

## Short communication: Algal leaf spot associated with *Cephaleuros virescens* (Trentepohliales, Ulvophyceae) on *Nephelium lappaceum* in Thailand

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**Abstract.** Sunpapao A, Pitaloka MK, Arikrit S. 2015. Algal leaf spot associated with *Cephaleuros virescens* (Trentepohliales, Ulvophyceae) on *Nephelium lappaceum* in Thailand. *Biodiversitas* 17: 31-35. Algal leaf spot disease of *Nephelium lappaceum* (rambutan) was observed in southern Thailand. The algae were isolated on Bold's basal medium (BBM) and identified based on appearance of the lesions, algal morphology and molecular properties. Characteristics of the filamentous thallus cells, sporangiophores, sporangia, gametes and zoospores were clarified. A portion of the 18S small subunit rRNA was amplified to validate the morphological identification by sequence similarity. To summarize the main results, the plant parasite causing algal leaf spot was identified as *Cephaleuros virescens*, and in sequencing-based phylogenetic analysis the *Cephaleuros* PSU-R5.1 isolate from rambutan grouped with the algae in genus *Cephaleuros*. This confirms *C. virescens* as a causal organism of algal leaf spot disease on rambutan in southern Thailand.

**Key words:** Green algae, leaf spot, morphology, *Nephelium lappaceum*, rRNA

### INTRODUCTION

Rambutan (*Nephelium lappaceum* Linn.) is a large-sized, evergreen tree belonging to the family Sapindaceae. It produces numerous fruits with a protuberant hairy surface. Rambutan is mostly cultivated in Southeast Asia and in other tropical areas. In Thailand, most rambutan cultivation is in the southern province of Surat Thani. Algal leaf spot disease is among the minor diseases commonly found in rambutan trees, exacerbated by the moist environment and long periods of high rainfall. Rambutan is subject to attack by several plant-parasitic organisms, some of which can be severe. This algal disease can damage plant leaves, fruits and stems, and cause economic loss. Rambutan fruits are well known to possess several health benefiting components. The most common algal parasites of woody plants belong to the genus *Cephaleuros*.

*Cephaleuros* species are filamentous green algae widely known as a parasite of higher plants. The genus belongs to the division of aquatic green algae (Chlorophyta), class Ulvophyceae, order Trentepohliales and family Trentepohliaceae (Guiry and Guiry 2015). They are aerial and need free water to germinate (Suto and Ohtani 2009). The genus *Cephaleuros* has been known to cause diseases with morphological characteristics similar to those caused by fungi. The disease caused by this alga manifests as bright orange spots on leaves and stems, similar to rust

fungi (Mann and Hutchinson 1907). Algae in this genus are widespread in tropical and subtropical areas causing damage on leaves, young stems and fruits of several host plants (Alfieri 1969). *Cephaleuros* has a relatively simple structure, but its classification based on morphology is complex (López-Bautista et al. 2006).

Though *Cephaleuros* species occur on the leaves of numerous economical plants (Brooks, 2004), *C. virescens* is the most frequently reported. The disease caused by this genus has been recorded in Hawaii (Rindi et al. 2005), Japan (Suto and Ohtani 2009), Florida (Marlatt and Campbell 1980; Marlatt and Alfieri 1981), America (Brooks 2003), Africa (Rindi et al. 2006) and Panama (Rindi et al. 2008). In Thailand, *Cephaleuros solutus* was the first to be reported as a causal agent of algal leaf spot disease (Pitaloka et al. 2014). Based on morphology and identification of its 18S small subunit rRNA, the algal leaf spot disease on Para rubber (*Hevea brasiliensis*) was attributed to *C. virescens* (Pitaloka et al. 2015), and on acacia (*Acacia auriculiformis*) it was attributed to *C. diffusus* (Sunpapao and Pitaloka 2015). Algal leaf spot is considered a minor disease in Thailand, so studies on the biology, ecology, distribution, taxonomy and pathogenicity of *Cephaleuros* are rare. Therefore, the aim of this research was to isolate and identify the species of *Cephaleuros* on rambutan based on its morphology and molecular analysis.

