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Two new records of *Ravenelia* species on legumes in Thailand

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Abstract *Ravenelia* species were found in legume plants (family Fabaceae) in Thailand. *Ravenelia stevensii* occurred on *Pterolobium macropterum*in Lampang and Khonkean provinces, and *Ravenelia neocaledoniensis* on *Vachellia farnesiana* in NakhonPathom province. They produced different sporulation stages and spore morphology on their hosts.*Ravenelia stevensii* produced spore stages including spermogonial, aecial, uredinial and telial stages in the same host (*P. marcopterum*). *Ravenelia stevensii* have prominently appendages on teliospore surface and its structure size was very closely to voucher specimens.The rust fungus, *R. neocaledoniensis* was found only uredinial stage on *V. farnesiana*. The species were identified confirmation bymorphology and phylogenetic analysis using 28S rDNA. The results showed that *R. neocaledoniensis* was grouped together with the sequence obtained from GenBank database. The four squences of *R. stevensii* were clustered and separated from others *Ravenelia* species with 100% bootstrap value supporting. These rustfungi and host taxa were reported as a new record not only in Thailand but also in Southeast Asia.

Keywords: Geographic distribution, Pucciniales, Rust fungi, Taxonomy

Introduction

The rust fungus, *Ravenelia* consists of some 200 species, although over 300 names have been reported in the literature (Farr and Rossman, 2019). The *Ravenelia* species occur on fabaceous plants in tropical and subtriopical regions of the world. In SoutheastAsia and Australasia, about 70 species have been reported (Farr and Rossman, 2019). In Thailand, however, only *R. japonica* Dietel& P. Sydow and *R. sessilis* Berkeley wererecorded on *Albizia* spp. (Lohsomboon *et al.*, 1988; Engkhaninun *et al.*, 2005).

During the rust survey in Thailand, *Ravenelia*spp.were found to cause disease on leaves of *Pterolobium macropterum* Kurz and *Vachellia fanesiana*(L.) Wight & Arn. *Pterolobium macropterum* Kurz, is a woody climberand classified in Fabaceae-Caesalpinioideae, distributed in South China

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through Southeast Asia including Thailand. This plant species is often found in mix deciduous forests and dry evergreen forests (Smitinand and Larsen, 1984).The host plant, *Vachellia fanesiana* (L.) Wight &Arn.(=*Acacia farnesiana*(L.) Wild), is classified in Fabaceae-Mimosoideae(Smitinand and Larsen, 1985)and widely planted as forage, ornamental or shade tree, and sometime used as herbals in Thailand.This studyaimed todescribe the two rust fungi and to determine their taxonomicidentify in the genus *Ravenelia*.

Materials and Methods

Sample collection and morphological observation

Rust-infected shoots and leaves of *P. macropterum* was collected in July through October in 2017, and February and June in 2019 in Lampang and Khonkaen provinces. Rust-infected leaves of *V. fanesiana* were collected in January and March 2018 in NakhonPathom province. The rust diseases wereobservedby using hand lens to confirm the fungal sporulation on the symptoms.The rust bearingleaves and twigs were dried and preserved as herbarium specimens. All of herbarium specimens were collected and deposited atDepartment of National Parks, Wildlife and Plant Conservation as National Parks Mycological Collection (BKF) and Kasetsart University at KampangSaen Campus Fungal Collection (KKFC). The type specimen of *R. stevensii* (PUR6216) was loaned from the Arthur Fungarium (PUR), Purdue Uuiversity.

For morphological observation and measurement, small pieces of the rust-bearingleaves were free-hands sectioned by lazer blade under a binocularmicroscope. The thin sections were placed on a microscope slide, mounted with lactophenal solution and covered with cover glass. Spores and associated fungal structures were scraped from the specimens and mounted in a drop of lactophenol solution on microscopic slide. The slide preparation was heated to boiling point to remove air bubble. The slide preparations were examine under a blight-field microscope (Olympus DP25). Twenty randomly selected spores and paraphyses were measured. Photomicrographs were taken and measured by microscope digital camera (Olympus DP25) with digital program (DP2-BSW).

