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Thielaviopsis spp. from Salak [*Salacca zalacca* (Gaerntn.)Voss] in Indonesia

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Abstract *Thielaviopsis* sp. Anamorph *Ceratocystis* sp. is an important agricultural pathogen fungal genus. Cultural characteristics showed two different groups recorded as brown and black colonies on the surface of the artificial media. Conidial structures showed morphology typical of *Thielaviopsis*. DNA sequences were generated for Internal Transcribed Spacer regions (ITS1 and ITS2). Morphology and molecular analyses showed the causal agents of rotten Salak were *Thielaviopsis paradoxa* and *T. ethacetica*. The descriptions of the fungi were illustrated in this study.

Keywords: *Ceratocystis*, fruit, molecular, morphology, sequence

Introduction

Salak is a fruit plant native to Indonesia. The Varieties of Salakare Bali, Condet, Pasaman, Pondoh and Sidempuan (Santoso *et al*., 2017). Whereas the good variety, the fruit is easily rotten, generally caused by fungi especially mold. The fungi commonly found in Salak namely, *Aspergillus* spp., *Ceratocystis* spp. (*Thielaviopsis* spp.), *Fusarium* spp. (Liptan, 2000). The identification of the contaminating molds is very useful to prevent and overcome the contamination of the fungus in the salak fruit, especially the fruit that is exported. From the previous experiment, there were two species of *Thielaviopsis* spp., one of which resembled *Bahusakala* sp. on morphology examination but with the aid of DNA sequencing, found *Thielaviopsis* sp.

Objectives of the research were to isolate and identify the *Thielaviopsis* species on Salak in Indonesia.

Materials and methods

Collection and isolation

Materials of this study were collected in Indonesia at fruit traditional market between April-October 2017, during field surveys for *Thielaviopsis*

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species occurring on salak (*Salacca zalacca*). The surveys were conducted at various cities of Bogor, Jakarta, Depok in Indonesia. Isolations were made by aseptically cutting aerial mycelium from the surfaces of infected rotten fruit with a sterile needle and transferring these to sterile SDA (Soboroud Dextrose Agar), amended with antibiotics. Sub culturing from single hypha tips purified isolates further and they were maintained on PDA (Potato Dextrose Agar).

DNA extraction, PCR and sequencing

DNA extraction, PCR and sequencing, DNA was extracted from 7-day-old cultures maintained on PDB (*Potato Dextrose Broth*) at 25 ℃. Mycelia of fungi were grown on GDP media is then harvested and extracted DNA using a nucleotide reagent PHYTOpure (Amersam LIFE SCIENCE). PCR amplification on ITS using primer ITS 4: 5'-TCC TCC GCT TAT TGA TAT GC-3 'and primer ITS 5: 5' - AGT AAA AGT CGT AAC AAG G-3' (White *et al*., 1990). Purification of product PCR was performed using PEG Precipitation methods (Hirashi, 1995) followed by cycle sequencing. The molecular analyses followed Wulandari *et al.* (2009). *Ceratocystis virescens*, isolate CMW 11164 was used as the out-group taxon in the analyses. Isolates used in this study listed in table 1.

Phylogenetics analysis

ITS sequences were aligned with MUSCLE (Edgar, 2004) as implemented in MEGA 7 and a neighbor-joining (NJ) phylogenetic tree was generated with MEGA. Representative sequences from each resulting group were submitted to BLASTN query in GenBank on NCBI (http://www.ncbi.nlm.nih.gov). The same BLASTN procedure was applied to the ITS sequences of all isolates obtained from the culture collections to confirm their identities. Datasets, including sequences generated in this study and relevant GenBank accessions. *Ceratocystis virescens*, isolate CMW 11164 was used as the outgroup taxon in the analyses. Alignments were constructed with MAFFT 6 (http://www.align.bmr.kyushu-u.ac.jp/ mafft/online/server/) and trimmed and assemble in Chromas Pro.

Results

Thielaviopsis Went, Meded. Proefstat. Suikerriet W. Java 5:4. 1893

= *Hughesiella* Bat. & A.F. Vital, Anals Soc. Biomol. Pernambuco 14: 141. 1956. (type species Hu. Euricol).

