

# DNA barcode of *Eurycoma longifolia* Jack (Simaroubaceae) from Sumatra, Indonesia based on *trnL-F* plastid sequence †

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**Abstract:** *Eurycoma longifolia* (Simaroubaceae) is a popular medicinal plant from the South-East Asian rainforest that often used as an aphrodisiac and anti malaria. The main supplies of *E. longifolia* from Indonesia is Sumatra. The increasing demand of this species for medicinal industries has led to illegal export in Indonesia. This study was aimed to identify specific nucleotides as molecular identity of *E. longifolia* from Sumatra based on DNA sequences of *trnL-F* plastid gene. Twenty four samples of *E. longifolia* were collected from three provinces in Sumatra to be sequenced. The aligned sequence of the *trnL-F* is 960 bp, and is an A/T rich region (A: 27.2%, T: 39.1%, C: 16.7% and G: 17.1%). The homology search using BLASTn of the genbank NCBI showed that the nucleotide composition of *E. longifolia* used in this study was similar to partial *trnL-F* region of *E. longifolia* MH751519 (100%) and KP995919 (99.76%), *Simaba morettii* MG599450 (99.76%), and *E. apiculata* GU593014 (99.76%). Close examination of the gene structure and composition showed that the DNA sequences of *E. longifolia* from Sumatra have five nucleotides variations that did not possessed by the reference *E. longifolia* as well other reference taxa used. All variations found were occurred mostly in the *trnL* intron region. When a phylogenetic analysis was performed, its results confirmed the correct identity of the samples species by their position was at the same clade as the other *E. longifolia*. The results from this study is expected to assist identification of herbal medicine containing *E. longifolia* from Sumatra, Indonesia.

**Keywords:** *Eurycoma longifolia*; Sumatra; medicinal plant; *trnL-F*; DNA barcode

## 1. Introduction

*Eurycoma longifolia* Jack is one of the most well known herbal medicinal plants in South-East Asian, popularly recognized as ‘Tongkat Ali’ or ‘Pasak Bumi’. The species is distributed in South Myanmar, Vietnam, Laos, Cambodia, Thailand, Peninsular Malaysia, Singapore, Sumatra, Borneo dan the Philippines [11], and may occurred in Bangladesh based on herbarium records of GBIF (Figure 1) [12]. The roots of this plant contained several active compounds of canthin, b-carboline alkaloids, derivate of *squalene tirucallane triterpenes*, *biphenylneolignans* and *quassinoids* [1] that has an antimicrobial [2], antimalarial [3], antidiabetic, antiulcer and anticancer [4-5], but mainly used as an aphrodisiac [6-7]. Currently, some of the products derived from this plants are found market in the

form of raw materials or packaged herbal products such as coffee, tea, capsules and tablets [8] as well as sweets [9].

This species is widely traded both domestic and internationally but not included within the CITES appendices [13]. In combination of the increasing market demand, a high price in the international market but low price from the farmer, make this species has been targeted for illegal exports in Indonesia. In addition to this, several industries herbal medicine prone to counterfeiting substitution, contamination, use of fillers [10]. Meanwhile several industries herbal medicine using this plants are prone to counterfeiting substitution, contamination, use of fillers [10]. The extraction of plants are usually taken from the wild by destructive harvesting of root pulling. If this continue to happen, the sustainability use the species cannot be warranty. In anticipating this, it is important to develop a system that can be used for on tracking and treacibility of the source plants. One of the approaches is to develop a DNA barcode as a specific identity for *E. longifolia* from Sumatra.

DNA barcode is a standard tool for species identification by using a fragment of DNA sequence of certain genes/regions [14-15]. The use of term DNA barcode was first raised in 2003 [16] and has gained worldwide attention in the scientific community since then [17]. Recently, the use of DNA barcode cover wide range of studies from the discovery a new species, even discriminating cryptic species, population diversity, food safety and conservation [18-20]. The CBOL proposed portions of two coding regions from the plastid (chloroplast) genome—*rbcL* and *matK*—as a “core barcode” for plants, to be supplemented with additional regions as required [20]. In this study we used *trnL-F* as an marker of choice for DNA barcode for *E. longifolia*. The *trnL-F* region is widely and often used for the study of molecular systematics due to its sufficient mutations to detect variations at specific and infra specific levels [21]. This region was one of the recommended regions for DNA barcoding analysis [21-22] as its meet all requirements identified by the CBOL of being suitable for DNA barcoding. Those loci were routinely retrievable with single primer pair, easy to obtain bidirectional sequence reads, and provided maximal discrimination among plant species [14].

