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Three Quinine and Cinchonidine producing *Fusarium* species from Indonesia

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Abstract

Five endophytic *Fusarium* isolates from *Cinchona calisaya* were characterized for their ability to produce cinchona alkaloids, such as quinine, quinidine, cinchonine and cinchonidine. Based on molecular identification using phylogenetic analysis of DNA sequence generated from the internal transcribed spacer (ITS) rDNA and the partial elongation factor (EF1–α) gene regions, these isolates were determined as *F. incarnatum* (strain IPBCC 15.1251 and IPBCC 15.1253), *F. solani* (strain IPBCC 15.1248 and IPBCC 15.1249) and *F. oxysporum* (strain IPBCC 15.1250). All strains, except *F. solani* strain IPBCC 15.1248, were detected capable in producing quinine and cinchonidine by using HPLC and UV–vis spectroscopy analyses. Only quinine was detected from the extract of the *F. solani* strain IPBCC 15.1248. This is the first report of *Fusarium* species capable of producing quinine and cinchonidine in synthetic liquid medium.

Key words – endophyte – fungi – malaria – phylogenetic – cinchona alkaloids

Introduction

Fusarium Link is an anamorphic hypomycetous fungus linked to *Gibberella* Sacc. as its teleomorphic state. The member of *Fusarium* is cosmopolitan found as saprobe, airborne, soilborne, endophyte and plant pathogen on a wide range of host plants, including several economically important crops such as tomatoes (Ignjatov et al. 2012), banana (Dita et al. 2010), cereals (Voigt et al. 2005), etc. Although member of *Fusarium* is more recognized as fungal pathogen on many economically important plants and on human (Guarro & Gene 1995, O'Donnell et al. 2009), however, they are also frequently isolated as endophytes from various plants and capable in producing secondary metabolites with medicinal and antimicrobial activities (Kour et al. 2008, Deng et al. 2009, Li et al. 2008, Tayung et al. 2011). Several species belonging to *Fusarium oxysporum* complex are also known causing human infections in immunocompromised patients (Hennequin et al. 1999). Currently, there are approximately 1132 legitimate names of *Fusarium* recognized in the Mycobank fungal databases (www.mycobank.org).

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Endophytic *Fusarium* has been well–known for their activities in inducing plant host resistance to pathogen or enhancing plant fitness to environmental stresses (Bacon & Yates 2006). Several studies on secondary metabolites discovery also found that many endophytic *Fusarium* species have been reported to produce metabolites with medicinal properties such as antimicrobial and anticancer activities (Shiono et al. 2007, Shweta et al. 2010, Tayung et al. 2011). Despite their agrochemical and biomedical importance, most of the potential endophytic *Fusarium* remain unidentified until species level, or phylogenetically uncharacterized.

In the last 10 years, application of phylogenetic species concept in determination of fungal endophyte diversity on various plants, either by using single gene or multigene analyses, becomes more important due to its robustness in revealing many cryptic fungal endophytes species. Traditional identification method of endophyte such as morphological and cultural characterization have been failed to identify many fungal endophytes even up to a familial level (Jeewon et al. 2013). Morphological and cultural approaches in identification of fungal endophyte species are problematic because mycelia pigmentation, shape and size of conidia are unstable and dependent on composition of media and environmental condition (Guo et al. 2001). Identification of *Fusarium* until species level (saprobes, pathogen or endpohyte) is currently involving combination of morphology species concept and phylogenetic species concept. Additional information from biological species concept such as vegetative compatibility groups (VCGs) and mating type is necessary for lower taxonomy identification such *forma speciales* (Leslie & Summerell 2006). Among them, phylogenetic species concept has been known as the most consistent concept for the identification of *Fusarium* members (Steenkamp et al. 2002). Currently, this approach involving combination of several gene regions such as the internal transcribed spacer (ITS) and the elongation factor 1α (EF–1α) regions (O'Donnell et al. 1998), β–tubulin *(*β–tub) and aminoadipate reductase gene regions (lys2) (Watanabe et al. 2011). Several new cryptic species of endophytic *Fusarium* were determined using this approach (Walsh et al. 2010).

In this study, six isolates of endophytic *Fusarium* from *Cinchona calisaya* Wedd. (common name: quina) [syn. *Cinchona ledgeriana* (Howard) Bern.Moens ex Trimen] (Rubiaceae), a medicinal plant for malarial fever disease, were characterized for their ability in producing cinchona alkaloids, such as quinine, quinidine, cinchonine, cinchonidine (Maehara et al. 2010). Identity of these isolates was determined using combination of morphology, cultural characteristics, and phylogenetic analysis based on DNA sequence generated from the ITS and the EF–1α gene regions.

Materials & Methods

Collection of samples

Specimen collection was conducted at quina plantation managed by the Research Center for Tea and Quina, Gambung, West Java, Indonesia in 29 September 2012. The specimen were collected from five individual healthy plants by cutting off asymptomatic flowers, leaves, petioles, stems, barks, and roots of *C. calisaya*. Five pieces of each organ were placed in zipped plastic bags. The plastic bags were sealed and labelled with the name of the host, collection site, date and collector/s. All materials were kept in ice boxes prior to isolation in the laboratory.

Isolation

The isolation protocol of endophytic fungi referred to the method described by Mostert et al. (2001) with modification. The materials were washed thoroughly in running tap water, and then surface-sterilized using 70% ethanol (EtOH) for 1 min, followed by soaking in sodium hypochlorite (NaOCl) 3% for 2 min, and 70% ethanol for 20 s. The samples were rinsed in sterile distilled water three times, and dried with sterile paper for at least 6 h. The sterile distilled water of the final rinse was poured onto the agar medium as a quality control of sterilization process.

After drying, samples were cut into segments approximately 0.5 × 1 cm and placed on the surface of Malt Extract Agar (MEA) medium (Difco, USA) (4 segments/petri dishes). All petri dishes were incubated at room temperature. Three replications were made for each sample. Hypal tips

growing out from the plated plant segments were immediately transferred onto Potato Dextrose Agar (PDA) (Difco, USA) plate. The growth of endophytic fungi mycelium was observed everyday, for about 30 d. The growing colonies were purified using hyphal tip isolation method to get a pure culture. Specimen herbarium was deposited at Hebarium Bogoriense (BO), Cibinong, under accession number BO 1913291. Culture isolates obtained in this study were deposited at the Bogor Agriculture University (IPB) culture collection (IPBCC), Dramaga, under accession number IPBCC.15.1250, IPBCC.15.1253, IPBCC.15.1251, IPBCC.15.1248 and IPBCC.15.1249 (Table 1).

