BIODIVERSITAS
Volume 16, Number 2, October 2015
Pages: 116-120

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d160202

Short Communication: A new record of plant parasitic green algae, *Cephaleuros diffusus* (Trentepohliaceae, Chlorophyta), on *Acacia auriculiformis* hosts in Thailand

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Manuscript received: 12 January 2015. Revision accepted: 13 May 2015.

Abstract. *Sunpapao A, Pitaloka MK. 2015. A new record of plant parasitic green algae,* Cephaleuros diffusus *(Trentepohliaceae, Chlorophyta), on* Acacia auriculiformis *hosts in Thailand. Biodiversitas 16: 116-120. Cephaleuros diffusus* algae were found to cause leaf spot disease of the host *Acacia auriculiformis*. The identification was based on morphological and molecular properties. The observed morphology identified the algae as *C. diffusus*. Transverse sections of the host leaves were used to quantify the disease severity of the host in terms of a four-point necrosis index. The host's lesions were subcuticular and subepidermal on the upper leaf surfaces. The identification of the algae was confirmed from molecular analysis, and a phylogenetic tree distinguished our samples from other *Cephaleuros* species. This is the first report of *C. diffusus* on *Acacia auriculiformis* in Thailand.

Key words: *Cephaleuros*, disease severity, morphology, necrosis, phylogenetic analysis

INTRODUCTION

The algae in the order Trenthepohliales are subaerial green algae, which are widespread and of diverse forms and habits in the tropical and the subtropical regions. This order is represented by the single family Trenthepohliaceae, which includes six genera: *Cephaleuros* Kunze, *Phycopeltis* Millardet, *Physolinum* Printz, *Stomatochroon* Palm, *Printzina* Thompson & Wujek, and *Trentepohlia* Mauritius. Among these, *Cephaleuros* is the most common and widespread algae found on vascular plants as well as on several economically important plants (Lopez-Bautista et al. 2002).

In most cases *Cephaleuros* is mistaken for a fungus, because the symptoms show erect, yellow to red filaments and hairs like a fruiting body that is raised on the leaf surface, which matches the characteristics of rust fungi (Marlatt and Alfieri 1981). The identification of *Cephaleuros* can be based on morphological characteristics, although they do not provide definitive separation to distinguish between the species. *Cephaleuros* grow in the subcuticular, subepidermal and intramatical leaf tissues, and necrosis is found beneath the alga thallus infection (Thompson and Wujek 1997). Thompson and Wujek (1997) concluded that the features most valuable in determining the species are: (i) thallus growth habit, (ii) manner of bearing head cell or sporangiate laterals, and (iii) the kinds of lesions produced. To confirm identification based on such features, molecular characterization is needed. However, pathogenicity tests of *Cephaleuros* as a plant pathogen have not been completed, probably due to the difficulties with producing zoospores for reinoculation on synthetic media (Chapman and Good 1983; Holcomb et al. 1998).

Acacia (*Acacia auriculiformis*) is a woody plant of the genus *Acacia*, originally from Australia, and spread to several countries including Thailand (Midgley 2007). This woody plant is now mostly found in commercial plantations in Southeast Asia. In Thailand acacia is also common in the wild, and is planted on the road sides to provide shade. The tropical regions where acacia is found are also in the spread range of *Cephaleuros*. The first report of *Cephaleuros* on acacia (Marlatt and Alfieri 1981) did not provide a specific species description. Furthermore, various pathological and physiological problems associated with a parasitic *Cephaleuros* infection still need confirming. Altogether, there is little prior research on the identification of *Cephaleuros* in Thailand. Therefore, the aim of this research was to characterize the *Cephaleuros* species on acacia in Thailand, based both on morphology and on molecular properties, and to estimate the disease severity in the acacia hosts using a necrosis index.

Materials and Methods

Sample collection and morphological identification

Algae: *Cephaleuros* sp., host: acacia (*Acacia auriculiformis*); locality: Pest Management field, Prince of Songkla University, Songkhla Thailand; collection: Dec 12, 2013, by Mutiara K. Pitaloka (PSU-A15). Macroscopic features of the parasitic algae were measured under a stereo microscope. The samples were prepared by hand sectioning, a piece of thallus was peeled from the leaf to observe the morphological characters of the thallus and

sporangiophores. Fresh thalli which producing gametangia or sporangia were placed on a drop of sterile water on a glass slide to observe the gametes and zoospore. All of the morphological characteristics were characterized under a light compound microscope. The identification based on morphological characteristics was referred to the descriptions in a monograph by Thompson and Wujek (1997). Specimens were deposited in the Culture Collection of the Pest Management Department, Prince of Songkla University, Thailand.

