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Ensuring Consumer Safety: Molecular Authentication of *Eurycoma longifolia* Derivative Products in the Wood Science and Technology Industry

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ABSTRACT

Eurycoma longifolia (*pasak bumi*) is a popular medicinal plant in Indonesia and is widely used in various products. Its high economic value has caused illegal harvesting and product falsification. Using molecular techniques, the authentication and traceability of *E. longifolia* derivatives can be controlled to ensure consumer safety. Therefore, this study aimed to authenticate the products and derivatives of *E. longifolia* (*pasak bumi*) produced, marketed, and consumed in Indonesia using molecular identification techniques. Genomic DNA from 37 leaf samples collected from the Sumatran mainland and the Riau Islands and six *E. longifolia* products were amplified and sequenced using *trn*L-*trn*F and internal transcribed spacer (ITS) regions. The results revealed that all leaf samples were indeed *E. longifolia* based on the markers used, with the six products, only the herbal tea product (sample code TCPB) was most likely derived from *E. longifolia* based on the two regions, suggesting that not all products labelled as *E. longifolia* in the market are authentic. The results also indicated that several other plants species are used as substitutes or adulterants, including *Simaba* spp., *Simarouba* spp., *Homalolepis* spp., *Vernonia gigantea*, *Elephantopus scaber*, *Gymnanthemum amygdalinum*, *Cyanthillium* spp., *Potentilla lineata*, *Ailanthus altissima*, *Geijera paniculata*, *Hannoa chlorantha*, and *Dalbergia* spp. *Klebsiella pneumoniae* bacteria were also identified in this study on the outer wooden cup of *E. longifolia* products. Therefore, this molecular approach is effective in identifying the authenticity of *E. longifolia* products, with *trn*L-*trn*F and ITS as the recommended DNA markers.

Keywords: authentication, DNA barcoding, *Eurycoma longifolia*, medicinal plant

1. INTRODUCTION

The current lifestyle trend leans towards natural-based practices, including healthcare and medicine (Coutinho Moraes *et al.*, 2015; Elkordy *et al.*, 2021; Insuasti-Cruz *et al.*, 2022). This shift is driven by a desire for holistic

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well-being and a growing recognition of the benefits of plant-derived substances. Plant-derived medicines offer sustainable alternatives to synthetic drugs (Elkordy *et al.*, 2021), align with environmental concerns (Theodoridis *et al.*, 2023), and reduce reliance on non-renewable resources (Verpoorte *et al.*, 2008). Advances in scientific research further support the importance of plant-based medicines as they uncover bioactive compounds with potential therapeutic applications (Gautam *et al.*, 2023; Nyakudya *et al.*, 2020). *Eurycoma longifolia* commonly referred to as *pasak bumi* or *tongkat ali* in the Indonesian and Malay languages is renowned for its medicinal properties in Southeast Asian countries such as Malaysia, Indonesia, and Vietnam (Bhat and Karim, 2010; Chua *et al.*, 2005; Evans Schultes, 1980; Kassim *et al.*, 2002). *E. longifolia* possesses a diverse array of bioactive compounds. These include *quassinoids*, *β-carboline alkaloids*, *canthin-6-one alkaloids*, *triterpene-type tirucallane*, *derivatives of squalene*, *eurycolactone*, *eurycomalactone*, *laurycolactone*, *biphenyl neolignan*, and *bioactive steroids* (Ang *et al.*, 2000; Kuo *et al.*, 2004; Mahfudh and Pihie, 2008; Miyake *et al.*, 2009; Tran *et al.*, 2014). The presence of these compounds contributes to the diverse pharmacological activities of *E. longifolia*, making it a subject of great interest for research and development of herbal products. This plant species grows naturally in Cambodia, Myanmar, and Thailand. In addition to its antimalarial, antipyretic, antiulcer, cytotoxic, and aphrodisiac properties (Bhat and Karim, 2010; Chua *et al.*, 2005; Evans Schultes, 1980; Kassim *et al.*, 2002) the root extracts have been traditionally used to enhance testosterone levels in men.

The significant value and affordability of this species has led to an increased market demand and intensive trade, both domestically and internationally (Sihotang and Rahmawati, 2019). Unfortunately, this high demand has led to illegal export practices and the potential overexploitation of *E. longifolia* in Indonesia (Susilowati *et al.*, 2021). The sustainable utilisation of this species is compromised by the persistence of current practices. Consequently, it is imperative to establish systems capable of monitoring and delineating the origins of plants and their derived products, as demonstrated by DNA barcoding (Abubakar *et al.*, 2018).

Molecular identification techniques, particularly DNA-based methods, play a pivotal role in the authentication and traceability of herbal products derived from *E. longifolia*. DNA markers have proven to be powerful tools for validating the authenticity and traceability of herbal and food products (Ganie *et al.*, 2015) as in the case of Korean ginseng (Jung *et al.*, 2014), manuka honey (McDonald *et al.*, 2018), *Orthosiphon stamineus* herbal medicine (Liow *et al.*, 2021), and processed hairtail fish products (Abdullah *et al.*, 2019). These methods provide a reliable means of verifying the presence and authenticity of plant materials in various commercial products, including dietary supplements, traditional medicine, and herbal preparations (Fadzil *et al.*, 2018; Gao *et al.*, 2010; Han *et al.*, 2016; Howard *et al.*, 2019; Noh *et al.*, 2018). By analysing specific genetic markers such as DNA barcodes, it is possible to differentiate *E. longifolia* from other related species and detect any adulteration or substitution that may occur in the market (Gao *et al.*, 2010; Han *et al.*, 2016).

Molecular identification methods have been widely used to confirm the presence of *E. longifolia* in commercial products. Studies have successfully employed techniques such as DNA sequencing, polymerase chain reaction (PCR), and DNA barcoding to assess the quality and authenticity of *E. longifolia*-based products (Abubakar *et al.*, 2018; Vejayan *et al.*, 2018). These molecular techniques provide accurate and reliable results, allowing for better regulation and control of the trade of *E. longifolia* products and ensuring consumer safety.

