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Phylogenetic and DNA barcoding study on *Dehaasia pugerensis* Koord. & Valeton, an endemic and critically endangered species from East Java, Indonesia

Aulia Hasan Widjaya^{1,2*}, Andi Salamah^{1*}, Tety Maryenti¹, Iyan Robiansyah³, Irfan Martiansyah⁴, Muhammad Rifqi Hariri⁵ and Weibang Sun⁶

Abstract

Background *Dehaasia pugerensis* Koord. & Valeton is an endemic plant species classified as critically endangered (CR) in Jember, Java. It thrives in arid, rocky forest regions, making it a significant genetic resource for plant conservation initiatives. This study aimed to analyze the genetics of *Dehaasia pugerensis* through a molecular approach utilizing DNA (deoxyribonucleic acid) barcoding technique. This experiment utilized three accessions from three populations in Puger Jember, East Java: Igir Pletes (IP), Watu Susu (WS), and Undak Sebanen (US), along with Klatakan (KT). Notably, samples from the WS accession were excluded from internal transcribed spacer (ITS) primers due to poor sequencing results and were substituted with samples from the US accession. A single sample from each population of *Dehaasia pugerensis* underwent DNA sequencing utilizing DNA barcoding markers from three chloroplast genes: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*), maturase K (*matK*), and the transfer RNA-histidine and the photosystem II protein D1 (*trnH-psbA*) intergenic spacer, as well as one nuclear ITS region.

Results Sequence analysis demonstrated identical base composition in the plastid genes *rbcL* and *matK*, minor variation in *trnH-psbA* intergenic spacer, and significant variation in the nuclear ITS region. This demonstrates that the evolutionary rate of the chloroplast genome is lower than that of the nuclear genome. The chloroplast and nuclear genomes exhibit significant differences in evolutionary rates, influenced by various internal and external factors such as inheritance mode, mutation rate, and evolutionary pressures. Phylogenetic analysis indicated that the three accessions clustered together within the same group, as determined by chloroplast genes and ITS region. The three accessions exhibit a close relationship with *Dehaasia hainanensis* and *Dehaasia incrassata*, as indicated by the genetic distance observed on the phylogenetic tree.

Conclusions DNA barcoding with *rbcL*, *matK*, *trnH-psbA* intergenic spacer, and ITS confirmed the identity of *Dehaasia pugerensis* and revealed key genetic diversity. Phylogenetic analysis grouped the three accessions (KT450, IP42, WS152) into a single clade, closely related to *Dehaasia hainanensis* and *Dehaasia incrassata*, indicating shared evolutionary traits. While *rbcL* and *matK* were genetically stable, *trnH-psbA* intergenic spacer and ITS showed notable variability, particularly in ITS, which revealed important genetic differences. These findings highlight the value

*Correspondence:

Aulia Hasan Widjaya

auli001@brin.go.id

Andi Salamah

salamah@sci.ui.ac.id

Full list of author information is available at the end of the article



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of molecular data in shaping conservation strategies for *Dehaasia pugerensis*, such as propagation, reintroduction, and seed banking.

Keywords Endemic species, Genetic resources, Nucleotide variation, Threatened species

1 Background

Dehaasia pugerensis Koord. & Valeton is an endangered species listed in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species as of 2021, categorized under Critically Endangered C2b.D ver.2.3 [1]. *Dehaasia pugerensis* plants are exploited for their economic potential in charcoal production, leading to increased hunting and significant threats to the species [2]. *Dehaasia pugerensis* is an endemic plant exclusive to Jember Regency, East Java [3]. This species was initially described by Koorders and Valeton in 1904, with the most recent specimens collected by Buwalda in 1940.

Dehaasia pugerensis was discovered at six of the sixteen surveyed locations in the Wuluhan and Ambulu subdistricts: Klatakan, Undak, Watu Susu, Igir Pletes, Papuma, and Tanggul Asri (Fig. 1). The sites are located a top Mount Watangan, with a total of 560 individuals documented. Among these, 194 individuals (34.6%) were in the adult phase [2]. The species’ habitat is affected by natural disturbances and human activities, with individuals located at elevations between 61 and 391 m above sea level. Helmanto and his crew in 2022 have commenced ex situ conservation efforts at the Bogor Botanic Gardens, where they have gathered 23 seeds and 15 seedlings [2].

The urgent conservation situation of *Dehaasia pugerensis* requires an immediate and thorough conservation action plan. The plan must be guided by essential

data regarding the species’ ecology and biology to ensure effectiveness. This entails examining the reproductive biology of *Dehaasia pugerensis* and doing deoxyribonucleic acid (DNA)-based molecular analyses to facilitate species identification and evaluate genetic diversity. Such research is crucial for formulating targeted conservation efforts.

DNA barcoding, a molecular technique extensively utilized for plant identification, has demonstrated efficacy in researching endangered species, particularly those within the Lauraceae family. Frequently utilized markers comprise the internal transcribed spacer (ITS), *matK*, *rbcL*, and *trnH-psbA* intergenic spacer [4]. These markers have been effectively utilized in barcoding research on Lauraceae genera including *Dehaasia*, *Litsea*, *Cinnamomum*, *Cassytha*, *Laurus*, *Lindera*, *Machilus*, *Neolitsea*, *Persea*, and *Phoebe*, as well as species such as *Sassafras tzumu* and *Actinodaphne quercina* [5–9]. Currently, there are no DNA barcoding data for *Dehaasia pugerensis*, rendering this research essential for substantiating conservation initiatives and alleviating the risk of extinction.

2 Materials and methods

2.1 Materials

The fresh leaves of *Dehaasia pugerensis* used in this investigation were obtained through exploratory activities. This study analyzed a total of three samples, with one representative sample from each of the main populations: Igir Pletes (IP), Watu Susu (WS), and Klatakan (KT). The Undak Sebanen (US) population was included as a backup sample. The sample from accession WS152 was omitted from ITS primers due to inadequate sequencing results and was replaced with accession US371 (Table 1).

