**Lithocarpus encleisocarpus** (Korth.) A. Camus - A newly recorded from Vietnam and its phylogenetic relationship based on genome-wide SNPs

Nguyen Van Ngoc*, and Hoang Thi Binh

Faculty of Biology, Dalat University, 01 - Phu Dong Thien Vuong, Dalat, Vietnam

*Correspondence: Nguyen Van Ngoc (email: ngocnv@dlu.edu.vn)

**Article info.**

Received 22 Aug 2022  
Revised 30 Sep 2022  
Accepted 06 Oct 2022

**ABSTRACT**

A newly recorded Lithocarpus (Fagaceae) species for the flora of Vietnam, namely Lithocarpus encleisocarpus (Korth.) A. Camus was reported in this study. This species is most 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 several 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, secondary veins 8-10 pairs. This study provides the phylogenetic relationship of *L. encleisocarpus* with its close 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.

**Keywords**

Fagaceae, Flora, Lam Dong, MIG-seq, NGS

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 itself, mainly from China (123 spp.; Huang et al., 1999) to continental Southeast Asia (169 spp. for Indo-China; Camus, 1948) including Vietnam (125 spp.; Ho, 2003; Ban, 2005; Ngoc et al., 2016, 2018, 2021), Thailand (60 spp.; Barnett, 1940; Phengklai, 2008; Strijk et al., 2014a, 2014b, 2019) and 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 Vietnam, *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 more 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 found several specimens of the genus *Lithocarpus* that do not resemble any species that have been recorded from Vietnam. Based on consulting the related taxonomic literature dealing with the flora of Vietnam (Ho, 2003; Ban, 2005) and neighboring countries (Huang et al., 1999; Phengklai, 2008) we confirmed those specimens were identified as *Lithocarpus encleisocarpus* (Korth.) A. Camus, which has not yet previously been reported in Vietnam.

In this paper, we report *L. encleisocarpus* as a newly recorded species for Vietnam 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 Vietnam were collected for molecular analysis. The materials including two samples of L. coaliitus Hickel & A. Camus, the species that have unique morphological features in the genus, were used as an outgroup in phylogenetic analysis. This study material includes a part of Ngoc et al. (2021) material. The detailed information on samples used for molecular analysis was shown in Table 1.

2.2. Morphological observation
The morphological traits of the L. encleisocarpus with its related species were examined by using taxonomic literature (Camus, 1931, 1938, 1942, 1943, 1945, 1948; Huang et al., 1999; Ho, 2003; Ban, 2005; Phengklai, 2008), specimens kept in the herbaria ANDA, BKF, DLU, HN, KAG, KYO, P, and VNM, and digitized plant specimen images available on the web of JSTOR Global Plants (https://plants.jstor.org/) and 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 the 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) and those sequences were deposited to the GenBank of NCBI for DNA barcode accession numbers generating.

The total genomic DNA of L. encleisocarpus and its related species was used as templates to perform the next-generation sequencing (NGS). MIG-seq library was constructed using the given protocols with the 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.

3. RESULTS

3.1. Taxonomic treatment

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

Synonyms: Quercus encleisocarpa Korth., Verh. Nat. Gesch. Ned. Bez., 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. Asiat. 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 so to 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. dahuoaensis* 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 pedicle, 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 Vietnam, so far known from 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., 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](Source: Nguyen Van Ngoc)
3.2. Phylogenetic relationship

The MIG-seq tree based on a 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. congtriosiens*, *L. braianensis*, *L. dahuoaiensis*, *L. stenopus*, *L. dalatensis*, *L. longipedicellatus*, *L. vinensis*, and *L. obovatifolius*.