Although previous studies have authenticated *E. longifolia* products, the derivative products of this species have become increasingly diverse (Abubakar *et al.*, 2018). In Indonesia, raw materials are extracted from the

wild because of the species' lack of cultivation or domestication (Pasaribu *et al.*, 2021; Suwardi *et al.*, 2022). This has led to a decrease in authentic raw materials, prompting the use of species falsely branded as *E. longifolia* (Li *et al.*, 2013; Mohammed Abubakar *et al.*, 2017). Consequently, the need for broader authentication efforts has been emphasised. Moreover, the diversity of adulterated materials varies between localities, regions, and countries based on the occurrence of complementary species (Soares *et al.*, 2017), necessitating specific authentication methods tailored to each geographical context. This study aimed to authenticate the products or derivatives of *E. longifolia* (*pasak bumi*) produced, marketed, and consumed in Indonesia using molecular identification techniques.

2. MATERIALS and METHODS

2.1. Plant material

A total of 37 leaf samples of *E. longifolia* were collected from the Sumatran mainland and Riau Islands, Indonesia (Table 1). Leaf samples were labelled, wrapped in sample envelopes, and dried with silica gel. In addition, six herbal products from the roots of *E. longifolia* (HPs) in various forms were also used (Table 2). In the present study, leaf samples of *E. longifolia* were used as the primary reference for authentication. These leaf samples served as crucial benchmarks for comparison with claimed derivative products of *E. longifolia*, including wood and roots. This approach allowed us to comprehensively evaluate and verify the authenticity of a wide range of *E. longifolia* products and provided valuable insights into the product origins and potential falsifications. The molecular analyses were performed at the Laboratory of Molecular Systematics, National Research and Innovation Agency, Indonesia.

2.2. DNA isolation, amplification, and sequencing

Leaf samples of *E. longifolia* were isolated using a Genomic DNA Mini Kit (Plant) from GeneAid, and products from *E. longifolia* were isolated using a DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA; HB-0542-003+1101205_PCard_DNY_Plant_Spi) following the manufacturer's protocol. Two molecular markers, namely *trn*L-*trn*F and the whole internal transcribed spa-

Table 1. List of sample collections of *Eurycoma longifolia* leaves from Sumatra mainland and Riau Islands

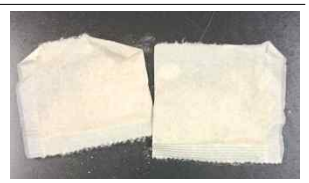
No	Sample code	Population	Location
1	02EL	Nagari Forest/Paru Village	Sijunjung District, Sijunjung Regency, West Sumatra Province
2	03EL	Nagari Forest/Paru Village	Sijunjung District, Sijunjung Regency, West Sumatra Province
3	04EL	Nagari Forest/Paru Village	Sijunjung District, Sijunjung Regency, West Sumatra Province
4	06EL	Nagari Forest/Timbulun Village	Sijunjung District, Sijunjung Regency, West Sumatra Province
5	07EL	Nagari Forest/Timbulun Village	Sijunjung District, Sijunjung Regency, West Sumatra Province
6	08EL	Nagari Forest/Timbulun Village	Sijunjung District, Sijunjung Regency, West Sumatra Province
7	09EL	Nagari Forest/Timbulun Village	Sijunjung District, Sijunjung Regency, West Sumatra Province
8	10EL	Nagari Forest/Timbulun Village	Sijunjung District, Sijunjung Regency, West Sumatra Province
9	A8-3	Riau A8-3	Ampang Delapan – Riau Province
10	R1EL	Rumbio	Kampar – Riau Province

Table 1. Continued

No	Sample code	Population	Location
11	R2EL	Rumbio	Kampar – Riau Province
12	1PEL	Papaso Village	Batang Lubu Sutan, Padang Lawas-North Sumatra Province
13	4PEL	Papaso Village	Batang Lubu Sutan, Padang Lawas-North Sumatra Province
14	5PEL	Papaso Village	Batang Lubu Sutan, Padang Lawas-North Sumatra Province
15	6PEL	Papaso Village	Batang Lubu Sutan, Padang Lawas-North Sumatra Province
16	7PEL	Papaso Village	Batang Lubu Sutan, Padang Lawas-North Sumatra Province
17	8PEL	Papaso Village	Batang Lubu Sutan, Padang Lawas-North Sumatra Province
18	9PEL	Papaso Village	Batang Lubu Sutan, Padang Lawas-North Sumatra Province
19	10PEL	Papaso Village	Batang Lubu Sutan, Padang Lawas-North Sumatra Province
20	PU1T	Tembesi Protected Forest	Tembesi, Sagulung District, Batam City, Riau Islands Province
21	PU3T	Tembesi Protected Forest	Tembesi, Sagulung District, Batam City, Riau Islands Province
22	PU4T	Tembesi Protected Forest	Tembesi, Sagulung District, Batam City, Riau Islands Province
23	PU2D	Duriangkang Protected Forest	Kabil, Nongsa District, Batam City, Riau Islands Province
24	PU4D	Duriangkang Protected Forest	Kabil, Nongsa District, Batam City, Riau Islands Province
25	PU7D	Duriangkang Protected Forest	Kabil, Nongsa District, Batam City, Riau Islands Province
26	PU8TN	Tiban Protected Forest	Tiban Lama, Sekupang District, Batam City, Riau Islands Province
27	PU10TN	Tiban Protected Forest	Tiban Lama, Sekupang District, Batam City, Riau Islands Province
28	PU12TN	Tiban Protected Forest	Tiban Lama, Sekupang District, Batam City, Riau Islands Province
29	PU7SH	Sei Harapan Protected Forest	Tanjung Riau, Sekupang District, Batam City, Riau Islands Province
30	PU14SH	Sei Harapan Protected Forest	Tanjung Riau, Sekupang District, Batam City, Riau Islands Province
31	PU21SH	Sei Harapan Protected Forest	Tanjung Riau, Sekupang District, Batam City, Riau Islands Province
32	PU8N	Nongsa Batam Protected Forest	Sambau, Nongsa District, Batam City, Riau Islands Province
33	PU11N	Nongsa Batam Protected Forest	Sambau, Nongsa District, Batam City, Riau Islands Province
34	PU13N	Nongsa Batam Protected Forest	Sambau, Nongsa District, Batam City, Riau Islands Province
35	PU2DG	Dangas Protected Forest	Tanjung Pinggir, Sekupang District, Batam City, Riau Islands Province
36	PU6DG	Dangas Protected Forest	Tanjung Pinggir, Sekupang District, Batam City, Riau Islands Province
37	PU9DG	Dangas Protected Forest	Tanjung Pinggir, Sekupang District, Batam City, Riau Islands Province

cer (ITS) regions, were selected for PCR amplification and DNA sequencing. The PCR amplification of the *trn*L-*trn*F region in *E. longifolia* leaf samples employed a set of universal primers, with the forward primer 'c' (5'-CGAAATCGGTAGACGCTACG-3') and reverse primer 'f' (5'-ATTTGAACTGGTGACACGAG-3'; Taberlet *et al.*, 1991). For the *E. longifolia* product sample, universal forward primer pair 'c' (5'-CGAAATCGGTAGAC

