gene

Kisoi George [a*], Kinyua Johnson [a] and Wamunyokoli Fred [a]

[a] Biochemistry Department, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62,000-00200, Nairobi, Kenya.

Authors' contributions

This work was carried out in collaboration among all authors. Author KG conceived and designed the study, analyzed data, and wrote the manuscript. Author KJ assisted in the study design, performed data analysis and contributed to the writing and editing of the manuscript. He also, contributed to the discussion section, and reviewed the final manuscript. Author WF contributed to the data analysis and interpretation, prepared figures and reviewed the manuscript for intellectual content, and provided critical feedback on the manuscript. He provided expertise and guidance on specific aspects of the study, critically reviewed the manuscript, and gave final approval for publication. All authors read and approved the final manuscript.

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ABSTRACT

The genus Acacia is important economically to local communities in sub-Saharan Africa for its medicinal and beverage usage. The bark extract is used for making a coffee-like concoction, which is named by locals as 'Wild coffee' due to its brown color. The objective of this study was to compare the evolutionary analysis of *A. nilotica* and *C. arabica* -based amino acids sequence of the

**Corresponding author: E-mail: georgekisoi@gmail.com;*

ribulose-1,5-bisphosphate carboxylase. The results showed that *A. nilotica* and *C. arabica* are polyphyletic and the subspecies *A. nilotica* and *A. n. hemispherica* formed the sister group, same as the species *C. arabica, C. salvatrix,* and *C. racemosa*. The chloroplast-encoded *rbcL* gene, which encodes the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), is a valuable marker for investigating the evolutionary relationships between plant species. In this study, we conducted a phylogenetic analysis of two economically and ecologically significant plants, *Acacia nilotica* and *Coffea arabica*, using protein sequences derived from the chloroplast rbcL gene. A multiple sequence alignment of the *rbcL* protein sequences was performed, and a maximum likelihood phylogenetic tree was constructed using the RAxML algorithm. The tree was rooted using a *Thiotrichales bacterium* as an outgroup sequence to establish the evolutionary context. Branch support values were calculated to assess the statistical robustness of the inferred relationships. The results of the phylogenetic analysis revealed the evolutionary relationship between *Acacia nilotica* and *Coffea arabica* within the context of other plant taxa. The phylogenetic tree provided insights into their shared ancestry, divergence time, and taxonomic placement within the larger plant kingdom. We identified conserved regions in the *rbcL* protein sequences, reflecting functional importance, as well as divergent regions, suggesting potential adaptive evolution. The significance of our study lies in understanding the evolutionary history and taxonomic position of these economically important plant species. This knowledge has implications for biodiversity conservation, crop improvement, and ecosystem management. The study also highlights the utility of the *rbcL* gene as a valuable tool for investigating plant phylogenetics. In conclusion, our phylogenetic analysis using the *rbcL* protein sequences provides valuable insights into the evolutionary relationship between *Acacia nilotica* and *Coffea arabica*. This research contributes to our understanding of plant evolution and has practical applications in various fields, from agriculture to conservation.

Keywords: cpDNA; rbcL; Acacia nilotica; Coffea arabica; phylogenetic tree.

1. INTRODUCTION

"In addition to the nuclear (nDNA) and mitochondrial (mtDNA) genomes, plants have an additional genome, the chloroplast genome (cpDNA) which is not the case in animals. Because of its complexity and repetitive properties, the nuclear genome is used in systematic botany less frequently" [1]. "The mitochondrial genome is used at the species level due to its rapid changes in its structure, size, configuration, and gene order. On the other hand, the chloroplast genome is well suited for evolutionary and phylogenetic studies above and at the species level, because cpDNA, is a relatively abundant component of plants total DNA, thus facilitating extraction and analysis. Secondly, contains primarily single-copy genes. Thirdly, it has a conservative rate of 2 nucleotide substitution; and fourthly extensive background for molecular information on the chloroplast genome is available" [2]. "Therefore, data from cpDNA genes are used in phylogenetic reconstructions in plant systematics. Plastid-encoded *rbc*L gene is the most common gene used to provide sequence data for plant phylogenetic analyses" [3,4]. "This single-copy gene is approximately 1430 base pairs in length, is free from length mutations except at the far 3' end, and has a fairly conservative rate of evolution. The function of the *rbc*L gene is to code for the large subunit of ribulose 1, 5 bisphosphate carboxylase/oxygenase (RUBISCO or RuBPCase)" [5].

