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## ***Lithocarpus encleisocarpus* (Korth.) A. Camus a newly recorded species for Viet Nam and its phylogenetic relationship based on genome-wide SNPs**

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### ABSTRACT

This study reports a newly recorded *Lithocarpus* (Fagaceae) species for the flora of Viet Nam, namely *Lithocarpus encleisocarpus* (Korth.) A. Camus. This species is morphologically similar to *L. dahuoaiensis* Ngoc & L.V. Dung in having a completely entire leaf margin, solitary cupule, long stalks of fruits, deeply cup-shaped or turbinate cupules, with a number of horizontal filiform lines, but it differs from the latter by having cupules almost completely covering the nut, surface of the cupule densely fulvous tomentose by stellate hairs, and secondary veins typically 8-10 pairs. This study provides the phylogenetic relationship of *L. encleisocarpus* with its closest species based on genome-wide SNPs. Taxonomic treatment, photographs, information on distribution and habitat, and the GenBank accession number for DNA barcodes of the species are also provided.

## 1. INTRODUCTION

*Lithocarpus* Blume, comprising more than 300 species (The Plant list 2013), is the second largest genus of the family Fagaceae in the world. While *Lithocarpus* is widespread from Japan and China to New Guinea, the center of its diversity can be found in continental Asia, mainly from China (123 spp.; Huang et al., 1999) to continental Southeast Asia (169 spp. for Indo-China; Camus, 1948) including Viet Nam (125 spp.; Ho, 2003; Ban, 2005; Ngoc et al., 2016, 2018, 2021), Thailand (60 spp.; Barnett, 1940; Phengkai, 2008; Strijk et al., 2014a, 2014b, 2019). One North American species (*Lithocarpus densiflorus* Hook. & Arn.), has recently been treated as a member of a new monotypic genus *Notholithocarpus* (Manos et al. 2008)

In Viet Nam, *Lithocarpus* is the most species-rich genus of the family Fagaceae. Approximately 125 species have been recorded, among which 48 are endemic to the country (Ho, 2003; Ban, 2005; Ngoc

et al., 2016, 2018, 2021). However, we often encounter species of *Lithocarpus* that are difficult to identify at the species level, indicating we need increased efforts to clarify its diversity and taxonomy accurately.

During our floristic research in Lam Dong and Khanh Hoa Provinces and surrounding areas (Fig. 1) from 2014 to the present, we discovered several specimens of the genus *Lithocarpus* that do not resemble previously recorded species from Viet Nam. Based on consulting the related taxonomic literature dealing with the flora of Viet Nam (Ho, 2003; Ban, 2005) and neighboring countries (Huang et al., 1999; Phengkai, 2008) we identified these specimens as *Lithocarpus encleisocarpus* (Korth.) A. Camus, which had previously never been reported in Viet Nam.

In this paper, we report *L. encleisocarpus* as a newly recorded species for Viet Nam and provide its

phylogenetic relationship with close species based on genome-wide SNP.

## 2. MATERIALS AND METHOD

### 2.1. Plant materials

A total of 40 samples of 22 species of putative taxa of *Lithocarpus* from, Khanh Hoa, Lam Dong, and other areas in Viet Nam were collected for molecular analysis. The materials including two samples of *L. coalitus* Hickel & A. Camus, the species that have unique morphological features in the genus, were used as an outgroup in the phylogenetic analysis. This study material includes a part of Ngoc et al. (2021) material. The detailed information on samples used for molecular analysis is shown in Table 1.

### 2.2. Morphological observation

The morphological traits of the *L. encleisocarpus* with its related species were examined with comparison to taxonomic literature (Camus, 1931, 1938, 1942, 1943, 1945, 1948; Huang et al., 1999; Ho, 2003; Ban, 2005; Phengkai, 2008), specimens are kept in the herbaria ANDA, BKF, DLU, HN, KAG, KYO, P, and VNM, and digitized plant specimen images are available on the web of JSTOR Global Plants (<https://plants.jstor.org/>) and the Chinese Virtual Herbarium (<http://www.cvh.org.cn/>).