The shape of structures and surface detailed of each spore stage from specimens were examined by using scanning electron microscope (SEM). The dusted spore and sori on host plant parts were fixed on double-sided adhesive tape on specimen holder, and coated with gold. There were examined under TOPCON-BT1505, Hitachi SU8020, and Hitachi S-4200 SEM (Hitachi, Tokyo, Japan) operating at 15 kV.

Molecular identification

DNA Extraction and PCR amplification

Spore mass from sorus on leaf such as teliospores or urediniospores were scraped and put on sterile slides, then crush between slides for breaking spore walls. Drop 40 µl of extraction buffer that contains 10 mMTris-HCLpH 8.3, 1.5 mM Mg Cl_2, 50 mM KCL, 0.01% SDS, this extraction buffer used for resolve spore debris and then transfer to 1.5 ml microtube. The spore solution was incubated at 37 ℃ 30 min, 60 ℃ 30 min, and 95 ℃ 10 min.

The tamplate DNA of rust fungi were amplified in D1/D2 region of 28S rDNA by using NL1 primer (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 primer (5'-GGTCCGTGTTTCAAGACGG-3')(Porter and Golding, 2012).PCR reaction were carried out in 50µl with 2x PCR Mastermix kit from abm company (Applied Biological Materials Inc.). This PCR kit is containingTaq DNA polymerase, dNTP and reaction buffer. The thermal cycling conditions set as 95 ℃ for 1 min followed by 35 cycle of 95 ℃ for 30 sec, 52 ℃ for 1 min, 72 ℃ for 1 min, with final extension at 72 ℃ for 1 min. The PCR products were checked in a 1% (W/V) agarose gel and purified usingQIAquick PCR Purification Kit (QIAGEN, Germany) and sequenced the purified DNA by Solgent Co., Ltd. (Korea).

Data analysis

Fungal DNA sequences were aligned together with sequences obtained from GenBank database by using Clustal W in software program MEGA 7.0 (Kumar*et al.*, 2016). Multiple alignments were analyzed based on the Neighbor-Joining (NJ)(Saitou and Nei, 1987) using the same program. Node support was evaluated by bootstrap analysis using 1000 replications in the same program (Felsenstein, 1985). The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site (Nei and Kumar, 2000).

Results

Taxonomy

***Raveneliastevensii* Arthur, Mycologia 7:178. 1915.**(Table 1, Figure 1)

Holotype: on *Acacia riparia* H.B.K., Puerto Rico, Guayanilla, 1913, F. L.Stevens No. 5881 (PUR6216)

Specimens examined: On *Pterolobium macropterum* Kurz,Muang district, Lampang, 31 July, 2016 (BKF P0015);Prabaht Arboretum, Muang district,

Lampang, 24 Oct, 2016 (BKF P0016); Maetha District, Lampang, C. Ayawong, 10 Feb. 2017 **(**BKF P0033 and BKF P0034); Muang District, Lampang, 11 Feb. 2017 (BKF P0028 and BKF P0030); Phuwiang district, Khonkean, 8 Jun. 2017 (BKF P0058)

