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Isolation and Identification of Two *Antrodia cinnamomea* Strains from Fruiting Bodies

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ABSTRACT

Antrodia cinnamomea strains ACTS1 and AC0623 were isolated from the fruiting bodies. The partial sequences of ribosomal RNA gene, including the internal transcribed spacers, ITS1-5.8S-ITS2, and 18S ribosomal RNA genes, have been sequenced and applied to identify these two strains. Four specific primer sets (NS1/NS2, NS3/NS4, NS5/NS8, and ITS1/ITS4) were utilized to perform PCR experiment of the ribosomal RNA genes. Comparison of partial nucleotide sequences (ITS1-5.8S-ITS2) with strains from different genus (*Antrodiella semisupina, Antrodiella romellii and Trametes versicolor*) was made. These DNA sequence data demonstrated that there is no difference among ACTS1, AC0623, BCRC 35396 and BCRC 35398. The colony morphology and growth characteristics of *Antrodia cinnamomea* strains in different culture media of agar plate were also made. No obvious microscopic difference of *A. cinnamomea* strains ACTS1, AC0623, BCRC 35396, and BCRC 35398 were observed. The results from this study suggested that strains ACTS1 and AC0623 isolated from fruiting bodies, and the strains BCRC 35396 and 35398, the original *A. cinnamomea* strain obtained from Food Industry Research and Development Institute (FIRDI) are the same species.

Key words: *Antrodia cinnamomea*, ribosomal RNA genes, polymerase chain reaction (PCR), fruiting bodies

INTRODUCTION

Antrodia cinnamomea, a native species in Taiwan, grows naturally on *Cinnamomum kanehirai* Hay, a kind of camphor tree grown only in Taiwan, and is commonly known as "Niu-chang-chih". This fungus forms fruit bodies that causes brown rot on *C. kanehirai*[1,2,3]. Aborigines living in Taiwan's mountain range have used this fungus for the treatment of food and drug intoxications, diarrhea, abnormal pains, hypertension and liver cancer in Taiwanese folk medicine[2,4,5,6]. The current price of fresh fruiting bodies of *A. cinnamomea* is estimated to be US$ 1500 per kg.

Economic incentives are effective for the development of artificial cultivation methods. Some organizations claim to have induced formation of fruiting bodies since early 2001. However, it seems that growth under such artificial environments still depends on luck. Nevertheless, submerged mycelia products have been recently marketed. We have isolated two strains ACTS1 and AC0623 from fruiting bodies of *A. cinnamomea* at Liou Kuei in southern Taiwan. In order to understand whether strains ACTS1 and AC0623 cultivated in Taiwan Sugar Research Institute (TSRI) and the BCRC 35396 and 35398 strains are the same, some molecular systematic techniques were made[7-22]. Random amplified polymorphic DNA (RAPD) markers with polymerase chain reaction (PCR) amplification of genomic DNA technique were also used for identification of *Cordyceps* species[17,23,24].

Based on PCR fragments of the whole ribosomal RNA gene (including the internal transcribed spacers [ITS1 and ITS2], plus 5.8S, 18S, and 28S ribosomal RNA genes) we can distinguish whether these strains have the same characteristics from the original species. The results of isolation and identification of these strains are described as follows.

MATERIALS AND METHODS

I. *Strains of Antrodia cinnamomea*

The strains of this study *A. cinnamomea* BCRC 35396 and *A. cinnamomea* BCRC 35398 were purchased from Bioresources Collection & Research Center (BCRC, FIRDI, Hsinchu, Taiwan). Strains of ACTS1 and AC0623 were isolated from fruiting bodies of *A. cinnamomea* at Liou Kuei in southern Taiwan as illustrated in Table 1.

II. *Isolation of Two Antrodia cinnamomea Strains from Fruiting Bodies*

Firstly the fruiting body was treated with antibiotics penicillin V or ampicillin to inhibit the growth of contaminants. Then it was cut into small pieces, put into the sterilized water and isolated from different dilutions of sterilized water on agar plate. Colonies were grown

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on plate for three weeks on M25 plate. Two strains of ACTS1 and AC0623 were isolated from fruiting bodies of *A. cinnamomea*.

III. *Antrodia cinnamomea Cultured Media*

A. cinnamomea strains ACTS1, AC0623, BCRC 35396, and BCRC 35398 were cultured and maintained at 28˚C on M25 agar plate (Merck, Germany). Mycelium obtained from the edge of 21-day-old colonies was used as the inoculum source. Other culture media such as PDA (potato dextrose agar, Difco, Becton Dickinson, USA), OAT (oatmeal agar, Difco, Becton Dickinson, USA), SGA (Sabouraud-2% glucose agar, Merck, Germany), and NA (nutrient agar, Merck, Germany) agar plate were used to make a comparison of colony growth. Cell mass production was carried out in 500-mL Hinton flask with rotary shaker of 110 rpm and 28˚C respectively. The M25 cultured media of *A. cinnamomea* strains are as follows: glucose 3%, bacto-peptone 1.5%, yeast extract 1.5%, malt extract 1.5%, $MgSO_4 \cdot 7H_2O$ 0.03%, KH_2PO_4 0.03% and K_2HPO_4 0.03%, pH 5.5.