2.2 Methods

DNA barcoding of *Dehaasia pugerensis* was performed utilizing the markers *rbcL*, *matK*, *trnH-psbA* intergenic

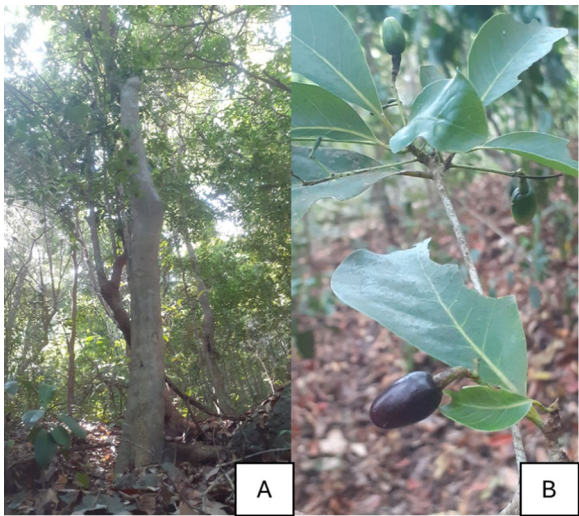


Fig. 1 *Dehaasia pugerensis*. A. Habit, B. Twig with mature fruit

Table 1 List of *Dehaasia pugerensis* accession number and location of origin

No	Species	Accession Number	Origin
1	<i>D. pugerensis</i>	IP42	Igir Pletes, Puger, Jember
2	<i>D. pugerensis</i>	WS152	Watu Susu, Puger, Jember
3	<i>D. pugerensis</i>	US371	Undak Sebanen, Puger, Jember
4	<i>D. pugerensis</i>	KT450	Klatakan, Puger, Jember

spacer, and ITS (Integrated DNA Technology, Singapore Science Park II, Singapore). DNA was extracted using the TianGen Plant Genomic DNA Kit (TIANGEN Biotech, Beijing, China) with modifications informed [9–11]. The polymerase chain reaction (PCR) was conducted in a 50 µl reaction mixture including 10 ng of DNA template, 1 µM of both forward and reverse primers, 25 µl of Taq from MyTaq™ HS Red Mix (Bioline, Tennessee, USA), and 17 µl of distilled water. The PCR protocol comprised an initial denaturation at 94 °C for 5 min, succeeded by 35 cycles of denaturation (94 °C for 15 s), annealing (52 °C for *rbcL*, 54 °C for *matK* and *trnH-psbA* intergenic spacer, and 58 °C for ITS for 15 s, Table 2), extension (72 °C for 10 s), and a concluding extension at 72 °C for 5 min, as delineated [12].

The PCR results were visualized on a 1% agarose gel (Vivantis Technologies, Selangor, Malaysia) by a Bio-Rad GelDoc system and subsequently sequenced by 1st Base (Singapore Science Park II, Singapore) via PT Genetika Science Indonesia. The *rbcL* and *matK* sequences were assembled into a contiguous sequence (contig), whereas only the forward strand was used for *trnH-psbA* intergenic spacer and ITS. The resulting contig sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis in National Center for Biotechnology Information (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to assess sequence homology and facilitate further analyses. An E-value of 0 and a percent identity exceeding 96% signified effective identification of the query sequence.

2.3 Data analysis

The ClustalW method [16] was employed for DNA sequence alignment. The alignment findings were scrutinized for possible nucleotide ambiguities arising from insertions, deletions, or substitutions, and requisite modifications were implemented to assure precision. The purified DNA sequences were subsequently utilized to create a phylogenetic tree. The ideal model for

phylogenetic reconstruction of each marker was determined utilizing the maximum likelihood method [17, 18]. Phylogenetic dendrograms trees generated utilizing the Tamura 3 (*matK*, *trnH-psbA* intergenic spacer, and ITS) and Jukes–Cantor (*rbcL*) model parameters [19, 20] and corroborated through 1,000 bootstrap repetitions [21]. All studies, encompassing parameter selection and tree construction, were conducted utilizing Molecular Evolutionary Genetics Analysis version 11 (MEGA 11) software [18].

3 Results

This study elucidates the genetic architecture of *Dehaasia pugerensis* through analysis of chloroplast (*rbcL*, *matK*, *trnH-psbA* intergenic spacer) and nuclear (ITS) markers. Nucleotide composition and GC content revealed contrasting patterns across loci (Table 3). The *rbcL* and *matK* markers exhibited high uniformity: *rbcL* showed identical nucleotide percentages (cumulative G + C = 44.8% over 373 bp) across accessions (IP42, WS152, KT450), while *matK* maintained consistent G + C content (37.3% over 610 bp). In contrast, *trnH-psbA* intergenic spacer and ITS displayed notable variability. For *trnH-psbA* intergenic spacer, IP42 and KT450 shared identical compositions, whereas WS152 showed minor deviations (G + C content 26.8% over 236 bp), and US371 lacked data due to sequencing issues. The ITS marker exhibited the highest diversity, with G + C content ranging from 62.5% to 65.2% (over 597 bp), underscoring its utility in detecting intraspecific variation.

Structural genomic variations were prominent in the ITS region (Table 4). Substitutions at positions 20 and 33 suggested point mutations driven by evolutionary pressures, while indels, particularly deletions in US371 (positions 568–580), highlighted structural divergence. Conserved regions (e.g., positions 110, 112, 115) indicated functional or evolutionary constraints, whereas mutation hot spots (positions 142, 145, 151) marked areas of genetic divergence. Phylogenetic inferences

Table 2 Primer information used in DNA barcoding analysis

No	Primer	Sequence (5'–3')	Ta (°C)	Reference
1	<i>rbcL</i> -F	ATGTACCACAAACAGAGACTAAAGC	52	[13]
	<i>rbcL</i> -R	GTAAATCAAGTCCACCRGC		
2	<i>matK</i> -3F	CGTACAGTACTTTGTGTTTACGAG	54	Kim unpublished
	<i>matK</i> -1R	ACCCAGTCCATCTGGAATCTTGTTTC		
3	<i>trnH-psbA</i> -f1	ACTGCCTTGATCCACTTGCC	54	[14]
	<i>trnH-psbA</i> -f2	CGAAGCTCCATCTACAAATGG		
4	ITS1-17SE	ACGAATTCATGGTCCGGTGAAGTGTTCG	58	[15]
	ITS2-26SE	TAGAATTCCTCCGGTTCGCTCGCCGTTAC		

