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FULL PAPER

# Morphology evolution and molecular phylogeny of *Pestalotiopsis* (Coelomycetes) based on ITS2 secondary structure

**Kyoko Watanabe · Takuya Nakazono · Yasunori Ono**

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**Abstract** The internal transcribed spacer 2 (ITS2) located between the 5.8S and 28S genes of the nuclear ribosomal gene cistron is conserved at the level of secondary structure rather than primary sequence. Within the fungal genus *Pestalotiopsis*, there were two types of ITS2 sequence patterns, and hence secondary structures, which were supported by high bootstrap values in phylogenies based on the ProfDist distance and Profile neighbor-joining algorithms. *Pestalotiopsis* consists of two groups that differ in color intensity of the spore as measured by optical density (OD) in three median cells of conidia comprised of five cells with one basal and two to four apical appendages. OD was quantified using a novel method with the publicly available software, Image J. OD values of species within one clade were high (dark OD >0.6), while OD values of species in the other clade were low (pale OD <0.6). However, knobbed-tipped appendages, which have been used to classify species of *Pestalotiopsis*, were observed in both clades. In the dark clade, knobbed-tipped appendage strains aggregated in one subclade, but in the pale group, these strains did not aggregate.

**Keywords** Classification · Coelomycetes · Conidial pigmentation · ITS2 secondary structure

K. Watanabe (✉) · T. Nakazono
College of Agriculture, Tamagawa University,
Machida, Tokyo 194-8610, Japan
e-mail: wkyoko@agr.tamagawa.ac.jp

Y. Ono
Discovery Science and Technology Department,
Drug Discovery and Biomedical Technology Unit,
Daiichi Sankyo RD Novare Co., LTD, Tokyo, Japan

## Introduction

The genus *Pestalotiopsis* contains endophytic and phytopathogenic fungal coelomycetes. Many species in this genus are useful in applied biology or agriculture, as biological control agents and microbial pesticides (Ando 1993), or as sources of therapeutic agents such as taxol (Strobel et al. 1996). *Pestalotiopsis* species are traditionally identified using morphological traits (Guba 1961; Steyaert 1949, 1953, 1961). The monographs by Guba (1961), Nag Raj (1993) and Sutton (1980) are the primary tools used for identification when molecular data are not available. In these monographs, the first key characteristic used to identify *Pestalotiopsis* species is the morphology of the tips of the appendages or variations in pigmentation among the three median cells, and the ultimate key characteristic is the size of the conidia and appendages.

Jeewon et al. (2003) reported that a phylogenetic tree of *Pestalotiopsis* based on ITS1, ITS2, and 5.8S rDNA consisted of three clades. They proposed that the variation in pigmentation (concolorous or versicolous) among the conidial cells is a useful trait for phylogenetic classification, and they rejected the use of color distinctions between umber olivaceous and fuliginous olivaceous within the versicolor group as described by Guba (1961).

Several researchers have produced phylogenetic trees to identify and assign taxonomic affiliations to *Pestalotiopsis* based on ITS1, ITS2, and 5.8S rDNA (Jeewon et al. 2003), *β-tubulin* (Wei et al. 2007), and *EF1α* (Watanabe 2009). These phylogenies all show similar topology to each other. Furthermore, phylogenetic trees based on combined ITS1, ITS2 and *β-tubulin* data without a model test for each gene also yielded the same tree topology as the genes above.

Recently, in eukaryotic organisms, ITS2 has been found to vary in both sequence and secondary structure (Müller

et al. 2004). Among eukaryotes, ITS2 has a common core consisting of three or four helixes, and this feature is useful as a marker for family level classification and megasystematics (Schultz et al. 2005). Moreover, Coleman (2007, 2009), Coleman et al. (2008), and Müller et al. (2007) have shown that the ITS2 sequence is species specific, and that compensatory base changes (CBC) in the secondary structure of ITS2 reflect recent and current evolutionary status, providing a tool for species identification. In this analysis, alignment of the ITS2 sequences was fixed by the matrix of secondary structure without any artificial steps, while the phylogenetic tree was constructed using 12 letters (4 nucleotides in 3 structural states; unpaired, paired left, paired right). This method avoids previous issues with alignment. Keller et al. (2010) showed that the RNA secondary structures of ITS2 improve the accuracy and robustness of phylogenetic trees. In existing methods, higher numbers of nucleotides result in greater robustness of results (Erixon et al. 2003), and combining data from different evolution markers may improve accuracy. However, combined genes need to be investigated using marker-specific phylogenetic procedures.

This study was conducted to: (1) assess phylogenetic relationships of *Pestalotiopsis* strains reconstructed by profile neighbor–joining coupled with ITS2 structure-specific sequence, as well as to produce reproducible phylogenetic trees for *Pestalotiopsis* based on ITS1, ITS2, and 5.8 S rRNA sequences; (2) examine significance of color intensity of three median cells in conidia as an objective trait (quantitative analysis of color) as a phylogenetic characteristic; and (3) resolve the position of strains with knobbed-tip appendages in the phylogeny of *Pestalotiopsis*.

## Materials and methods

### Samples

Twenty-one strains of *Pestalotiopsis* (Table 1) isolated from several sources and preserved at the Microorganisms Section (MAFF) of the NIAS Genebank, National Institute of Agrobiological Science (NIAS, Tsukuba, Japan) and at Tamagawa University (TAP) were maintained on potato dextrose agar (PDA, Eiken, Tochigi, Japan) at 25°C. These strains were used for phylogenetic analysis, color intensity analysis of three median cells, and observation of tip morphology of appendages.

### DNA extraction and ITS1, ITS2, and 5.8S rDNA amplification

DNA from each fungal strain was extracted from 7-day-old cultures on PDA using a QIAGEN DNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The primer pair ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) (White et al. 1990) was used for PCR amplification of ITS1, ITS2 and 5.8S rDNA. Amplification was performed with 1 cycle of 94°C for 1 min and 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. PCR products were purified with ExoSAP-IT (GE Healthcare Japan, Tokyo, Japan) directly or after subcloning. For subcloning, a TOPO (R) cloning kit (Invitrogen, Tokyo, Japan) was used in accordance with the manufacturer's instructions. Purified products were sequenced using an ABI 310 DNA sequencer (ABI, Tokyo, Japan).