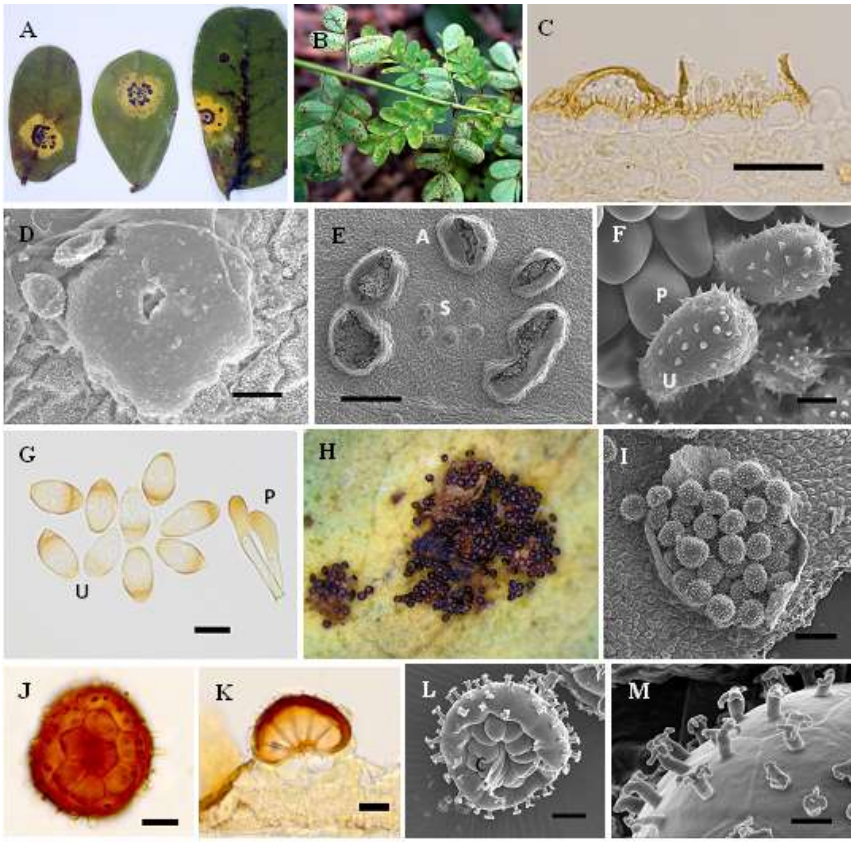

Figure 1. Sori and spores of *Ravenelia stevensii*on *Pterolobium macropterum***:** A, D-F (BKF P0015), B, G, (BKFP0016), C, I (BKF P0028), H, J, K (BKF P0034), L (BKF P0030), M (BKF P0033) A. Spermogonia and aecia on leaves, B. Symptom on leaves C. Cross section of spermogonia D. Spermogonia (SEM), E. A group of spermogonia and aecia (SEM), F. Urediniospores (SEM), G. Urediniospores and paraphyses, H. Telia on abaxial leaf surface, I. Telium (SEM), J. Teliospore, K. Cross section of teliospore, L. Bottom view of teliospore and cysts (SEM), M. Appendages with forked tubercules. Bars: C 50 µm, D 10 µm, E 300 µm, F 5 µm, G 20 µm, I 100 µm, J–K 20 µm, L 15 µm, M 5 µm

Table 1.Comparison of the morphology of the *Pterolobium* rust fungus and *Ravenelia stevensii*

Life cycle stages		*Pterolobium* rust fungus (This study)	*Ravenelia stevensii*		
			Arthur (1915) (holotype)	**Cummins (1978)**	**Berndt (2002)**
Spermogoinial stage	Spermogonium size	47–255 × 23–96 µm	—	—	—
Aecial stage	Aeciospore size	23–45 × 12–21 µm	—	—	—
	Aeciospore wall thickness	3–9 µm at the apex; 0.6–2 µm at the side	—	—	—
	Urediniospore size	19–39× 11–21 µm	25–30 × 8–13 µm	(18–)22–28(–30) × (8–)9–13 µm	25–39 ×13–19 µm
Uredinial stage	Urediniospore wall thickness	2–8 µm at the apex	1 µm or less at side	1 µm at side	ca. 1.5 µm at side
	Number and distribution of germ pores	2–4, equatorial	4, equatorial	4(5–6), equatorial	5–6, more or less equatorial
	Paraphysis size	30–74 µm long, 8–16 µm wide	37–45 µm long, 9–12 µm wide	9–12 µm wide	—
	Paraphysis wall thickness	—	2–5 µm at the apex	2–6 µm at the apex	—
Telial stage	Teliospore size	49–83×53–75 µm	40-63 µm across	(40–)50–70(–75) µm across	—
	Probasidium cell number	3–6 cells across	3–6 cells across	(3)4–5(6) cells across	—
	Probasidium cell wall thickness	1.8–6 µm	—		—
	Teliospore surface ornamentation	2–5 tubercles per a probasidium, apically 2–4 forked, 3–10 µm long, 2–5 µm wide	1–3 tubercles per a probasidium, apically 2–3 forked, 6-19 µm long, 2–3 µm wide	1–4 tubercles per a probasidium, apically 2–4 forked, (6–)10–13(–18) µm long, 2–4 µm wide	—

