Alkaloid profile of endophytic *Diaporthe* spp. from Cinchona calisaya

Profil alkaloid endofit Diaporthe *spp. dari Cinchona calisaya*

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Abstract

Endophytic fungi have been known as potential source of bioactive compound, similar to their host. This study was conducted to identify endophytic *Diaporthe* spp. isolated from *Cinchona calisaya* and to explore their potential in producing alkaloids, particularly quinine. A total of 39 strains of *Diaporthe* spp. were identified using ITS1-5.8S-ITS2 and EF1-α gene. Seventeen strains have species name and they represent eight species of *Diaporthe* i.e. *D. cynaroidis, D. endophytica, D. ganjae, D. gardeniae, D. litchicola, D. phaseolorum, D. pseudomangiferae,* and *D. rhoina*. The remaining 22 strains represents 15 groups of unidentified *Diaporthe* sp. High performance liquid chromatography analyses of chloroform extract of 21-days old static cultures of all fungi in potato dextrose broth showed that these strains produce totally 82 different alkaloids. Each strain producedabout 2-38 different alkaloids. Sixteen out of 39 strains (41%), i.e. one strain each of *D. endophytica*, *D. gardeniae*, *D. phaseolorum*, and *D. pseudomangiferae*, 2 strains of *D. litchicola*, and 10 strains of unidentified *Diaporthe* were able to produce quinine. The quinine concentration ranges from 1.1 mg/l to 155.2 mg/l. By using Jaccard's similarity index 0.44 as the cutting score, UPGMA analyses of alkaloid profile of these strains shows that these strains forms 23 clusters. Alkaloid profile clustering does not support the phylogenetic grouping. This indicates that the alkaloid profile is strain dependent. Some potential strains should be further investigated for optimization of their quinine production.

Keywords: *Cinchona calisaya*, *Diaporthe* spp., endophytes, quinine

Abstrak

Cendawan endofit merupakan salah satu sumber senyawa bioaktif yang potensial seperti inangnya. Penelitian ini bertujuan untuk mengidentifikasi Diaporthe *spp. asal* Cinchona calisaya *dan menganalisis potensi produksi alkaloidnya, terutama kuinin. Sebanyak 39 strain* Diaporthe *spp. diidentifikasi menggunakan sekuen daerah ITS1-5.8S-ITS2 and gen EF1-α. Tujuh belas strain termasuk ke dalam 8 spesies* Diaporthe *yaitu* D. cynaroidis, D. endophytica, D. ganjae, D. gardeniae, D. litchicola, D. phaseolorum, D. pseudomangiferae, and D. rhoina. *Dua puluh dua strain lainnya mewakili 15 grup* Diaporthe *spp. Analisis* High performance liquid chromatography *terhadap*

ekstrak kloroform dari kultur cendawan pada agar kentang cair yang diinkubasi secara statik selama 21 hari menunjukkan bahwa strain-strain tersebut menghasilkan 82 jenis alkaloid. Puncak-puncak pada kromatogram pada waktu retensi yang berbeda menunjukkan jenis alkaloid yang berbeda. Setiap strain dapat memproduksi 2-38 alkaloid yang berbeda. Sebanyak 16 dari 39 strain (41%), yaitu masing-masing satu strain dari D. endophytica, D. gardeniae, D. phaseolorum dan D. pseudomangiferae,*2 strain* D. litchicola, *dan 10 strain* Diaporthe *spp. dapat menghasilkan kuinin. Konsentrasi kuinin berkisar antara 1,1 mg/ml sampai 155,2 mg/ml. Berdasarkan batas nilai index kemiripan (Jaccard)= 0,44, analisis UPGMA profil alkaloid strain-strain ini menunjukkan bahwa strain-strain ini terbagi dalam 23 kelompok. Kelompok berdasarkan profil alkaloid tidak mendukung pengelompokan berdasarkan filogenetik. Hal ini menunjukkan bahwa profil alkaloid tersebut bergantung pada strainnya. Strain-strain potensial perlu dipelajari lebih lanjut dalam optimasi produksi kuininnya.*

Kata kunci: Cinchona calisaya, Diaporthe *spp., endofit, kuinin*

INTRODUCTION

Quina tree (*Cinchona* spp.) is widely known as a medicinal plant. The bark of this tree has antimalarial compound namely quinine (Taylor, 1975). In addition to antimalarial drug, quinine can be usedasraw material in pharmacy, as food colorant, and beverage flavor (Santoso *et al.,* 2004). Therefore, some species of *Cinchona* spp. has been cultivated in several countries for quinine production. One of the commercial clone for quinine production is *C. calisaya* (syn. *C. ledgeriana*).

Quinine industry in Indonesia has been established before Second World War, and Indonesiahad ever been recognized as the world's largest supplier of quinine (Susilo, 2011). Starting 2003, the Quina tree plantation area has decreased and consequently the tree populationhas also declined. In 2011, Quina plantation area was only about 3.886 hectares in West Java, and the production met only 30-50% demand of the industry (Susilo, 2011). This shortage is overcome by importing bark flakes. Although replanting program is being undertaken, alternative sources for quinine production have to be investigated. Among those alternatives is endophytic fungi obtained from Quina tree.

