Leptoxyphium kurandae - New record of insect gut associated sooty mould fungus from India

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Abstract
During a survey to study insect associated fungi, a sooty mould fungus, Leptoxyphium kurandae found in a gut of insect (Dusky Cotton bug) from Western Ghats, Junnar, India was isolated. It is characterized by elongated synnemata consisting of hyphae with bulbous base and at apex an open terminal funnel shaped conidiogenous zone. The hyphae composed of cylindrical cells, constricted at septa and covered with thick mucilaginous layer. Based on morphological characters and sequence comparison of the internal transcribed spacer (ITS) of rDNA and large subunit of ribosome (LSU), the fungus was identified as Leptoxyphium kurandae, a species not previously known from India.

Key words – Dusky cotton bug – ITS – LSU – Synnemata

Introduction
Western Ghats are major diversity hotspot for both flora & fauna. Plant diversification has been implicated as a major factor affecting the diversity of bugs, beetles & other insect groups (Farrell 1998). Insect associated fungi & also their crucial role in nutritional requirement for insect had remained under study (Suh et al. 2005). Due to less studies on insect gut associated fungi, survey was conducted in Western Ghats to isolate fungi. Species of Leptoxyphium causes a kind of sooty mould & belongs to the family Capnodiaeae. They are found to be restricted to the glandular trichomes of leaves. Leptoxyphium occurs worldwide in form of sooty mould & frequently in pure colonies (Hughes 1976). Though Leptoxyphium is plant pathogenic fungi, the association is not observed in insects so far. Recently two new species, Leptoxyphium madagascariense (Cheewangkoon et al. 2009) on leaves of Eucalyptus camaldulensis & L. kurandae (Crous et al. 2011) on leaves of Eucalyptus sp. were described. Literature survey of compilation of Indian fungi shows that so far six species from genus Leptoxyphium are recorded earlier from India which includes L. axillatum (Hughes 1976) on leaves of Albizia lebbeck (L.); L. bahiense (Batista 1963) on leaves of Gossypium barbadense (L.), G. arboreum (L.), G. hirsutum (L.); L. fumago (Srivastava 1982) on leaves of Kydia pinnata (L.), Ilex diopyrena (L.), Alnus nepalensis (L.), Mangifera indica (L.), Quercus leucotrichophora (L.), Coix lycrima-jobi (L.), Rhododendron arboreum (L.); L. graminum Patouillard (Spegazzini 1918) on Citrus sp.; L. longispora (Jain & Mehta 2004) on leaves of Hibiscus rosa-sinensis (L.) & Leptoxyphium sp. (Spegazzini 1918) on
Crescentia pinnata (L.); L. zeae (Mukerji & Khanna 1983) on leaves of Zea mays (L.) (Mukerji et al 1983, Bilgrami et al 1991, Jain et al. 2004, Jamaluddin et al. 2004). However, L. kurandae is not reported from India. We obtained a culture of Leptoxyphium kurandae from the gut of Dusky Cotton bug (Lygaeidae) which feeds on leaves (Vennila 2007).

Materials & Methods

Isolates and morphology

Bug was collected from leaves directly and transported alive to the laboratory. It was surface disinfected with 95% ethanol and rinsed in 0.7% saline solution before removal of the gut and dissected out aseptically. Gut was suspended in 0.7% saline, homogenized by using pestle which was used as stock. Microbial load was reduced using serial dilution by 10 fold diluting stock solution. One unit of stock was removed and added in next tube containing 9 unit of 0.7% saline called 1st dilution ie. $10^{-1}$ and dilutions are made up to $10^{-4}$ which were then spread on to the 2% PDA plates supplemented by antibiotics. Isolated fungal colonies were purified on 2% PDA plates. Out of these, one was selected for further study on the basis of molecular data. A herbarium specimen was deposited in the Ajrekar Mycological Herbarium (AMH), India (AMH 9616); a culture was deposited at Microbial Culture Collection (MCC), India (MCC 1085) and in Centraalbureau voor Schimmelcultures (CBS), Netherlands (CBS 135836).

For morphotaxonomic studies and photomicrographs, Olympus BX53 microscope (Olympus, Japan) was used fitted with ProgRes C5 camera (Jenoptik, USA). For microscopic examination, fungal colony was mounted on glass slides with lactic acid cotton blue (Hi-Media). Measurements of all taxonomically relevant parameters were made by Jenoptik, USA. Colony characteristics in culture were studied on 5 different media: 2% malt extract agar (MEA), potato dextrose agar (PDA), potato carrot agar (PCA) and Czapek dox agar (CDA). To test optimum temperature for growth, culture plates PDA, CDA, MEA, PCA, 2% OA were incubated at 5 – 45 ± 2°C temperatures range.

