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An In Silico Approach for Evaluation of ITS, *rbc*L, and *psb*A-*trn*H for DNA Barcoding of *Eugenia* spp.

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ABSTRACT

The genus Eugenia is known to be complex with intricate synonyms and taxonomy, and morphological identification is often unreliable due to overlapping characteristics and environmental influences, particularly in the flowers. DNA barcoding provides a way around this problem, as it can identify specimens using very short gene sequence fragments obtained from a small number of tissues. The method used in this research is the in silico dengan menggunakan barcode DNA ITS, *rbc*L, dan *psb*A-*trn*H dari spesies *Eugenia* spp yang ditemukan di NCBI GenBank. The successful reconstruction of the phylogenetic tree from the three regions, including ITS, *rbc*L, and *psb*A-*trn*H shows that several species of *Eugenia* spp. are divided into 2 clades. Research successfully analyzed *Eugenia* plant relationship with using the ITS, *rbc*L, and *psb*A-*trn*H gene sequences in silico based shows that several species of *Eugenia* spp. are divided into 2 clades. In general, high bootstrap values are shown by phylogenetic trees based on the ITS region.

***Studi In Silico Eugenia spp. Berdasarkan DNA Barcode ITS, rbc*L, *dan psb*A-*trn*H**

ABSTRAK: *Genus Eugenia dikenal kompleks dengan sinonim dan taksonomi yang rumit, serta identifikasi morfologis yang sering tidak dapat diandalkan karena karakter yang tumpang tindih dan dipengaruhi oleh lingkungan, terutama pada bagian bunga. Barcoding DNA memberikan jalan keluar dari masalah ini, karena dapat mengidentifikasi spesimen menggunakan fragmen urutan gen yang sangat pendek yang diperoleh dari sejumlah kecil jaringan. Metode yang digunakan dalam penelitian ini adalah in silico dengan menggunakan barcode DNA ITS, rbc*L*, dan psbA-trnH dari spesies Eugenia spp yang ditemukan di NCBI GenBank. Keberhasilan rekonstruksi pohon filogenetik dari tiga wilayah antara lain ITS, rbc*L*, dan psbA-trnH menunjukkan bahwa beberapa spesies Eugenia spp. dibagi menjadi 2 clade. Penelitian yang berhasil menganalisis hubungan tanaman Eugenia dengan menggunakan rangkaian gen ITS, rbc*L*, dan psbA-trnH secara silico menunjukkan bahwa beberapa spesies Eugenia spp. dibagi menjadi 2 clade. Secara umum nilai bootstrap yang tinggi ditunjukkan oleh pohon filogenetik berdasarkan wilayah ITS.*

INTRODUCTION

Genus *Eugenia* is the largest genus of Neotropical Myrtaceae. It is also the most species-rich angiosperm genus in Brazil, the second-most species-rich genus of trees in the and the most species-rich tree genus in the ombrophilous forest that surrounds the Atlantic coast of Brazil, locally referred to as the "Mata Atlântica" (Araujo et al., 2021);(da Costa et al., 2020). The natural distribution of *Eugenia* ranges from southern Mexico, Cuba and the Antilles to Uruguay and Argentina, with a small number of species (ca. 20%) in Africa, Southeast Asia and the Pacific.

Eugenia and *Myrcianthes* O.Berg comprise a single clade, named the "Eugenia group" Guollo et al. (2024) investigated the classical infra-generic groups of *Eugenia* and provided suites of morphological characters with which to distinguish them. The resulting topology Silverio et al. (2024) that a monophyletic *Eugenia* can only be preserved by including the traditional Neotropical genera Calycorectes O.Berg, Hexachlamys O.Berg, Phyllocalyx O.Berg and Stenocalyx O.Berg. Within *Eugenia* s.l. and Myrcianthes, nine clades were identified as morphologically diagnosable groups (Zahro et al., 2023). *Eugenia* morphology and current phylogenetic and taxonomic understanding of the "*Eugenia* group" considering recent nomenclatural updates are summarised below. Clade numbers refer to the work where more details are provided (Pittarelli et al., 2021).

The Myrtaceae family presents a multitude of challenging and thought-provoking issues in terms of nomenclature and systematic classification (Cardoso & Sajo, 2004). The genus *Eugenia*, which was named after Francois *Eugene* de Savoie-Carignan, a prominent figure in art, science, and literature during the 17th and 18th centuries, has posed significant challenges and sparked debates in the field of angiosperm taxonomy (Foresti et al., 2024). Since the era of Linnaeus, numerous species from both the Old and New World have been classified under the genus *Eugenia*. According to Defaveri et al. (2011), approximately 2500 species were identified as members of, or reassigned to, this particular genus by Low et al. (2022) , further reported the publication of 100 additional binomial names under *Eugenia* by 1950. Furthermore, Index Kewensis documented an extra 200 names by Santos et al. (2022) introduced 22 new names in a recent publication. Approximately 35 generic names, both legally and invalidly published, which are derived from different OldWorld taxa, have been or have the potential to be consolidated into *Syzygium* P. If the taxonomic classification of *Syzygium* is revised to *Eugenia*, as has been undertaken by numerous scholars, the inclusion of these 35 or more names could significantly increase the count of generic synonyms associated with *Eugenia* s. I. to approximately 70 (Mbobo et al., 2023). This is due to the fact that over 30 primarily American genera Rachmah et al. (2023) have been regarded as congeneric with *Eugenia* s. s. by different researchers. The genus *Eugenia* has evidently become cumbersome, giving rise to a complex synonymy and intricate taxonomy (Mahmoud et al., 2021). There exist three distinct perspectives concerning the distribution of *Eugenia* and its Old World counterparts, as documented in historical literature (Riaz & Abid, 2021).