Spermogonia aggregate or scattered, type 7, 23–(46.6)–96 × 47–(106.4)–255 µm surrounded by aecia, Aecia uredo-type, on upper leaf, subepidermal, erumpent, paraphysate, and located close to telia, aeciospores pedicellate, broadly ellipsoid or broadly obovoid, widen at apex, light brown, 12–(16.4)–21 × 23–(32.4)–45 µm; Apical wall 3–(6.3)–9 µm thick; Side wall 0.6–(1.1)–2 µm thick; germ pores 2–3 and equatorial; Paraphysesclavate or spathulate, 8–(12.4)–16 × 30–(55.7)–74 µm; Uredinia on both leaf surfaces, subepidermal, scattered, erumpent, with peripheral paraphyses; urediniospores broadly obovoid or ellipsoidal with truncate base,, brown, light brown or yellowish brown, thickened at apex, and 10–(16.4)–20 x 19–(27.8)–39 µm in size; the wall 2–(5.7)–8 µm thick; germ pores 2–4 and equatorial; paraphyses light brown,clavate or spathulate. Telia on both sides of leaf, mostly on lower leaf, aggregate or scattered, subculicular erumpent, without paraphysis; teliospore heads discoid, red or reddish brown, appendicular surface, 53–(62.5)–75 × 49–(71.1)–83 µm, 3–6 cells across; probasidia one–celled, thick walls, and10–(16.0)–27 ×18–(26.4)–35 µm in size; the wall 1–(4.2)–6 µm thick, appendage tubular, branched or2- to 4- forked at the apex 1–(2.5)–5 × 3–(6.0)–10 µm; cysts globose, hygroscopic, pendent, multiseriate and deciduous, pedicel short and multihyphal.

Host and geographic distribution: on *Acacia riparia* Kunth: Colombia (Pardo-Cardona, 1998): Puerto Rico (Arthur, 1915;1917: Cummins, 1978: Stevenson, 1975); Virgin Island (Stevenson, 1975); on *Acacia* sp.: Mexico (Cummins, 1978; Gallegos and Cummins, 1981); on *Pterolobium macropterum* Kurz: Thailand (This study).

Ravenelia neocaledoniensis B. Huguenin**,** Bull.Trimestriel Soc. Mycol. Fr. 82:263. 1966, (Table 2, Figure 2).

Specimen examined: on *Vachelliafarnesiana*(L.) Wight &Arn. (=*Acacia farnesiana*(L) Wild.), KamphaengSaen District, NakhonPathom ,8 Jan. 2018.C. Ayawong, (BKF P0065).

Spermogonia and aecia not seen, Urediniacircular, small less than 1 mm on both side of leaves, subepidermal and erumpent; urediniosporesobovoid or ellipsoidal or pyriform to clavate with truncate base, echinulate and thick-walled; 15–(17.5)–20 × 25–(31.0)–43 µm; apical wall 1.2–(1.9)–3.3 µm thick; sided wall 0.6–(0.9)–1.4 µm thick; germ pores 3–5 and equatorial or scattered; uredinial paraphyses geniculate, peripheral, and 7–(9.8)–12 x 26–(47.3)–68 µm in size; the wall 1.6–(2.9)–4.7 µm thick. Telia and teliospore not seen.

Host and geographic distribution: on *Vachellia farnesiana* (L.) Wight &Arn. (=*Acacia farnesiana*(L) Wild.): New Caledonia (Huguenin 1966a,

Huguenin 1966b; Mouchacca and Horak 1998); Australia (MacTaggart*et al*. 2015); on *Acacia* sp. Australia (Berndt, 2002).