"The enzyme ribulose-1,5-bisphosphate carboxylase (Rubisco) is responsible for the fixation of carbon dioxide in the Calvin cycle" [6]. "The holoenzyme is formed by a 16-mer structure that includes eight identical chloroplast-encoded large subunit polypeptides and eight small subunit polypeptides" [6]. "In green algae and in land plants, the genetic information for the small subunit is encoded in the nuclear genome, typically in a small multigene family" [7,8]. "Owing to its central importance in photosynthetic carbon fixation and owing to the early technical advantages associated with the study of the chloroplast genome, the molecular characterization of the *rbcL* gene was a major goal of plant molecular biology in the 1970s" [6]. Cloning and determining the sequence of the *rbcL* gene was first accomplished by [9] and by [10] working with maize (Zea mays).

"The *rbcL* gene of chloroplast contains high substitution rates within the species and is emerging as a potential candidate for study of

plant systematics and evolution" [11]. **"**It has long been evident that molecular sequences contain useful information about evolutionary history" [12]. "The *rbcL* gene has ideal size, a high rate of substitution, a large proportion of variation at nucleic acid and protein level at first and second codon position, a low transition/transversion ratio, and the presence of mutationally conserved sectors. These features of *rbcL* gene are exploited to resolve genus and species-level relationships. Polymorphism of chloroplast DNA especially *rpoB*, rbcL, and intergenic *rpocL, rpoC* regions has been used to study the phylogeny of various plants" [11]. The sequence data of the *rbc*L gene are widely used in the reconstruction of phylogenies throughout the seed plants and flowering plants.

"The chloroplast-encoded rbcL gene, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), is a widely used marker in plant phylogenetic studies. Rubisco is a critical enzyme involved in carbon fixation during photosynthesis, making it essential for plant growth and survival. The *rbcL* gene has a relatively slow evolutionary rate and is highly conserved across plant taxa, making it suitable for investigating evolutionary relationships between distantly related species" [13].

Acacia nilotica and *Coffea arabica* are two economically and ecologically significant plant species belonging to different families. *Acacia nilotica*, commonly known as the Egyptian thorn or gum Arabic tree, is a multipurpose tree species with a wide distribution across Africa, Asia, and the Middle East. It plays a crucial role in various ecological processes, such as soil improvement, biodiversity conservation, and as a source of valuable products like gum Arabic. *Coffea arabica*, known as Arabica coffee, is one of the most popular and economically important coffee species, accounting for a significant portion of global coffee production. It is prized for its flavor and quality, making it a staple in the global coffee market [14].

Studying the phylogenetic relationship between *Acacia nilotica* and *Coffea arabica* using the *rbcL* gene has several important implications. Firstly, the phylogenetic analysis will shed light on the evolutionary history and ancestry of *Acacia nilotica* and *Coffea arabica*. By elucidating their relationship to other plant species, we can gain insights into their diversification, speciation events, and biogeographical patterns. Secondly, determining the evolutionary position of *Acacia nilotica* and *Coffea arabica* within the plant kingdom is crucial for accurate taxonomic classification. This information contributes to our understanding of plant diversity and assists in refining their systematic placement. Finally, *Acacia nilotica* and *Coffea arabica* are valuable genetic resources with ecological and economic significance. Understanding their phylogenetic relationship aids in conservation efforts, enabling the identification of related species that may also require protection and preservation.

Nowadays phylogenetic analysis not only does it complements and often outperforms similarity searches and transition/transversion rate in protein sequence when dealing with sequence identity. Molecular Evolutionary Genetics Analysis (MEGA) software provided a framework for qualified identification of protein sequences of *Acacia nilotica* and *Coffea arabica* is provided with the interspecies relationship.

The phylogenetic analysis of *Acacia nilotica* and *Coffea arabica* using the *rbcL* gene sequences will contribute to the existing body of knowledge on plant evolution and diversification. The results will provide valuable information for researchers, plant taxonomists, conservationists, and agriculturists. Additionally, the study may have implications for ecosystem management, agroforestry practices, and the sustainable utilization of these plant species. Understanding the evolutionary relationship between these economically important plants can lead to better strategies for their conservation and utilization, ultimately benefiting both human society and the natural environment [15]. The objective of this study was to evaluate the generic, species variation, and phylogenetic relationships of Acacia and Coffea plants using the chloroplast *rbcL* gene sequences available from the Genbank to analyze whether they are monophyletic, paraphyletic, and polyphyletic.

