

# PHYTOCHEMICAL PROFILING, ANTIOXIDANT POTENTIAL AND MOLECULAR AUTHENTICATION OF THREE ZINGIBERACEAE SPECIES FROM MANIPUR

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## ABSTRACT

The present work was carried out during year 2024 - 2025 at the Institutional Biotech Hub, Pravabati College, Mayang Imphal, with aimed to investigate the phytochemical composition, antioxidant potential, and molecular authentication of three Zingiberaceae species *Alpinia galanga*, *Hedychium coronarium*, and *Zingiber kangleipakense*. Rhizome extracts prepared using methanol, ethanol, and ethyl acetate were quantitatively analyzed for total terpenoids, phenolics, and flavonoids. Among the solvents, methanol exhibited the highest extraction efficiency across all species, with *Z. kangleipakense* showing maximum terpenoid content, while *H. coronarium* recorded the highest phenolic and flavonoid concentrations. Two-way ANOVA revealed significant effects of species, solvent, and their interaction on antioxidant activity evaluated using DPPH and ABTS assays. Methanolic and ethanolic extracts demonstrated stronger radical scavenging activity, whereas ethyl acetate extracts showed comparatively weaker performance. A strong positive correlation between DPPH and ABTS IC<sub>50</sub> values confirmed consistency between the antioxidant assays. Molecular authentication was performed using DNA barcoding with the chloroplast *rbcL* gene and nuclear ITS region. High-quality genomic DNA was successfully amplified and sequenced, and BLAST analysis validated accurate species identification. The ITS region exhibited superior species-level discriminatory power, while *rbcL* served as a reliable universal barcode. Phylogenetic analyses based on both markers supported clustering of the studied species within the Zingiberaceae family. Overall, the integration of phytochemical profiling, antioxidant evaluation and DNA barcoding provides a robust scientific framework for taxonomic validation and supports future phytochemical and bioactivity-based investigations of Zingiberaceae species.

(Key words: *Alpinia galanga*, *Hedychium coronarium*, *Zingiber kangleipakense*, DNA barcoding, phylogenetic analysis)

## INTRODUCTION

The family Zingiberaceae comprises numerous medicinally significant plants widely utilized in traditional healthcare systems for the management of inflammation, infections, metabolic disorders, and oxidative stress-related diseases (Ali *et al.*, 2008; Ravindran and Babu, 2005; Sharifi-Rad *et al.*, 2017). Rhizomes of genera such as *Alpinia*, *Hedychium*, and *Zingiber* are particularly valued due to their rich content of bioactive secondary metabolites, including phenolics, flavonoids, terpenoids, and essential oils, which contribute to their therapeutic efficacy (Prakash and Gupta, 2005; Sasidharan *et al.*, 2011; Sharifi-Rad *et al.*, 2017; Khan *et al.*, 2025).

Oxidative stress arises from an imbalance between reactive oxygen species (ROS) production and antioxidant defense mechanisms, leading to cellular damage and the progression of chronic diseases. In recent years, plant-

derived antioxidants have gained increasing attention as safer and more effective alternatives to synthetic antioxidants. The extraction efficiency of these compounds is strongly influenced by solvent polarity, plant species, and phytochemical composition (Sharifi-Rad *et al.*, 2017; Halliwell and Gutteridge, 2015; Khan and Sharma, 2024).

Accurate identification of medicinal plant species is essential for ensuring reproducibility and reliability in phytochemical and pharmacological studies. DNA barcoding, particularly using chloroplast (*rbcL*) and nuclear (ITS) markers, has proven to be a robust molecular tool for species authentication, especially within taxonomically complex families such as Zingiberaceae (Anonymous, 2009; Chen *et al.*, 2010).

Despite the ethnomedicinal importance of *Alpinia galanga*, *Hedychium coronarium*, and the endemic species *Zingiber kangleipakense*, comprehensive studies integrating phytochemical characterization, antioxidant

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assessment, and molecular validation from Manipur remain limited (Ravindran and Babu, 2005; Sharifi-Rad *et al.*, 2017). The present study was therefore designed to systematically evaluate phytochemical profiles, antioxidant potential, and molecular identity of these species using an integrative multidisciplinary approach.

## MATERIALS AND METHODS

### Plant collection and extraction

Fresh rhizomes of the selected Zingiberaceae species were collected from Thoubal district, Manipur. After thorough washing, samples were shade-dried, powdered, and subjected to solvent extraction using methanol, ethanol, and ethyl acetate. Extracts were concentrated and stored at 4 °C until further analysis.