Table 2. List of sample collections of *Eurycoma longifolia* herbal products

No	Sample code	Product	Serving form	Source	Photo
1	TCPB	Herbal tea	Root chips	Shopee*: Herbal healing official	
2	GDPB	Inner wooden cup	Solid root	Shopee: Fatya_chan	
3	GLPB	Outer wooden cup	Solid root	Shopee: Fatya_chan	
4	APBC	Tea from root	Root powder	Shopee: Fatya_chan	
5	KPB	Herbal capsules	Root powder	Shopee: Syahira Bodher	
6	SAPB	Dry root chips	Root chips	Shopee: Fatya_chan	

* Shopee is the largest e-commerce platform in Southeast Asia.
TCPB: herbal tea product, GDPB: wooden cup, GLPB: wooden cup, APBC: herbal tea product, KPB: herbal capsules, SAPB: dry root chips.

GCTACG-3') and reverse primer 'd' (5'-GGG GATAGA GGGACTTGAAC-3'), forward primer 'e' (5'-GGTTCA AGTCCCTCTATCCC-3'), and reverse primer 'f' (5'-AT TTGAACTGGTGACACGAG-3'; Taberlet *et al.*, 1991) were used. The PCR amplification of genomic DNA from *E. longifolia* leaf samples and its products from the ITS region was performed using universal primers. The forward primer pair 'ITS 5P' (5'-GGAAGGAGAAGTC GTAACAAGG-3') and reverse primer 'ITS 8P' (5'-CACGCTTCTCCAGACTACA -3') were utilised in this procedure (Möller and Cronk, 1997).

The preparation of the PCR mixture for genomic DNA from leaf samples involved a total volume of 12.5 μL. This comprised 6.25 μL of 2x My Taq HS Red Mix PCR buffer (Bioline, Meridian Bioscience, Newtown, OH, USA), 0.25 μL each of the forward and reverse primers, 4.75 μL of nuclease-free water (QIAGEN), and 1 μL of the DNA template. The PCR mixture for genomic DNA derived from *E. longifolia* products had a total volume of 13.5 μL. This included 0.375 μL of both the forward and reverse primers (5 pmol each), 0.25 μL of Taq DNA polymerase (Toyobo, Tokyo, Japan), 2.5 μL of dNTP (Toyobo), 6.25 μL of PCR buffer KOD FX Neo (Toyobo), 2.75 μL of nuclease-free water (QIAGEN), and 1 µL of the DNA template (10 ng/μL). The amplification reactions were conducted using the Sedi G Thermo Cycler (Wealtec Bioscience, New Taipei City, Taiwan) under optimal conditions: initial denaturation at 94℃ for 3 min, denaturation at 94℃ for 30 secs, annealing at 55℃ for 30 secs, extension at 72℃ for 1 min and 30 secs, and a final extension at 72℃ for 4 min. The PCR amplification process was done over 35 cycles.

PCR products were visualised on a 1% agarose gel (Agarose Vivantis, Shah Alam, Malaysia) and stained with GelRed (Biotium, Hayward, CA, USA) using an electrophoretic process that lasted 30 min at 100 volts. After the electrophoresis process was completed, the target bands were photographed using a gel documentation system (Bioinstrument, ATTO Biosystems, Amherst, NY, USA). The amplified PCR products were sent to a 1st Base company for Sanger sequencing.

2.3. Data analysis

The compiled sequences from the forward and reverse sequences were integrated using ATGC version 4.3.5. The nucleotide composition of the *trn*L-*trn*F and ITS 5P-8P genes was assessed using MEGA X software (Kumar *et al.*, 2016). To ascertain homology and identity, samples were aligned with the GenBank DNA database using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLASTn analysis identified the sequence with the highest percentage of similarity as the one most closely related to the query sequence. To reconstruct the phylogenetic tree, samples from this study were juxtaposed with reference sequences obtained from NCBI GenBank (https://www.ncbi.nlm.nih.gov/). Bayesian inference was conducted using MrBayes 3.6 (Huelsenbeck and Ronquist, 2001) integrated as plugins in Geneious Prime version 2021.0, with the HKY85 substitution model and a distributed gamma rate variation. The analysis involved two independent Markov chain (MCMC) runs of four chains for 1,100,000 generations, with tree topologies sampled every 200 generations. Burn-in periods, determined by the SD of split frequency values, were set to 100,000 generations, considering values < 0.95 as strongly and weakly supported (Devecchi *et al.*, 2018).

3. RESULTS and DISCUSSION

3.1. Sequence homology and identity of *Eurycoma longifolia* HPs

The amplicon sizes of the *trn*L-*trn*F chloroplast and ITS genes from the 37 leaf samples were 957 and 845 bp, respectively. The results of the BLASTn analysis of

the *trn*L-*trn*F and ITS regions showed that the *E. longifolia* leaf samples in the study had 99%–100% similarity with the *Eurycoma* sample in GenBank (Table 3). Thus, the BLASTn analysis results confirmed the identity of *E. longifolia* samples collected from the Sumatra mainland and Riau Islands used in this study.