(Source: Ngoc et al., 2016)
This group has morphologically distinct from the rest of this study's samples by its completely solitary fruit, long pedicel, cupule with minute scales, and cover most of the nut. *Lithocarpus encleisocarpus* has a close genetic relationship with *L. congtriensis*, *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. giganathophyllus* 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                  |
|--------------------------|---------------------------------|-----------------------------|
| *L. braianensis*         | Ngoc et al. V3240 (DLU, FU)     | Bidoup-Nui Ba NP            |
| *L. campylepites*        | Ngoc et al. V3245 (DLU, FU)     | Bidoup-Nui Ba NP            |
| *L. coailitus*           | Ngoc et al. V4191, V10140 (DLU, FU) | Bidoup-Nui Ba NP       |
| *L. congtriensis*        | Ngoc et al. NAF200, NAF200A (DLU); Tagane et al. V9102 (DLU, FU) | Bidoup-Nui Ba NP     |
| *L. corneus*             | Yahara et al. V2957 (DLU, FU)   | Bach Ma NP                  |
| *L. dahuiaoensis*        | Ngoc et al. V5404A & V5404B (DLU, FU) | Dong Nai NR                |
| *L. dalatensis*          | Tagane et al. V9106 (DLU, FU, KAG) | Bidoup-Nui Ba NP         |
| *L. ducampii*            | Ngoc et al. V6029 (DLU, FU)     | Xuan Mai, Ha Noi            |
| *L. enclosurecarpus*     | Tagane et al. V1627 (DLU, FU)   | Hon Ba NR                   |
| *L. garrettianus*        | Yahara et al. V1267, V1556, V1556A (DLU, FU) | Hon Ba NR                  |
| *L. giganathophyllus*    | Ngoc et al. V3200, V3266 (DLU, FU) | Bidoup-Nui Ba NP        |
| *L. gougerotae*          | Bon & Quang V6055 (DLU)         | Ba Vi NP                    |
| *L. hongiaoensis*        | Ngoc et al. V3235 (DLU, FU)     | Bidoup-Nui Ba NP            |
| *L. laoticus*            | Ngoc et al. V3193 (DLU, FU)     | Bidoup-Nui Ba NP            |
| *L. longipedicellatus*   | Nguyen et al. V3813 (DLU, FU)   | Vu Quang NP                 |
| *L. obovatifolius*       | Yahara et al. V2714, V2983 (DLU, FU) | Bach Ma NP                |
| *L. pachylepis*          | Ngoc et al. V4567, V4843 (DLU, FU) | Hoang Lien NP            |
| *L. stenopus*            | Ngoc et al. V3875 & V3875A (DLU, FU) | Vu Quang NP            |
| *L. touranensis*         | Yahara et al. V10004 (DLU, FU, KAG) | Bidoup-Nui Ba NP       |
| *L. vinhensis*           | Ngoc et al. V3591, V3787 (DLU, FU) | Vu Quang NP               |
| *L. vuquangensis*        | Yahara et al. V5743, V5938 (DLU, FU) | Vu Quang NP            |
| *L. xylocarpus*          | Tagane et al. V4337; Ngoc et al. V8464 (DLU, FU) | Bidoup-Nui Ba NP      |

NP = National Park; NR = Nature Reserve.
ACKNOWLEDGMENT

The authors wish to thank our colleagues at Kyushu University and Tohoku University (Japan) for their help in experimenting in the laboratory. Our gratitude goes to the curators and staff of the following herbaria, BKF, DLU, FU, HN, K, KYO, P, and VNM for making their materials accessible.

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.03-2018.325.
REFERENCES

Ban, N. T. (2005). Fagaceae. In N. T. Ban (Eds.), Checklist of plant species of Vietnam 2. (pp. 227–271). Agricultural Publishing House, Hanoi.

Barnett, E.C. (1944) Keys to the Species Groups of Quercus, Lithocarpus, and Castanopsis of Eastern Asia, with Notes on their Distribution. Transactions of the Botanical Society of Edinburgh, 34(1), 159–204. doi: 10.1080/13594864409441557

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics, 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Camus, A. (1931). Sur quelques genres de Fagaces. Riviera Scientifique, 18, 37–42.

Camus, A. (1938). Fagacées nouvelles de l’asie orientale. Notulae systematicae (Paris), 6(4), 178–185.

Camus, A. (1942). Fagacées asiatiques nouvelles. Bulletin du Muséum National d’Histoire Naturelle Series, II 4(5), 357–360.

Camus, A. (1943). Lithocarpus (Fagacées) nouveaux d’Annam. Bulletin de la Société Botanique de France 90(4–6), 84–85. https://doi.org/10.1080/00378941.1943.10837497

Camus, A. (1945). Espèces et variétés nouvelles du genre Lithocarpus. Bulletin de la Société Botanique de France 92(4–6), 82–84. https://doi.org/10.1080/00378941.1945.10834409

Camus, A. (1948). Les Chènes: Monographie du genre Quercus et Lithocarpus. Chènes Atlas (Vol. 3). Paul Lechevalier & fils.

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: An analysis tool set for population genomics. Molecular Ecology, 22, 3124–3140. https://doi.org/10.1111/mec.12354

Darriba, D., Taboada, G.L., Doallo, R., & Posada, D. (2012). jModelTest 2: More models, new heuristics and parallel computing. Nature Methods, 9(8), e772. https://doi.org/10.1038/nmeth.2109

Doyle, J.J., Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin, 19, 11–15.

Dunning, L. T. & Sawolainen, V. (2010). Broad-scale amplification of matK for DNA barcoding plants, a technical note. Botanical Journal of the Linnean Society, 164, 1–9.

Ho, P. H. (2003). An Illustrated Flora of Vietnam (Vol. 2). Young Publishing House, Ho Chi Minh City.

Huang, C.J., Zhang, Y.T., Bartholomew, B. (1999). Fagaceae. In W. Zhengyi, P. H. Raven, H. Deyuan, (Eds.), Flora of China. Volume 4, (pp. 333–369). http://www.efloras.org

Kress, W. J., Erickson, D. L., Jones, F. A., Swenson, N. G., Perez, R., Sanjur, O., & Bermingham, E. (2009). Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. Proceedings of the National Academy of Sciences, 106, 18621–18626.

