Transformation of *Cinchona* Alkaloids into 1-*N*-Oxide Derivatives by Endophytic *Xylaria* sp. Isolated from *Cinchona pubescens*

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The microbial transformation of four *Cinchona* alkaloids (quinine, quinidine, cinchonidine, and cinchonine) by endophytic fungi isolated from *Cinchona pubescens* was investigated. The endophytic filamentous fungus *Xylaria* sp. was found to transform the *Cinchona* alkaloids into their 1-*N*-oxide derivatives.

Key words *Cinchona pubescens*; endophyte; *Xylaria* sp.; microbial transformation; *Cinchona* alkaloid; antimalarial activity

Cinchona alkaloids have been used as drugs for the treatment of several diseases. Quinine is much popular as an antimalarial drug against the erythrocyte stage of the parasite. However, to the best of our knowledge, no research on the endophytes isolated from *Cinchona* plants has been reported. We predicted that endophytic microbes living in *Cinchona* plants would transform *Cinchona* alkaloids into their chemical derivatives. In the present paper, we report the microbial transformation of *Cinchona* alkaloids by the endophytic fungus *Xylaria* sp. which was isolated from the young stems of *Cinchona pubescens* VAHL. (Rubiaceae).[1)]

The young stems of *Cinchona pubescens* collected in West Java, Indonesia, were cut into pieces *ca.* 1 cm in length and the exterior was sterilized with 70% EtOH and 5.3% sodium hypochlorite. Then the pieces were placed on corn-meal malt agar (CMMA) containing chroramphenicol and incubated at 27 °C for 3 d. Then individual colonies on the plates were transferred to potato dextrose agar (PDA) and incubated again at 27 °C for 5 d with periodic checks of purity to obtain a total of 10 endophytic filamentous fungi.

Through several screening tests for the microbial transformation of *Cinchona* alkaloids by the 10 endophytic fungi, it was found that the filamentous fungus *Xylaria* sp.[2)] transforms four *Cinchona* alkaloid hydrochloride salts, *i.e.*, quinine hydrochloride (**1**·HCl), quinidine hydrochloride (**2**·HCl), cinchonidine hydrochloride (**3**·HCl), and cinchonine hydrochloride (**4**·HCl), into chemical derivatives in potato dextrose broth (PDB) medium.

Each cultivation medium including the fungus bodies was homogenized and extracted with $CHCl_3$. The $CHCl_3$-soluble portion was separated by HPLC on Hibar LiChrosorb NH_2 to afford **1a** (90% yield) from **1**·HCl, **2a** (71% yield) from **2**·HCl, **3a** (82% yield) from **3**·HCl, and **4a** (52% yield) from **4**·HCl, respectively. Among those transformation products, **1a**, **2a**, and **3a** were identified by comparisons of their physicochemical data (mp, $[\alpha]_D$, ^{1}H-, ^{13}C-NMR, IR, and UV) with those of quinine 1-*N*-oxide,[3—5)] quinidine 1-*N*-oxide,[3—6)] and cinchonidine 1-*N*-oxide,[3,7)] respectively.

4a, which was transformed from cinchonine hydrochloride (**4**·HCl), showed similar absorptions to those of **4** in the IR and UV spectra. The ^{13}C-NMR spectrum (in CD_3OD) also showed similar signal patterns to those of **4**, except the chemical shifts of 2-C (δ 65.0), 6-C (δ 66.6), and 8-C (δ 74.0) observed in lower magnetic fields than those of 2-C (δ 50.3), 6-C (δ 50.9), and 8-C (δ 61.3) for cinchonine (**4**). From the above findings, it was assumed that **4a** might be cinchonine 1-*N*-oxide, which was previously prepared from **4** by Dodin *et al.*[7)]