Type species: *Thielaviopsis ethacetica* Went, Meded. Proefstat. Suikerriet W. Java 5: 4. 1893

Emended generic diagnosis. *Ascomatal bases* globose, light brown, display dark as result of aleurioconidia and distinctly digitate or stellate appendages. *Ascomatal necks* long, tapering to apex, dark grey. *Ostiolar hyphae* divergent, hyaline. *Asci* dehiscent. *Ascospores* aseptate, ellipsoidal, hyaline with sheath. *Conidiophores* lageniform, solitary, occasionally aggregate in synnemata. *Primary conidia* aseptate, cylindrical, hyaline. *Secondary conidia* aseptate, cylindrical to oblong, hyaline becoming grey, thick-walled. *Aleurioconidia* subglobose, oblong or ovoid, thick-walled, forms holoblastically, singly or in chains, grey-brown.

Thielaviopsis ethacetica Went, Meded. ProefstnSuikerriet W. Java 5: 4. 1893. Fig. 1

= *Endoconidiumfragrans*Delacr., Bull. Soc. Mycol. Fr. 9:184. 1893

= *Catenulariaechinata*Wakker in Wakker& Went, de Ziekten van het Suikerriet op Java, EJ Brill, Leiden p 196. 1898.

Ascomatal bases fully or partially submerged in substrata, mostly globose, appearing dark in old cultures when surrounded with aleurioconidia and ascomatal appendages. *Ascomatal appendages* stellate or digitate, mostly restricted to aerial parts of partially submerged ascomatal bases. *Ascomatal necks* dark mouse gray, erect. *Ostiolar hyphae* hyaline, divergent. *Asci* not observed. *Ascospores* not observed. *Conidiophore*s mostly hyaline, phialidic, lageniform, mononematous with enteroblasticconidium ontogeny, solitary. *Primary conidia* hyaline, aseptate, cylindrical, 7–16 × 4–7 µm. *Secondary conidia* aseptate, initially hyaline, turning grayish sepia, thick-walled at maturity, cylindrical to oblong, 5–10 × 2–6 µm. *Aleurioconidia* produced holoblastically, singly or in chains, grayish sepia to umber, granulated, thick-walled, subglobose, oblong or ovoid.

Colonies on *SDA* initially hyaline to white, progressively darkening, turning yellow, or brown in the center and white in the edge after 10 d, reverse brown. Mycelium aerial and submerged, hyphae hyaline, smooth, often terminating as conidiophores, septate, no constriction at septa.

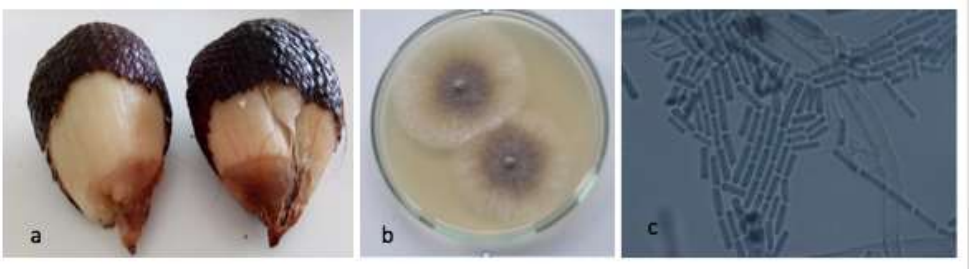

Figure 1. *Thielaviopsis ethacetica.* a. Fruit rot with brown mycelia, b. Brown colony in *SDA*, c. Conidia (M : 1000×)

Thielaviopsis paradoxa (De Seynes) Höhn., Hedwigia 43: 295. 1904. Fig. 2
Basionym: *Sporochisma paradoxum* De Seynes, Rech. Hist. Nat. Veg. Inf. 3: 30. 1886

= *Sporoschismaparadoxum* de Seynes, Recherches pour Servira` l'HistoireNaturelle des Ve´ge´tauxInfe´rieurs 3:30. 1886. (basionym)
= *Chalaraparadoxa* (de Seynes) Sacc., Syll. Fung. 10:595. 1892.
= *Ceratostomellaparadoxa* (de Seynes) Dade, Trans. Br. Mycol. Soc. 13:191. 1928.
= *Ophiostomaparadoxum* (de Seynes) Nannf., In Melin&Nannf., SvenskaSkogsvFo¨r. Tidskr. 32:408. 1934.
= *Endoconidiophoraparadoxa* (de Seynes) R.W. Davidson, J. Agric. Res. 50:802. 1935.
= *Stilbochalaradimorpha*Ferd. &Winge, Bot. Tidsskr.30:220. 1910.

