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# Cultural studies coupled with DNA based sequence analyses and its implication on pigmentation as a phylogenetic marker in *Pestalotiopsis* taxonomy

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ABSTRACT

Previous phylogenetic studies based on DNA sequence data have partially resolved taxonomic relationships among *Pestalotiopsis* species. There are still some morphological characters whose phylogenetic significance have not been assessed properly due to limited taxon sampling, in particular the degree of pigmentation of median cells. In this study, the stability of pigmentation of median cells of conidia in *Pestalotiopsis* species was evaluated in subculture, and a molecular phylogenetic analysis was conducted on 45 strains belonging to 26 species in order to reappraise the pigmentation of median cells for its significance in the taxonomy of *Pestalotiopsis*. Phylogenetic relationships were inferred from nucleotide sequences in ITS regions (ITS1, 5.8S and ITS2) and β-tubulin 2 gene (*tub2*). The results showed that pigmentation of median cells was stable and it could be a key character in the taxonomy of *Pestalotiopsis* species. Instead of "concolorous" and "versicolor" proposed by Steyeart (1949), "brown to olivaceous" and "umber to fuliginous" are described and proposed in this paper.



## 1. Introduction

*Pestalotiopsis* was established by Steyeart (1949) for species with conidia that are five-celled, with three intermediate coloured cells and hyaline end cells, and with two or more apical appendages arising from the apical cell. Molecular studies have shown that *Pestalotiopsis* is a monophyletic genus (Jeewon et al., 2002).

Inter-specific delineation has been mainly based on morphology of the conidia (Steyeart, 1949; Guba, 1961; Nag Rag, 1993), conidiogenesis (Sutton, 1980) and teleomorph association (Barr, 1975, 1990; Zhu et al., 1991; Metz et al., 2000). Approximately 225 species of *Pestalotiopsis* have been described (CABI Bioscience Database, 2009), and many of them exist as plant pathogens, plant endophytes or saprobes.

In recent years, molecular data has been used in the identification and classification of *Pestalotiopsis* species (Jeewon et al., 2003, 2004; Wei and Xu, 2004; Hu et al., 2007; Liu et al., 2007; Wei et al., 2007; Espinoza and Briceño, 2008; Keith, 2008; Luan et al., 2008; Zhou et al., 2009; Tejesvi et al., 2009). Jeewon et al. (2003) assessed the phylogenetic importance of morphological characters based on ITS sequence analysis. To further resolve taxonomic issues, Hu et al. (2007) analysed a combined dataset DNA sequence data from the ITS region and β-tubulin gene and found that the combined genes were more appropriate in solving taxonomic relationships.

Pigmentation of median cells was recognised as an important character for identification and classification of *Pestalotiopsis*, and "concolorous" and "versicolor" was proposed by Steyeart (1949) and Guba (1961) as two main types of pigmentation of median cells. However, there were still some discrepancies regarding the taxonomic importance of pigmentation in delimiting species. For instance, Griffiths and Swart (1974) considered pigmentation of median cells as an important character in taxonomy of *Pestalotiopsis funerea* and *P. triseta*. This result was confirmed by Hu et al. (2007), who studied conidial characters of 37 strains isolated from *Pinus armandii* and *Ribes* sp. and suggested that pigmentation is a reliable trait for inter-specific classification. In another study carried out by Wei et al. (2006), it was found that pigmentation changed in different cultures but appeared stable when cultured on autoclaved carnation leaves with potato dextrose agar (PDA) medium. Whether pigmentation is a stable character has been a matter of taxonomic debate. Purohit and Bilgrami (1968) showed

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that during cultural studies, individual species presented concolorous or versicolor in different generations. This was consistent with the results of Satya and Saksena (1984), who observed that *Pestalotiopsis glandicola* and *P. versicolor* var. *polygoni* produced spores with different colour intensities in culture and on different hosts and the authors argued that colour contrast of median cells was not a dependable character. A similar phenomenon was also reported by Purohit and Bilgrami (1969) who examined more than 100 pathogenic isolates of *Pestalotiopsis*.