Table 3 Nucleotide composition of four DNA barcoding markers in three *Dehaasia pugerensis* accessions

Accessions	<i>rbcL</i> (%)					Total	<i>matK</i> (%)					Total
	T	C	A	G	G+C		T	C	A	G	G+C	
IP42	30.0	22.0	25.2	22.8	44.8	373	29.3	18.0	33.3	19.3	37.3	610
WS152	30.0	22.0	25.2	22.8	44.8	373	29.3	18.0	33.3	19.3	37.3	610
KT450	30.0	22.0	25.2	22.8	44.8	373	29.3	18.0	33.3	19.3	37.3	610
Accessions	<i>trnH-psbA</i> intergenic spacer (%)					Total	ITS (%)					Total
	T	C	A	G	G+C		T	C	A	G	G+C	
IP42	25.4	11.0	47.9	15.7	26.7	236	16.5	31.7	21.0	30.8	62.5	568
WS152	25.1	11.1	48.1	15.7	26.8	235	-	-	-	-	-	-
US371	-	-	-	-	-	-	14.6	34.1	20.2	31.1	65.2	540
KT450	25.4	11.0	47.9	15.7	26.7	236	17.1	29.5	20.6	32.8	62.3	597

Notes: Accession WS152 was not used with ITS primers due to poor sequencing results, so accession US371 was replaced

Table 4 Nucleotide variation of the nuclear DNA ITS region in three *Dehaasia pugerensis* accessions

Accession	20	33	34	35	37	38	39	44	46	48	73	75	110	112	114	115	121	122	142	145	151	154	157
IP42	T	G	C	C	G	C	G	G	G	C	T	G	T	T	A	T	C	C	G	G	C	G	C
US371	G	A	.	T	C	T	C	C	C	.	C	T	C	C	G	C	.	T	.	.	T	A	.
KT450	G	A	G	T	C	T	C	C	C	T	C	T	C	C	G	C	A	T	T	A	T	A	T
Accession	166	169	175	180	183	190	191	192	193	203	204	205	215	216	219	221	224	229	231	234	239	241	243
IP42	-	-	-	-	-	-	-	-	-	G	G	A	C	T	C	A	C	A	C	G	C	C	G
US371	C	C	G	C	G	C	G	C	C	A	T	G	T	A	.	.	.	C	A	C	.	.	.
KT450	A	A	A	A	C	A	A	G	G	A	T	G	T	A	G	T	G	C	A	C	A	A	T
Accession	247	249	254	257	260	266	267	268	270	274	275	278	283	288	289	290	292	293	294	295	296	300	303
IP42	C	G	T	A	C	T	T	T	T	G	C	A	A	A	C	G	A	T	A	T	C	C	T
US371	T	C	C	G	G	A	A	C	A	.	G	T	G	G	A	A	T	G	C	A	G	.	G
KT450	T	.	G	T	G	A	G	C	A	T	.	T	G	G	A	A	T	G	C	A	G	A	G
Accession	304	307	313	314	316	319	325	328	334	339	340	342	346	349	351	352	353	361	362	363	365	367	380
IP42	C	A	A	T	A	A	G	G	C	C	T	G	G	A	T	T	G	A	C	A	T	A	T
US371	T	.	G	A	T	T	T	A	.	G	A	A	C	G	C	C	.	G	G	C	C	G	G
KT450	T	T	G	A	T	T	T	A	T	G	G	.	A	C	G	C	C	T	G	C	A	T	C
Accession	383	389	390	391	392	400	405	407	413	421	422	428	432	434	436	437	438	444	447	450	463	467	468
IP42	A	C	T	T	G	T	T	C	A	C	G	C	G	A	A	C	T	G	C	G	G	A	C
US371	.	.	G	C	C	C	C	.	T	T	A	T	A	.	G	G	C	A	.	C	.	T	.
KT450	C	G	G	G	.	C	C	G	T	T	A	T	.	C	G	.	C	T	G	.	A	T	G
Accession	469	471	478	482	483	488	489	494	495	497	498	511	512	514	516	519	523	524	525	527	528	529	531
IP42	C	T	G	G	A	G	A	T	T	C	T	A	G	A	T	A	C	G	A	T	G	C	C
US371	T	C	A	A	G	C	G	G	C	.	G	.	A	G	.	G	A	.	.	C	T	G	A
KT450	T	C	A	.	G	C	G	G	A	A	G	G	.	G	A	G	A	A	T	C	T	G	T
Accession	532	533	534	539	540	542	545	554	555	556	558	559	560	561	562	563	567	568	569	570	572	577	587
IP42	G	A	C	A	A	C	T	G	T	A	C	G	A	T	T	A	C	C	A	C	A	T	C
US371	.	C	.	C	T	.	C	A	A	C	T	A	C	C	G	C	.	-	-	-	-	-	-
KT450	T	C	G	G	T	A	C	.	.	C	T	C	C	C	G	C	G	G	G	A	T	C	G
Accession	588	590	591	592	598	599	600	604	609	611	612	614	616	618	620	621	622	623	624	627	628	630	
IP42	G	G	A	A	C	G	C	G	G	A	C	G	G	G	C	C	G	A	T	C	A	G	
US371	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
KT450	A	A	C	T	G	C	T	C	C	G	T	A	A	C	G	G	A	C	C	A	G	T	

Notes: The number above the nucleotide code is the nucleotide site number on the sequence. Red boxes indicate insertion-deletion mutations.

The number above the nucleotide code is the nucleotide site number on the sequence. Red boxes indicate insertion-deletion mutations

from ITS suggested a shared ancestral sequence among accessions, with US371 representing a distinct lineage, while IP42 and KT450 exhibited closer genetic ties, likely due to recent divergence or shared selective pressures.