Algal culture and isolation

Algal thalli were isolated from the leaves and cultured on Bold's basal medium (BBM) (Bischoff and Bold 1963; Andersen 2005). The isolation method followed Suto and Ohtani (2011) with some adaptations. Leaves with fresh thalli were washed under running water for five minutes and soaked in water for one hour, wiped with cotton wool, dipped in 70% ethanol, and rinsed with sterile water three times. For isolation, a small pieced of thalli was peeled from the leaf surface and placed on agar medium.

DNA extraction, PCR amplification and DNA sequencing

The filament-like colonies were harvested from BBM and subjected to DNA extraction by the CTAB method. The amplification of 18s nuclear small subunit rDNA (18s nsu rDNA) by PCR used universal primers PNS1-forward (5' CCAAGCTTGAATTCGTAGTCATATGCTTGTC 3') (Hibbett 1996) and NS41-reverse (5' CCCG TGTTGAGTCAAATTA 3'). The PCR reaction mixture had a final 50 µL reaction volume containing 10 pmol of each primer, 2x DreamTaq Green PCR Master Mix (Thermo Scientific), and 50 ng of template DNA. The amplification was carried out in a BIO-RAD T100^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following program: one cycle of denaturation step for 3 min at 95°C; 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; followed by the final extension step of 10 min at 72°C. The PCR products were visualized by agarose gel electrophoresis. The PCR products of 18s rDNA portion were sequenced at the Scientific Equipment Center, Prince of Songkla University, Songkhla, Thailand, by automated DNA sequencing with ABI Prism 377 (Applied Biosystems, USA), using the same primers used in the PCR reaction. The sequences obtained were compared with known sequences of *Cephaleuros* available in GenBank (The National Center of Biological Information, USA) by using BLAST search. Phylogenetic and molecular evolutionary analyses sequence query results were conducted using CLUSTAL W and the software package MEGA6.

Disease severity

A four-point necrosis index (Brooks 2004) was used to assess the disease severity in the acacia hosts of the parasitic algae. The index labeling criteria were: (0) No necrosis, (1) superficial necrosis of the cell layer beneath the algae thallus, with or without tissue hyperplasia, (2) necrosis of > 1 cell layer but not full leaf thickness, with or without tissue hyperplasia, erosion, or suberin formation, and (3) necrosis from upper to lower leaf surface, including "*shot hole*" symptoms.

Results and Discussion

To characterize the algal leaf spot disease causal organism, infected samples were collected. The most obvious symptom was orange tufts found on the upper leaf surfaces, constituting thalli of the green alga. Lesions were raised spots with no discoloration around the spot on the upper leaf surface (Figures 1.A and 1.B). The epidermal cells and palisade cells were necrotic, being brown to dark brown beneath the thalli (Figures 1.G and 1.H). The thalli growth was subcuticular and subepidermal on the upper leaf surfaces, with open narrow filaments. The radial extension of the thalli ranged from random to periodic structures, with equal dichotomy of the marginal apical cell. Filamentous-like ramuli were compacted, raising a thin layer on the upper leaf surface of 1-5 mm diameter. The filamentous cells ranged from short cylindrical to irregularly shaped, 7.5-42.5 µm long and 5-17.5 µm wide (Figure 1.D). The width to length ratio (W/L) was 1-6, and as the irregular and open filamentous growth becomes congested, the larger cells extended from the filament, while the small cells raised the setae, sporangiophores, or initial gamentangia. Some of the small cells produced a small, dense, irregularly shaped expanse by close dichotomies, which raised one to two celled setae. The setae grew from short cylindrical filaments, one to five celled, 16.5-280 µm long and 5-7.5 µm wide.