Given the ubiquitous nature of *Pestalotiopsis* species and its occurrence as plant pathogens, a reevaluation of the taxonomic significance of pigmentation is warranted. The objectives of this study are to (1) assess whether pigmentation of median cells is stable or not; (2) investigate whether successive subculturing affects the degree of pigmentation; and (3) infer phylogenetic relatedness of *Pestalotiopsis* species, especially those with different degree of pigmentation and knobbed apical appendages.

## 2. Materials and methods

### 2.1. Culture and observation

A total of 27 strains belonging to 14 species were observed on their consecutive subcultures for three generations (Table 1). All the strains tested were cultured on autoclaved carnation leaves

**Table 1**
List of fungi with their hosts, habitat and Genbank accession numbers used in this study.

| Taxon | Isolates | Host | GenBank accession numbers | |
|---|---|---|---|---|
| | | | ITS | β-Tubulin |
| *Ingroup* | | | | |
| A | | | | |
| *Pestalotiopsis aquatica* | PSHI2002Endo321 | *Podocarpus macrophyllus* (Thunb.) D. Don. | AY687303 | DQ333571 |
| *Pestalotiopsis clavispora* | PSHI2002Endo389 | *Camellia sinensis* O. Ktze | AY682929 | DQ333572 |
| *Pestalotiopsis coffeae* 1 | PSHI2004Endo108 | *Zalacca wallichiana* Salacca. | DQ789387 | DQ657895 |
| *Pestalotiopsis coffeae* 2 | PSHI2004Endo483 | *Caryota ochlandra* Hance | DQ789386 | DQ657894 |
| *Pestalotiopsis crassiuscula* | PSHI2002Endo356 | *Podocarpus macrophyllus* (Thunb.) D. Don. | AY687868 | DQ333574 |
| *Pestalotiopsis foedans* 1 | PSHI2004Endo487 | *Bruguiera sexangula* (Lour.) Poir. | DQ813420 | DQ657897 |
| *Pestalotiopsis foedans* 2 | PSHI2004Endo409 | *Aegiceras coniculatum* (L.) Blanco | DQ813418 | DQ657898 |
| *Pestalotiopsis lamberiae* | PSHI2004Endo86 | *Neodypsis decaryi* Jum | DQ813422 | DQ657901 |
| *Pestalotiopsis milletiae* 1 | PSHI2004Endo125 | *Tamarindus indica* Linn. | DQ813424 | DQ657902 |
| *Pestalotiopsis milletiae* 2 | PSHI2004Endo127 | *Tamarindus indica* Linn. | DQ813425 | DQ657871 |
| *Pestalotiopsis paeoniae* | PSHI2002Endo8801 | *Taxus yunnanensis* Cheng et L.K. Fu. | AY687311 | DQ333581 |
| *Pestalotiopsis paeoniicola* | PSHI2002Endo3502 | *Podocarpus nagi* | AY687310 | DQ333582 |
| *Pestalotiopsis palmarum* 1 | PSHI2004Endo458 | *Zalacca wallichiana* Salacca. | DQ813426 | DQ787836 |
| *Pestalotiopsis palmarum* 2 | PSHI2004Endo454 | *Roystonea regia* (H.B.K.) Cook. | DQ813427 | DQ787837 |
| *Pestalotiopsis pampeana* | PSHI2004Endo94 | *Pachira macrocarpa* Walp. | DQ813428 | DQ657876 |
| *Pestalotiopsis pauciseta* | PSHI2004Endo120 | *Tamarindus indica* Linn. | DQ813429 | DQ787838 |
| *Pestalotiopsis photiniae* | PSHI2002Endo403 | *Camellia sasanqua* (Thunb.) | AY682942 | DQ333583 |
| *Pestalotiopsis subcuticularis* | PSHI2002Endo882 | *Taxus yunnanensis* | AY687878 | DQ333584 |
| *Pestalotiopsis versicolor* | PSHI2004Endo124 | *Tamarindus indica* L. | DQ334862 | DQ333585 |