IV. *Extraction of Genomic DNA*

Genomic DNA was prepared using plant DNAzol Reagent[25] (Invitrogen, USA). The procedures were as follows.

(I) *Extration*

Plant tissues of *A. cinnamomea* were pulverized in liquid nitrogen using mortar and pestle. As the samples were grinded, liquid nitrogen was replenished in the mortar 2 to 3 times. The resulting homogenous frozen powder was then transferred to a microcentrifuge tube containing plant DNAzol. Every gram of plant tissue needed 3 mL of plant DNAzol. The mixture was mixed thoroughly by gentle inversion a few times before incubation at 25˚C and shaking for 5 min. The mixture was then added to 0.3 mL of chloroform and mixed vigorously. After incubation at 25˚C for another 5 min, the mixture was centrifuged.

(II) *Centrifugation*

Following extraction, the extract was centrifuged at 12,000 × g for 10 min. The supernatant was transfer to a fresh tube.

(III) *DNA Precipitation*

The collected supernatant was mixed with 0.23 mL of absolute ethanol. The compound was mixed by inverting the tube 6 to 8 times before storage at room temperature for 5 min. Precipitated DNA was separated by centrifugation at 5,000 × g for 4 min.

Table 1. Source and collection number of *Antrodia cinnamomea* strains in this study

Species	Collection number	Source
Antrodia cinnamomea	BCRC 35396	BCRC[a], Taiwan
Antrodia cinnamomea	BCRC 35398	BCRC, Taiwan
Antrodia cinnamomea fruiting body	ACTS1	Liou Kuei, Taiwan isolated by TSRI[b], Taiwan
Antrodia cinnamomea fruiting body	AC0623	Liou Kuei, Taiwan, isolated by TSRI, Taiwan

[a]BCRC: Bioresources Collection & Research Center, Hsinchu, Taiwan.
[b]TSRI: Taiwan Sugar Research Institute, Tainan, Taiwan.

Table 2. Primers used in this study[21]

Primer designation	Primer sequences (5'→3')	Tm (˚C)
NS1	GTAGTCATATGGCTTGTCTC	54
NS2	GGCTGCTGGCACCAGACTTGC	62
NS3	GCAAGTCTGGTGCCAGCAGCC	62
NS4	CTTCCGTCAATTCCTTTAAG	56
NS5	AACTTAAAGGAATTGACGGAAG	49
NS8	TCCGCAGGTTCACCTACGGA	64
ITS1	TCCGTAGGTGAACCTGCGG	62
ITS4	TCCTCCGCTTATTGATATGC	58

(IV) *DNA Wash*

1. *Plant DNAzol-ethanol Wash*

Plant DNAzol–ethanol wash was prepared by mixing 1 volume of DNAzol with 0.75 volume of 100% ethanol. A quantity of 0.3 mL of Plant DNAzol-ethanol wash solution was mixed with the DNA precipitate by vortexing. After 5 min, the mixture was subjected to centrifugation at 5,000 × g for 4 min.

2. *Ethanol Wash*

After the DNAzol wash solution was removed, the DNA pellet was washed by vigorously mixing with 0.3 mL of 75% ethanol. The mixture was then centrifuged at 5,000 × g for 4 min.

(V) *DNA Solubilization*

After ethanol layer was removed by decanting, the remaining ethanol was further removed with a micropipette that was allowed to sit vertically for about 2 min. The remaining DNA pellet was air dried and then dissolved in 70 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). If DNA pellet is difficult to dissolve, 8 mM NaOH solution can be used instead of TE buffer. In a typical DNA preparation, the DNA solution is cloudy and

may contain insoluble material, which can be removed by centrifugation at 12,000 × g for 4 min.

V. *Polymerase Chain Reaction (PCR) of rRNA Gene*

The fragment of ITS1-5.8S-ITS2, 18S rRNA gene were amplified by polymerase chain reaction. The quantitative analysis of DNA was obtained using OD260/280 after extraction of genomic DNA. Fifty ng of DNA samples from each strain were taken, and the concentration of $MgCl_2$ is 1.5 mM. Four specific primer sets (NS1&NS2, NS3&NS4, NS5&NS8, and ITS1&ITS4) were utilized to perform PCR experiments of the ribosomal RNA genes. Table 2 shows the primers used in this study. The reaction condition of PCR is shown as follows: reaction volume: 100 μL, dNTP concentration: 200 μM, primer concentration: 0.5 μM, and dosage of Taq polymerase (Promega, USA): 0.1 U/μL. The procedure of PCR reaction was began with 95˚C for 5 min. and then heated for 1 min. for denaturation. The annealing temperature is 57˚C (for 1 min) and the elongation temperature is 72˚C (for 2 min) for 40 cycles. The PCR products were separated by electrophoresis in a 2.0% agarose gel, 100 V voltage in 0.5 times TBE (50 mM Tris base, 45 mM boric acid, and 0.5 mM EDTA) buffer (Invitrogen, USA) and then stained with ethidium bromide. The gel was observed and photographed under ultraviolet light.