This present study was aimed to develop a DNA barcode for *E. longifolia* from Sumatra using the *trnL-F* region. We expect to discover nucleotide variations that some of which were specific for samples from Sumatra. The results from this study is expected to assist identification of herbal medicine containing *E. longifolia* from Sumatra, Indonesia.

## 2. Materials and Methods

### 2.1. Samples

Twenty-four leaves samples were collected from Sumatra mainland and Riau islands (Table 1) dried in silica gel for further use.

**Table 1.** Sources of samples of *E. longifolia* from Sumatra Indonesia and reference taxa used for the phylogenetic analysis.

No	Species	Locality	Sample code, Genbank accession	Reference
1	<i>Eurycoma longifolia</i>	North Sumatra, Indonesia	1PEL	This study
2	<i>Eurycoma longifolia</i>	North Sumatra, Indonesia	2PEL	This study
3	<i>Eurycoma longifolia</i>	North Sumatra, Indonesia	4PEL	This study
4	<i>Eurycoma longifolia</i>	North Sumatra, Indonesia	5PEL	This study
5	<i>Eurycoma longifolia</i>	North Sumatra, Indonesia	6PEL	This study
6	<i>Eurycoma longifolia</i>	North Sumatra, Indonesia	7PEL	This study
7	<i>Eurycoma longifolia</i>	North Sumatra, Indonesia	8PEL	This study
8	<i>Eurycoma longifolia</i>	North Sumatra, Indonesia	9PEL	This study
9	<i>Eurycoma longifolia</i>	North Sumatra, Indonesia	10PEL	This study
10	<i>Eurycoma longifolia</i>	West Sumatra, Indonesia	02EL	This study
11	<i>Eurycoma longifolia</i>	West Sumatra, Indonesia	03EL	This study
12	<i>Eurycoma longifolia</i>	West Sumatra, Indonesia	04EL	This study

13	<i>Eurycoma longifolia</i>	West Sumatra, Indonesia	06EL	This study
14	<i>Eurycoma longifolia</i>	West Sumatra, Indonesia	07EL	This study
15	<i>Eurycoma longifolia</i>	West Sumatra, Indonesia	08EL	This study
16	<i>Eurycoma longifolia</i>	West Sumatra, Indonesia	09EL	This study
17	<i>Eurycoma longifolia</i>	West Sumatra, Indonesia	10EL	This study
18	<i>Eurycoma longifolia</i>	Riau, Sumatra, Indonesia	ES1EL	This study
19	<i>Eurycoma longifolia</i>	Riau, Sumatra, Indonesia	ES2EL	This study
20	<i>Eurycoma longifolia</i>	Riau, Sumatra, Indonesia	ES3EL	This study
21	<i>Eurycoma longifolia</i>	Riau, Sumatra, Indonesia	A81EL	This study
22	<i>Eurycoma longifolia</i>	Riau, Sumatra, Indonesia	A83EL	This study
23	<i>Eurycoma longifolia</i>	Riau, Sumatra, Indonesia	R1EL	This study
24	<i>Eurycoma longifolia</i>	Riau, Sumatra, Indonesia	R2EL	This study
25	<i>Eurycoma longifolia</i>	Kuala Lumpur, Malaysia	MH751519	[22]
26	<i>Eurycoma longifolia</i>	Unknown	KP995519	[23]
27	<i>Eurycoma apiculata</i>	Unknown	GU593014	[24]
28	<i>Simaba morettii</i>	French Guiana	MG599405	[25]
29	<i>Odyendyca gabonensis</i>	Gabon	MG599427	[25]
30	<i>Simaba glabra</i>	Mato Grosso, Brazil	MG599404	[25]
31	<i>Perriera madagascariensis</i>	Unknown	GU593020_	[24]
32	<i>Simaba monophylla</i>	Kaieteur Plateau, Guyana	MG599402	[25]

## 2.2. Isolation of DNA, Amplification and sequencing of *trnL-F*

The total genomic DNA was isolated using Genomic DNA Mini Kit (Plant) from GeneAid following the manufacturer's protocol.