## MATERIALS AND METHODS

### Morphological characters

Thirty algal leaf spot samples ( $n=30$ ) of rambutan were collected on August, 2014 from the Pest Management field, Faculty of Natural Resources, Prince of Songkla University, Thailand. Algal thalli were removed from fresh leaves and preliminarily observed under stereomicroscope. The shape, size, color and growth of algal thalli and their lesions were described and photographed. Taxonomic characters, including gametangia, gametes, zoosporangia, zoospores and setae were evaluated using the dichotomous key of Thompson and Wujek (1997). Algal specimens were deposited in the culture collection of the Pest Management Department, at the Prince of Songkla University, Thailand.

### Algal culture, isolation and DNA extraction

Algal cultures were obtained based on the methods of Suto and Ohtani (2011). Leaves with fresh thalli were washed under running water for one hour, wiped with sterile cotton wool, dipped in 70% ethanol and rinsed with sterile distilled water. After that, small thalli (2-3 mm) were removed with a sterile razor blade and placed on Bold's basal medium (BBM; Bischoff and Bold 1963; Andersen 2005), and incubated at 20°C in a light: dark cycle of 12:12 hours, for two to six months. Algal colonies were then scraped from the BBM medium with a glass slide and put into a microcentrifuge tube. DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method following a prior report (Kollar et al. 1990).

### Amplification of 18S nuclear small subunit rRNA by PCR

The extracted DNA was amplified with PCR using universal primers for a conserved sequence of 18S nuclear small subunit rRNA. The primer pairs were: PNS1-forward (5' CCAAGCTTGAATTCGTAGTCATATGCTTGTC 3') (Hibbett 1996) and NS41-reverse (5' CCCGTGTTGAGTCAAATTA 3'). The PCR used a final 50- $\mu$ l reaction volume containing 10 pmol of each primer, 2x DreamTaq Green PCR Master Mix (Thermo Scientific), and 50 ng of template DNA. An initial denaturation step for 3 min at 95°C was followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 50°C, and extension for 1 min at 72°C, with a final extension step of 10 min at 72°C. The PCR products were visualized by agarose gel electrophoresis. The 1 kb GeneRuler DNA ladder (Thermo Scientific) was used as DNA marker.

### DNA sequencing and phylogenetic analyses

The PCR products of the 18S nuclear small subunit rRNA gene region were bidirectionally sequenced at the Scientific Equipment Center, Prince of Songkla University by automated DNA sequencing with ABI Prism 377 (Applied Biosystems, USA), using the same primers as in the PCR reaction. Phylogenetic analysis of the 18S rRNA sequences from the *Cephaleuros* samples included known *Cephaleuros* sequences from the GenBank database for comparison. The phylogenetic and molecular evolutionary

analyses were conducted using CLUSTAL W and the software package MEGA6.