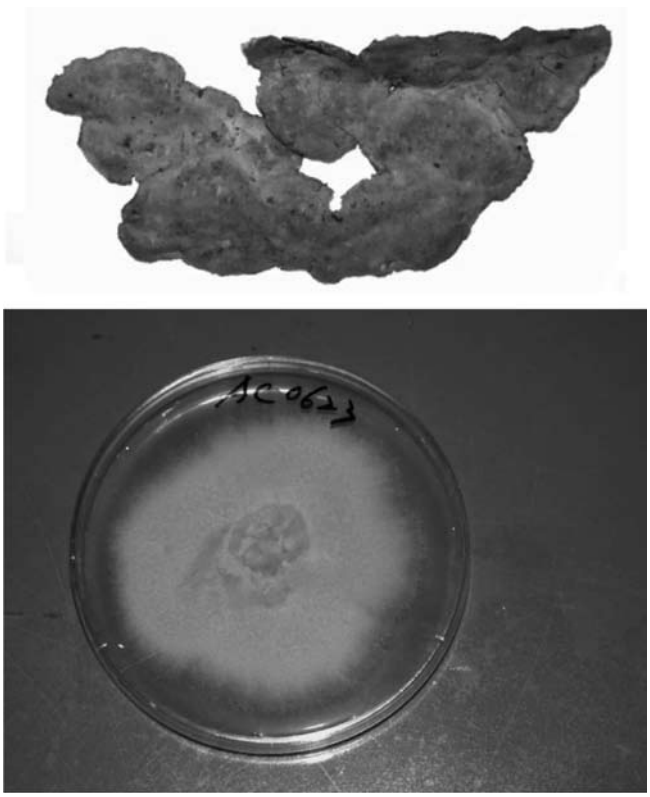

Figure 1. The fruiting body of *Antrodia cinnamomea* strain AC0623 and colonies grow 3 weeks on M25 agar plate.

RESULTS

I. *Isolation of Two Antrodia cinnamomea Strains from Fruiting Bodies*

Figure 1 shows the fruiting body of *A. cinnamomea* strain AC0623 and colonies grew 3 weeks on M25 agar plate (Merck, Germany). The comparison of strains ACTS1, AC0623, BCRC 35396 and BCRC 35398 on M25, PDA, OAT, SGA, and NA agar plate are shown in Table 3.