Amplification of *trnL-F* region by the PCR technique was using a universal pair primer of 'c' for forward 5' (CGAAATCGGTAGACGCTACG) and 'f' (ATTTGAACTGGTGACACGAG) [26]. A PCR mixture of a total volume of 12.5 µL consisted of 5 pmole each of forward and reverse primer and 10 ng/µL of DNA template. The PCR reaction was performed in a Takara with the optimum condition of the following: a pre-denaturation at 94°C for 2 min, 30 cycles composed a denaturation at 94°C for 30 s, annealing at 52°C for 3 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The reaction was repeated in 40 cycles. The amplified bands were visualized on 1.5% agarose stained with GelRad. Electrophoresis was executed with 50 volts for 60 min in 1x TBE buffer. The target *trnL-F* bands were visualised under the UV light using AttoBioinstrument. The amplicons were then sequenced using Sanger sequencing at the First Base company.

## 2.3. Data analysis

The *trnL-F* sequence results were assembled using contig editor on ATGC software package version 4.3.5. (Genetyx Co. , Japan). The forward and reverse of each sequence were observed carefully to ensure there was no mismatch on consensus sequence produced. The nucleotide composition of the *trnL-F* gene were evaluated using MEGA 7.0 software [27]. Samples were examined their homology and identity by using BLAST nucleotide on GeneBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Data from the GenBank were downloaded in FASTA format form. Both data from this research and from GenBank were processed and put together using MEGA software [27]. The data obtained were aligned using Muscle in MEGA7 [27]. The genetic distance estimation data was analyzed using Pairwise Distance with the Kimura 2-parameter model [28] also in MEGA7. The phylogenetic tree was reconstructed using the Maximum-Likelihood method with 1000 bootstrap replicates [29] with the Kimura 2-Parameter (K2P) model in the MEGA7.

## 3. Results

### 3.1. The sequence homology and identity of *E. longifolia* from Sumatra

The amplicon size of the *trnL-F* chloroplast gene from the 24 samples from this study was 961 bp, consisted of 469 bp *trnL* (its exons and majority of intron) and 492 *trnF* gene and the intergenic

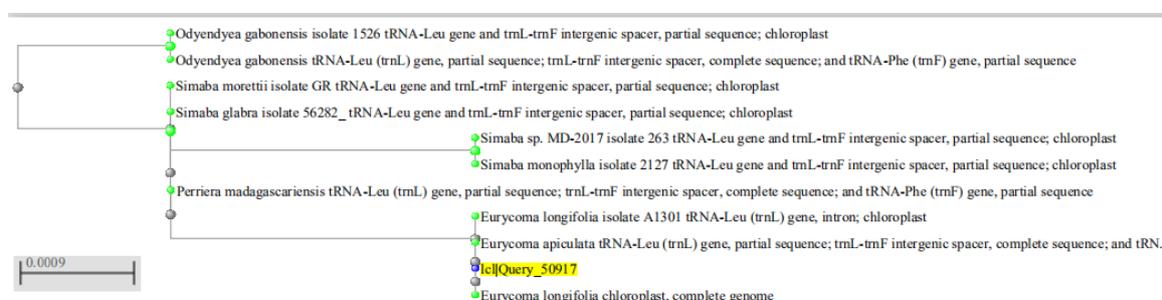
spacer between *trnL* and *trnF*. Of the 469 *trnL* region, 417 bp were homologous to many sequences from the GenBank, 18 of which were having similarity of more than 99% (data not shown). Eight reference species were further used to build phylogenetic tree based on distance analyses (Figure 2) to assess the phylogenetic position of *E. longifolia* samples from Sumatra, thus determining its identity.