Endophytic fungi have been known to produce bioactive compounds originally from their host (Strobel *et al.,* 1993, Zhao *et al.,* 2010). For example, taxol is naturally produced by *Taxus brevifolia* and now is produced by various endophytes from different medicinal plants (Gangadevi *et al.,* 2008a, 2008b, Visalakchi & Muthumary, 2010). Endophytic fungi of *Cinchona* spp. have been reported by several researchers (Simanjuntak *et al.,* 2002, Mumpuni *et al.,* 2004, Winarno, 2006, Maehara *et al.,* 2010). A single strain of a closely related *Diaporthe phaseolorum*, an endophytic fungi from *C. ledgeriana* (syn. of *C. calisaya*), was reported to produce quinine and its related substances (Maehara *et al.,* 2012). Prior to this study, a number of specimens of endophytic *Diaporthe* from *C. calisaya* Wedd. (syn. *C. calisaya* var. *ledgeriana* Howard) has been collected (data unpublished). The collection was not limited to those from bark and young stems but from all parts of the tree. Based on phylogenetic study using Internal Transcribed Spacer (ITS) region, the collection consisted of *D. beckhausii, D. eucalyptorum, D. infecunda, D. hongkongensis, D. litchicola, D. phaseolorum, D. psoraleae,*

D. pseudomangiferae, and some unidentified species of *Diaporthe* (data unpublished). Re-identification of these *Diaporthe* using other marker such as EF1-α is still needed. Further, bioactive compoundsin those collected species has not been explored. Therefore, this study was aimed to reconfirm the identity of endophytic *Diaporthe* spp. isolated from leaf, petiole, twig, bark, and fruit of *C. calisaya* and to analyze its potential for alkaloid production, particularly quinine.

MATERIALS AND METHODS

Sampling and fungal endophyte isolation

Flowers, leaves (with petioles), stems, barks, and roots of *C. calisaya* werecollected from trees grown in quina plantation managed by the Research Center for Tea and Quina, Gambung, West Java, Indonesia (29 September 2012). The plantation is located at 7°8'35.78"S, 107°30'59.55"E and 1.400 m asl. Five pieces of each healthy organ were taken from 5 individual plants and placed in zipped plastic bags. The collecting bags were sealed and labelled with the name of the host tree, collection site, date, and collector(s). All specimens were kept in ice boxes prior to isolation in the laboratory.

Isolation

The fungal endophytes were isolated using isolation protocol described by Mostert *et al*. (2001) with modification. The samples were first washed in running tap water, then surface-sterilized using 70% ethanol for 1 min, followed by soaking in 3% sodium hypochlorite for 2 min, and 70% ethanol for 20 s. The samples were rinsed three times in sterile distilled water, 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 approximately1 × 2 cm and placed on the surface of Malt Extract Agar (MEA) (Difco, USA) (4 segments/petri dish). All petri dishes were incubated at room condition. Three replications were made for each sample. The growth of endophytic fungi mycelium were observed every day, for about 30 d. The growing colonies were purified using hyphal tip isolation method to get a pure culture. The cultures were kept in Bogor Agriculture University Culture Collection (IPBCC). Cultures having characteristics resembling morphotypes of *Diaporthe* were then selected for further investigation.

Identification of the fungi

Identification was done by phylogenetic analyses on the bases of combined ITS1-5.8S-ITS2 and EF gene sequences. Genomic DNA from 7-d fungal mycelia grown in 5 ml of Potato Dextrose Broth (PDB) (Difco, USA) were harvested using Phytopure™ DNA extraction kit (GE Health care, UK) following the manufacturer's protocol.

The primer pairs of ITS5 (forward)(5' –TCCTCCGCTTATTGATATGC–3') and ITS4 (reverse) (5'–TCCGTAGGTGAACCTGCGC–3') (White *et al.*, 1990) were used to amplify the Internal Transcribed Spacer (ITS) region including 5.8S rDNA. Amplification was done using Polymerase Chain Reaction (PCR) method performedin

a 25 µl reaction volume as follow: 10 µl nuclease free water, 12.5 µl DreamTaq® green master mix (Thermo scientific, USA), 0.5µl of forward and reverse primer, 0.5 µl DMSO, and 1 µl DNA template. The PCR condition for 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, 90s at 72°C extension, and 5 min at 72°C for the final extension.

EF gene was amplified using EF1-α 728F (5'-CATCGAGA AGTTCGAGAAG-G-3') and EF1-986R (5'-TACTTGAAG-GAACCCTTACC-3') primers. Amplification of EF gene was performed in 25 µl reaction volumes containing nuclease free-water 8.75 µl, Gotaq green master mix (Promega, USA) 12.5 µl, forward and reverse primer 0.625 µl for each primer, DMSO 0.5 µl and DNA template 2 µl. The PCR condition was 94°C for 5 min for initial denaturation, followed by 40 cycles of 30 s at 94°C for denaturation, 30 s at 52°C for annealing, 30s at 72°C for extension, and 7 min at 72°C for the final extension. All PCR reactions were conducted using T100 thermal cycler (Bio-Rad, USA).

