



## The first phylogenetic study of *Kiliophora* (Fungi, Anamorphic Xylariales)

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### Abstract

*Kiliophora* Kuthub. & Nawawi (Type: *K. fusispora* Kuthub. & Nawawi) was first described based on conidiophores bearing spindle-shaped conidia. Only two species have been reported worldwide, viz, *K. fusispora* and *K. ubiensis* Kuthub. & Nawawi. During the study of fungal diversity on *Shorea* spp. in Indonesia, we found *K. ubiensis* and successfully obtained pure isolate of this fungus through single spore isolation method. Since the taxonomy placement of this genus in the subphylum Pezizomycotina is unknown, phylogenetic analyses was carried out based on Internal Transcribed Spacer of ribosomal DNA sequence by using Maximum Parsimony method. The phylogenetic tree suggested that genus *Kiliophora* should taxonomically be placed in the family Amphisphaeriaceae (Ordo Xylariales). This report is the first finding of *K. ubiensis* from Indonesia.

**Key words** – Amphisphaeriaceae – ITS – Phylogenetic – Taxonomy – Xylariales

### Introduction

*Kiliophora* Kuthub. & Nawawi (1993) (type: *K. fusispora*) was erected to replace the hyphomycete genus *Danaea* Caneva & Rambelli (1981) (type: *D. ubiensis* Caneva & Rambelli) (Kuthubutheen & Nawawi 1993). Kuthubutheen & Nawawi (1993) noted that the generic name of *Danaea* was illegitimate according to the Code article 64.1, due to its contradiction with *Danaea* Sm. (1793), a tropical fern endemic to South America. The name of *Kiliophora* was based on conidiophores bearing spindle shaped conidia. This genus is characterised by having enteroblastic, polytretic, and discrete conidiogenous cells arising directly and from about midway up the setiform conidiophores, and acropleurogenous, hyaline–pale brown, fusiform to spindle-shaped conidia (Kuthubutheen & Nawawi 1993). The taxonomic position of *Kiliophora* within Subphylum Pezizomycotina has been unknown since the first publication of this genus.

Recently, rapid progress in molecular phylogenetic analyses have resolved the taxonomy position of several uncertain fungal genera within the Kingdom Fungi. During the study of decaying wood and leaf litter of *Shorea* spp. in Bogor (West Java, Indonesia), we found hyphomycete specimen which morphologically resembles characteristics of *K. ubiensis*. Single spore isolation was successfully done to obtain the pure culture of this specimen, and therefore, the molecular phylogenetic analyses based on ITS (Internal Transcribed Spacer) rDNA sequence was carried out to examine the phylogenetic position of *Kiliophora* within the Subphylum Pezizomycotina.

## Materials & Methods

### Morphological examination

Specimen of *K. ubiensis* on leaf litter of *Shorea* spp. was collected from research forest area managed by the Center for International Forestry Research (CIFOR), located at the Situ Gede village, Bogor, West Java, Indonesia. The presence of fungal fruiting structures on leaf litter of *Shorea* spp. was examined by dissecting microscope (Olympus® SZX7, Japan). Compound microscope (Olympus® CX41, Japan) was used to determine microscopic structures. Materials were mounted in water for microscopic examination, measurement of the structures, and for photographing. Shear's solution (Kirk et al. 2008) was used for permanent fixation. 30 conidia, 10 conidiogenous cells, and 10 conidiophores were measured. Species identification was carried out based on Seifert et al. (2011). Single conidia isolation was carried out using Water Agar (WA) according to the method described by Choi et al. (1999) with certain modification. Herbarium specimen was deposited at Herbarium Bogoriense (BO), Indonesia. Culture was preserved at Bogor Agricultural University Culture Collection (IPBCC).