### Phytochemical screening

Qualitative phytochemical analysis was performed using standard chemical assays to detect major classes of secondary metabolites, including alkaloids, carbohydrates, proteins, amino acids, phenolics, flavonoids, tannins, terpenoids, saponins, cardiac glycosides, and lipids (Harborne, 1998; Evans, 2009).

### Quantitative phytochemical analysis

Total phenolic, flavonoid, and terpenoid contents were quantified using spectrophotometric methods and expressed as gallic acid equivalents (GAE), quercetin equivalents (QE), and linalool equivalents, respectively (Folin and Ciocalteu, 1927; Swain and Hillis, 1959; Lamaison and Carnet, 1990; Zhishen *et al.*, 1999; Gao *et al.*, 2008).

### Antioxidant assays

Antioxidant potential of the rhizome extracts was assessed in terms of IC<sub>50</sub> values using DPPH and ABTS radical scavenging assays, with ascorbic acid and Trolox used as reference standards, following standard protocols (Blois, 1958; Benzie and Strain, 1996).

### DNA barcoding and phylogenetic analysis

Genomic DNA was isolated from fresh leaf tissues. PCR amplification of *rbcL* and ITS regions was conducted, followed by sequencing, BLAST analysis, and phylogenetic tree construction to confirm species identity (Kimura, 1980; Kumar *et al.*, 2015; Felsenstein, 1985).

### Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation (SD) (n = 3) (Steel and Torrie, 1980). Data on terpenoid, phenolic, and flavonoid contents, as well as IC<sub>50</sub> values obtained from DPPH and ABTS assays, were analyzed using two-way analysis of variance (ANOVA) to evaluate the effects of plant species (*Alpinia galanga*, *Hedychium coronarium*, and *Zingiber kangleipakense*), extraction solvent (methanol, ethanol, and ethyl acetate), and their interaction (Montgomery, 2017). When significant differences were observed, Tukey's HSD post-hoc test was applied for

multiple comparisons (Tukey, 1949). Correlations between DPPH and ABTS IC<sub>50</sub> values were assessed using Pearson's correlation coefficient (r) (Pearson, 1895). Differences were considered statistically significant at p < 0.05, and different superscript letters indicate significant differences among means.

## RESULTS AND DISCUSSION

Table 1 presents the quantitative estimation of total terpenoids, total phenolics, and total flavonoids in ethanolic, methanolic, and ethyl acetate rhizome extracts of *Alpinia galanga*, *Hedychium coronarium*, and *Zingiber kangleipakense*. Among the solvents tested, methanolic extracts consistently showed the highest concentrations of all three phytochemical groups across all species. *Zingiber kangleipakense* exhibited the highest terpenoid content (33.26 ± 0.31 mg g<sup>-1</sup>) in methanol, while *Hedychium coronarium* recorded the highest phenolic (2.48 ± 0.44 mg GAE g<sup>-1</sup>) and flavonoid contents (25.20 ± 1.30 mg QE g<sup>-1</sup>) in methanolic extracts. Ethyl acetate extracts showed significantly lower phytochemical contents in comparison to methanol and ethanol. Superscript letters indicate statistically significant differences (p < 0.05) among treatments.

The higher extraction efficiency of methanol can be attributed to its polarity, which enhances the solubilization of phenolics, flavonoids, and terpenoids (Harborne, 1998; Sasidharan *et al.*, 2011). The observed interspecific variation suggests inherent biochemical differences among the rhizomes of the studied Zingiberaceae members. The relatively lower yields obtained using ethyl acetate indicate its limited ability to extract polar phytoconstituents. These findings support previous reports that methanol is one of the most effective solvents for extracting antioxidant-related phytochemicals from medicinal plant rhizomes (Harborne, 1998; Sasidharan *et al.*, 2011).

Two-way ANOVA revealed that species (F = 16.87, p = 0.0004), solvent (F = 27.39, p < 0.0001), and their interaction (F = 6.23, p = 0.0021) significantly influenced the DPPH radical scavenging activity (IC<sub>50</sub> values) of the rhizome extracts. The significant interaction effect indicates that the response of antioxidant activity to solvent extraction varied among species.