Table 2. Description of morphological characteristicsof *R. neocaledoniensis* compared with each spore structure morphology of the Thai specimen ((BKFP0065) and specimens from New Caledonia and Australia

Spore stage	New Caledonia (Huguenin, 1966a)	Australian specimens (Shivas*et al*., 2014)	Thai specimen (BKFP0065)
Uredinia	Uredinia not seen	on both leaf surfaces and stems, subepidermal, erumpent, round, reddish brown	circular, reddish brown, < 1 mm on both sides of leaf surface (upper leaf or abaxial), erumpent
Urediniospores	Uredinospores not seen	ellipsoidal or obovoid, yellowish brown, 26–35 × 15–20 µm; wall 1–2 µm thick, apex slightly thickened, finely verruculose, with 5–6 sometimes scattered or equatorial germ pores.	obovoid or ellipsoidal or pyriform with truncate base, echinulate, 15–20 ×25–43 µm, thick wall; apical walls 1–3 µm; sided walls 0.6 –1.4 µm, 3–5 equatorial germ pores or scattered
Paraphyses	Telialparaphyses brown.	Uredinialparaphyses present, peripheral, cylindrical to clavate, yellowish brown to reddish brown, 44–64 µm (at thickest point) × 8–10 µm; walls 2.5–5.0 µm thick.	Uredinialparaphyses geniculate, peripheral, thick wall; 8–12 × 26–68 µm,; walls 2–5 µm
telia and teliospore heads	Teliospores: 85 × 105 µm diam., 3-10 cysts and pedicelate	Telia on lower leaf surface and stems, black. Teliospores in complex discoid heads, 4 or more celled, reddish brown to brown, 66–119 µm, composed of 18 to over 24 cells, each cell 16–19 × 25–28 µm, height of teliospore is 60–70 µm, smooth, pedicel persistent, up to 48 µm.	Telia and teliospore head not seen
spermogonia, aecia and aeciospores	Spermogonia: 70-100 µm; Aecia on young branches, erumpent, with psuedoperidia, oblong, 21–25 µm, pale yellow. Aecidiospore:24–30 × 14–20 µm, wall 1.5–2 µm thick, apical wall 2.5–3 µm thick, 6–11 germ pores and scattered.	Aecia occur on stems causing witches' brooms, amphigenous, red, peridium present. Aeciospores globose, subglobose or irregular, hyaline to red, 22–26 × 15–21 µm; wall 3.5–5 µm thick, smooth.	Spermogonia and aecia not seen

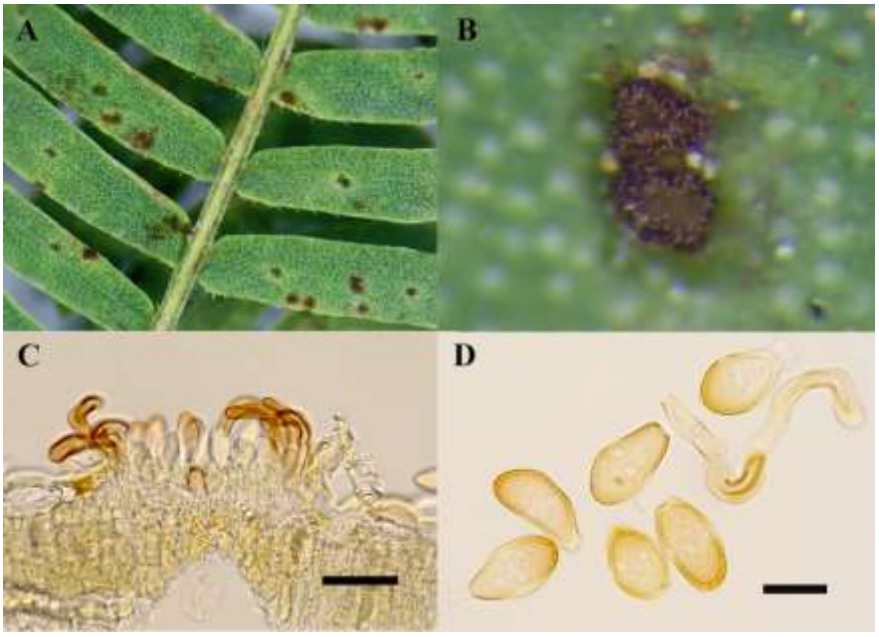

Figure 2. Sori and spores of *Ravenelia neocaledoniensis* (BKFP0065): A. Symptom on leaves, B. Sori on upper leaf surface, C. Cross section of uredinium, D. urediniospores and paraphyses. Bars: C 50 µm, D 20 µm