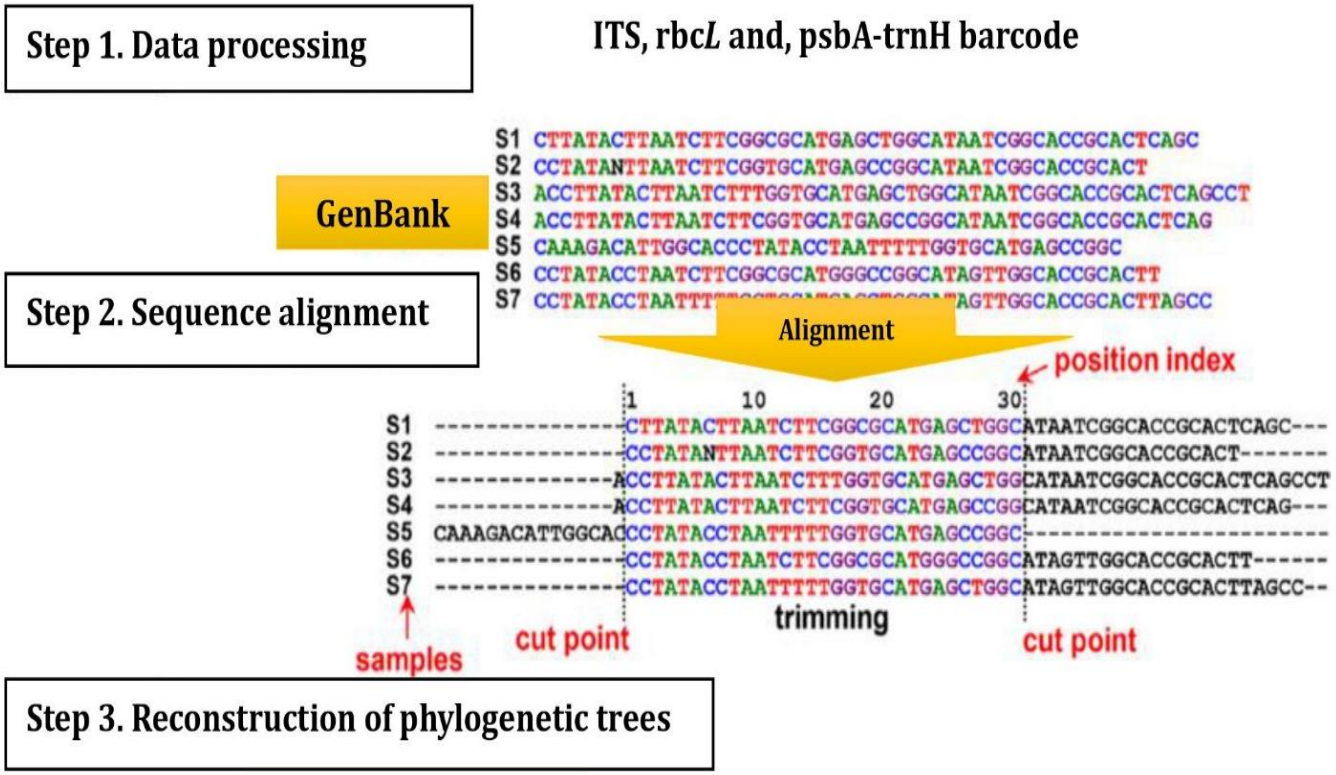
Since 2003, DNA barcode has been used intensively for organism identification. DNA barcode is a short standardized DNA region used to identify organisms as specific as species level (Widiani et al., 2021); (Kamelia et al., 2024). This method possesses several advantages in the comparison to traditional methods such as high repeatability and stability, applicability to any developmental stages of organism, and ability to identify target organisms after cooked or processed (Tefu et al., 2023);(Bare et al., 2022). Similar in silico studies in the Myrtaceae family in the

species *Psidium guajava* and *Eucalyptus globulus* showed microsatellite loci with high Blast e-values or hits (Medeiros et al., 2021).

Syzygium is a difficult genus to be classified as a result of character relatively little morphology, although consistently link a species to in certain species groups well (Zahro et al., 2023). Matter This is also supported by the overlap the names *Syzygium* and *Eugenia* thus occur major revision of the genus Eugenia into *Syzygium* (Badou et al., 2020). Identify species of the genus *Syzygium* accurately use the method conventional with relatively difficult morphology and can take a long time (Reis et al., 2021). This can due to lack of knowledge regarding vegetation an lack of floral character and fruit required for identification (Laha et al., 2020). Sequence-based species identification Short DNA (barcode) is a method what is considered fast, can be accountable and consistent so it is really needed for acceleration Identify the species of an organism especially *Syzygium* (Jamdade et al., 2022). Therefore, the main aims of this in silico study were to evaluate the species resolution ability of ITS, *rbc*L, and *psb*A-*trn*H in *Eugenia* spp. available on the National Center for Biotechnology Information (NCBI). This study may contribute to the use of proper molecular sequences as effective barcoding markers for identification of specific species of *Eugenia* spp., serving for breeding, conservation, and diversity research of this crop plant.