### Phylogenetic analysis based on ITS1, ITS2, and 5.8S rDNA sequences

The basic data set for each locus, ITS1, ITS2, or 5.8SrDNA, consisted of 42 taxa: 40 *Pestalotiopsis* strains (Tables 1, 2) and 2 *Seiridium* species used as outgroups, *S. cardinale* (Petr.) Nag Raj & W.B. Kendr. and an unidentified *Seiridium*. The *S. cardinale* sequence, AF409995, had previously been used as the outgroup in an analysis of *Pestalotiopsis* sequences described in Liu et al. (2007) and Hu et al. (2007). Sequences (around 540 bp) were assembled and aligned using Clustal W (Thompson et al. 1994 in BioEdit, http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) and were optimized manually. Gaps were treated as missing data (93 site removed). Phylogenetic analysis of sequences was conducted using Mega 4 (Tamura et al. 2007). Maximum-parsimony (MP) phylogenetic trees were reconstructed using the close-neighbor-interchange algorithm (Nei and Kumar 2000) with search level 3 with equal character weights, as implemented in Mega4. The initial trees were obtained with the random addition of sequences (100 replicates). The strength of the internal branches from the resulting tree was tested by bootstrap (BS) analysis (Felsenstein 1985) using 1,000 replications in both distance and parsimony analyses.

### ITS2 sequence and secondary structure prediction

The sequence and secondary structure of ITS2 from 39 *Pestalotiopsis* strains and two *Seiridium* species, *S. cerdinera* and an unidentified *Seiridium*, were analyzed as described in Schultz and Wolf (2009). ITS2 sequences derived from PCR products amplified with the ITS5 and ITS4 primer pair were annotated according to previous methods (Eddy 1998; Keller et al. 2009) and were searched for predicted secondary structures (Schultz et al. 2005) found in the ITS2 database (Schultz et al. 2006; Selig et al. 2008). The predicted secondary structure of ITS2 from *Seiridium* sp. was used as a template for the homology-modeling

**Table 1** List of strains used in this study for molecular analysis and measuring of color density with species name, host, and accession numbers of the ITS1, ITS2, and 5.8S rDNA sequences

| Species | Host | Strain no. | Accession no. |
|---|---|---|---|
| *P. adusta* (Ellis & Everh.) Steyaert 1 | *Lilium* sp. | TAP 08K0013 | AB491976 |
| *P. aletridis* (Pat.) G.C. Zhao & N. Li | *Ixora chinensis* Lam. | MAFF 237039 | AB491762 |
| *P. calabae* (Westend.) Steyaert | *Cocos nucifera* L. | MAFF 237935 | AB491977 |
| *P. disseminata* (Thüm.) Steyaert 1 | *Pieris japonica* (Thunb.) D. Don | MAFF 241681 | AB491979 |
| *P. foedans* (Sacc. & Ellis) Steyaert | *Diospyros kaki* Thunb | MAFF 238089 | AB612120 |
| *P. glandicola* (Castagne) Steyaert | *Machilus thunbergii* Sieb. & Zucc. | TAP 12O048 | AB482205 |
| *P. gracilis* (Kleb.) Steyaert | *Itea oldhamii* K. Schneid. | MAFF 240470 | AB491978 |
| *P. ixorae* (Rangel) Bat. & Peres | *Ixora* sp. | MAFF 237036 | AB491975 |
| *P. maculans* (Corda) Nag Raj 1 | *Camellia japonica* L. | TAP 17O055 | AB482217 |
| *P. neglecta* (Thüm.) Steyaert 1 | *Pieris japonica* (Thunb.) D. Don | MAFF241853 | AB491980 |
| *P. neglecta* 2 | *Kalmia latifolia* L. | MAFF 241682 | AB491981 |
| *P. neglecta* 3 | *Pieris japonica* (Thunb.) D. Don | TAP 99M112 | AB482211 |
| *P. neglecta* 4 | *Pieris japonica* (Thunb.) D. Don | MAFF241857 | AB498063 |
| *P. pallidotheae* Kyoko Watan. & Yas. Ono | *Pieris japonica* (Thunb.) D. Don | MAFF 240993 | AB482220 |
| *P. palustris* Nag Raj | *Pieris japonica* (Thunb.) D. Don | MAFF241730 | AB482215 |
| *P. palmarum* (Cooke) Steyaert | *Spondias mombin* L. | MAFF 240487 | AB491982 |
| *P. podocarpi* (Dennis) X.A.Sun & Q.X.Ge | *Pandanusodoratissimus* L. F. | MAFF 240479 | AB491983 |
| *P. theae* (Sawada) Steyaert 1 | *Camellia sinensis* (L.) Kuntze | MAFF 752011 | AB482210 |
| *P. theae* 2 | *Rhaphiolepis indica* (L.) Lindl. ex Ker var. *umbellata* (Thunb. ex Murray) Ohashi | MAFF 238515 | AB482203 |
| *Pestalotiopsis* sp. 1 | *Gardenia jasminoides* Ellis | MAFF 240500 | AB491984 |
| *Pestalotiopsis* sp. 2 | Broad-leaved tree | MAFF 238514 | AB482219 |

MAFF, strains preserved at NIAS Genebank, National Institute of Agrobiological Science; TAP, strains preserved at Tamagawa University

algorithm provided with the ITS2 database (Wolf et al. 2005). Homology modeling was performed according to the default settings (ITS2PAM50 matrix, transfer helixes with 75% similarity, gap open penalty of 20, gap extension penalty of 4) for transfer helixes. The ITS2-specific scoring matrix automatically aligned sequences and structures simultaneously using 4SALE (Seibel et al. 2006, 2008). A ProfDist neighbor-joining (PNJ) tree was constructed, and a sequence-structure ProfDist neighbor-joining analysis was conducted using the ProfDist database and algorithm (Friedrich et al. 2005; Wolf et al. 2008).