## RESULTS AND DISCUSSION

### *Cephaleuros virescens* Kunze ex. E. M. Fries

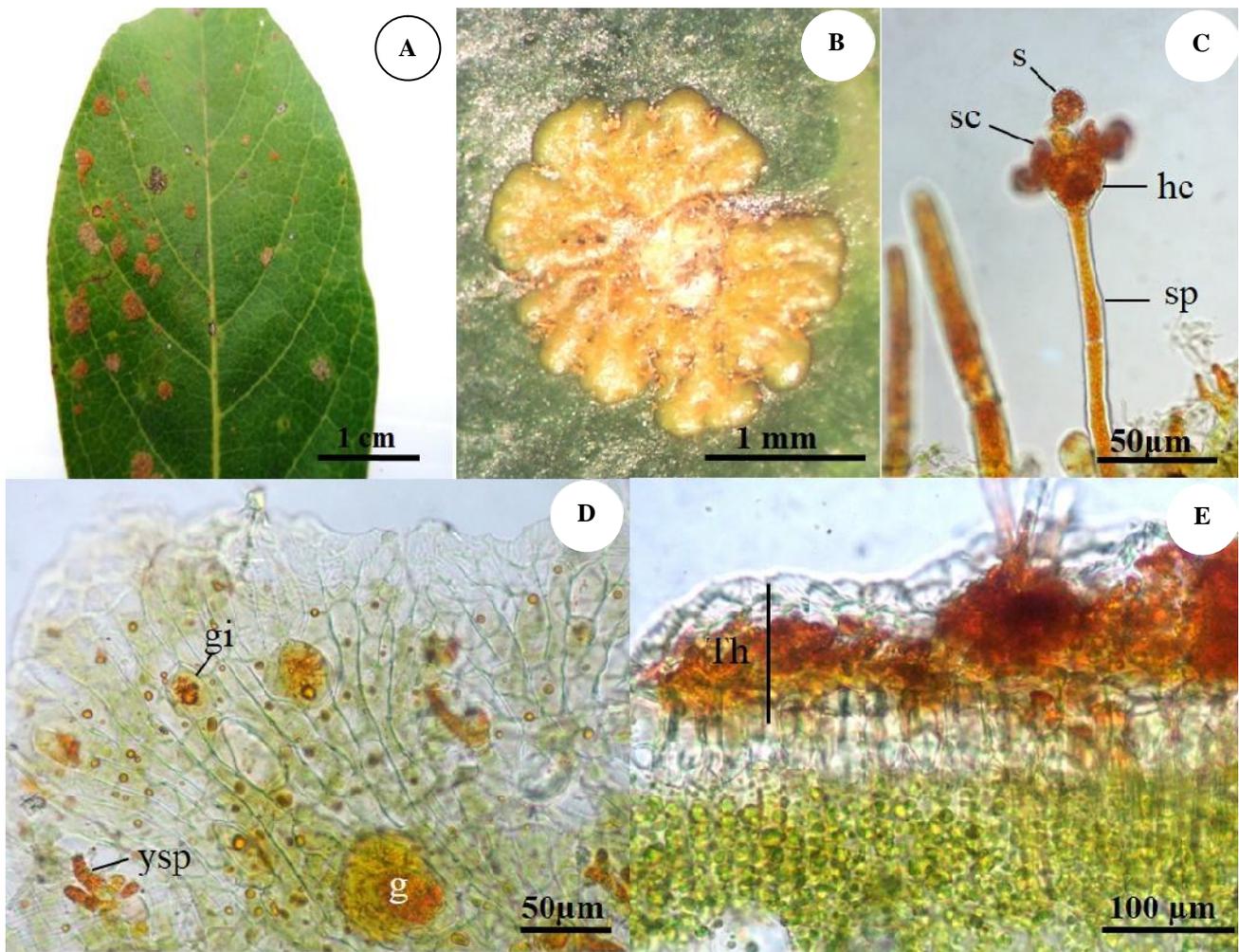
**Synonyms:** *Mycoidea parasitica* Cunningham, *Phylactidium tropicum* Möbius, *Cephaleuros albidus* Karsten, *Cephaleuros laevis* Karsten, *Cephaleuros mycoidea* Karsten, *Cephaleuros candelabrum* Schmidle, *Cephaleuros pulvinatus* Schmidle, *Chrooderma endophytica* F.E. Fritsch, *Phycopeltis hawaiiensis* J.W. King, and *Cephaleuros virescens* f. *sessilis* Islam (Guiry and Guiry 2015).