Gamentangia were spherical in shape, dark orange, developed in a cluster of 3-7 cells, 12.5-32.5 µm long and 12.5-22.5 µm wide (Figure 1.C). Gametes were obbiconic to spherical, 5-10 µm long and 5-7.5 µm wide, with biflagella 15-20 long. Zoospores were obovoid and some had a bullet shape, 10-16.25 µm long and 5-8.75 µm wide with biflagella 15-25 µm long (Figure 1.F). The sporangiophores were sparsely produced on the upper leaf surface, being cylindrical, erect, solitary or in a tuff of three or more, 250-440 µm long and 10-12.5 µm wide (Figure 1.E). Three to five of head cells developed terminally on the sporangiophores bearing two to four sporangia laterally, with both sporangia and their sulfutory cells. The sporangia were spherical to elliptical, 12.5-27.5 µm long and 10-20 µm wide, yellow to dark orange. Based on the key species referred to in the monograph of Thompson and Wujek (1997), the fifteen collected algal samples were identified as *Cephaleuros diffusus.*

A *Cephaleuros* species with a wide range of hosts is *C. virescens.* In this study, we compared *C. virescens* to *C. diffusus*, and their distinguishing characteristics are summarized in Table 1. The thalli of *C. diffusus* formed raised spots, whereas *C. virescens* forms circular discs without gaps crenate or entire margin. The thallus growth habit of *C. diffusus* was open and filamentous, whereas for *C. virescens* this is pseudoparenchymatous. Both species bear head cells terminally.