PCR products were electrophorised 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.

Nucleotide sequences obtained from the respective primer pairs (ITS5 and ITS4) were examined and edited by direct examination using ChromasPro 1.41 software (Technelysium Pty Ltd., Australia). Phylogenetic analysis was conducted using the neighbor joining (NJ) method in MEGA 5.1 with 1.000 bootstrap cycle.

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 1.000 random sequence additions was performed to find the optimum tree. The stepwise addition option was set as random and the 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 using bootstrap (BS) analysis (Felsenstein, 1985) on 1.000 replications. BS values of 50% or higher than that were shown. Random sequence addition was used in the bootstrap analysis. All sites were treated as unordered and unweighted, and the gaps were treated as missing data. The partition homogeneity test (Farris *et al.*, 1994) on 1.000 replicates, 10 random addition sequence replicates, and TBR branch swapping was conducted by using PAUP* to determine whether ITS and EF1–α datasets were conflicting. A significance level of P = 0.01 was adopted for this test (Cunningham, 1997). The most parsimonious tree was refined using Tree Graph 2 software (Stöver & Müller, 2010).

Alkaloid production and extraction

Each of the pure cultures was re-cultivated on PDA (Potato Dextrose Agar) at room temperature for 7 d. Three pieces (0.5×0.5 cm^2) of mycelial plugs were inoculated into flask (500 ml) containing 200 ml PDB with an initial pH of 6.0. The

cultures were incubated in static condition for 21 d in room temperature. The mycelial mass was filered using Whatman paper no. 1. The secondary metabolites were extracted separately both from biomass and filtrate by adding 100 ml (v/v) chloroform ($CHCl_3$) (Simanjuntak *et al.*, 2002; Winarno, 2006) and 0.1 N NaOH (v/v), followed by homogenizing using separating funnel and allowed to stand a few seconds to form two layers. The upper and the bottom layer were separated. The bottom layer was taken out and the same volume of new chloroform was given to extract the remaining metabolites. Each fraction was then collected and concentrated using rotary evaporator (Buchi, Switzerland) at 45°C and 60 rpm. The extracts were stored at 4°C as stock solution for alkaloid analysis.

HPLC analysis

The fractions were analyzed quantitatively using high performance liquid chromatography types Perkin Elmer Series 200 UV-Vis detector, equipped with Ascentis C18 column, with KH_2PO_4 20 mM pH 2.5: CH_3CN = 75%: 25% as eluent, at flow rate of 1.20 ml/min and detected in 234 nm wavelength.

$$\text{Concentration of quina (mg/l)} = \frac{\text{area sample}}{\text{area standard}} \text{ x } [\text{Standard}]$$

Data analysis

Unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis was applied on the basis of Jaccard's coefficient using Multivariate Statistical Package software (MVSP) version 3.13r for dendrogram reconstruction. Jaccard's coefficient was calculated based on the presence of the alkaloidal peak.

RESULT AND DISCUSSION

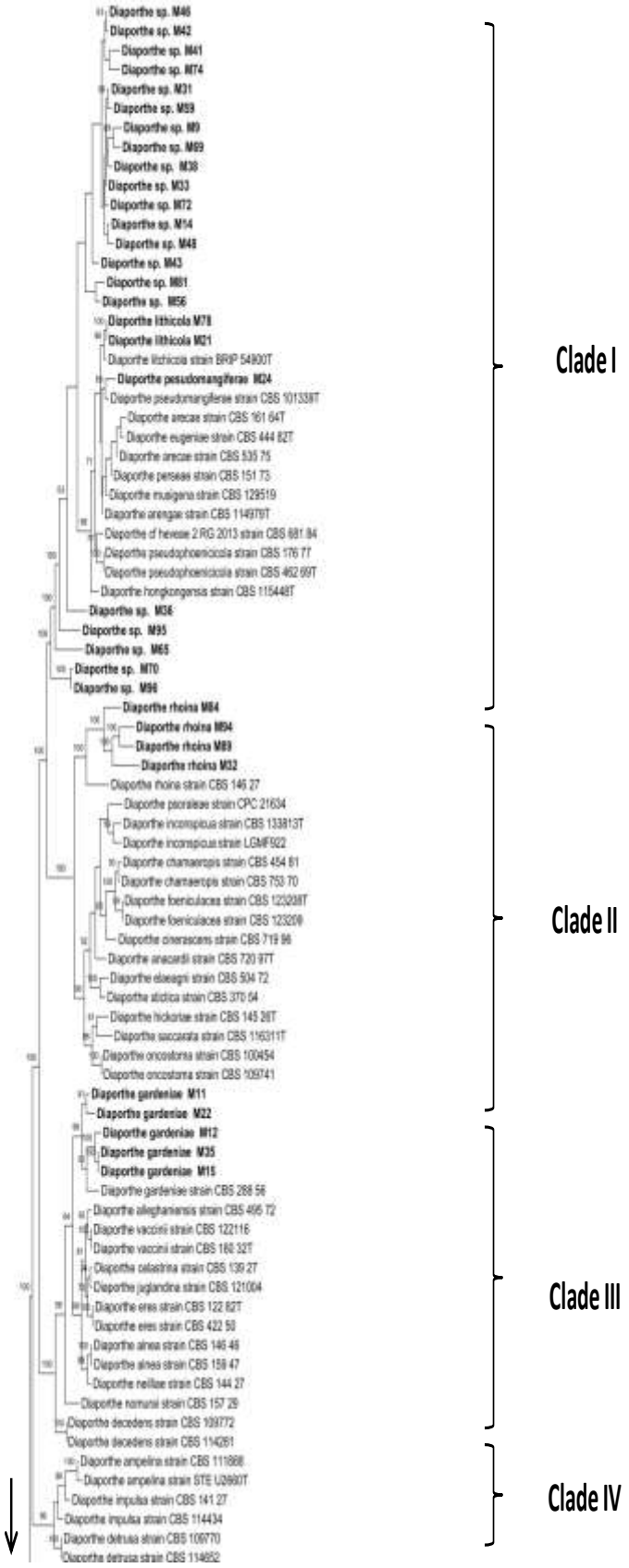
This study represents the first step for linking the biodiversity of endophytic *Diaporthe* spp. from *C. calisaya*, their taxonomic identities, and phylogenetic positions with their alkaloid profile. Therefore, the correct name for each is needed. A total of 39 strain of endophytic *Diaporthe* spp. from various organs of *C. calisaya* were studied (Table 1). Seventeen strains can be identified into species level. These consist of *D. cynaroidis*, *D. endophytica*, *D. eucalyptorum*, *D. ganjae*, *D. gardenia*, *D. litchicola*, *D. phaseolorum*, *D. pseudomangiferae*, and *D. rhoina*. The remaining 22 strains are still unidentified and they represent 15 clusters. According to Gomes *et al.* (2013) additional gene marker such as TUB, ACT and HIS are needed for identification certain species of *Diaporthe*.