METHOD

The method used in this research is in silico. An in silico is an analysis method that uses a computational technology approach through a database to identify genetic variations and genetic relationships of a species (Santos et al., 2022). Flowchart of in cilico analysis method (Figure 1): Step 1—data processing, Step 2—sequence alignment, and Step 3— reconstruction of phylogenetic trees. The detailed procedures are explained in the following sections.



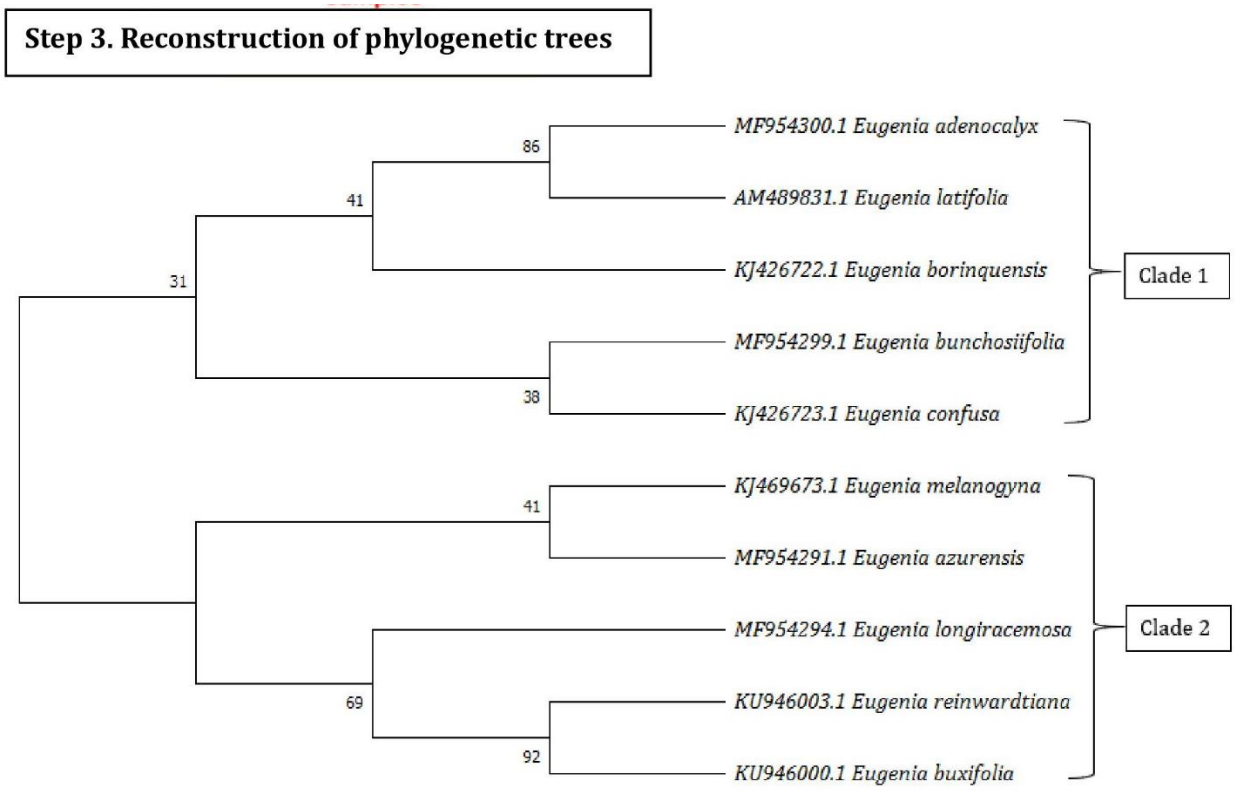


Figure 1. Flowchart of in cilico analysis method

1. Search for nucleotide sequences on the NCBI GenBank

The nucleotide sequence of *Eugenia* spp. was obtained from the NCBI (National Center for Biotechnology Information) GenBank database. Sequence searches were carried out with the keywords: a) *Eugenia* spp. ITS (Internal Transcribed Spacer); b) *Eugenia* spp. *rbc*L (ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit); and c) *Eugenia* spp. *psb*A-*trn*H.

Eugenia spp. sequences from ITS, *rbc*L, and *psb*A-*trn*H were each downloaded in top 10 sequences. Next, all the sequences are downloaded in FASTA format for analysis.

2. Sequence alignment using MEGA X

An alignment process then carries out *Eugenia* spp. sequences downloaded. The alignment process uses the MEGA X application. The alignment process is carried out to analyze a species homology level and identify sequences that have the potential as barcodes.

3. Reconstruction of phylogenetic trees

Reconstruction of the phylogenetic tree of *Eugenia* spp. was carried out using the MEGA X. After that, genetic distance analysis was carried out from the *Eugenia* spp. sequence alignment data using the MEGA X application. The phylogenetic tree was reconstructed using the neighbor-joining statistical method, the 1000 times bootstrap method, the p-distance substitution model including transitions and transversions, and complete deletion (Kapli et al., 2020);(Wibberg et al., 2021);(Salem et al., 2023).

RESULTS AND DISCUSSION

Percent Identity of *Eugenia* spp.

This research using DNA barcode sequence ITS (Internal Transcribed Spacer), *rbc*L (ribulose-1,5-bisphosphate carboxylase /oxygenase large subunit), and *psb*A-*trn*H from the *Eugenia* spp. species found in NCBI GenBank. The sequences used in this study are the ITS gene (Table 1), *rbc*L gene (Table 2), and *psb*A-*trn*H gene (Table 3).