Table 1 Genbank accession number and strain code/culture collection number of *Fusarium* species used in this study.

Species	**Strain**	**GenBank accession number**	
		ITS	**EF1–α**
Fusarium solani	IPBCC 15.1248	LC026135	LC049293
Fusarium solani	IPBCC.15.1249	LC026136	LC049294
Fusarium incarnatum	IPBCC 15.1251	LC026133	LC049292
Fusarium incarnatum	IPBCC 15.1252	LC026134	LCO49295
Fusarium incarnatum	IPBCC 15.1253	LC026132	LC049291
Fusarium oxysporum	IPBCC 15.1250	LC026138	LCO49072
Fusarium acuminatum	NRRL 52789	JF740933	JF740857
Fusarium asiaticum	MAFF 240264	AB586991	AB674269
Fusarium asiaticum	NRRL 26156	NR 121320	AF212452
Fusarium avenaceum	MAFF 239206	AB587016	AB674293
Fusarium cerealis	NRRL 25491	AF006340	AF212465
Fusarium cerealis	MAFF 241212	AB820717	AB820701
Fusarium decemcellulare	MAFF 238421	AB587017	AB674294
Fusarium decemcellulare	MAFF 238422	AB587018	AB674295
Fusarium dimerum	CBS 632 76	AB586995	AB674273
Fusarium dimerum	MAFF 237465	AB586996	AB674274
Fusarium equiseti	NRRL 26419	GQ505688	GQ505599
Fusarium equiseti	MAFF 236434	AB586999	AB674277
Fusarium equiseti	MAFF 236723	AB587000	AB674278
Fusarium incarnatum	MAFF 236521	AB586988	KF255493
Fusarium graminearum	MAFF 240270	AB586992	AB674270
Fusarium kyushuense	MAFF 237645	AB587019	AB674296
Fusarium kyushuense	NRRL 6490	FSU85545	AB674297
Fusarium larvarum	CBS 169 30	AB586984	AB674265
Fusarium larvarum	CBS 638 76	AB586985	AB674266
Fusarium langsethiae	CBS 113234	AB587021	AB674298
Fusarium langsethiae	FRC T 1000	AB587023	AB674300
Fusarium lateritium	MAFF 235344	AB587004	AB674281
Fusarium merismoides	MAFF 236504	AB586998	AB674276
Fusarium oxysporum	CBS 133023	KF255448	KF255492
Fusarium oxysporum	CBS 127 73	KF913730	KF913725
Fusarium poae	FRC T 0796	AB586983	AB674301
Fusarium poae	MAFF 305947	AB587024	AB674302
Fusarium phyllophilum	CBS 216 76	AB587006	AB674283
Fusarium solani	CBS 132898	KF255440	KF255484
Fusarium solani	NRRL 28579	DQ094383	DQ246910
Fusarium solani	MAFF 238538	AB587013	AB674290
Fusarium subglutinans	ATCC 38016	AB587008	AB674285
Fusarium sporotrichioides	CBS 119839	AB587026	AB674304
Fusarium sporotrichioides	MAFF 236639	AB587027	AB674305
Fusarium sporotrichioides	ATCC 34914	AB587025	AB674303
Fusarium tricinctum	ATCC 38183	AB587028	AB674264
Fusarium tricinctum	CBS 393 93	AB587029	AB674263
Fusarium tricinctum	MAFF 235551	AB587030	AB674262
Fusarium verticillioides	CBS 576 78	AB587010	AB674287
Fusarium verticillioides	MAFF 240085	AB587012	AB674289
Penicillium citrinum	AX4602	KJ413363	KJ476397

Morphological and cultural characterization

Morphological–based determination of *Fusarium* referred to the identification key described by Leslie & Summerell (2006). Colony characterization of each isolate was conducted to the 7 days old isolates growth on the PDA medium. The colony characteristics observed include diameter, color of the surface and reverse, margin, and texture. Microscopic structures, such as macroconidia, microconidia, conidiogenous cells and chlamydospore were examined by using Olympus BX53 light microscope (OLYMPUS, Japan) under 1000× magnification using immersion oil. Shear's solution was used as mounting medium (Kirk et al. 2008). Thirty measurements were taken to all microscopic characters.

Molecular characterization

DNA isolation, PCR amplification and sequencing

Genomic DNA from 7 d fungal mycelia growth in 5 mL of Potato Dextrose Broth (PDB) (Difco, USA) were harvested using Phytopure™ DNA extraction kit (GE Healthcare, UK) following the manufacturer's protocol. Amplification of genomic DNA was done using Polymerase Chain Reaction (PCR) method performed in a 25 mL reaction volume as follow: 10 µL nuclease free water, 12.5 µL DreamTaq® green master mix (Thermo scientific, USA), 0.5 µL of forward and reverse primer, 0.5 µL DMSO and 1 µL DNA template. The primer pairs of ITS5 (forward) (5'–TCCTCCGCTTATTGATATGC–3') and ITS4 (reverse) (5'–TCCGTAGGTGAACCTGCGC–3') (White et al. 1990) were used to amplify the ITS region including 5.8S rDNA. The PCR condition for the ITS region was set as follow: 90 s at 95°C for initial denaturation, followed by 35 cycles of 30 s at 95°C denaturation, 30 s at 55°C annealing, 90 s at 72°C extension and 5 min at 72°C for the final extension. For the amplification of partial translation EF–1α, the primer pairs of EF1–728F (5'–CATCGAGAAGTTCGAGAAGG–3') and EF1–986R (5'–TACTTGAAGGAACCCTTACC–3') (Carbone & Kohn 1999) were employed. PCR reaction was performed in 25 mL as follow: nuclease free water 8.75 µL, Gotaq® green master mix (Promega, USA) 12.5 µL, 0.625 µL for each forward and reverse primer, DMSO 0.5 µL and DNA templete 2 µL. The PCR condition was set as follows: 94°C for 5 min for initial denaturation, followed by 35 cycles of 30 s at 94°C denaturation, 30 s at 52°C annealing, 30 s at 72°C extension and 7 min at 72°C for final extension. All PCR reactions were performed using T100 thermal cycler (Bio–Rad, USA). PCR products were electrophorized in a 1% (w/v) agarose gel soaked in 1× TAE buffer at 100V for 30 min. 1 kb DNA ladder was used as a marker during the electrophoresis. The gel was soaked in EtBr (ethidium bromide) for 30 min prior to UV light examination using Gel Doc XR system (Bio–Rad, USA). Purified PCR products were sent to 1stBASE (Malaysia) for sequencing.