Genetic distance analyses across markers delineated evolutionary relationships within *Dehaasia*. Using *rbcL*, conspecific accessions (KT450, IP42, WS152) showed zero genetic distance (Table 5), indicating clonal or near-identical lineages. In contrast, *Dehaasia incrassata* (LC736465.1) and *Dehaasia microcarpa* (MG817008.1) exhibited maximal divergence (0.019 and 0.014, respectively), reflecting distinct evolutionary trajectories. *Dehaasia hainanensis* (NC_068504.1) showed minimal distance (0.003)

from *Dehaasia pugerensis*, suggesting recent speciation or gene flow.

The *matK* marker reinforced interspecific relationships: *Dehaasia cuneata* (AJ247160.2) displayed significant divergence (1.357), while *Dehaasia firma* (LC736938.1) and *Dehaasia incrassata* (LC736937.1) showed closer affinity (0.003–0.005, Table 6). The *trnH-psbA* intergenic spacer revealed pronounced genus-wide diversity (genetic distances: 1.220–1.390), with *Dehaasia pugerensis* accessions (WS152, IP42) showing no divergence, contrasting sharply with the high variability in *Dehaasia incrassata* (Table 7). ITS-based distances (0.149–0.418) further highlighted intrageneric diversity, with *Dehaasia hainanensis* diverging markedly (0.079) from congeners (Table 8).

Table 5 Genetic distance of samples on *rbcL* region

No	Accession Sample	1	2	3	4	5	6	7	8	9	10	11	12
1	<i>Dehaasia pugerensis</i> _KT450												
2	<i>Dehaasia pugerensis</i> _IP42	0,000											
3	<i>Dehaasia pugerensis</i> _WS152	0,000	0,000										
4	NC_068504.1_ <i>Dehaasia hainanensis</i>	0,003	0,003	0,003									
5	MG817008.1_ <i>Dehaasia microcarpa</i>	0,014	0,014	0,014	0,011								
6	AB925910.1_ <i>Dehaasia poilanei</i>	0,003	0,003	0,003	0,000	0,011							
7	AB925550.1_ <i>Dehaasia cuneata</i> var. <i>longifolia</i>	0,005	0,005	0,005	0,003	0,008	0,003						
8	AB925315.1_ <i>Dehaasia cuneata</i>	0,005	0,005	0,005	0,003	0,008	0,003	0,000					
9	LC736473.1_ <i>Dehaasia brachybotrys</i>	0,014	0,014	0,014	0,011	0,000	0,011	0,008	0,008				
10	LC736470.1_ <i>Dehaasia membranacea</i>	0,014	0,014	0,014	0,011	0,000	0,011	0,008	0,008	0,000			
11	LC736465.1_ <i>Dehaasia incrassata</i>	0,019	0,019	0,019	0,016	0,014	0,016	0,019	0,019	0,014	0,014		
12	LC736199.1_ <i>Dehaasia firma</i>	0,011	0,011	0,011	0,008	0,008	0,008	0,011	0,011	0,008	0,008	0,008	

Table 6 Genetic distance of samples on *matK* region

Accession Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 <i>Dehaasia pugerensis</i> _KT450														
2 <i>Dehaasia pugerensis</i> _IP42	0,000													
3 <i>Dehaasia pugerensis</i> _WS152	0,000	0,000												
4 NC_068504.1_ <i>Dehaasia hainanensis</i>	0,000	0,000	0,000											
5 AJ247160.2_ <i>Dehaasia cuneata</i>	1,357	1,357	1,357	1,357										
6 LC736939.1_ <i>Dehaasia brachybotrys</i>	1,329	1,329	1,329	1,329	0,005									
7 LC736938.1_ <i>Dehaasia firma</i>	1,336	1,336	1,336	1,336	0,005	0,003								
8 LC736937.1_ <i>Dehaasia incrassata</i>	1,342	1,342	1,342	1,342	0,007	0,005	0,005							
9 LC736936.1_ <i>Dehaasia membranacea</i>	1,329	1,329	1,329	1,329	0,005	0,000	0,003	0,005						
10 MW849612.1_ <i>Dehaasia incrassata</i>	1,330	1,330	1,330	1,330	0,007	0,002	0,005	0,007	0,002					
11 MH332636.1_ <i>Dehaasia firma</i>	1,336	1,336	1,336	1,336	0,007	0,005	0,005	0,007	0,005	0,007				
12 AB924704.1_ <i>Dehaasia cuneata</i>	1,342	1,342	1,342	1,342	0,002	0,003	0,003	0,005	0,003	0,005	0,005			
13 AB925278.1_ <i>Dehaasia poilanei</i>	1,335	1,335	1,335	1,335	0,005	0,007	0,007	0,008	0,007	0,008	0,008	0,003		
14 AB924928.1_ <i>Dehaasia cuneata</i> var. <i>longifolia</i>	1,342	1,342	1,342	1,342	0,002	0,003	0,003	0,005	0,003	0,005	0,005	0,000	0,003	

Table 7 Genetic distance of samples on *trnH-psbA* intergenic spacer region

No	Accession Sample	1	2	3	4	5	6	7
1	LC737514.1_ <i>Dehaasia membranacea</i>							
2	LC737515.1_ <i>Dehaasia incrassata</i>	0,075						
3	LC737516.1_ <i>Dehaasia firma</i>	0,052	0,056					
4	LC737517.1_ <i>Dehaasia brachybotrys</i>	0,025	0,051	0,025				
5	<i>Dehaasia pugerensis</i> _KT450	1,372	1,220	1,293	1,320			
6	<i>Dehaasia pugerensis</i> _WS152	1,390	1,232	1,288	1,335	0,000		
7	<i>Dehaasia pugerensis</i> _IP42	1,372	1,220	1,293	1,320	0,000	0,000	

Table 8 Genetic distance of samples on ITS region

No	Accession Sample	1	2	3	4	5	6
1	AF272268.1_ <i>Dehaasia incrassata</i>						
2	FJ719308.1_ <i>Dehaasia hainanensis</i>	0,079					
3	AY934890.1_ <i>Dehaasia cuneata</i>	0,071	0,042				
4	<i>Dehaasia pugerensis</i> _KT450	0,327	0,354	0,345			
5	<i>Dehaasia pugerensis</i> _US371	0,225	0,251	0,243	0,149		
6	<i>Dehaasia pugerensis</i> _IP42	0,391	0,418	0,409	0,388	0,286	

Phylogenetic clustering using chloroplast markers identified close relationships between *Dehaasia firma* and *Dehaasia brachybotrys* (distance: 0.025), implying recent divergence, whereas *Dehaasia incrassata*'s distinctness (distances: 0.075–0.056 from *Dehaasia membranacea* and *Dehaasia firma*) suggested unique adaptive evolution. These results underscore the genus's dynamic evolutionary history, with conserved loci (*rbcL*, *matK*) stabilizing species identification and hyper-variable regions (*trnH-psbA* intergenic spacer, ITS) resolving fine-scale diversity.