### DNA extraction, PCR (Polymerase Chain Reaction) amplification and sequencing

A total genomic DNA was extracted from 5-days old fungal mycelia cultured in Potato Dextrose Broth (PDB) using REDEExtract-N-Amp<sup>TM</sup> Seed PCR Kits (Sigma, USA), following the manufacture's protocol. Primer pairs of ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') was used for DNA amplification of ITS region, including the 5.8S rDNA region (White et al. 1990). The PCR amplification condition was performed in 25 µl reaction volume as follows: nuclease free water 10µl, GoTaq<sup>®</sup>Green Master Mix (Promega, Madison, USA) 12.5µl, 0.5µl for each primer, DMSO (Dimethyl Sulfoxide) 0.5µl, and DNA template 1µl. PCR was performed by TaKaRa thermocycler (TaKaRa, Japan) using the following PCR parameters: initial denaturation at 95°C for 90s, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s, elongation at 72°C for 90 s, and final extension at 72° C for 5 min. The characterization of PCR product was performed via agarose gel electrophoresis on a TAE 1% agarose gel containing Ethidium Bromide (EtBr) as the staining agent. The PCR product was sent to 1stBASE (Malaysia) for sequencing. The new ribosomal DNA sequence has been deposited in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) under accession number KF056850. The GenBank accession numbers of the other sequences and taxa used to construct the phylogenetic tree were shown in fig. 2.

### Sequence alignment and phylogenetic analysis

Sequence obtained from the respective primers (ITS5 and ITS4) was assembled and manually edited using ChromasPro 1.41 software (Technelysium Pty Ltd., South Brisbane, Australia). Dataset of Xylariales used by Jaklitsch & Voglmayr (2012) was employed in the multiple alignment with 42 additional sequences retrieved from GenBank (NCBI, DDBJ). The multiple alignment was conducted using MUSCLE (Multiple Sequence Comparison by Log-Expectation) (Edgar 2004) implemented in MEGA (Molecular Evolutionary Genetics Analysis) version 6.0 (Tamura et al. 2013). Phylogenetic analyses were conducted using the maximum parsimony (MP) method in PAUP\* 4.0b10 (Swofford 2002). The MP analysis was performed with the heuristic search option using the 'tree-bisection-reconstruction' (TBR) algorithm with 1000 random sequence additions to find the optimum tree. The stepwise addition option set as random and maximum tree number was set at 500. Tree length (TL), consistency index (CI), retention index (RI), related consistency index (RC), and homoplasy index (HI) were also calculated. The Kishino-Hasegawa (KH) likelihood test (Kishino & Hasegawa 1989) was carried out to compare the best tree topology obtained by the nucleotide sequence data with a constrained tree. The strength of the internal branches of the phylogenetic tree in MP analysis was tested with bootstrap (BS) analysis (Felsenstein 1985) using 1000 replications. BS values of 50 % or higher than that are shown. Random sequence addition was used in the bootstrap analysis. All sites were treated as unordered

and unweighted, and gaps treated as missing data. *Diaporthe eres* (JQ807445), *D. helianthi* (AJ312365), and *D. ambigua* (AY485744) were used as outgroups. TreeGraph 2 (Stöver et al. 2010) was used to refine the phylogenetic tree.

## Results

### Taxonomy

#### *Kiliophora ubiensis* Khutub. & Nawawi

Fig. 1a–d

Colonies on the decaying leaves, scattered, mostly solitary, rarely in groups. Mycelium immersed in the substratum, 2–4 μm wide. Conidiophores macronematous, mononematous, dark brown, becoming paler toward the rounded apex, simple, erect, thick-walled, setiform, septate, up to 210 μm long, 5.5–7 μm wide. Conidiogenous cells formed from about the middle part of conidiophores through minute pores, enteroblastic, polytretic, discrete, hyaline, globose to subglobose, smooth, 3–5 μm wide, initially simple and solitary, then proliferating repeatedly through one or more of the conidiogenous loci in the previously cell to generate branched chains of conidiogenous cells. Conidia 7–17 × 2–4 μm, biconic, fusiform, aseptate, hyaline to subhyaline, smooth, solitary. Colonies on PDA (Potato Dextrose Agar) ± 4cm after 14 days, dark-brown, thick, smooth with slightly irregular margin, covered with slim white mycelium, reverse black, no sporulation found.