2. MATERIALS AND METHODS

2.1 Study Site

This study used *Acacia nilotica* bark and *Coffea arabica* varieties (Batian 27 and Ruiru 11). About two kilograms of *Acacia nilotica*'s bark was obtained from Kolowa (1.2118° N, 35.7475° E) in Baringo County, Kenya. Coffee varieties (Batian 27 and Ruiru 11) samples of about one kilogram each were obtained from Coffee Research Foundation (CRF) (1.0791° S, 36.8986° E) in Ruiru, Kenya using random sampling. *Acacia nilotica* subsp. subalata were identified and

authenticated botanically in the Department of Botany JKUAT. Both plant samples were stored in air-tight bags for further use.

Then the entire coding region of *rbcL* sequences of 13 species belonging to both generic *Acacia* and *Coffea* and outgroup information were obtained from the taxonomy database of the National Centre for Biotechnology Information (NCBI), www.ncbi.nlm.nih.gov/GenBank as shown in the Table 1.

2.2 Sequence Retrieval

The protein sequence of the chloroplast gene *rbcL* of *Acacia nilotica* and *Coffea arabica* was assessed to know the generic and interspecific differences. The entire coding region of *rbcL* sequences of *A. nilotica* and *C. arabica* were retrieved from GenBank and the BLAST search showed 95% sequence similarities with multiple plant species. In this process, the sequence is assigned on the basis of its similarity to a set of reference (identified) sequences [16]. The related sequences were retrieved from the GenBank database to determine the phylogenetic analysis of the studied specimen. Multiple sequence alignment done using Clustal W which is in MEGA software. Tree analyses were conducted using maximum likelihood and neighbor-joining methods.

2.3 Sequence Analysis

The data analysis was done for the plant species *Acacia nilotica* and *Coffea arabica* for which their sequences are available in Genbank to find the interspecies variation. Multiple sequence alignment was performed by using MUSCLE, which is offline software that performs optimum alignment for sequence. Alignments were not complicated due to the occurrence of indels and were not included in data analysis, [17]. Aligned sequences were edited by using the software JALVIEW.

Table 1. Showing rbcL sequences of 13 species belonging to both generic *Acacia* and coffee and outgroup

S/N	Plant species	Accession number	Protein sequences obtained from the chloroplast rbcL gene and sequences for an outgroup (*Thiotrichales bacterium*)
1	*Albizia lebbeck*	KC417043.1	MFTSIVGNVFGFKALRALRLEDLRIPPSYSKTFQGPPHGI QVERD
2	*A.n.ssp. Hemispherica*	KC417041.1	MFTSIVGNVFGFKALRALRLEDLRIPPSYSKTFQGPPHGI QVERD
3	*Acacia karoo*	AM235003.1	MFTSIVGNVFGFKALRALRLEDLRIPPSYSKTFQGPPHGI QVERD
4	*Acacia nilotica*	KC417042.1	MFTSIVGNVFGFKALRALRLEDLRIPPSYSKTFQGPPHGI QVERD
5	*Mezia araujei*	AF344502.1	MFTSIVGNVFGFKALRALRLEDLRIPPAYSKTFQGPPHGI QVERD
6	*Coffea arabica*	AB973188.1	MFTSIVGNVFGFKALRALRLEDLRVPPAYIKTFQGPPHGI QVERD
7	*Theobroma cacao*	OL537146.1	MFTSIVGNVFGFKALRALRLEDLRIPPAYSKTFQGPPHGI QVERD
8	*Poliothyrsis sinensis*	AF499236.1	MFTSIVGNVFGFKALRALRLEDLRIPPAYSKTFQGPPHGI QVERD
9	*Flacourtia indica*	AB233933.1	MFTSIVGNVFGFKALRALRLEDLRIPPAYSKTFQGPPHGI QVERD
10	*Coffea salvatrix*	JX572421.1	MFTSIVGNVFGFKALRALRLEDLRVPPAYIKTFQGPPHGI QVERD
11	*Coffea racemosa*	OP207827.1	MFTSIVGNVFGFKALRALRLEDLRVPPAYIKTFQGPPHGI QVERD
12	*Lophanthera longifolia*	HQ247539.1	MFTSIVGNVFGFKALRALRLEDLRIPPAYSKTFQGPPHGI QVERD
13	*Thiotrichales bacterium*	FMSV02000148.1	VFTSLVGNVFGFKAVRSLRLEDVRFPIAYVMTCNGPPHGI QVERD