Phylogenetic analysis

The multiple aligned D1/D2 sequences of 28S rDNA consisted of 17 sequences representing 12 taxa with 589 total lengths. The sequences in this study are *R. japonica* (BKF P0057), *R. neocaledoniensis* (BKF P0065), *R. ornata* (BKF P0023, BKF P0114), and *R. stevensii* (BKF P0034, BKF P0059, BKF P0028, BKF P0030). The phylogenetic treewas reconstructed with Neighbor-Joining (NJ) method involved 17 nucleotide sequences. The sequences in this study clustered within species group, the out-group from GenBank (NCBI) were separated. *Sphaerophragmium* sp. (BRIP56910), *Aecidium physalidis* (EPI910180), *Gymnosporangium hemisphaericum* (NYBG237061), *Uromycladium falcatarium* (BRIP57990), *Pileolaria terebinthi* and *Phakopsora pachyrhizi* were included to be outgroup of *Ravenalia* species. All positions with less than 95% site coverage were eliminated. That was, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 385 positions in the final dataset. The NJ tree revealed that *R. neoclaedoniensis* (BKF P0065) was grouped together with the sequence of *R. neoclaedoniensis*

(BPI56907) supporting by 100% bootstrap value. *Ravenelia stevensii* specimens including BKF P0034, BKF P0059, BKF P0028 and BKF P0030 were separated from others *Revenelia* species and clustered together with highly supporting by 100% bootstrap value.

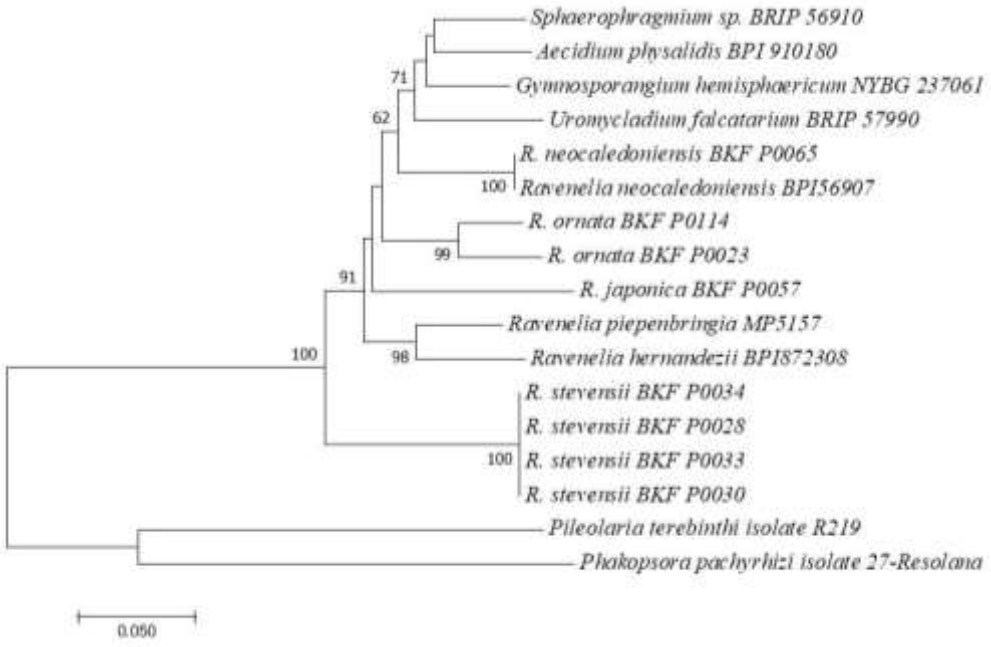

Figure 3. Neighor-joining tree of *Ravenelia* species and other rust fungi based on 28S rDNA sequences data. Bootstrap values above 50% from 1000 replicates are indicated on the corresponding branch