## Color intensity analysis

Samples from cultures grown on PDA were subcultured on PDA or on leaf agar (Kishi 1994) consisting of boiled leaves of *Hydrangea macrophylla* (Thunb.) Sieb. f. *macrophylla* (Wilson) Hara overlaying water agar and exposed to natural light. Erumpent acervuli formed under the epidermis, breaking through the upper layer and becoming patelliform. Conidia produced from acervuli 1 month after inoculation were mounted in water. An Olympus BX51 microscope with an LBD filter and Olympus DP70 digital system were used to capture images of the conidia using default settings. The white balance setting was used for automatic optimization, but this protocol also works without adjusting white balance.

The optimum number of pixels (1,360 × 1,024 pixels and RGB color) was determined by preliminary analysis (data not shown). Image J software developed at the National Institutes of Health (http://rsb.info.nih.gov/ij/) was used to measure the color density of the three median cells in conidia. Color density was measured by the dot blot method in Image J (http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels). Conidia oriented in the vertical direction were selected with the rectangle tool. The lines of a rectangle were positioned to just outside the cell wall boundaries of the three median cells of a typical conidium. A rectangle of the same size was placed on a background area (no conidia) to select the first box to be used as a control. Note that in the software, the word "lane" is used instead of "box," as this software was written for gel scanning. A second box containing the three median cells of the conidium was created (Fig. 1). Profile plots were then generated for each box.

**Table 2** GenBank accession numbers for the ITS1, ITS2, and 5.8S rDNA sequences used in molecular analysis

| Species | Strain no. | Accession no. | References |
|---|---|---|---|
| *Pestalotiopsis acaciae* (Thüm.) K. Yokoy. & S. Kaneko | TAP 31O086 | AB482207 | Watanabe et al. (2010) |
| *P. adusta* 2 | TAP 32O089 | AB482212 | Watanabe et al. (2010) |
| *P. crassiuscula* Steyaert 1 | TAP 23O074 | AB482222 | Watanabe et al. (2010) |
| *P. crassiuscula* 2 | TAP 01O037 | AB482208 | Watanabe et al. (2010) |
| *P. disseminata* 2 | TAP 29O082 | AB482213 | Watanabe et al. (2010) |
| *P. disseminata* 3 | MAFF 238347 | AB482214 | Watanabe et al. (2010) |
| *P. fici* Steyaert 1 | TAP 34O099 | AB482201 | Watanabe et al. (2010) |
| *P. fici* 2 | TAP 39O129 | AB482200 | Watanabe et al. (2010) |
| *P. funerea* (Desm.) Steyaert | | AF405299 | Jeewon et al. (2002) |
| *P. japonica* (Syd.) Steyaert | TAP 11O047 | AB482204 | Watanabe et al. (2010) |
| *P. kunmingensis* J.G. Wei & T. Xu | | AY373376 | Jeewon et al. (2004) |
| *P. longiseta* (Speg.) K. Dai & Ts. Kobay. | MAFF 752008 | AB482206 | Watanabe et al. (2010) |
| *P. karstenii* (Sacc. & P. Syd.) Steyaert | | AY681476 | Wei et al. (2004)[a] |
| *P. maculans* 2 | | AF405296 | Jeewon et al. (2002) |
| *P. microspora* (Speg.) Bat. & Peres | | AY687882 | Wei et al. (2004)[a] |
| *P. neglecta* 5 | TAP 08O038 | AB482216 | Watanabe et al. (2010) |
| *P. neglecta* 6 | TAP 20O063 | AB482209 | Watanabe et al. (2010) |
| *P. olivacea* (Guba) G.C. Zhao & J. He | | DQ417182 | Wei et al. (2006)[a] |
| *P. theae* 3 | TAP 36O105 | AB482202 | Watanabe et al. (2010) |
| *Seiridium cardinale* (Petr.) Nag Raj & W.B. Kendr. | | AF409995 | Jeewon et al. (2003) |
| *Seiridium* sp. | MAFF 238468 | AB482221 | Watanabe et al. (2010) |

MAFF, strains preserved at NIAS Genebank, National Institute of Agrobiological Science; TAP, strains preserved at Tamagawa University

[a] Direct submission

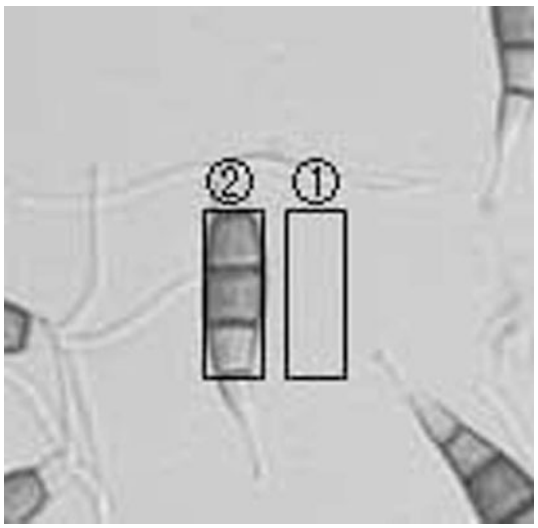


**Fig. 1** Example of the use of Image J software for measuring the OD value of the three median cells of conidia formed on the leaf agar. ① rectangle on the background to select the first box. ② rectangle to align with the outer cell walls of the three median cells for second box

The Image J program was used to measure the intensity of gray-scale color. Each spore was scanned transversely giving a plot where the cell walls (least translucent) showed up as peaks. Light colored areas below the peaks represent the pixel count. The area above the peaks represents the absence of pixels. The control box in a background area was also analyzed and the pixel count compared to the box with the spore, to give an indication of the spore color intensity.