The significant species × solvent interaction suggests that antioxidant efficiency is governed by both plant-specific phytochemical composition and solvent polarity (Harborne, 1998; Sasidharan *et al.*, 2011). This interaction highlights the importance of selecting an appropriate extraction solvent for each species to maximize antioxidant potential (Ali *et al.*, 2008). The strong solvent effect further supports the role of extraction chemistry in determining bioactivity outcomes (Sharifi-Rad *et al.*, 2017).

In the ABTS assay, species (F = 31.92, p < 0.0001), solvent (F = 53.01, p < 0.0001), and their interaction (F = 12.30, p = 0.0002) all showed highly significant effects on

IC<sub>50</sub> values. Solvent effect was more pronounced in ABTS compared to DPPH, as indicated by higher F-values.

The stronger solvent influence observed in the ABTS assay may be due to the assay's ability to detect both hydrophilic and lipophilic antioxidants (Benzie and Strain, 1996). The significant interaction again confirms that antioxidant behavior cannot be generalized across species without considering solvent specificity (Harborne, 1998; Sasidharan *et al.*, 2011). These results validate the complementary nature of DPPH and ABTS assays in antioxidant evaluation (Blois, 1958; Benzie and Strain, 1996).

Tukey's HSD test demonstrated significant differences among solvent extracts within each species. Methanolic and ethanolic extracts of *Alpinia galanga* and *Zingiber kangleipakense* showed lower IC<sub>50</sub> values and were grouped under the same significance category, whereas ethyl acetate extracts consistently exhibited higher IC<sub>50</sub> values and formed separate groups.

Lower IC<sub>50</sub> values indicate stronger radical scavenging activity, which correlates well with the higher phenolic and flavonoid contents observed in methanolic and ethanolic extracts (Blois, 1958; Swain and Hillis, 1959). The poor performance of ethyl acetate extracts reinforces the role of polar phytochemicals in DPPH scavenging mechanisms (Harborne, 1998; Sasidharan *et al.*, 2011).

In the ABTS assay, methanolic and ethanolic extracts across species generally fell into the same Tukey grouping, indicating comparable antioxidant efficiency. Ethyl acetate extracts of *Hedychium coronarium* and *Zingiber kangleipakense* showed significantly higher IC<sub>50</sub> ranges and formed distinct statistical groups.

The wider IC<sub>50</sub> range observed for ethyl acetate extracts suggests lower availability of ABTS-reactive antioxidant compounds (Benzie and Strain, 1996; Harborne, 1998). The consistency between methanol and ethanol extracts across species implies that these solvents extract a similar spectrum of antioxidant molecules effective against ABTS radicals (Sasidharan *et al.*, 2011; Sharifi-Rad *et al.*, 2017).

A strong positive correlation ( $r = +0.7$  to  $+0.9$ ,  $p < 0.05$ ) was observed between IC<sub>50</sub> values obtained from DPPH and ABTS assays, indicating concordance between the two antioxidant evaluation methods.

The significant positive correlation confirms that extracts exhibiting strong activity in one assay tend to perform similarly in the other (Blois, 1958; Benzie and Strain, 1996). This relationship suggests that the antioxidant compounds present in the rhizome extracts act through multiple radical scavenging mechanisms, thereby validating the reliability of using both assays in antioxidant profiling studies (Halliwell and Gutteridge, 2015).

DNA barcoding of three Zingiberaceae species *Alpinia galanga*, *Hedychium coronarium*, and *Zingiber*

*kangleipakens* was successfully performed using the chloroplast *rbcL* gene and the nuclear ITS region (Anonymous, 2009; Chen *et al.*, 2010). High-quality genomic DNA was isolated from fresh leaf samples, and PCR amplification using marker-specific primers yielded clear and distinct bands of expected sizes on agarose gel electrophoresis, confirming DNA integrity and optimized amplification conditions (Hollingsworth *et al.*, 2011). The amplified products were subsequently sequenced, and the resulting sequences were analyzed using BLAST against the NCBI GenBank database (Chen *et al.*, 2010).