Ascomatal bases fully or partially submerged in substrata, mostly globose, partially or completely covered by aleurioconidia and ascomatal appendages. *Ascomatal appendages* digitate, (mostly on exposed areas of ascomatal bases. Ascomatal necks erect, long wide at apices; bases of the necks occasionally swollen, forming collar-like structures. *Ostiolar hyphae* hyaline, divergent. *Asci* not observed. *Ascospores* not observed. *Conidiophores* hyaline to grayish sepia, phialidic, lageniform, mononematous with enteroblasticconidium ontogeny, commonly solitary, but occasionally aggregated in synnemata, variable in size. *Primary conidia* hyaline, aseptate, cylindrical, 8–20 × 4–6 µm. *Secondary conidia* aseptate, initially hyaline, turning grayish sepia to umber, thick-walled when mature, cylindrical to oblong. *Aleurioconidia* produced holoblastically, singly or in short chains, dark mouse umber, granulated, thick-walled, and mostly oblong to subglobose, 4–8 × 8–16 µm.

Colonies on *SDA* initially hyaline to white, becoming dark green-olivaceous or black after 10 days, reverse black. Mycelium aerial, submerged, hyphae hyaline, smooth, often terminating as conidiophores, septate, no constriction at septa.

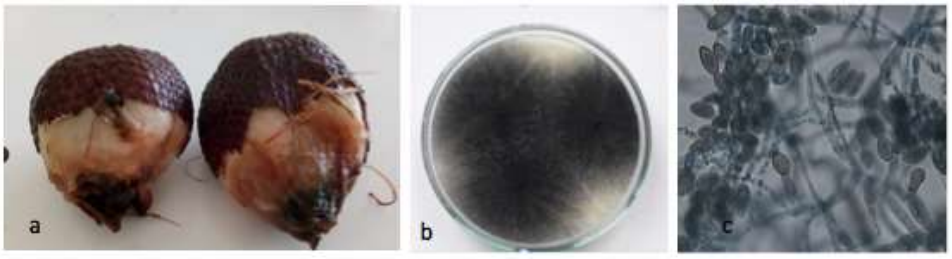

Figure 2. *Thielaviopsis paradoxa.* a. Fruit rot with brown to black mycelia, b. Black colony in *SDA*, c. Conidia (M : 1000×)

Molecular phylogenetic analyses

The dataset contains thirteen sequences of *Thielaviopsis* species, *Ceratocystis virescens* as an outgroup (Fig. 3). Species name, culture number, host name, genBank accession no and origin of the species are presented in table 1.

Table 1. Isolates used in this study

Species name	**No of culture**	**Host**	**GenBank Accession No**	**Origin**
			ITS	
Ceratocystis paradoxa s. str. = *Thielaviopsis paradoxa*	CMW 36689 (**Epitype**)	*Theobroma cacao*	JX518342	Cameroon
	CMW 36642	*Theobroma cacao*	JX518346	Cameroon
	NFW 234	*Salacca zalacca*	MH094197 (In this study)	Indonesia
Ceratocystis ethacetica =*Thielaviopsis ethacetica*	CMW 37775 = IMI 50560 **(epitype)**	*Ananas comasus*	JX518341	Malaysia
	CMW 3671	*Saccharum* sp.	JX518351	South Africa
	NFW 235	*Salacca zalacca*	MH094196 (in this study)	Indonesia
Ceratocystis cerberus	CMW 36668 **(type)**	*Elaeis guineensis*	JX518348	Cameroon
	CMW 36641	*Elaeis guineensis*	JX518345	Cameroon
	CMW 35021	*Theobroma cacao*	JX518355	Cameroon
Ceratocyctis euricoi	CMW 28537= CBS 893.70 **(Type)**	*Cocos nucifera*	JX518335	Brazil
	CMW 8790	*Cocos nucifera*	JX518327	Indonesia
	CMW 8799	*Cocos nucifera*	JX518328	Indonesia
Ceratocystis virescens	CMW 11164	*Fagus americanum*	U75624.1	USA

1/CABI: Commonwealth Agricultural Bureaux International Bioscience, formerly International Mycological Institute (IMI), CBS: CentraalbureauvoorSchimmelcultures (Westerdijk Institute), CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, NFW: Nilam Fadmaulidha Wulandari Collection.