Table 1. Top 10 Percent Identity of *Eugenia* spp. sequences ITS Regions

No.	Scientific Name	Query Cover	E-Value	Percent Identity	Accession
1.	*Eugenia bunchosiifolia*	100%	0.0	100.00%	MG707978.1
2.	*Eugenia stigmatosa*	100%	0.0	98.13%	MG.708067.1
3.	*Eugenia speciosa*	100%	0.0	97.70%	KX789274.1
4.	*Eugenia luschnathiana*	100%	0.0	97.56%	KX789272.1
5.	*Eugenia ruschiana*	100%	0.0	97.42%	KX789280.1
6.	*Eugenia bacopari*	100%	0.0	97.28%	KJ187608.1
7.	*Eugenia wentii*	100%	0.0	97.27%	KX789273.1
8.	*Eugenia pluriflora*	100%	0.0	97.14%	MG708034.1
9.	*Eugenia hiemalis*	100%	0.0	97.14%	KJ187623.1
10.	*Eugenia macrocalyx*	100%	0.0	97.13%	FJ037852.1

Table 2. Top 10 Percent Identity of *Eugenia* spp. sequences *rbc*L Regions

No.	Scientific Name	Query Cover	E-Value	Percent Identity	Accession
1.	*Eugenia cereja*	100%	0.0	100.00%	MG718099.1
2.	*Eugenia bunchosiifolia*	100%	0.0	100.00%	MG718096.1
3.	*Eugenia subavenia*	100%	0.0	99.86%	MG718113.1
4.	*Eugenia prasina*	100%	0.0	99.86%	MG718111.1
5.	*Eugenia mosenii*	100%	0.0	99.86%	MG718106.1
6.	*Eugenia cuprea*	100%	0.0	99.86%	MG718102.1
7.	*Eugenia cerasiflora*	100%	0.0	99.72%	MG718260.1
8.	*Eugenia verticillata*	100%	0.0	99.72%	MG718116.1
9.	*Eugenia brevistyla*	100%	0.0	99.72%	MG718095.1
10.	*Eugenia supraaxillaris*	100%	0.0	99.72%	MG718114.1

Table 3. Top 10 Percent Identity of *Eugenia* spp. sequences psbA-trnH

No.	Scientific Name	Query Cover	E-Value	Percent Identity	Accession
1.	*Eugenia bunchosiifolia*	100%	0.0	100.00%	MF954299.1
2.	*Eugenia adenocalyx*	100%	0.0	97.60%	MF954300.1
3.	*Eugenia latifolia*	100%	0.0	97.41%	AM489831.1
4.	*Eugenia melanogyna*	100%	0.0	97.23%	KJ4696731.1
5.	*Eugenia reinwardtiana*	100%	0.0	97.04%	KU946003.1
6.	*Eugenia confusa*	100%	0.0	96.86%	KJ426723.1
7.	*Eugenia buxifolia*	100%	0.0	96.86%	KU946000.1
8.	*Eugenia longiracemosa*	100%	0.0	96.49%	MF954294.1
9.	*Eugenia azurensis*	100%	0.0	96.49%	MF954291.1
10.	*Eugenia boringuensis*	100%	0.0	96.32%	KJ426722.1

Reconstruction of the Phylogenetic Tree

The NCBI GenBank contains a lot of genetic information, including the DNA sequence of an organism and the nucleotide length and accession number for each organism. Based on searches, many *Eugenia* spp. species are available at NCBI. This data was obtained from the isolation of the ITS, *rbc*L, and *psb*A-*trn*H genes (Winandari et al., 2024). Each *Eugenia* spp. sequence from the ITS, *rbc*L, and *psb*A-*trn*H genes underwent the NCBI BLAST and alignment process in the MEGA X application as identification data for phylogenetic tree reconstruction. The

BLAST process was carried out to determine the level of similarity of the sequences studied with the sequences contained in NCBI GenBank. A total of 10 species with the highest percent identity and query cover values were selected and downloaded in FASTA format. Next, the downloaded sequences are subjected to alignment and phylogenetic tree reconstruction in NCBI GenBank.

The phylogenetic tree was reconstructed using the Neighbor-joining statistical method, the 1000 times bootstrap method, the p-distance substitution model including transitions and transversions, and complete deletion (Salsabila et al., 2023). Phylogenetic trees show evolution in living things from the same ancestor (Hu et al., 2020). Phylogenetic trees are a combination of molecular biology and statistics. Phylogenetic trees are often used to explain paralogy and orthology through molecular approaches (Zhang et al., 2021). Phylogenetic tree reconstruction uses bootstrap values with 1000x replications to reconstruct accurate phylogenetic tree results (Kapli et al., 2021). Phylogenetic trees show the relationship between species. According to research by Letunic & Bork (2021) kinship is an interspecies or intraspecies status that has a high percentage of similarities and indicates that species can be said to be related (Smith, 2020). Phylogenetic tree reconstruction, namely the ITS gene (Figure 2), *rbc*L gene (Figure 3), and *psb*A-*trn*H gene (Figure 4).