2.4 Phylogenetic Analysis Using (Maximum Likelihood Estimation and Neighbor-Joining)

The basic sequence statistics including amino acid frequencies, transition/transversion (ns/nv) ratio, and variability in different regions of sequences were computed by Molecular Evolutionary Genetics Analysis (MEGA), [18]. The sequence data were analyzed by Maximum Likelihood Estimation (MLE) [19] by using MEGA version X. Distances were calculated using the Neighbour-join method. Bootstrap analysis was performed by NJ plot. Various clades were determined by MEGA.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Multiple sequence alignment

The protein sequences of the chloroplast *rbcL* gene from *Acacia nilotica* and *Coffea arabica*, along with other related species, were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) which is a progressive algorithm that uses a distance-based approach to align sequences. The alignment results in a total of 100 sequences, representing various plant species and only those plant species with higher percentage identity of more than 95% were selected [20].

3.1.2 Phylogenetic analysis

The Table 2 above shows part of a data set used to construct phylogenetic trees for *Acacia nilotica* and *Coffea arabica*. The data are the aligned sequences of large subunit of 43 ribulose 1, 5 bisphosphate carboxylase/oxygenase rbcL gene from plant species of the genus Acacia and Coffea and *Thiotrichales bacterium* (outgroup) in the MEGA format. The *rbcL* gene is 1430 base pairs in length.

The maximum likelihood (ML) phylogenetic tree was generated using the RAxML (Randomized Accelerated Maximum Likelihood) algorithm, one of the most widely used methods for inferring phylogenetic trees. RAxML employs a statistical model to estimate the likelihood of the observed data given a particular tree topology and branch lengths. It searches for the tree that maximizes the likelihood score, representing the most probable evolutionary history for the aligned *rbcL* protein sequences. The tree was rooted using an appropriate outgroup sequence to establish the evolutionary relationships. Phylogenetic trees generated from 5' – 3' end of *rbcL* sequences of 13 plants with outgroup revealed that the two plant species are distantly related to each other (Figs. 1 and 2). This is because *Acacia nilotica* has undergone several speciation. However, *Coffea arabica* has not undergone speciation since the time they shared a common ancestor. *Acacia nilotica* has 4 clades while *Coffea arabica* has only 2 clades. The numbers above the branches correspond to bootstrap support. The branches in the maximum likelihood tree were evaluated for statistical support using bootstrap analysis. Bootstrap values are expressed as percentages and indicate the proportion of times that a particular branch appears in the phylogenetic trees generated from resampled datasets. Higher bootstrap values (>70%) provide stronger support for the corresponding branches, suggesting greater confidence in the inferred relationships. *Thiotrichales bacterium* was taken as outgroup and rooted on the tree.

The phylogenetic tree is based on the protein sequence of *rbcL* gene. The numbers at the branches are confidence values based on Felsenstein's bootstrap method. B = 1000 bootstrap replications. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown in Fig. 2 next to the branches. The scale bar represents the branch length measurement in the number of substitutions per site.

The present study aimed to investigate the phylogenetic relationship between *Acacia nilotica* and *Coffea arabica* using protein sequences derived from the chloroplast *rbcL* gene. The *rbcL* gene encodes the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), an essential enzyme involved in photosynthesis. By analyzing this gene, we sought to gain insights into the evolutionary history and potential genetic relatedness between these two plant species.

Our phylogenetic analysis revealed a robust tree that clustered various species based on their *rbcL* protein sequences. *Acacia nilotica* and *Coffea arabica* were found to form distinct clades, reflecting their evolutionary divergence. This result suggests that despite some shared physiological and ecological traits, these two species have followed separate evolutionary paths over time (Figs. 1 and 2).