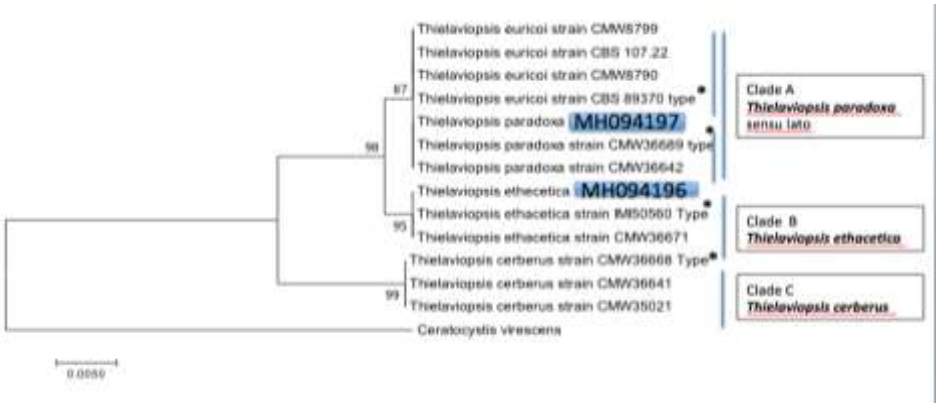

Figure 3. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA7. *: Type species; blue box isolates found in this study.

Discussion

Morphological species concept is characterized a species by body shape and other structural features and is applied to asexual and sexual organisms and useful when information on gene flow is unknown. Since it is subjective, researcher may disagree on which features to use to distinguish a species. Phylogenetic species concept here, species is defined as the smallest group of individuals with a common ancestor, forming a single branch of the "tree of life". This compared numerous characteristics, especially morphology and molecular sequences, with those of other organisms. The difficulty with this concept is determined the degree of difference necessary to indicate a separate species. *Thielaviopsis* spp. on salak is characterized by its morphological and molecular characteristics (Mbenoun *et al*., 2014).

Thielaviopsis species can cause severe disease in plants with widespread around the globe. The fungus produces two different types of asexual spore, endoconidia and chlamydospores. The sexual state rarely found in nature, as *Ceratocystis*. The fungus causes diseases of banana, pineapple, sugarcane, ornamental plants as well as palm (Elliott, 2018).

Thielaviopsis trunk rot of palm (Elliott, 2018); *Thielaviopsis paradoxa* bud rot of *Hyophorbe lagenicaulis* (Soytong *et al*., 2005); *Thielaviopsis paradoxa* complex on oil palm and cacao (Mbenoun *et al*., 2014); *Thielaviopsis paradoxa* stem rot in *Dracaena marginata* (Dos Santos *et al*., 2012). *Thielaviopsis* can also be found on stem, leaves and fruits, causing premature fruit drop (Kile, 1993). Results of this study provided the fungi isolated from Salak.

The other study was to determinate the pathogenic and genetic diversity of *Thielaviopsis paradoxa* from palm oil in Colombia, Ecuador and Brazil. Research showed that the genetic diversity of the isolates is intermediate and predominant compare to *Cerocystis* species. Population structure analyses of RAPD data suggested that all the isolates in this study belonged to a single population. Data on pathogen diversity will provide information about population structure and breeding strategies (Alvarez *et al*., 2012).

Mbenon *et al*. (2014) conducted research on *Ceratocystis paradoxa* complex with multigene phylogenetic analyses of the ITS, β-tubulin and TEF-1α gene loci and combination of morphology and mating study, found four species recognized in the genus, i.e *C. paradoxa*, *C. etachetica*, *C. cerberus*, and *C. musarum*. *C. paradoxa* complex comprises greater species diversity, i.e *C. paradoxa* sensu stricto (sens.stric.) and two of *C. paradoxa* sensu lato (s. lato).

Thielaviopsis genus presented in the numerous important plants pathogens of mainly monocotyledons plants, as the causal agent of many fruit and vegetable rot, pineapple. Research conducted by Wijesinghe *et al.* (2010), fungicidal used from *Trichoderma asperellum* has advantage for the pineapple (*Anansas comasus*) rot. The formulation of *T. asperellum* might be one of the agents for biocontrol for pineapple fruit rot (Wijesinghe *et al.*, 2010). Althought *Thielaviopsis* is one of plant pathogenic fungi, but it has also beneficial funtions as a good candidate for cellulose producer (Sari *et al*., 2017).

The occurance of *Thielaviopsis* spp. in Salak (*Salacca zalacca*)

Thielaviopsis spp. is occurring in Salak because of contamination from soils that have chlamydospore of *Thielaviopsis*. The disease incidence is found frequently when the fruit have wound. Furthermore, the seller or farmer in several traditional fruit markets in Indonesia, especially in West Java and Jakarta, used to apply the fruits with the fertilizer that contains soil and cow dung. The farmer believes the use of fertilizer (soil and cow dung) can enhance and prolong of fruit life (pers. comm.).

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