4 Discussion

The multilocus DNA barcoding analysis of *Dehaasia pugerensis* from Puger, East Java, integrated four genetic markers—chloroplast *rbcL*, *matK*, *trnH-psbA* intergenic spacer, and nuclear ITS—to resolve taxonomic and evolutionary relationships. Sequence composition analyses revealed distinct structural and compositional profiles across loci, with chloroplast markers exhibiting shorter sequences and lower GC content (*rbcL*: 373 bp, GC=22.0–22.8%; *matK*: 610 bp, GC=18.0–19.3%; *trnH-psbA* intergenic spacer: 235–236 bp, GC=11.1–15.7%) compared to the nuclear ITS region (540–597 bp, GC=30.8–34.1%). This disparity in GC content aligns with prior studies on *Durio testudinarius* Becc. [22], where chloroplast genes, constrained by their roles in photosynthesis and plastid function, are under stronger purifying selection, favoring AT-rich regions for efficient

replication and transcription. The AT bias in chloroplast genomes may reflect evolutionary optimization for reduced mutational load in highly conserved photosynthetic pathways, where even minor nucleotide changes could disrupt critical enzymatic functions (e.g., ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity in *rbcL*). Conversely, the ITS region's higher GC content likely stabilizes ribosomal ribonucleic acid (rRNA) secondary structures through enhanced base pairing, mitigating thermal denaturation in the species' tropical habitat [23]. Such thermostability is critical for maintaining ribosomal integrity under fluctuating environmental conditions, while ITS hypervariability—driven by relaxed selection and recombination in noncoding spacers—facilitates rapid adaptation to ecological stressors. These compositional differences underscore an evolutionary trade-off: Chloroplast genomes prioritize functional conservation to preserve photosynthetic efficiency, while nuclear ribosomal regions accommodate plasticity to enhance genetic diversity and adaptive potential. This dichotomy highlights the complementary roles of chloroplast and nuclear markers in evolutionary studies, with the former stabilizing taxonomic identification and the latter illuminating microevolutionary dynamics.

Nucleotide variation analyses highlighted contrasting evolutionary pressures across loci, underscoring the divergent functional and adaptive roles of chloroplast versus nuclear genomes. Chloroplast markers

(*rbcL*, *matK*, *trnH-psbA* intergenic spacer) exhibited complete sequence conservation across *Dehaasia pugerensis* accessions (KT450, IP42, WS152), a pattern attributed to stringent purifying selection acting on genes critical for photosynthetic efficiency and plastid integrity [24–26]. For instance, *rbcL* encodes the large subunit of RuBisCO, an enzyme central to carbon fixation, where even minor mutations could disrupt catalytic activity, while *matK* regulates intron splicing in chloroplast transcripts, necessitating evolutionary stability. The absence of polymorphisms in these loci reflects their conserved roles in maintaining essential cellular functions, with selection pressures eliminating deleterious variants to preserve metabolic homeostasis.

In stark contrast, the nuclear ITS region displayed hypervariability, with 183 polymorphic sites (142 substitutions, 41 indels) across accessions. This plasticity arises from the dynamic evolutionary mechanisms governing ribosomal DNA (*rDNA*), including unequal crossing over during meiosis and gene conversion—processes amplified in multigene families like ITS due to concerted evolution [27]. Such mechanisms drive rapid sequence homogenization within populations while permitting divergence between lineages. Structural variations, such as the 13-bp deletion in accession US371 (positions 568–580), may perturb ribosomal RNA (*rRNA*) secondary structure or interaction with ribosomal proteins. However, compensatory mutations in adjacent regions or epigenetic modifications (e.g., cytosine methylation) could buffer functional impacts, allowing these variants to persist neutrally or under balancing selection. The ITS region's capacity to accumulate mutations without catastrophic fitness consequences positions it as a powerful tool for resolving fine-scale intraspecific divergence, such as population bottlenecks caused by habitat fragmentation or founder effects during range expansion [28–30]. For example, allele specific ITS variants could delineate cryptic subpopulations adapted to microhabitats within East Java's heterogeneous landscapes, offering insights into local adaptation and gene flow dynamics. This duality—conservation in chloroplasts versus plasticity in nuclear ribosomal deoxyribonucleic acid (*rDNA*)—illustrates how genomic compartmentalization shapes evolutionary trajectories, with chloroplast markers anchoring taxonomic clarity and ITS illuminating the microevolutionary forces driving diversification.

Among the four markers analyzed, *matK* exhibited the highest representation in the NCBI GenBank database, underscoring its established utility and standardization as a core DNA barcoding locus in plant phylogenetic studies [25, 31]. As a chloroplast gene encoding a maturase involved in intron splicing, *matK* combines moderate sequence length (~610 bp) with sufficient variability

to resolve species-level divergence while retaining conserved regions critical for primer design and alignment [25]. Its widespread adoption is further reflected in its consistent GC content (18.0–19.3%), which aligns with the AT-rich nature of chloroplast genomes, optimizing amplification efficiency across diverse plant taxa. The prevalence of *matK* data in public repositories like GenBank highlights its role as a benchmark for comparative phylogenomics, enabling large-scale meta-analyses of evolutionary relationships and biodiversity assessments [31]. This dominance contrasts with markers such as *trnH-psbA* intergenic spacer or ITS, which, despite their utility in fine-scale divergence studies, face challenges in standardization due to length heterogeneity or multicopy complications. The robust availability of *matK* sequences not only facilitates taxonomic clarity in understudied genera like *Dehaasia* but also reinforces its integration into global barcoding initiatives, such as the Consortium for the Barcode of Life (CBOL), which prioritizes loci balancing universality and discriminative power [25]. Nevertheless, this reliance on *matK* underscores the need for complementary nuclear markers (e.g., ITS) to resolve complex evolutionary scenarios, such as hybridization or incomplete lineage sorting, where chloroplast data alone may yield incomplete phylogenetic signals.

