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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 posses 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, Baijnath, 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; Larrondo 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) cryoboxes, 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 TCC GCT 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, H_2O (Sterile Ultra Pure Water, Sigma) qsp 25 µl. The thermo-cycling 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 94ºC, 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 FavorPrepTM 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

Arthrinium rasikravindrae S.M. Singh, L.S. Yadav, P.N. Singh, R. Sharma & S.K. Singh, *Mycotaxon* **122**: 449-460, 2012 **(Figs 1-3)**

(≡ *Arthrinium rasikravindrii* Shiv M. Singh, L.S. Yadav, P.N. Singh, Rahul Sharma & S.K. Singh, in Singh *et al*. *Mycotaxon* **122**: 452 (2012).

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).

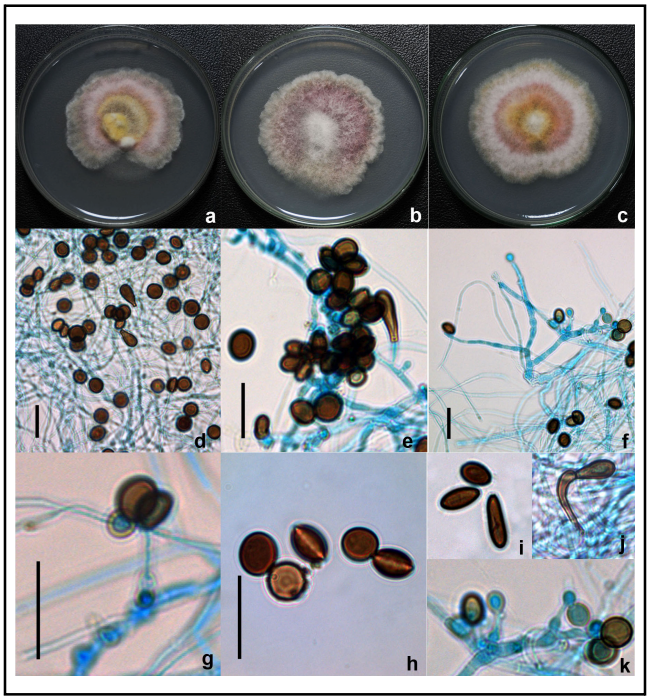

Fig 1. *Arthrinium rasikravindrae* NFCCI 4158. a. Colony on MEA at 25°C, b. Colony on CMA at 25°C, c. Colony on PCA at 25°C, d. Variation in conidial shape, e. Magnified view of globose to elongated conidia, f. conidia attached with conidiogenous cells, g. Terminal conidigenous cells arising from conidiophores, h. Lenticular conidia with germ slits, i. Magnified view of subglobose to elongated conidia, j. Anomalous conidium (cigar shaped), k. Magnified view of condiophores bearing terminal conidiogenous cells and conidia. Scale Bars = 20 µm.

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. rasikravindrae* (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 rasikravindrae* is characterized by producing lenticular (ovoid) and elongate to clavate condia in face view, which are thick/double walled, brown to pale olivaceous having prominent truncate base and 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 rasikravindrae* (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. rasikravindrae* (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. rasikravindrae* CBS:337.61 (GenBank Accession No. KF144961). Data available in NCBI GenBank indicates that there are 20 entries made so far from different hosts and geographical regions in the world, like Switzerland, China, Netherlands, Thailand, Norway, Brazil, Japan, and India (**Table I**).

