Morphology, phylogeny and ex situ conservation of Arthrinium rasikravindrae (Apiosporaceae: Xylariales): a new record from India

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ABSTRACT

This paper deals with identification, characterization and documentation of an interesting isolate exhibiting unique morphological characters on different artificial nutrient media isolated as phylloplane fungus. This isolate was identified based on morphological, cultural, molecular sequence data. Phylogenetic analysis was conducted using ITS region and 28S rDNA gene regions. Results revealed that, the present isolate belongs to the genus Arthrinium and is closely related to Arthrinium rasikravindrae Singh et al. (2012) reported from soil collected from the Arctic Archipelago Svalbard, Norway. To our knowledge, this is the first report of documentation of Arthrinium rasikravindrae isolated as saprophyte from India. As a part of ex situ conservation, this taxon is preserved for long term in National Fungal Culture Collection of India (NFCCI) following cryopreservation method.

Keywords: Arthrinium, biodiversity, conservation, systematics, India, Xylariales

INTRODUCTION

After establishment, the genus Arthrinium Kunze ex Fr. became heterogeneous assemblage due to addition of cryptic species to it and hence taxonomy was revisited from time to time by researchers for resolving the taxonomic ambiguities (Seifert et al., 2011; Crous and Groenewald, 2013). Review of Index Fungorum (2017) reveals the presence of about 70 entries under Arthrinium. They are isolated from widely distributed natural substrates like air, sand, dried or decaying aerial plants, algae, insect gut, home dust, beach sands, etc. (Crous and Groenewald, 2013; Sharma et al., 2012; Réblová et al., 2016; Dai et al., 2016). Many of its species possess biotechnological potential, reported to produce various chemical substances, like extrolites with antimicrobial properties. Some of them exhibit toxicity against pathogenic bacteria, fungi, and human cancer cell lines (Agut and Calvo, 2004; Klemke et al., 2003; Aissaoui et al., 1999) and cutaneous infections in humans (Rai, 1989; Zhao et al., 1990; Hoog et al., 2000).

The present fungus was isolated during the course of selective isolation of unusual or rare fungi, their identification and ex situ conservation from natural substrates (Karandikar et al., 2015; Singh et al., 2015; 2017; Singh and Singh, 2016). A pigment producing colony was found growing on potato dextrose agar without sporulation during initial period. Later on after about 25 days small pin-head like dark crust formation appeared irregularly in plate culture. Its microscopic observation revealed the presence of dark brown lenticular conidial structure having hyaline equatorial germ slit, and several balloon-shaped, anomalous conidia were also observed. Based on these features the isolate was initially accommodated to the genus Arthrinium. Though, a few morphological characters of this isolate showed similarity with A. phaeospermum (Corda) Ellis (Ellis, 1976) while others were different. Therefore, the identity of this isolate was further determined by sequence analyses and phylogeny, as Arthrinium rasikravindrae Singh, Yadav, Singh, Sharma & Singh (Singh et al., 2012).

MATERIALS AND METHODS

Sampling, Selective Isolation and Morphological Characterization: During survey of personal garden at Simbal, Bajnath, Himachal Pradesh, India some chilli plants expressing disease symptoms were collected and brought to laboratory in the form of semi-dried herbarium. The sample was subjected to moist incubation chamber and selective isolation into pure culture, following standard procedures. Briefly, different fungal fruiting structures growing on moist incubated leaf samples were picked up with the help of stereomicroscope (NIKON SMZ1500 aided with Digi-CAM) and inoculated on potato dextrose agar. Simultaneously, leaf was washed with sterile water and inoculated on PDA. The inoculated Petri-plates were kept for incubation at 25°C. After 48 hrs plates were observed regularly for emerging colonies and their selection. Following this procedure, the present isolate together with other selected colonies were transferred to agar slants. Further, comparative study of colony morphology was done on 3-different culture media, malt extract agar (MEA), corn meal agar (CMA) and potato carrot agar (PCA). Methuen handbook of colour was referred for recording colony colours (Kornerup and Wanscher, 1978). Sporulating cultures were identified based on morphology using standard literatures (Ellis, 1971; 1976; Carmichael et al., 1980; Domsch et al., 1980; Larondo and Calvo, 1990; 1992). Photographs and microscopic details were recorded from specimens mounted in lactophenol-cotton blue and distilled water using Carl Zeiss Image Analyzer 2 (Germany) microscope. Fungal structures were measured with software Axiovision Rel. 4.8. A pure culture is deposited and accessioned as NFCCI 4158 in the National Fungal Culture Collection of India (NFCCI-WDCM 932), MACS Agharkar Research Institute, Pune, India.