### 2.3. Phylogenetic analysis

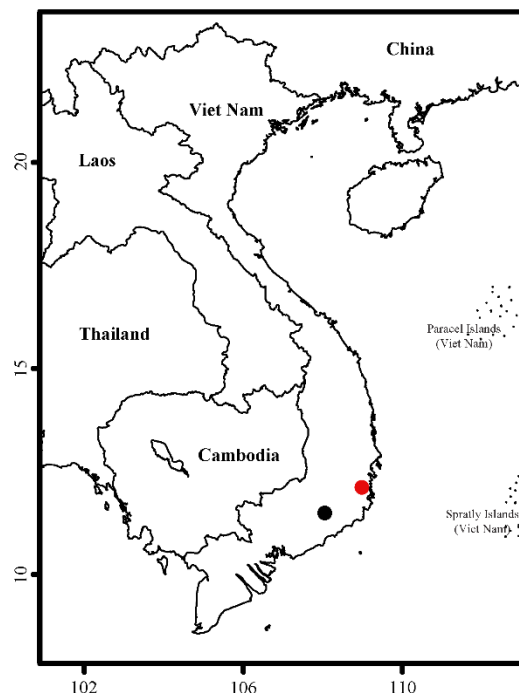
**DNA Extraction:** The CTAB method (Doyle and Doyle, 1987) with minor modifications described in Toyama et al. (2015) was used for DNA extraction of 40 *Lithocarpus* samples.

**DNA Barcoding:** Three DNA regions of *L. encleisocarpus* (ITS, matK, and rbcL) were sequenced according to published protocols (Rohwer et al., 2009; Kress et al., 2009; Dunning and Savolainen, 2010). Sequences were deposited to the GenBank of NCBI for DNA barcode accession numbers generation.

The total genomic DNA of *L. encleisocarpus* and its related species was used as templates to perform the next-generation sequencing (NGS). The MIG-seq library was constructed using the given protocols with modifications that had been previously published (Suyama and Matsuki, 2015; Suyama et al., 2021; Ngoc et al., 2021).

For the phylogenetic analysis, the MIG-seq raw data was pretreated by using Trimmomatic 0.39 (Bolger et al., 2014) and then the Stacks 2.41 pipeline

(Catchen et al., 2013; Rochette et al., 2019) was used for de novo SNP discovery with the parameters set as described by Takata et al. (2019) and Ngoc et al. (2021). The GTR+G model was used as recommended by jMrModeltest 2.1.10 (Darriba et al., 2012) to construct a maximum likelihood tree of genome-wide SNPs data set using RAxML ver. 8.2 (Stamatakis, 2014). Bootstrap support was determined by performing 1000 replicates.



**Figure 1. Distribution map of *Lithocarpus encleisocarpus* in Viet Nam. Black dot denotes Di Linh, Lam Dong Province; Red dot denotes Hon Ba Nature Reserve, Khanh Hoa Province**

## 3. RESULTS

### 3.1. Taxonomic treatment

*Lithocarpus encleisocarpus* (Korth.) A. Camus, Rivièra Sci. 18: 40 1931 (Fig. 2).

**Synonyms:** *Quercus encleisocarpa* Korth., Verh. Nat. Gesch. Ned. Beziit., Bot. 209 (1844); *Quercus encleisocarpa* var. *divergens* A. DC., Prodr. 16(2): 103 (1864); *Cyclobalanus encleistocarpa* (Korth.) Oerst., Vidensk. Meddel. Dansk Naturhist. Foren. Kjøbenhavn 1866: 81 (1866); *Quercus encleisocarpa* var. *aperta* H. W. Kung ex Hook. f., Fl. Brit. India 5: 617 (1888); *Pasania encleisocarpa* (Korth.) Gamble, J. Asiatic Soc. Bengal, Pt. 2, Nat. Hist. 75: 449 (1915); *Synaedrys encleisocarpa*

(Korth.) Koidz., Bot. Mag. (Tokyo) 30: 186 (1916); *Castanopsis encleisocarpa* (Korth.) Rehder, J. Arnold Arbor. 1: 122 (1919).