Phylogenetic reconstructions, rooted with *Phoebe lanceolata* (Nees) as an outgroup, resolved *Dehaasia pugerensis* accessions into a monophyletic clade (bootstrap support >50%) with negligible intraspecific divergence (genetic distance = 0.000), indicative of recent colonization or clonal propagation in fragmented habitats. This genetic homogeneity suggests limited gene flow among populations [32], potentially exacerbated by anthropogenic habitat fragmentation in East Java, where isolated subpopulations may rely on vegetative reproduction to maintain genetic cohesion. Interspecific comparisons reveal nuanced evolutionary relationships: Minimal divergence from *Dehaasia hainanensis* (0.003, *rbcL/matK*) supports a sister-species relationship likely shaped by Pleistocene vicariance, wherein climatic fluctuations during glacial cycles isolated ancestral populations across Sundaland, driving allopatric speciation (Fig. 2) [33]. In contrast, stronger affinity to *Dehaasia incrasata* (ITS/*trnH-psbA* intergenic spacer) in accession IP42 (Fig. 3) implies adaptive introgression—a phenomenon where selective gene flow introduces advantageous alleles (e.g., drought tolerance) across species boundaries—or convergent evolution under shared ecological pressures, such as adaptation to calcium-rich soils prevalent in their native habitats.

Genetic distance metrics further delineated genus-wide evolutionary trajectories, revealing a

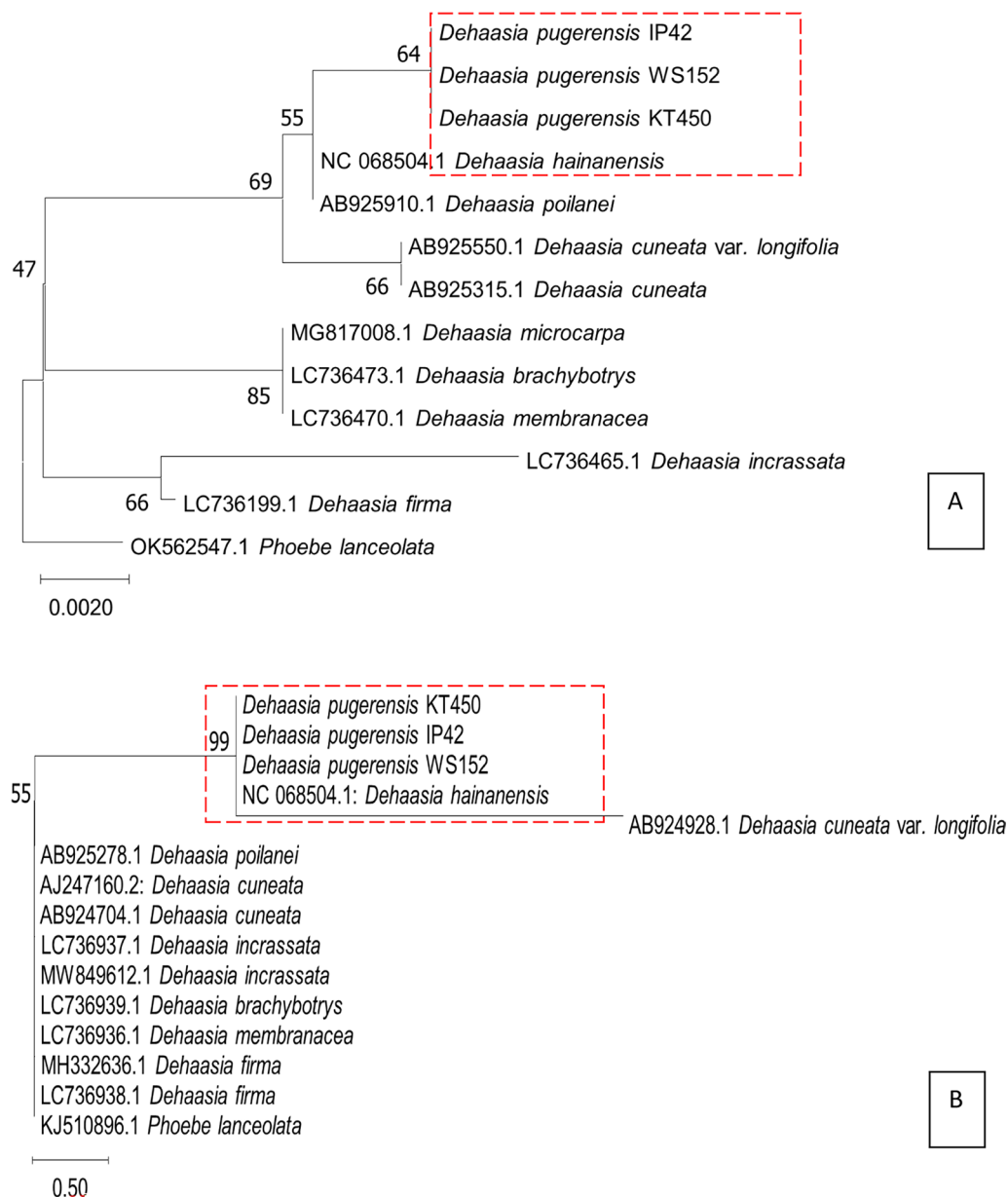


Fig. 2 Phylogenetic tree using the maximum likelihood method with a bootstrap of 1,000 replicates based on the *rbcL* (a) and *matK* (b) genes. The numbers above branches represent bootstrap frequencies. The code behind the species' name is the accession number in the NCBI Gene Bank. The red-striped box shows the grouping of three *D. pugerensis* accessions. The scale bar indicates the size of the genetic distance