In this combined analyses of ITS and EF 1-α sequences, the data set consisted of 147 taxa with 1280 total characters and *Diaporthella corylina* CBS 121124 is used as an outgroup. Of these characters, 494 characters were constant, 222 were parsimony uninformative and 564 were parsimony informative. Following a heuristic search using PAUP, 958 most parsimonious trees were retained (length= 6517 steps, CI= 0.236, RI= 0.651, RC= 0.154, HI= 0.764) of which one is shown in Fig. 1. A partition homogeneity test showed that ITS and EF1-α could be combined (P= 0.647) into single analysis. In the most parsimonious tree, 10 clades are formed (Fig 1).

Clade I consists of 23 strains of *Diaporthe* spp and within this clade thereare 2 sub-clades, and some single lineage groups. Sub-clade I consists of 19 strains of *Diaporthe* spp. that do not cluster with any reference.

TABLE 1
Thirty nine strains of endophytic *Diaporthe* spp. in *Cinchona calisaya* under study

Species	Source	Strain	Accesion number	
			ITS	EF
Diaporthe sp. M9	Twig	IPBCC 15.1286	LC041055	LC050454
Diaporthe sp. M14	Twig	IPBCC 15.1291	LC041028	LC050469
Diaporthe sp. M31	Twig	IPBCC 15.1278	AB899786	AB900129
Diaporthe sp. M33	Twig	IPBCC 15.1282	AB899787	AB900130
Diaporthe sp. M36	Leaf	IPBCC 15.1279	LC041046	LC050463
Diaporthe sp. M38	Twig	IPBCC 15.1284	LC041019	LC050471
Diaporthe sp. M41	Twig	IPBCC 15.1311	LC041054	LC050455
Diaporthe sp. M42	Twig	IPBCC 15.1277	LC041035	LC050459
Diaporthe sp. M43	Petiole	IPBCC 15.1276	AB899784	AB900127
Diaporthe sp. M45	Leaf	IPBCC 15.1284	LC041023	LC050466
Diaporthe sp. M46	Petiole	IPBCC 15.1294	AB899785	AB900131
Diaporthe sp. M48	Twig	IPBCC 15.1310	LC041053	LC050483
Diaporthe sp. M56	Fruit	IPBCC 15.1296	LC041022	LC050484
Diaporthe sp. M59	Twig	IPBCC 15.1284	LC041023	LC050486
Diaporthe sp. M65	Root	IPBCC 15.1290	LC041057	LC050456
Diaporthe sp. M69	Twig	IPBCC 15.1287	LC041056	LC050467
Diaporthe sp. M70	Twig	IPBCC 15.1304	LC041034	LC050473
Diaporthe sp. M72	Twig	IPBCC 15.1280	LC041038	LC050485
Diaporthe sp. M74	Leaf	IPBCC 15.1280	LC041029	LC050461
Diaporthe sp. M81	Bark	IPBCC 15.1295	LC041021	LC050457
Diaporthe sp. M95	Twig	IPBCC 15.1301	LC041027	LC050468
Diaporthe sp. M96	Twig	IPBCC 15.1305	LC041048	LC050476
D. cynaroidis M54	Fruit	IPBCC 15.1274	LC041031	LC050472
D. endophytica M90	Leaf	IPBCC 15.1312	AB899789	AB900123
D. ganjae M71	Twig	IPBCC 15.1340	LC041037	LC050478
D. gardeniae M11	Petiole	IPBCC 15.1339	LC041017	LC050460
D. gardeniae M22	Leaf	IPBCC 15.1307	LC041030	LC050470
D. gardeniae M12	Twig	IPBCC 15.1310	LC041062	LC050462
D. gardeniae M35	Petiole	IPBCC 15.1275	LC041051	LC050474
D. gardeniae M15	Twig	IPBCC 15.1308	LC041049	LC050477
D. litchicola M78	Fruit	IPBCC 15.1297	AB899788	AB900128
D. litchicola M21	Fruit	IPBCC 15.1314	LC041026	LC050475
D. phaseolorum M10	Fruit	IPBCC 15.1319	LC041043	LC050458
D. phaseolorum M40	Twig	IPBCC 15.1318	LC041040	LC050482
D. pseudomangiferae M24	Twig	IPBCC 15.1299	LC041041	LC050480
D. rhoina M84	Twig	IPBCC 15.1322	LC041047	LC050464
D. rhoina M94	Petiole	IPBCC 15.1320	LC041018	LC050465
D. rhoina M89	Twig	IPBCC 15.1288	LC041058	LC050479
D. rhoina M32	Twig	IPBCC 15.1323	LC041050	LC050481


