Phylogenetic relationships of *Astrocystis eleiodoxae* sp. nov. (Xylariaceae)

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An ascomycete with morphological similarities to *Astrocystis* (Xylariaceae) was collected from the peat swamp palms *Eleiodoxa conferta* and *Licuala longicalycata* in southern Thailand and is introduced here. The new taxon is characterized by carbonaceous black stromata on persistent white hyphae, and brown ascospores surrounded by a thin mucilaginous sheath and with a longitudinal germ slit. No anamorph was observed in nature or in culture. Phylogenetic relationships were investigated based on ITS1-5.8S-ITS2, partial LSU and SSU rDNA sequences using maximum parsimony and Bayesian analyses. Phylogenetic analyses of the ITS regions places the taxon in Xylariaceae, in a clade comprising *Astrocystis eleiodoxae* and *Stilbohypoxylon elaeicola* with good support and a sister group to *Astrocystis, Kretzschmaria, Rosellinia* and *Xylaria* species, with moderate support. LSU and SSU rDNA data places *A. eleiodoxae* in a clade with *A. cocoës, Rosellinia necatrix* and *Stilbohypoxylon elaeicola* in the *Xylariaceae.* The data indicates a relationship between *A. eleiodoxae* and *Stilbohypoxylon elaeicola*. There are no other *Astrocystis* sequence data available in GenBank, and based on molecular data shown here and morphological data we described *Astrocystis eleiodoxae* as a new species.

Key words – molecular phylogeny – new species – palm fungi – *Stilbohypoxylon*

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Introduction

A study of saprobic fungi on the peat swamp palms *Eleiodoxa conferta* and *Licuala longicalycata* (Pinnoi et al. 2006; Pinruan et al. 2007) yielded a new xylariaceous ascomycete with morphological similarities to *Astrocystis*, *Nemania* and *Stilbohypoxylon*. Taxa from palms within these and related genera have been reviewed by Smith et al. (2001) and based on morphological characteristics our taxon most closely resembles *Astrocystis*.

Astrocystis is a genus mostly confined to monocotyledons and has uni- rarely multi-peritheciate stromata, which may develop beneath the host cuticle and appear superficial. The asci have a relatively short stipe and the ascal ring is relatively small, amyloid and stopper-shaped (Smith et al. 2001). A key to six accepted species was provided by Smith et al. (2001).

This study introduces a new species, *Astrocystis eleiodoxae* and explores the phylo-

genetic relationships of this and related species based on rDNA sequences of the ITS1-5.8S-ITS2 and partial LSU and SSU genes.

Methods

Sample collection, fungal isolation

Decaying petioles of the palm *Eleiodoxa conferta* were collected from Sirindhorn Peat Swamp Forest, Thailand. Palm material was placed in sterile plastic bags, returned to the laboratory and incubated in moist plastic boxes at 25°C. Fungi were observed under a stereomicroscope, and then measured and illustrated under a compound microscope. All morphological measurements were carried out in sterile water, with a mean from 25 measurements for most characters. Melzer's reagent was used to test the amyloidity of the apical ring and 10% KOH for testing the dehiscence of the perispore. A single spore technique was used for isolation of the species. Axenic cultures were grown on potato dextrose agar medium (PDA) for 2−3 weeks and used for the molecular study. Herbarium specimens and living cultures are deposited in the BIOTEC Herbarium (BBH) and Culture Collection (BCC), respectively.