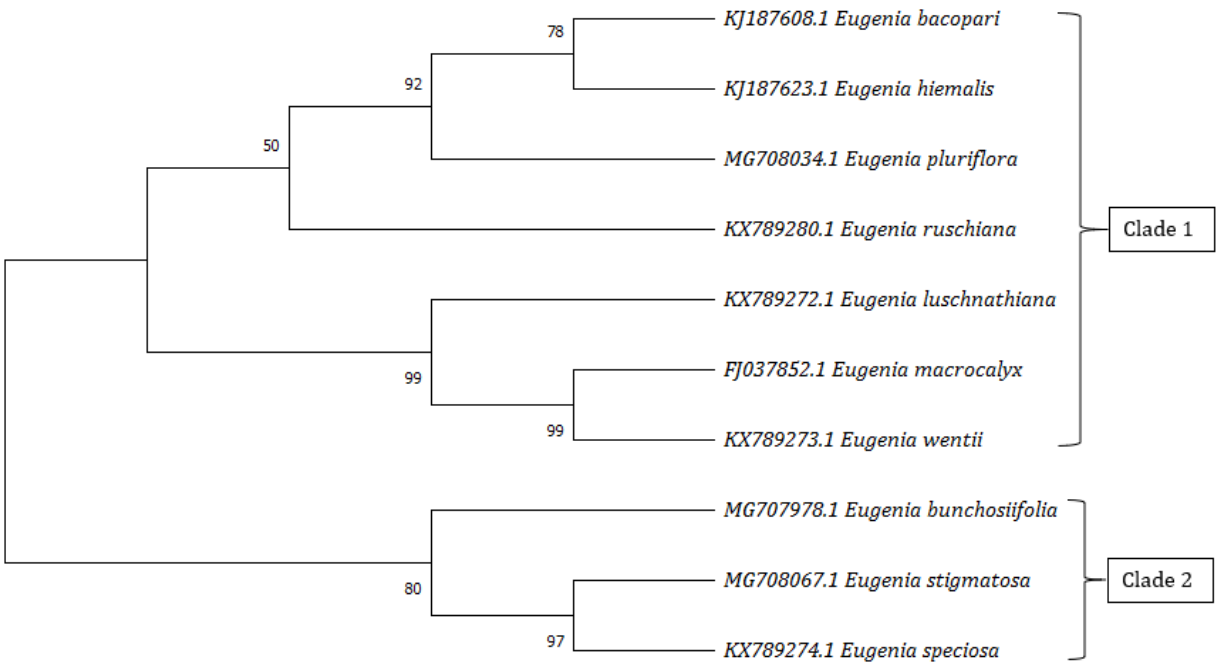


Figure 2. Phylogenetic tree reconstruction based on ITS using the Neighbor-joining statistical method, 1000 times bootstrapping, and p-distance substitution model

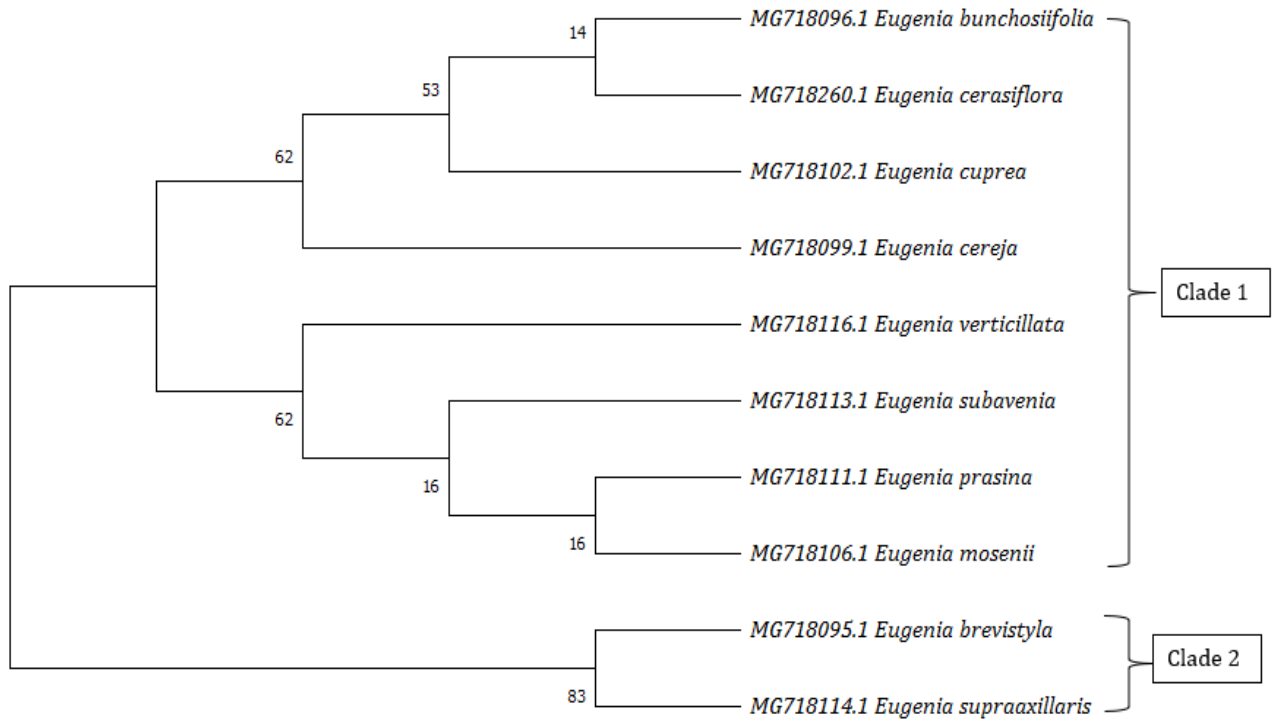


Figure 3. Phylogenetic tree reconstruction based on *rbc*L using the Neighbor-joining statistical method, 1000 times bootstrapping, and p-distance substitution model