Teleomorph – unknown.

Known distribution – Southeast Asia (Indonesia, Malaysia), Africa (Ivory Coast).

Material examined – Indonesia, West Java, Bogor, Situ Gede village, CIFOR forest area, on decaying leaf of *Shorea* sp., September 2012, Israwati Harahap, BO22692 (monoconidial isolate IPBCC 131080) (GenBank accession number: KF056850).

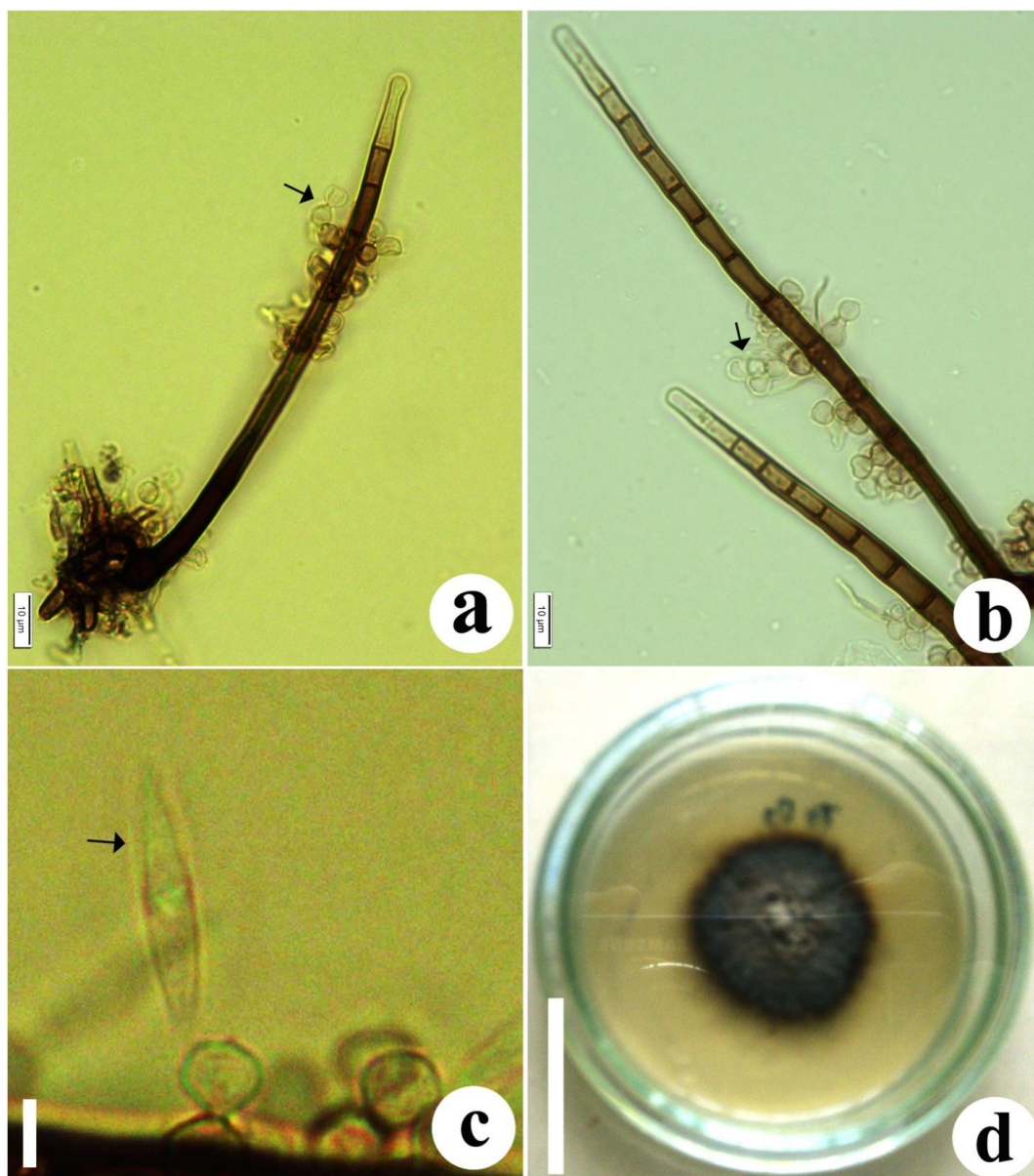
Notes – the morphological differences of *K. fusispora* and *K. ubiensis* were elucidated in detail by Khutubutheen & Nawawi (1993), supported by precise line drawings and microphotographs. The current specimen found in Indonesia apparently resembles *K. ubiensis* due to its conidiogenous cells consistently proliferating repeatedly through one or more of the conidiogenous loci in the previously formed cell to produce branched chains of conidiogenous cells (observation from about 30 individual fruiting bodies) (Fig. 1). In *K. fusispora*, the proliferation of conidiogenous cells occurs occasionally (Kuthubutheen & Nawawi 1993). Another morphological characters resemble *K. ubiensis* includes narrower setiform conidiophores (210 × 5.5–7 μm vs. 200 × 6–9 μm of *K. fusispora*), and smaller conidia (7–17 × 2–4 μm vs. 30–37 × 6–8 μm of *K. fusispora*) (Kuthubutheen & Nawawi 1993).