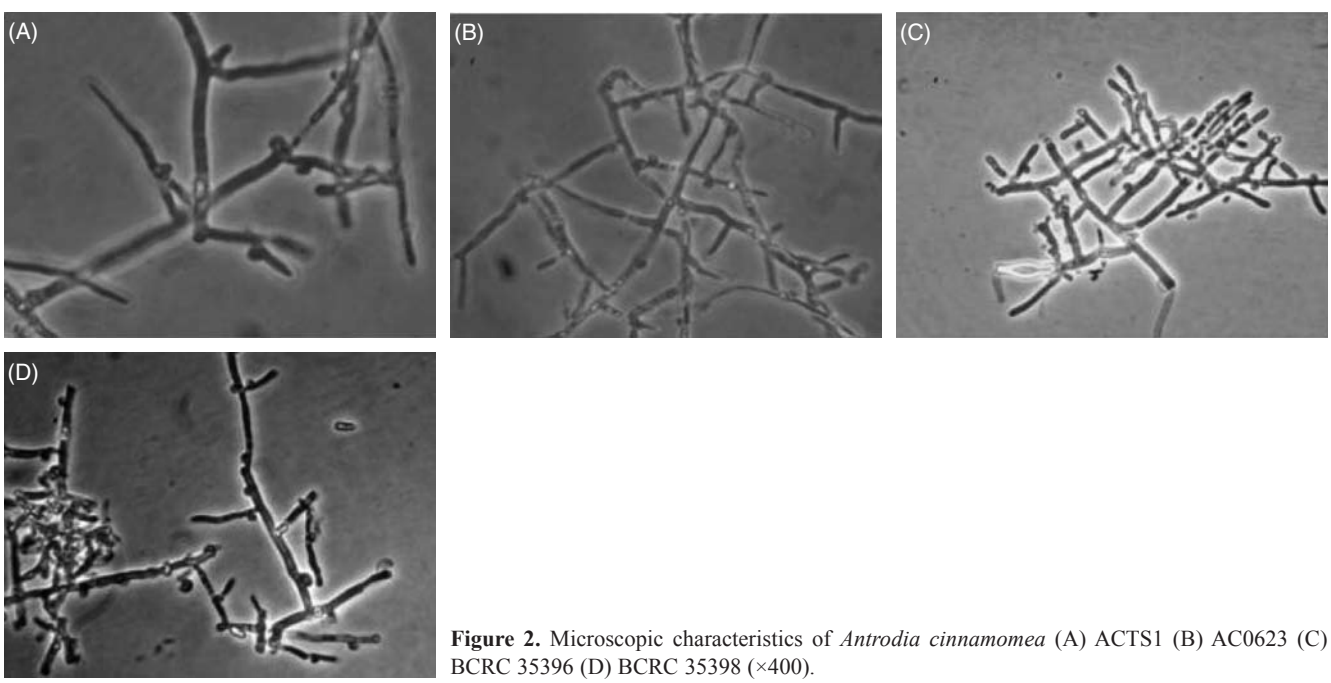

Figure 2. Microscopic characteristics of *Antrodia cinnamomea* (A) ACTS1 (B) AC0623 (C) BCRC 35396 (D) BCRC 35398 (×400).

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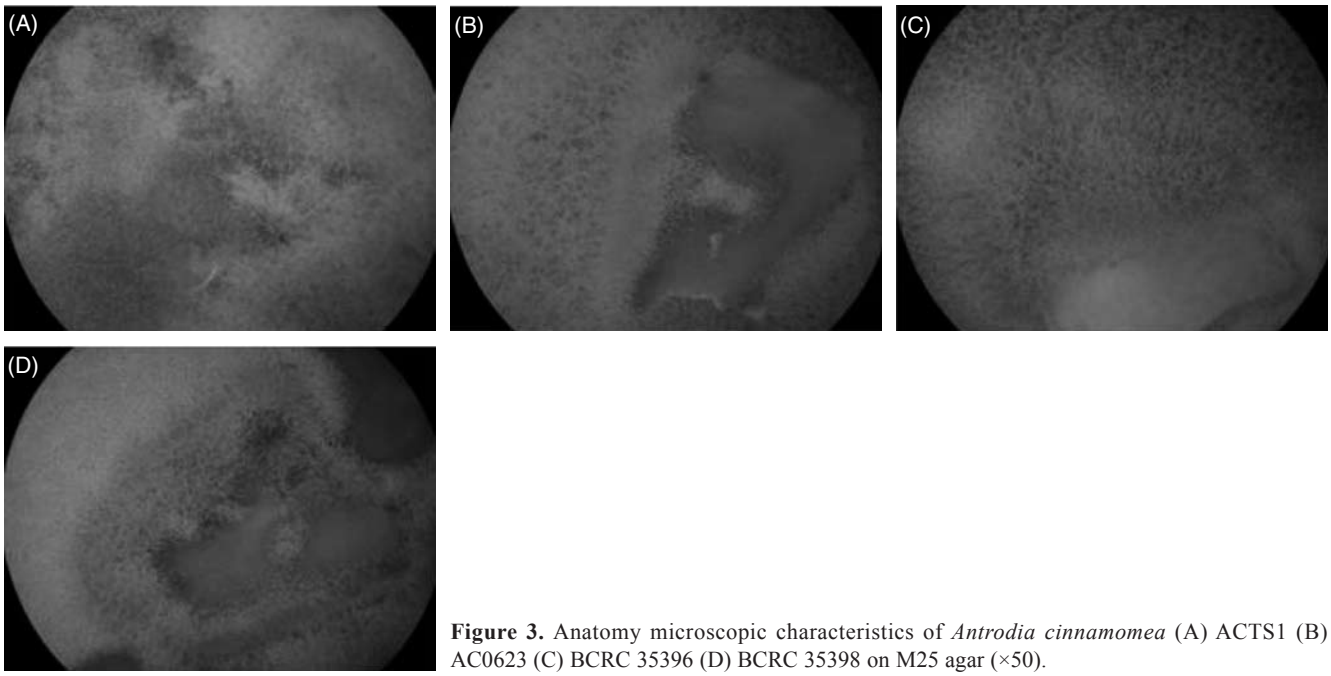

Figure 3. Anatomy microscopic characteristics of *Antrodia cinnamomea* (A) ACTS1 (B) AC0623 (C) BCRC 35396 (D) BCRC 35398 on M25 agar (×50).

Table 3. Diameter of the colonies of *Antrodia cinnamomea* strains in different media

Medium \ Strain	ACTS1	AC0623	BCRC 35396	BCRC 35398
M25	5.0 cm[a]	6.5 cm	6.0 cm	5.0 cm
PDA	4.5 cm	5.5 cm	5.5 cm	5.3 cm
OAT	4.5 cm	4.5 cm	5.3 cm	6.5 cm
SGA	4.3 cm	4.5 cm	4.0 cm	5.0 cm
NA	—[b]	—	—	—

[a]cm: the length were measured when the colonies reached their maximum diameter of *A. cinnamomea* strains within 21 days after inoculated on the plates.
[b]No growth.

The growth rate of *A. cinnamomea* on M25 agar was the fastest among all combination tested. The microscopic traits of these strains are shown in Figure 2, and the anatomy microscopic characteristics of *A. cinnamomea* are depicted in Figure 3.

