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RESEARCH ARTICLE **OPEN ACCESS**

Morphological and DNA-based Classification of *Cyathus* sp. Isolates from Thailand (Basidiomycota, Nidulariaceae)

Waraporn Sutthisa[1]* and Niwat Sanoamuang[2,3]

[1]Department of Biology, Faculty of Science, Mahasarakham University, Kantarawichai District, Maha Sarakham Province, 44150 Thailand. [2]Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon, Kaen University, Khon Kaen, 40002 Thailand. [3]Applied Taxonomic Research Center, Khon Kaen University, Khon Kaen, 40002 Thailand.

Abstract

Cyathus sp. isolates from three areas in Thailand (Khon Kaen University, Nam Nao National Park and Phu Ruea, Loei Province) were morphologically characterized by their peridiocarp, peridioles and basidiospores. This allowed to assign most isolates to five *Cyathus* species: *C. berkeleyanus, C. earlei, C. pallidus, C. stercoreus* and *C. striatus*. Phylogenetic analysis of ribosomal ITS sequence data yielded three groups of *Cyathus* isolates and unidentified species group. The *Pallidum* group including KKUNN1, is closely related to *C. berkeleyanus* (DQ463355.1), KKUITN2 (KU202745) and KKUITN3 (KU202751) are closely related to *C. pallidus* (DQ463356.1). The *Ollum* group includes *C. africanus* and *C. hookeri*. The *Striatum* group, such as KKUITP2 (KU202744) and KKUITP3 (KU202743) are closely related to *C. stercoreus* (DQ463356.1). The LSU sequence data suggest that KKULN2 and KKULN3 are closely related to *C. pallidus* (DQ463336.1), whereas KKULP2 and KKULP3 are closely related to *C. stercoreus*.

Keywords: *Cyathus*, ribosomal internal transcribed spacer (ITS), large subunit ribosomal DNA (LSU rDNA), peridiocarp

***Correspondence:** waraporn.s@msu.ac.th

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INTRODUCTION

The bird's nest fungi (phylum Basidiomycota, agaricoid clade, family Nidulariaceae) comprize five genera, viz. *Crucibulum*, *Mycocalia*, *Nidularia*, *Nidula* and *Cyathus*[1-4]. Typically, these genera produce small fruiting bodies on soil, animal droppings or organic matter[5]. The presence of gray to black peridioles with funicular cords distinguishes *Cyathus* from the other nidulariacean genera[6]. *Cyathus* species are inedible mushrooms that absorb nutrients into their cells by releasing enzymes to decompose organic matter. The basidiocarp of *Cyathus* sp. is funnel shaped with peridioles and a funicular cord. The basidiocarp are off-white color covered with fluffy hair and the reason that makes it different from other species because of the peridium surface is folded and contains subspherical basidiospores[7,8]. There are 45 species in this genus[3], which have spread around the world, especially in warm and hot areas[5]. There are doubts about the propriety of the old classification system based on the traditional taxonomy. Basidiocarps examination presented a large amount variation between the various collections, and morphological identification. Observations show that all culture collections were classified using morphology of basidiocarp. So, Martin *et al.*[9] collected *Cyathus* from Cape Verde and identified by using morphology and molecular data. They obtained a new species, *Cyathus lignilantanae* sp. nov. Whereas, Amazonian *Cyathus* species have a new species such as *Cyathus albinus* with light color hirsute exoperidium contrasting with a dark brown emplacement and basidiospores ovoid to elliptical and the other three such as *C. amazonicus, C. earlei* and *C. triplex*, are recorded for the first time from their localities[10]. The extinct category of the Red List of Threatened Fungi of Japan found that *Cyathus badius* and *C. boninensis* are included. The last and the only collections of both species were made in 1936, and no additional samples have been collected since then. Nowadays the samples were collected and morphological comparisons with the holotype and phylogenetic analyses based on ribosomal ITS, LSU and concatenated dataset placed *C. badius* in a highly contributed clade with *C. parvocinereus*[11]. In northern Thailand, 16 species of the five nidulariacean genera have been collected[6], including the new species *Cyathus subglobisporus* which on the basis of ITS and LSU nucleotide sequence data was assigned to the *Striatum* species group[6]. Yet, despite these few previous studies, the species diversity of the family Nidulariaceae, and of the genus *Cyathus* in particular, in Thailand remains poorly documented. Therefore, we report here on the identification of 12 specimens of *Cyathus* from NE Thailand, based on morphological data combined with ITS and LSU nucleotide sequences.

