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Addition of two new species of *Pestalotiopsis* to the fungal diversity in India

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Two new coelomycetous fungi viz. *Pestalotiopsis anacardii* sp. nov. and *Pestalotiopsis termitarii* sp. nov., one is causing leaf spot on mango (*Mangifera indica* L.) and another is growing as saprophyte on termite nest in the tea agro-ecosystem were isolated from two different geographic locations of India. Both the fungi were producing black, carbonaceous acervuli. Conidia were fusiform, straight to curved, 4-5 celled, crowned with situlae. Exterior cells hyaline, intermediate cells are variably coloured (pale brown to black). Simple and hyaline pedicels present, attached to the base of the conidia. *Pestalotiopsis anacardii* is morphologically distinguished from similar species such as *P. albomaculans*, *P. palmarum*, *P. crassiuscula*, *P. photiniae* and *P. virgatula* in respect to size of condia, number and size of setulae. *Pestalotiopsis termitarii* is also distinct from similar species viz. *Pestalotiopsis versicolar*, *P. microspora*, *P. sydowiana* and *P. virgatula* on the basis of different morphological characters. Regions of the ITS (ITS1 and ITS2) were amplified from genomic DNA using PCR. DNA characters were analysed using maximum parsimony and maximum likelihood criteria. Phylogenetic analyses indicate that both the species are also phylogenetically distinct from similar species.

Key words: Coelomycetous fungi, internal transcribed spacer, new species, *Pestalotiopsis*, phylogeny, taxonomy

INTRODUCTION

The genus *Pestalotiopsis* constitutes one of the more commonly collected genera of leaf spot causing in plants. *Pestalotiopsis* species are an important group of endophytic fungi (Okane *et al.*, 1998; Suryanarayanan *et al.*, 1998; Cannon and Simmons, 2002; Toofanee and Dulymamode, 2002; Wei and Xu, 2003, 2004; Kumar and Hyde, 2004; Photita *et al.*, 2004; Wang *et al.*, 2005; Gonthier *et al.*, 2006). This anamorphic genus has been studied extensively by Steyaert (1949, 1955, 1961) and Guba (1961). While Guba (1961) adopts a broader generic concept, Steyaert (1949) restricts *Pestalotia* to a single species and reassigned most of the remaining species of *Pestalotia* to *Pestalotiopsis* Steyaert, and *Truncatella* Steyaert, while a majority of the species remained unassigned. Sutton (1961) has fully discussed the arguments opposing or supporting either approach and gave evidence favouring the rearrangement proposed by Steyaert (1949).

Pestalotiopsis is a complex genus and consists of members difficult to classify at the species level. At present, inter-specific delineation of this genus is based on morphology of the conidia (Guba, 1961; Nag Rag, 1993), conidiogenesis (Sutton, 1980) and teleomorph association, which has been described for only a few species (Barr, 1975; 1990; Metz *et al.*, 2000; Zhu *et al.*, 1991). Since the establishment of the genus (Steyaert, 1949), numerous taxonomic studies have been conducted in an attempt to devise a suitable classification scheme for the different species (Guba, 1961; Nag Rag, 1993; Sutton, 1980).

Hughes (1953) and Kendrick (1979) have pointed out that developmental features of conidia and conidiophores should be given more importance in taxonomic studies. This concept has also been advocated by Sutton (1980), who has suggested that a more rationale and natural classification of

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coelomycetous fungi would be one based on conidiogenesis.

The relation with morphological characters and phylogenetic significance have been demonstrated and discussed by Jeewon *et al.* (2003) and Wei *et al.* (2005). Molecular studies have indicated that *Pestalotiopsis* species isolated from same hosts are not necessarily related (Jeewon *et al.*, 2004; Wei, 2004; Liu *et al.*, 2007). It has been proposed that when a new *Pestalotiopsis* species is described, morphological characters should be taken into account rather than host association and molecular phylogenetic information is also necessary to prove that the taxon is unique from other known species (Jeewon *et al.*, 2004; Wei and Xu 2004, Liu *et al.*, 2007).

As part of an ongoing study on the fungal diversity on different hosts from India, we have isolated the two fungal species from different host. These fungi have been identified as genus *Pestalotiopsis*. But both the fungi are differing in some of the characters from the reported species.

MATERIALS AND METHODS

Sources of fungal species : These two species were collected from one infected mango leaf (*Mangifera indica* L.) and another from termite nest in the tea agro-ecosystem.