**Description:** Evergreen tree 15–20 m tall; young branchlets, rufous tomentose by adpressed, stellate hairs, soon glabrous, greyish brown in vivo and blackish brown in sicco, sparsely lenticellate; terminal buds subglobose, ca. 8–10 mm long, scales broadly ovate. Leaves alternate, blades broadly elliptic to slightly ovate, ca. 12–15 × 4–6 cm, thin-coriaceous, broadest about the middle; base slightly rounded to acute, margin entire, apex 1–2 cm acuminate or caudate, glabrous adaxially, densely glaucous adpressed stellate-hairy abaxially; midrib slightly impressed above, distinctly raised below; secondary veins 8–10 pairs, more or less prominent beneath, at an angle of 45–60 degree from the midrib, tertiary veins subscalariform, invisible to faintly visible on both surfaces; petioles ca. 5–15 mm long. Flowers not seen. Infructescences erect, woody, 10–15 cm long, rachis densely adpressed hairy. Acorn solitary, ovoid-globose, 15–20 mm in height, 19–23 mm in diam. (including cupule); fruiting stalk 10–15 mm long. Cupules, cup-shaped, wall thinner than 1 mm, crackled, outside fulvous tomentose by stellate hairs, almost completely covering the nut, 12–14 mm in height, 19–23 mm in diam., bractlets small triangular, obscure, more or less concentric. Mature nut 14–18 mm in height, 18–22 mm in diam., densely white tomentose; scar created by cupule at the base is concave; wall

woody, crackled; apex abruptly acuminate, ca. 1.5–2 mm in height.

*Lithocarpus encleisocarpus* (Fig. 2) is morphologically similar to *L. dahuoiensis* Ngoc & L. V. Dung (Fig. 3) in having an utterly entire leaf margin, solitary cupule, long stalks of fruits, deeply cup-shaped or turbinate cupules with the number of horizontal filiform lines. But *L. encleisocarpus* is distinct by its leaf blades pubescent then glabrescent abaxially; much longer pedicel, cupules almost completely covering the nut, outside densely fulvous tomentose by stellate hairs; secondary veins 8–10 pairs.

**Phenology.** Mature fruits were collected in June.

**Distribution and habitat.** In Viet Nam, so far known in Di Linh District, Lam Dong Province and Hon Ba Nature Reserve, Khanh Hoa Province. We found less than ten mature individuals distributed in the evergreen forest (Fig. 1).

**GenBank accession No.** *Ngoc et al. V3263*: LC712429 (rbcL), LC712428 (matK), ON606016 (ITS).

**Additional specimens examined:** VIET NAM: Khanh Hoa Province, Hon Ba Nature Reserve, alt. 220 m, 12°07' 24.19"N, 109°00' 13.01"E, 14 July 2014, *Tagane S., Kanemitsu H., Dang V.S., Tran H., Loi X.N., Thach N.D., Dinh N., Hieu P.N.H. V3194* (DLU, FU). Lam Dong, Di Linh, Gung Re, alt. 1100 m, 11°28'23.5"N, 108°03'58.9"E, 21 June 2015, *Ngoc N.V., Luong D., Hoang B. V3263* (DLU, FU).



**Figure 2.** *Lithocarpus encleisocarpus*: A – Branch with infructescence, B – Infructescence, C - Adaxial surface of mature Leaf, D - Immature fruits. All photos taken by Nguyen Van Ngoc



**Figure 3.** *Lithocarpus dahuoaiensis* Ngoc & L. V. Dung. A Leafy twig B Buds C Petiole D Abaxial surface of mature Leaf E Infructescence F Mature fruit G Cupule (left) and bottom of nut (right) H Vertical sections of nut. All photos from Ngoc et al. (2016).

### 3.2. Phylogenetic relationship

The MIG-seq tree based on genome-wide SNPs dataset (40 accessions and a total of 32036 SNPs) showed the phylogenetic relationship between *L. encleisocarpus* with its related (Fig. 4). The phylogenetic tree included three major clades (except outgroup *L. coalitus*) almost supported by 100% bootstrap values.

*Lithocarpus encleisocarpus* was placed in clade 1 which was supported by 100% bootstrap value including *L. congtrioensis*, *L. braianensis*, *L. dahuoaiensis*, *L. stenopus*, *L. dalatensis*, *L. longipedicellatus*, *L. vinensis*, and *L. obovatifolius*. This group is morphologically distinct from the rest of this study's samples by its completely solitary fruit, long pedicel, and the cupule with minute scales, which cover almost of the nut. *Lithocarpus encleisocarpus* has a close genetic relationship with *L. congtrioensis*, *L. braianensis*, and *L.*

*dahuoaiensis*, these species nested in a highly supported monophyletic group (98% bootstrap value).