spectrum of divergence shaped by ecological and historical forces. Conserved lineages, such as *Dehaasia hainanensis* and *Dehaasia poilanei* (genetic distance=0.000–0.008), reflect recent speciation or recurrent hybridization in sympatric zones [34], likely facilitated by overlapping flowering phenologies and shared pollinators, underscoring the role of ecological opportunity in driving rapid radiation within the genus. In contrast, divergent taxa like *Dehaasia*

incrassata (0.075–0.019) and *Dehaasia microcarpa* (0.014) represent relict lineages characterized by elevated genetic distances. These adaptations likely emerged during Miocene aridification events, positioning these species as evolutionary holdovers of past climatic regimes. Outliers such as *Dehaasia cuneata* exhibited marked divergence (1.357, *matK*), signaling ecological specialization through niche partitioning in lowland rainforests. Collectively, these patterns highlight the interplay

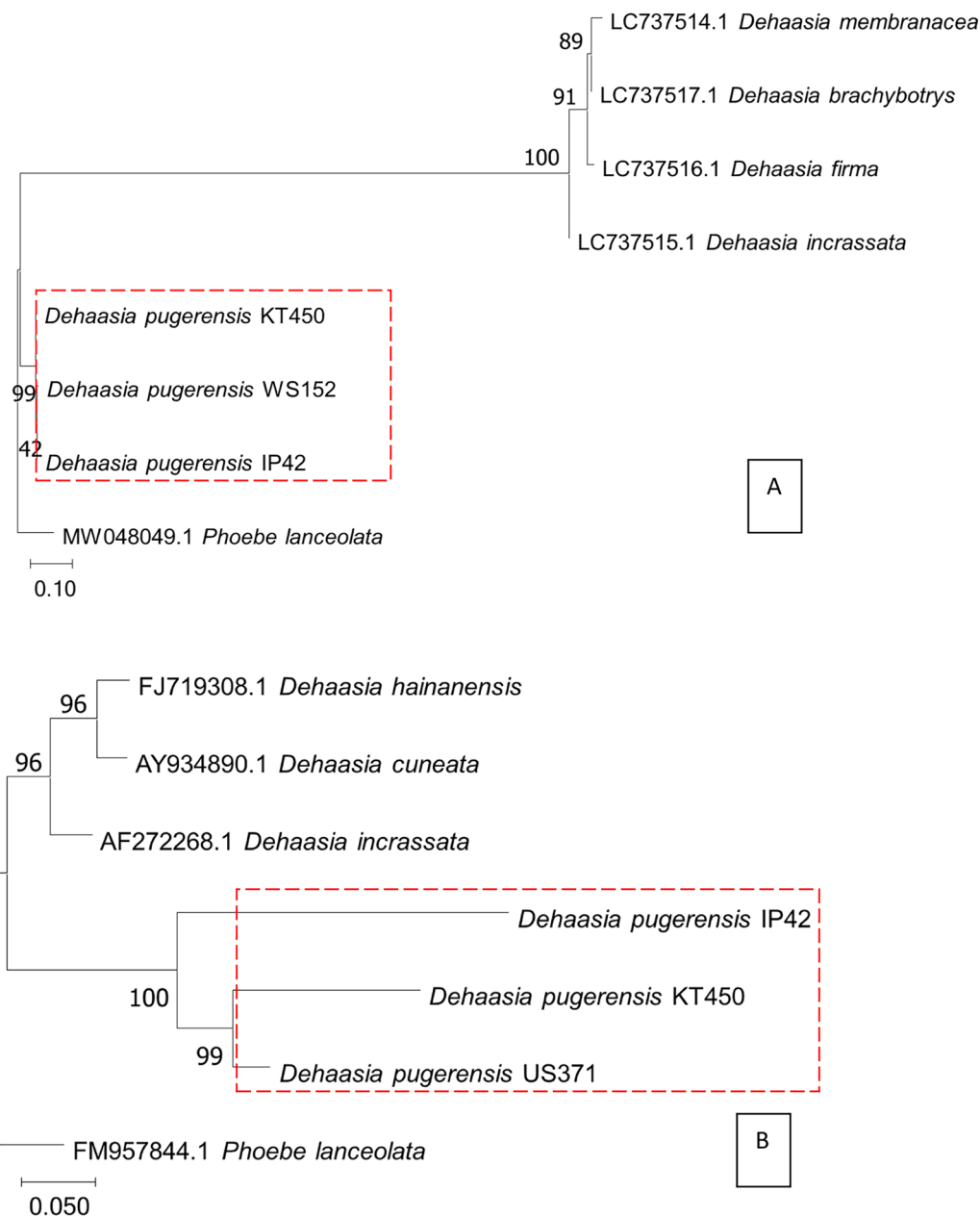


Fig. 3 Phylogenetic tree using the maximum likelihood method with a bootstrap of 1,000 replicates based on the *trnH-psbA* intergenic spacer region (a) and ITS core (b). The numbers above branches represent bootstrap frequencies. The code behind the species' name is the accession number in the NCBI Gene Bank. The red-striped box shows the grouping of three *D. pugerensis* accessions. The scale bar indicates the size of the genetic distance

of historical climatic shifts, ecological specialization, and reproductive strategies in sculpting the genetic architecture of the *Dehaasia* genus.

Despite low bootstrap support (<50%) for deeper phylogenetic nodes—a common challenge in rapidly radiating lineages—the high confidence in conspecific clustering underscores the utility of multilocus data in resolving recent radiations. For instance, conflicting

topologies between chloroplast (*rbcL/matK*) and nuclear (ITS/*trnH-psbA* intergenic spacer) markers in *Dehaasia pugerensis* accession IP42 may reflect incomplete lineage sorting or chloroplast capture, wherein plastid genomes are introgressed between species without nuclear gene flow [35]. These complexities highlight the necessity of integrating multiple markers to disentangle hybridization histories and reticulate evolution. The

findings collectively emphasize the dynamic interplay of neutral processes (e.g., drift) and selective forces shaping *Dehaasia* diversification, while advocating for conservation strategies that prioritize evolutionarily distinct lineages (e.g., *Dehaasia incrassata*) and mitigate genetic erosion in genetically depauperate taxa like *Dehaasia pugerensis*.