**Table 2.** The results of homology search of the *trnL*-F sequence.

Accession number	Species	% similarity
MH751519	<i>Eurycoma longifolia</i>	100
MG599405	<i>Simaba morettii</i>	99.76
KP995519	<i>Eurycoma longifolia</i>	99.76
MG599427	<i>Odyendyca gabonensis</i>	99.76
GU593014	<i>Eurycoma apiculata</i>	99.76
GU593020	<i>Perriera madagascariensis</i>	99.52
MG599404	<i>Simaba glabra</i>	99.28
MG599402	<i>Simaba monophylla</i>	99.28

One of the sequence reference was derived from a complete chloroplast genome sequence of *E. longifolia* (GenBank accession MH751519) originated from Kuala Lumpur Malaysia [22]. When performing the blast nucleotide search, the query used was accession P1EL and it was 100% similar to this reference sequence, this indicates the query (P1EL) has the same haplotype as the reference (MH751519). Meanwhile the samples of *E. longifolia* have more than 99% similarity to the eight reference taxa (Table 2).

When a phylogenetic distance-tree based analysis was performed using sequence query of P1EL (Table 1), it formed a group together with the other reference *E. longifolia* (Figure 2). Thus, this confirmed the identity of the P1EL representing all the samples used in this study.



**Figure 1.** A phylogenetic tree distance based on Fast minimum evolution with max sequence difference set as 0.75 showing that the query sequence (highlighted in yellow) was identified as *E. longifolia* as it was located at the same group as the other *E. longifolia* accessions.

### 3.2. Nucleotide composition and variations

The DNA sequence of *trnL*-F of *E. longifolia* from Sumatra are A/T rich region (Table 2), composed 27.2 % thymine (T), 16.6% cytosine (C), 39.0% adenine (A), and 17.1% guanine (G). The composition of these nucleotides was similar to those reference sequences (Table 3) as well as in *Prunus armeniaca* (Rosaceae) [30].

The total single-base substitution (point mutations) found in of the 961 bp the *trnL*-F region observed in Sumatra samples were five, located at position 52, 55, 135, 421, and 742, while mutation at position 161 and 371 were found only in reference taxa (Table 3). There was also one indels event of one to two A repeat, but this was excluded from the discussion due to its ambiguity. The shared nucleotides among the samples used in this study was started from position 53 to 469 containing *trnL* intron region. Thus the first and the last point mutations (at position 52 and 742) were inclusively refer to samples from Sumatra. The first base mutation was observed in position 52, a transition

of A --> G, found mainly in samples from the North Sumatra, few from West Sumatra and one from Riau. The second was, a transversion from C --> T found in samples from Sanglap Riau in position 55. The third point mutation was another transition from T--> C observed in three populations from Riau (AS, R and ES) at position 135. The fourth was an transversion of G --> T in position 161 that seemed to be an autapomorph nucleotide for *Simaba monophylla*. Another transition of C --> T was found in position 371 belonging to non-genus *Eurycoma*. Another transversion from G --> T found in position 421 recorded from five samples (06EL to 10EL, Table 3) from the West Sumatra. The last one transversion from C--> G was observed in the intergenic spacer between *trnL* and *trnF* gene (position 742), and this mutation were observed in the same five samples from the West Sumatra.

**Table 2.** Nucleotide composition and variation found in the *trnL*-F sequence of *Eurycoma longifolia* from and the reference accessions.