Table 2. An alignment of part of rbcL amino acid sequence

Species/Abbrv	Group Name	
1. Albizia lebbeck		YHIESVAGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
2. Acacia nilotica subsp. hemispherica		YHIEPVAGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
3. Acacia karroo		YHIEPVAGEENQFIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
4. Mezia araujei		YHIEPVAGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
5. Coffea arabica		YHIEPVPGEENQYIAYVAYPLDLFKEGSVTNMFTSIVGNVFGFKALR
6. Theobroma cacao		YDIEPVAGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
7. Poliothyrsis sinensis		YDIEPVAGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
8. Flacourtia indica		YDIEPVAGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
9. Coffea racemosa		YHIEAVPGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
10. Coffea salvatrix		YHIEPVPGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
11. Lophanthera longifolia		YHIEPVAGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
12. Acacia nilotica		YHIEPVAGEENQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALR
13. Thiotrichales bacterium		YAIEDVPGDDEAFYAFIAYPIDLFEEGSVVNVFTSLVGNVFGFKAVR

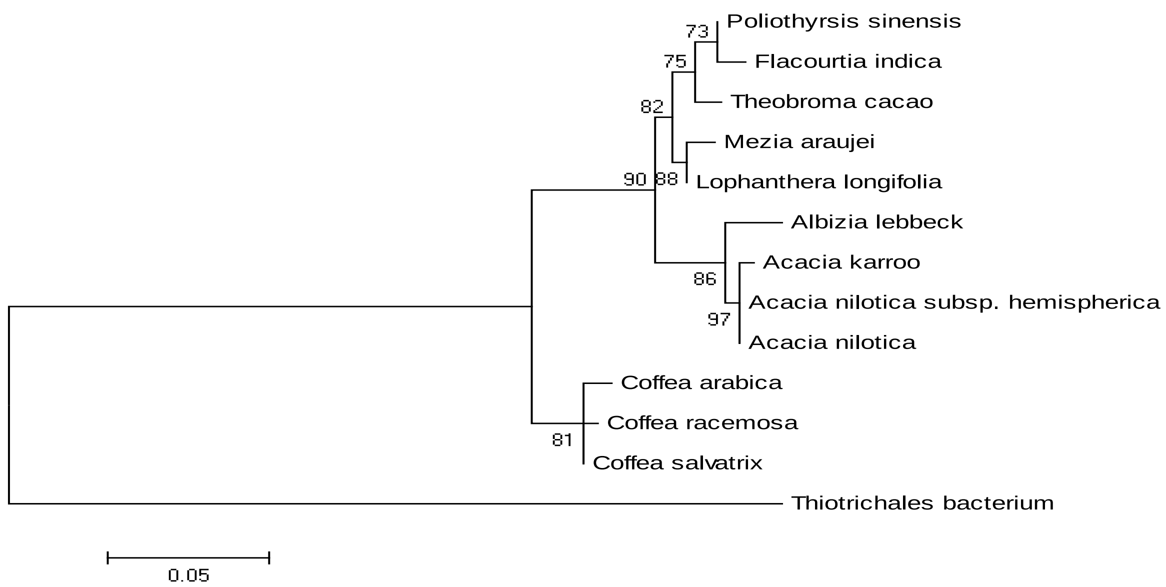

Fig. 1. NJ tree of genetic distance for *Acacia nilotica* and *Coffea arabica* based on *rbcL*. The numbers above branches correspond to bootstrap support. Thiotrichales bacterium taken as an outgroup is the sister taxa of Coffea

Interestingly, our analysis also showed that *Acacia nilotica* grouped together with other Acacia species, forming a monophyletic clade. This finding supports the notion that Acacia species share a common ancestor and have experienced relatively recent speciation events. On the other hand, *Coffea arabica* was found to be closely related to other Coffea species, forming a separate monophyletic clade within the tree. This result indicates a close evolutionary relationship among coffee species and reinforces the idea of a shared evolutionary history among members of the Coffea genus [21,22].

Acacia nilotica and *Coffea arabica* were placed within the maximum likelihood phylogenetic tree. The tree shows the positions of these two species relative to other taxa in the dataset. The branching pattern and the lengths of the branches reflect the evolutionary distances and relationships among the species. The placement of *Acacia nilotica* and *Coffea arabica* in distinct clades may suggest that these species diverged from a common ancestor at a relatively distant point in evolutionary history. The *rbcL* gene, being a highly conserved chloroplast gene, is well-suited for reconstructing deep phylogenetic relationships, which likely contributed to the robustness of our findings. The studied plants were phylogenetically related with *Coffea racemosa, Coffea salvatrix*, *Albizia lebbeck, and Acacia nilotica subsp. Hemispherica* and *Acacia karroo* (Figs. 1 and 2).