The endophytic fungus *Xylaria* sp. was submitted to DNA analysis of the 18S, ITS-1, 5.8S, and ITS-2 rDNA regions. Comparison of the base sequences with those of the authentic *Xylaria enteroleuca* (CBS 651.89),[8)] analyzed by us, showed 99.9% similarity for the 18S region [*X. enteroleuca* (CBS 651.89): 3 gaps/2455 bp] and 100% similarity for the ITS1-5.8S-ITS2 region (total 530 bp). These findings indicate that the endophytic *Xylaria* sp. is very close to *X. en-*

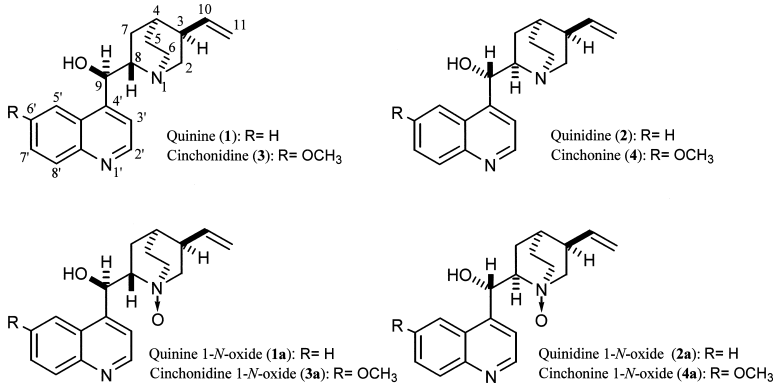

Fig. 1

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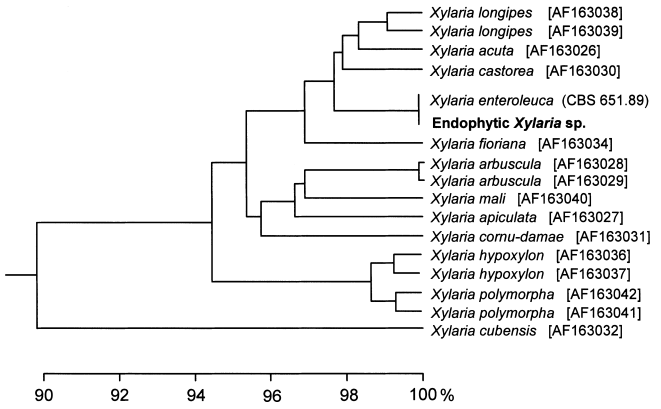

Fig. 2. Phylogenetic Relationships in the ITS1-5.8S-ITS2 rDNA Region among 16 *Xylaria* Fungi and the Endophytic Fungus *Xylaria* sp.

Table 1. Antimalarial Activity of 1-*N*-Oxide Derivatives (**1a**, **2a**, **3a**, **4a**)

	IC_{50} against *P. falciparum*	Breeding ratio for FM3A cells (%)
Quinine 1-*N*-oxide (**1a**)	1.3×10^{-5} M	83 (4.1×10^{-5} M)
Quinidine 1-*N*-oxide (**2a**)	1.2×10^{-5} M	71 (3.9×10^{-5} M)
Cinchonidine 1-*N*-oxide (**3a**)	—	100 (4.2×10^{-5} M)
Cinchonine 1-*N*-oxide (**4a**)	2.1×10^{-5} M	100 (4.6×10^{-5} M)
Quinine (**1**)	1.5×10^{-7} M	58 (4.7×10^{-5} M)

teroleuca.

Interestingly, the authentic *X. enteroleuca* (CBS 651.89) also transformed *Cinchona* alkaloids (**1**·HCl, **2**·HCl, **3**·HCl, and **4**·HCl) into their 1-*N*-oxides in 94% yield for **1a**, 70% yield for **2a**, 60% yield for **3a**, and 71% yield for **4a**, respectively.

Siebers-Wolff *et al.*[3] reported the microbial transformation of *Cinchona* alkaloids, from **1** to **1a** (conversion yield 8.6% by *Microsporium gypseum*, 1.3% by *Pelliccularia filamentosa*), from **2** to **2a** (9.2% by *Xylaria digitata*, 1.0% by *Mycobacterium smegmatis*), and from **3** to **3a** (4.7% by *Pelliccularia filamentosa*). However, they did not mention any microbes that transformed cinchonine (**4**) into the 1-*N*-oxide (**4a**).