Finally, the optical densities were obtained with the formula "1 - (Lane 2/Lane 1)" to reflect pixel count. Data were collected from 20 conidia from each strain and were analyzed by one-way ANOVA and Tukey-HSD. Within species, optical density (OD) values from conidia grown on PDA at 23°C for 1 month were compared with those formed on the leaf agar in order to assess the differences in pigmentation on the two substrates. OD values of conidia from leaf agar cultures were used for the statistical analysis.

## Morphological observation

MAFF and TAP strains were cultured on leaf agar as noted above. Microscopic characteristics of mature conidia were observed with an Olympus DX 50 microscope equipped with Nomarski optics, and images were captured with a DP 20 digital system (Olympus). Specimens were mounted in Shear's reagent (10 g sodium acetate, 200 ml glycerol, 300 ml ethanol, and 1,000 ml distilled water, Chupp 1940).

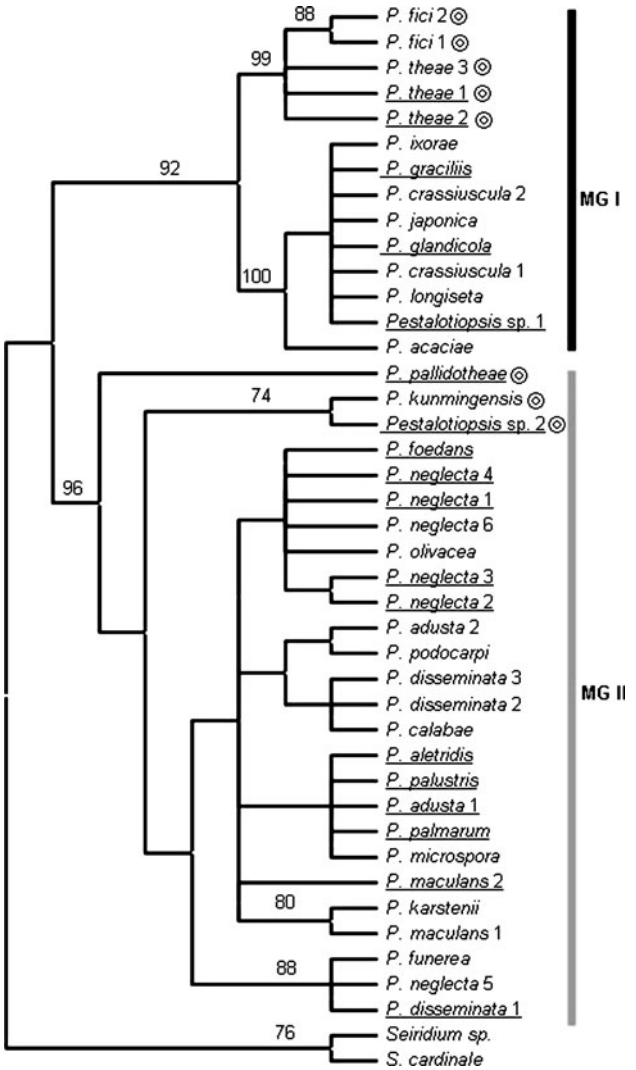


**Fig. 2** Consensus tree inferred from 506 most parsimonious trees of *Pestalotiopsis* based on DNA sequence data from ITS1, ITS2, and 5.8S rDNA. Branches corresponding to partitions reproduced in less than 50% of trees are collapsed. Sample data are listed in Tables 1 and 2. The consistency index is 0.674157, the retention index is 0.940083, and the composite index is 0.689969 for all sites and parsimony-informative sites. Bootstrap values of more than 70% ($n = 1000$ replicates) are indicated at the corresponding nodes. *Underlined species* were used for the conidial color density assays. *Black bar* indicates Molecular Group I (MG I) and *grey bar* indicates Molecular Group II (MG II). ◎ indicates species with knobbed tip appendage

## Results

### Molecular analysis of ITS1, ITS2 and 5.8S rRNA

MP analysis of the ITS1, ITS2, and 5.8S rRNA sequences resulted in 506 trees of 109 steps, the consistency index was (0.674157), the retention index was (0.940083), and the composite index was 0.689969 (0.633764) for all sites and parsimony-informative sites (in parentheses). There were a total of 448 positions in the final data set (93 positions were deleted), out of which 53 were parsimony informative. Tree topologies of all MP trees were very similar, differing only slightly at the terminal branches. The consensus tree inferred from all MP trees is shown in Fig. 2.

Two groups, referred to as Molecular Groups I (MG I) and II (MG II), emerged from this analysis, with bootstrap values exceeding 90% (Fig. 2). MG I consisted of species such as *P. gracilis* (Kleb.) Steyaert, *P. ixorae* (Rangel) Bat. & Peres, and *P. theae* (Sawada) Steyaert, while MG II consisted of species such as *P. adusta* (Ellis & Everh.) Steyaert, *P. foedans* (Sacc. & Ellis) Steyaert, and *P. neglecta* (Thüm.) Steyaert.

MG I and MG II had very similar 5.8S rDNA sequences, but the groups were clearly differentiated by a 40- to 50-bp gap in ITS1, which was similar to the results reported by Liu et al. (2010), and a few gaps and unique sequences were seen in ITS2 (data not shown).

### ITS2 secondary structure prediction and a phylogenetic tree based on ITS2 secondary structure

The ITS2 of all species examined ranged from 153 to 171 bp in length. There were four helices (helix I, II, III, and IV) in the secondary structures of ITS2. Molecular morphometrics of predicted ITS2 secondary structure identified two clades, called secondary structure I (SS I) and secondary structure II (SS II), that were supported by bootstrap values over 94. SS I included *P. gracilis, P. ixorae*, and *P. theae*, and SS II included *P. adusta*, *P. foedans*, and *P. neglecta* (Fig. 3a). There were U–U mismatches in the second helix of all species, and the third helix was longest. The second helix in SS I had a conserved "GCT TAGTGTTGGGAA" motif at the left side of the helix. In contrast, helix II in SS II had a "GCTTAGTGTTGGGAG" motif. The *Seiridium* species used for outgroup comparison had a predicted ITS2 secondary structure more similar to that of SS I than that of SS II (data not shown). The calculated evolutionary distance between SS I and SS II (Fig. 3b) suggested that SS I has a closer relationship with *Seiridium* than with SS II.