DNA extraction, amplification and sequencing

DNA extraction was performed by following a modified protocol as defined and outlined previously (Jeewon et al. 2004, Wang et al. 2005, Pinruan et al. 2007, Promputtha et al. 2007). Three different regions of the rDNA gene (characterised by different rates of evolution) were amplified. Primer pairs NS1 (5′−GTAGTCATATGCTTGTCTC−3′) and NS4 (5′−CTTCCGTCAATTCCTTTAAG−3′) primer pairs were used for the small 18S subunit (White et al. 1990). LROR (5′−ACCCGCTGAACTTAAGC−3′) and LR5 (5′−TCCTGAGGGAAACTTCG−3′) primer pairs were used to amplify a segment of the large 28S subunit (about 900 nucleotides) (Vilgalys & Hester 1990). ITS4 (5′−TCCTCC GCTTATTGATATGC−3′) and ITS5 (5′−GGA AGTAAAAGTCGTAACAAGG−3′) as defined by White et al. (1990) were used to generate about 550 nucleotides from the complete ITS including 5.8S regions. The amplification conditions were performed in a 50 μL reaction volume as follows: 1 × PCR buffer, 0.2 mM each dNTP, 0.3 M of each primer, 1.5 mM $MgCl_2$, 0.8 units Taq Polymerase and 10 ng DNA. PCR parameters for all the regions were as follows: initial denaturation 94ºC for 3 minutes, 35 cycles of 94ºC for 1 minute, 52ºC for 50 seconds, 72ºC for 1 minute, final extension of 72ºC for 10 minutes. Characterization of PCR products was done via agarose gel electrophoresis on a 1% agarose gel containing ethidium bromide as the staining agent. DNA sequencing was performed using primers as mentioned above in an Applied Biosystem 3730 DNA Analyzer at the Genome Research Centre (University of Hong Kong).

Phylogenetic analysis

DNA sequences were aligned using BioEdit (Hall 2005) and Clustal X 1.83 (Thompson et al. 1997) with other sequences obtained from GenBank. A blast search was performed to find the possible sister groups of the newly sequenced taxa. In addition, fungal members from different families of the order Xylariales and related orders were also included in the analyses. Phylogenetic analyses were performed using PAUP* version 4.0b10 (Swofford 2002). Prior to phylogenetic analysis, ambiguous sequences at the start and the end were deleted and gaps manually adjusted to optimize alignment. Maximum parsimony analyses were conducted using heuristic searches as implemented in PAUP, with the default options method. Analyses were done under different parameters including unweighted parsimony, and weighted parsimony criteria. Clade stability was assessed in a bootstrap analysis with 1000 replicates, random sequence additions with maxtrees set to 1000 and other default parameters as implemented in PAUP*. Kishino-Hasegawa (KH) tests (Kishino & Hasegawa 1989) and Templeton tests (Templeton 1983), were performed in order to determine whether trees inferred from the different tree building methods were significantly different. The Bayesian analyses were conducted with the Markov chains run for 1000000 generations.

Trees were viewed in Treeview (Page 1996). The nucleotide sequences reported in this paper have been deposited in GenBank.

Results

Astrocystis eleiodoxae A. Pinnoi, E.B.G. Jones & K.D. Hyde, **sp. nov.** Figs 1–14 MycoBank 513077.

Etymology – *eleiodoxae* refers to the palm host.

Stromata perithecilis similia, superficialis, solitaria vel gregaria, atra, carbonacea, 825–1375 µm diam. × 250–375 µm alta, subglobosa. Peridio 22.5–62.5 µm latus, aliquot strata ex compressus cellular, atro. Paraphysa 2 µm latus, filamentosae, septatae, numerosa. Asci 107.5–155 × 6.2–10 µm, 8-spori, cylindrici, pedicellati, unitunicati, truncata ad apicem, 5 × 2.5 µm, annulo apicali in liquore iodato Melzeri cyanescente. Ascosporae brunnae, inequilaterallis ellipsoidae, 17.5–23 × 4.5–6.2 µm, exiguus curvata, unicellulae, leavia piries.

Holotypus – BBH9822.

Stromata semi-superficial, solitary or gregarious, black, shiny, carbonaceous, without apparent KOH-extractable pigments, synnemata absent; covering 1–3 perithecia; in vertical section 825–1375 µm diam., 250–375 µm high, subglobose (Figs 1–3). Peridium 22.5–62.5 µm ($\overline{x}$ = 32 µm, n = 15) wide, comprising several layers of compressed cells, black (Fig. 3). Paraphyses 2 µm wide, filamentous, septate, numerous and embedded in a gelatinous matrix (Figs 2, 4). Asci 107.5–155 × 6.2–10 µm ($\overline{x}$ = 131 × 8.5 µm, n = 25), 8−spored, cylindrical, relatively short, apically truncate with a 5 × 2.5 µm wedge-shaped, J+, subapical ring (Figs 5–8). Ascospores 17.5–23 × 4.5–6.2 µm ($\overline{x}$ = 19 × 5 µm, n = 25), uniseriate, brown, inequilaterally ellipsoidal, slightly curved, unicellular, smooth-walled, germ slit full-length and with a thin mucilaginous sheath (Figs 9–14).