Isolation and sporulation : The surface of leaf samples was washed with running tap water and sterilized with 75% ethanol (60 seconds), 1:3 dilutions of water and NaClO (5 minutes) and 75% ethanol (30 seconds) (Zheng and Jiang, 1995). Samples were washed three times with sterilized water, cut into about 0.5-0.6 cm segments and transferred to PDA medium in Petri-dishes. For the second species, 1 g termite nest soil was taken and isolation was done by soil plate method (Warcup, 1950). Plates were incubated at 25 ^{0}C for 3-20 days and checked regularly. When mycelial growth and spores were observed, further isolation were carried out by hyphal tipping and single spore isolation following the methods as outlined by Choi *et al.* (1999) and Lacap *et al.* (2003) and their morphological characters were observed. The morphology was compared with other species based on literature (Guba, 1971).

DNA extraction, PCR amplification and DNA sequencing : For the molecular analysis, the total genomic DNA was extracted from fresh fungal mycelia following a modified protocol of Doyle and Doyle (1987). The ITS region was amplified by PCR, using primers ITS 1 and ITS 4 (White *et al.*, 1990). The PCR reactions were performed in a volume of 50 µl under standard conditions (Innis et al., 1990). Automated sequencing of the purified PCR products was performed in both directions using the ITS 1 and ITS 4 primers on a 3100-Avant Genetic Analyzer. Nucleotide sequences were edited with the SEQMAN II v. 3.61 programs from the DNASTAR software package (LASERGEN).

ITS region analysis : Totally 27 *Pestalotiopsis* strains belonging to different species were used for phylogenetic analysis of ITS region (Tables 1). The sequences were aligned with Clustal X software (Thompson *et al.*, 1997) and the results were adjusted manually where necessary to maximize alignment. The alignment data were subsequently used for maximum-parsimony (MP) analysis, in which searches for most parsimonious trees were conducted with Mega 4.1 (Tamura *et al.*, 2007). For each search, 1000 replicates of random stepwise sequence addition were performed and 100 trees were saved per replicate. Gaps were treated as missing data. Homologous sequence positions were treated as a discrete character with four possible unordered states (A, G, C, or T), and equally weighted parsimony (with a transition:tranversion ratio of 1:1) was included in the parsimony analysis. Optimal trees were identified using heuristic searches based on 1000 random addition replicates retaining clades compatible with the 50% majority-rule in the bootstrap consensus tree.

RESULTS

Morphology : Both the new fungi viz. *P. anacardii* and *P. termitarii* were producing black, carbonaceous acervuli. Conidia were fusiform, straight to curved, 4-5 celled, crowned with situlae. Exterior cells hyaline, intermediate cells are variably coloured (pale brown to black). Simple and hyaline pedicels present, attached to the base of the conidia. On the basis of literature, both the fungi were identified as genus *Pestalotiopsis*. But both the fungi were differing in some of the characters from the reported species.

Table 1: List of fungi with their host, habitat and accession number used in this study.