MATERIALS AND METHODS

Microorganisms and morphological identification

Twelve isolates of *Cyathus* sp. were collected from three parts of NE Thailand, including Khon Kaen University (KKU1, KKU2, KKU3, KKU4, KKU5, KKU6), Nam Nao National Park (KKUNN1, KKUITN2, KKUITN3) and Phu Ruea, Loei Province (KKULP1, KKUITP2, KKUITP3), all samples were sent to the Plant Pathology Laboratory, Khon Kaen University. Voucher specimens were deposited at Khon Kaen University Culture Collection (KKU). The isolates were identified using the methodologies proposed by Fungi Keys, Mushroom Expert[2,12,13]. Macroscopic features were described from fresh basidiocarps with a stereomicroscope (Carl Zeiss). Peridioles of the basidiospores were cross-sectioned and mounted with 3% KOH solution for observation under a microscope (Zeiss Primo Star) with AxioVision Rel.4.8.2. Thirty basidiospores were measured[14,15].

DNA analyses

Each isolate was cultured in potato dextrose broth (PDB), shaked at 120 rpm for 7 days. Subsequently, mycelia were collected and stored at -20°C for 24 hours. Total genomic DNA was extracted following Zang[13]. The universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ribosomal ITS region[16] and NL1 (5'-GCATATCAATAAGCGGAGGAAA AG-3') and NL4 (5' GGTCCGTGTTTCAAGACGG-3') to amplify large subunit ribosomal DNA (LSU rDNA) region[17]. PCR reaction mixtures and running conditions were as described by Sutthisa and Sanoamuang[18]. PCR amplicons were examined by gel electrophoresis[19] and sent to First BASE Laboratories Sdn. for sequencing.

DNA sequence alignment and phylogenetic analysis

ClustalX2 was used to align nucleotide sequences according to its default settings[20,21]. *Cyathus* sp. comprise of 10 species for ITS, 12 species for LSU and four other bird's nest fungi obtained from GenBank (Table 1) with. PAUP v.4.0b101 were used for phylogenetic analysis following Swofford[22], Felsenstein[23], Huelsenbeck and Ronquist[24] and Page[25] methods.

RESULTS

Morphological characterization

The morphological identification of *Cyathus* sp. isolates was based on Fungi Keys, Mushroom Expert[2,12,13]. The isolates were classified as follows:

***Cyathus berkeleyanus* (Tul. & C. Tul.) Lloyd**

Isolate KKUNN1. Peridium campanulate to infundibuliform, 11.0-13.0 mm high and mouth 7.0-80 mm wide. Exoperidium hairy, covered tomentum, pale brown to brown. Endoperidium brown with conspicuously plicate. The diameter of peridioles 1.5-2.5 mm, with dark brown to black color, ellipsoid or lenticular in outline. Basidiospores smooth, hyaline, ellipsoid, 9.0-12.0 x 5.0-7.0 µm, thin walled, 1-2.5 µm (Fig. 1).

***Cyathus earlei* Lloyd**

Isolate KKUPR1. Peridium infundibuliform, 4.9-8.1 mm high, mouth 1.2-5.5 mm wide. Hairy exoperidium with brown and sable color and filled with tomentum. Endocardium brownish grey to grey, unruffle to slightly plicate. Diameter of peridioles 1.3-1.9 mm, peridioles dark grey to black, circular or lenticular in outline. Basidiospores smooth, hyaline, elliptical to ovoid, 8.6-20.1 x 7.9-11.1 µm, thin walled, 1.27–2.54 µm (Fig. 2).