### Phylogenetic analyses

Alignment of the ITS region contained 100 sequences and 736 total characters, of which 207 characters are constant, 122 characters are variable and parsimony-uninformative, 407 characters are parsimony-informative. From a total 525 of equally parsimonious trees generated by the MP analysis, the best parsimonious tree was generated in 3313 steps (CI= 0.326, RI= 0.598, RC= 0.195, HI= 0.674). The phylogenetic tree confirmed the placement of *K. ubiensis* within family Amphisphaeriaceae. The sequence of *K. ubiensis* showed a close phylogenetic relationship to *Polyscytalum fecundissimum* (EU035441) and *Phlogicylindrium* spp. [*Ph. eucalypti* (DQ923534), *Ph. eucalyptorum* (EU040223), *Ph. eucalyptorum* (EU040222), *Ph. uniforme* (JQ044426)] with 54% BS (Fig. 2).

### Discussion

In the original publication of the genus *Kiliophora*, Khutubutheen & Nawawi (1993) did not mention any information regarding its taxonomic position. At the moment, *Kiliophora* is noted as hypomycetous fungus anamorphic of Pezizomycotina due to lack of information of teleomorphic state (Kirk et al. 2008). Our BLAST result of *K. ubiensis* ITS sequence showed highest similarity to



**Fig. 1** – a–d *Kiliophora ubiensis*. a Proliferating conidiogenous cells (arrow). b Branched proliferation of conidiogenous cells (arrow). c Conidium (arrow). d 14-days old colony on PDA. – Bars c = 4μm, d = 40mm.

sequences belonging to *Phlogicylindrium* spp., *Beltrania* spp., *Beltraniella portoricensis* (GU905993), and *Polyscytalum fecundissimum* (EU035441) with only 89–90% similarity (data not shown). These genera have been known as anamorphic of Amphisphaeriaceae. Among them, only genus *Phlogicylindrium* belongs to coelomycetous fungus (Crous et al. 2007). Based on the BLAST result, we constructed preliminary phylogenetic analyses involving large number sequences of taxa belong to Pezizomycotina. The analyses showed that *Kiliophora* nested in the Ordo Xylariales (data not shown). This information was further used to construct molecular phylogenetic analyses involving members of Xylariales. The phylogenetic trees clearly showed that sequence of *K. ubiensis* nested within the members of Amphisphaeriaceae (Fig. 2). However, the sequence of *K. ubiensis* showed no distinct association to recognized teleomorphic genera within Xylariales.