Sequencing attempts for some root or root chip products (GLPB and SAPB; Table 2) using the *trn*L-*trn*F region were unsuccessful despite three repetitions of the DNA extraction procedure with several modifications. The sequence readings generated for both products were highly unreadable, resulting in only four products being displayed in the amplification results using the *trn*L-*trn*F region (Table 4). These unreadable products were excluded from further analysis. GLPB and SAPB are products that physically consist of solid woody root or root chips and are sold as *E. longifolia* woody root. DNA extraction from wood is more challenging than that from fresh leaves (Liepelt *et al.*, 2006; Swetha *et al.*, 2014), thus potentially complex modifications in the DNA extraction process may be required to yield more pure genomic DNA, leading to higher success rates during sequencing. This is especially important if the wood material used in product manufacturing is not authentic *E. longifolia* but rather substitute species that are more abundant and easily accessible. To mask the characteristics of the substitute species, manufacturers often employ additional processes involving various chemicals that can affect the appearance of the substitute material (Psifidi *et al.*, 2015). With such processes, DNA extraction becomes more difficult because of the need to eliminate numerous chemical compounds to obtain pure DNA for amplification in the subsequent stages. Modifications to the DNA extraction process are crucial for enhancing the purity and quality of genomic DNA extracted from wood products (Fatima *et al.*, 2018; Finkeldey *et al.*, 2010). These modifications include optimising the extraction protocol for wood samples, pre-treating samples to remove contaminants, employing physical disruption methods, and using specialised DNA purification kits (Dilley and Chapman, 2019; Rachmat *et al.*, 2024; Rachmayanti *et al.*, 2006; Verbylaitė *et al.*, 2010).

The *trn*L-*trn*F BLASTn sequence of *E. longifolia* products showed that only the TCPB (root chips) pro

Table 3. Reference sequences with the closest similarity to *Eurycoma longifolia* leaf samples in the study based on BLASTn analysis for *trn*L-*trn*F and ITS regions

No	Region	Similarity	Accession	% Similarity	% Query cover	Reference
1	*trn*L-*trn*F	*Eurycoma apiculata*	GU593014	100.00	100	Muellner-Riehl *et al*., 2016
		E. longifolia	MH751519	99.79	100	Ng *et al*., 2019
		Simarouba versicolor	MG599400	99.16	99	Devecchi *et al*., 2018
		Perriera madagascariensis	GU593020	98.96	100	Muellner-Riehl *et al*., 2016
		Homalolepis morettii	MG599405	98.96	100	Devecchi *et al*., 2018
2	ITS	*E. longifolia*	MN715379	98.16	83	Unpublished
		E. longifolia	MG643109	98.98	81	Devecchi *et al*., 2018
		Ailanthus altissima	OX327691	88.62	100	Unpublished
		Simaba guianensis	MG643102	93.19	81	Devecchi *et al*., 2018
		Homalolepis cuneata	MG643083	93.18	81	Devecchi *et al*., 2018

ITS: Internal transcribed spacer.

Table 4. Reference sequences with the closest similarity to *Eurycoma longifolia* products in the study based on BLASTn analysis GenBank for both the *trn*L-*trn*F and ITS regions

Region	No.	Sample code	No. Accession	% Similarity	% Query cover	Species	References
*trn*L-*trn*F	1	APBC	NC_063303.1	98.89	99	*Dalbergia obtusifolia*	Unpublished
			MH547571.1	100.00	95	*Dalbergia latifolia*	Lee *et al*., 2019
			MN251247.1	98.70	99	*Dalbergia cochinchinensis*	Song *et al*., 2019
			MN251245.1	98.70	99	*Dalbergia hupeana*	Song *et al*., 2019
			MN251242.1	98.70	99	*Dalbergia sissoo*	Song *et al*., 2019
	2	GDPB	MG599402.1	92.65	99	*Simaba monophylla*	Devecchi *et al*., 2018
			MG599406.1	92.48	99	*Simarouba glauca*	Devecchi *et al*., 2018
			MG599436.1	92.46	99	*Homalolepis subcymosa*	Devecchi *et al*., 2018
			MG599404.1	92.46	99	*Homalolepis glabra*	Devecchi *et al*., 2018
			MG599403.1	92.46	99	*Homalolepis pumila*	Devecchi *et al*., 2018
	3	KPB	GU818102.1	98.83	100	*Vernonia gigantea*	Pelser *et al*., 2010
			NC_061907.1	98.83	99	*Elephantopus scaber*	Unpublished
			NC_053851.1	98.44	99	*Gymnanthemum amygdalinum*	Zhou *et al*., 2021
			MZ958830.1	98.05	99	*Cyanthillium cinereum*	Unpublished
			OK040129.1	98.05	99	*C. cinereum*	Unpublished
	4	TCPB	MH751519.1	99.67	100	*E. longifolia*	Ng *et al*., 2019
			MG599405.1	99.49	97	*Homalolepis morettii*	Devecchi *et al*., 2018
			MG599406.1	98.81	97	*S. glauca*	Devecchi *et al*., 2018
			MG599407.1	98.48	97	*Homalolepis suffruticosa*	Devecchi *et al*., 2018
			MG599416.1	98.80	97	*Simaba pubicarpa*	Devecchi *et al*., 2018
ITS 5P-8P	1	KPB	JX564720.1	98.82	89	*E. scaber*	Devecchi *et al*., 2018
			MT914283.1	97.70	92	*Potentilla lineata*	Liang, 2020
			DQ813304.1	97.18	94	*E. scaber*	Unpublished
			KP052671.1	97.28	92	*E. scaber*	Unpublished
			JN407429.1	97.52	90	*E. scaber*	Unpublished
	2	SAPB	OX327710.1	85.89	100	*Ailanthus altissima*	-
			OX327691.1	85.89	100	*A. altissima*	-
			DQ787413.1	86.38	93	*Simarouba amara*	Unpublished
			MN082855.1	83.69	99	*Geijera paniculata*	Duretto *et al*., 2020
			MN715379.1	85.73	87	*E. longifolia*	Unpublished

