Curr Microbiol (2018) 75:84–91
https://doi.org/10.1007/s00284-017-1353-4

Comparative Study on Whole Genome Sequences of *Aspergillus terreus* (Soil Fungus) and *Diaporthe ampelina* (Endophytic Fungus) with Reference to Lovastatin Production

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Received: 23 August 2016 / Accepted: 31 August 2017 / Published online: 6 September 2017

Abstract Lovastatin is a competitive inhibitor of the enzyme hydroxymethyl glutaryl coenzyme A reductase (HMGR) in cholesterol biosynthetic pathway and hence used in the treatment of hyperlipidemia. In a previous study, we report a tropical soil isolate, *Aspergillus terreus* (KM017963), which produces ample amount of lovastatin than its counterpart that are endophytic in origin. Bioinformatic analysis of whole genome sequence of *A. terreus* (AH007774.1), a soil isolate revealed the presence of gene cluster (AF141924.1 & AF141925.1) responsible for lovastatin production, whereas endophytic fungi including a strain of *A. terreus* showed no homology with the lovastatin gene cluster. The molecular study was also carried out targeting PCR amplification of the two important genes, *lov*E (a regulatory gene) and *lov*F (transcriptional regulatory factor) in genomic and c-DNA of soil and endophytic fungi. Expression of the two genes was successful in *A. terreus* (KM017963), whereas the same was not achieved in endophytic fungi. To further validate our above findings, in the present study, the whole genome sequencing of *A. terreus* and a selected endophytic fungus, *Diaporthe ampelina* (Phomopsis) was performed. Lovastatin gene cluster, when aligned on the consensus sequence of both genomes, the entire lovastatin gene cluster was detected in a single scaffold (1.16) of *A.terreus* genome. On the contrary, there was a complete absence of lovastatin gene cluster in the genome of *D. ampelina* (an endophyte). The probable reasons for the absence of lovastatin gene cluster in endophytic fungi are discussed.

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Introduction

Lovastatin ($C_{24}H_{36}O_5$) is a potent drug which has been largely used for lowering blood cholesterol and it was the first statin drug approved by the United States Food and Drug Administration (USFDA) in 1987. It competitively inhibits the enzyme, hydroxyl methylglutaryl coenzyme A (HMG-CoA) reductase which catalyzes the reduction of HMG-CoA to mevalonate, a rate-limiting step of cholesterol biosynthesis. Lovastatin is produced as a secondary metabolite by several fungi such as *Aspergillus terreus, Penicillium citrinum, Monascus ruber*, and *Pleurotus ostreatus* [29]; however, commercial production of lovastatin is achieved from *Aspergillus terreus* (ATCC 20542) through submerged fermentation [8]. Further, endophytic fungi are also the proven sources of secondary metabolites with pharmaceutical importance. Therefore, they are exploited to grow as axenic cultures on synthetic media under controlled conditions (devoid of interaction with host plant) for the production of commercially valuable secondary metabolites with anticancer, antioxidant, anti-inflammatory, antiparasitic, antiviral, and antimicrobial properties. However, there are very few reports on the production of lovastatin by endophytic fungi [19, 22, 23].

A total of 17 genes together make a complete lovastatin gene cluster that include nonaketide synthase (LNKS by *lov*B), diketide synthase (LDKS by *lov*F), enoyl reductase (*lov*C), transesterase (*lov*D), HMG-CoA reductase (ORF8), regulatory (*lov*E and ORF13), and cytochrome P450 monooxygenase. Only three genes, i.e., *lov*E, *lov*F, and *lov*D are completely sequenced and widely studied amidst other genes of the cluster. Functionality of these genes could have been largely predicted by sequence comparison.

Lovastatin production is chiefly regulated by *lov*E and deletion of the same results in the termination of lovastatin

production, whereas gene duplication increases its yield by 5- to 7-fold [9, 10]. *Lov*E genes isolated from *A. terreus* (CCTCC AF93208) was cloned into the vector pMD-19T (TaKaRa) for the overexpression of lovastatin and sequencing studies confirmed the presence of *lov*E gene in transformants [9]. Thus, gene transfer studies of *lov*E indicate a pivotal role of *lov*E in cross platform gene transfer [2]. Furthermore, as noted, *Lov*F gene encodes an enzyme, LDKS which interacts with the transesterase enzyme (encoded by *lov*D) and catalyzes the attachment of the 2-methylbutyric acid to monacolin J to form the functional lovastatin [7]. Cloning and overexpression of *lov*D into *E.coli* for direct synthesis of simvastatin (a semisynthetic derivative of lovastatin) from monacolin J (a precursor of lovastatin) was also reported [31, 32]. Also, comparative studies on lovastatin production by SmF and SSF have been reported at molecular level in soil fungi [2]. Whole genome sequencing of the strain, *A. terreus* (ATCC-20542), was largely reported for the first time by Askenazi et al. [1].