**Fig. 2** – Phylogenetic tree generated from Maximum Parsimony (MP) analyses of the ITS rDNA involving 107 sequences from the ordo Xylariales including outgroups. Family placement of the sequences were referred to Lumbsch & Huhndorf (2007), Kirk et al. (2008), Tanaka et al. (2011), and Jaklitsch & Voglmayr (2012). Percentage bootstrap support ( $\geq 50\%$ ) is shown on the branches.

The tree generated from ITS region showed a close phylogenetic affinity of *K. ubiensis* to the members of *Phlogicylindrium* and *Polyscytalum* (Fig. 2). However, *Phlogicylindrium* spp. are morphologically distinct from *K. ubiensis* by having conidiomata, cylindrical–hyaline conidia, and not denticulate conidiogenous cells. The setae in *Phlogicylindrium* spp. is also absent. Members of *Phlogicylindrium* have been known as foliar plant pathogens (Summerell et al. 2006, Crous et al. 2007, 2011), while the members of *Kiliophora* have been known as saprobes on decayed wood (Khutubutheen & Nawawi 1993). Another phylogenetically close taxon, *P. fecundissimum*, is morphologically similar to *K. ubiensis* due to having sympodial–denticulate conidiogenous cells, but differs to *K. ubiensis* due to lacking of setae and conidia formed in acropetal chains (Ellis 1971).

Four hyphomycetous genera—*Beltrania* Penz., *Beltraniella* Subram., *Beltraniopsis* Bat. & J.L. Bezerra, and *Subramaniomyces* Varghese & V.G. Rao—were morphologically found comparable to *Kiliophora* (Seifert et al. 2011). Morphological characteristics of dark and sterile setae, with denticulus conidiogenous cells that formed from the conidiophores, and biconic conidia of *Beltrania*, *Beltraniella*, and *Beltraniopsis* clearly resemble the genus *Kiliophora* (Seifert et al. 2011). However, these beltranioid fungi are morphologically distinct from *Kiliophora* by having brown conidia with hyaline equatorial band. The genus *Subramaniomyces* is similar to *Kiliophora* by having denticulus conidiogenous cells and biconic conidia, but differs due to lacking of setae and having basal ramoconidia (Seifert et al. 2011). In our phylogenetic analyses, *K. ubiensis* was found paraphyletic to the sequences of *S. fusisaprophyticus* (Matsush.) P.M. Kirk (EU040241), *Beltraniella portoricensis* (GU905993), and *Beltrania* spp. within family Amphisphaeriaceae (Fig. 2). Shirouzu et al. (2010) previously also reported the placement of the beltranioid fungi—*Beltrania*, *Beltraniella*, *Beltraniopsis*—and *S. fusisaprophyticus* within family Amphisphaeriaceae (Xylariales), and noted that *Beltraniella* is linked to *Pseudomassaria* Jacz. (Shirouzu et al. 2010, Hyde et al. 2011). All of these hyphomycetous genera having denticles conidiogenous cells that formed from the conidiophores.

According to Seifert et al. (2011), another hyphomycetous genera such as *Chaetopsina* Rambelli, *Chaetopsis* Grev., *Zanclospora* S. Hughes & W.B. Kendr., and *Spondylocladiopsis* M.B. Ellis are also morphologically similar to *Kiliophora* by having setiform conidiophores, conidiogenous cells formed from the conidiophores, and amero, hyaline conidia. However, *Kiliophora* is distinguishable from *Chaetopsina*, *Chaetopsis*, *Zanclospora*, and *Spondylocladiopsis* due to its denticle and non–phialide conidiogenous cells and biconic conidia with minute polar extensions. Currently, *Chaetopsina* and *Zanclospora* are placed in Nectriaceae (Hypocreales) (Luo & Zhuang 2010) and Chaetosphaeriaceae (Chaetosphaeriales) (Fernández et al. 2006), respectively, based on morphology and molecular phylogenetic analyses of ribosomal DNA. The taxonomical status of *Chaetopsis* and *Spondylocladiopsis* have been noted as *incertae sedis* in fungal taxonomy (Hyde et al. 2011).

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