Table 4. Continued

Region	No.	Sample code	No. Accession	% Similarity	% Query cover	Species	References
ITS 5P-8P	3	TCPB	MG643109.1	98.06	88	*E. longifolia*	Devecchi *et al*., 2018
			MN715379.1	96.98	87	*E. longifolia*	Unpublished
			MG643102.1	92.39	88	*Simaba guianensis*	Devecchi *et al*., 2018
			MG643083.1	92.24	88	*Homalolepis cuneata*	Devecchi *et al*., 2018
			MG643108.1	92.09	88	*Hannoa chlorantha*	Devecchi *et al*., 2018
	4	GLPB UPPER	CP115837.1	100.00	99	*Klebsiella pneumoniae*	Unpublished
			CP115930.1	100.00	99	*K. pneumoniae* subsp. *pneumoniae*	Unpublished
			CP095426.1	100.00	99	*K. pneumoniae*	Tang *et al*., 2023
			CP095428.1	100.00	99	*K. pneumoniae*	Tang *et al*., 2023
			CP115714.1	100.00	99	*K. pneumoniae*	Unpublished

ITS: internal transcribed spacer, APBC: herbal tea product, GDPB: wooden cup, KPB: herbal capsules, TCPB: herbal tea product, GLPB: wooden cup, SAPB: dry root chips.

duct was similar to *E. longifolia* MH751519.1 (99.67%). However, the GDPB product was similar to *Simaba monophylla* MG599402.1 (92.65%), *Simarouba glauca* MG599406.1 (92.48%), and *Homalolepis* species such as *Homalolepis subcymosa* MG599436.1 (92.46%), *Homalolepis glabra* MG599404.1 (92.46%), *Homalolepis pumila* MG599403.1 (92.46%), which belongs to the same family as *E. longifolia*, namely Simaroubaceae (Table 4). The two products, TCPB and GDPB, were similar to *E. longifolia* and the Simaroubaceae family sequences from GenBank in the *trn*L-*trn*F sequence, indicating that these products were most likely derived from *E. longifolia*. Meanwhile, the other root powder products (APBC and KPB) had dissimilarities with the sequences of *E. longifolia* or other species in the Simaroubaceae family in GenBank, indicating that the products may not be derived from *E. longifolia.*

The ITS region amplification was only successful for four products: TCPB, GLPB, KPB, and SAPB. However, the two remaining *E. longifolia* herbal products, GDPB and APBC, failed to be amplified, resulting in messy and unreadable sequences. The results of the ITS BLAST sequencing of *E. longifolia* products showed that only TCPB was similar to *E. longifolia* MG643109.1 (98.06%) and MN715379.1 (96.98%) from GenBank. In addition, the TCPB product was similar to *Simaba guianensis* MG643102.1 (92.39%), *Homalolepis cuneata* MG643083.1 (92.24%), and *Hannoa chlorantha* MG643108.1 (92.09%) which belong to the Simaroubaceae family (Table 4). The other products (KPB, SAPB, and GLPB) were dissimilar to the *E. longifolia* GenBank sequence. This pattern indicates that the TCPB product was most likely derived from *E. longifolia* and other products were derived from other species that are not found in the Simaroubaceae family. ITS analysis in this study also indicates the identification of *Klebsiella pneumonia* bacteria in GLPB (wooden cup product), urging consumers to exercise caution in the purchase and use of wooden cups as consumable items, given their potential to host such bacteria.

Of the six products analysed, only TCPB (an herbal tea product) was identified as an *E. longifolia* derivative based on the *trn*L-*trn*F and ITS regions, whereas the other products were derived from different plant species, indicating the potential for product mislabelling or falsification. Therefore, the DNA markers used in this study (*trn*L-*trn*F and ITS regions) were effective in tracing the authenticity of the product.

Compared to fresh plant DNA, neither the *trn*L-*trn*F nor the ITS regions were successfully amplified for all products. This inability to achieve successful DNA amplification may be attributed to DNA degradation during the manufacturing process. Various secondary metabolites, including polysaccharides, glycoproteins, polyphenolic compounds, and pigments, originating from raw materials in diverse plant tissues and organs, have been established as factors that impede DNA extraction, amplification, and sequencing processes (Zahra *et al.*, 2016). Additionally, the degradation of DNA at primer annealing sites may contribute to unsuccessful amplification of the targeted DNA barcode regions, leading to reaction failure (Newmaster *et al.*, 2013).

Various plant species which were mixed and packaged in powder form, chips, or fine particles also contributed to amplification failure using the two selected DNA regions or products that failed to be amplified with the previous primer pairs, which had a sequence length of approximately 900 base pairs, and the use of a new primer pair for both targeted regions may be necessary to amplify shorter lengths (Little and Jeanson, 2013). Similar outcomes were observed in a previous study that had successful amplification using a shorter fragment of 500bp (Abubakar *et al.*, 2018). Several studies have suggested that shorter DNA barcodes are more efficacious for the identification and authentication of herbal products because of their enhanced amplification efficiency (Little, 2014; Little and Jeanson, 2013).

Both products, presented as *E. longifolia* herbal tea with the code TCPB, were conclusively identified as *E. longifolia* through both the *trn*L-*trn*F region and ITS. This unequivocally establishes that the tea in question was sourced from the botanical elements of *E. longifolia*. The raw material for tea, comprising leaves, is notably more abundant than the *E. longifolia* products derived from the roots. *E. longifolia* tea products, distinguished by their leaf utilisation, have emerged as environmentally friendly options. This is primarily because of the sustainable nature of leaf harvesting, setting it apart from products relying on root extraction, an inherently destructive process that leads to the demise of the plant. Consequently, *E. longifolia* tea products derived from leaves have heightened potential for authenticity when juxtaposed with their counterparts that utilise root organs or stems.

The claim that the wooden cup originated from the root of *E. longifolia* remains unsubstantiated, as evidenced by both the *trn*L-*trn*F marker and ITS analysis. The cup, which measures 9 cm in diameter, requires raw material from *E. longifolia* roots with diameters exceeding 9 cm. Morphologically and botanically, the assertion that the cup is derived from the roots of *E. longifolia* appears exaggerated. Considering the morphological aspects and botanical characteristics observed during field surveys on the Sumatran mainland and the Riau Islands, *E. longifolia* is a small tree with an average diameter of less than 15 cm and a height not exceeding 15 m. The production of a wooden cup with a 9 cm diameter would necessitate an extensive supply of large *E. longifolia* roots, which is inconsistent with the abundance of *E. longifolia* individuals in their natural habitat in Sumatra. Molecular identification of the wooden cup product revealed that the substituted wood used in its construction belongs to a different genus within the same family. From the perspectives of availability, rarity, and conservation status, the substituted type was more accessible and abundant.