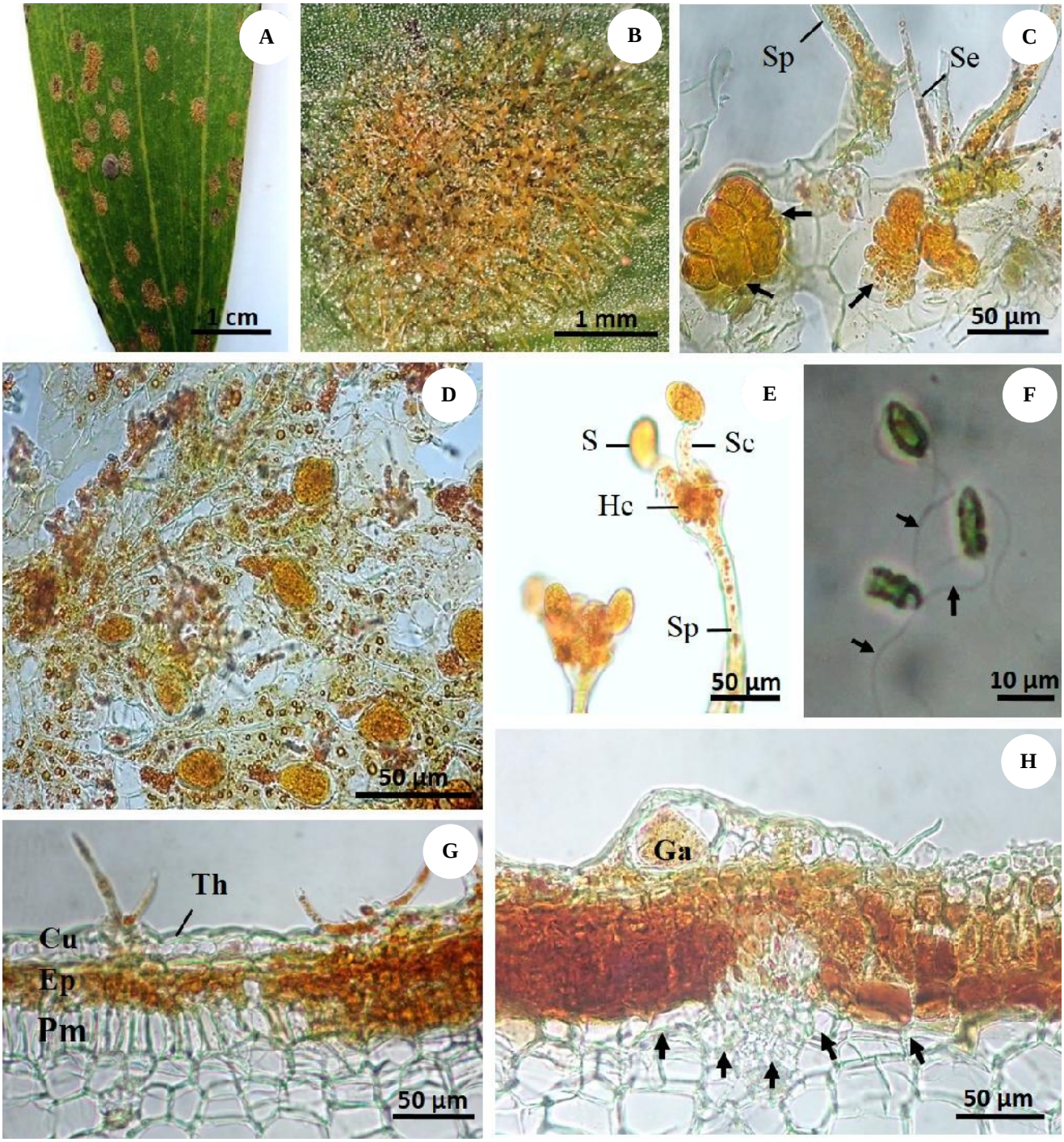

Figure 1. A. Lesions with *C. diffusus* parasitic algae on an acacia leaf, B. Thalli on the leaf surface, C. Clusters of gametangia (arrows) along with sporangiophores (Sp) and setae (Se), D. Filaments of *C. diffusus* with radial expansion, E. Sporangiophores terminally bearing the head cell (Hc) with suffultory cells (Sc) and sporangia (S), F. Zoospores with biflagella (arrows), G. Transverse section of *C. diffusus* showing thallus growth through to leaf tissue causing necrosis of cuticle and epidermis and of some palisade cells, H. Necrosis on the leaf tissue and protective corky tissue (arrows), Cu: cuticle, Ep: epidermal, Pm: palisade mesophyll, Th: thallus, Ga: gametangia.

To estimate disease severity, assessment on a four-point necrosis index was conducted. Ten transverse leaf sections from different plants were observed. The thalli grew subcuticularly and subepidermally through the leaf tissue, and some thalli colonized beneath the cuticle. Necrosis caused by *Cephaleuros* only occurred beneath of the thalli, while some lesions showed tissue hyperplasia without any necrosis. Due to these observed symptom characteristics, the necrosis index of a leaf was scored as 2 with necrosis of > 1 cell layer but not of full leaf thickness, with or without tissue hyperplasia, erosion, or suberin formation.

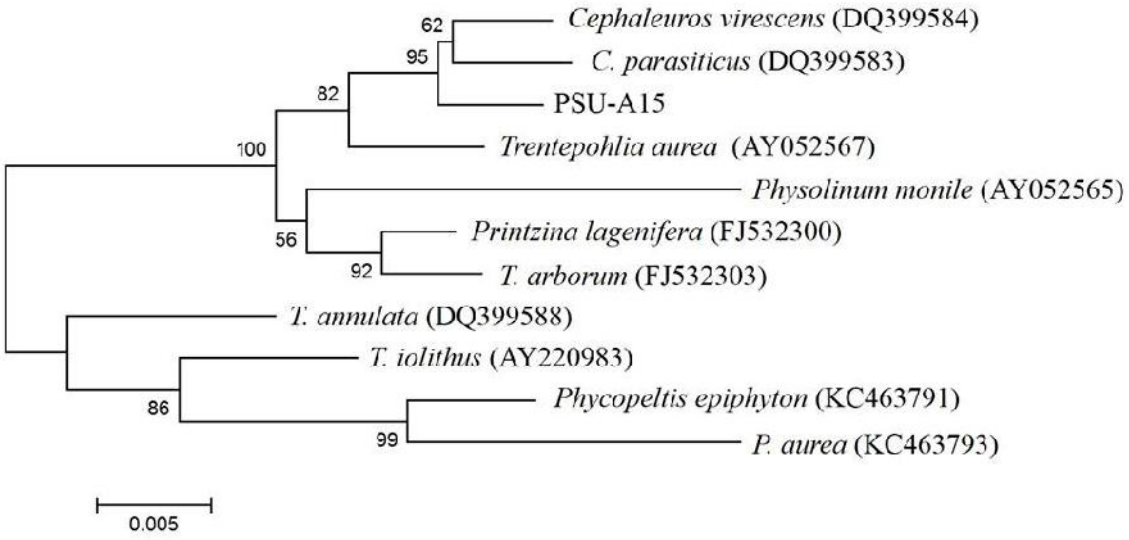

Figure 2. A phylogenetic tree showing genotypic comparison of a current *Cephaleuros diffusus* (PSU-A15) sample with highly sequence-similar species from GeneBank, using segments of 18s rDNA. The bootstrap values are shown on the branches, and the GenBank accession numbers are shown in parentheses. The comparison indicates that the PSU-A15 sample represents *Cephaleuros*, as a species distinct from *C. virescens* and *C. parasiticus*.