Diaporthe sp. M46
Diaporthe sp. M42
Diaporthe sp. M41
Diaporthe sp. M74
Diaporthe sp. M31
Diaporthe sp. M59
Diaporthe sp. M9
Diaporthe sp. M69
Diaporthe sp. M38
Diaporthe sp. M33
Diaporthe sp. M72
Diaporthe sp. M14
Diaporthe sp. M48
Diaporthe sp. M43
Diaporthe sp. M81
Diaporthe sp. M56
Diaporthe litchicola M78
Diaporthe litchicola M21
Diaporthe litchicola strain BRIP 54900T
Diaporthe pesudomangiferae M24
Diaporthe pseudomangiferae strain CBS 101339T
Diaporthe arecae strain CBS 161 64T
Diaporthe eugeniae strain CBS 444 82T
Diaporthe arecae strain CBS 535 75
Diaporthe perseae strain CBS 151 73
Diaporthe musigena strain CBS 129519
Diaporthe arengae strain CBS 114979T
Diaporthe cf hevese 2 RG 2013 strain CBS 681 84
Diaporthe pseudophoenicicola strain CBS 176 77
Diaporthe pseudophoenicicola strain CBS 462 69T
Diaporthe hongkongensis strain CBS 115448T
Diaporthe sp. M36
Diaporthe sp. M95
Diaporthe sp. M65
Diaporthe sp. M70
Diaporthe sp. M96
Diaporthe rhoina M84
Diaporthe rhoina M94
Diaporthe rhoina M89
Diaporthe rhoina M32
Diaporthe rhoina strain CBS 146 27
Diaporthe psoraleae strain CPC 21634
Diaporthe inconspicua strain CBS 133813T
Diaporthe inconspicua strain LGMF922
Diaporthe chamaeropis strain CBS 454 81
Diaporthe chamaeropis strain CBS 753 70
Diaporthe foeniculacea strain CBS 123208T
Diaporthe foeniculacea strain CBS 123209
Diaporthe cinerascens strain CBS 719 96
Diaporthe anacardii strain CBS 720 97T
Diaporthe elaeagni strain CBS 504 72
Diaporthe sticica strain CBS 370 54
Diaporthe hickoriae strain CBS 145 26T
Diaporthe saccarata strain CBS 116311T
Diaporthe oncostoma strain CBS 100454
Diaporthe oncostoma strain CBS 109741
Diaporthe gardeniae M11
Diaporthe gardeniae M22
Diaporthe gardeniae M12
Diaporthe gardeniae M35
Diaporthe gardeniae M15
Diaporthe gardeniae strain CBS 288 56
Diaporthe alleghaniensis strain CBS 495 72
Diaporthe vaccinii strain CBS 122116
Diaporthe vaccinii strain CBS 160 32T
Diaporthe celastrina strain CBS 139 27
Diaporthe juglandina strain CBS 121004
Diaporthe eres strain CBS 122 82T
Diaporthe eres strain CBS 422 50
Diaporthe alnea strain CBS 146 46
Diaporthe alnea strain CBS 159 47
Diaporthe neilliae strain CBS 144 27
Diaporthe nomurai strain CBS 157 29
Diaporthe decedens strain CBS 109772
Diaporthe decedens strain CBS 114261
Diaporthe ampelina strain CBS 111888
Diaporthe ampelina strain STE U2660T
Diaporthe impulsa strain CBS 141 27
Diaporthe impulsa strain CBS 114434
Diaporthe detrusa strain CBS 109770
Diaporthe detrusa strain CBS 114852
Clade I
Clade II
Clade III
Clade IV