### Analysis of color intensity of conidia

The OD values of the three median cells of conidia in each strain are shown in Table 3. Except for *P. calabae* (Westend.) Steyaert, *P. adusta*, *P. neglecta* 2, and *Pestalotiopsis* sp. 1, the values differ significantly between cells obtained from cultures on PDA and cells from leaf agar ($P < 0.05$). In general, OD values were higher for cells on leaf agar, except for *P. foedans*, *P. neglecta* 2, *P. palmarum* (Cooke) Steyaert, *P. theae* 1, and *Pestalotiopsis* sp. 2.

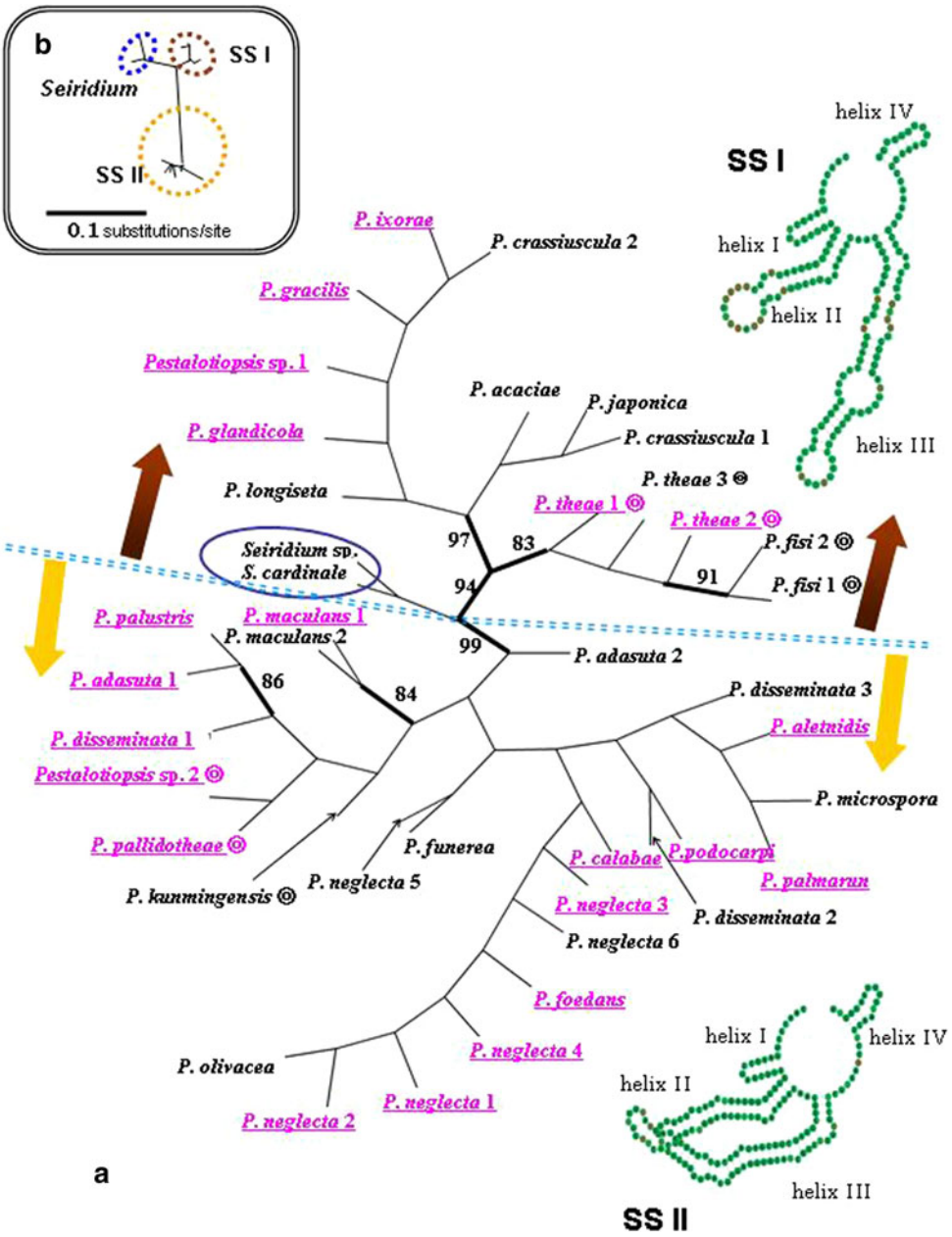


**Fig. 3** Neighbor–joining phylogeny of *Pestalotiopsis* based on comparisons of ITS2 rDNA and secondary structure (**a**). The *dotted line* divides the genus into two clades based on bootstrap values over 90% and on features of the ITS2 secondary structures. The tree is unrooted, but *Seiridium cardinale* is the appropriate outgroup, according to some reports (e.g., Liu et al. 2007). Branch thickness reflects bootstrap values calculated with ProfDist neighbor-joining. *Bold branches* indicate more than 80% bootstrap support. The evolutionary distances between these groups are shown in **b**. *Underlined species* (*pink*) were used for the color intensity assays of three median cells. ⊙ indicates species with knobbed tip appendage

Statistical analysis of OD values of the three median cells of conidia from strains cultured on leaf agar yielded two groups: OD I [OD value >0.62: *P. glandicola* (Castagne) Steyaert, *P. gracilis*, *P. ixorae*, *P. theae* 1, *P. theae* 2, and *Pestalotiopsis* sp. 1] and OD II (OD value <0.57: *P. adusta* 1, *P. aletridis* (Pat.) G.C. Zhao et N. Li., *P. calabae*, *P. disseminata* (Thüm.) Steyaert, *P. foedans*, *P. maculans* 1, *P. neglecta* 1, 2, 3, 4, *P. pallidotheae* Kyoko Watan. & Yas. Ono, *P. palmarum*, *P. palustris* Nag Raj, *P. podcarpi* (Dennis) X. A. Sun & Q. X. Ge, and *Pestalotiopsis* sp. 2) (Fig. 4). The visually determined 'dark color' strains coincided with OD I, and 'pale color' strains coincided with OD II. However, color intensity of conidia on PDA did not show the difference between OD groups such as *P. gracilis* and *P. ixorae*.