**Host:** *Nephelium lappaceum* Linn.

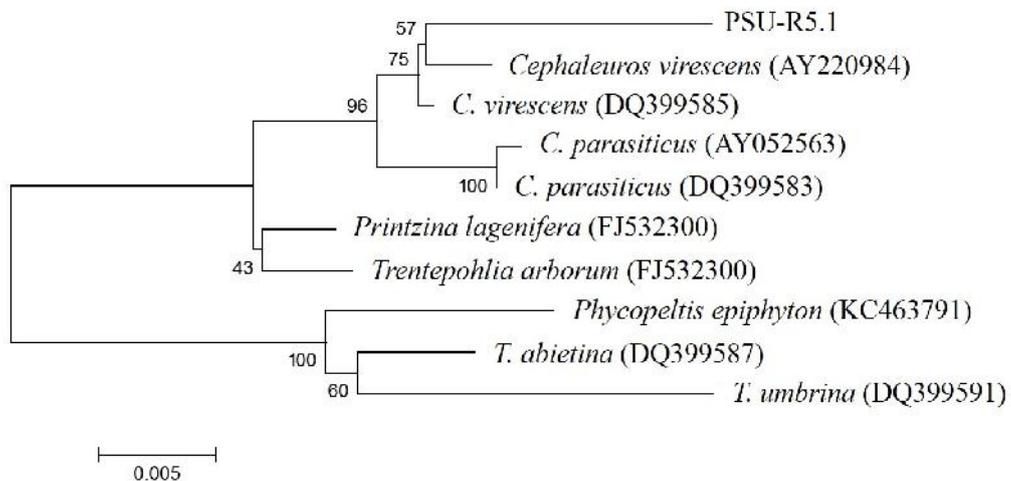
Fresh thalli or lesions appeared separately or in small groups on the leaves of rambutan in late August 2014 when rain was frequent and the weather was warm. The symptoms were orange to brown small circular scurf on the leaves, approximately 1-4 mm in diameter (Figure 1.A-1.B). Transverse sectioning demonstrated that the thalli were subcuticular with subepidermal growth through leaf tissue, and caused necrosis of the epidermal cells (Fig 1.E). Filamentous cells of the algae were long and cylindrical, 95.5-147.5 (mean, 85.5)  $\mu$ m long  $\times$  17.5-37.5 (24.5)  $\mu$ m wide, with length/width (L/W) ratio of 1: 3-7 (Figure 1.D). Setae were short filaments of two to five cells, 17.5-50 (27.5)  $\mu$ m long  $\times$  2.5-7.5 (5.5)  $\mu$ m wide.

Sporangiophores developed from thalli on the upper leaf surface. They were cylindrical, three to five cells, erect, solitary, 252-430 (320.5)  $\mu$ m long  $\times$  10-20 (17)  $\mu$ m wide (Figure 1.C). Head cells developed terminally and produced four sporangia, each on a sporangiate lateral (Figure 1.C). Sporangia were elliptical, 20-30 (25.5)  $\mu$ m long  $\times$  15-22.5 (18.5)  $\mu$ m wide (Figure 1.C). Gametangia were spherical to elliptical, 37.5-50 (45)  $\mu$ m long  $\times$  30-37.5 (32.5)  $\mu$ m wide and produced beneath the cuticle (Figure 1.D). Gametes were spheroidal, 7-10 (5.5)  $\mu$ m long  $\times$  5-7 (6)  $\mu$ m wide with flagella 13-17  $\mu$ m long. Zoospores were elliptical, 10-13 (11.5)  $\mu$ m long  $\times$  5-7 (6.5)  $\mu$ m wide with flagella 16-22  $\mu$ m long. Based on these results and using the monograph of Thompson and Wujek (1997), the thirty samples of the algae causing leaf spot disease on rambutan were identified as *Cephaleuros virescens*. We also compared the distinguishing characteristics of *C. virescens* and *C. diffusus* (Table 1).

Fresh thalli cultured on BBM grew slowly, producing 3-to 5-mm diameter greenish colonies of tufted filaments in two to six months. To confirm the morphological identification, one isolate (PSU-R5.1) was selected for molecular analysis. PCR was conducted to amplify a portion of 18S rRNA using the PNS1 and NS41 primer pair. The nucleotide sequence analysis of the *Cephaleuros* 18S rRNA using BLAST search revealed that our partial sequence was 1,052 bases long. This nucleotide sequence was compared to known *Cephaleuros* spp. and other algal genera in the NCBI (the National Center for Biotechnology Information) databases and deposited in GenBank with accession number (AB971690). A 98% sequence identity