Phylogenetic analysis

Nucleotide sequences obtained from the respective primer pairs (ITS5 and ITS4, EF1–728F and EF1–986R) were examined and refined by direct examination using Chromas Pro 1.41 software (Technelysium Pty Ltd., Australia). Newly ITS and EF 1–α sequences of endophytic *Fusarium* from *C. calisaya* were aligned with DNA sequence from 43 *Fusarium* isolates of Watanabe et al. (2011) using MUSCLE (Edgar 2004) implemented in MEGA 6 (Tamura et al. 2013). *Penicillium chrysogenum* strain CBS 906.70 was used as outgroup in the analyses. Regions designated as ambiguously aligned were excluded from the analyses. GeneBank accession number, strain code and taxon names used in this study are showed in Table 1.

Phylogenetic analysis was conducted using the maximum parsimony (MP) method in PAUP* 4.0b10 (Swofford 2002). The heuristic search option using the 'tree–bisection–reconstruction' (TBR) algorithm with 1000 random sequence additions was performed 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 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. The partition homogeneity test (Farris et al. 1995) with 1000 replicates, 10 random addition sequence replicates, and TBR branch swapping was conducted by using PAUP* to determine whether ITS and EF–1α datasets were in conflict. A significance level of P = 0.01 was adopted for this test (Cunningham 1997). TreeGraph 2 software (Stöver & Müller 2010) was used to refine the phylogenetic tree.

Cinchona alkaloids analyses

Determination of cinchona alkaloids was qualitatively and quantitatively carried out according to the method described by Simanjuntak et al. (2002). Each *Fusarium* isolate was inoculated into 200 mL PDB medium pH ±6.0 in 500 mL Erlenmeyer flasks. The cultures were incubated with static methods for 21 d at room temperature. After 21 d, biomass and filtrate of fungal isolates from the fermentation flasks were homogenized and extracted with the addition of chloroform p.a. ($CHCl_3$, ≥99.8%) as a solvent solution. This step was carried out three times for each sample. Each fraction was collected and concentrated by rotary evaporator at a temperature of 45°C and 60 rpm rotation. The extracts of the endophytic fungus were analyzed quantitatively by high performance liquid chromatography (HPLC) types Perkin Elmer Series 200 UV–Vis detector, nonpolar ascentis® C18 HPLC column, 5 µm, L × I.D. 25 cm × 4.6 mm, eluent KH_2PO_4 20 mM pH 2.5: CH_3CN = 75%: 25%, and a flow rate at 1.20 mL/minute. HPLC condition was set as follow: column Cosmosil 5C18-MS-II (4.6 × 150 mm), mobile phase using Methanol : Acetonitril (80 : 20), flow rate at 1.0 mL/min, column temperature at 40°C and detection wavelength at 210 nm. The concentration of cinchona alkaloids were calculated as follows:

Concentration ($mg.L^{-1}$) = (Sample Area × Concentration of Standard) : Standard Area

To affirm the HPLC results, analysis of absorption spectra were carried out by using UV–Vis spectrophotometer (Germany). Absorption spectra of metabolites present in the extract were determined by making the absorption scan in range from 190 nm to 400 nm.

Results

Morphological and cultural characterization

The morphology and cultural characteristics of six *Fusarium* isolates from *C. calisaya* on PDA medium after 7 d incubation were described in Table 2. The colony caharacteristics of *Fusarium* isolates strain IPBCC 15.1251 and IPBCC 15.1253 were slightly different to those of strain IPBCC 15.1248, strain IPBCC 15.1249 and strain IPBCC 15.1250 due to producing pink to light red pigmentation on the PDA medium, and microcospically having aseptate macroconidia after 7 d incubation. In addition, *Fusarium* isolate strain IPBCC 15.1250 producing purple to dark purple pigmentation on the medium, while strain IPBCC 15.1248 and strain IPBCC 15.1249 did not produce any pigmentations.

Phylogenetic analysis

The partition homogeneity test of the two datasets–ITS and part of EF–1α regions–showed that significant conflict exist between the phylogenies of the individiual dataset (P < 0.01). Therefore, we constructed the datasets separately as individual phylogenetic analysis. In the parsimony analysis of ITS sequence dataset, the alignment contained 49 sequences and 489 total characters, of which 257 characters are constant, 60 characters are variable and parsimony–uninformative, 172 characters are parsimony–informative. All characters have equal weight. The best parsimonious tree was generated in 555 steps (CI = 0.647, RI = 0.862, RC = 0.558, HI = 0.353). The endophytic *Fusarium* sequences from *C. calisaya* were divided into three distinct lineages (Fig. 1). Sequence of *Fusarium* sp. strain IPBCC 15.1251, IPBCC 15.1252 and IPBCC 15.1253 nested in the clade containing *F. equiseti–F. incarnatum* complex (*F. equiseti* strain MAFF 236434, strain MAFF 236723, strain NRRL 26419T and *F. incarnatum* strain MAFF 236521) with 99% BS, sequence of strain IPBCC 15.1250 forming monophyletic clade with members of *F. oxysporum* (*F. oxysporum* strain CBS 127.73 and strain CBS

133023T) (BS = 90%), and sequence of strain IPBCC 15.1248 and IPBCC.15.1249 nested in the clade containing *F. solani* sequences (*F. solani* strain CBS 132898, strain NRRL 28579T and *F. solani* f. *mori* strain MAFF 238538) with 100% BS.