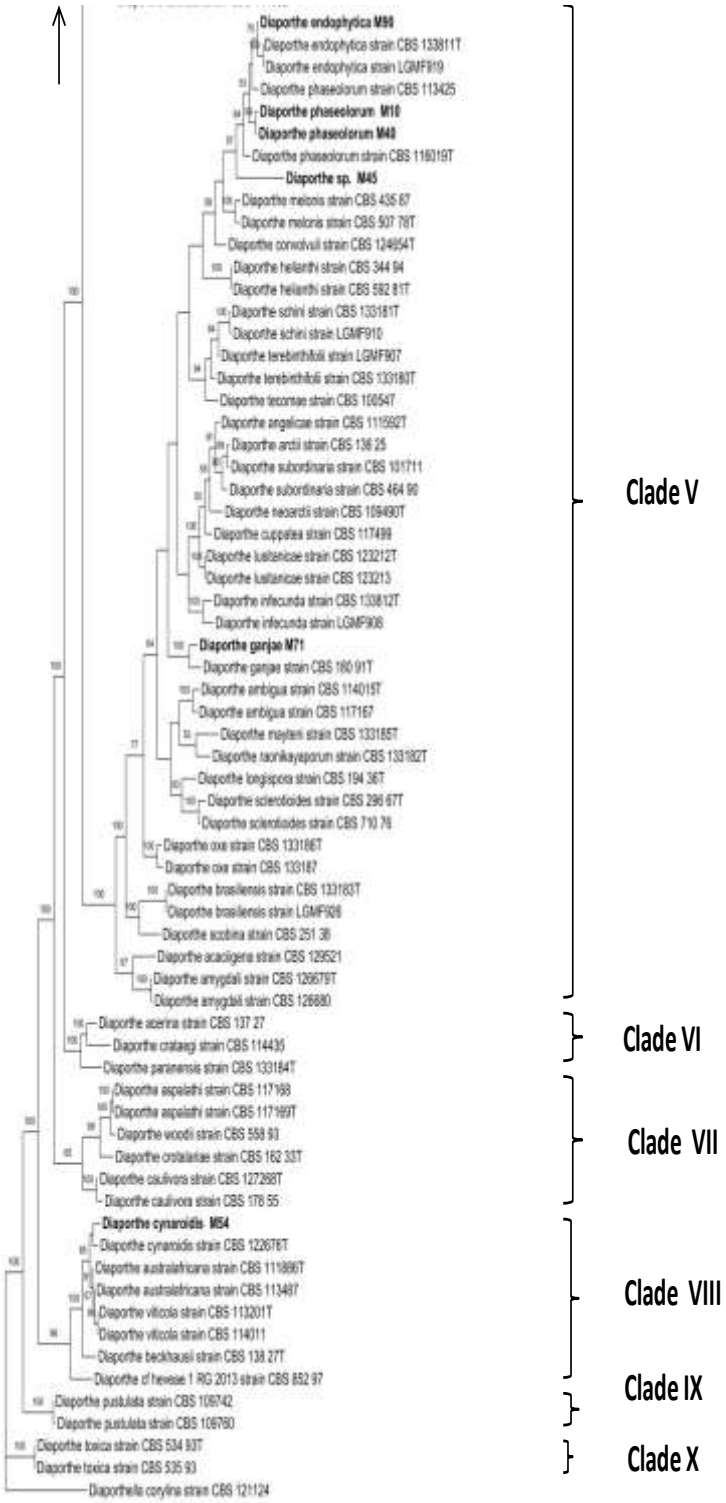


FIGURE 1
Maximum-parsimony tree showing a relationship between endophytic fungi of *Diaporthe* spp. and references based on the sequences of combine between ITS5-5.8S-ITS4 of nuclear rDNA and EF1-α gene. The tree was rooted with *Diaporthella corylina* CBS 121124. Bootstrap value > 50% (1000 replicates) are shown at the branches.