Phylogenetic Analysis: The ITS and LSU alignments were independently used to confirm species resolution for present isolate. The evolutionary history was inferred by using the maximum likelihood method (MLM) based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood -2147.7851 for ITS and -1607.5240 for LSU are shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

In case of ITS sequences, the rate variation model allowed for some sites to be evolutionarily invariable ([+I], 57.1850% sites). The analysis involved 36 nucleotide sequences. There were a total of 517 positions in the final dataset. In case of LSU discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5321). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 75.8786% sites). The analysis involved 34 nucleotide sequences. There were a total of 712 positions in the final dataset. For both ITS and LSU all positions with less than 80% site coverage were eliminated. That is, fewer than 20% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016) (**Figs. 2 & 3**). Analysis of both the genes placed the present isolate of *Arthrinium* NFCCI 4158 {(GenBank MF461066 (ITS), MF461172 (LSU)}with *Arthrinium rasikravindrae* Singh *et al*. (2012).

Conservation of microbial genetic resources on a sustained basis has become a strategic requirement for supporting basic and applied research. Standard text of CBD also includes microbes as biological matter (CGIAR 2001). Primary objectives of preserving and storing of microbial strains are intended to maintain organisms in a viable state to ensure their morphological, physiological, and genetic stability under laboratory conditions. Various methods are being practiced for long-term preservation and maintenance of these microorganisms to serve various purposes. Several methods with modifications in recipes are in use. Maintenance of fungal cultures in paraffin oil is simple, effective and probably one of the oldest methods for long term preservation. This technique has been modified from time to time to suit the specific requirements. Later on, its effectiveness to very diverse group of organisms, like bacteria, algae, fungi and yeast strains, were studied and reaffirmed (Ajello *et al*., 1951; Annear, 1956). In principle, sterile mineral oil prevents desiccation, and is reported to diminish gas exchange which substantially reduces the metabolism of fungal strains to be stored. By correctly applying this method, culture (s) can be maintained for years together, which may vary from a few weeks to about 14 years, and in exceptional cases, up to 32 years at 15°-20°C, which also facilitates its survival to unusual temperature variations (Cavalcanti, 1991; Silva *et al.*, 1994). Though, this is one of the oldest methods, it is widely accepted for cultures especially not amenable to freezing or freeze drying. Besides, as an advantage this technique is comparatively low-cost and technologically simple and reduces incidence of mite infestations. Though, no single method is complete for preserving all groups of microbes, their cryopreservation below -130°C is generally regarded as safe, barring a few exceptions. However, success of cryopreservation depends on factors, like type of materials, choice of the cryoprotectant,

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

Isolate	Host	Genbank Accesion No.	Country of Origin
A. rasikravindrae UASWS1483**	*Platanus* x *acerifolia*	KT722600	Jussy, Switzerland
A. rasikravindrae UASWS1481**		KT722598	
A. rasikravindrae UASWS1477**		KT722594	
A. rasikravindrae UASWS1470**		KT722587	
A. rasikravindrae OUCMBI110096**	*Sargassum thunbergii*	KP269008	Qingdao, China
A. rasikravindrae OUCMBI110088**		KP269000	
A. rasikravindrae CBS:337.61**	*Cissus* sp.	KF144961	Netherlands
A. rasikravindrae CPC:21602 **	Rice	KF144915	Thailand
A. rasikravindrae NFCCI 2144	Soil	NR_119932	Svalbard: Ny-alesund, Norway
A. rasikravindrae UFMGCB 9620	*Kappaphycus alvarezii*	KX788181	Brazil
A. rasikravindrae	Bamboo	KU872134	Thailand
A. rasikravindrae MFLU 15-1227		KU872133	
A. rasikravindrae MFLUCC 11-0616		KU863132	
A. rasikravindrae MFLUCC 15-0203		KU863131	
A. rasikravindrae MAFF 410785*	Unknown	AB220273	Japan
A. rasikravindrae MAFF 305708*	Unknown	AB220272	Japan
A. rasikravindrae IFO 6575*	Unknown	AB220266	Japan
A. rasikravindrae DLEN2008007*	Unknown	GU266274	Dalian, China
A. rasikravindrae L10-2-2*	*Oryza granulata*	HM008625	China
A. rasikravindrae L3-4-2*	*Oryza granulata*	HM008624	China
A. rasikravindrae NFCCI 4158	Phylloplane of *Capsicum* sp.	MF461172	India
		MF461066	

* Submitted as *A. phaeospermum* in NCBI now designated as *A. rasikravindrae* (Singh *et al.* 2012).
** These entries were made as *A. rasikravindrii* which is corrected here as *A. rasikravindrae* following the correct epithet available in Index Fungorum (www.species fungorum.org).