The claim regarding *E. longifolia* root tea in powdered form (APBC) has also been identified as not

originating from the *E. longifolia* plant but rather from the wood organ of the rosewood plant (*Dalbergia*). The falsification of the powdered form is particularly straightforward, given that the powder lacks distinctive morphological features. The introduction of a bitter taste into the product can be artificially achieved, or by natural substitution of ingredients, such as those found in various species within the *Dalbergia* genus, which already possess a naturally bitter taste.

The claim that the herbal capsules contained *E. longifolia* in powdered form was disproved, as confirmed by both the *trn*L-*trn*F region and ITS analysis. Both regions recognised the product as potentially derived from different plant species, including *Vernonia gigantea*, *Elephantopus scaber*, *Chanthilium cinereum*, *Gymnanthemum amugdalium*, and *Potentilla cireata*. DNA marker-based identification suggested that the herbal capsules were a composite of different plant species. In Indonesia, the identified plant species constituting the herbal capsules are commonly used by the public as traditional herbal medicines or supplements. These plants are abundant on forest floors because of their herbaceous nature, and some have been cultivated intensively in home gardens or on larger scales. Despite being derived from plant species with similar herbal properties, labelling the product as *E. longifolia* is misleading and constitutes a fraudulent practice. Labelling products originating from *E. longifolia* leads to a significantly higher market value, creating a deceptive economic advantage, as products labelled based on factual information about actual plant ingredients may have a lower market value.

Similarly, the claim regarding the herbal tea made from *E. longifolia* roots, presented in the form of thin root shavings, lacks support from the molecular identification conducted in this study. The results of our molecular analysis indicated that the source of the raw material for the product resembled *Alianthus altissima*, *Simarouba amara*, and *Geijera paniculata*. Additionally, the product is suggested to be a composite of several types of raw materials, all of which are known for their herbal properties in Indonesia.

3.2. Phylogenetic analyses

Following the sequence identity results of all samples, these results were verified by phylogenetic analysis. The results of the phylogenetic tree reconstruction using the *trn*L-*trn*F gene (Fig. 1) with *Castela erecta* (GU593013.1) as the outgroup (Clayton *et al*., 2010, unpublished) indicated that TCPB clustered together with the *E. longifolia* leaf sample sequence, with a branch support of 0.9298. Additionally, the construction of trees in Mr. Bayes using the ITS gene (Fig. 2) with *Castela emoryi* (MF963835.1) as the outgroup (Thornhill *et al*., 2017) revealed that TCPB clustered with *E. longifolia* leaf samples and *E. longifolia* (MG643109.1) sequences from GenBank, with a branch support of 0.5944. Conversely, other product samples, namely KPB, GDPB, GLPB, SAPB, and APBC, were not grouped together with the *E. longifolia* leaf samples used in this study or *E. longifolia* in the NCBI database.

Using *trn*L-*trn*F primers, BLASTn analysis revealed that among those tested, only the TCPB sample exhibited 100% similarity to *E. longifolia* species (Ng *et al.*, 2019; MH751519). BLASTn also identified other TCPB species (Table 4), indicating the possibility of adulteration with other substitutes or products. The presence of substituted products may result in barcodes that differ from the labels assigned to the tested herbal products (Abubakar *et al.*, 2018; Newmaster *et al.*, 2013). As shown in Fig. 1, Bayesian analysis indicated that among the tested herbal products samples, only TCPB formed a monophyletic clade that was strongly supported (0.9909) by the *E. longifolia* reference sequence derived from this study. 02EL, 06 EL, 07 EL, 08 EL, 09EL, 10EL, 5PEL, 6 PEL, 7 PEL, A83EL, PU 10TN, PU12TN, PU14 SH, PU21 SH, PU2DF, PU3T, PU4T, PU6DG, PU7D,