DNA extraction, PCR and sequencing

Genomic DNA was isolated from fungal mycelium grown on PDA, using QIAamp® DNA Mini Kit (Qiagen, Inc., Valencia CA) as per manufacturer’s instructions and quantified by NanoDrop spectrophotometer (ND-1000, Thermo scientific, USA). Two universal primer pairs ITS1-ITS4 (White et al. 1990) and LR7-5.8S (Vilgalys & Hester 1990) were used separately for amplification of the internal transcribed spacer (ITS1-5.8S-ITS2) region and D1/D2 region of the large subunit (LSU) rRNA gene respectively by using PE 9700 thermocycler (PE Applied Biosystems, Singapore). The PCR was carried out by standard techniques with the following cycling program: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min and hold on 20°C. The purified double-stranded PCR product was used as templates for sequencing with an ABI 3730 xl DNA sequencer using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA). The sequence homology was carried by using BLASTn search to compare it with available sequences of Leptoxyphium in GenBank database (Zhang et al. 2000).

Leptoxyphium kurandae Crous & R.G. Shivisa, Persoonia, 26: 145, 2011

MycoBank MB560176

Optimum temperature for growth was 20–25°C. Mycelium consisting of grey-brown hyphae with 3.6–9.1 μm diam, septate, branched, constricted at septa, forming hyphal ropes, thick-walled, smooth and frequently encased in mucoid sheath. Conidiomata (Fig. 2E) synnematous, separate or in clusters of 2–3, erect, straight to slightly flexuous; bulbous base brown 25.1–39.5 × 25.6–28.1 μm; ($\bar{x} = 32.7 \times 26.47 \mu m$, n = 10), cylindrical part dark olivaceous brown 23.3–67.7 × 11.8–23.3 μm; ($\bar{x} = 44.8 \times 15.5 \mu m$, n = 10), and after 3 months (Fig. 2D) 518.1–1278 × 9.57–12.83 μm; ($\bar{x}$
hyphal apex 22.3–65.7 × 19–68.9 μm; \( \overline{x} = 44.2 \times 38.3 \mu m, n = 10 \), Conidiophores (Fig. 1A-C) subcylindrical to subulate, 0–2-septate, 3.5–29.6 × 1.0–3.3 μm; \( \overline{x} = 15.8 \times 1.8 \mu m, n = 15 \), tightly aggregated in apical part of synnemata. Conidiogenous cells intercalary, terminal, phialidic, 1.4–5.1 × 0.5–2.1 μm; \( \overline{x} = 2.4 \times 1.0 \mu m, n = 10 \), tapering, with parallel to wall and visible collarette. Conidia (Fig. 1D) broadly ellipsoid with rounded ends, 0-1 septate, eguttulate, hyaline, smooth, 4.1–11.7 × 1.3–3.9 μm; \( \overline{x} = 6.8 \times 2.2 \mu m, n = 50 \), aggregating at apex of synnemata in drop of liquid (Fig. 1E).

Sexual stage – Not known

**Fig. 1** – Leptoxyphium kurandae MCC 1085. A: apical structure; B-C: conidiophore & conidiogenous cells; D: conidia; E: conidia produced in drop of liquid at top of synnemata. Bars A-D 20 μm; E 100 μm.

**Fig. 2** – Developmental stages of formation of synnematous conidiophores structure of L. kurandae MCC 1085 in artificial culture media. Bars A-C, E 20 μm; D 100 μm. A: Cells differentiation; B: rearrangement of cells; C: formation of synnematous conidiophore structure; D: Complete developed synnematous structure; E: Phase contrast image of synnematous conidiophores with hyphal apices
Culture characteristics - (25°C in dark, after 2 wks): Colonies were slow growing and spreading, moderate aerial mycelium and even margins, reaching 28 mm diam after 2 wk; on malt extract agar surface olivaceous grey VI’’’i (121), outer region isabelline III’’’i (65), and pale hazel IV’’’i (88) in reverse; on oatmeal agar surface olivaceous grey VI’’’i (121); on potato-dextrose agar surface dark greenish olivaceous IV’’’i (90), grey olivaceous V’’’i (107) in outer region and reverse (Rayner 1970).

Material examined – India, Pune (Western Ghats), Maharashtra, from gut of Dusky Cotton Bug, 10 January 2013, Swapnil Kajale (Holotype, AMH 9616; culture, MCC 1085, CBS 135836). Gene sequences: KF826942 (ITS), KF826943 (LSU)

Notes – The genus Leptoxyphium Speg. typified by Leptoxyphium graminum (Pat.) Speg. mainly characterized by the synnemata arising from helically twisting hyphae or ropes of repent hyphae with terminal conidiogenous zone. Hyphae composed of cylindrical cells & mucilaginous hyphal outer walls (Hughes 1976). This genus has been expanded to include eighteen legitimate species according to mycobank.