The alignment of partial EF–1α composed of 49 sequences and 345 total characters, of which 72 characters are constant, 61 characters are variable and parsimony–uninformative, 212 characters are parsimony–informative. All characters have equal weight. The best parsimonious tree was generated in 892 steps (CI= 0.565, RI= 0.792, RC= 0.448, HI= 0.435). The placement of six endophytic *Fusarium* sequences in the phylogenetic tree generated from partial EF–1α dataset is similar to that of the ITS tree (Fig. 2). Sequence of *Fusarium* sp. strain IPBCC 15.1251, IPBCC 15.1252 and IPBCC 15.1253 nested in the clade containing *F. equiseti–F. incarnatum* complex with 95% BS, sequence of strain IPBCC 15.1250 forming monophyletic clade with *F. oxysporum* clade (BS = 99%), and sequence of strain strain IPBCC 15.1248 and IPBCC.15.1249 nested in the *F. solani* clade with 97% BS. Based on the phylogenetic trees (ITS and partial EF–1α) analyses, the sequence of *Fusarium* strain IPBCC 15.1250 was determined as *F. oxysporum*, and the *Fusarium* sequence strain IPBCC 15.1248 and IPBCC.15.1249 were determined as *F. solani*. However, these datasets were failed to resolve the species name of *Fusarium* spp. strain IPBCC 15.1251, IPBCC 15.1252 and IPBCC 15.1253.

In order to resolve the identity of the *Fusarium* spp. strain IPBCC 15.1251, IPBCC 15.1252 and IPBCC 15.1253, we conducted separate phylogenetic analysis based on partial EF–1α sequence involving these three sequences with 42 sequences belonging to *F. equiseti–incarnatum* complex used by Castellá and Cabañes (2014). Sequence of *F. asiaticum* strain NRRL 26156 (GenBank accession number: AF212452) was used as outgroup. All genbank accession number, strain code and taxon names used in this analysis were showed in Fig. 3. The alignment of this dataset composed of 46 sequences and 255 total characters included in the analysis, of which 144 characters were constant, 43 characters were variable and parsimony–uninformative, 68 characters were parsimony–informative. All characters have equal weight. The best parsimonious tree was generated in 204 steps (CI = 0.667, RI = 0.851, RC = 0.567, HI = 0.333). The phylogenetic tree showed that sequence of *Fusarium* spp. strain IPBCC 15.1251, IPBCC 15.1252 and IPBCC 15.1253 formed monophyletic clade with *F. incarnatum* NRRL 34004 (GQ505628) (BS = 71 %). This clade nested within the large monophyletic clade containing sequences belong to *F. incarnatum sensu stricto* (*s.str.*) (BS = 83 %). Based on this analysis, *Fusarium* spp. strain IPBCC 15.1251, IPBCC 15.1252 and IPBCC 15.1253 were determined as *F. incarnatum*.

Cinchona alkaloids analyses

The HPLC analysis showed that four strains were capable in producing quinine, viz, *F. incarnatum* strain IPBCC 15.1253 and strain IPBCC 15.1251; *F. oxysporum* strain IPBCC 15.1250; and *F. solani* strain IPBCC 15.1248 and strain IPBCC.15.1249. It was demonstrated by having similar retention time (Rt) with the quinine standard (± 2.2 min) (Fig. 4). The concentration of quinine produced by the *Fusarium* isolates in this study range from 0.7 to 0.9 mg/L, while cinchonidine range from $2.5 \times 10^{-5}$to 1.0×10^{-4} mg/L after 21 d incubation (Table 3).

Among them, *F. incarnatum* strain IPBCC 15.1253 and strain IPBCC 15.1251; *F. oxysporum* strain IPBCC 15.1250; and *F. solani* strain IPBCC.15.1249 were also capable in producing cinchonidine in synthetic medium. It was shown by having small peak at 1.9 min retention time similar to cinchonidine standard (Fig. 4). Because the peak in HPLC analysis was not really clear, we carried out UV–Vis spectroscopy analysis to affirm the HPLC results. UV–Vis spectra showed close retention time between the peaks of quinine and cinchonidine standards at ± 239 nm and ± 280 nm, respectively (Fig. 5a). This figure also showed that the cinchonidine spectrum was weaker than quinine in absorbing UV. Based on the UV–Vis analysis, quinine and cinchonidine were detected from the extracts of *F. incarnatum* strain IPBCC 15.1253 and strain IPBCC 15.1251; *F. oxysporum* strain IPBCC 15.1250; and *F. solani* strain IPBCC.15.1249, but cinchonidine peak was in low resolution (Figs. 5b–f). This is probably due to low concentration of cinchonidine in the fungal extracts, particularly in the extracts of *F. oxysporum* strain IPBCC 15.1250 (Fig. 5b) and *F. solani* strain IPBCC 15.1249 (Fig. 5e).

Table 2 Morphological and cultural characteristics of endophytic *Fusarium* from *C. calisaya* on PDA after 7 d incubation.

Character	***F. incarnatum* strain IPBCC.15.1253**	***F. incarnatum* strain IPBCC.15.1251**	***F. oxysporum* strain IPBCC.15.1250**	***F. solani* strain IPBCC.15.1248**	***F. solani* strain IPBCC.15.1249**
Colony	cottony, aerial	cottony, aerial	cottony, immersed	cottony, aerial	cottony, immersed
Diameter of colony (cm)	4.2	4.8	4.3	5.8	4.8
Conidiophore	long and single	long and single	short	long and single	long and single
Conidiogenous cell (µm)	monophialide, 1.5 × 4.5	monophialide, 1.4 × 4.3	monophialide, 1.9 × 6.7	monophialide, 3.3 × 5.9	monophialide, 2.7 × 9.3
Microconidia (µm)	oblong to ovoid, aseptate, 2.9−3.9 × 6.1−8.6	oblong to ovoid, aseptate, 8.7−11 × 15.4−25.7	oval to elliptical, aseptate, 8.8−12.9 × 25.4−34.6	oval, aseptate, 2.9−5.2 × 13.2−17.2	oval, aseptate, 2.9−8.7 × 8.9−20.9
Macroconidia (µm)	oblong to elliptical, aseptate, 3.6−4.2 × 11.9−20.9	oblong to elliptical, aseptate, 3.5−11 × 13.7−45.3	obovate, 3-6 septate, 8.5−11.8 × 31.9−70.4	obovate, septate (4-6), 19.9−38.6 × 3.4-5.9	obovate, septate, 3.4−11.4 × 19.9−62.6
Chlamydospore (µm)	present, obovoid, intercalar, 6.3 × 8.5	present, globular, intercalar and terminal, 5.3 −7.9 × 7.1−9.7	present, globular, intercalar, terminal, 6.1 − 10.6 × 5.4 − 11.4	present, oval, intercalar, 4.5−6.5 × 7.3−8.1	present in chain or single, oval, intercalar, 4.6−8.8 × 6.5−9.2 ,
Color	white to pink	white to pink	purple/dark purple	white to pale	white to pale
Source	Petiole	fruit	bark	Twig	twig