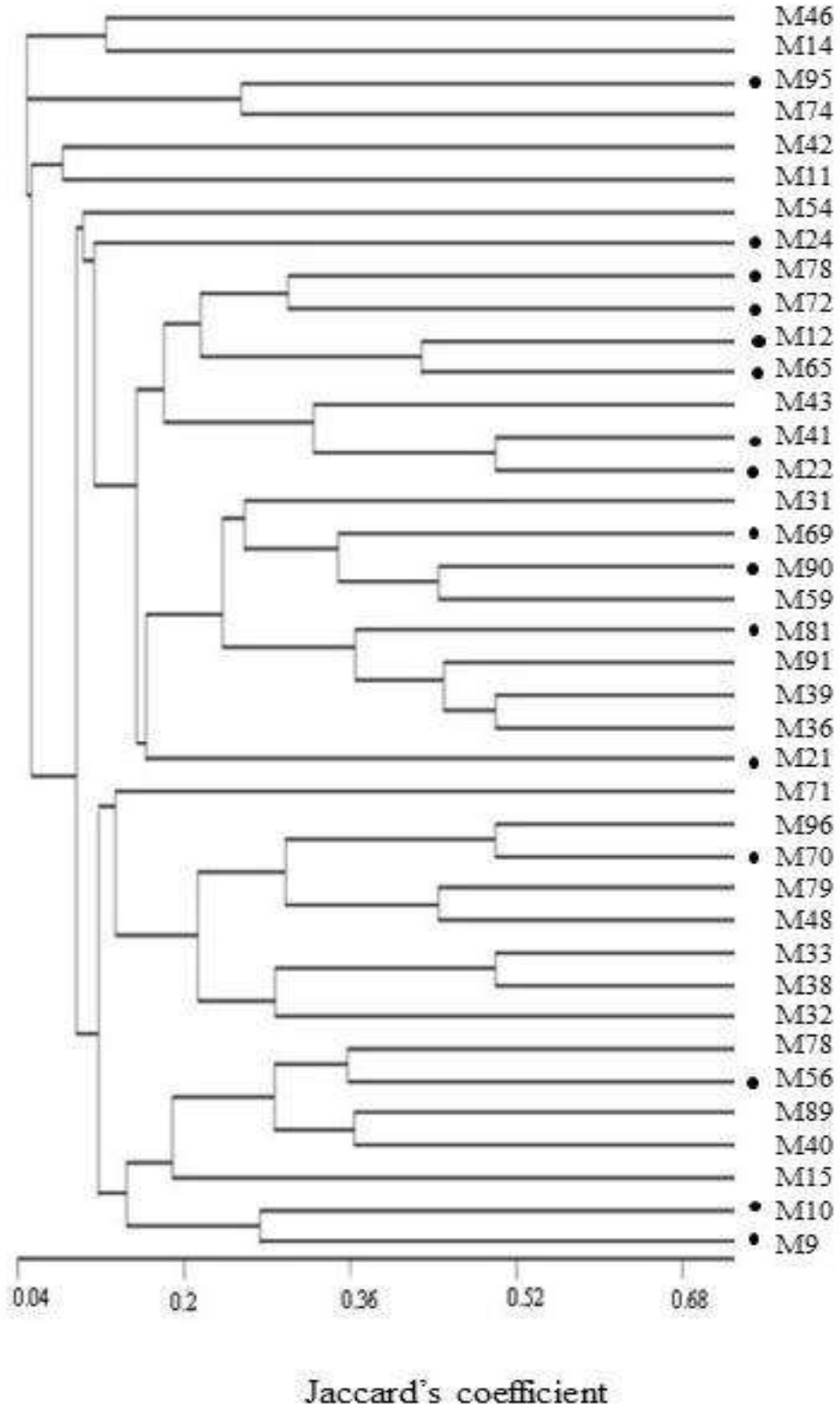


FIGURE 2
Clusters of endophytic *Diaporthe* spp. based on the similarity on their alkaloid profile.

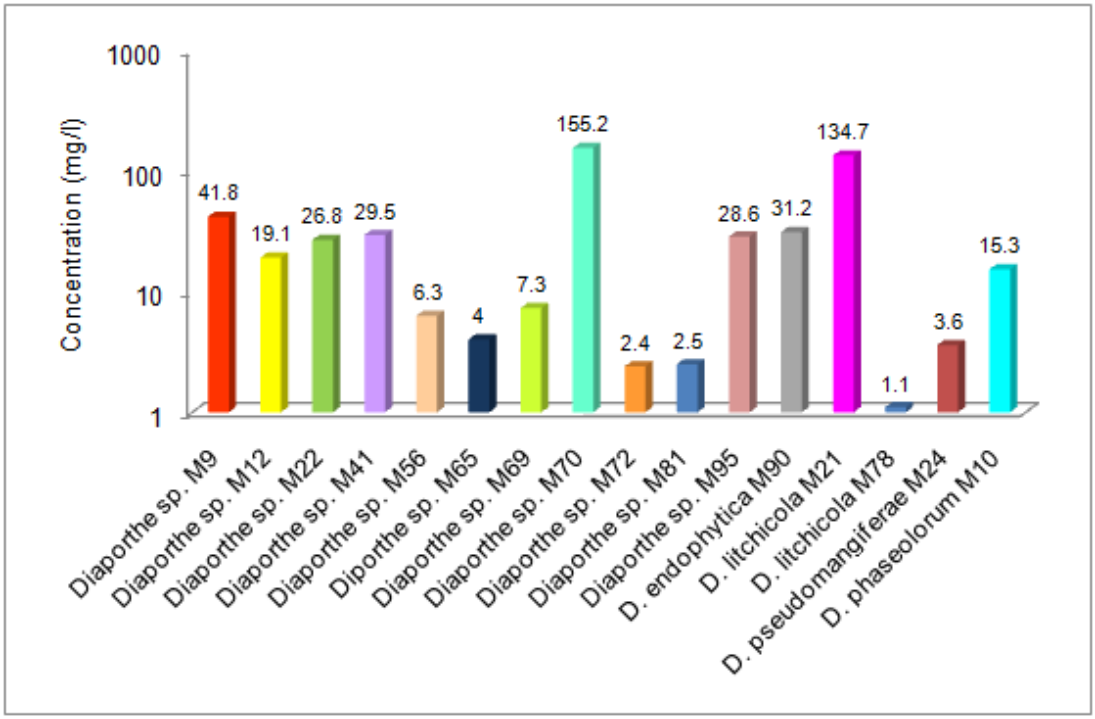


FIGURE 3
Quinine production of *Diaporthe* spp.