To determine the detailed mechanism of microbial oxidation by the *Xylaria* sp., three reactions in PDB were conducted: 1) cultivation of the fungus with bubbling of nitrogen gas; 2) transformation by the filtrate of cultivated medium of the fungus; and 3) transformation without the fungus (as a blank test). Under all conditions, the alkaloids (**1**·HCl, **2**·HCl, **3**·HCl, and **4**·HCl) were not transformed into their 1-*N*-oxides. Therefore the microbial oxidation of *Cinchona* alkaloids may be carried out by an endoenzyme system, not by an exoenzyme system, using molecular oxygen.

Finally, it should be noted that quinine 1-*N*-oxide (**1a**), quinidine 1-*N*-oxide (**2a**), and cinchonine 1-*N*-oxide (**4a**) weakly inhibit the proliferation of the malaria parasite pathogen *Plasmodium falciparum* (a chloroquine-resistant strain).[9]

Experimental

Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-360 digital polarimeter. FAB-MS were recorded on JEOL SX-102A spectrometer, IR spectra on a Shimadzu FT-IR 8500 spectrometer, and UV spectra on a Hitachi U-3500 spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained with a JEOL JNM-Lambda 500 spectrometer operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C nuclei, respectively; chemical shifts are reported in parts per million relative to that of tetramethylsilane ($\delta=0$) as an internal standard, and coupling constants are given in Hertz. PCR amplification was carried out with an ABI GeneAmp PCR System 9700. DNA sequencing was performed with an ABI PRISM 310 Genetic Analyzer. HPLC was carried out with a TOSOH PD-8020. Column chromatography was carried out using Kieselgel 60 (230—400 mesh, Merck). TLC on silica gel 60F_{254} (Merck) was used to ascertain the purity of the compounds. The spots were visualized by spraying with Dragendorff's reagent.

Plant Material The young stems of *C. pubescens* Vahl. were collected in the Cibodas Botanical Garden in West Java, Indonesia, in August 1998, and identified at the Herbarium Bogoriense, Research Centre for Biology-LIPI, Indonesia.

Isolation of Endophytic Fungi from *C. pubescens* The young stems were cut into pieces (*ca.* 1 cm in length) and washed under tap water for 10 min. The pieces were treated with 70% ethanol for 1 min, 5.3% sodium hypochlorite for 5 min, and 75% ethanol for 0.5 min, and then cut into two pieces. The cut stems were placed on the plating medium [CMMA containing chroramphenicol (0.05 mg/ml)], and incubated at 27 °C. After 3 d, individual colonies were transferred to PDA and then incubated again at 27 °C for 5 d with periodic checks for purity. The 10 isolated endophytic filamentous fungi were stored on PDA at −80 °C.

Microbial Transformation of *Cinchona* Alkaloids in PDB by the Endophytic Fungus *Xylaria* sp. Endophytic *Xylaria* sp. was inoculated into PDB solution [4.8 g of PDB (DIFCO) and 200 ml of water] in four 500-ml Erlenmeyer flasks and then shaken at 120 rpm at 27 °C. After one day, a small amount (2 mg each) of *Cinchona* alkaloids [quinine hydrochloride (**1**·HCl), quinidine hydrochloride (**2**·HCl), cinchonidine hydrochloride (**3**·HCl), and cinchonine hydrochloride (**4**·HCl)] were added to each reaction medium in the Erlenmeyer flasks and shaking continued. After 3 d, *Cinchona* alkaloids (20 mg each of **1**·HCl, **2**·HCl, **3**·HCl, and **4**·HCl) were again added to each reaction medium.