| *Pestalotiopsis virgatula* | PSHI2004Endo415 | *Bruguiera gymnorrhiza* (L.) Poir. | DQ813435 | DQ787841 |
| *Pestalotiopsis westerdijkii* | PSHI2004Endo98 | *Allamanda cathartica* L. | DQ137856 | DQ137862 |
| B | | | | |
| *Pestalotiopsis fici* | PSHI2004Endo334 | *Zalacca wallichiana* Salacca. | DQ789388 | DQ657896 |
| *Pestalotiopsis theae* 1 | PSHI2001path205 | *Camellia sinensis* O. Ktze | AY681479 | DQ137870 |
| *Pestalotiopsis theae* 2 | PSHI2002Endo310 | *Camellia nitidissima* Chi | AY681480 | DQ137871 |
| *Pestalotiopsis theae* 4 | PSHI2004Endo46 | *Dracontomelon duperreanum* Pierre | DQ813432 | DQ141534 |
| *Pestalotiopsis theae* 5 | PSHI2004Endo80 | *Lucuma nervosa* A. DC. | DQ813433 | DQ787843 |
| *Pestalotiopsis theae* 6 | PSHI2001path099 | *Camellia caudata* Wall. | AY681478 | DQ787842 |
| C | | | | |
| *Pestalotiopsis adusta* 1 | PSHI2001path020 | *Podocarpus macrophyllus* var. maki | AY687298 | DQ657884 |
| *Pestalotiopsis adusta* 2 | PSHI2004Endo420 | *Bruguiera gymnorrhiza* (L.) Poir. | DQ789377 | DQ657885 |
| *Pestalotiopsis bicolor* 1 | PSHI2004Endo143 | *Hyophorbe lagenicaulis* Mart. | DQ789380 | DQ657888 |
| *Pestalotiopsis bicolor* 2 | PSHI2004Endo144 | *Hyophorbe lagenicaulis* Mart. | DQ789381 | DQ657889 |
| *Pestalotiopsis heterocornis* 1 | PSHI2002Endo303 | *Camellia japonica* L. | AY687874 | DQ137867 |
| *Pestalotiopsis heterocornis* 2 | PSHI2002Endo408 | *Camellia sasanqua* (Thunb.) | AY681492 | DQ137866 |
| *Pestalotiopsis heterocornis* 3 | PSHI2002Endo391 | *Podocarpus macrophyllus* (Thunb.) D. Don. | AY681491 | DQ137865 |
| *Pestalotiopsis karstenii* 1 | PSHI2002Endo402 | *Camellia sasanqua* (Thunb.) | AY681476 | DQ137860 |
| *Pestalotiopsis karstenii* 2 | PSHI2002Endo201 | *Camellia japonica* L. | AY681472 | DQ137858 |
| *Pestalotiopsis karstenii* 3 | PSHI2002Endo353 | *Camellia japonica* L. | AY681474 | DQ137859 |
| *Pestalotiopsis kunmingensis* | PSHI2002Endo766 | *Podocarpus macrophyllus* (Thunb.) D. Don. | AY373376 | DQ333576 |
| *Pestalotiopsis microspora* 1 | PSHI2002Endo747 | *Camellia sinensis* O. Ktze | AY681484 | DQ333579 |
| *Pestalotiopsis microspora* 2 | PSHI2002Endo1015 | *Pinus massoniana* Lamb. | AY681485 | HM536979 |
| *Pestalotiopsis neglecta* 1 | PSHI2002Endo401 | *Podocarpus nagi* (Thunb.) Zoll et Mor. | AY682932 | DQ141530 |
| *Pestalotiopsis neglecta* 2 | PSHI2002Endo404 | *Camellia nitidissima* Chi | AY682933 | DQ141531 |
| *Pestalotiopsis neglecta* 3 | PSHI2002Endo502 | *Taxus chinensis* (Pilg.) Rehd. var. *mairei* (Lemee et levl.) Cheng et L.K. Fu | AY681486 | DQ141532 |
| *Pestalotiopsis olivacea* 1 | PSHI2002Endo696 | *Camellia sasanqua* (Thunb.) | AY687883 | DQ333580 |
| *Pestalotiopsis olivacea* 2 | PSHI2002Endo839 | *Podocarpus nagi* (Thunb.) Zoll et Mor. | AY681488 | DQ787834 |
| *Outgroup* | | | | |
| *Sordaria alcina* | CBS 109460 | – | AY681198 | AY681232 |
| *Sordaria tomento-alba* | CBS 260.78 | – | AY681195 | AY681229 |

*Note*: '–' no information available.