Our preliminary biochemical and bioinformatic studies revealed that endophytic fungi do not produce lovastatin and as well do not harbor lovastatin genes in its genome, respectively [3, 4, 20]. Further, investigations with 10 endophytic fungi (*Colletotrichum.* sp., *Aspergillus terreus*, *Sordaria.* sp., *Curvularia.* sp, *Pithomyces.* sp., *Pestalotiopsis.* sp., *Phomopsis* sp., *Phoma.* sp., *Podospora* sp., *Botrytis.* sp.,) which included *Diaporthe ampelina (Phomopsis)*, revealed the complete absence of key genes (*lov*E and *lov*F) of lovastatin biosynthetic cluster [4]. For further validation of above results, a whole genome sequencing study was undertaken with *D. ampelina,* representing the endophytic fungi, for comparing with that of a lovastatin-producing soil isolate *A. terreus* (KM017963).

Materials and Methods

Isolation of endophytic fungi

Endophytic fungus, *Diaporthe ampelina* from the plant *Commiphora wightii* and soil fungus *Aspergillus terreus* (KM017963) from the tropical soil collected from Tumkur district, Karnataka, India, were isolated as described previously [20].

Reinfection of Plants with Endophytic Fungi

Seeds of *Commiphora wightii*, Sapindales, and *Burseraceae* (a medicinal plant) were purchased from Dhanvanthrivana, Jnanabharathi campus, Bangalore University, Bangalore, India for reinfection with *D. ampelina*. Reinfection of the plants with isolated endophytic fungus (*D. ampelina*) was performed on the plantlets of *Commiphora wightii* as described previously [4, 17].

Culturing of Fungal Strains

Spore suspensions ($10^7/10^8$ spores/ml) of test organisms [*A. terreus* (KM017963) and *D. ampelina*] were inoculated to 100 ml of Potato Dextrose Broth (PDB). Flasks were kept on orbital shaker at 120 rpm at 30 °C for 5 days followed by extraction of genomic DNA from fungal mycelia [4].

Isolation of Genomic DNA by Cetyltrimethylammonium Bromide (cTAB) Method

One gram of the obtained fungal mycelia was ground in the presence of liquid nitrogen to break the intact cell walls of fungi. The cTAB extraction buffer was added and incubated for 10 min at 65 °C. Separation and precipitation of DNA were performed with phenol:chloroform:isoamylalcohol (25:24:1) and isopropanol, respectively. The obtained DNA was dissolved in 50 µl of molecular grade water and stored at 4 °C [33].

Qualitative and Quantitative Analysis of Genomic DNA

Quality of DNA was checked on 1% agarose gel (loaded 5 µl) for a single intact band. The gel was run at 110 V for 30 min. To determine the DNA quantity, 1 µl of each DNA sample was loaded in Nanodrop-2000 to find A260/280 ratio. Further, the required concentration of DNA for whole genome sequencing was determined using Qubit® 3.0 Fluorometer [27].

Preparation of 2 × 125 HiSeq 2500 library

The paired-end sequencing library was prepared using Illumina TruSeq Nano DNA HT Library Preparation Kit (USA). Genomic DNA (200 ng) was fragmented by Covaris method to generate a mean fragment distribution of 3500 bp. Covaris shearing generates dsDNA fragments with 3′ or 5′ overhangs. The fragments were then subjected to end-repair. This process converts the overhangs resulting from fragmentation into blunt ends using end-repair mix. The 3′ to 5′ exonuclease activity of this mixture removes the 3′ overhangs and the 5′ to 3′ polymerase activity fills in the 5′ overhangs. A single 'A' nucleotide is added to the 3′ ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3′ end of the adapter provides a complementary overhang for ligating

the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation. Indexing adapters were ligated to the ends of the DNA fragments, preparing them for hybridization onto a flow cell. The ligated products were purified using SP beads supplied in the kit. The size-selected product was PCR amplified as described in the kit protocol [27].

Quantity and Quality Check (QC) of Library on Bioanalyzer

The amplified library was analyzed for its Quantity and quality in Bioanalyzer-2100 (Agilent Technologies, USA) using High Sensitivity (HS) DNA chip as per the manufacturer's instructions [27].

Cluster Generation and Sequencing

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyzer profile, library was loaded onto HiSeq 2500 for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions on HiSeq 2500. The kit reagents were used in the binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment [27].