Species	Nucleotide percentage (%)*				Position of point mutations**							
	T	C	A	G	52	55	135	161	371	421	742	
<i>Eurycoma longifolia</i> 02EL	27.5	16.6	39.0	16.9	A	C	T	G	C	G	C	
<i>Eurycoma longifolia</i> 03EL	27.5	16.6	39.0	16.9	.	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 04EL	27.5	16.6	39.0	16.9	.	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 06EL	27.5	16.6	39.0	16.9	G	.	.	.	.	T	G	
<i>Eurycoma longifolia</i> 07EL	27.5	16.6	39.0	16.9	G	.	.	.	.	T	G	
<i>Eurycoma longifolia</i> 08EL	27.2	16.6	39.0	17.1	G	.	.	.	.	T	G	
<i>Eurycoma longifolia</i> 09EL	27.2	16.6	39.0	17.1	G	.	.	.	.	T	G	
<i>Eurycoma longifolia</i> 10EL	27.2	16.6	39.0	17.1	G	.	.	.	.	T	G	
<i>Eurycoma longifolia</i> 1PEL	27.2	16.6	39.0	17.1	G	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 2PEL	27.2	16.6	39.0	17.1	G	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 4PEL	27.2	16.6	39.0	17.1	G	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 5PEL	27.2	16.6	39.0	17.1	G	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 6PEL	27.2	16.6	39.0	17.1	G	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 7PEL	27.2	16.6	39.0	17.1	G	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 8PEL	27.2	16.6	39.0	17.1	G	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 9PEL	27.2	16.6	39.0	17.1	.	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> 10PEL	27.2	16.6	39.0	17.1	.	.	.	.	.	.	.	
<i>Eurycoma longifolia</i> R1EL	26.9	16.8	39.2	17.1	.	.	C	.	.	.	.	
<i>Eurycoma longifolia</i> R2EL	27.2	16.6	39.0	17.1	G	.	C	.	.	.	.	
<i>Eurycoma longifolia</i> ES1EL	27.2	16.6	39.0	17.1	.	T	C	.	.	.	.	
<i>Eurycoma longifolia</i> ES2EL	27.2	16.6	39.0	17.1	.	T	C	.	.	.	.	
<i>Eurycoma longifolia</i> ES3EL	27.2	16.6	39.0	17.1	.	T	C	.	.	.	.	
<i>Eurycoma longifolia</i> A81EL	26.9	16.8	39.2	17.1	.	.	C	.	.	.	.	
<i>Eurycoma longifolia</i> A83EL	26.9	16.8	39.2	17.1	.	.	C	.	.	.	.	
<b>Average</b>	27.2	16.7	39.1	17.1								
<i>Eurycoma longifolia</i> KP995519	27.2	16.6	39.2	17.1	-	.	.	.	.	.	-	
<i>Eurycoma longifolia</i> MH751519	27.2	16.6	39.0	17.1	-	.	.	.	.	.	-	

<i>Eurycoma apiculata</i> GU593014	27.2	16.6	39.2	17.1	-	.	.	.	.	.	-
<i>Perriera madagascariensis</i>											
GU593020	27.4	16.3	39.2	17.1	-	.	.	.	T	.	-
<i>Simaba monophylla</i> MG599402	27.6	16.3	39.2	16.8	-	.	.	T	T	.	-
<i>Simaba glabra</i> MG599404	27.3	16.3	39.3	17.0	-	.	.	.	T	.	-
<i>Simaba morettii</i> MG599405	27.5	16.4	39.0	17.1	-	.	.	.	T	.	-
<i>Odyendyaea gabonensis</i>											
MG599427	27.5	16.6	39.0	16.9	-	.	.	.	T	.	-
<b>Average (total samples)</b>	<b>27.3</b>	<b>16.6</b>	<b>39.1</b>	<b>17.0</b>							

Note: \*: inferred from 417 bp of *trnL* intron region; \*\*: inferred from 960 bp complete *trnL*-F region, the aligned *trnL* intron share with all samples started at position 53.

### 3.3. Phylogenetic tree reconstruction

The results of the phylogeny tree reconstruction using Maximum Likelihood showed unresolved topology with relatively low bootstrap support (BS) (Figure 2). Five species of non-*Eurycoma* (A) are separated into different lineage with 66% BS. The remaining samples of *E. longifolia* samples from Sumatra as well as reference *E. longifolia* have unresolved position on the topology except for samples from Riau (B) and West Sumatra (C) supported by 64% and 61% bootstrap value respectively.