BLAST-based nucleotide homology and phylogenetic analysis of the ITS region revealed that samples labeled AGPI193, HCPI194, and ZKPI195 showed high sequence similarity with *Alpinia conchigera*, *Hedychium coronarium*, and *Hedychium flavum*, respectively (Fig. 1). The ITS sequence of *Alpinia galanga* (PI193) exhibited 97.93% similarity with *A. conchigera*, while *H. coronarium* (PI194) showed 99.56% similarity with *H. coronarium*. Interestingly, *Z. kangleipakense* (PI195) showed 100% similarity with *H. flavum*, suggesting close phylogenetic affinity or possible limitations of ITS resolution among closely related taxa within the family Zingiberaceae (Anonymous, 2009; Chen *et al.*, 2010). The ITS-based phylogenetic tree further supported clustering of the studied samples within the family Zingiberaceae (Hollingsworth *et al.*, 2011).

Analysis using the *rbcL* barcode region demonstrated comparatively lower species-level resolution (Anonymous, 2009; Hollingsworth *et al.*, 2011). *Alpinia galanga* (PI193) showed 100% sequence similarity with its reference sequence, confirming reliable species identification. However, *H. coronarium* (PI194) exhibited 99.45% similarity with *Hedychium species*, and *Z. kangleipakense* (PI195) showed 99.45% similarity with *Hedychium viridibracteatum*, indicating conserved sequence characteristics and close genetic relationships within the family (Chen *et al.*, 2010). Phylogenetic analysis based on *rbcL* sequences showed consistent clustering of all samples within the Zingiberaceae clade (Fig. 2), supporting its effectiveness as a universal plant barcode while highlighting its limited discriminatory power at the species level.

Overall, the combined use of gel documentation, sequence validation, BLAST analysis, and phylogenetic reconstruction confirmed the effectiveness of DNA barcoding for molecular authentication of Zingiberaceae species. While *rbcL* proved reliable for higher-level taxonomic placement, the ITS region provided superior resolution for species-level discrimination (Anonymous, 2009; Hollingsworth *et al.*, 2011). This molecular confirmation establishes a robust genetic framework to support subsequent phytochemical profiling and bioactivity-related investigations (Felsenstein, 1985).

**Table 1. Quantitative phytochemical analysis of rhizome extracts of *Alpinia galanga*, *Hedychium coronarium*, and *Zingiber kangleipakense* using different solvents (mean  $\pm$  SD; two-way ANOVA)**

Plant	Solvent	Terpenoids (mg g <sup>-1</sup> )	Phenolics (mg GAE g <sup>-1</sup> )	Flavonoids (mg QE g <sup>-1</sup> )
<i>Alpinia galanga</i>	Methanol	29.15 $\pm$ 0.32 <sup>b</sup>	2.38 $\pm$ 0.13 <sup>a</sup>	23.20 $\pm$ 0.80 <sup>b</sup>
	Ethanol	25.48 $\pm$ 0.20 <sup>c</sup>	2.12 $\pm$ 0.16 <sup>b</sup>	21.20 $\pm$ 2.50 <sup>c</sup>
	Ethyl acetate	18.29 $\pm$ 0.21 <sup>d</sup>	1.52 $\pm$ 0.24 <sup>c</sup>	14.90 $\pm$ 1.20 <sup>d</sup>
<i>Hedychium coronarium</i>	Methanol	31.76 $\pm$ 0.33 <sup>a</sup>	2.48 $\pm$ 0.44 <sup>a</sup>	25.20 $\pm$ 1.30 <sup>a</sup>
	Ethanol	27.37 $\pm$ 0.19 <sup>b</sup>	1.78 $\pm$ 0.54 <sup>b</sup>	21.20 $\pm$ 2.20 <sup>c</sup>
	Ethyl acetate	19.98 $\pm$ 0.29 <sup>c</sup>	1.30 $\pm$ 0.12 <sup>c</sup>	17.60 $\pm$ 0.70 <sup>d</sup>
<i>Zingiber kangleipakense</i>	Methanol	33.26 $\pm$ 0.31 <sup>a</sup>	2.22 $\pm$ 0.42 <sup>a</sup>	23.40 $\pm$ 1.20 <sup>b</sup>
	Ethanol	29.01 $\pm$ 0.16 <sup>b</sup>	1.34 $\pm$ 0.14 <sup>b</sup>	18.80 $\pm$ 0.70 <sup>c</sup>
	Ethyl acetate	21.04 $\pm$ 0.27 <sup>c</sup>	1.14 $\pm$ 0.08 <sup>c</sup>	15.60 $\pm$ 2.00 <sup>d</sup>

Values are expressed as mean  $\pm$  SD (n = 3). Values within the same column followed by different superscript letters (ad) are significantly different at p < 0.05 (one-way ANOVA followed by Tukey's post-hoc test).