After cultivation for 2 weeks, each medium including the fungus bodies was homogenized and extracted with $CHCl_3$. Each $CHCl_3$ layer was separated by HPLC [Hibar LiChrosorb NH_2, 5 μm, 4.6×250 mm; CH_3CN:H_2O=95:5; flow rate, 1.0 ml/min; detector, UV (210 nm)] to afford **1a** (18.7 mg, 90% conversion yield from **1**·HCl), **2a** (14.7 mg, 71% conversion yield from **2**·HCl), **3a** (16.9 mg, 82% conversion yield from **3**·HCl), and **4a** (10.7 mg, 52% conversion yield from **4**·HCl). When *Cinchona* alkaloids (22 mg each of **1**·HCl, **2**·HCl, **3**·HCl, and **4**·HCl) were added at once to the reaction medium, the conversion yields decreased.

Among the microbial transformation products, **1a**, **2a**, and **3a** were identified as quinine 1-*N*-oxide,[3—5] quinidine 1-*N*-oxide,[3—6] and cinchonidine 1-*N*-oxide,[3,7] respectively, by comparisons of the physicochemical properties including the specific rotations. ^{1}H- and ^{13}C-NMR data of **1a**, **2a**, and **3a** are given here, since the spectra in CD_3OD have not yet been reported.

Quinine 1-*N*-Oxide (**1a**): ^{1}H-NMR (500 MHz, CD_3OD) δ: 1.71 (1H, m, 7-Ha), 1.95 (1H, m, 4-H), 2.03 (1H, m, 5-Ha), 2.39 (1H, m, 5-Hb), 2.43 (1H, m, 7-Hb), 2.96 (1H, m, 3-H), 3.21 (1H, ddd, J=3.1, 6.6, 12.7 Hz, 2-Ha), 3.34 (1H, m, 6-Ha), 3.46 (1H, dd, J=5.5, 11.0 Hz, 8-H), 3.72 (1H, dd, J=11.0, 12.7 Hz, 2-Hb), 3.94 (3H, br s, 6′-OC$\underline{H}_3$), 4.17 (1H, m, 6-Hb), 5.02 (1H, dd, J=0.9, 10.3 Hz, 11-Ha), 5.08 (1H, dd, J=0.9, 17.2 Hz, 11-Hb), 5.79 (1H, ddd, J=7.0, 10.3, 17.2 Hz, 10-H), 6.23 (1H, br s, 9-H), 7.40 (1H, dd, J=2.7, 9.2 Hz, 7′-H), 7.78 (1H, d, J=4.6 Hz, 3′-H), 7.83 (1H, d, J=2.7 Hz, 5′-H), 7.93 (1H, d, J=9.2 Hz, 8′-H), 8.68 (1H, d, J=4.6 Hz, 2′-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: 20.90 (7-C), 27.64 (5-C), 28.77 (4-C), 42.38 (3-C), 56.54 (6′-O$\underline{C}H_3$), 59.98 (6-C), 64.14 (9-C), 71.08 (2-C), 74.95 (8-C), 102.92 (5′-C), 116.85 (11-C), 120.09 (3′-C), 123.57 (7′-C), 127.56 (4′a-C), 131.23 (8′-C), 139.54 (10-C), 144.55 (8′a-C), 148.09 (2′-C), 149.31 (4′-C), 159.88 (6′-C).

Quinidine 1-*N*-Oxide (**2a**): ^{1}H-NMR (500 MHz, CD_3OD) δ: 1.38 (1H, br dd, J=10.6, 10.6 Hz, 7-Ha), 1.90 (1H, br s, 4-H), 1.98 (1H, m, 5-Ha), 2.07 (1H, m, 5-Hb), 2.74 (1H, br dd, J=10.6, 10.6 Hz, 7-Hb), 2.92 (1H, br dd, J=7.9, 17.9 Hz, 3-H), 3.41 (1H, m, 2-Ha), 3.42 (1H, m, 6-Ha), 3.55 (1H, br dd, J=10.6, 10.6 Hz, 8-H), 3.59 (1H, m, 6-Hb), 3.96 (3H, s, 6′-OC$\underline{H}_3$), 4.32 (1H, ddd, J=2.7, 7.6, 12.2 Hz, 2-Hb), 5.19 (1H, d, J=10.4 Hz, 11-Ha), 5.23 (1H, d, J=17.4 Hz, 11-Hb), 6.22 (1H, ddd, J=7.9, 10.4, 17.4 Hz, 10-H), 6.74 (1H, br s, 9-H), 7.42 (1H, dd, J=2.4, 9.2 Hz, 7′-H), 7.69 (1H, d, J=2.4 Hz, 5′-H), 7.79 (1H, d, J=4.6 Hz, 3′-H), 7.94 (1H, d, J=9.2 Hz, 8′-H), 8.69 (1H, d, J=4.6 Hz, 2′-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: 21.21 (7-C), 27.42 (5-C), 29.14 (4-C), 42.54 (3-C), 56.58 (6′-O$\underline{C}H_3$), 64.53 (9-C), 64.73 (2-C), 66.19 (6-C), 73.69 (8-C), 102.74 (5′-C), 117.15 (11-C), 120.18