Discussion

Based on BLASTn search of NCBI’s GenBank nucleotide database, the closest hits using the ITS & LSU sequences of L. kurandae MCC 1085 are showed in Table 1. The sequences of ITS & LSU domain confirms the closest species in terms of pairwise sequence similarity (using BLASTn search tool) as L. kurandae (99% similarity), whereas the L. madagascariense with 98% similar.

Table 1 - NCBI-BLAST hits (ITS & LSU) against Leptoxyphium kurandae MCC 1085

<table>
<thead>
<tr>
<th>IT S</th>
<th>Similarity (%)</th>
<th>LSU</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptoxyphium kurandae (JF951150)</td>
<td>99</td>
<td>Leptoxyphium kurandae (JF951170)</td>
<td>99</td>
</tr>
<tr>
<td>Leptoxyphium sp. TMS-2011(HQ631026)</td>
<td>99</td>
<td>Microxyphium citri (AY004337)</td>
<td>99</td>
</tr>
<tr>
<td>Leptoxyphium madagascariense (GQ303277)</td>
<td>98</td>
<td>Leptoxyphium fumago (GU214430)</td>
<td>99</td>
</tr>
<tr>
<td>Polychaeton citri (GU214649)</td>
<td>91</td>
<td>Leptoxyphium madagascariense (GQ303308)</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Capnodium coffeae (GU214400)</td>
<td>98</td>
</tr>
</tbody>
</table>

There is variation in morphological characters viz. size of hyphal apex, cylindrical part, conidiogenous cells and also branched synnematous structures showing slight variation in morphological features from L. kurandae. Also the present species is very much different than L. madagascariense (Table 2), but rDNA sequence comparisons (Table 1) showed that our isolate is indeed L. kurandae with 99% similarity. As observed morphologically after 3 months, successive proliferated fructifications were seen of which a length of cylindrical part increased drastically high as 518.1–1278 x 9.57–12.83 μm in pure culture. Also in the old culture multiple secondary synnemata arise from single conidiophore of a conidiomata to form a branched structure (Fig. 2D). No reports were found that insect gut is a habitat of Leptoxyphium other than the insect honeydew (sugar rich liquid). The present fungus might have entered in the gut of bug through the leaves on which it was feeding or living. The role of Leptoxyphium is not known in gut of insect whether pathogenic or symbiotic so far but it might be harmful to insect because of some phenolic compounds produced by Leptoxyphium (Singh et al. 2005). Sequencing of rDNA shows that our isolate is Leptoxyphium kurandae, a species not previously recorded from insect gut. Moreover, there is no previous record of L. kurandae from India. The isolate is reported for the first time from gut of Dusky cotton bug which is a common pest on cotton and leaves (Vennila 2007). Therefore, the present fungus is reported as new record from insect gut and also from India.
Table 2- Comparison of closely identical *Leptoxyphium* species.

<table>
<thead>
<tr>
<th>Parameters</th>
<th><em>L. kurandae</em> MCC 1085</th>
<th><em>L. kurandae</em> CBS 129530</th>
<th><em>L. madagascariense</em> CBS 120605</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conidia</td>
<td>4.1‒11.7 × 1.3‒3.9 μm</td>
<td>(4‒)6‒7(‒9) × 2‒3 μm</td>
<td>4.5‒5 × 3‒3.5 μm</td>
</tr>
<tr>
<td>Conidiophore</td>
<td>3.5‒29.6 × 1.0‒3.3 μm</td>
<td>15‒25 × 2‒3 μm</td>
<td>-</td>
</tr>
<tr>
<td>Conidiogenous cells</td>
<td>1.4‒5.1 × 0.5‒2.1 μm</td>
<td>7‒10 × 2‒2.5 μm</td>
<td>≤1 × 2.8 μm</td>
</tr>
<tr>
<td>Bulbous base</td>
<td>25.1‒39.5 × 25.6‒28.1 μm</td>
<td>30‒50 × 25‒35 μm</td>
<td>-</td>
</tr>
<tr>
<td>Cylindrical part</td>
<td>23.3‒67.7 × 11.8‒23.3 μm</td>
<td>60‒100 × 12‒15 μm</td>
<td>(200‒)250(‒300) × (8‒)10‒12(‒15)μm</td>
</tr>
<tr>
<td>Hyphal apex</td>
<td>22.3‒65.7 × 19‒68.9 μm</td>
<td>30‒50 × 25‒40 μm</td>
<td>35‒50 × 35‒60 μm</td>
</tr>
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</table>

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References


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