Species GenBank	Source of Culture	Host/ Geographic Origin/Disease	Accession No
Pestalotiopsis adusta	ICMP 5434	*Digitalis purpurea*, New Zealand, leaf spot	AF409955
Pestalotiopsis aquatica	HKUCC 8311	*Leucospermum* sp., S. Africa, leaf spot	AF409956
Pestalotiopsis bicilia	BRIP 25718	*Xanthorrhoea* sp., Australia, leaf spot	AF409973
Pestalotiopsis dichaeta	BRIP 25627	*Bletia* sp., Australia, Grey Blight	AF409987
Pestalotiopsis funerea	ICMP 7314	*Cupressocyparis leylandii*, New Zealand, Canker	AF405299
Pestalotiopsis gracilis	HKUCC 8320	*Scaevola hainanensis*, Hong Kong, China, NA	AF409962
Pestalotiopsis longisetula	STE-U 1771	*Leucospermum* sp., Cecily, Leaf spot	AF409971
Pestalotiopsis sinensis	BRIP 25617	*Chrysalidocarpus lutescens*,	AF409966
Pestalotiopsis	HKUCC 8326	Australia, NA *sydowiana Protea mellifera*, S. Africa, NA	AF409970
Pestalotiopsis theae	HKUCC 7982	*Protea mellifera*, S. Africa, Na	AF405297
Pestalotiopsis uvicola	BRIP 25613	*Verticordia* sp., Australia, Collar rot	AF409994
Pestalotiopsis versicolor	BRIP 14534	*Psidium guajava*, Australia	AF405298
Pestalotiopsis virginiana	HKUCC 8380	*Polygonum multiflorum*, HongKong, NA	AF409959
Pestalotiopsis vismiae	HKUCC 8328	*Leucospermum* sp., Hawaii, USA, leaf spot	AF409977
Pestalotiopsis sp. EN2	HKUCC 8370	*Scaevola hainanensis*, Hong Kong, China, NA	AF409964
Pestalotiopsis sp. EN4	HKUCC 8372	*Scaevola hainanensis*, Hong Kong, China, NA	AF409983
Pestalotiopsis sp. 4	BRIP 25624	*Nepenthes khasiana*, Australia	AF409989
Pestalotiopsis sp. EN5	HKUCC 8373	*Scaevola hainanensis*, Hong Kong, China, NA	AF409960
Pestalotiopsis sp. EN6	HKUCC 8374	*Scaevola hainanensis*, Hong Kong, China, NA	AF409981
Pestalotiopsis sp. 7	HKUCC 8325	*Leucospermum* sp., S. Africa,NA	AF409979
Pestalotiopsis sp. EN7	HKUCC 8375	*Scaevola hainanensis*, Hong Kong, China, NA	AF409982
Pestalotiopsis sp. 8	HKUCC 8324	*Leucospermum* sp., S. Africa, leaf spot	AF409961
Pestalotiopsis sp. EN8	HKUCC 7984	*Scaevola hainanensis*,Hong Kong, China, NA	AF405294
Species GenBank	Source of Culture	Host/ Geographic Origin/Disease	Accession No
Pestalotiopsis sp. 9	STE-U 1755	*Leucospermum* sp., S. Africa,leaf spot	AF40998
Pestalotiopsis sp. EN9	HKUCC 8319	*Scaevola hainanensis*, Hong Kong, China, NA	AF409963
Pestalotiopsis sp. EN10	HKUCC 7985	*Scaevola hainanensis*, Hong Kong, China, NA	AF409978
Pestalotiopsis sp. EN 14	HKUCC 3183	*Kandelia candel*, Hong Kong,China, NA	AF409965

The new species *Pestalotiopsis anacardii* isolated from the leaves of mango are similar to *Pestalotiopsis albomaculans, P. mangiferae, P. virgatula, P. crassiuscula, P. photiniae* and *P. anacardii* (Table 2). However, *P. anacardii* has a large conidium length/width ratio as compared to other similar species and can also be distinguished on the basis of number of setulae (2-3) and size of setulae (20-33µ).

The other new fungus *Pestalotiopsis termitarii* isolated from termite nest differs from the similar species of *Pestalotiopsis* viz. *Pestalotiopsis versicolar, P. microspora, P. sydowiana* and *P. antennaeformis* (Table 3) in having 4-5 no.of cells, 15-25µ size setulae, 2 darker and 1 pale intermediate cells and the conidial size of 15-35x6-11µ.

Table 2: Morphological characteristics of *Pestalotiopsis anacardii* compared with similar *Pestalotiopsis* species

Species	No. of cells	Median cells	Conidial size	No. of Setulae	Setulae size
Pestalotiopsis albomaculans	5	Upper two dark, Lowerone umber	17-20 X7.5-9 µm	3	12-22 µm
P. mangiferae	5	Upper two dark, Lower one umber	22-26 X 8-11 µm	3	19-26 µm
P. virgatula	5	Upper two dark, Lowerone umber	21-24 X 6-8 µm	2	8-10 µm
P. crassiuscula	5	Upper two umber, Lower one olivaceous	22-26 X 7-9 µm	2-3	13-32 µm
P. photiniae	5	Three intermediate cell dark	20-24 X 7-8 µm	3	17-34 µm
P. anacardii	5	Upper two dark, Lower one umber	24-36 X 7-11 µm	2-3	20-33 µm

The unit of length and width of conidia and apical appendages is µm.