***Cyathus pallidus* Berk. & M.A.Curtis**

Isolates KKUITN2 and KKUITN3. Peridium campanulate, 6.2-13.1 mm high, mouth 2.8-6.9 mm wide. Exoperidium hairy, cream to brown, covered with tomentum. Endoperidium pale brown, flat to slightly plicate, lightly bright, not differ from exterior. Peridioles 1.6-2.9 mm in diameter, dark grey to black, circular or lenticular in outline. Basidiospores smooth, hyaline, sub-

Table 1. All the isolates with their origin and GenBank accession numbers

Taxon	Origin	GenBank accession number	
		ITS	LSU
Cyathus sp.	China	KJ195660.1	EF613554.1
C. africanus	China	DQ463340.1	DQ463330.1
C. annulatus	USA	NR 119588.1	-
	Thailand	-	DQ463332.1
C. berkeleyanus	China	DQ463355.1	-
C. crassimurus	USA	NR 119587.1	-
C. helenae	Canada	-	DQ463334.1
C. jiayuguanensis	China	-	DQ463325.1
C. olla f. *anglicus*	USA	-	DQ463326.1
C. pallidus	China	DQ463356.1	DQ463336.1
C. poeppigii	China	-	DQ463339.1
C. renweii	China	NR 119589.1	DQ463333.1
C. setosus	Jamaica	-	DQ463331.1
C. stercoreus	UK	EU784193.1	-
	China		DQ463338.1
C. striatus	USA	DQ486697.1	-
	Germany		DQ071742.2
Crucubulum laeve	Germany	DQ071701.2	DQ071771.1
Mycocalia denudata	USA	DQ911596.1	-
Nidula sp.	China	KC763972.1	-
Nidularia balachowskii	Israel	-	JX436154.1
Sphaerobolus sp.	USA	AY654738.1	AY654737.1

globose to elliptical, 6.8-14.5 × 6.0-8.1 µm, thin walled 1–1.27 µm (Fig. 3).

***Cyathus stercoreus* (Schwein.) de Toni**

Isolates KKUITP2 and KKUITP3. Peridium cone-shaped to goblet-like or a narrow inverted cone, 6.6-9.6 mm high, with mouth diameter 4.1-7.3 mm. Endoperidium yellowish brown, quite shaggy or wooly (at least when young). Endoperidium gray to black and smooth. Peridioles 1.9-2.8 mm in diameter, dark gray to black, ellipsoid or lenticular in outline. Basidiospores smooth, hyaline, sub-globose to ovoid (Fig. 4).

***Cyathus striatus* (Huds.) Willd**

Isolate KKU1, KKU2, KKU3, KKU4, KKU5 and KKU6**.** Peridium funnel-shaped, 8.0-10 mm high, mouth diameter 7.0-9.0 mm. Exoperidium

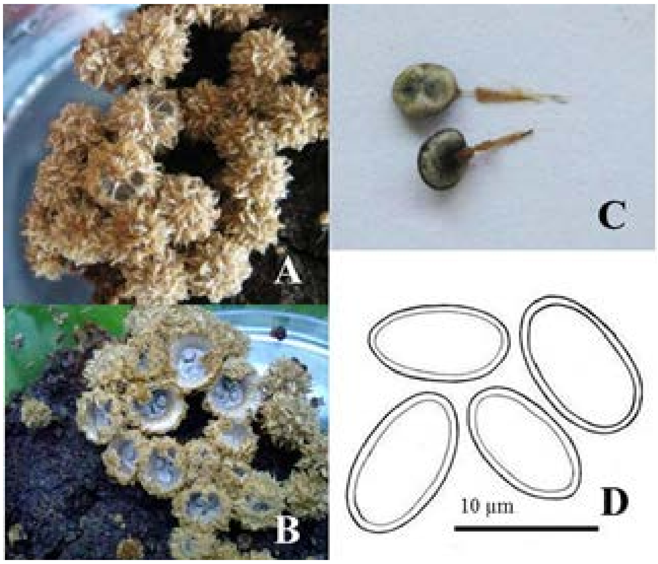

Fig. 1. *Cyathus berkeleyanus* KKUNN1 – A-B, Basidiocarps; C, Peridioles; D, Basidiospores.