II. *Comparison of the PCR Products of Ribosomal RNA Genes among the Antrodia cinnamomea Strain ACTS1, AC0623, BCRC 35396, and BCRC 35398*

Four specific primer sets (NS1/NS2, NS3/NS4, NS5/NS8, and ITS1/ITS4) were utilized to perform PCR amplification of the ribosomal RNA genes (including 18S and ITS1-5.8S-ITS2 rRNA gene). The results are shown in Figure 4. The fragment length and location with specific primer sets to perform PCR amplification are shown as follows:
The PCR product of NS1 & NS2: 550 bp (18S rRNA gene)
The PCR product of NS3 & NS4: 600 bp (18S rRNA gene)
The PCR product of NS5 & NS8: 650 bp (18S rRNA gene)

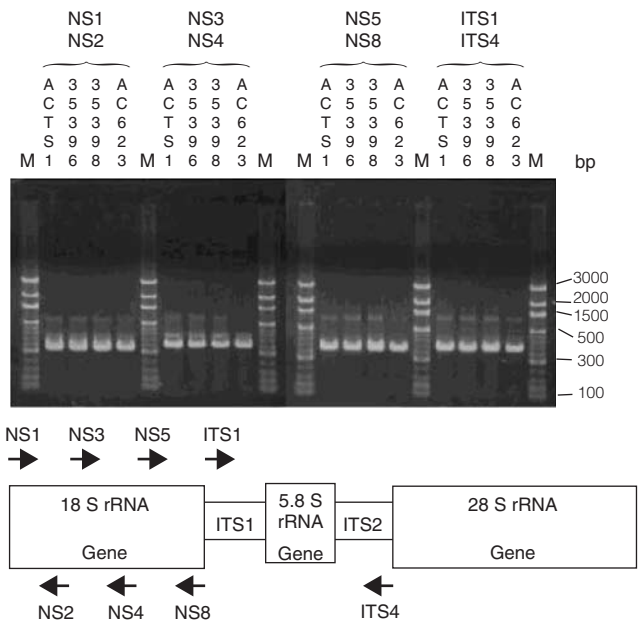

Figure 4. Comparison of the PCR products of ribosomal RNA genes among the *Antrodia cinnamomea* strain ACTS1, AC0623 (TSRI), and BCRC 35396, BCRC 35398 strain (FIRDI). PCR products were separated by electrophoresis in a 2.0% agarose gel and then stained with ethidium bromide. The gel was observed and photographed under ultraviolet light. The100-bp ladder (M) as molecular weight standards is shown in intervals.

The PCR product of ITS1& ITS4: 700 bp (ITS1-5.8S-ITS2 rRNA gene)

III. *DNA Sequence Analysis of Antrodia cinnamomea Strain*[16] *and Others*

Four specific primer sets (NS1/NS2, NS3/NS4, NS5/

NS8, and ITS1/ITS4) were utilized to perform primer for direct DNA sequence of PCR product. Ribosomal RNA genes of ACTS1, BCRC 35396, BCRC 35398, and AC0623 were used for DNA sequence analysis. Based on the ribosomal RNA genes (18S and ITS1-5.8S-ITS2 rRNA genes) and four specific primer sets, the length of PCR product after reaction was 556 bp, 602 bp, 659 bp and 682 bp respectively. The complete sequence of 18S rRNA, ITS1, 5.8S rRNA and ITS2 were obtained. The results of the DNA sequences analysis among ACTS1, AC0623, BCRC 35396, and BCRC 35398 strains are shown in Table 4.

Table 4. Similarity of DNA sequences of partial ribosomal RNA genes [containing partial 18S ribosomal RNA gene, internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, internal transcribed spacer 2 (ITS2), and partial 28S ribosomal RNA gene] among 4 *Antrodia cinnamomea* strains including ACTS1 (AY378092), AC0623 (AY378093), BCRC 35396 (AY378094), and BCRC 35398 (AY378095)

Strain	AC0623	BCRC 35396	BCRC 35398	ACTS1
ACTS1	100%	99.8%	99.9%	100%
AC0623	100%	99.8%	99.9%	100%
BCRC 35396	99.8%	100%	99.9%	99.8%
BCRC 35398	99.9%	99.9%	100%	99.9%

From the sequence analysis of *Antrodia cinnamomea*[26], 18S rRNA is shown to be a high conservative region (about 1700 bp) and the similarity between BCRC 35396 and BCRC 35398 strains was over 98%. Since the region of partial nucleotide sequence ITS1-5.8S-ITS2 (about 680 bp) is non-conservative evolutionally, it is suitable for species identification. Partial nucleotide sequences (ITS1-5.8S-ITS2) were compared with those of the strains from different genus (*Antrodiella semisupina, Antrodiella romellii, and Trametes versicolor*). The sequences were analyzed