Manos, P.S., Cannon, C.H., Oh, S.H. (2008) Phylogenetic relationships and taxonomic status of the paleoendemic Fagaceae of Western North America: Recognition of a new genus, Notholithocarpus. Madroño, 55, 181–190. doi: 10.3120/0024-9637-55.3.181

Ngoc, N.V., Dung, L.V., Tagane, S., Binhm, H.T., Son, H.T., Trung, V.Q., & Yahara, T. (2016). Lithocarpus dahuoaiensis (Fagaceae), a new species from Lam Dong Province, Vietnam. Phytotaxa, 69, 23–30. https://doi.org/10.3897/phytotaxa.69.9821

Ngoc, N. V., Hung, N. V., Binh, H. T., Tagane, S., Toyama, H., Son, H.T., Ha, T.V., & Yahara, T. (2018) Lithocarpus vuquangensis (Fagaceae), a new species from Vu Quang National Park, Vietnam. Phytotaxa, 95, 15–25. https://doi.org/10.3897/phytotaxa.95.21832

Ngoc, N.V., Binh, H.T., Nagahama, A., Tagane, S., Toyama, H., Matsuo, A., Suyama, Y., & Yahara, T. (2021). Morphological and molecular evidence reveals three new species of Lithocarpus (Fagaceae) from Bidoup-Nui Ba National Park, Vietnam. Phytotaxa, 186, 73–92. https://doi.org/10.3897/phytotaxa.186.69878

Phenglklai, C. (2008) Fagaceae. In T. Santisuk, K. Larsen, (Eds.), Flora of Thailand 9(3) (pp. 179–410). The Forest Herbarium, Bangkok.

Rochette, N. C., Rivera-Colon, A. G., & Catchen, J. M. (2019). Stacks 2: Analytical methods for paired-end sequencing improve RADseq-based population genomics. Molecular Ecology, 28, 4737–4754. https://doi.org/10.1111/mec.15253

Rohwer, J. G., Li, J., Rudolph, B., Schmidt, S. A., van der Werrf, H., & Li, H. W. (2009). Is Persea (Lauraceae) monophyletic? Evidence from nuclear ribosomal ITS sequences. Taxon, 58, 1153–1167.

Stamatakis, A. (2014). RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics (Oxford, England), 30(9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Strijk, J., Sirimongkol, S., Rueangruea, S., Ritphet, N., & Chumnumroon, V. (2014a). Lithocarpus orbicarpus (Fagaceae), a new species of Stone Oak from Phang Nga province, Thailand. PhytoKeys, 34, 33–46. doi: 10.3897/phytotaxa.34.6429

Strijk, J. S., Rueangruea, S., Sirimongkol, S., & Suddee, S. (2014b). Lithocarpus corneus (Fagaceae), a new record for the Flora of Thailand. Thai Forest Bulletin (Botany), 42, 1–5.
Strijk, J. S., & Son, H. T. (2019). *Lithocarpus gigantophyllus* (Fagaceae), a new record from Loei province (Thailand). *Thai Forest Bulletin (Botany)*, 47(2), 145–151.

The Plant List. (2013). *Version 1.1. Published on the Internet*. http://www.theplantlist.org/

Suyama, Y., & Matsuki, Y. (2015). MIG-seq: An effective PCR-based method for genome-wide single-nucleotide polymorphism genotyping using the next-generation sequencing platform. *Scientific Reports*, 5, e16968. https://doi.org/10.1038/srep16963

Suyama, Y., Hirota, S.K., Matsuo, A., Tsunamoto, Y., Mitsuyuki, C., Shimura, A., & Okano, K. (2021). Complementary combination of multiplex high-throughput DNA sequencing for molecular phylogeny. *Ecological Research*, 1–11. https://doi.org/10.1111/14401703.12270

Takata, K., Taninaka, H., Nonakam M., Iwasem F., Kikuchi, T., Suyama, Y., Nagai, S., & Yasuda, N. (2019). Multiplexed ISSR genotyping by sequencing distinguishes two precious coral species (Anthozoa: Octocorallia: Coralliidae) that share a mitochondrial haplotype. *PeerJ*, 7, e7769. https://doi.org/10.7717/peerj.7769

Toyama, H., Kajisa, T., Tagane, S., Mase, K., Chhang, P., Samreth, V., Ma, V., Sokh, H., Ichihasi, R., Onoda, Y., Mizoue, N., & Yahara, T. (2015) Effects of logging and recruitment on community phylogenetic structure in 32 permanent forest plots of Kampong Thom, Cambodia. *Philosophical Transactions of the Royal Society B. Biological Sciences*, 370(1662), 1–13. https://doi.org/10.1098/rstb.2014.0008.