Table 2. DNA Primers Used for PCR and rDNA Sequencing

Primer	Sequence (5′-3′)	Application	Location
18SFm3xy	GCC ATG CAT GTC TAA GTA TAA GCA	18S region amplification 18S region sequencing	18S rDNA
18SRm2	TCA GTA TTT ATT GTC ACT ACC TCC	18S region sequencing	18S rDNA
FG18SF2	GGT AAT TCC AGC TCC AAT AGC GTA	18S region sequencing	18S rDNA
FG18SR6	ACT GTC CCT ATT AAT CAT TAC GGC	18S region sequencing	18S rDNA
FG18SF8	AGC ATG GAA TAA TAG AAT AGG ACG	18S region sequencing	18S rDNA
18SRm1	GTC CAT TGT AAC ATC ACC AGC GTT	18S region sequencing	18S rDNA
FG18SF5	TAA ACT ATG CCG ATT GGG GAT C	18S region sequencing	18S rDNA
18SRm3	CTG TCA ATC CTC ATT GTG TCT GGC	18S region sequencing	18S rDNA
18SFm5xy	GGA GCC TGC GGC TTA ATT TG	18S region sequencing	18S rDNA
FG18SR2	CAC TGA GCC ATT CAA TCG GTA GTA	18S region amplification 18S region sequencing	18S rDNA
FG18SF9	GTG GTG CAT GGC CGT TCT TAG TTG	ITS1-5.8S-ITS2 amplification ITS1-5.8S-ITS2 sequencing	18S rDNA
FG18SF10	TCT GTG ATG CCC TTA GAT GTT CTG	ITS1-5.8S-ITS2 sequencing	18S rDNA
FG28SR1	CAG CAT TCC CAA ACT ACT CGA CTC	ITS1-5.8S-ITS2 sequencing	28S rDNA
FG28SR2	CTT TCG ATC ACT CTA CTT GTG CGC	ITS1-5.8S-ITS2 amplification ITS1-5.8S-ITS2 sequencing	28S rDNA

(3′-C), 123.57 (7′-C), 127.58 (4′a-C), 131.32 (8′-C), 139.18 (10-C), 144.62 (8′a-C), 148.14 (2′-C), 149.21 (4′-C), 159.94 (6′-C).