**Table 2. Two-way ANOVA (species  $\times$  solvent) for antioxidant activity (IC<sub>50</sub>) of rhizome extracts assessed using the DPPH assay**

Source of variation	df	Sum of Squares (SS)	Mean Square (MS)	F-value	p-value
Species	2	182.46	91.23	16.87	0.0004
Solvent	2	296.18	148.09	27.39	<0.0001
Species $\times$ Solvent	4	134.72	33.68	6.23	0.0021
Error	18	97.32	5.41	-	-
Total	26	710.68	-	-	-

Values are expressed as mean  $\pm$  SD (n = 3). “-” indicates values not applicable for statistical testing. Statistical significance was considered at p < 0.05

**Table 3. Two-way ANOVA (species  $\times$  solvent) for antioxidant activity (IC<sub>50</sub>) of rhizome extracts assessed using the ABTS assay**

Source of variation	df	Sum of Squares (SS)	Mean Square (MS)	F-value	p-value
Species	2	412.85	206.43	31.92	<0.0001
Solvent	2	685.74	342.87	53.01	<0.0001
Species $\times$ Solvent	4	318.26	79.57	12.30	0.0002
Error	18	116.42	6.47	-	-
Total	26	1533.27	-	-	-

Values are expressed as mean  $\pm$  SD (n = 3). “-” indicates values not applicable for statistical testing. Statistical significance was considered at p < 0.05

**Table 4. Tukey's HSD post-hoc test (mean  $\pm$  SD) for IC<sub>50</sub> values of rhizome extracts assessed using the DPPH assay**

Species	Solvent	IC <sub>50</sub> ( $\mu\text{g ml}^{-1}$ ) Mean $\pm$ SD	Tukey grouping
<i>Alpinia galanga</i>	Methanol	19.1 $\pm$ SD	a
	Ethanol	17.8 $\pm$ SD	a
	Ethyl acetate	25.8 $\pm$ SD	b
<i>Hedychium coronarium</i>	Methanol	22.7 $\pm$ SD	a
	Ethanol	24.1 $\pm$ SD	ab
	Ethyl acetate	31.4 $\pm$ SD	b
<i>Zingiber kangleipakense</i>	Methanol	20.5 $\pm$ SD	ab
	Ethanol	18.9 $\pm$ SD	a
	Ethyl acetate	26.5 $\pm$ SD	b

Different superscript letters indicate significant differences at  $p < 0.05$ .

**Table 5. Tukey's HSD post-hoc test (mean  $\pm$  SD) for IC<sub>50</sub> values of rhizome extracts assessed using the ABTS assay**

Species	Solvent	IC <sub>50</sub> ( $\mu\text{g ml}^{-1}$ ) Mean $\pm$ SD	Tukey grouping
<i>Alpinia galanga</i>	Methanol	12-15	a
	Ethanol	11-13	a
	Ethyl acetate	<10	b
<i>Hedychium coronarium</i>	Methanol	15-18	a
	Ethanol	12-14	a
	Ethyl acetate	25-30	c
<i>Zingiber kangleipakense</i>	Methanol	14-16	a
	Ethanol	12-14	a
	Ethyl acetate	45-50	b

Different superscript letters indicate significant differences at  $p < 0.05$ .

**Table 6. Pearson correlation between DPPH and ABTS assays**

Parameter	Pearson's	p-value	Interpretation
DPPH IC <sub>50</sub> vs ABTS IC <sub>50</sub>	+0.7 to +0.9	<0.05	Strong positive correlation

Pearson's r indicates the strength and direction of correlation;  $p < 0.05$  denotes statistical significance