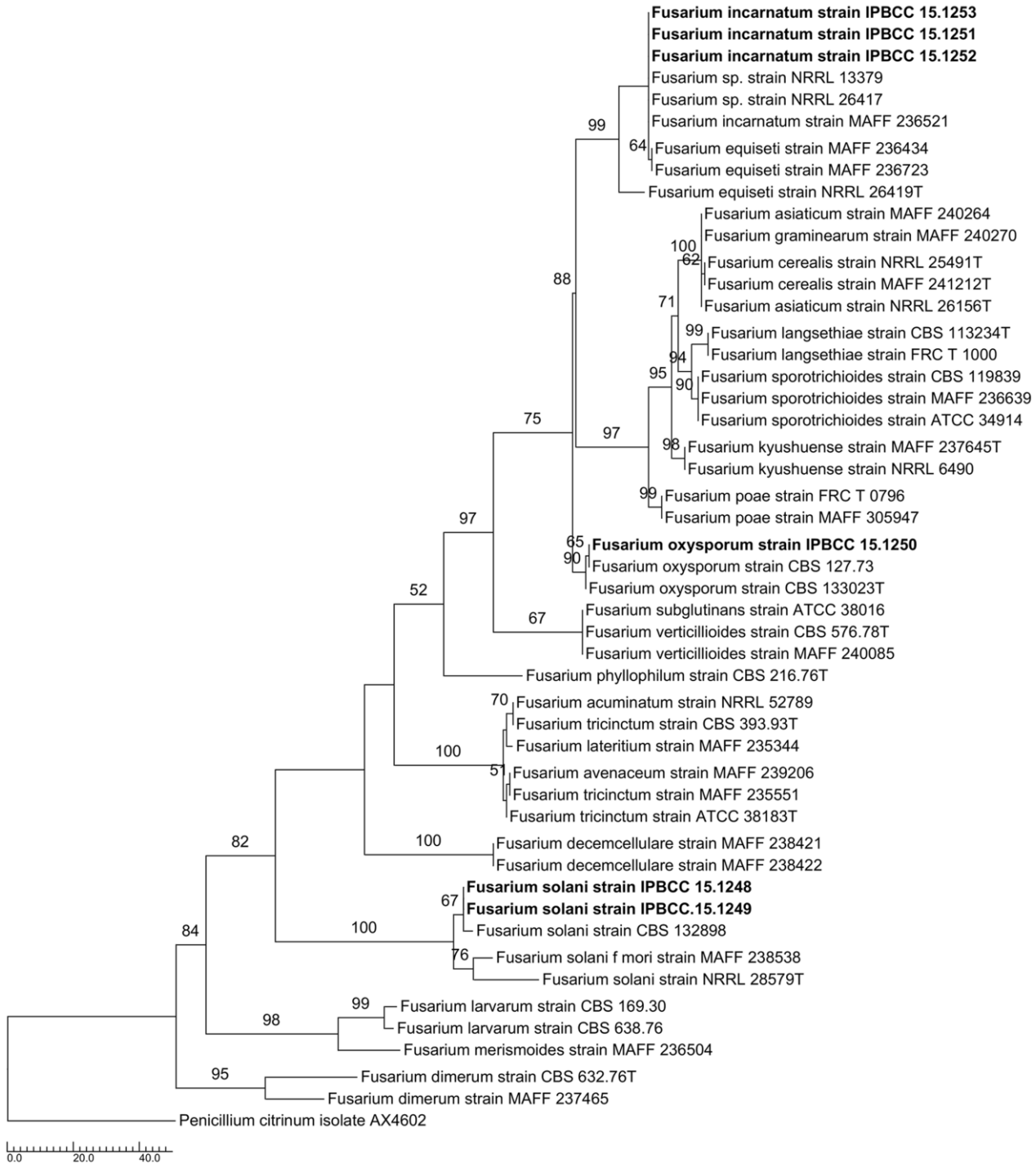

Fig. 1 – Maximum Parsimony (MP) tree showing relationship between endophytic *Fusarium* spp. from *C. calisaya* with related species based on the ITS rDNA sequences. Bootstrap value > 50% is shown at the branches node.

Discussion

It was predicted by several researchers that each of about 300,000 species of plants in terrestrial ecosystem is likely associated with more than one species of bacterial and fungal endophytes (Strobel & Daisy 2003). With their capability in producing various bioactive compounds, fungal endophytes has gained more attention in discovery of new secondary metabolites, or as an alternative source to replace plants as bioactive plant secondary metabolites producer due to their ability to synthesize similar or the same natural products produced by the plants (Kusari & Spiteller 2011). Despite this