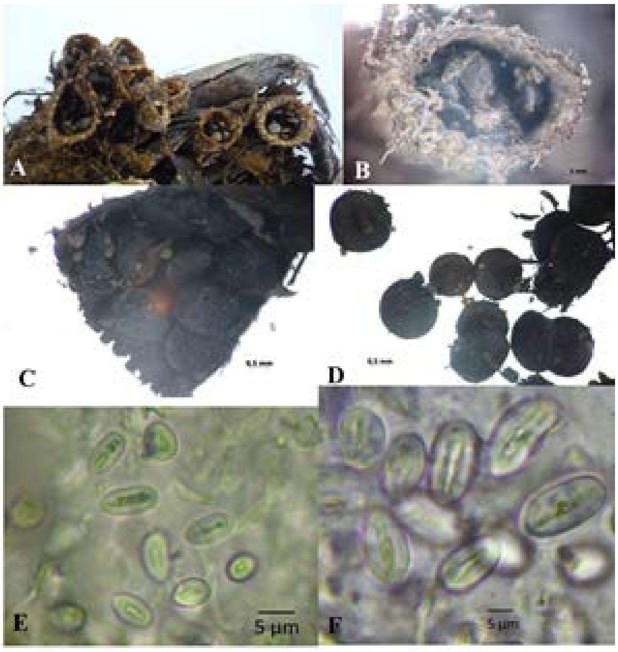

Fig. 2. *Cyathus earlei* KKUPR1 – A-B, Basidiocarps; C-D, Peridioles; E-F, Basidiospores.

shaggy to hairy, with tufts of hairs, brown to reddish brown, covered with tomentum, woolly. Endoperidium grayish brown, external wall plicate, covered with tomentum, lid typically white, disappearing with maturity. Peridiole diameter 1.5-2.5 mm, peridioles dark grey to black, ellipsoid or lenticular in outline. Basidiospores spheroidal to oval shaped, hyaline, unruffled, 16.0-20.0 × 10.0-12.0 µm, the wall was thick with 2-3 µm (Fig. 5).

DNA sequence characterization

The ITS dataset involved 26 isolates representing 22 *Cyathus* taxa, with *Crucibulum leave, Mycocalia denudata, Nidula* sp. and *Sphaerobolus* sp. as outgroup. Our phylogenetic analyses showed three groups of *Cyathus* isolates and an unidentified species group. The *Pallidum* group including KKUNN1 and is closely related to *C. berkeleyanus* (DQ463355.1), KKUITN2 and KKUITN3 are closely related to *C. pallidus* (DQ463356.1). The *Ollum* group includes *C. africanus* and *C. hookeri*. The *Striatum* group, such as KKUITP2 and KKUITP3, are closely related to *C. stercoreus* (DQ463356.1). Unidentified group including KKUPR1 is related closely to *Cyathus* sp. (KJ195660.1). However, KKU1, KKU2, KKU3, KKU4, KKU5 and KKU6 are not related to *Cyathus* taxa and other bird's nest fungi tested in this study (Fig. 6). The LSU dataset included 27 isolates representing 24 *Cyathus* taxa, with *Crucibulum leave, Nidularia balachowskii* and *Sphaerobolus* sp. as outgroup. Our phylogenetic analyses showed that KKULN2 and KKULN3 are closely related to *C. pallidus*

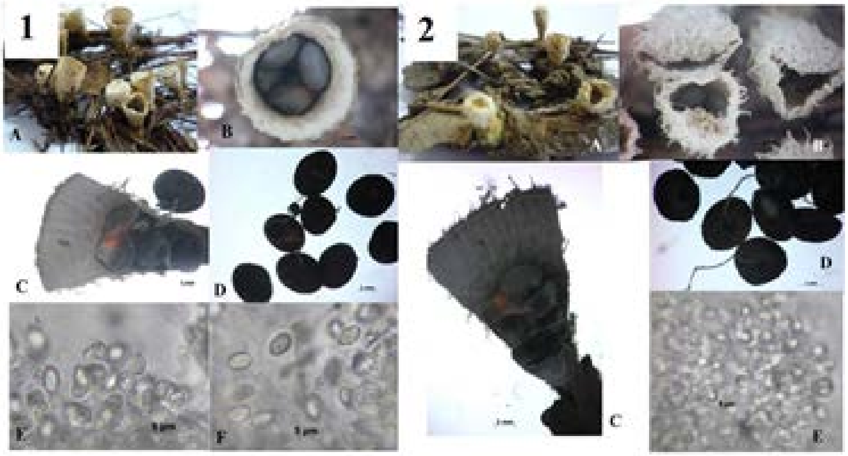

Fig. 3. *Cyathus pallidus* - 1, KKUITN2/KKULN2; 2, KKITN3/KKULN3; A-B, Basidiocarps; C-D, Peridioles; E-F, Basidiospores.