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BCRC35396              TCCGTAGGTGAACCTGCGGAAGGACCATTATTGTAT----TTGAAAGGGG
BCRC35716              TCCGTAGGTGAACCTGCGGAAGGACCATTATTGTAT----TTGAAAGGGG
AC0623                 TCCGTAGGTGAACCTGCGGAAGGATCATTATTGTAT----TTGAAAGGGG
ACTS1                  TCCGTAGGTGAACCTGCGGAAGGATCATTATTGTAT----TTGAAAGGGG
BCRC35398              TCCGTAGGTGAACCTGCGGAAGGATCATTATTGTAT----TTGAAAGGGG
Antrodiella semisupina TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGAACTTGGGCATAG
Antrodiella romellii   TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGAACTTGGGCAAAG
Trametes versicolor    TCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGT---TTTGAAACGAG

BCRC35396              TTGTAGCTGACCTCCTCTTGAAAAGGGGGGAGGTATGTGCACACCTCT-G
BCRC35716              TTGTAGCTGACCTCCTCTTGAAAAGGGGGGAGGTATGTGCACACCTCTTG
AC0623                 TTGTAGCTGACCTCCTCTTGAAAAGGGGGGAGGTATGTGCACACCTCT-G
ACTS1                  TTGTAGCTGACCTCCTCTTGAAAAGGGGGGAGGTATGTGCACACCTCT-G
BCRC35398              TTGTAGCTGACCTCCTCTTGAAAAGGGGGGAGGTATGTGCACACCTCT-G
Antrodiella semisupina TTGTAGCTGGCCTC---------AGCAATGAGGCATGTGCACAC-TGT-G
Antrodiella romellii   TTGTAGCTGGCCTC---------AGCAATGGGGCATGTGCACAC-TTT-G
Trametes versicolor    TTGTAGCTGGCCTT---------C----CGGGGCATGTGCACGC-TCT-G

BCRC35396              TTCATTCATATTCTCTCACACCTGTGCATGCTTTGTAGGTTGGTTTTGAA
BCRC35716              TTCATTCATATTCTCTCACACCTGTGCATGCTTTGTAGGTTGGTTTTGAA
AC0623                 TTCATTCATATTCTCTCACACCTGTGCATGCTTTGTAGGTTGGTTTTGAA
ACTS1                  TTCATTCATATTCTCTCACACCTGTGCATGCTTTGTAGGTTGGTTTTGAA
BCRC35398              TTCATTCATATTCTCTCACACCTGTGCATGCTTTGTAGGTTGGTTTTGAA
Antrodiella semisupina TTCATCCAC-CCTTCACACCTCTGTGCACTTCTCATGGGTTTGGGTCAAG
Antrodiella romellii   TTCATCCAC-C-TTCACACCACTGTGCACTTCTCATGGGTC-GGGTTGCG
Trametes versicolor    CTCATCCAC-TCT----ACCCCTGTGCACTTACTGTAGGTTGGCGTGGGC

BCRC35396              TGGTTGTCTTCTCTGATGGAGACAGCTGTTTTGACCTTCCTATGTTTTTT
BCRC35716              TGGTTGTCTTCTCTGATGGAGACAGCTGTTTTGACCTTCCTATGTTTTTT
AC0623                 TGGTTGTCTTCTCTGATGGAGACAGCTGTTTTGACCTTCCTATGTTTTTT
ACTS1                  TGGTTGTCTTCTCTGATGGAGACAGCTGTTTTGACCTTCCTATGTTTTTT
BCRC35398              TGGTTGTCTTCTCTGATGGAGACAGCTGTTTTGACCTTCCTATGTTTTTT
Antrodiella semisupina T---------CTGAAATATGGCGAAGCCC-----CCTTCTCATGTGTTTT
Antrodiella romellii   T---------CTGAAATATGGCAAAGCCC-------TTCTCATGTGTTTT
Trametes versicolor    T---------CCTTAACGGGAGCATTCTG-----CCGGCCTATGTATACT
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BCRC35396               AAATTGACTCCGTATCAGTTACAGAATGTA----TGTTGCGTGTAACGCA
BCRC35716               AAATTGACTCCGTATCAGTTACAGAATGTA----TGTTGCGTGTAACGCA
AC0623                  AAATTGACTCCGTATCAGTTACAGAATGTA----TGTTGCGTGTAACGCA
ACTS1                   AAATTGACTCCGTATCAGTTACAGAATGTA----TGTTGCGTGTAACGCA
BCRC35398               AAATTGACTCCGTATCAGTTACAGAATGTA----TGTTGCGTGTAACGCA
Antrodiella semisupina  AAACACACACTATACAAGTTTTAGAATGTAACAATCATGCGTC-AATGCA
Antrodiella romellii    AC-CACACACT--ACAAGTTTTAGAATGTAACAATCATGCATT-AATGCA
Trametes versicolor     ACAAACACTTTA---AAGTATCAGAATGTAA-----ACGCGTCTAACGCA