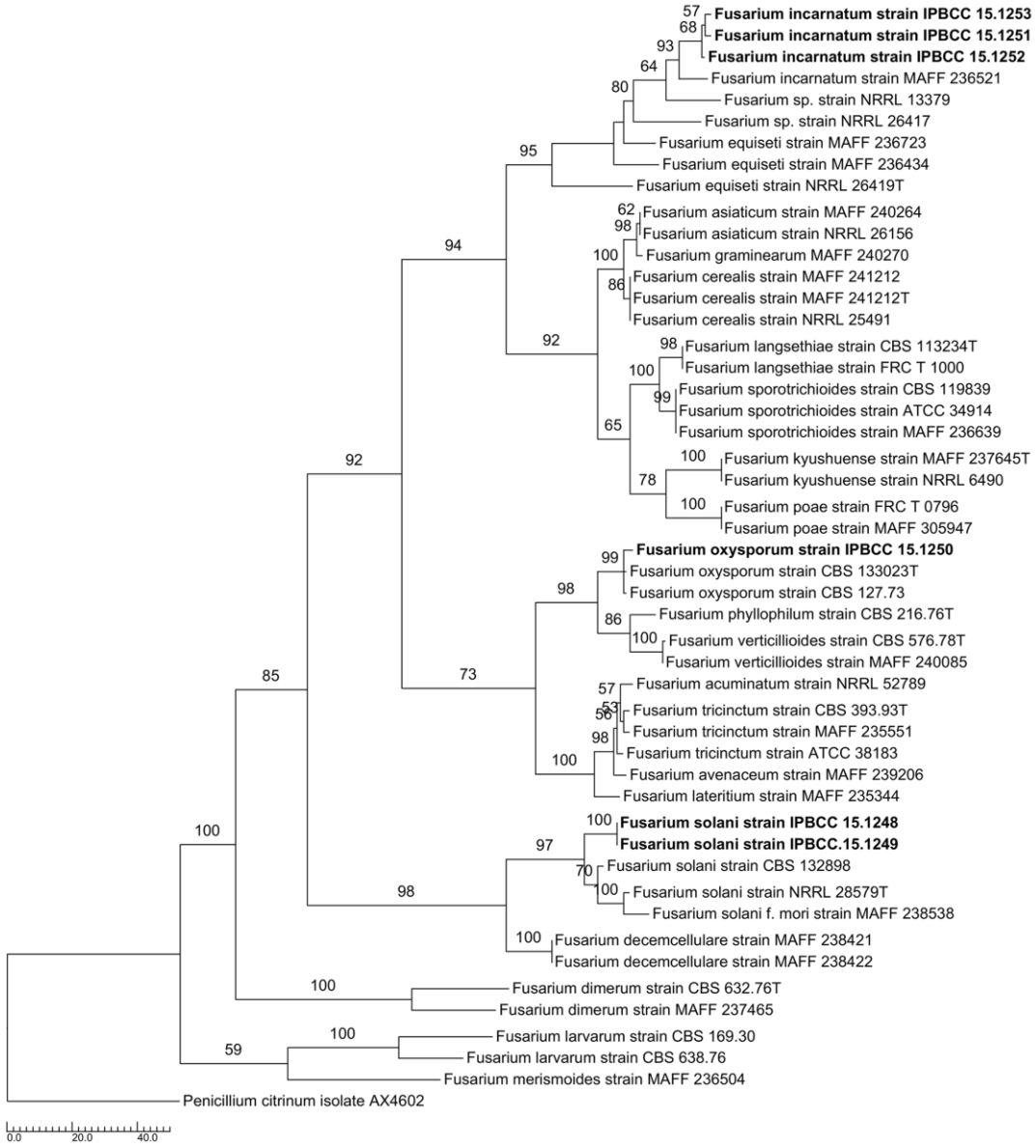

Fig. 2 – Maximum Parsimony (MP) tree showing relationship between endophytic *Fusarium* spp. from *C. calisaya* with related species based on the EF–1α sequences. Bootstrap value > 50% is shown at the branches node.

potential, majority of the potential endophytic fungi remain taxonomically uncharacterized (Huang et al. 2009). Therefore, efforts on discovery of new secondary metabolites from fungal endophytes must be carried out in line with the fungal endophytes taxonomical study to reveal their true scientific name identity.

Quinine is widely recognized as one of *Cinchona* alkaloids produced as secondary metabolites from the bark of *Cinchona* plants (*Cinchona* spp.). Since its finding in 1820, quinine has been used as the main medication for malaria disease due to its effectivity against erythrocytic stage of the parasite *Plasmodium falciparum* (Wijnsma & Verpoorte 1988). One of the earlier report indicating *Cinchona* alkaloids production by fungal endophytes was published by Simanjuntak et al. (2002). In further report, taxonomical identity of common fungal endophyte capable of producing quinine was determined belonging to the genus *Diaporthe* (anamorph: *Phomopsis*) (Maehara et al. 2012). Different genera of endophytic fungi such as *Arthrinium*, *Fomitopsis*, *Penicillium*, *Schizophyllym* and *Xylaria* were also reported as potential quinine producer (Shibuya et al. 2003, Agusta et al. 2005, Maehara et al. 2010).