Colonies of *A. eleiodoxae* on PDA at 25ºC are relatively fast growing, white, effuse, producing globose structure, black, carbonaceous in the center within 1 month but not sporulating.

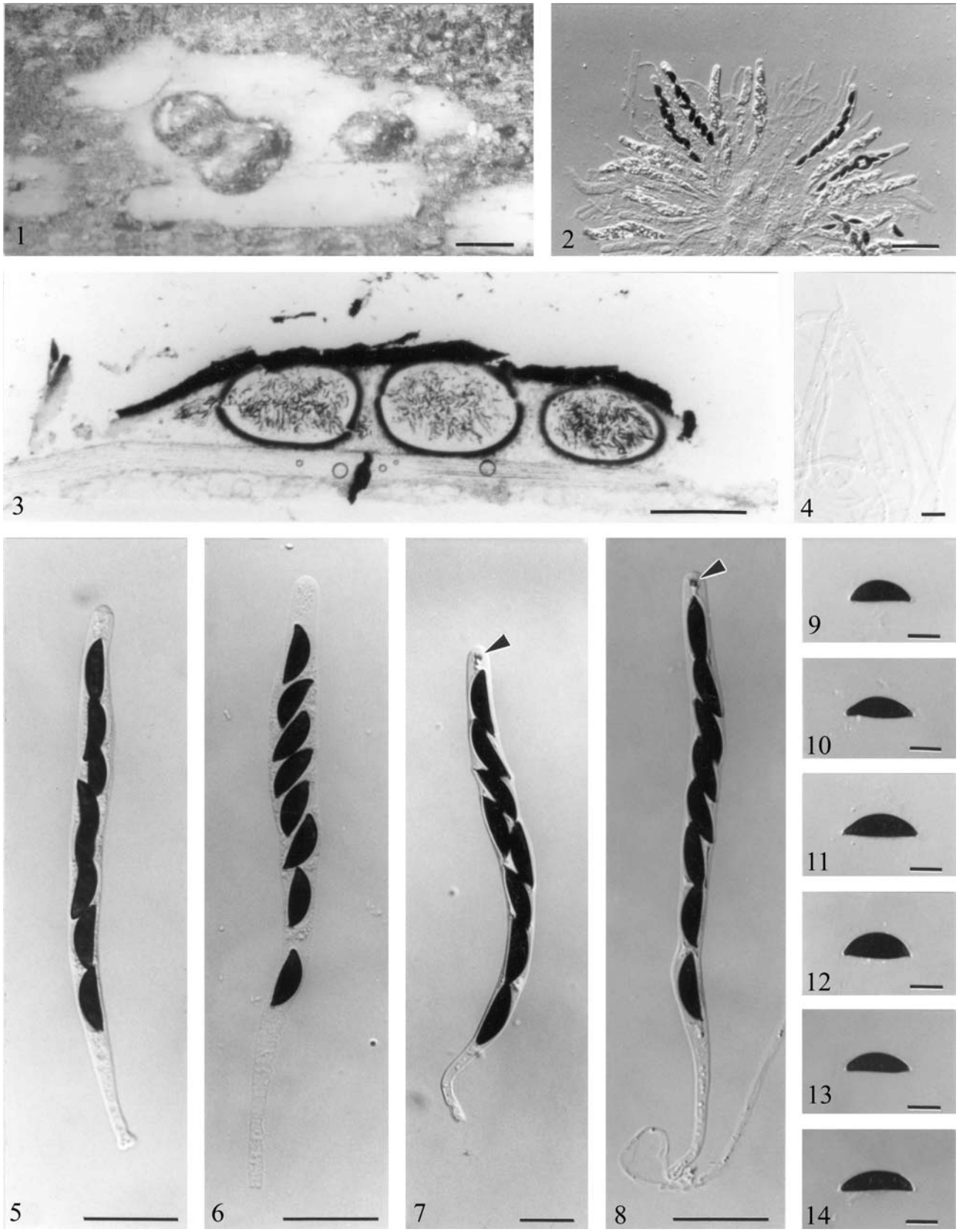
Material examined – Thailand, Narathiwat, Sirindhorn Peat Swamp Forest, on submerged petiole of *Eleiodoxa conferta*, 12 May 2001, A. Pinnoi, BBH 9822 (holotype) – ex-type cultures BCC 12874 and BCC 12875; ibid., on submerged petiole of *Eleiodoxa conferta*, 22 June 2001, A. Pinnoi, BBH 9825 – cultures BCC 12512.