Fig. 4. *Cyathus stercoreus* - 1, KKUITP2/KKULP2; 2, KKUITP3/ KKULP3; A-B, Basidiocarps; C-D, Peridioles.

(DQ463336.1) as the *Pallidum* group. KKULP2 and KKULP3 are closely related to *C. stercoreus* (DQ483338.1) as the *Striatum* group. Therefore, KKU1, KKU2, KKU3, KKU4, KKU5, KKU6, KKUNN1 and KKUPR1 are not related to any *Cyathus* species in this study (Fig. 7).

Fig. 5. *Cyathus striatus* - A-F, Basidiocarp: A, KKU1; B, KKU2; C, KKU3; D, KKU4; E, KKU5; F, KKU6; G, Peridioles; H, Basidiospores.

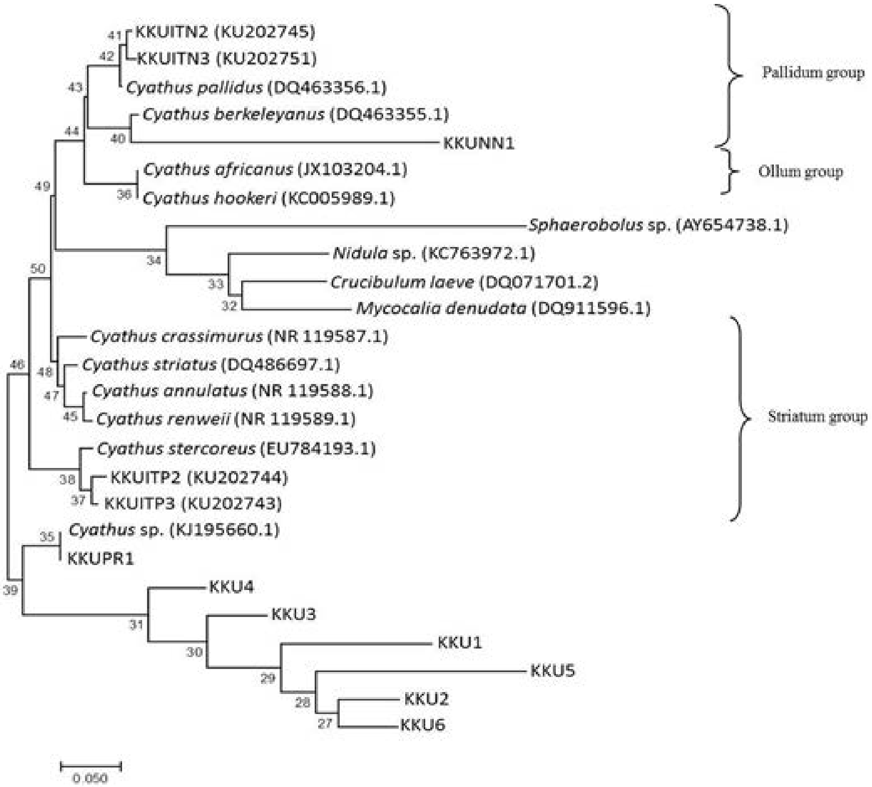

Fig. 6. Phylogenetic relations of *Cyathus* based on rDNA sequences including ITS-1, 5.8S and ITS-2 regions by neighbor-joining method. Numbers at branches are bootstrap support values based on 1000 replications.