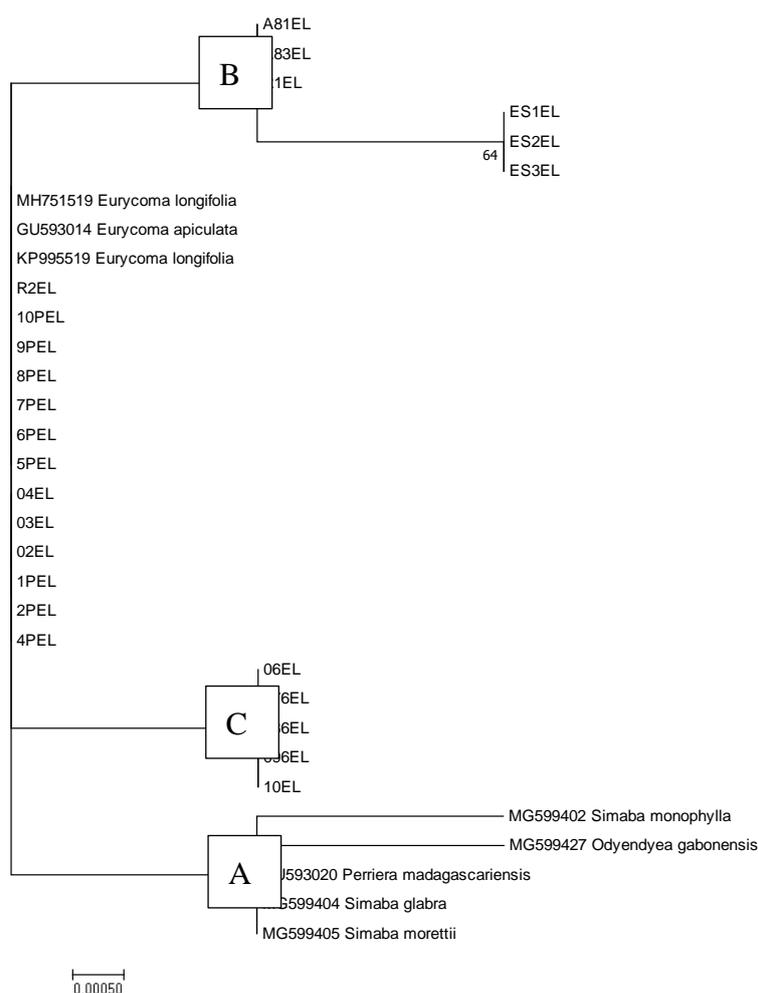


Figure 2. A molecular phylogenetic tree by Maximum Likelihood method using 32 *trnL* intron sequences. Branch supports was inferred using 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

#### 4. Discussion

The partial sequence of the *trnL*-F region is an A/T rich region, particularly from the *trnL* intron, resulted in this study, has confirmed the identity of the samples as *E. longifolia*. The complete cp genome that has been produced recently has helped this confirmation as the reference sequence originated from Kuala Lumpur Malaysia has the identical *trnL* intron as query sample derived from the North Sumatra. The relatively closer geographic distance between Kuala Lumpur and North Sumatra may have been in coincidence with the identical *trnL* intron sequence. In addition, the nucleotide from the North Sumatra did not possessed unique substitution, hence the sequence composition have more in common than population from the West Sumatra and Riau. This idea was confirmed by the unresolved phylogenetic position of the North Sumatran samples and reference sample from Kuala Lumpur. Examination of nucleotide variation has revealed five single base substitutions possessed by the samples from Sumatra and two may be unique to Riau. Even though this deduction was derived only one region (*trnL*-F), the pattern seemed to be consistent in 24 samples, particularly referring to the point mutations recorded only from Riau.

#### 5. Conclusions

In a total of 961 bp *trnL*-F sequence used in this study, we discovered five point mutations specifically possessed by samples of *E. longifolia* from the West Sumatra dan Riau. Of the five nucleotides, four were found in the *trnL* intron and one in the intergenic spacer between *trnL* and *trnF* gene. Two point mutations were observed from some samples from West Sumatra and all samples from Riau respectively. Thus, the Riau clades are the only groups of *E. longifolia* that were resolved in the phylogenetic analysis. It is suggested that the *trnL* intron can be used as one of potential markers for establishing DNA barcode for *E. longifolia* from Indonesia. It is recommended to use more DNA barcode markers with a similar mutation rates as *trnL* intron to complement this present results.

**Supplementary Materials:** The following are available online at <http://www.xxxxx>, Figure S1: title, Table S1: title, Video S1: title.

**Author Contributions:** All authors contributed equally in the preparation of research and the manuscript.

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