Ex situ Conservation (long term preservation and maintenance): The pure and identified culture of Arthrinium rasikravindrae NFCCI 4158 has been preserved for long term following different methods like preservation in paraffin oil (Onions and Smith, 1984). In addition, cryopreservation method was used for long term maintenance of pure culture in
liquid nitrogen. Briefly, the selected culture was grown on two different media, PDA and MEA. After appropriate growth quality check was done by slide preparation and microscopy. The 5-mm plugs were cut out with sterilized cork borer and aseptically transferred to already labeled cryovials containing 10% glycerol. Tightly capped cryovials were placed in Nalgene® freeze containers filled with isopropanol. The whole set was kept in -70°C in deep freezer for 4 hrs for freezing of samples (1°C/min). The frozen cryovials containing samples were placed in pre-cooled (-70°C) cryoboxyes, and then transferred to their respective racks. Then loaded racks were finally transferred to cryocan filled with liquid nitrogen (Singh and Baghela, 2017).

**DNA extraction, Amplification and Phylogeny:** Genomic DNA was isolated from pure colony grown on potato dextrose agar plate after 4 days of growth following a simple and rapid DNA extraction protocol (Aamir et al., 2015) using FastPrep® 24 tissue homogenizer (MP Biomedicals GmbH, Germany). The amplification of internal transcribed spacer region 1, 5.8 ribosomal RNA gene and internal transcribed spacer region 2 was achieved using the primers ITS 4: 5’TCC TGC TAT TGA TAT GC 3’ and ITS 5: 5’GGA AGT AAA AGT CGT AAC AAG G 3’ (White et al., 1990). Partial ribosomal nuclear large subunit (nucLSU) was amplified using primers LROR: 5’ACC CGC TGA ACT TAA GC and LR7: 5’ TAC TAC CAC CAAGAT CT 3’ (Vilgalys and Hester, 1990) using Applied Biosystems ProFlex PCR System. PCR was performed in a 25 μl reaction using 2 μl template DNA (10-20 ng), 0.5 U Taq DNA polymerase (Genei, Bangalore, India), 2.5 μl 10X Taq DNA polymerase buffer, 0.5 μl 200 μM of each dNTP (Genei, Bangalore, India), 1μl 10 pmol primer, H₂O (Sterile Ultra Pure Water, Sigma) qsp 25 μl. The thermocycling conditions involved an initial denaturation at 94°C for 4 min, followed by 35 cycles of 1 min at 94°C, 30 sec at 50°C, 1 min at 72°C and final extension at 72°C for 8 min for ITS region where as in case of partial nucLSU conditions involved 5 min denaturation step at 94°C, followed by 30 cycles of 1 min at 94°C, 50s at 52°C and 1.2 min at 72°C with a final 7 min extension step at 72°C. The PCR products were purified with FavorPrep™ PCR Purification Kit. Purified PCR product of these marker genes was subjected to direct sequencing using BigDye® Terminator v3.1 Cycle sequencing Kit and ABI 3100 DNA analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA).

The sequence was analyzed using the gapped BLASTn search algorithm and aligned to the nearest neighbours. Sequences were submitted in NCBI GenBank accession numbers MF461066 (ITS) and MF461172 (LSU). A maximum likelihood tree based on pairwise alignment of sequences was constructed using MEGA 7 with 1000 bootstrap replications (Kumar et al., 2016).

**TAXONOMY**


Conidiophores arising mostly from swollen basal cells (4.0 μm wide), micro to semi-macronematous, mononematous, unbranched, straight to flexuous, smooth-walled, hyaline to sub-hyaline arising from lateral hyphae 7.5-16 × 1-1.75 μm. Conidia variable in shape and size acropleurogenous; lenticular conidia are globose to ovoid in face view, 8.75-13 × 7-12 μm; elongated: cylindrical to clavate conidia, 16-25.75 × 6-12.5 μm, smooth and double walled, brown to pale olivaceous, base truncate with equatorial germ slit.

**Culture characters:** Colonies grew faster at 25°C on MEA, attained a diameter of 55-57 mm after 5-days. The initial colour of colonies was orange grey (5B2) in centre which later turned to pinkish (11A2) and orange grey (5B2) near periphery, velvety, margin irregular, zonate. The colony reverse was greyish brown (11E3) to greyish red (11C5). Colonies grew well at 25°C on CMA, attained a diameter of 45-50 mm after 5-days. The initial colour of colonies was whitish (11A1) turning greyish rose (11B4), floccose, margin irregular. The colony reverse was greyish rose (11B5) to greyish orange (5B3). Colonies grew well at 25°C on PCA, attained a diameter of 44-46 mm after 5-days. The initial colour of colonies was orange grey (5B2), which later turned to greyish red (9B4) and brownish orange (5C3) near periphery, velvety, margin irregular. The colony reverse was dark blonde (5D4).

