

## Molecular phylogeny of endophytic *Dendryphiella*: In quest of finding out ancestor of important rice seed micro-flora

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### ABSTRACT

Thirteen endophytic non sporulating fungi present in healthy seeds of rice cultivars, popular in coastal India, were isolated by 'host tissue transplant' method. Those were identified by the molecular technique and phylogenetic analysis by Phylogeny fr software was done. The phylogeny tree of 32 taxa including 27 *Dendryphiella* species and five *Cochliobolus miyabeanus* spp. was rendered. There were two major groups. Five *Cochliobolus miyabeanus* spp. (out group) were together the smaller cluster whereas all the *Dendryphiella* species with same internal node. The *Dendryphiella* group was further subdivided in two sub groups where 10 marine *Dendryphiella* and 17 terrestrial endophytic *Dendryphiella* grouped in two different sister groups. The high bootstrap values for each subcluster justified their grouping in the proper bunch. This analysis indicated that marine and 'terrestrial endophytic *Dendryphiella*' had common ancestors.

**Key words:** Endophyte; *Dendryphiella*; Molecular Phylogeny; fungal taxonomy; rice seed micro-flora

Endophytes constitute major seed microflora of rice crop. Plant-symbiotic fungi influence the structure and function of all terrestrial ecosystems, but significant portions of endophytic isolates consist of sterile mycelia. Consequently it was difficult to identify the endophyte by traditional morphology based methods. Therefore these important inhabitants of ecosystem were ignored by seed microbiologists in past. Now fungal identification is possible by using ribosomal DNA (rDNA) sequences and this molecular technique has become one of the most useful tools (Crous et al., 2009). It is reported that the endophytes contribute to minimize the effects of biotic and abiotic stresses in ecofriendly manner (Rodriguez et al., 2009; Dhua et al., 2011; Sethi et al., 2015). The term endophyte is applied to fungi which live within plant tissues, for all or part of their life cycle and cause no apparent infections. This definition excludes the mycorrhizal fungi. The aim of this study was to identify the endophytic seed microflora of rice cultivars and examine the taxonomic composition as well as the

evolutionary origin of these fungi. Better understanding of taxonomy and ecology of these important endophytes will enable the ecofriendly management of rice diseases.

### Isolation and identification of endophytic *Dendryphiella* from rice seeds

Thirteen endophytic *Dendryphiella* present in healthy rice seeds were isolated by 'host tissue transplant' method. Cultures were grown on MS broth (Murashige & Skoog, 1962) and DNA was extracted (Dhua et al., 2011). Internal Transcribed Spacer (ITS) region of Ribosomal DNA was amplified. The amplified product(s) were sequenced and the sequence alignment was done for identifying the microbes. The NCBI-GenBank was searched using the Mega-BLAST algorithm for each query sequence and the top hits were recorded. If top hits were unidentified then selection of top 'named species' was done and their FASTA files were retrieved from Gen-Bank database. Bootstrap test was done in MEGA 4 (Tamura and Kumar, 2004; Tamura et al., 2007) and phylogeny was

studied by UPGMA method to identify the query isolate. The nucleotide sequences of identified query culture(s) were submitted to NCBI - GenBank and accession numbers were obtained.

### Phylogeny of endophytic *Dendryphiella*

*Cochliobolus miyabeanus* c.o. brown spot disease of rice (Table 2) was included as an out group while studying the phylogeny of endophytic *Dendryphiella*. The FASTA files of seventeen nucleotide sequences of endophytic *Dendryphiella* associated with rice seeds / other terrestrial plants and ten marine *Dendryphiella* spp. were retrieved from NCBI database (Table 1). The information about the substrate and geographic origin of Marine *Dendryphiella* species included in this study were retrieved from Dissertation of dela Cruz (2006). The molecular phylogeny of 32 taxa including 27 *Dendryphiella* accessions and 5 *Cochliobolus miyabeanus* sequences were analyzed in Phylogeny fr platform.