Cinchonidine 1-*N*-Oxide (**3a**): ^{1}H-NMR (500 MHz, CD_3OD) δ: 1.67 (1H, dddd, J=2.7, 2.7, 11.0, 13.1 Hz, 7a-H), 1.95 (1H, dd, J=2.9, 5.6 Hz, 4-H), 2.02 (1H, m, 5-Ha), 2.33 (1H, dddd, J=2.7, 6.4, 11.6, 11.6 Hz, 5-Hb), 2.39 (1H, m, 7-Hb), 2.95 (1H, m, 3-H), 3.17 (1H, ddd, J=3.5, 6.4, 12.8 Hz, 2-Ha), 3.30 (1H, m, 6-Ha), 3.41 (1H, dd, J=5.8, 11.0 Hz, 8-H), 3.68 (1H, dd, J=11.0, 12.8 Hz, 2-Hb), 4.18 (1H, dddd, J=3.5, 3.5, 11.6, 11.6 Hz, 6-Hb), 5.00 (1H, d, J=10.4 Hz, 11-Ha), 5.07 (1H, d, J=17.1 Hz, 11-Hb), 5.77 (1H, ddd, J=7.0, 10.4, 17.1 Hz, 10-H), 6.74 (1H, s, 9-H), 7.68 (1H, dd, J=8.4, 8.4 Hz, 6′-H), 7.79 (1H, dd, J=8.4, 8.4 Hz, 7′-H), 7.85 (1H, d, J=4.6 Hz, 3′-H), 8.06 (1H, d, J=8.4 Hz, 8′-H), 8.50 (1H, d, J=8.4 Hz, 5′-H), 8.85 (1H, d, J=4.6 Hz, 2′-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: 21.16 (7-C), 27.70 (5-C), 28.74 (4-C), 42.31 (3-C), 60.18 (6-C), 64.23 (9-C), 71.51 (2-C), 75.17 (8-C), 116.72 (11-C), 120.15 (3′-C), 124.87 (5′-C), 126.49 (4′a-C), 128.51 (6′-C), 129.96 (8′-C), 130.94 (7′-C), 139.61 (10-C), 148.63 (8′a-C), 150.92 (2′-C), 150.93 (4′-C).

The physicochemical properties of cinchonine 1-*N*-oxide (**4a**) are given here, since Dodin *et al.*[7] reported only ^{1}H-NMR (in DMSO-d_6) and MS spectra for **4a**.

Cinchonine 1-*N*-Oxide (**4a**): Colorless plates, mp 173—174 °C (ethyl acetate and methanol), $[\alpha]_D$ +161.6° (c=0.43, in MeOH at 22 °C). IR (KBr) cm^{-1}: 3160, 3078, 1638, 1519, 1508, 760. UV (EtOH) nm (ε): 226 (29000), 284 (4200), 301 (3200), 315 (2500). ^{1}H-NMR (500 MHz, CD_3OD) δ: 1.32 (1H, m, 7a-H), 1.87 (1H, br s, 4-H), 1.94 (1H, m, 5-Ha), 2.04 (1H, m, 5-Hb), 2.68 (1H, m, 7-Hb), 2.89 (1H, m, 3-H), 3.30—3.37 (2H, 2-Ha and 6-Ha), 3.46 (1H, dd, J=9.3, 9.3 Hz, 8-H), 3.52 (1H, m, 6-Hb), 4.33 (1H, ddd, J=2.8, 8.0, 12.1 Hz, 2-Hb), 5.18 (1H, d, J=10.4 Hz, 11-Ha), 5.22 (1H, d, J=17.4 Hz, 11-Hb), 6.21 (1H, ddd, J=7.9, 10.4, 17.4 Hz, 10-H), 6.85 (1H, br s, 9-H), 7.67 (1H, dd, J=8.5, 8.5 Hz, 6′-H), 7.79 (1H, dd, J=8.5, 8.5 Hz, 7′-H), 7.86 (1H, d, J=4.4 Hz, 3′-H), 8.06 (1H, d, J=8.5 Hz, 8′-H), 8.40 (1H, d, J=8.5 Hz, 5′-H), 8.85 (1H, d, J=4.4 Hz, 2′-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: 21.31 (7-C), 27.43 (5-C), 29.18 (4-C), 42.53 (3-C), 64.69 (9-C), 64.97 (2-C), 66.59 (6-C), 73.96 (8-C), 117.07 (11-C), 120.29 (3′-C), 124.76 (5′-C), 126.55 (4′a-C), 128.53 (6′-C), 130.01 (8′-C), 130.93 (7′-C), 139.31 (10-C), 148.68 (8′a-C), 150.96 (2′-, 4′-C). FAB-MS m/z: 311 $[M+H]^+$. High-resolution FAB-MS m/z: Calcd for $C_{19}H_{23}N_2O_2$: 311.1760 $[M+H]^+$. Found: 311.1764.