Strains (Fig 1). Two strains (M78 and M21) of *D. litchicola* and *D. litchicola* strain BRIP 54900 form sub-clade II with 100% BS.

Diaporthe sp. M24 is in one clade with *D. pseudomangiferae* CBS 101339 with 85% BS. The other *Diaporthe* such as strain M36, M95, M65, M70, and M96 form single lineage group that are not clustered with any reference strains, and thus strains could not be named.

Clade II consists of *D. rhoina* M84, M94, M89, M36 and *D. rhoina* CBS 14627 that has 100% BS. Clade III consists of *D. gardeniae* M11, M22, M48, M35, M15 and *D. gardeniae* CBS 288 46 with BS 96%. None of the specimens of *Diaporthe* is in clade IV. In clade V, strain M90 is identified as *D. endophytica* since it is in one clade with *D. endophytica* CBS 13381 and LGMF 919 with 70% BS. *Diaporthe* strain M10 and M40 are considered as *D. phaseolorum*, eventhough their clustering with *D. phaseolorum* CBS 116019 supported with low BS value (53%). Strain M45 is a sister clade to *D. melonis* CBS 43587 and 50778, and thus is unidentified. Strain M71 is *D. ganjae* since they form one clade with *D. ganjae* CBS 18091 (BS 100%). In clade VIII, strain M54 form one clade with *D. cynaroidis* CBS 122676 that is supported by low BS value. None of the specimens fall within clade VI and VII, IX and X.

All strains studied were able to produce alkaloids. Based on their unique retention time, about 82 different alkaloids were detected. One strain is able to produce 2-38 alkaloids with different concentrations in which one of them is quinine. According to Zhang *et al.* (2012) various quinoline and isoquinoline alkaloids were produced by

endophytic fungi. However, alkaloid-producing *Diaporthe* is rarely reported. A few examples have been identified, such as*D. phaseolorum,* an endophytic fungi from *C. ledgeriana* that excreted quinine (Maehara *et al.,* 2012), *Diaporthe* sp. from *Rhizophora stylosa* produced isochromophilones (Zang *et al.,* 2012) and *Phomopsis* sp. from *Allamanda cathartica* gives lactone alkaloid (Nithya *et al*., 2011). These alkaloids are not present as a single component in the crude extract.

UPGMA analyses using alkaloids profiles of all strains resulted in 23 clusters (Fig. 2) using similarity index of 0.44 as the cutting score. This grouping is hardly depicted the phylogenetic relationship. Of those cluster, only *Diaporthe* sp. M70-96 group is in accordance with their phylogenetic clustering. Therefore, this result indicates that alkaloid production is not species dependent but strain dependent.

Of the alkaloids detected, quinine is present in 16 strains out of 39 strains of endophytic *Diaporthe* spp. studied. The concentration is between 1.1 mg/l and 155.2 mg/l (Fig 3). Quinine is the major alkaline alkaloids in 5 strains tested, i.e. *Diaporthe* sp. M70, *D. litchicola* M21, *Diaporthe* sp M9, and *D. endophytica* M90 Of those quinine-producing strains, produces relatively high concentration of quinine, i.e. 155.2 mg/l, 134.7 mg/l, 41.8 mg/l, and 31.2 mg/l, respectively (Fig 3) comparing to those reported by Maehara *et al.* (2012), i.e. 60-100 µg/l.

The quinine-producing *Diaporthe* found in this study were isolated either from twig, fruit, leaf, petiole, root, and bark of *C. calisaya,* with twig as the most common habitat. This finding indicates that *Diaporthe* from organs other than young stem (Maehara *et al.,* 2011) are also able to produce quinine. This also shows that non-primary quinine accumulating organs such as bark may inhabit quinine-producing endophytic fungi.In addition to quinine, other alkaloids such as quinidine, cinchonine and cinchonidine are expected to present in the metabolite extract. Further investigation on these alkaloids is needed.

Other chemicals from endophytic fungi may also have antimalarial activities. Isaka *et al.* (2007, 2010) reported that pullularin A, B and C from culture of endophytic *Pullularia* sp. and sesquiterpenoids from *Xylaria* sp. exhibited antimalarial activities. They inhibited *Plasmodium falciparum* K1 with IC_{50} 3.6 µg/ml. Furthermore, Romero *et al.* (2008) isolated Butyrolactone V compound from endophytic *Xylaria* sp., with potential activity against *P. falciparum.* Haritakun *et al.* (2010) also isolated endophytic *Aspergillus terreus* that showed anti-malarial activity with IC_{50} 7.9µg/ml.

CONCLUSION

In conclusion, endophytic *Diaporthe* from *C. calisaya* consisted of eight species including some unidentified *Diaporthe*. All strains were able to produce alkaline alkaloids, but only 41% strains were able to produce quinine up to 200 mg/ml. Quinine production is not species dependent but strain dependent as the alkaloid profile varies depending on strains. These findings imply on the potential use of *Diaporthe* as alkaline alkaloids producers including quinine in sustainable bases. Further investigation should be emphasized on bioprospect assessment of the potential strains.

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