Table 3: Morphological characteristics of *Pestalotiopsis termitarii* compared with similar *Pestalotiopsis* species

Species	No. of cells	Median cells	Conidial size	No. of Setulae	Setulae size
Pestalotiopsis versicolar	5	Upper two dark, Lower one olivaceous	22-27 X 7.5-9.5 µm	3-4	17-27 µm
P. microspora	5	Median three cells olivaceous	19-24 X 5-7 µm	3	3-15 µm
P. sydowiana	5	Upper two dark, Lower one olivaceous	23-29 X 8-9.5 µm	3-4	23-40 µm
P. virgatula	5	Upper two dark, Lower one umber	21-24 X 6-8 µm	2	8 10 µm
P. antennaeformis	5	Upper two dark, Lower one umber	21-36 X 6.5-9 µm	2-4 knob-bed	13-50 µm
P. termitarii	4-5	Upper two dark, Lower one umber	15-35 X 6-11 µm	2-3	15-25 µm

The unit of length and width of conidia and apical appendages is µm.

Phylogenetic analyses : The ITS dataset of 29 taxa resulted in data matrix of 484 sites. Maximum-Parsimony analysis yielded 47 most parsimonious trees with tree length (TL) 330 steps, consistency index (CI) 0.762500, retention index (RI) 0.953995 and composite index 0.899068. The strict consensus tree was constructed (Fig. 1) in which *Pestalotiopsis anacardii* and *P. termitarii* did not cluster together with any references.

The ITS sequence obtained from *P. anacardii* and *P. termitarii*, consisting of the two spacer, have size of 530 and 509 bp. The results of the ITS region sequence similarity comparisons showed that *P. anacardii* had similarities with *P. albomaculans, P. palmarum, P. crassiuscula, P. photiniae* and *P. virgatula* (92-93%). *Pestalotiopsis termitarii* had also similarities with *Pestalotiopsis versicolar, P. microspora, P. sydowiana* and *P. virgatula* (90-91%). Molecular results support that *Pestalotiopsis anacardii* and *P. termitarii* are new species which are distinguished from *P. karstenii, P. heterocornis* and other *Pestalotiopsis* species.

TAXONOMY

***Pestalotiopsis anacardii* Kamil *et al.* sp. nov. Fig.2**

Myco Bank: **MB 518688**

Gen Bank: **HM852518**

Etymology: Referring to the family of the host on which the species inhabit.

Fungus, grown on mango leaves, with acervuli forming on mango which are erumpent, pustule-like, unilocular, oval or irregular in outline. *Conidiogenous cells* discrete or integrated, lageniform to ampulliform or subcylindrical, colorless, smooth-walled, 13-23.4 x 2.3-5.2 mm with up to 3 proliferations. *Conidia* fusiform, long cylindri-

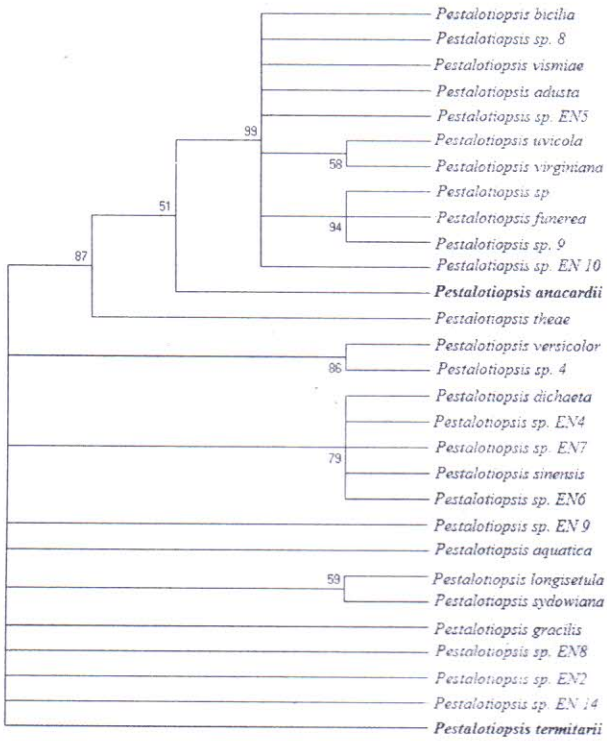

Fig 1. Strict consensus tree of 47equally parsimonious trees generated from the ITS region (ITS1, 5.8S and ITS2) sequences of 29 strains showing the relationship of *Pestalotiopsis anacardii* and *P. termitarii* with reference taxa. The tree rooted with *Bartalinia robillardoides* (TL = 330, CI 0.762500, RI = 0.953995, Composite index = 0.899068). Bootstrap values greater than or equal to 50% are shown at branches.