### Identification of endophytic fungi associated with the rice cultivars grown in Coastal Odisha

It is difficult to identify the non spore producing fungi by traditional morphology based methods. The ITS regions is used in fungal taxonomy because they are fairly diverse and vary between species within the genus (Guo et al., 2003; Crous et al., 2009; Wang et al., 2015). Here *Dendryphiella* was found to be associated with

different rice cultivars. Thirteen isolates of *Dendryphiella* associated with the rice cultivars grown in coastal region of India were identified by molecular techniques during this investigation and their Gen Bank accession numbers were as follows: KJ563122; KJ563118; KC832507; KC832508; KC832509; KJ563119; KC832510; KT582010; KT582011; KT582012; KT582014; HM572292 (Table 1).

*Dendryphiella* is a genus of fungi belonging to the class Dothideomycetes, order Pleosporales and family Pleosporaceae. The *Cochliobolus miyabeanus* and *Alternaria* both belong to the family Pleosporaceae but it is estimated that >14% of GenBank sequences of *Alternaria* species are misclassified (Woudenberg et al., 2013). Hence at the time of identification of endophytic cultures by Mega-BLAST *algorith*m, one or two sequences of *Alternaria* appearing in top 20 hits (showing significant alignment with the query sequences) were not considered during this analysis. *Alternaria* was not included as out group in phylogenetic study also.

### Phylogeny of endophytic *Dendryphiella*

The phylogeny of endophytic *Dendryphiella* from rice seeds was studied to develop the better understanding of their taxonomy and ecology. Phylogeny fr platform which proposes a pipeline already set up to run and connect programs recognized for their accuracy and

**Table 1.** Terrestrial endophytic *Dendryphiella* species included in this study.

<i>Dendryphiella</i> sp.	NCBI-GenBank Accession	Substrate	Geographic origin
FV39-II	KJ563122	Seeds of rice cultivar Mirchamat	India
KARUNA-3	KJ563118	Seeds of rice cultivar Karuna	India
FV9	KC832507	Seeds of rice cultivar Jaifulla	India
FV16	KC832508	Seeds of rice landrace Kharkoili	India
FV22	KC832509	Seeds of rice landrace Chinger	India
Savitri-4	KJ563119	Seeds of rice variety Savitri	India
Swarna-1	KC832510	Seeds of rice variety Swarna	India
crri.1	KT582010	Seeds of rice land race Mayurkantha	India
crri.2	KT582011	Seeds of rice land race Mayurkantha	India
crri.4	KT582012	Seeds of rice var. Ranjeet	India
crri.6	KT582014	Rice seeds	India
crri.34		Seeds of rice land race Champa	India
EN 12	HM572292	Seeds of rice land race Mayurkantha	India
BR354	FJ971840	Seeds of <i>Oryza sativa</i> L. var. KDML105	Thiland
NBRC 100153	DQ307315	Perennial weed <i>Eleocharis kuroguwai</i>	Ohwiin rice eco-system Japan
UOC.PTS.RDWW.12	KT150268	Calamus thwaitesii	Sri Lanka
DX-FOF2	KC871034	<i>Oryza rufipogon</i>	China

**Table 2.** *Cochliobolus miyabeanus* species used in this study as out group.

Strain of <i>Cochliobolus miyabeanus</i>	NCBI-GenBank Accession	Substrate	Geographic origin
Palawan-Bipolar PG10	DQ300199	Rice Leaves infected with brown spot disease	IRRI, Philippines
88	JN093305	Rice Leaves infected with brown spot disease	Jodhpur, India
NBRC 100216	GU222692	Leaf Spot on Switchgrass	Mississippi
Bi-4	JN943401	-	Japan
	JQ517483	Leaf Spot on grass species	Iran

speed. There MUSCLE is for multiple alignment, and alignment curation is done by G blocks.