Table 1. Characteristics for distinguishing between *Cephaleuros diffusus* (PSU-A15) and *C. virescens*, based on phenotype and behavior.

Characters		***Cephaleuros diffusus* (PSU-A15)**	***Cephaleuros virescens* (Suto and Ohtani 2009)**
Host		*Acacia auriculiformis*	*Magnolia grandiflora* & *Persea thunbergii*
Thalli	Shape	Raised spot	Circular disk, without gaps, crenate or entire margin
	Diameter (mm)	1-5	1-8
	Growth habit	Open filamentous	Pseudoparenchymatous
Filamentous cells	Shape	Short cylindrical to irregular	Long cylindrical
	Length × width (µm)	7.5-42.5 × 5-17.5	22-79 × 7-24
	L/W ratio	1-6	2.7-4.4
	Branching manner	Dichotomous	Equal dichotomy
Setae	Shape	Cylindrical filament	1) Slender filament; 2) short bunt tipped filament
Gametangia	Shape	Spherical	Spherical or elliptical
	Length × width (µm)	12.5-32.5 × 12.5-22.5	29-58 × 18-43
Gametes	Shape	Obbiconic to spherical	Ellipsoidal to fusiform
Sporangiophores	Length × width (µm)	250-440 × 10-12.5	70-240 × 12-14
	Head cell placement	Terminally	Terminally
Sporangia	Shape	Spherical to elliptical	Elliptical
	Length × width (µm)	12.5-27.5 × 10-20	17-27 × 15-21
Zoospores	Shape	Obovoid	Ellipsoidal to broad fusiform
	Length × width (µm)	10-16.25 × 5-8.75	7-11 × 4.5-6.5
Lesions	Discoloration	Absent	Absent

The *Cephaleuros* species are known as plant parasitic algae on several woody plants, attacking leaves, branches, stems, or fruit of the plant. The *Cephaleuros* on acacia has been recorded in Florida (Marlatt and Alfieri 1981), although while the samples were described as *Cephaleuros* no taxonomy of the genus was reported. Those samples were reported as *Cephaleuros* sp. on *Acacia auriculiformis*. However, in this current study, we describe the species of *Cephaleuros* found on the leaves of acacia and identified as *C. diffusus*.

Thompson and Wujek (1997) concluded that the morphological features most valuable for determining the species of *Cephaleuros* are the thallus growth habit, the manner of bearing the head cell or sporangia laterally, and the kind of lesion produced. However, they stated that the dimensions of filamentous cell, gametangium, were not always definitive, and the color of algae is unhelpful in

identification since it varies with exposure. This makes phenotypic identification ambiguous because the characteristics simply are not definitively distinctive in general. Therefore, genotypic observations combined with phylogenetic analysis are important for confidence in the species identification. One colony sample of *C. diffusus* was harvested from BBM and subjected to DNA extraction, PCR amplification and DNA sequencing. BLAST searches in GenBank indicated that the present algae were similar to and grouped along with *Cephaleuros*. The sequence of the PCR product was deposited in the GenBank database with accession number AB972267. A phylogenetic tree from neighbor joining analysis shows that *C. diffusus* (PSU-A15) is closely related to *Cephaleuros*, and well separated from *C. virescens* and *C. parasiticus* (Figure 2). This corroborates the tentative identification from morphological observations with a genomics based identification that we consider definitive. The current research is the first to definitively associate *C. diffusus* with algal leaf spot on acacia in Thailand. This report constitutes a new record of occurrence, symptomatology, morphology and molecular characterization of *C. diffusus* associated with algal leaf spot disease on acacia.

ACKNOWLEDGEMENTS

The authors would like to thank the Center of Excellence in the Agricultural and Natural Resources Biotechnology (CoE-ANRB), the Faculty of Natural Resources, Prince of Songkla University, Songkla, Thailand for funding and facilities. The copy-editing service of RDO/PSU and the helpful comments of Dr. Seppo Karrila are gratefully acknowledged.

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