SSU based phylogenies

The 18S dataset contained 25 taxa including 830 characters with 114 parsimony informative sites, 62 parsimony uninformative sites and 654 constant characters, with *Dothidea sambuci* as the outer group (not shown). Unweighted parsimony analysis (with gap treated as missing character), which yielded 6 parsimonious trees of 903.7 steps with CI, RI, RC and HI of 0.667, 0.771, 0.514 and 0.333 respectively. Bootstrap values (generated from 1000 replicates) and Bayesian posterior probabilities were generated from 1000000 generations. *Astrocystis eleiodoxae* is a member of the family Xylariaceae, order Xylariales and clustered with *Stilbohypoxylon elaeicola*, *Rosellinia necatrix* and *Astrocystis cocoës* with high support, with *Xylaria hypoxylon* as a sister group. The *Astrocystis* species are in a monophyletic sub-clade (Xylariaceae) with Amphisphaeriaceae as a sister group in the Xylariales clade. Phylogenetically the Xylariales are distinct from other orders included in the analysis, with high support (92%).

LSU based phylogenies

The 28S DNA matrix consisted of 33 taxa with *Dothidea sambuci* as an outgroup. The aligned dataset was 835 characters, out of which 207 were parsimony informative, 77 parsimony uninformative and 551 constant characters. The tree shows 737 steps with CI, RI, RC and HI of 0.541, 0.713, 0.386 and 0.459, respectively (not shown). Bootstrap values (generated from 1000 replicates) and Bayesian posterior probabilities were generated from 1000000 generations. *Astrocystis eleiodoxae* and *S. elaeicola* grouped with *Rosellinia necatrix* and *Astrocystis cocoës* with high bootstrap support and Bayesian posterior probabilities support of 100%. *Astrocystis eleiodoxae* and *S. elaeicola* clustered together with weak parsimony bootstrap support and Bayesian posterior probabilities. *Stilbohypoxylon quisquiliarum* was distantly placed from



Figs 1–14 – Light micrographs of *Astrocystis eleiodoxae* (from holotype). **1** Stroma on natural substrata. **2** Squash mount illustrating asci and paraphyses. **3** Section of stroma with ascomata. **4** Paraphyses. **5–6** Asci with relatively short stipe. **7–8** Asci with J+ stopper-shaped apical ring (arrowed). **9–14** Ascospores. Bars 1 = 1 mm, 2 = 100 µm, 3 = 1 mm, 4 = 5 µm, 5–8 = 25 µm, 9–14 = 10 µm.

Astrocystis eleiodoxae and *Stilbohypoxylon elaeicola* and grouped with *Xylaria* and *Nemania* species in a poorly supported subclade.

ITS based phylogenies

The ITS data consisted of 36 taxa with *Diatrype disciformis* as an outgroup. The aligned dataset was 772 characters, out of which 330 were parsimony informative, 114 parsimony uninformative and 328 constant characters (Fig. 15). Clade A comprises two *Xylaria*, two *Kretzschmaria* species and *Stilbohypoxylon quisquiliarum*. Clade B consists of *Astrocystis* and *Rosellinia* species and *Halorosellinia oceanica*. Clade C includes two strains of *Astrocystis eleiodoxae* that are monophyletic and *Stilbohypoxylon elaeicola* with high bootstrap support. Clade D is a well supported *Nemania* group, while clade E constitutes *Nemania maritima*, *N. confluens*, *Rosellinia aquila*, *R. pepo* and *Astrocystis cocoës*. Clade F (*Daldinia* species) and G (*Hypoxylon* and *Annulohypoxylon* species) are basal to the family (Fig. 15).