Data generation on HiSeq 2500

The data were generated on HiSeq using 2×125 bp chemistry. The raw reads were filtered using Trimmomatic (v 0.35) with quality value QV > 30 and other contaminants such as sequencing adapters were trimmed [27].

Parameters considered for filtration are as follows:

- Adapter trimming was performed.
- SLIDINGWINDOW: Perform a sliding window trimming of 20 bp, cutting once the average quality within the window falls below a threshold of 30.
- LEADING: Cut bases off the start of a read, if below a threshold quality of 30.
- TRAILING: Cut bases off the end of a read, if below a threshold quality of 25.
- MINLENGTH: Drop the read if it is below 100 bp length.

Mapping Reads to the Reference Genome for *A. terreus*

The *A. terreus* genome and gene information (GFF3 file) were downloaded from Ensembl Fungi database (ftp://ftp.ensemblgenomes.org/pub/fungi/release-30/fasta/aspergillus_terreus).

High quality reads were mapped against *A.terreus* genome with size of 29.3 Mb using BWA mem (Version: 0.7.12-r1039) with default parameters including, minimum seed length: 19, penalty for mismatch: 4 etc.

Mapping was performed in two following steps:

(a) Indexing of reference genome
(b) Aligning filtered reads to the reference index

The alignment was obtained in BAM file format, which was used for further downstream analysis including gene annotation and SNP discovery [27].

Denovo Assembly and Scaffolding for *D.ampelina*

High quality reads were assembled using ABySS (Version: 1.5.2) and SSPACE (Version: 3.0) with default parameter.

Assembly was performed in two following steps:

- *Assembly of High Quality Reads* This step was performed by AbySS assembler to generate contigs
- *Scaffolding of Contigs* This step was performed by SSPACE for scaffolding, in which contigs were processed for gap filling by the reads to improve the assembly and generate scaffolds.

Gene Annotation for *A. terreus*

Genes were obtained from GFF3 annotation file of *A. terreus* (reference genome-ftp://ftp.ensemblgenomes.org/pub/fungi/release-30/fasta/aspergillus_terreus).

Gene Prediction for *D.ampelina*

Genes were predicted from the assembled scaffolds using Agustus (Version: 3.2.1) with default parameters, using species model as *Saccharomyces cerevisiae* S288C [27].

Gene Annotation for *D.ampelina*

Functional annotation of the genes was performed using BLASTx program, which is a part of National Center for Biotechnology Information (NCBI) BLAST-2.3.0+ standalone tool. BLASTx finds the homologous sequences for the genes against NR (non redundant protein database) [27].

Single Nucleotide Polymorphisms (SNPs) Discovery for *A. terreus*

Putative SNPs were called from the mapping file generated by BWA-mem program in BAM format. Standard pipeline of samtools mpileup was used with default parameter, but minimum depth was set to 15 to call SNPs and INDELs [27].

Identification of Lovastatin Biosynthesis Cluster Genes

Lovastatin biosynthesis gene cluster was downloaded from NCBI with accession AF141924.1 and AF141925.1. A total number of 17 genes were present in both the accession numbers. Lovastatin biosynthetic cluster genes were aligned on the scaffolds of the draft genome assembly [27].

Results and Discussion

Lovastatin is an anticholesterolemic drug which is widely used worldwide. Researchers have screened several microbes for the production of lovastatin, a secondary metabolite, from various niches [26]. Although, there are many endophytic fungi that are being exploited for various pharmaceutically valuable secondary metabolites, there are a very few reports of lovastatin being produced by endophytic fungi [19, 22]. A study demonstrated that the endophytic fungi are poor producers of lovastatin since none of the 54 endophytic fungi isolated by us from medicinal plants produced lovastatin even after prolonged growth period [20]. It was also observed that *A. terreus*, isolated as an endophyte, did not produce lovastatin, whereas a soil strain of the same fungus produced significant levels of lovastatin under identical growth conditions [21].

The bioinformatic study of whole genome sequences of endophytic fungi and a soil fungus, *A. terreus* (sequences obtained from NCBI genbank) for the presence of lovastatin gene cluster (AF141924.1&AF141925.1) was carried out. Whole genome sequence of *A. terreus* (AH007774.1), a soil isolate revealed the presence of lovastatin gene cluster showing 100% homology, whereas all other endophytic fungi including a strain of *A.terreus* (NT165934.1) showed no homology with the lovastatin gene cluster [3].