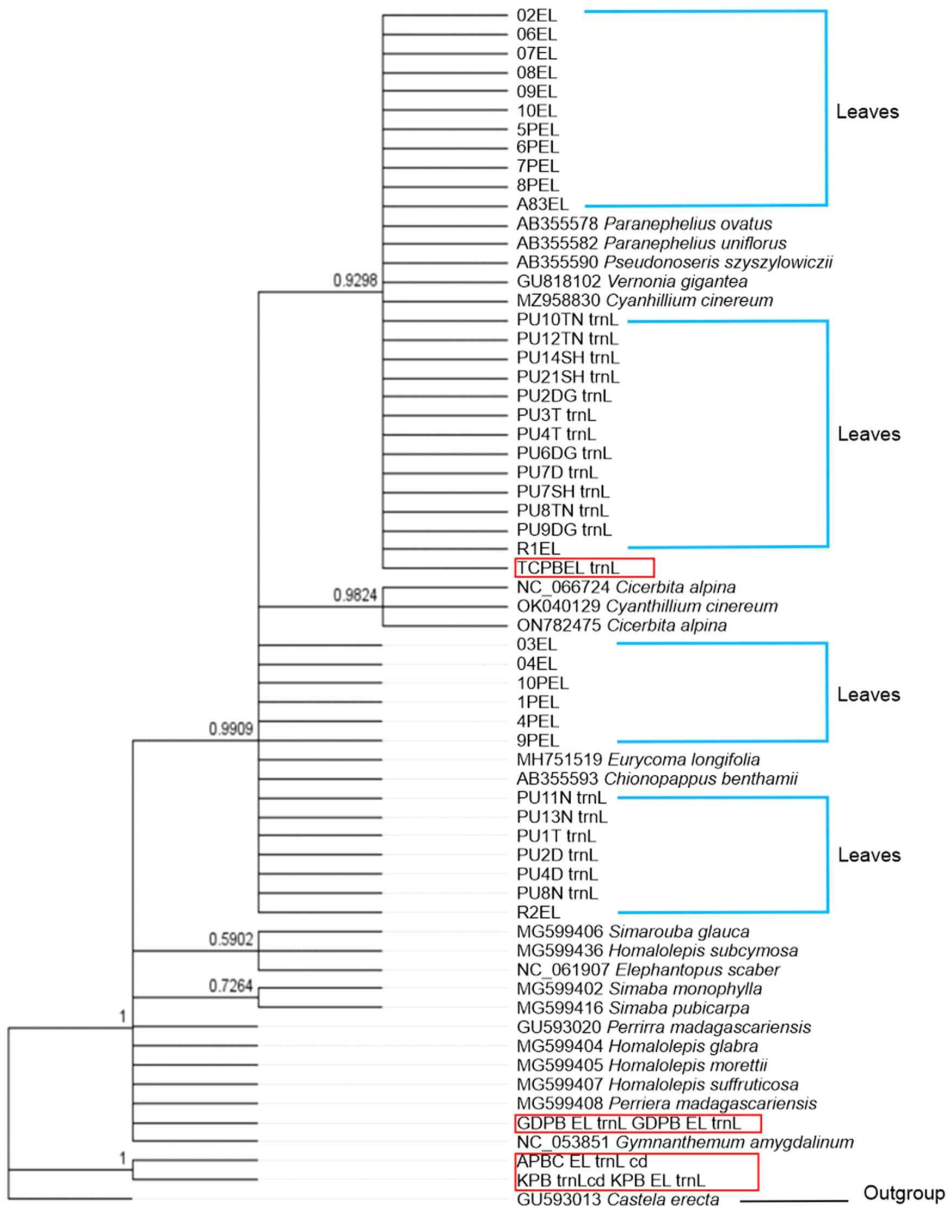


Fig. 1. A phylogenetic tree generated by *trn*L-*trn*F sequences based on Bayesian inference (Fig. created from Geneious version 2023.0 created by Biomatters. Available from: https://www.geneious.com). The number above branches are posterior probability values. The sample code corresponds to Table 1.

PU7SH, PU8TN, PU9DG, R1EL and other sequences of *E. longifolia* obtained from the GenBank repository (MH751519.1). This study demonstrated the authenticity of the tested herbal product samples. However, several

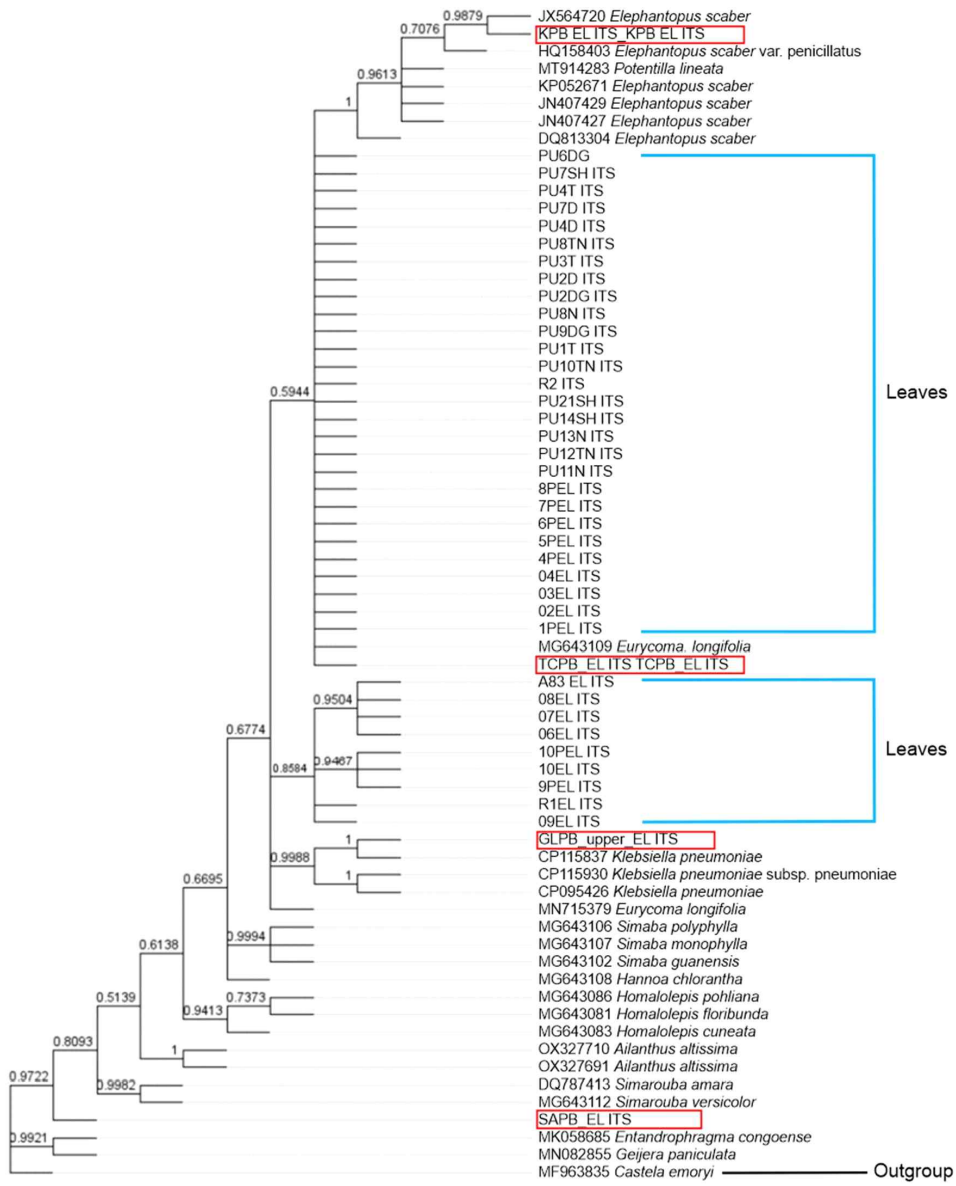


Fig. 2. A phylogenetic tree generated by ITS sequences based on Bayesian inference (Fig. created from Geneious Prime version 2023.0 created by Biomatters. Available from: https://www.geneious.com). The number above branches are posterior probability values. The sample code corresponds to Table 1. ITS: internal transcribed spacer.

species were clustered in the same clade: *Paranephelius ovatus* AB355578, *Paranephelius uniflorus* AB355582, *Pseudonoseris szyszylowiczii* AB355590, *V. gigantea* GU818102, *C. cinereum* MZ958830. These findings

suggest that while *trn*L-*trn*F can be adequately amplified across a diverse range of plants, its variability may not be sufficient for species discrimination, as suggested by de Groot *et al.* (2011).