PhyML is for phylogeny and finally TreeDyn for tree rendering and reconstruction of a robust phylogenetic tree from a set of sequences. The G blocks program eliminated poorly aligned positions and divergent regions. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (Dereeper et al., 2008; Dereeper et al., 2010). The phylogeny tree of 32 Texas including 27 *Dendryphiella* species and five *Cochliobolus miyabeanus* spp. was rendered during this study. There were two major clusters in phylogenetic tree and five *Cochliobolus miyabeanus* spp. as out group (Table 2) clubbed together in the smaller cluster. Whereas, all *Dendryphiella* species had same internal node. Thus clustering of 27 *Dendryphiella* spp. in a different branch corroborates the identity of *Dendryphiella* spp. included here (Fig. 1). This indicated that marine and 'terrestrial endophytic *Dendryphiella*' had common ancestors. The *Dendryphiella* group was further subdivided in to

two sub groups where 10 marine *Dendryphiella* (Table 3) and 17 terrestrial endophytic *Dendryphiella* grouped in two different sister groups. The high bootstrap values for each subcluster justified their grouping in proper bunch (Fig. 1).

The *Dendryphiella* sp. NBRC 100153 was identified and patented by Japanese and it had 94% identity with rice endophytes *Dendryphiella* sp. FV39-II, *Dendryphiella* sp. Karuna-3, *Dendryphiella* sp. BR354, *Dendryphiella* sp. FV9, *Dendryphiella* sp. FV16, *Dendryphiella* sp. FV22, *Dendryphiella* sp. Savitri-4, *Dendryphiella* sp. Sarala-1 and *Dendryphiella* sp. Swarna-1. The query coverage was 92-100% and E value was zero with above mentioned sequences (Table).

Basically the genus *Dendryphiella* is among the most commonly isolated saprobe, cosmopolitan marine fungi having been isolated from soil samples in salt marshes (Pugh, 1962a; Pugh, 1962b; Pugh & Beeftink. 1980) or from living or decaying seaweeds (Genilloud et al., 1994), seagrasses (Newell & Fell,

**Table 3.** Marine *Dendryphiella* species used in this study.

Organism	Strain	NCBI-GenBank Accession	Substrate*	Geographic*origin
<i>Dendryphiella arenaria</i>	TUBS.Den35	DQ307304	unknown substrate	Gozo, Malta (Mediterranean Sea)
<i>Dendryphiella arenaria</i>	TUBS.Den25	DQ307298	<i>Ceramium</i> sp.	Fort de Soto, FL., USA (Gulf of Mexico)
<i>Dendryphiella salina</i>	TUBS 10	DQ307293	<i>L. digitata</i> (drift samples)	Helgoland, Germany (North Sea)
<i>Dendryphiella salina</i>	TUBS Den29	DQ307301	<i>Glaux maritima</i> L. Cuxhaven	Germany (North Sea)
<i>Dendryphiella arenaria</i>	NBRC.32140	DQ307321	driftwood	Teshio, Hokkaido, Japan(Sea of Japan)
<i>Dendryphiella arenaria</i>	TUBS 02	DQ307290	<i>Fucus</i> sp. (dried sample)	Rügen Island, Germany (Baltic Sea)
<i>Dendryphiella arenaria</i>	CBS181.58	DQ411539	coastal sand under <i>Ammophila arenaria</i>	Gironde, France (European Atlantic Coast)
<i>Dendryphiella salina</i>	TUBS 05	DQ307292	<i>P. urceolata</i>	Friedrichsort, Germany (Baltic Sea)
<i>Dendryphiella arenaria</i>	TUBS Den32	DQ307303	unknown substrate	Crete, Greece (Mediterranean Sea)
<i>D. arenaria</i>	TUBS Den26	DQ307299	unknown substrate	Fort de Soto, FL, USA (Gulf of Mexico)