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Colonies grew faster at 25°C on OMA, attained a diameter of 67-69 after 5-days. The initial colour of colonies was whitish (3A1) to pale yellow (3A3), floccose, margin irregular, producing pale yellow colour (3A2) exudates. The colony reverse was pale yellow (3A3).

**Teleomorph:** Not observed.

**Distribution:** India, Switzerland, China, Netherlands, Thailand, Norway, Brazil and Japan.

**Collection examined:** Himachal Pradesh, Baijnath, Simbal (31.9754 N° 76.6507 E") from phylloplane of Capsicum sp. 18.11.2016, S. Rana, NFCCI 4158, GenBank MF461066 (ITS), MF461172 (LSU).

**DISCUSSION**

Singh et al. (2012) described *Arthrinium rasikravindrii*. Later on, the specific epithet was orthographically corrected to *A. rasikravindriae* (Art. 60C.1 of the code). The current name mentioned in Index Fungorum (http://www.indexfungorum.org) and Mycobank (http://www.mycobank.org/) is being accepted and used. This taxon was described based on the asexual morph from a culture isolated from soil collected in Arctic Archipelago Svalbard, Norway. Micro and macro morphological characters of the present isolate revealed presence of heteromorphic conidia. *Arthrinium rasikravindriae* is characterized by producing lenticular (ovoid) and elongate to equatorial germ slit. Present isolate also produce colour pigment in agar culture at optimal condition of 25°C. Overall morphological characters recorded in present isolate showed similarity with original description of *Arthrinium rasikravindriae* (Singh et al., 2012), except minor differences in dimensions of fruiting structures. Since species level identification is difficult in *Arthrinium* when only the asexual morph is available (Crous and Groenewald, 2013). Also conidial characters are not considered as useful identifying feature due to variation in morphology depending on growth conditions and habitats (Crous and Groenewald, 2013). Therefore, the identity of present isolate was re-confirmed based on sequence analysis and phylogeny.

A BLAST search of ITS sequences via the NCBI database indicated that the ITS sequence of *Arthrinium* isolate NFCCI 4158 is closest to type species, *A. rasikravindriae* (GenBank accession No NR_119932; JF326454), with 99.45% identity (543/546 bp with one gap). Similarly, the LSU sequence of *Arthrinium* isolate NFCCI 4158 showed 99.86% identity (715/716 bp with one gap) to that of *A. rasikravindriae* CBS:337.61 (GenBank Accession No. KF144961). Data available in NCBI GenBank indicates that there are 20 entries on factors, like type of materials, choice of the cryoprotectant, etc. However, success of cryopreservation depends on factors, like type of materials, choice of the cryoprotectant,
cooling and thawing rates, etc. Preservation of cultures between -190 and -196°C either in liquid or vapour phase (of liquid nitrogen) gives excellent results. The cryopreservation method is being practiced at NFCCI as one of the best method of long term preservation of fungal cultures.

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REFERENCES


Table 1: Sequences of Arthrinium rasikravindrae available in NCBI till date.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host</th>
<th>Genbank Access No.</th>
<th>Country of Origin</th>
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<tr>
<td>A. rasikravindrae UASWS1483**</td>
<td>Platanus x acerifolia</td>
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<td></td>
<td>KF722598</td>
<td>Switzerland</td>
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<td>A. rasikravindrae UASWS1478**</td>
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<td>KF722574</td>
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<td>A. rasikravindrae OLC-MBH110096**</td>
<td>Geranium thunbergii</td>
<td>KF290900</td>
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<td>Phyllotoma of Capsicum sp.</td>
<td>MF461132</td>
<td>India</td>
</tr>
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</table>

**SCC codes added as A. phaeospermum in NCBI now designated as A. rasikravindrae (Singh et al. 2012).

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REFERENCES


Fig 2. Molecular phylogenetic tree inferred from the DNA sequence data for ITS of Arthrinium rasikravindrae NFCCI 4158. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was rooted to Arthrinium puccinoides CBS 549.86 (AB220253) and Nigrospora sphaerica 9038 (GQ919077). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Fig 3. Molecular phylogenetic tree inferred from the DNA sequence data for LSU of Arthrinium rasikravindrae NFCCI 4158. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was rooted to Robillarda sessilis CBS 276.78 (KR873286). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.
Africa. Consultative Group on International Agricultural Research, Washington, DC.


Index Fungorum, 2017. www.indexfungorum.org