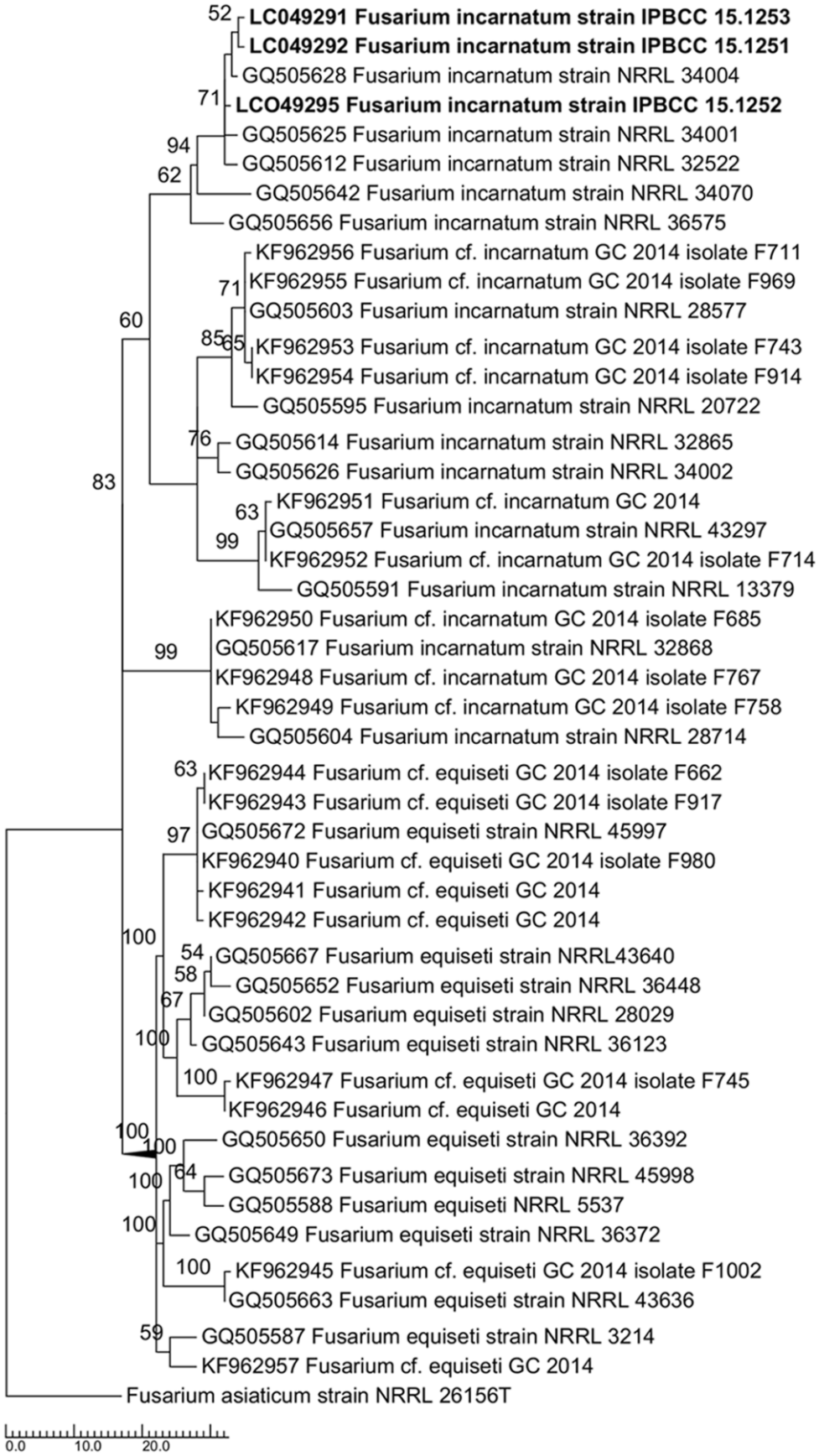

Fig. 3 – Maximum Parsimony (MP) tree showing phylogenetic affinities of *Fusarium* isolates strain IPBCC 15.251, IPBCC 15.252 and IPBCC 15.253 within *Fusarium incarnatum-equiseti* complex. Bootstrap value > 50% is shown at the branches node.

Table 3 HPLC analysis of *Cinchona* alkaloids from endophytic *Fusarium* spp.

			Alkaloids			
			Quinine	**Quinidine**	**Cinchonine**	**Cinchonidine**
Retention time (Rt) standard			2.2	2.6	1.7	1.9
F. incarnatum	IPBCC 15.1251	Area	41398776	-	-	1701
		Concentration (mg/L)	0.8	-	-	3.6×10^{-5}
	IPBCC 15.1253	Area	47547190	-	-	5125
		Concentration (mg/L)	0.9	-	-	1×10^{-4}
F. oxysporum	IPBCC 15.1250	Area	46398275	-	-	1172
		Concentration (mg/L)	0.9	-	-	2.4×10^{-4}
F. solani	IPBCC 15.1248	Area	45494218	-	-	-
		Concentration (mg/L)	0.9	-	-	-
	IPBCC 15.1249	Area	34190841	-	-	1181
		Concentration (mg/L)	0.7	-	-	2.5×10^{-5}

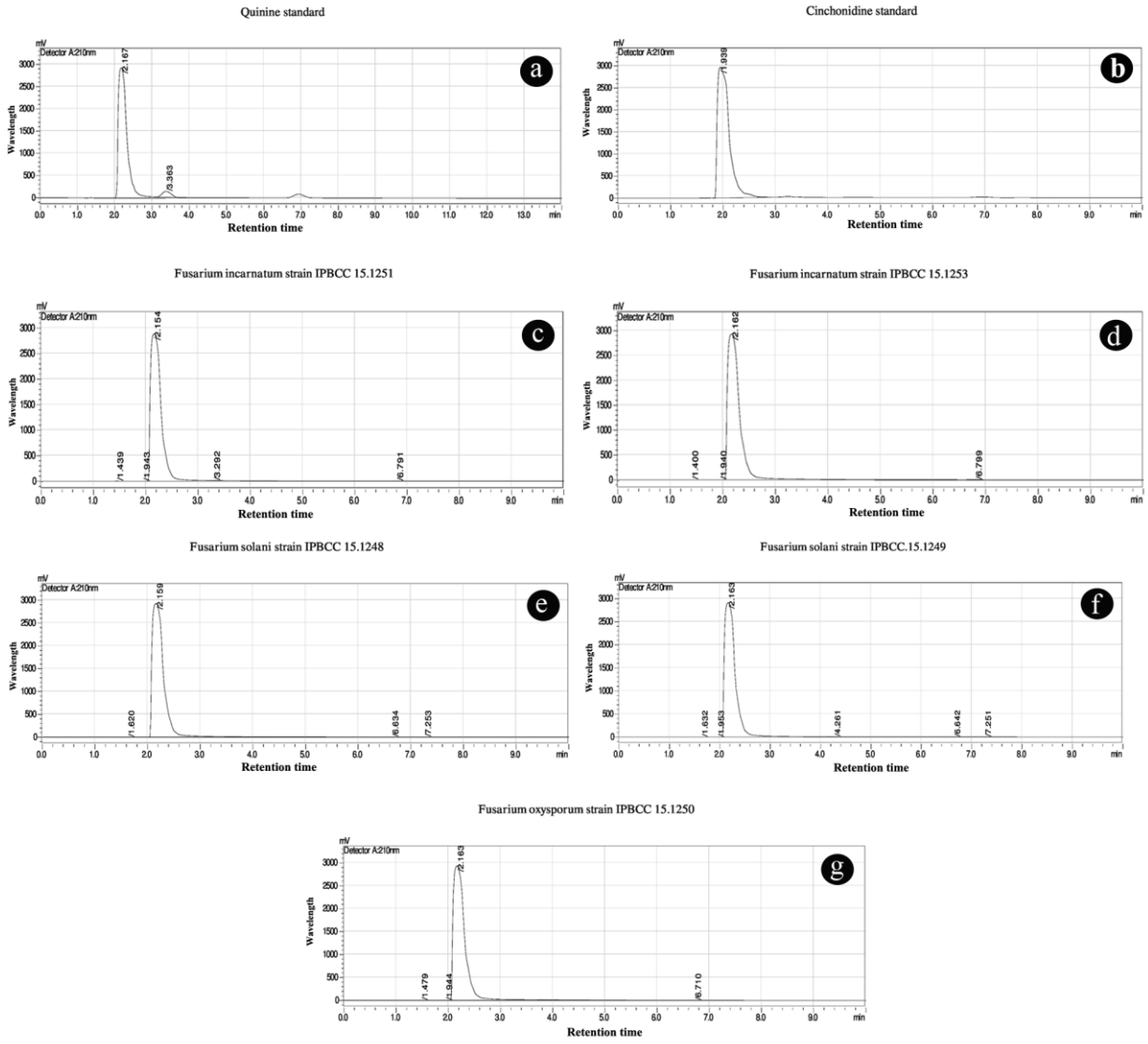

Fig. 4a–**g** – HPLC profiles of *Fusarium* isolates in this study: **a** quinine standard. **b** cinchonidine standard. **c** *F. incarnatum* strain IPBCC 15.1251. **d** *F. incarnatum* strain IPBCC 15.1253. **e** *F. solani* strain IPBCC 15.1248. **f** *F. solani* strain IPBCC 15.1249. **g** *F. oxysporum* strain IPBCC 15.1250.