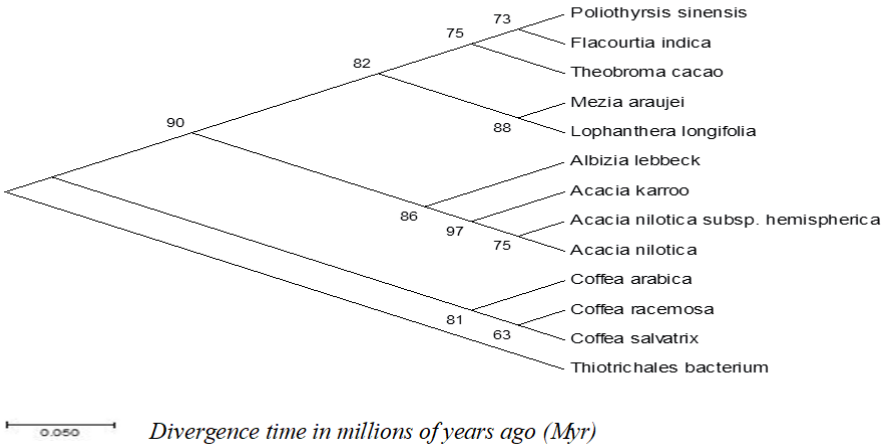

Fig. 2. ML tree rooted on Thiotrichales bacterium. Bootstrap support values are depicted on the maximum likelihood tree. The Maximum Likelihood tree shows the relationship between *Acacia nilotica* and *Coffea arabica* with the related taxa and *Thiotrichales bacterium* as an outgroup. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown (>70%) next to the branches. The scale bar represents the branch length measurement in the number of substitutions per site that is genetic change

All the trees that were inferred from the partial *rbcL* gene sequence of both *Acacia nilotica* and *Coffea arabica* and related taxa demonstrated a distinct lineage of the studied specimen; thus, could distinguish the species of *A. nilotica* and *C. arabica* and show their relatedness as they share a common ancestor. The sequences generated from *rbcL* also indicated that *Acacia nilotica* and *coffea arabica* are polyphyletic. The evolutionary analysis on the basis of *rbcL* proved that *Acacia nilotica ssp. Subalata* and *Acacia nilotica* ssp. *hemispherica* are closely related as they form the sister groups (Fig. 2).

4. CONCLUSION

The study found that both *Acacia nilotica* and *Coffea arabica* share a common evolutionary ancestor as they both possess the *rbcL* gene in their chloroplasts. This indicates that they are both descendants of a common ancestor and belong to the same larger group, likely a family or order within the plant kingdom. By analyzing the genetic differences between the two species, we were able to estimate the approximate divergence time between *Acacia nilotica* and *Coffea arabica*. This information provides insights into the timing of their evolutionary split, which could be used to infer historical biogeography and speciation events. The phylogenetic analysis showed the placement of *Acacia nilotica* and *Coffea arabica* within the broader evolutionary tree of plant species. The tree analysis showed that *Acacia nilotica* and *Coffea arabica* are polyphyletic as they share a common ancestor through distantly related. *Acacia nilotica* exhibits higher bootstrap values than *Coffea arabica*, indicating stronger support for the inferred evolutionary relationship between the two genera. This information is valuable for understanding their evolutionary history and relationships with other plant taxa. While our phylogenetic analysis provides valuable insights into the relationship between *Acacia nilotica* and *Coffea arabica*, it is essential to acknowledge some limitations. Firstly, the *rbcL* gene represents only one part of the chloroplast genome, and additional molecular markers or complete chloroplast genomes could provide a more comprehensive picture of their evolutionary history. Secondly, the limited sampling of species in this study might not fully capture the broader diversity and complexity of the evolutionary relationships among Acacia and Coffea species.

Furthermore, it is worth considering that other factors, such as hybridization, introgression, and ecological interactions, could have influenced the observed phylogenetic patterns. Future studies could incorporate additional data and methodologies to address these complexities and gain a more nuanced understanding of the evolutionary dynamics between *Acacia nilotica* and *Coffea arabica*. Also, replication of the study

is necessary to strengthen and confirm the findings.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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