Combined 28S and ITS1-5.8S-ITS2 based phylogenies

The combined dataset consisted of 27 taxa with *Pestalotiopsis versicolor* as an outgroup. The aligned dataset was 1746 characters. The tree obtained was 737 steps with CI, RI, RC and HI of 0.583, 0.510, 0.297 and 0.417, respectively. Six Xylariaceae clades are identified, all with high bootstrap support (Fig. 16). Clade F is basal to the family and comprises *Annulohypoxylon* and *Hypoxylon* species in subclades, while clade E consists of *Biscogniauxia* species. Clade A has high support with *Rosellinia* and *Astrocystis* species. Clade B comprises *Astrocystis eleiodoxae* which clusters with *S. elaeicola* with weak support. *Halorosellinia oceanica* and *Rosellinia necatrix* form a sister group. Clade D supports *Stilbohypoxylon quisquiliarum*, *Kretzschmaria deusta* and *Xylaria grammica*. Three *Nemania* species constitute a well-supported monophyletic Clade C that is phylogenetically distinct from the other xylariaceous genera.

Discussion

Astrocystis eleiodoxae possesses several morphological characters which it shares with the other six *Astrocystis* species. These include a raised stroma under which the ascomata develop, an ascus with a relatively short stipe, a J+ wedge-shaped subapical ring and brown ascospores with a germ slit. It is however different from any of the accepted species in the monograph of the genus by Smith et al. (2001) and is thus described here as a new species.

Sequences data from 18S rDNA and 28S rDNA confirm the monophyly of the two *Astrocystis eleiodoxae* strains isolated on different occasions, and are well positioned in the Xylariaceae. The species clustered with *Astrocystis cocoës* and *Rosellinia necatrix* with high Bayesian posterior probabilities support 99% (data not shown).

Tang et al. (2007, 2009) have used various genes to test their use in separating taxa in the Xylariaceae. The phylogeny of several xylariaceous genera has also been evaluated using protein-coding genes, such as β-tubulin and α-actin genes (Hsieh et al. 2005). These gene regions may be particularly useful since limited success has been achieved in delineating genera and resolving generic relationships based on ribosomal DNA genes (Sánchez-Ballesteros et al. 2000, Smith et al. 2003, Triebel et al. 2005, Peláez et al. 2008). Unfortunately this study could not utilize these genes as there are no sequences in GenBank representing the genes of genera discussed here. The phylogenetic results therefore provide little indication of the genera to which this new taxon belongs, especially as the identification of those sequences used from GenBank in this study could not be verified (Zhang et al. 2008).

Petrini (2004) undertook a revision of *Stilbohypoxylon* accepting ten species and providing a key to the taxa. In her paper an earlier epithet '*elaeicola*" in *Rosellinia elaeicola* Henn. was used to represent *S. moelleri* Henn and *Astrocystis cocoës* was considered a synonym. *Stilbohypoxylon* stromata are characterized by spine-like synnemata covered with yellow granules (Fröhlich & Hyde 2000). As stromata mature