*Lithocarpus hongiaoensis*, *L. vuquangensis*, *L. garrettianus*, *L. laoticus*, *L. xylocarpus*, *L. ducampii*, *L. corneus*, *L. pachylepis*, and *L. giganthophyllus* were formed in the clade 2 and supported by 97% bootstrap value. This group comprises species that have clustered cupules (3 or 5) rarely solitary, cupules have robust scales.

Four samples of the remaining species including *L. gougerotae*, *L. touranensis*, and *L. campylolepis* clustered in a highly supported monophyletic group (clade 3, 100% bootstrap value). This putative taxon is morphologically different from the rest of the *Lithocarpus* genus with solitary and spiny-cupule, long spiny scales, cupule covers almost 4/5 of the nut, and the nut is covered by a velvet layer.

**Table 1. List of specimen vouchers that were used for phylogenetic analysis**

Species	Vouchers	Localities
<i>L. braianensis</i>	Ngoc et al. V3240 (DLU, FU)	Bidoup-Nui Ba NP
<i>L. campylolepis</i>	Ngoc et al. V3245 (DLU, FU)	Bidoup-Nui Ba NP
<i>L. coalitus</i>	Ngoc et al. V4191, V10140 (DLU, FU)	Bidoup-Nui Ba NP
<i>L. congtrioensis</i>	Ngoc et al. NAF200, NAF200A (DLU); Tagane et al. V9102 (DLU, FU)	Bidoup-Nui Ba NP
<i>L. corneus</i>	Yahara et al. V2957 (DLU, FU) Nguyen et al. V3305 (DLU, FU) Ngoc et al. V3194 (DLU, FU)	Bach Ma NP Vu Quang NP Bidoup-Nui Ba NP
<i>L. dahuoaiensis</i>	Ngoc et al. V5404A & V5404B (DLU, FU)	Dong Nai NR
<i>L. dalatensis</i>	Tagane et al. V9106 (DLU, FU, KAG)	Bidoup-Nui Ba NP
<i>L. ducampii</i>	Ngoc et al. V6029 (DLU, FU) Tagane et al. V1627 (DLU, FU)	Xuan Mai, Ha Noi Hon Ba NR
<b><i>L. encleisocarpus</i></b>	Ngoc et al. V3263 (DLU, FU)	Bidoup-Nui Ba NP
<i>L. garrettianus</i>	Yahara et al. V1267, V1556, V1556A (DLU, FU)	Hon Ba NR
<i>L. giganthophyllus</i>	Ngoc et al. V3200, V3266 (DLU, FU)	Bidoup-Nui Ba NP
<i>L. gougerotae</i>	Bon & Quang V6055 (DLU)	Ba Vi NP
<i>L. hongiaoensis</i>	Ngoc et al. V3235 (DLU, FU)	Bidoup-Nui Ba NP
<i>L. laoticus</i>	Ngoc et al. V3193 (DLU, FU)	Bidoup-Nui Ba NP
<i>L. longipedicellatus</i>	Nguyen et al. V3813 (DLU, FU)	Vu Quang NP
<i>L. obovatifolius</i>	Yahara et al. V2714, V2983 (DLU, FU)	Bach Ma NP
<i>L. pachylepis</i>	Ngoc et al. V4567, V4843 (DLU, FU) Ngoc et al. V3187 (DLU, FU)	Hoang Lien NP Bidoup-Nui Ba NP
<i>L. stenopus</i>	Nguyen et al. V3875 & V3875A (DLU, FU) Yahara et al. V2739 (DLU, FU)	Vu Quang NP Bach Ma NP
<i>L. touranensis</i>	Yahara et al. V10004 (DLU, FU, KAG)	Bidoup-Nui Ba NP
<i>L. vinhensis</i>	Ngoc et al. V3591, V3787 (DLU, FU)	Vu Quang NP
<i>L. vuquangensis</i>	Yahara et al. V5743, V5938 (DLU, FU)	Vu Quang NP
<i>L. xylocarpus</i>	Tagane et al. V4337; Ngoc et al. V8464 (DLU, FU)	Bidoup-Nui Ba NP

NP = National Park; NR = Nature Reserve.