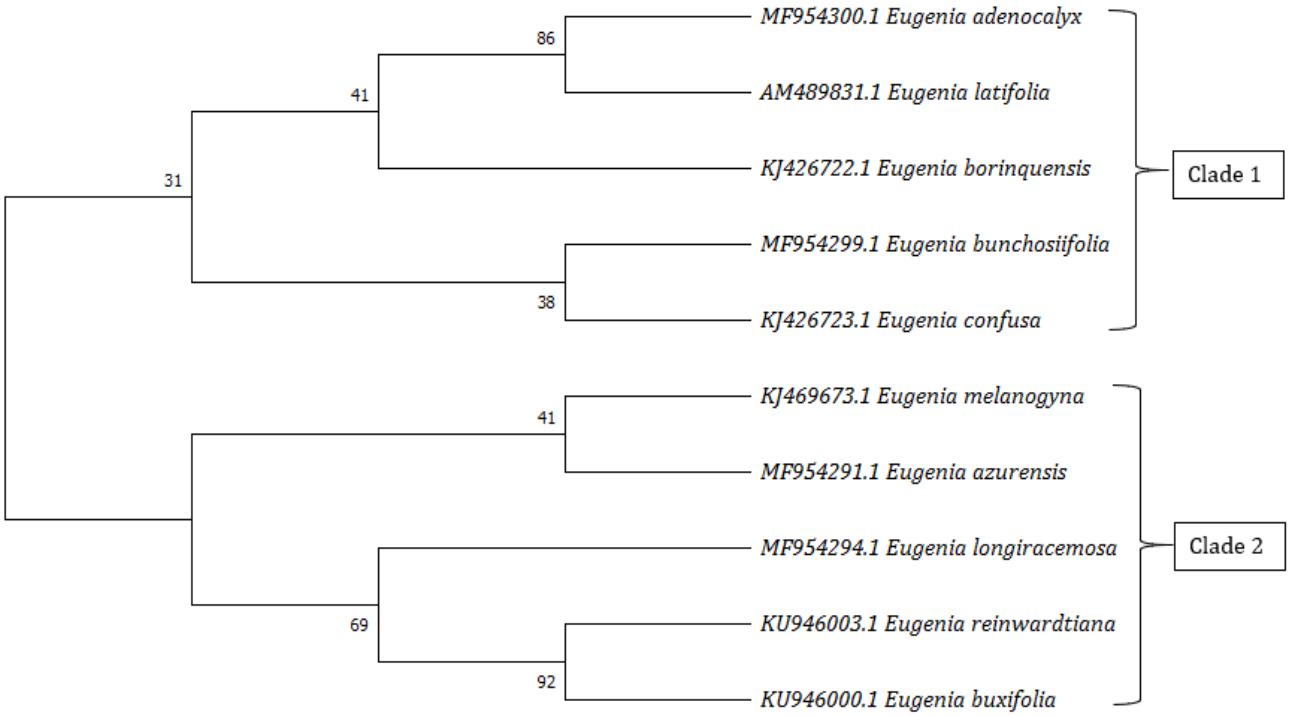


Figure 4. Phylogenetic tree reconstruction based on *psb*A-*trn*H using the Neighbor-joining statistical method, 1000 times bootstrapping, and p-distance substitution model

The successful reconstruction of the phylogenetic tree from the three regions, including ITS (Figure 2), *rbc*L (Figure 3), and *psb*A-*trn*H (Figure 4) shows that several

species of *Eugenia* spp. are divided into 2 clades. According to Arita et al. (2021), this clade was formed because there are nucleotide sequences that are similar to each other, and nucleotide sequences that tend to be the same will group together in one clade (Shulgina & Eddy, 2023). The phylogenetic tree formed is monophyletic, meaning that the group originates from the same ancestor and inherits genetic, biochemical, and morphological characteristics from all its descendants. This is what makes monophyletic members closely related to each other (Martin et al., 2021). This statement is by the phylogenetic tree formed, namely that all members come from the same genus, namely the genus *Eugenia*.

Based on Figure 2, the phylogenetic tree based on the ITS region is divided into two clades. ITS Clade 1 is a large clade because it consists of seven species, including *E. bacopari, E. hiemalis, E. pluriflora, E. ruschiana, E. luschnathiana, E. macrocalyx,* and *E. wentii*. ITS Clade 2 consists of three species, *namely E. bunchosiifolia, E. stigmatosa*, and *E. speciosa*. Figure 3 shows a phylogenetic tree based on the *rbc*L region divided into two clades. Clade 1 *rbc*L is a large clade because it consists of eight species, namely *E. bunchosiifolia, E. cerasiflora, E. cuprea, E. cereja, E. verticillata, E. subavenia, E. prasina,* and *E. mosenii*. Clade 2 *rbc*L is a small clade because it only consists of two species, namely *E. brevistyla* and *E. supraaxillaris*. Based on Figure 4, the phylogenetic tree based on the *psb*A-*trn*H region consists of two clades, each clade has the same number of species members. Clade 1 *psb*A-*trn*H consists of *E. adenocalyx, E. latifolia, E. borinquensis, E. bunchosiifolia*, and *E. confusa*. Clade 2 *psb*A-*trn*H consists of *E. melanogyna, E. azurensis, E. longiracemosa, E. reinwardtiana*, and *E. buxifolia*.