Further, to validate the above in silico result, studies were undertaken at molecular level. The lovastatin gene cluster includes *Lov*E and *lov*F, the two important genes involved in the lovastatin biosynthetic pathway. *Lov*E gene encodes a transcription factor which regulates the expression of genes that involve in the biosynthetic pathway, whereas *lov*F gene encodes a diketide synthase (LDKS), one of the two polyketide synthases in lovastatin biosynthesis. PCR amplification of *Lov*E and *lov*F was performed using genomic DNA and constructed complementary DNA [13, 16, 30] obtained from the lovastatin producing fungus, *A. terreus* (KM017963) and 10 endophytic fungi isolated by us from medicinal plants. Results of this approach yielded amplification products (amplicons) for *lov*E (1512 kb), *lovF* (749 bp), and a fragment of 18S Ribosomal RNA (300 bp, housekeeping gene) with *A. terreus* (KM017963),whereas the similar expression of genes (*Lov*E and *lov*F) was not detected in endophytic fungi (except a fragment of 18S Ribosomal RNA genes). These results clearly indicate that endophytic fungi do not express lovastatin biosynthetic genes that could be due to the absence of genes (*lov*E or *lov*F) in genomic DNA (unpublished data) as well as c-DNA [4] of all the endophytic fungi.

Therefore, results of previous biochemical [20], bioinformatics [3], and molecular level [4] studies categorically concluded that the endophytic fungi may not be the

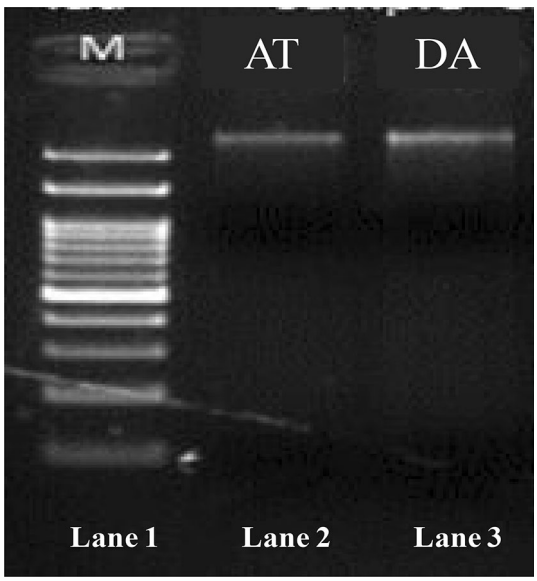

Fig. 1 Agarose (1%) gel electrophoresis of genomic DNA. *Lane 1* (M): marker; *Lane 2* (AT): genomic DNA of *Aspergillus terreus; Lane 3* (DA): genomic DNA of *Diaporthe ampelina*

Table 1 Gene statistics of *Diaporthe ampelina*

Description	*D. ampelina*
Total size of the assembly (bp)	905
Number of Scaffolds	59,496,072
Average size of scaffolds (bp)	65,742
Scaffold N50 (bp)	134,716
Maximum size of scaffold (bp)	796,877
Minimum size of scaffold (bp)	204

potential candidates for lovastatin production. To further confirm the above result, whole genome analysis of a soil fungus, *A. terreus* was carried out and compared with that of an endophytic fungus (*D. ampelina*). The genome of an industrial lovastatin producing *A. terreus* strain (ATCC 20542) is already available in the database; however, genome sequencing was done with *A. terreus,* isolated by us from tropical soil and compared with that of an endophytic fungus (*D. ampelina*) for authentication.

Whole genome sequencing of *A. terreus* and *D. ampelina* (Fig. 1) (with 2 × 125 bp chemistry) was performed using HiSeq 2500. We constructed and sequenced a paired-end library to obtain filtered reads of 20,116,834 (5 GB) for *A. terreus*. The raw data of *A. terreus* contained negligible adapter sequences and phred quality score almost more than 30 for each base in the sequences. The raw data of *D. ampelina* were quality filtered using Trimmomatic-v 0.35 and 17,006,041 high quality reads (4.23 GB) were obtained. The high quality reads were assembled using AbySS (Version:1.5.2) and SSPACE (Version:3.0) as a result, 59 Mb in 905 scaffolds were assembled with N50 of 134,716 bp for *D. ampelina*. For *A. terreus,* the maximum and minimum sizes of genes were 1,430,898 bp and 212 bp, respectively, with average gene length of 1945 bp, whereas for *D. ampelina*, it was 4,886 bp and 201 bp, respectively, with average gene size of 889 bp. The total of 24,672 genes for *D. ampelina* was predicted using Agustus (Version: 3.2.1) and a total of 5202 genes were obtained for *A. terreus* (Tables 1, 2). Reads (91.78%) of *A. terreus* were mapped to the reference genome with 96.88% coverage and a total number of 25,151 SNPs and 2,644 INDELs were discovered using standard pipeline of samtools