BCRC35396               T--ATTGTATAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAA
BCRC35716               T--ATTGTATAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAA
AC0623                  T--ATTGTATAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAA
ACTS1                   T--ATTGTATAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAA
BCRC35398               T--ATTGTATAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAA
Antrodiella semisupina  T--ATAATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAA
Antrodiella romellii    T--ATAATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAA
Trametes versicolor     TCTATAATACAACTTTTAGCAACGGATCTCTTGGCTCTCGCATCGATGAA

BCRC35396               GAACACGGCGAAATGTGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
BCRC35716               GAACACGGCGAAATGTGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
AC0623                  GAACACGGCGAAATGTGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ACTS1                   GAACACGGCGAAATGTGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
BCRC35398               GAACACGGCGAAATGTGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
Antrodiella semisupina  GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
Antrodiella romellii    GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
Trametes versicolor     GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC

BCRC35396               ATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCTGAGGAGCATGC
BCRC35716               ATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCTGAGGAGCATGC
AC0623                  ATTGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCTGAGGAGCATGC
ACTS1                   ATTGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCTGAGGAGCATGC
BCRC35398               ATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCTGAGGAGCATGC
Antrodiella semisupina  ATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGC
Antrodiella romellii    ATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGC
Trametes versicolor     ATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGC

BCRC35396               CTGTTTGAGTGTCATGGAATTATCAACCCTTTTGACTTTTTGT-------
BCRC35716               CTGTTTGAGTGTCATGGAATTATCAACCCTTTTGACTTTTTGT-------
AC0623                  CTGTTTGAGTGTCATGGAATTATCAACCCTTTTGACTTTTTGT-------
ACTS1                   CTGTTTGAGTGTCATGGAATTATCAACCCTTTTGACTTTTTGT-------
BCRC35398               CTGTTTGAGTGTCATGGAATTATCAACCCTTTTGACTTTTTGT-------
Antrodiella semisupina  CTGTTTGAGTGTCATGGTATTCTCAACCCTGCTACATTTTTTTGAAAGAT
Antrodiella romellii    CTGTTTGAGTGTCATGGTATTCTCAACCCTGCTACATTTTTT--------
Trametes versicolor     CTGTTTGAGTGTCATGGAATTCTCAACTT--ATAAATCCTTGTG------

BCRC35396               -TGAATGGGCTTGGATTTGGAGGGTTAAATTGCTGGCTCTT----CTTTT
BCRC35716               -TGAATGGGCTTGGATTTGGAGGGTTAAATTGCTGGCTCTT----TTTTT
AC0623                  -TGAATGGGCTTGGATTTGGAGGGTTAAATTGCTGGCTCTT----TTTTT
ACTS1                   -TGAATGGGCTTGGATTTGGAGGGTTAAATTGCTGGCTCTT----TTTTT
BCRC35398               -TGAATGGGCTTGGATTTGGAGGGTTAAATTGCTGGCTCTT----TTTTT
Antrodiella semisupina  GTTGCTTGGCTTGGACTTGGAGGTTT--ATTGCTGGCATTCAACTTGTTT
Antrodiella romellii    GTAGCTGGGCTTGGACTTGGAGGT----ATTGCCGGTGTTC---TCTTTT
Trametes versicolor     ATCTATAAGCTTGGACTTGGAGGC-----TTGCTGGCCCTT---------
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BCRC35396               T-GATTCAGCTCCTCTTGAATGCATTAGCTTGAACCCTTGTGGATTGACC
BCRC35716               T-GATTCAGCTCCTCTTGAATGCATTAGCTTGAACCCTTGTGGATTGACC
AC0623                  TTGATTCAGCTCCTCTTGAATGCATTAGCTTGAACCCTTGTGGATTGACC
ACTS1                   TTGATTCAGCTCCTCTTGAATGCATTAGCTTGAACCCTTGTGGATTGACC
BCRC35398               T-GATTCAGCTCCTCTTGAATGCATTAGCTTGAACCCTTGTGGATTGACC
Antrodiella semisupina  GAACGCGGGCTCCTCTGAAATGCATTAGCTGGAATGT-TACCGAGCATGA
Antrodiella romellii    GAACGCGGGCTCCTCTGAAATGCATTAGCTGGAATGT-TACCGAGCATGA
Trametes versicolor     -GCGGTCGGCTCCTCTTGAATGCATTAGCTTGATTCCGTACGGATCGGCT

BCRC35396               TTATCGGTGTGATAGTCATCTATGCCGTGGTTGTCTGAGGTGGGATCGGC
BCRC35716               TTATCGGTGTGATAGTCATCTATGCCGTGGTTGTCTGAGGTGGGATCGGC
AC0623                  TTATCGGTGTGATAGTCATCTATGCCGTGGTTGTCTGAGGTGGGATCGGC
ACTS1                   TTATCGGTGTGATAGTCATCTATGCCGTGGTTGTCTGAGGTGGGATCGGC
BCRC35398               TTATCGGTGTGATAGTCATCTATGCCGTGGTTGTCTGAGGTGGGATCGGC
Antrodiella semisupina  TT--CAATGTGATAATTGTCTACGTTGCTTCAACTTGGTATTAATTCTGT
Antrodiella romellii    TT--CAATGTGATAATTGTCTACGTTGCTTCAACTTGGTATTAATTCTGT
Trametes versicolor     CT--CAGTGTGATAATTGTCTACGCTGT---GACCGTGAAGTGTTTTGGC