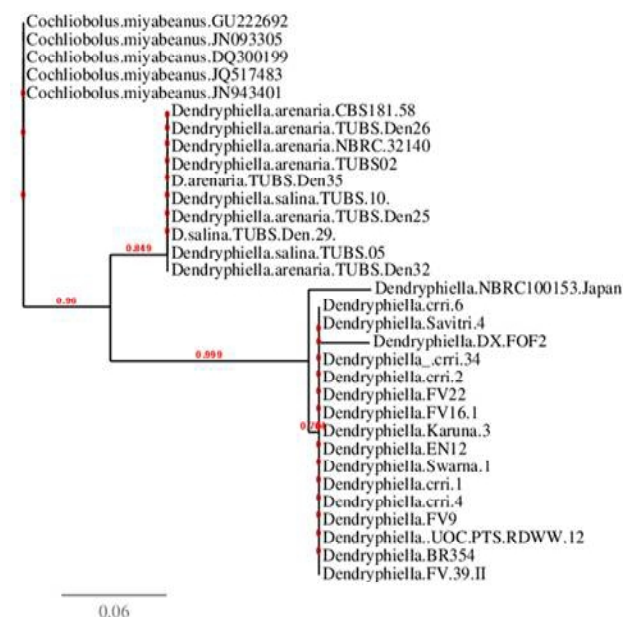
\*Substrate and Geographic origin of Marine *Dendryphiella* species included in this study were retrieved from dela Cruz (2006).

**Table 4.** Database sequences searched by NCBI- Mega BLAST and showing pair-wise similarities with fungal isolate *Dendryphiella* sp. NBRC 100153.

Database sequences showing similarity		Maxi. Score	Total score	Query coverage	E value	Maximum Identity
Accession	Description					
KJ563122	<i>Dendryphiella</i> sp. FV39-II	754	754	100 %	0	94%
KJ563118	<i>Dendryphiella</i> sp. KARUNA-3	749	749	99%	0	94%
FJ971840	<i>Dendryphiella</i> sp. BR354	747	747	99%	0	94%
KC832507	<i>Dendryphiella</i> sp. FV9	745	745	99%	0	94%
KC832508	<i>Dendryphiella</i> sp. FV16	743	743	99%	0	94%
KC832509	<i>Dendryphiella</i> sp. FV22	739	739	99%	0	94%
KJ563119	<i>Dendryphiella</i> sp. Savitri-4	737	737	100%	0	94%
KJ563120	<i>Dendryphiella</i> sp. Sarala-1	730	730	97%	0	94%
KC832510	<i>Dendryphiella</i> sp. Swarna-1	682	682	92%	0	94%

1980; Newell, 1981). The obligate marine fungi grow and produce spores exclusively in estuarine or marine habitats (Kohlmeyer, 1974). Among the most commonly isolated marine fungi are two species belonging to the genus *Dendryphiella* i.e., *D. arenaria* and *D. salina*. Both are known to be of marine origin. However, some of the fungal species from terrestrial or freshwater areas able to grow in estuarine or marine environments were defined as facultative marine fungi (Hyde et al., 2000). The phylogeny studies during this investigation concurred most of the earlier observation as all the accessions of marine *D. salina* and *D. arenaria* clustered together in the phylogenetic tree drawn in

present study. Large-scale shifts in habitat during evolution require populations to respond to new selective pressures. The species of 'terrestrial endophytic' *Dendryphiella* also may be of marine origin. The shifts by microbial symbionts are often associated with bursts of species diversification driven by the exploitation of new adaptive zones (Chaverri and Samuels, 2013). The phylogenetic analysis in this study revealed that endophytic *Dendryphiella* from healthy rice seeds clustered together irrespective of geographic location of their origin. Another attention grabbing finding was that *Dendryphiella* sp. NBRC 100153 though isolated from a perennial weed *Eleocharis kuroguwai* Ohwiin (Patent No. JP8119817-A) found in irrigated rice area, was close to the *Dendryphiella* species from rice seeds. The phylogenetic analysis in present study confirmed that the endophytic fungi present in rice seeds were *Dendryphiella* species. Efficacy of metabolites of 'marine *Dendryphiella*, may be tested against rice pathogens and their effect on rice plants is to be studied to develop strategies / products for ecofriendly management of rice diseases and at the same time harvesting more rice.

**Fig.1.** Phylogeny tree depicting relation between endophytic and marine *Dendryphiella* species.

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