## Morphological characteristics of tip appendage of conidia

Among the strains used here, *P. fisi, P. theae* Steyaert, *P. pallidotheae*, and *Pestalotiopsis* sp. 2 had knobbed-tipped appendages (Fig. 5), and although *P. kunmingensis* J.G. Wei & T. Xu was not observed in this study, this strain has knobbed-tipped appendages according to Wei and Xu (2004).

**Table 3** Color density (OD value) of the median cells of conidia measured using Image J software

| OD | Species | Strain no.[a] | On PDA | | On Leaf agar | | $P < 0.05$[c] |
|---|---|---|---|---|---|---|---|
| | | | Ave. | SD[b] | Ave. | SD | |
| OD I[d] | | | | | | | |
| | *P. glandicola* | TAP 12O048 | – | – | 0.624 | 0.033 | – |
| | *P. gracilis* | MAFF 240410 | 0.556 | 0.038 | 0.643 | 0.026 | * |
| | *P. ixorae* | MAFF 237036 | 0.576 | 0.038 | 0.65 | 0.045 | * |
| | *P. theae* 1 | MAFF 752011 | 0.716 | 0.038 | 0.68 | 0.021 | * |
| | *P. theae* 2 | MAFF 238515 | – | – | 0.643 | 0.035 | – |
| | *Pestalotiopsis* sp. 1 | MAFF 240500 | 0.679 | 0.034 | 0.691 | 0.033 | |
| OD II[d] | | | | | | | |
| | *P. adusta* 1 | TAP 08K0013 | 0.494 | 0.043 | 0.521 | 0.029 | * |
| | *P. aletridis* | MAFF 237039 | 0.442 | 0.07 | 0.552 | 0.026 | * |
| | *P. calabae* | MAFF 231935 | 0.528 | 0.032 | 0.534 | 0.055 | |
| | *P. disseminata* 1 | MAFF 241681 | 0.491 | 0.049 | 0.512 | 0.022 | |
| | *P. foedans* | MAFF 238089 | 0.55 | 0.041 | 0.501 | 0.043 | * |
| | *P. maculans* 1 | TAP 17O055 | 0.539 | 0.025 | 0.550 | 0.028 | |
| | *P. neglecta* 1 | MAFF 241853 | 0.503 | 0.035 | 0.536 | 0.034 | * |
| | *P. neglecta* 2 | MAFF 241857 | 0.544 | 0.033 | 0.531 | 0.045 | |
| | *P. neglecta* 3 | TAP 99M112 | – | – | 0.491 | 0.032 | – |
| | *P. neglecta* 4 | MAFF 241857 | 0.476 | 0.036 | 0.476 | 0.032 | |
| | *P. pallidotheae* | MAFF 240993 | 0.591 | 0.037 | 0.567 | 0.031 | * |
| | *P. palmarum* | MAFF 240481 | 0.572 | 0.022 | 0.549 | 0.029 | * |
| | *P. palustris* | MAFF 241930 | 0.520 | 0.042 | 0.552 | 0.029 | * |
| | *P. podocarpi* | MAFF 240479 | 0.518 | 0.04 | 0.544 | 0.038 | * |
| | *Pestalotiopsis* sp. 2 | MAFF 238514 | 0.538 | 0.018 | 0.519 | 0.031 | * |

[a] MAFF: strains preserved at NIAS Genebank, National Institute of Agrobiological Science. TAP: strains preserved at Tamagawa University

[b] Standard deviation from 20 replicates

[c] Asterisk (*) indicates significant difference at the $P < 0.05$ level, according to Tukey-HSD

[d] OD I: Optical density group I is "pale" (OD < 0.6), OD II: optical density group II is "dark" (OD > 0.6). Both are determined by conidia on leaf agar

## Discussion

In monographs such as that of Steyaert (1949), pigmentation of the median cells of conidia has been identified as an important criterion for taxonomic classification of *Pestalotiopsis*. Guba (1961), Nag Raj (1993), and Sutton (1980) also used pigmentation criteria for classification. On the other hand, Satya and Saksena (1984) showed that conidia exhibited different color intensities on different substrates, and they suggested that unaided visual estimation of color density is not effective for species identification. Jeewon et al. (2003) showed that *Pestalotiopsis* consists of three main clades and that pigmentation of the median cells of conidia, appendage morphology, and molecular systematics based on ITS1, ITS2, and 5.8S rDNA sequences are all highly useful traits for phylogenetic analysis and species identification.

In this study, sequence analysis of ITS1, ITS2, and 5.8S rDNA identified two main clades, MG I and MG II (Fig. 2). Furthermore, analysis of the ITS2 sequence and secondary structure identified two groups, SS I and SS II. The ITS2 secondary structure, which has been found to be highly correlated with taxonomic classification (Coleman 2009), showed two distinct secondary structures. SS I was completely different from SS II and appeared to be, evolutionarily, very distant (Fig. 3a, b). The ITS2 sequence and secondary structure have also been found to be highly correlated with taxonomic classification (Coleman 2009).