Microbial Transformation of *Cinchona* Alkaloids in PDB by *X. enteroleuca* *X. enteroleuca* was inoculated into PDB solution [4.8 g of PDB (DIFCO) and 200 ml of water] in four 500-ml Erlenmeyer flasks and then shaken at 120 rpm at 27 °C. **1a** (19.5 mg, 94% conversion yield), **2a** (13.2 mg, 70% conversion yield), **3a** (12.4 mg, 60% conversion yield), and **4a** (14.7 mg, 71% conversion yield) were obtained from 22 mg each of **1**·HCl, **2**·HCl, **3**·HCl, and **4**·HCl through a procedure similar to that used for the microbial transformation by the endophytic fungus *Xylaria* sp.

DNA Extraction and PCR Amplification The endophytic fungus *Xylaria* sp. was ground down in saline-EDTA buffer (400 μl, 0.15 M NaCl, 0.1 M EDTA·2Na) containing alumina powder (5 mg) and 10% SDS (20 μl) was added. The suspended liquid was heated at 60 °C for 10 min. After cooling, phenol (400 μl) was added to the mixture and then submitted to centrifugation at 15000 rpm for 5 min. The lower phase was collected and treated with 70% EtOH. The whole mixture was again submitted to centrifugation, and the precipitates were collected and dried under reduced pressure, then treated with RNase (0.5 μg, Sigma) in TE buffer solution [50 μl, TE buffer: 10 mM Tris–HCl, 1 mM EDTA·2Na (pH 8.0)] at room temperature for 10 min. After the removal of RNA was confirmed by electrophoresis, amplification was performed in a reaction mixture of 50 mM Tris–HCl buffer (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 1.0% Triton X-100, 2.4 mM $MgCl_2$, 100 mM of each dNTP (Takara), 0.3 μM of each primer (Table 2, 18S region; 18SFm3xy and FG18SR2, ITS1-5.8S-ITS2 region; FG18SF9 and FG28SR2) with about 1 ng of genomic DNA and 1 unit of the *Taq* DNA Polymerase (Promega). The reaction mixture was placed in a thermal cyclic programmed for 0.5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 0.5 min at 50 °C, 1.5 min at 72 °C, and a final extension at 72 °C for 10 min. The PCR product was checked by electrophoresis in 1% agarose gel with ethidium bromide 0.5 mg/ml. The presence of a single bright band in each lane confirmed successful amplification. The PCR product was suspended in 95% EtOH and placed at −80 °C for 27 min, and then the whole was submitted to centrifugation at 15000 rpm for 15 min. After removal of the supernatant, the residue was resuspended in 250 μl of 70% EtOH and centrifuged at 15000 rpm for 5 min, and the precipitates were collected and dried under reduced pressure to obtain a DNA template.

DNA Sequencing and Phylogenetic Analyses Amplification was performed in a reaction mixture of 1 unit of BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (ABI), 0.3 μM of each primer (Table 2) and 15 ng of the DNA template. The reaction mixture was placed in a thermal cyclic programmed for 1 min at 96 °C, followed by 25 cycles of 0.5 min at 96 °C, 0.25 min at 50 °C, 4 min at 60 °C, and a final extension at 60 °C for 10 min. One microliter of 6 M aqueous ammonium acetate and 30 μl of 99.5% EtOH were added to the reaction mixture and the whole was shaken briefly and then allowed to stand in an ice-water bath for 15 min. The mixture was centrifuged at 14000 rpm for 15 min. The supernatant was removed and the residue was suspended in 200 μl of 70% EtOH and centrifuged at 14000 rpm for 10 min. The resulting precipitates were collected and dried under reduced pressure to afford the cloned DNA fragments. Those fragments were analyzed using a DNA sequencer. Sequence alignment, a similarity matrix, and a neighbor-joining phylogenetic tree were created on a Macintosh personal computer using the DNASTAR program (DNASTAR Inc.) and the CLUSTAL V software pakage.[10,11]

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