Bootstrap values are shown in numbers located on each branch of the phylogenetic tree. The phylogenetic tree based on the ITS region has a high bootstrap value (80-99%) and there are only two less high bootstrap values (50-78%). In contrast, the phylogenetic tree based on the *rbc*L region has bootstrap values that tend to be weak (14-62%) and only has one high bootstrap value (83%). Meanwhile, the phylogenetic tree based on the *psb*A-*trn*H region has a high bootstrap value (86-92%) and a weak bootstrap value (31-69%). In general, high bootstrap values are shown by phylogenetic trees based on the ITS region. The bootstrap value shows the high accuracy of the branching formed. According to Canay et al. (2021) the greater the bootstrap value, the higher the level of confidence in the results of the phylogenetic tree reconstruction. Phylogenetic trees consist of lines indicating the evolutionary distance between species and common ancestry (Wibberg et al., 2021). The length and shortness of the line formed show the evolutionary distance. The longer the line, the further the evolutionary distance and the species has more modern characters. Meanwhile, the shorter the line formed, the more primitive the character of a species (Zhang et al., 2021).

Phylogenetic tree reconstruction using the neighbor-joining method. According to research by Kapli et al. (2020) the principle of this method is to select sequences that when combined will produce the best estimate of the length of the closest branch showing the real distance between sequences. The phylogenetic tree was tested statistically using the bootstrap method with 1000 replications. According to research by Kapli et al. (2021) bootstrap values of 100 to 1000 replications are used to predict the confidence level of a phylogenetic tree. In line with research by Hu et al. (2020)which states that a large bootstrap value indicates

the higher accuracy of a phylogenetic tree which is based on the distribution of characters in the data which is strongly influenced by random effects (Villaverde et al., 2020).

The alignment results in the ITS region show a lot of genetic variation in each *Eugenia* spp. species compared to the *rbc*L and *psb*A-*trn*H regions. This is because ITS is a nuclear locus. In contrast to the chloroplast or mitochondrial DNA regions, crossing over often occurs at the nuclear locus, which causes recombinants to have a lot of genetic variation. The *rbc*L and *psb*A-*trn*H regions have a high level of homology so they are not suitable for use as DNA barcodes for *Eugenia* spp. DNA barcodes *rbc*L and *psb*A-*trn*H are chloroplast DNA originating from maternal offspring. Chloroplast DNA has a low level of genetic recombination so according to the alignment results *rbc*L and *psb*A-*trn*H have a high level of homology (Wang et al., 2020);(Letunic & Bork, 2021).

Estimates of Evalutionary Divergence in Sequence *Eughenia* spp.

Genetic distance is the level of gene differences (genomic differences) of a population or species which is measured through numerical quantities. Genetic distance is a parameter used to see the genetic diversity of the species being studied. The genetic distance value ranges from 0 – 1, a value of 0 indicates that the observed species is very closely related, while a value of 1 indicates that the observed species is very distantly related (Wang et al., 2020).

The genetic distance between *Eugenia* spp. in this study ranges from 0.000 - 0.032 as seen in Table 4. The lowest genetic distance observed in the *Eugenia wentii* species was 0.000 while the highest genetic distance was seen in the *Eugenia hiemalis* species. The results of the genetic distance based on the ten highest ITS sequences used can be concluded that *Eugenia wentii* and *Eugenia luschnathiana* have a close relationship. Meanwhile, *Eugenia pluriflora* and *Eugenia hiemalis* have a distant relationship.

The genetic distance between *Eugenia* spp. in this study ranges from 0.000 - 0.004 as seen in Table 5. The lowest genetic distance observed in the *Eugenia wentii* species was 0.000 while the highest genetic distance was seen in the *Eugenia pluriflora* and *Eugenia hiemalis* species. The results of the genetic distance based on the ten highest *rbc*L sequences used can be concluded that *Eugenia prasine*, *Eugenia mosenii*, and *Eugenia verticillata* have a close relationship. Meanwhile, *Eugenia supraaxillaris* has a distant relationship.

The genetic distance between *Eugenia* spp. in this study ranges from 0.000 - 0.042 as seen in Table 6. The lowest genetic distance observed in the *Eugenia latifolia* species was 0.000 while the highest genetic distance was seen in the *Eugenia longiracemosa* species. The results of the genetic distance based on the ten highest *psb*A-*trn*H sequences used can be concluded that *Eugenia adenocalyx* and *Eugenia latifolia* have a close relationship. Meanwhile, *Eugenia longiracemosa* and *Eugenia azurensis* have a distant relationship.

Based on research on the genetic distance between plant samples with relatives *Syzygium* counted using the Kimura-2-Parameter method on MEGA6 (Kimura, 1980). Results shows the genetic distance between pakoba and jamun is 0.002. According to Giaretta et al. (2022), the lower the genetic distance value between two organisms, the closer they are the kinship between the two. Results this indicates that it is possible large pakoba and jamblang plants closely related, and even inclined as the same or constituted species subspecies. Genetic distance between munches with *Syzygium cumini* which is 0.010. Mark this is much larger than the intermediate value pakoba and jamun.

Genetic distance value between bombongan and other *Syzygium* obtained from GenBank ranged between 0.005-0.010. Assess the interspecies distance between genus *Syzygium*, namely 0.000-0.011. Range this is very small in comparison the results of the interspecies genetic distance were 0.0023-0.0291 for Acacia plants (Batiha et al., 2022), average genetic distance interspecies 0.042±0.0030 for plants from the Myristicaceae family de Paulo et al. (2020), as well as the average genetic distance interspecies 0.0093±0.0081 for plants Rosaceae (Kostikova & Petrova, 2021), Based on these results, the ability of the matK gene to separate *Syzygium* plants in terms of this is low (Aung et al., 2020).