In 2003, Jeewon et al. suggested that the genus *Pestalotiopsis* consists of three main clades based on ITS1, ITS2, and 5.8S rDNA sequences analyses. Their results, however, could also be interpreted to mean that species of *Pestalotiopsis* were divided into two main clades and that one of the main clades consisted of two subclades. Their research group (Liu et al. 2010) later revised the phylogenetic tree into two main clades, even though they used combined ITS1, ITS2, and *β-tubulin* data without a model test for each gene. In our results, as there were two distinct ITS2

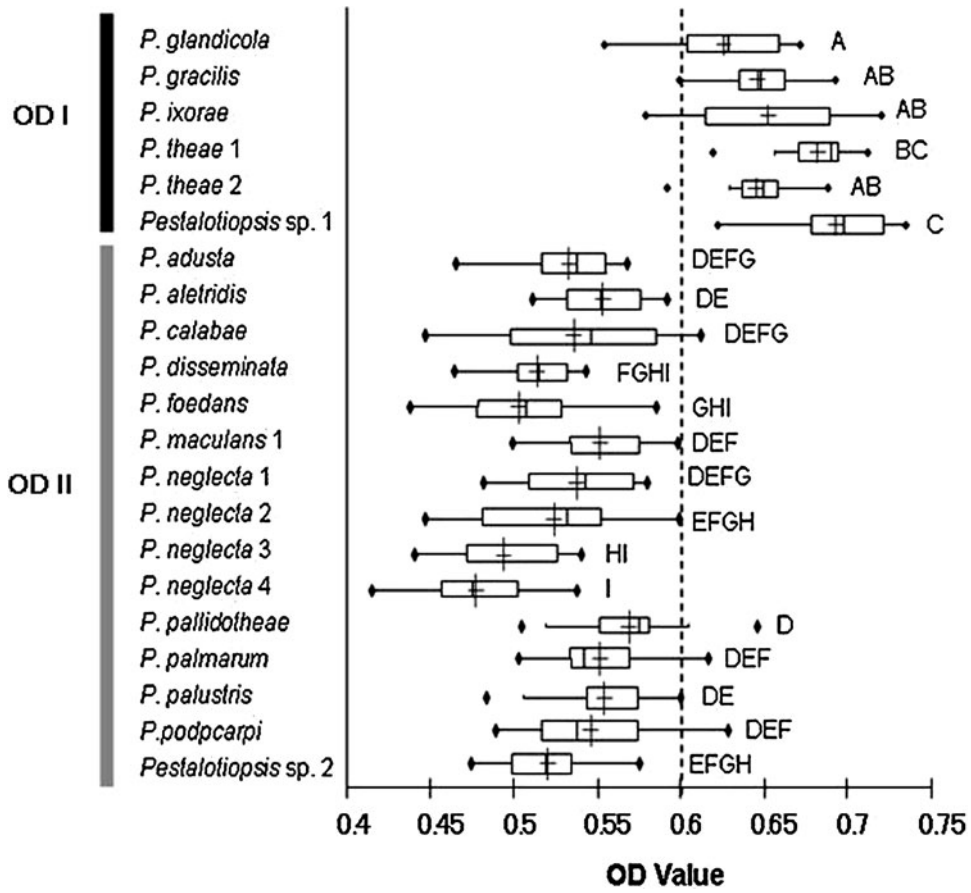


**Fig. 4** Box plots of OD values of the median cells of conidia grown for 1 month on leaf agar. *Different* letters indicate statistically significant differences between means at $P < 0.05$ according to Tukey's HSD. *Longitudinal edges* of *box plots* indicate lower quartile (*left edge*), median, and upper quartile (*right edge*). *Plus symbols* geometric average, *diamonds* maximum or minimum value

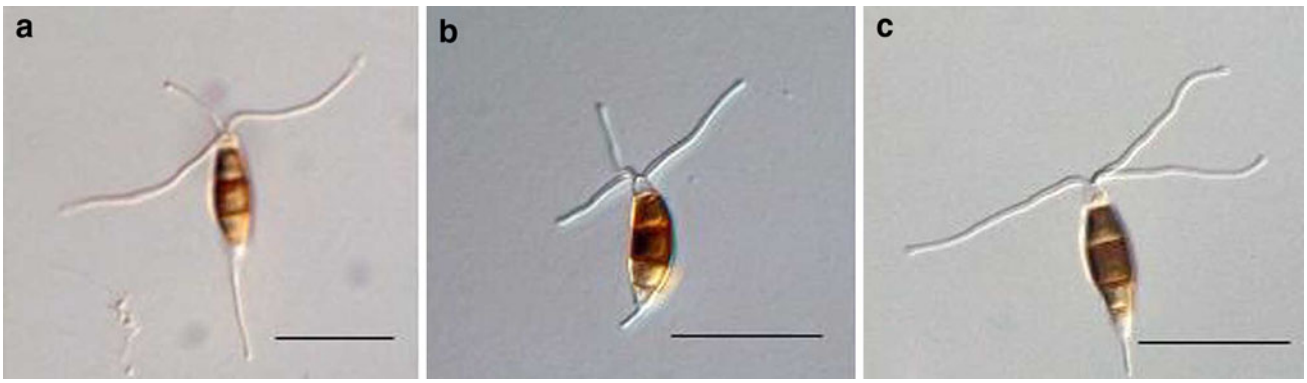


**Fig. 5** Conidia with knobbed-tipped appendages. **a** *P. plidetheae*, **b** *Pestalotiopsis* sp. 2, **c** *P. theae* 2. *Bars* 20 μm

secondary structures in *Pestalotiopsis*, the genus could be said to have two main monophyletic groups, and these reflected the difference in the color of three median conidia cells.

Cell color in conidia was previously determined subjectively by eye as a criterion for species identification. This traditional procedure can result in incorrect species identification because some species have appendages of the same size and the same tip morphology, and differ only in the color of the three median cells of conidia. For these criteria, "color" assessment was not based on an objective, color-chart-dependent method to assess the color of pigmented cells in *Pestalotiopsis*.

There have been a number of examples of difficulties associated with *Pestalotiopsis* species identification using conidial cell color as the sole identifying trait. *Pestalotiopsis theae* has concolorous and brown or yellow–brown median cells according to the monograph of Guba (1961). However, Jeewon et al. (2003) reported that *P. theae* belongs in a group with brown concolorous pigmentation

that had diverged within a larger clade having versicolorous pigmentation based on molecular analysis of ITS1, ITS2, and 5.8S rDNA. They concluded that variation in pigmentation among median cells, such as concolorous or versicolorous median cells, is a sound diagnostic characteristic for distinguishing species. However, the phylogenetic trees showed that the concolorous group can be divided in two, one of which (containing *P. theae*) diverged from the versicolorous group as a subgroup of the versicolorous group. Jeewon et al. (2004) later showed a phylogenetic tree topology similar to their results in 2003 based on ITS1, ITS2, and 5.8S rDNA.