The genetic homogeneity observed in *Dehaasia pugerensis* (e.g., identical ITS sequences in accessions IP42 and KT450) raises critical concerns about inbreeding depression and diminished adaptive capacity, as reduced allelic diversity limits the species' ability to respond to environmental stressors such as pathogen outbreaks or climate shifts. This vulnerability necessitates urgent conservation interventions, including ex situ seed banking and the restoration of habitat corridors to reconnect fragmented populations [36], thereby facilitating gene flow and restoring evolutionary potential. Conversely, divergent taxa like *Dehaasia incrassata*—harboring unique alleles associated with drought tolerance, such as enhanced abscisic acid biosynthesis or cuticular wax deposition—represent evolutionary reservoirs critical for genus-wide resilience. These adaptive traits, likely honed during historical aridification events, could inform breeding programs aimed at enhancing stress tolerance in congeners. Furthermore, the hypervariability of the ITS region highlights untapped biotechnological potential; structural variations, such as indels in rRNA spacers, may modulate secondary metabolite synthesis, including alkaloids or terpenoids with pharmacological properties relevant to anticancer or antimicrobial drug discovery [37–39].

However, genomic databases remain disproportionately skewed, with only 18% of *Dehaasia* species represented [40], creating significant gaps in understanding the genus's evolutionary and adaptive breadth. Prioritizing underrepresented taxa (e.g., *Dehaasia firma*, *Dehaasia brachybotrys*) in future sequencing efforts will clarify unresolved polytomies and illuminate adaptive radiation, particularly in understudied clades. Integrating genetic data with ecological niche modeling could identify climate refugia—areas where microclimatic stability may buffer species from anthropogenic warming—while isotopic or edaphic data layers could reveal soil nutrient adaptations critical for habitat restoration. Concurrently, community-based agroforestry initiatives, such as cultivating *Dehaasia* species alongside cash crops, may buffer endemic habitats from encroachment while providing economic incentives for local stakeholders. By synthesizing genomic, ecological, and socioeconomic frameworks, this multidisciplinary approach can safeguard *Dehaasia*'s genetic legacy, ensuring its role in both ecosystem stability and biotechnological innovation.

While this study offers important genetic insights into *Dehaasia pugerensis*, several limitations should be acknowledged. The reliance on *rbcL*, *matK*, *trnH-psbA* intergenic spacer, and ITS may not fully capture genetic variation, as the low mutation rates of *rbcL* and *matK* could underestimate intraspecific diversity. Additionally, the small sample size (three accessions) may not adequately represent the species' genetic breadth. Furthermore, the use of only plastid and nuclear ribosomal markers, while informative, may not fully reflect the genetic diversity present in more complex genomic regions. Although phylogenetic analysis grouped *Dehaasia pugerensis* with *Dehaasia hainanensis* and *Dehaasia incrassata*, further validation using nuclear markers or whole-genome sequencing is needed. The study also does not address ecological adaptation, potential hybridization, or broader conservation concerns. Finally, the study's focus on molecular data alone neglects the potential influence of environmental factors on genetic diversity and evolutionary patterns. Future research should incorporate larger sample sizes, additional genetic markers, and ecological data to provide a more comprehensive understanding of *Dehaasia pugerensis* and its evolutionary significance.

5 Conclusions

DNA barcoding using the markers *rbcL*, *matK*, *trnH-psbA* intergenic spacer, and ITS confirmed the species' identity and revealed important genetic diversity. Phylogenetic analyses grouped the three *Dehaasia pugerensis* accessions (KT450, IP42, WS152) into a single clade with strong bootstrap support, revealing high genetic similarity and evolutionary proximity to *Dehaasia hainanensis* and *Dehaasia incrassata*. These results indicate shared adaptive traits or recent divergence among these species. While *rbcL* and *matK* showed uniform sequences, indicating genetic stability, the *trnH-psbA* intergenic spacer and ITS markers exhibited significant variability among the different accessions, suggesting evolutionary differences. The ITS region revealed notable genetic variations that could impact the species' adaptation and traits. These results highlight the critical role of molecular data in shaping effective conservation strategies to protect *Dehaasia pugerensis* in its natural habitat.

List of Abbreviations

BLAST	Basic local alignment search tool
CBOL	Consortium for the barcode of life
CR	Critically endangered
DNA	Deoxyribonucleic acid
IP	Igir pletes
ITS	Internal transcribed spacer
IUCN	International union for conservation of nature
KT	Klatakan
<i>matK</i>	Maturase K
MEGA 11	Molecular evolutionary genetics analysis version 11

NCBI	National center for biotechnology information
PCR	Polymerase chain reaction
<i>rbcl</i>	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
<i>rDNA</i>	Ribosomal deoxyribonucleic acid
<i>rRNA</i>	Ribosomal ribonucleic acid
<i>RuBisCO</i>	Ribulose-1,5-bisphosphate carboxylase/oxygenase
<i>trnH-psbA</i>	The transfer RNA-histidine and the photosystem II protein D1
US	Undak sebanen
WS	Watu susu

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Author contributions

AHW conducted the initial preparation and sequence data analysis. AS, TM, and IR meticulously designed and coordinated all experiments. IM and MRH conducted and validated the sequence analysis. AHW prepared the manuscript draft. AHW, AS, TM, IR, IM, MRH, and WS reviewed and approved to the final document. All authors equally contributed as main contributor of this manuscript.

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Data availability

No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

¹Department of Biology, University of Indonesia, Depok, Indonesia. ²Research Center for Applied Botany, National Research and Innovation Agency, Bogor, Indonesia. ³Research Center for Ecology and Ethnobiology, National Research and Innovation Agency, Bogor, Indonesia. ⁴Research Center for Applied Botany, National Research and Innovation Agency, Bogor, Indonesia. ⁵Research Center for Biosystematics and Evolution, National Research and Innovation Agency, Bogor, Indonesia. ⁶Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China.

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