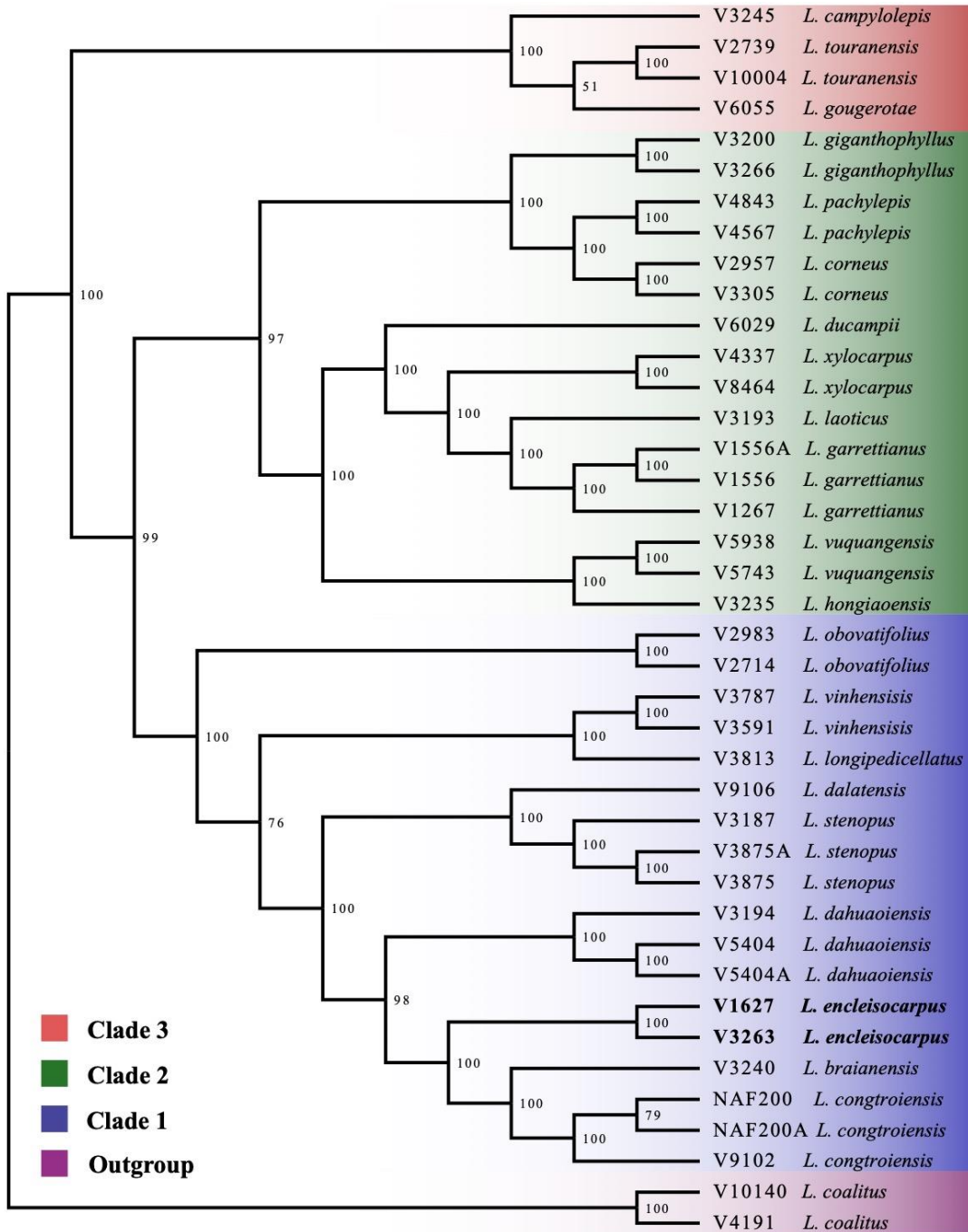


Figure 4. The phylogenetic relationship of *Lithocarpus encleisocarpus* with its related based on SNPs data set from MIG-seq

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