Wei and Xu (2004) reported that *P. kunmingensis* was a new species based on versicolorous median cells, but Liu et al. (2007) constructed a phylogenetic tree based on ITS1, ITS2 and 5.8S rRNA, and *β-tubulin* sequences and designated *P. hainanensis* A.R. Liu, T. Xu & L.D. Guo as a distinct species; their tree placed *P. kunmingensis* in a sister clade derived from one with concolorous pigmentation.

Recently, Watanabe and Nakazono (2009) reported that a preliminary objective method for color intensity based on Image J was useful and that it reflected two clades of the molecular phylogenies based on ITS1, ITS2, 5.8S rDNA, *β-tubulin*, and *EF1α*, respectively, and that it emphasized the importance of pigmentation for identification in these refracted phylogenetic trees. Furthermore, Watanabe (2009) pointed out possible misidentification of *Pestalotiopsis* strains stored in public culture collections. Some *Pestalotiopsis* collections remain misidentified because previous researchers have used the pigmentation of three median cells of conidia, a relatively subjective criterion, as an important step in morphological classification. For example, the only morphological difference between *P. pallidotheae* and *P. theae* is the pigmentation of median cells; nevertheless, these species are in completely different phylogenetic groups based on analysis of ITS1, ITS2, and 5.8S rDNA sequences (Watanabe et al. 2010).

In the absence of molecular systematic data, using only this morphological criterion may result in the misclassification of species. *Pestalotiopsis palumarum* in this study, and that in reports by Jeewon et al. (2003) and Liu et al. (2010), were associated with different sister taxa. This antinomy also indicates the difficulties and limitations of identification without objective traits. In this study, we were unable to resolve these differences, but evidence indicates that they are different species.

Liu et al. (including Jeewon, 2010) rejected their previous speculations (Jeewon et al. 2003) and put forward the notion that "color" pigmentation of three median cells, such as "color" and brown to olivaceous and umber to fuliginous by the color chart, is the key to taxonomy. However, as they used the color chart of Ridgway (1912) to assess the pigmentation of median cells as phylogenetic markers, their results are still unreliable, since the color chart (Ridgway 1912) is unsatisfactory (Hawksworth 1974) and color input through microscopy was assessed without color management. Thus, as this "color" was unable to overcome the issue faced by traditional descriptions such as Guba (1961), an objective method for assessment the color is needed.

Instead of using a "color" criterion, our study shows that a new criterion, 'color intensity' (i.e., pale or dark color, as assessed using Image J software), reflects the groups derived from molecular analyses and type of ITS2 secondary structures. We confirmed the importance of the color of the three median cells in phylogenetic analyses by objective traits, and further defined the relationship between tip appendages and the phylogenetic tree. For assessing the color of median cells of conidia, we focused on color intensity and attempted to use an objective measurement using conidia from strains cultured on leaf agar. The threshold pigmentation value of 0.6 distinguished OD I and OD II. All species of high color intensity in the median cells (OD I; observable dark cells) were in MG I and SS I, and all species characterized by low color intensity in median cells (OD II; observable pale cells) were in MG II and SS II. Indeed, both groups were genetically distant when compared with *Seiridium* (Fig. 3b). The color intensity in the median cells of the conidia reflects the phylogeny of *Pestalotiopsis* and can be a useful criterion for species identification in the absence of molecular data.

On the other hand, the relationship with the morphology of the appendage tip is not clear cut. With respect to presence or absence of the knobbed-tipped appendage, the dark group (clade), which included *P. theae* and *P. fici*, aggregated in one subclade (SS I/MG I/OD I), but not the other (SS II/MG II/OD II). These results indicate that color intensity of three median cells is related to phylogeny, but the relationship with the morphology of the appendage tip is not clear.

Jeewon et al. (2003) noted that species with knobbed-tipped appendages and brown concolorous median cells fall into one monophyletic group. This group is part of a group that is equivalent to the SS I/MG I/OD I group we identified, and they concluded that the knobbed-tipped appendage appears to have evolved once during the evolution of *Pestalotiopsis* species. The existence of species with knobbed-tipped appendages in other clades has been demonstrated by some researchers (e.g., Liu et al. 2007; Watanabe et al. 2010), and this is supported by the present study. In the SS II/MG II/OD II group, *P. kunmingensis* and *Pestalotiopsis* sp. 2 did not aggregate into one subclade with *P. pallidotheae*. These results lead to two hypotheses about the manifestation of evolution of knobbed appendage. One is that the knobbed appendages are the result of

convergent evolution because the knobbed appendage is a polyphyletic characteristic in the rDNA tree. Another is that the knobbed appendage is a plesiomorphic trait because the taxa with knobbed-tipped appendages in the MG II group share the basal nodes. This traditional trait for species identification is useful, but the evolutionary relationships are not clear, yet.

*Seiridium* species are generally used as an outgroup in the molecular analyses of *Pestalotiopsis* species. However, the ITS2 secondary structure observed in the SS I group of *Pestalotiopsis* was more similar to that of the *Seiridium* outgroup species than to that of the SS II group. The morphologies of *Pestalosphaeria*, the teleomorph of *Pestalotiopsis*, and *Lepteutypa*, the teleomorph of *Seiridium*, are similar (Samuels et al. 1987), as are their phylogenetic relationships based on 28S rDNA sequence comparisons and morphological observations (Jeewon et al. 2002). Our analysis of phylogeny based on ITS2 secondary structure is consistent with the conclusions of Jeewon et al. (2002) and suggests that the genera allied with *Pestalotiopsis* should be reconsidered using an analysis of ITS2 secondary structure.

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