BCRC35396               TTCTAATGGTGCAAGTCCCTTCAGGGGGATGATTTTCTAATGACCTTCTG
BCRC35716               TTCTAATGGTGCAAGTCCCTTCAGGGGGATGATTTTCTAATGACCTTCTG
AC0623                  TTCTAATGGTGCAAGTCCCTTCAGGGGGATGATTTTCTAATGACCTTCTG
ACTS1                   TTCTAATGGTGCAAGTCCCTTCAGGGGGATGATTTTCTAATGACCTTCTG
BCRC35398               TTCTAATGGTGCAAGTCCCTTCAGGGGGATGATTTTCTAATGACCTTCTG
Antrodiella semisupina  TTCAGCTTCTAACCGTCCCCTTTGCGGGACAATATCT---TGAACATCTG
Antrodiella romellii    TTCAGCTTCTAACCGTCCGCA----AGGACAATATCT---TGAACATCTG
Trametes versicolor     --GAGCTTCTAACCGTCCATT----AGGACAACTTCT---T-AACATCTG

BCRC35396               ACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCG
BCRC35716               ACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCG
AC0623                  ACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCG
ACTS1                   ACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCG
BCRC35398               ACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCG
Antrodiella semisupina  ACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCG
Antrodiella romellii    ACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCG
Trametes versicolor     ACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCG

BCRC35396               GAGGA
BCRC35716               GAGG-
AC0623                  GAGGA
ACTS1                   GAGGA
BCRC35398               GAGGA
Antrodiella semisupina  GAGGA
Antrodiella romellii    GAGGA
Trametes versicolor     GAGGA
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Figure 5. Comparison of DNA sequences of partial ribosomal RNA genes (containing partial ITS1, 5.8S rRNA gene, and partial ITS2) among 5 *Antrodia cinnamomea* strains including ACTS1 (AY378092), AC0623 (AY378093), BCRC 35396 (AY378094), BCRC 35398 (AY378095), and BCRC 35716 (AJ496402), as well as *Antrodiella semisupina* (AF126903), *Antrodiella romellii* (AF126902), and *Trametes versicolor* (AY309015) strains.

and are shown in Figure 5. The results from this study suggest that the two strains (ACTS1 and AC0623) isolated from fruiting bodies are of the same species, *A. cinnamomea*. The nucleotide sequences reported in this and other paper have been submitted to the GenBank with accession numbers AY378092 (ACTS1), AY378093 (AC0623), AY378094 (BCRC 35396), AY378095 (BCRC 35398), AJ496402 (BCRC 35716)[26], AY309015 (*Trametes versicolor*), AF126902 (*Antrodiella romellii*)[27], and AF126903 (*Antrodiella semisupina*)[27].

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DISCUSSION

PCR primers that target conserved regions of fungal rRNA genes, amplifying sequence-variable fragments of genes or intervening noncoding regions, have been used for sequence comparisons. Interspecies variability is also manifested in the fragment size of the internal transcribed spacer 1 and 2 (ITS1 and ITS2, respectively) regions[28]. In order to identify *A. cinnamomea* rapidly and correctly, the molecular biology methods for *A. cinnamomea* species and strains analysis were used[26]. Firstly, they used 18S rRNA gene, ITS gene and intergenic spacer of rRNA, and Mn-superoxide dismutase gene[26]. In addition, phylogenetic analysis methods, Neighbor Joing method, Parsimony method and Maximum Likelihood method were also used to analyze evolution relation of *A. cinnamomea*[26]. The specific primer sets based PCR fragments of the whole ribosomal RNA gene [including the internal transcribed spacers (ITS1 and ITS2), 5.8S, and 18S ribosomal RNA genes] combined partial nucleotide sequence (ITS1-5.8S-ITS2) analysis with other genus for negative control could be applied to investigate whether the strain cultivated in Taiwan Sugar Research Institute (TSRI) is the species named *A. cinnamomea*. Comparison of colony morphology and growth characteristics of *Antrodia cinnamomea* strains in different culture media of agar plates was made. The growth rate of *A. cinnamomea* on M25 agar was the fastest among all combinations tested. Nutrient agar medium was not suitable for *A. cinnamomea* strains. The results demonstrate that there is no difference in the eletrophoresisgram of the amplified PCR products between strains ACTS1, AC0623, BCRC 35396, and BCRC 35398. No obvious microscopic difference of *A. cinnamomea* strains ACTS1, AC0623, BCRC 35396, and BCRC 35398 was observed in this study. The major biologically active component triterpenoids will be in conjunction with our continuing research from the cultured *A. cinnamomea* mycelia.

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