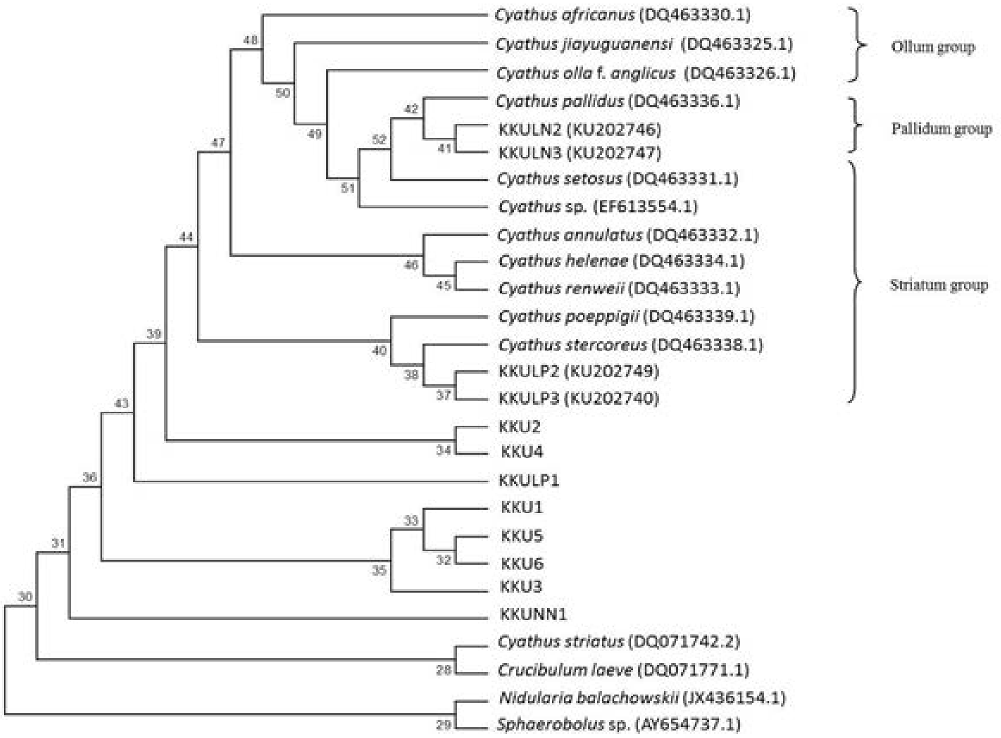

Fig. 7. Phylogenetic relations of *Cyathus* based on rDNA sequences including LSU regions by neighbor-joining method. Numbers at branches are bootstrap support values based on 1000 replications.

DISCUSSION

Bird's nest fungi identification is a matter of careful, sometimes microscopic, inspection of the morphological features. In this study we identified *Cyathus* sp. using morphological characters, including peridiocarp, peridiole and basidiospore, under stereo- and compound-microscopes. The results showed that KKUNN1 was *C. berkeleyanus*; KKUPR1 was *C. earlei*; KKUITN2 and KKUITN3 were *C. pallidus;* KKUITP2 and KKUITP3 were *C. stercoreus* and KKU1, KKU2, KKU3, KKU4, KKU5 and KKU6 were *C. striatus*. Similarly, *Cyathus morelensis* with taxonomic opinion, basidiocarp images and peridiole, basidiospores and peridiocarp illustrations. Additionally, the four species that registered from Brazil including *C. intermedius, C. montagnei, C. setosus* and *C. triplex* was first reported from northeastern Brazil[2]. Inspection of holotype of *C. setosus,* found that spore dimensions different from explained in the original publication.

The DNA sequence data of ITS and LSU, were consistent with the morphological identifications of KKUITN2 (*C. pallidus*), KKUITN3 (*C. pallidus*), KKUITP2 (*C. stercoreus*) and KKUITP3 (*C. stercoreus*). DNA identification may remain undecided if there are insufficient well-identified reference sequences in GenBank and BOLD. For example, BLAST searches in GenBank shown 72-82% identity with *C. striatus*. In addition, we amplified the SSU sequence of all isolates when compared to the GenBank database it showed that all of them were closely related with *C. striatus* with 99% identity.

Hence, the DNA data were not informative in this study. Alternately, three infrageneric groups consist of *Ollum, Pallidum* and *Striatum* was classified by using datasets of ITS and LSU. Still these three species groups can be separated on morphological basis. In northern Thailand, bird's nest fungi have been collected before and this leaded even to the description of a new species, viz.*C. subglobisporus*[22]. *Cyathus olla* was keep in culture collections for morphological and molecular studies. First, fruiting body were examined and the results show the much variation between the various collections, and found that classification using morphological characteristics conflicts with current taxonomy feature of species. All field collected accessions basidiocarp were classified by morphology. A new species described as *C. olla* f. *brodiensis*[26] distinction from *C. olla* by using morphology and RAPD analysis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Both author(s) have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

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