A smaller genetic distance indicates that the level of kinship is closer, whereas a long genetic distance suggests that the level of kinship is increasingly distant. According to Ningrum et al. (2020), analysis at the DNA level will provide genetic distance estimation results much more accurately than locus analysis biochemical and other methods.

The primary rationale for using deep DNA sequence phylogenetic studies is that changes occur in nucleotide bases according to time, so it will be able to estimate the speed of evolution that is happening and will reconstruct the evolutionary relationship between one group of organisms with others (Ningrum et al., 2020). DNA sequences are starting to be widely studied and researched by world taxonomic practitioners to be used as characters in phylogenetic research. They have several advantages, because DNA sequences offer accurate data through better homology testing of existing characters (Enagbonma et al., 2019). DNA sequences also provide many character states because of the significant differences in the rate of change of nucleotide bases in different loci. DNA sequences have been proven to produce more natural kinship relationships (Arita et al., 2021);(Madduppa et al., 2022).

Table 4. Estimates of evalutionary divergence in ITS sequence pairs between 10 species of *Eugenia* spp.

No	Species	1	2	3	4	5	6	7	8	9	
1.	*Eugenia bunchosiifolia*										
2.	*Eugenia stigmatosa*		0.016								
3.	*Eugenia speciosa*		0.019	0.010							
4.	*Eugenia macrocalyx*		0.025	0.026	0.029						
5.	*Eugenia luschnathiana*		0.023	0.022	0.028	0.013					
6.	*Eugenia ruschiana*		0.022	0.026	0.026	0.029	0.028				
7.	*Eugenia bacopari*		0.023	0.025	0.028	0.022	0.020	0.022			
8.	*Eugenia wentii*		0.025	0.026	0.029	**0.000**	0.013	0.020	0.022		
9.	*Eugenia pluriflora*		0.023	0.025	**0.031**	0.026	0.022	0.022	0.010	0.026	
10.	*Eugenia hiemalis*		0.023	0.029	**0.032**	0.026	0.025	0.023	0.007	0.026	0.011

Table 5. Estimates of evalutionary divergence in *rbc*L sequence pairs between 10 species of *Eugenia* spp.

No	Species	1	2	3	4	5	6	7	8	9
1.	*Eugenia cereja*									
2.	*Eugenia bunchosiifolia*	0.001								
3.	*Eugenia subavenia*	0.001	0.001							
4.	*Eugenia prasina*	0.001	0.001	0.000						
5.	*Eugenia mosenii*	0.001	0.001	0.000	0.000					
6.	*Eugenia cuprea*	0.001	0.001	0.002	0.002	0.002				
7.	*Eugenia cerasiflora*	0.001	0.001	0.002	0.002	0.002	0.002			
8.	*Eugenia verticillata*	0.001	0.001	0.000	0.000	0.000	0.002	0.002		
9.	*Eugenia brevistyla*	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.001	
10.	*Eugenia supraaxillaris*	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.002	0.001

Table 6. Estimates of evalutionary divergence in *psb*A-*trn*H sequence pairs between 10 species of *Eugenia* spp.

No	Species	1	2	3	4	5	6	7	8	9
1.	*Eugenia bunchosiifolia*									
2.	*Eugenia adenocalyx*	0.028								
3.	*Eugenia latifolia*	0.028	0.000							
4.	*Eugenia melanogyna*	0.030	0.009	0.009						
5.	*Eugenia reinwardtiana*	0.036	0.015	0.015	0.013					
6.	*Eugenia confusa*	0.032	0.013	0.013	0.015	0.020				
7.	*Eugenia buxifolia*	0.036	0.015	0.015	0.013	0.007	0.020			
8.	*Eugenia longiracemosa*	**0.042**	0.020	0.020	0.022	0.020	0.024	0.020		
9.	*Eugenia azurensis*	**0.038**	0.016	0.016	0.015	0.020	0.018	0.020	0.030	
10.	*Eugenia borinquensis*	0.036	0.015	0.015	0.016	0.022	0.020	0.022	0.028	0.024

CONCLUSIONS AND SUGGESTIONS

Research successfully analyzed *Eugenia* plant relationship with using the ITS, *rbc*L, and *psb*A-*trn*H gene sequences in silico based shows that several species of *Eugenia* spp. are divided into 2 clades. In general, high bootstrap values are shown by phylogenetic trees based on the ITS region. The alignment results in the ITS region show a lot of genetic variation in each *Eugenia* spp. species compared to the *rbc*L and *psb*A-*trn*H regions. This is because ITS is a nuclear locus. The *rbc*L and *psb*A-*trn*H regions have a high level of homology so they are not suitable for use as DNA barcodes for *Eugenia* spp. Chloroplast DNA has a low level of genetic recombination so according to the alignment results *rbc*L and *psb*A-*trn*H have a high level of homology. Given the research findings that the ITS region shows higher genetic variation compared to the *rbc*L and *psb*A-*trn*H regions, it is recommended to use ITS sequences as the primary molecular marker in taxonomic and phylogenetic studies of *Eugenia spp.*. Additional markers may be needed to obtain a more comprehensive understanding of genetic variation within this genus. Furthermore, considering the high level of homology in the *rbc*L and *psb*A-*trn*H sequences, further research could consider developing or identifying other genetic markers that are more variable and informative for DNA barcoding of *Eugenia* spp.. Continued studies could also explore factors influencing genetic variation in these species, including environmental factors and local adaptation.

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