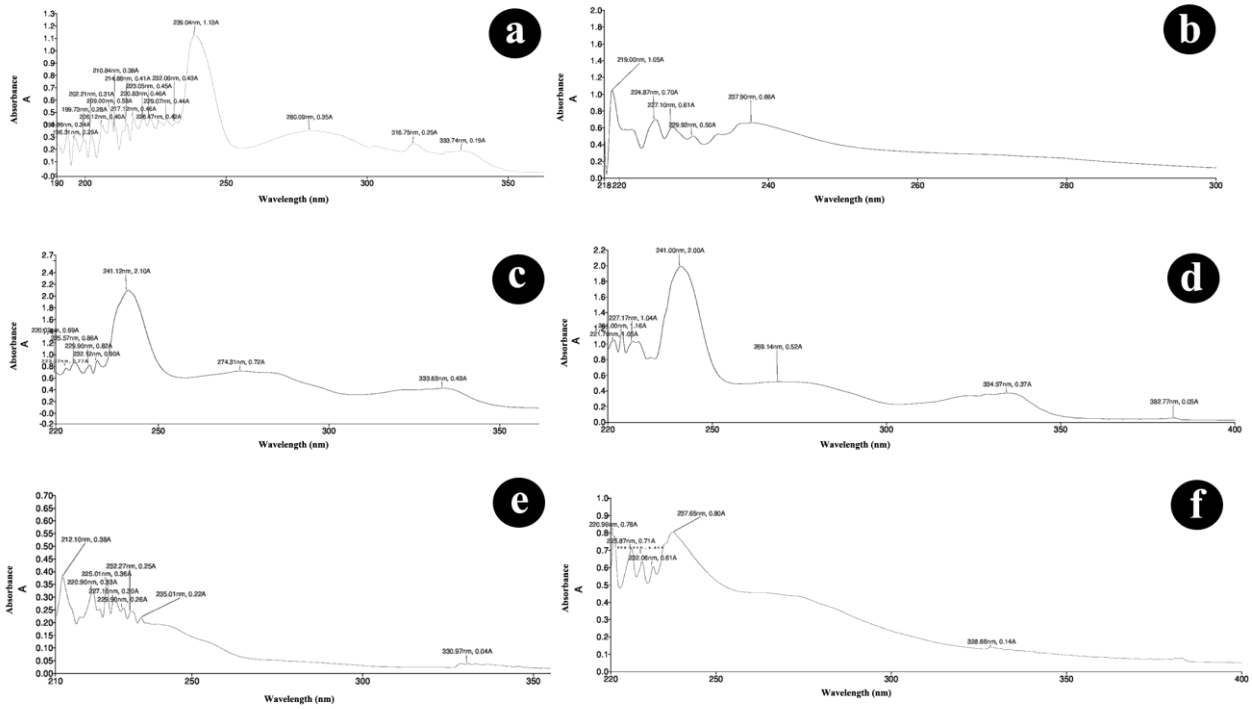

Fig. 5a–f – UV–Vis profiles of *Fusarium* isolates in this study: **a** quinine and cinchonidine mixed standard. **b** *F. oxysporum* strain IPBCC 15.1250. **c** *F. incarnatum* strain IPBCC 15.1251. **d** *F. incarnatum* strain IPBCC 15.1253. **e** *F. solani* strain IPBCC 15.1249. **f** *F. solani* strain IPBCC 15.1248.

In this study, three *Fusarium* species are reported as the new fungal endophytes capable of producing quinine, namely, *F. incarnatum* strain IPBCC 15.1253 and strain IPBCC 15.1251, *F. oxysporum* strain IPBCC 15.1250, and *F. solani* strain IPBCC 15.1248 and strain IPBCC.15.1249. These *Fusarium* isolates, except *F. solani* strain IPBCC 15.1248, were also capable in producing cinchonidine. All of these *Fusarium* species were isolated from different types of plant tissue. *Fusarium incarnatum* strain IPBCC 15.1251 was originally isolated from fruit, while *F. incarnatum* strain IPBCC 15.1253 was isolated from petiole, *F. oxysporum* strain IPBCC 15.1250 from bark, and *F. solani* strain IPBCC.15.1249 from twig (Table 2). This result not only showed that *Fusarium* endophytes from quina capable in producing similar *Cinchona* alkaloids produced by their host, but also revealed that the *Cinchona* alkaloids can be produced by fungal endophytes such as *Fusarium* spp. from different plant organs. The concentrations of quinine produced by *Fusarium* spp. from the current study were also higher than fungal endophytes from the previous studies, such as *Diaporthe* sp. CLF–J (AB505415), *Diaporthe* sp. CLF–M (AB505418) and *Arthrinium* sp. (AB505426) (Maehara et al. 2012, 2013). According to Maehara et al. (2013), endophytic fungi from *Cinchona* were capable in producing principal *Cinchona* alkaloids such as quinine, quinidine and cinchonine at concentration >50 µg/L. Since the highest quinine contents reside in the *Cinchona* bark (Song et al. 2009), it is unexpected that non–bark *Fusarium* endophyte was also produced quinine as high as the *Fusarium* endophyte isolated from bark.

Phylogenetic analysis of quinine–producing *Fusarium* species showed that there are more than one species of the genus *Fusarium* exist as endophyte within plant tissues of *C. calisaya*. The finding of more than one species from a single fungal genus occupied the same species of host plant, in fact, was not uncommon as several authors had previously reported (Niekerk et al. 2005, Santos & Phillips 2009, Santos et al. 2011, Thompson et al. 2011). The current study also found that a single species of endophyte can occupies different type of plant organs as showed by several morphotypes of *F. incarnatum* isolated from petiole (strain IPBCC.15.1253), fruit (strain IPBCC.15.1251) and bark (strain

IPBCC.15.1252). Eventually, enormous potential of fungal endophyte diversity as source of secondary metabolites discovery is demonstrated in this study.

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