BLASTn analysis of the ITS barcoding regions indicated that only one of the tested herbal product samples (TCPB) exhibited a > 88% match with *E. longifolia* (Table 4). Nevertheless, the BLASTn results revealed that TCPB harboured a DNA barcode from a different species, contrary to the labelled sample information. TCPB also has 87%–88% similarity with *S. guianensis* MG643102.1, *H. cuneata* MG643083.1, and *H. chlorantha* MG643108.1. Referring to Fig. 2, the Bayesian analysis of the ITS region in *E. longifolia* revealed that one of the tested HP samples (TCPB) formed a cluster with the *E. longifolia* reference sequence established in the current study (PU6DG, PU7SH, PU4T, PU7D, PU4D, PU8TN, PU3T, PU2D, PU2DG, PU8N, PU9DG, PU1T, PU10TN, R2, PU21SH, PU14SH, PU13N, PU12TN, PU11N, 8PEL, 7PEL, 6PEL, 5PEL, 4PEL, 04, 03, 02, 1 PEL) and other *E. longifolia* sequences retrieved from the GenBank database (MG643109). This result indicated that TCPB did contained *E. longifolia*. Other tested HP samples were positioned outside the clade of the reference samples, suggesting that these herbal medicinal products (HMPs) harboured plant species that were different from those declared on their packaging labels.

The Bayesian results for both *trn*L-*trn*F and ITS demonstrated that the other five HP-tested samples clustered not with *E. longifolia* but with *S. glauca*, *Homalolepis subcymora*, *E. scaber*, and *Simaba*. *S. glauca* is a flowering tree that is native to Florida, South America, and the Caribbean. The tree is well suited to warm, humid, and tropical regions such as Indonesia. *E. scaber* (locally known as tapak liman) is a plant that can be easily found in Indonesia, including Southeast Celebes (Choi and Hwang, 2005).

The Bayesian analysis results aligned with those of the BLASTn, demonstrating that the tested HPs containing *E. longifolia* clustered together, while those lacking *E. longifolia*, contrary to the labelled information, formed a distinct clade. Thus, both the BLASTn and Bayesian tree methods effectively differentiated between HMPs containing *E. longifolia* and those without HMPs (Zhao *et al.*, 2013).

In the context of DNA barcoding in this study, only one of the six tested HPs (TCPB) was identified as authentic based on the criteria from BLASTn and Bayesian tree analyses, indicating possible counterfeiting or adulteration in five of the tested HPs. As emphasised by Zhao *et al.* (2013), any new method for identifying traditional medicines should prioritise accuracy, digitisation, repeatability, simplicity, and practicality. Compared with traditional methods, holds significant importance because of its ability to identify species in various forms, such as dried herbs, leaves, and roots, without being restricted by morphological characteristics.

Our study suggests that the *trn*L-*trn*F and ITS regions are promising DNA barcodes for authenticating *E. longifolia* HMPs because of their short sizes. An ideal marker should be short enough to amplify degraded DNA, produce high-quality sequences, and possess robust variability for sample discrimination (Li *et al.*, 2015; Vijayan and Tsou, 2010). The ITS region, confirmed by various researchers, including (Gao *et al.*, 2010; Han *et al.*, 2016), stands out as one of the best DNA barcode regions for the identification and authentication of medicinal plants. Chen *et al.* (2010) conducted an extensive study to identify the ITS2 region as the most suitable for medicinal plant identification, with a success rate of 92.7% in 6,600 samples and closely related species. In another study by He *et al.* (2012), which analysed 109 samples with five DNA barcode regions, the ITS and ITS2 regions were found to be the most effective among the tested regions.

This study marks the pioneering use of DNA barcode molecular identification technology to identify *E. longi-*

folia and its adulterants, thereby extending the application of *trn*L-*trn*F and ITS sequences to medicinal plants, particularly in Indonesia. Mislabelling observed in certain products constitutes a form of fraud, as consumers rely on product labels for expected benefits. Olivar *et al.* (2016) exemplified this by documenting instances where *Vitex negundo* (Lamiaceae) was substituted with contaminants, such as *Moringa olifeira* (Moringaceae) and *Mormodica charantia* (Cucurbitaceae), despite being medicinal plants, as revealed through DNA barcoding. Similar occurrences emerged in the current study, where products labelled as *E. longifolia* actually contained other species, such as *Homalolepis*, *Elephantopus*, and *Simaba*, potentially harbouring different natural compounds compared to *E. longifolia*. Notably, the identification of *Dalbergia* sp. as a substituted species in a product claimed to be derived from the roots of *E. longifolia* is intriguing, given its classification into a different plant family and its lack of known aphrodisiac benefits. This indirect form of adulteration, which involves the use of abundant and easily accessible materials to maximise profits, has been documented by Satheeshkumar *et al.* (2016).

Authenticating commercial products with incorrect species poses challenges, particularly when their forms are altered, such as powdered capsules or dried plant materials for tea. These alterations compromised the morphological characteristics and diminished the aroma due to volatile evaporation. Nevertheless, utilising an appropriate DNA extraction method capable of yielding high-purity DNA allows for amplification even from minute DNA samples. The Bayesian tree analysis in the present study effectively distinguished between *E. longifolia* and the other species.

4. CONCLUSIONS

DNA barcoding is a rapid and reliable method for authenticating herbal products derived from *E. longifolia*. This study showed that herbal products of *E. longifolia* available in the Indonesian market are subject to adulteration or substitution potentially stemming from inaccuracies in species identification. This study also strongly advocates the prompt integration of DNA barcoding as a mechanism for identifying instances of species adulteration to improve the production of high-quality herbal products. Consequently, DNA barcoding should be included in the quality control measures of government certification bodies to facilitate a comprehensive evaluation of product authenticity and to ensure the safety of the herbal products consumed. This will provide nuanced insights into the safety and efficacy of these products.

CONFLICT of INTEREST

No potential conflict of interest relevant to this article was reported.

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