cal, 5 septate, 24-36 x 7-11 mm; 3 intermediate coloured cells strongly contrasted, the upper two of them fuliginous, the lowest coloured cell light brown, together 15-28 mm long; apical cell conic,

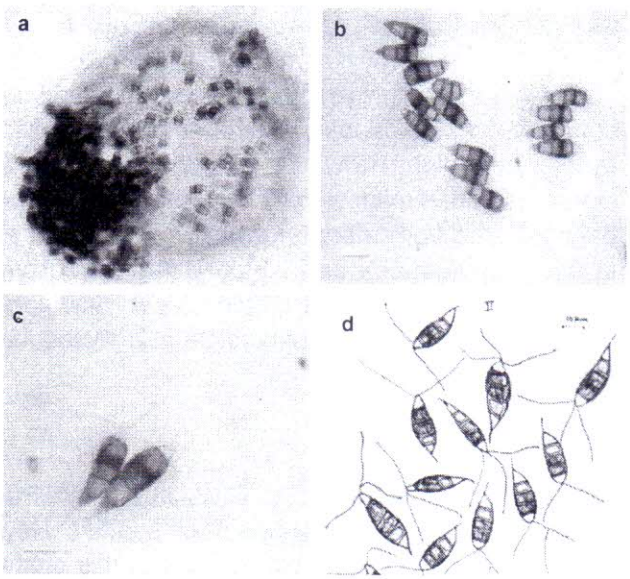

Fig. 2. *Pestalotiopsis anacardii* **a.** Pseudopycnidia, **b, c.** Conidia on potato dextrose agar. **d.** Camera lucida drawing of conidia. Bars: b=10 µm, c= 20 µm, and d= 10 µm.

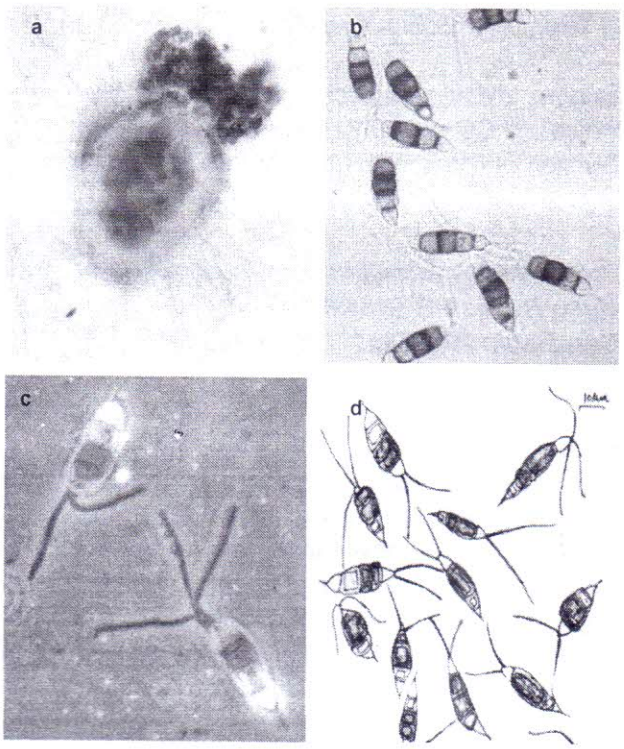

Fig. 3. *Pestalotiopsis termitarii* **a.** Pseudopycnidia, **b, c.** Conidia on potato dextrose agar. **d.** Camera lucida drawing of conidia. Bars: b=10 µm, c,d = 20 µm, and e = 10 µm.

coloureless, smooth, 3.3-6.5 mm long; basal cell obconical, smooth-walled, coloureless, 5.9-10.4 mm long; apical appendages tubular, unbranched, 2-3 in no., 20-33 mm long; basal appendage centric, 6-8 mm long.

Fungus acerval erumpens, pustula, monoloculus, pralis vel inaequalis in ambitus. Cellulae conidiogenae in conidiophorus discretus, lageniformis ad. Ampulliformis Cylindricus,yaline, glabro-tunicatus, 13-23.4 X 2.3-5.2 µm cum ad 3 Proliferus ; conidia fusiformis macro- cylindricus,5 septata. 24-36 X 7-11 µm;3 cellulae- mediae coloratae, super two fuligineus, infirmus cella clarus brunneae, 15-28 µm longi ; cellulae apicalis conicus, hyaline, glaber, 3.3-6. 5 µm longi; cellulae basales obcounicus, glabro- tuni catus, hyaline, 5.9-10.4 µm longi; apicalis setulis 2-3, tubularis, disbicornis, 20-33 µm longis; basales pediceli 6-8 µm longis.