**Figure 1.** A. Leaf spot lesions caused by *Cephaleuros virescens* on the upper leaf surface of rambutan, B. entire, lobed thallus, C. erect sporangiophores of *C. virescens*, each with four sporangia laterals consisting of a suffultory cell and attached sporangium, D. entire closed-ramulate thallus with young sporangiophore (y)sp, gametangia initial (gi) and gametangia (g), E. transverse section of a rambutan leaf showing the thallus (Th) of *C. virescens* and associated necrotic tissue



**Figure 2.** Phylogenetic tree based on 18S rRNA sequences of *Cephaleuros* (PSU-R5.1) shows closely related *Cephaleuros* cases with less similarity to other cases. The tree was constructed with the neighbor-joining method, and the GenBank accession numbers are presented in parentheses

**Table 1.** Comparison of phenotypic characteristics between *Cephaleuros virescens* (PSU-R5.1) and *C. diffusus*

Characters		<i>Cephaleuros virescens</i> (PSU-R5.1)	<i>Cephaleuros diffusus</i> (Sunpapao & Pitaloka 2015)
Host		<i>Nephelium lappaceum</i>	<i>Acacia auriculiformis</i>
Thalli	Shape	Raised spot, circular disk	Raised spot
	Diameter (mm)	1-4	1-5
	Growth habit	Pseudoparenchymatous	Open filamentous
Filamentous cells	Shape	Long cylindrical	Short cylindrical to irregular
	Length × wide (µm)	95.5-147.5 (85.5) × 17.5-37.5 (24.5)	7.5-42.5 (30.5) × 5-17.5 (12.5)
	L/W ratio	3-7	1-6
	Branching manner	Equal dichotomous	Dichotomous
Setae	Shape	Cylindrical filament	Cylindrical filament
	Length × wide (µm)	17.5-50 (27.5) × 2.5-7.5 (5.5)	16.5-280 (150) × 5-7.5 (5.5)
Gametangia	Shape	Elliptical	Spherical
	Length × wide (µm)	37.5-50 (45) × 30-37.5 (32.5)	12.5-32.5 (17.5) × 12.5-22.5 (20.5)
Gametes	Shape	Spheroidal	Obconic to spherical
	Length × wide (µm)	7-10 (5.5) × 5-7 (6)	5-10 (7.5) × 5-7.5 (6)
Sporangiophores	Length × wide (µm)	252-430 (320.5) × 10-20 (17)	250-440 (350.5) × 10-12.5 (10.5)
	Head cell placement	Terminal	Terminal
Sporangia	Shape	Elliptical	Spherical to ellipsoid
	Length × wide (µm)	20-30 (25.5) × 15-22.5 (18.5)	12.5-27.5 (15.5) × 10-20 (12.5)
Zoospores	Shape	Elliptical	Obovoid
	Length × wide (µm)	10-13 (11.5) × 5-7 (6.5)	10-16.25 (13.5) × 5-8.75 (7.5)
Lesions	Discoloration	Absent	Absent

confirmed the isolate as a member of the *Cephaleuros* genus. A phylogenetic analysis of sequences imported from GenBank indicated that Thai isolate PSU-R5.1 was closely related to the genus *Cephaleuros*, but well separated from the other species (Figure 2).

In this study, the organism isolated from rambutan leaf spot was characterized based on its morphological characteristics and molecular properties. Algae in genus *Cephaleuros* have reportedly caused diseases in numerous plant species in various habitats (Joubert and Rijkenberge 1971; Marlatt and Alfieri 1981; Chapman and Good 1983; Holcomb 1986; Thompson and Wujek 1997; Suto and Ohtani 2009; Pitaloka et al. 2015; Sunpapao and Pitaloka 2015). The algae were consistently found growing beneath the host cuticle. The tissue beneath thalli was necrotic, and no necrotic spots were observed without thalli. Cross-sections through thalli suggested the plant tissue necrosis was spreading from the thalli into healthy tissues. However, the lesions were rather small and caused only minor damage to *Nephelium lappaceum*. Even small algal spots can cause a yield reduction if there are enough of them to reduce photosynthesis or trigger premature leaf senescence.

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