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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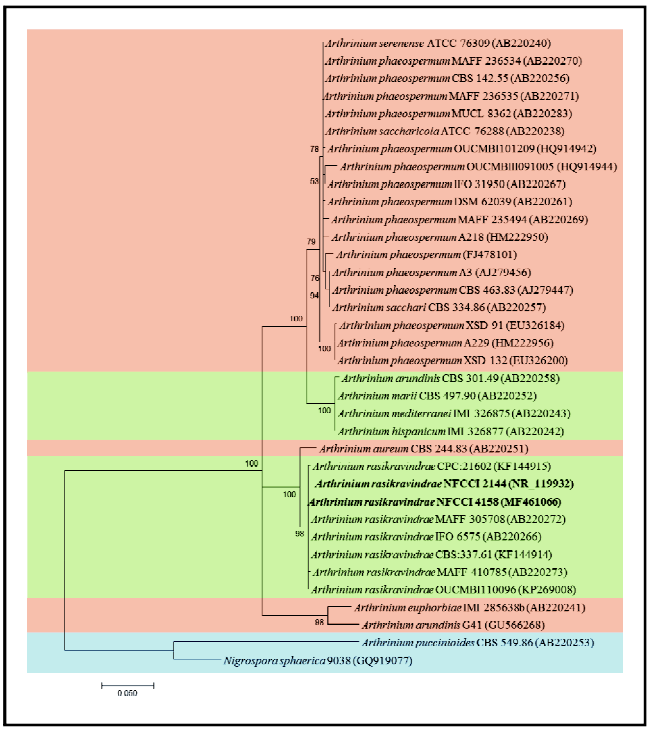

Fig 2. Molecular phylogenetic tree inferred from the DNA sequence data for ITS of *Arthrinium rasikravindrae* NFCCI 4158. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was rooted to *Arthrinium puccinioides* CBS 549.86 (AB220253) and *Nigrospora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

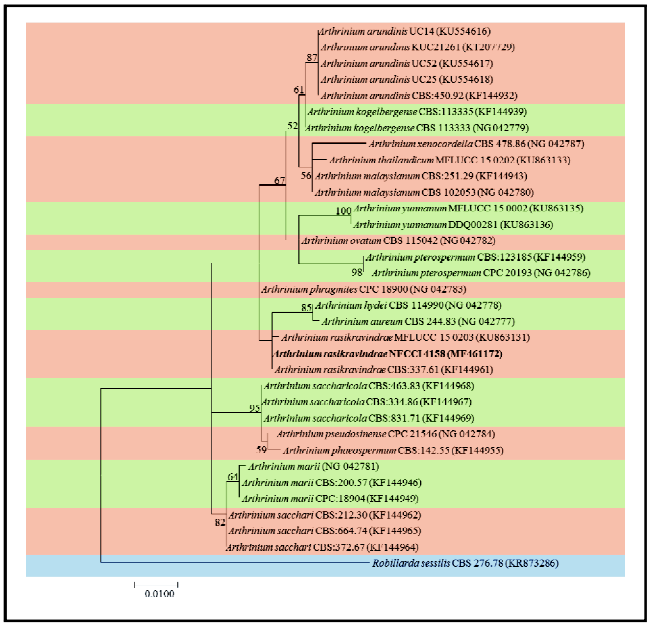

Fig 3. Molecular phylogenetic tree inferred from the DNA sequence data for LSU of *Arthrinium rasikravindrae* NFCCI 4158**.** The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was rooted to *Robillarda sessilis* CBS 276.78 (KR873286). The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

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