Discussion

In this study, host of *Ravenelia stevensii* was in the genus *Pterolobium*, it is the member of subfamily Caesalpinioidae. And, there was different host reported from America, *Acacia* spp. in subfamily Mimosoideae.There are the same in characteristics of teliospore surface ornamentation, showed2- to 4-forked projection, in addition to the teliosporesize and its probasidium number that showed the taxonomic identity of the rust fungus on *Pterolobium* in Thailand as *R. stevensii* on *Acacia* in the America. *Pterolobium macropterum* is a new host for the fungus.Spermogonialand aecial stages of this species were not described in the original description by Arthur (1915). *Ravenelia stevensii* has been known only the producing of uredinial and telial stages in the Ameicas (Arthur, 1915; 1917; Baxer, 1966; Cummins, 1978; Gallegos and Cummins,

1981; Pardo-Cardona, 1998; Stevenson, 1975). In this study, connection of four spore stages in an autoeciousmacrocyclic life cycle was confirmed by continued field observations. It foundspermogonial and aecial stages which occurred in the rainy season that an average temperature more than 30°C (July through October), uredinial stage in the end of October, and telial stage in cool season (January and February), approximately temperature was below 20°C.The first report of *R. neocaledoniensis* was found on *A. farnesiana* (as *V. Farnesiana* in New Caledonia Island, South Pacific by Huguenin (1966b). Subsequently, Walker (1983) considered that *Ravenelia* on *A. farnesiana* in Australia was an exotic fungus and possible it was introduced from Central America where *Ravenelia* species were distributed on hosts in Americas, Asia and Africa. Berndt (2002) reported that *R. neocaledoniensis* was described the uredinial stage for the first time in Australia. Shivas*et al.* (2014) reported that the fungus infected *V. farnesiana* in Australia. We observed only the uredinial stage of *R. neocaledoniensis* in Thailandbut the pycnial stage, aecial stage and telial stage were not produced.For this, however, the climate in Thailand where their hosts werelocatedin tropical zone, and there are different from Australia and New Caledonia.

Currently DNA sequence data are not available for comparison to verify the morphology-based identity of *R. stevensii* in Thailand and the Americas. However, 28S rDNA sequence analysis was done with four specimens of *R. stevensii* in this study. Although, no sequence information of this species deposited in the GanBank database, the cluster analysis supported these four sequences in the same group (100% bootstrap value) and separated from other species. While, *R. neocaledoniensis*(BKF P0065)sequence was clustered with the sequence (BPI56907) obatianed from database. Grandhe and Kuvalekar (2007) constructed the phylogenetic tree based on 18S rDNA sequence analysis of *R. esculenta* and related rust species. The cluster analysis revealed that *R. esculenta* and *Gymnosporangium* shared a common ancestry, although *R. esculenta* is an autoecious on angiosperm and the genus *Gymnosporangium* is heteroecious with pycnia, aecia on angiosperm and uredia, telia on gymnosperm. Ebinghaus and Begerow (2018) described two new rust species, *R. piepenbringiae* and *R. hernandezii* (Pucciniales) on *Senegalia* spp. (Fabaceae) from the Neotropics (Panama, Costa Rica) using morphological characteristics and molecular phylogenetic analyses based on 28S rDNA sequence data. It was suggested that a clade including rusts on neotropical *Senegalia* species, such as *R. cohniana*, *R. hernandezii* sp.nov.and *R. piepenbringiae* sp.nov., displays a sister group to two neotropically distributed rusts which infect non-*Senegalia* hosts such as *R. echinata* var. *ectypa*(on *Calliandraformosa*) and *R. havanensis* (on *Enterolobium contortisiliquum*). A second clade included *Ravenelia* species

on *Senegalia* spp. from paleotrpical origin, appeared only distantly related to the former species cluster. These studies showed that there was phylogenetic correlation between molecular and morphological data.

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