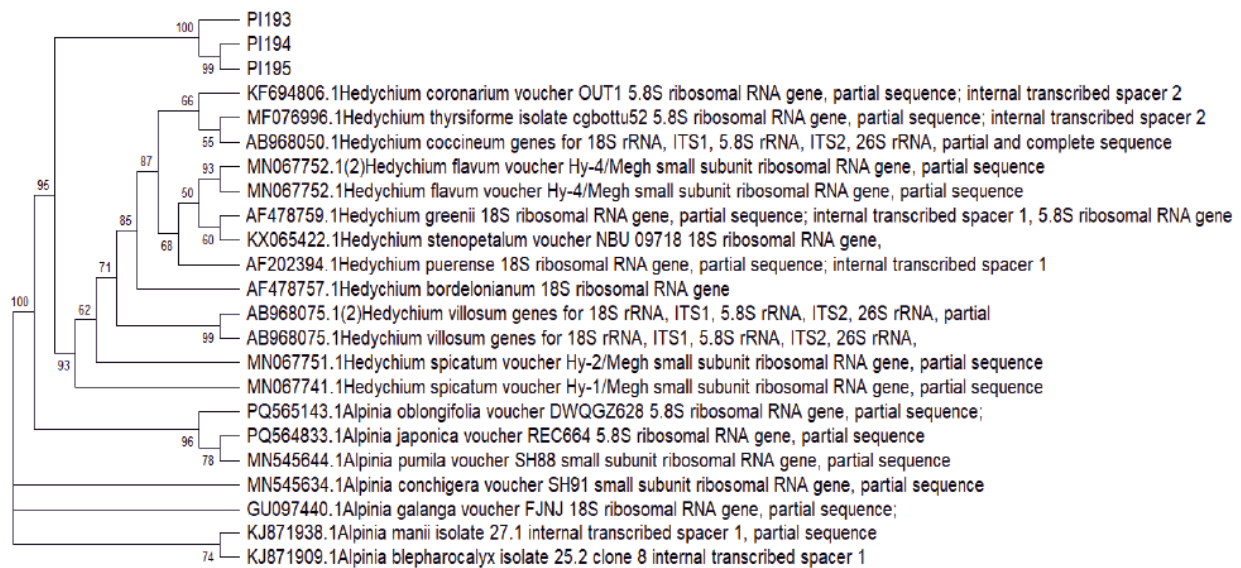


Figure 1. ITS-based phylogenetic relationships among studied Zingiberaceae species.

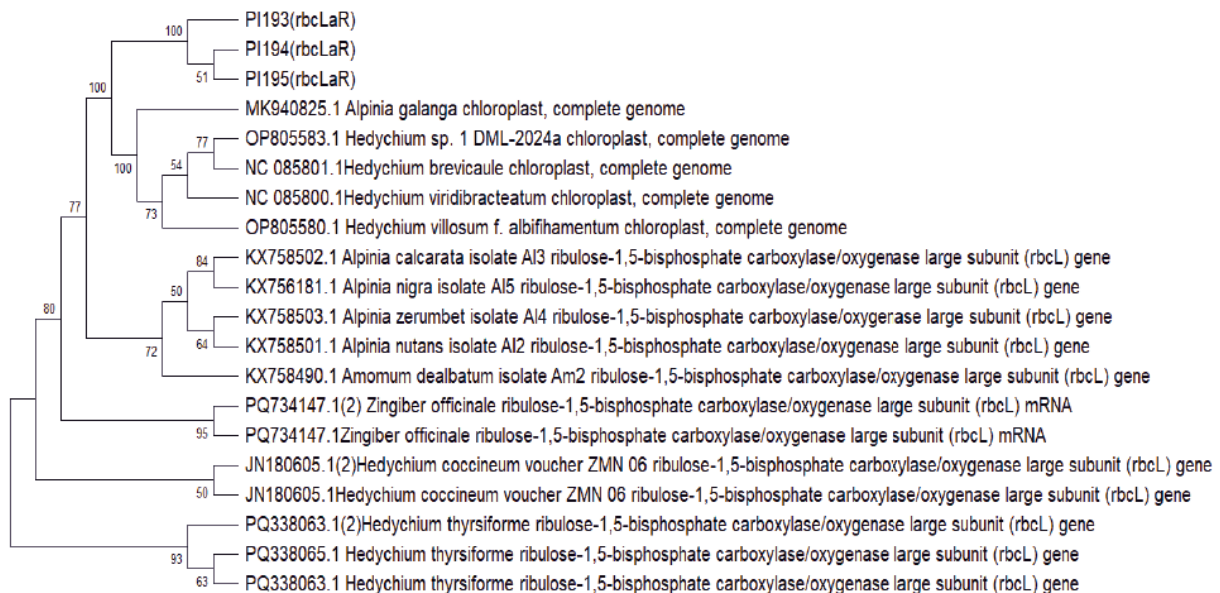


Figure 2. rbcL-based phylogenetic relationships among studied Zingiberaceae species.

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**Rec. on 03.01.2026 & Acc. on 28.01.2026**