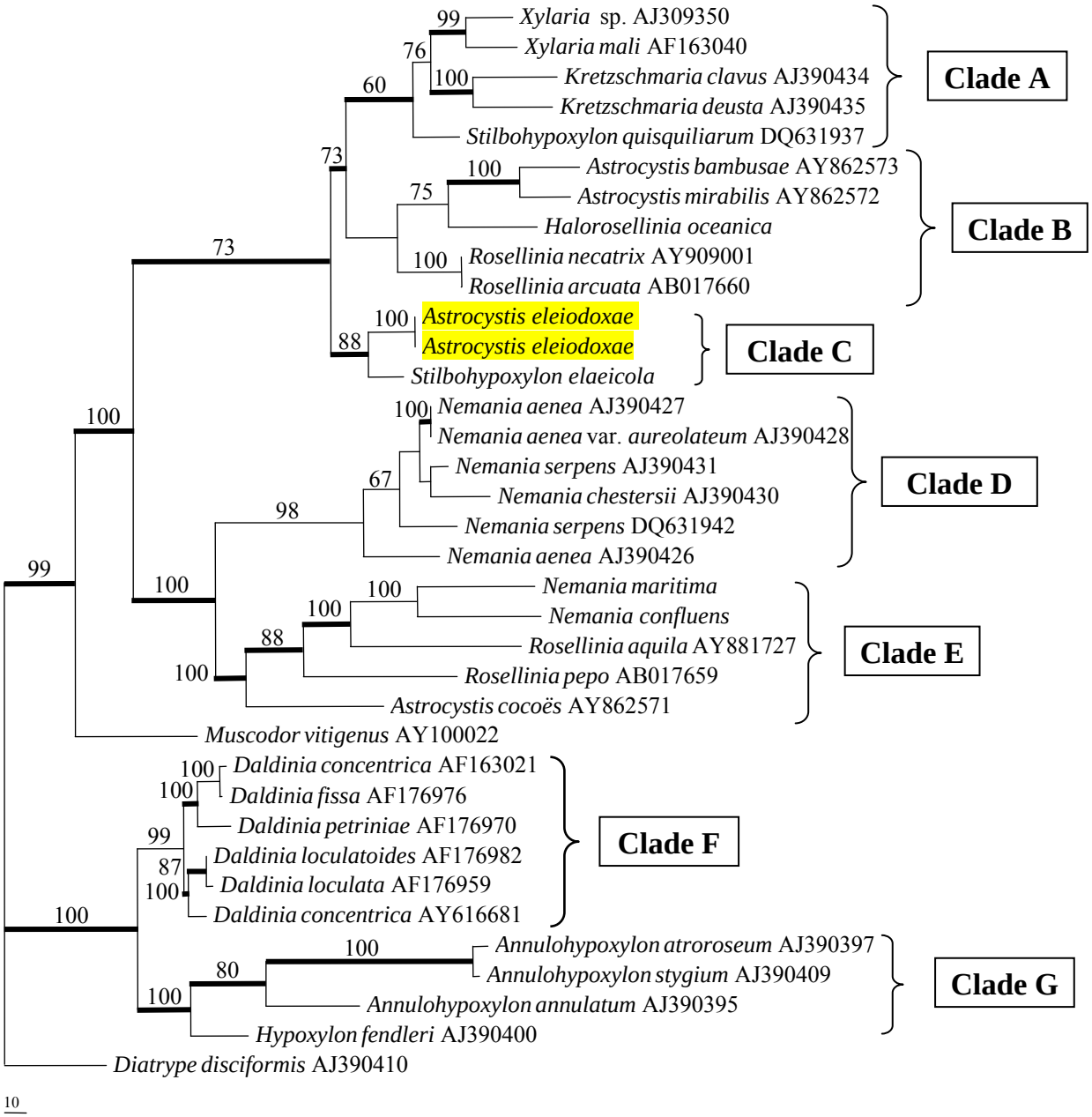


Fig. 15 Phylogenetic tree based on ITS1-5.8S-ITS2 sequences. The tree is rooted with *Diatrype disciformis* and constructed under un-weighted maximum parsimony criterion. The number at each branch point represents percentage bootstrap support calculated from 1000 replicates and Bayesian posterior probabilities (thickened branches). Branch lengths are proportional to the numbers of nucleotide substitutions and are measured by scale bar (Bar; 10 % sequence divergence).

these may be lost and this may lead to confusion in identification. Petrini (2004) found these spines to be present in some collections but not others while in the material examined by Smith et al. (2001) spines were lacking.

In the case of the new species described here, there were no spines or yellow granules at any stage of development and for this reason the taxon is best placed in *Astrocystis*. Whether *Astrocystis cocoës* belongs in *Astrocystis* or *Stilbohypoxylon* has yet to be resolved. Furthermore, whether *Stilbohypoxylon* species should all be transferred to *Astrocystis* needs consideration following re-examination of the type of *Astrocystis, A. mirabilis* Berk. & Broome. What is clear is that the phylogeny of these tropical xylariaceous species is a long way from being resolved and requires chemical as well as molecular data.