Colony on PDA is white in colour, cottony, fruitbodies ink-like, more or less gregarious, reverse of the culture yellow white.

Habitat/Distribution: Known to inhabit living leaf

of *Mangifera indica*, Gujarat, India.

Extype: INDIA, Gujarat, endophyte of *Mangifera indica*, (ITCC no. 6524), (deposited in the ITCC, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India).

Pestalotiopsis termitarii Kamil et al. sp. nov. Fig. 3
Myco Bank: MB 518696.
Gen Bank: HM852517

Fungus, grown on termite mounds, with fruiting bodies (simple acervuli) black and carbonaceous, is found in tea agro-ecosystem. *Conidiogenous cells* discrete or integrated, lageniform to ampulliform or subcylindrical, colourless, smooth-walled, 13-23.4 × 2.3-5.2 µm with up to 3 proliferations. *Conidia* fusiform, long cylindrical, 4- 5 septate, 15-35 X 6-11 µm; 3 intermediate coloured cells strongly contrasted, the upper two of them fuliginous, the lowest coloured cell light brown, gattulate, second cell 7-13 µm long; apical cell conic, colourless, smooth, 3.3-6.5 µm long; basal cell obconical, smooth-walled, colorless, 5.9-10.4 µm long; apical appendages tubular, unbranched, 2-3 in no., 15-25 µm long; basal appendage centric, 5-7 µm long.

Fungus a cervuv erumpens, pustula, mmloculus, oralis vel inaequalis in ambitus. Cellulae conidiogenna in conidiophores discretus, lageniformis ad. Ampullifomis Cylindricus, hyaline, glabro-tunicatus, 13-23.4 X 2.3-5.2 µm cum ad 3 Proliferus; conidia fusiformis macro- cylindricus,4-5 septata. 15-35 X6-11 µm;3 cellulae- mediae coloratae, super tm fuligineus, infirmus cella clarus brunneae guttulate, 7-13 µm longi ; cellulae apicalis conicus, hyaline, glaber, 3.3-6.5 µm longi; cellulae basales obcunicus, glabro- tni catus, hyaline, 5.9-10.4 µm longi; apicalis setulis 2-3, tubularis, disbicornis, 15-25 µm longis; basales pediceli 5-7 µm longis.

Colony on PDA is white in colour, cottony, fruitbodies ink-like, more or less gregarious, reverse of the culture yellow white.

Habitat/Distribution: Known to inhabit assaprophyte on termite nest in the tea agro-ecosystem, Assam, India.

Extype : INDIA, Assam, saprophyte on termite nest in the tea agro-ecosystem, (ITCC no. 6233), (deposited in the ITCC, Division of Plant Pathology, Indian Agricultural Research Institute, INRI, New Delhi, India).

DISCUSSION

On the basis of phylogenetic analyses *Pestalotiopsis* species can be divided into two groups corresponding to their morphological characters. In first group the median colourous cells are umber to fuliginous, but in second group median colourous cells are brown to olivaceous. Although *P. anacardii* and *P. termitarii* are similar in some morphological characters, they belong to two different groups on the gene phylogenetic trees.

Griffiths and Swart (1974) recognized that differences in pigmentation of median cells were of taxonomic significance. This corroborated with the results of the Sutton (1961). However, in other studies, pigmentation of the median cells was shown to be unreliable for differentiating certain *Pestalotiopsis* species and argued that colour contrast of median cells is not a dependable character (Purohit and Bilgrami, 1968). Purohit and Bilgrami (1968) suggested that this genus should be studied under uniform conditions.

In our study, all the *Pestalotiopsis* strains tested for phylogenetic analyses based on ITS region sequences support pigmentation of median cells as an important taxonomic character in *Pestalotiopsis* (Jeewon *et al.* 2003; Wei *et al.*, 2005; Liu *et al.*, 2007). This result also demonstrated that endophytic *Pestalotiopsis* species are not specific to their host plant. However, all the *Pestalotiopsis* spp. which are similar to the new species, have not been isolated from mango leaves and termite nest in our investigations and which is reported from these host, those are not matching with new species on the basis of morphological and molecular analysis.

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