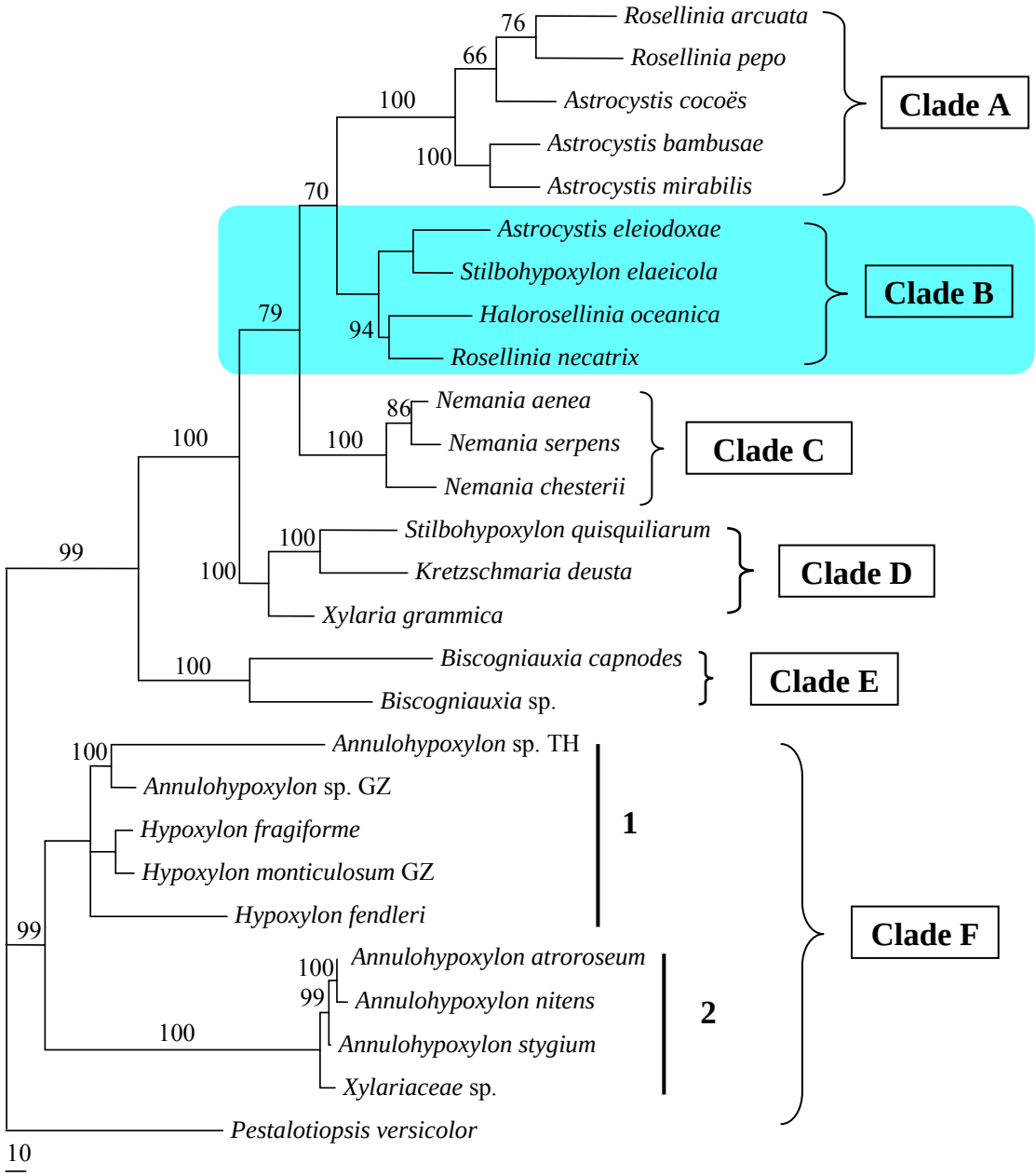


Fig 16. Phylogenetic tree based on combined 28S and ITS1-5.8S-ITS2 sequences. The tree is rooted with *Pestalotiopsis versicolor* and constructed under un-weighted maximum parsimony criterion. The number at each branch point represents percentage bootstrap support calculated from 1000 replicates and Bayesian posterior probabilities (thickened branches). Branch lengths are proportional to the numbers of nucleotide substitutions and are measured by scale bar (Bar; 10 % sequence divergence).

Petrini (2004) reduced *Astrocystis cocoës* to synonymy with *S. elaeicola*, but molecular data presented here does not support this taxonomic assignment. Ju & Rogers (2002) have suggested that *N. maritima* and *N. confluens* are not well placed in the genus *Nemania* and in the ITS data set they group with *Rosellinia aquila*, *R. pepo* and *Astrocystis cocoës*. The phylogenetic relationships of both these groups warrant further study.

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