Table 2 Gene statistics of *Aspergillus terreus*

Description	*A. terreus*
Number of genes	5202
Average gene length	1945
Maximum gene length	1,430,898
Minimum gene length	212

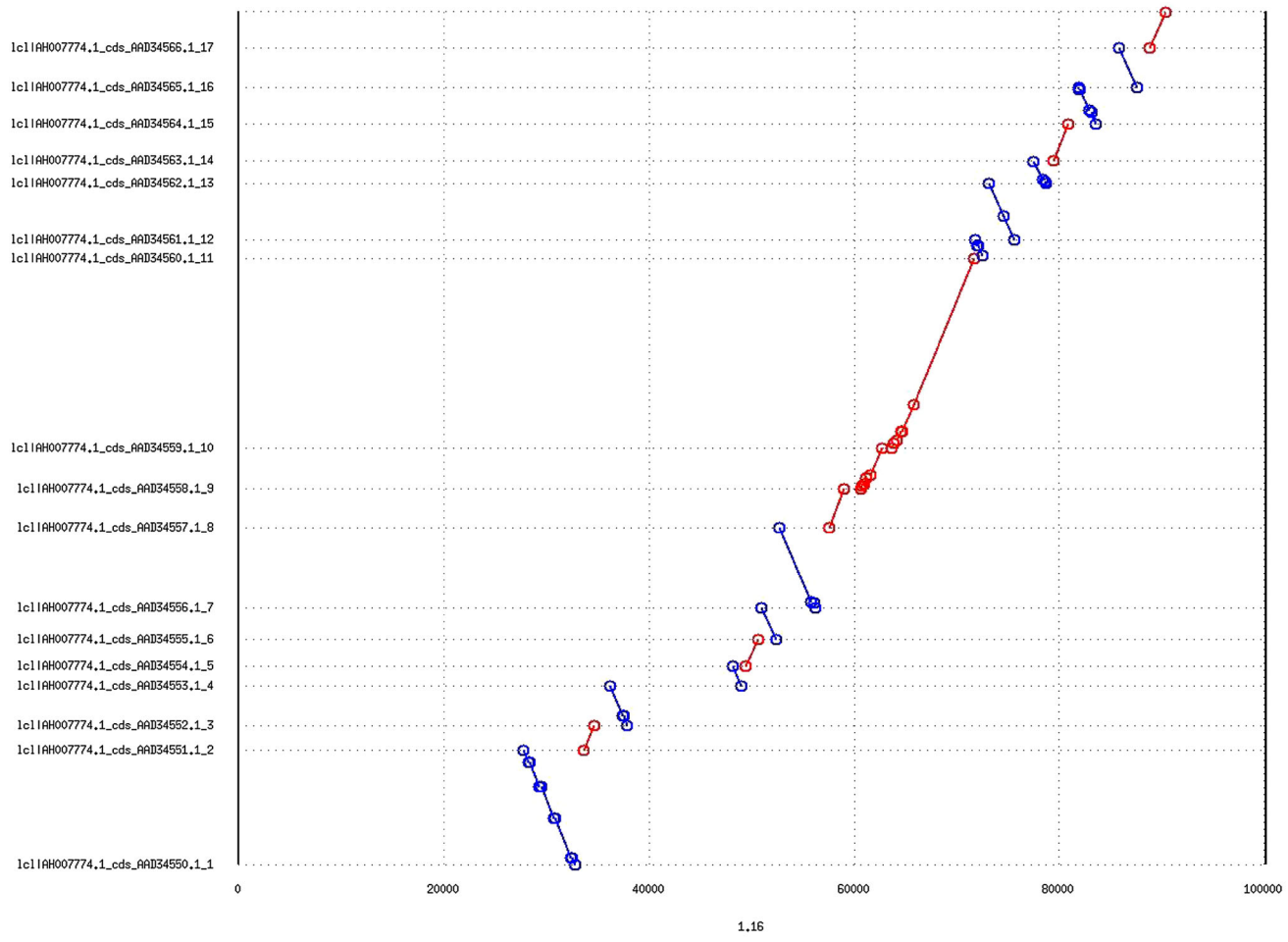

Fig. 2 Dotplot for lovastatin biosynthetic gene cluster mapping against consensus scaffold 1.16

Table 3 Mapping details of lovastatin cluster genes on consensus sequences

Gene accession	Consensus sequence ID	Consensus start	Consensus end	Gene start	Gene end	Identity score (%)
AAD34550.1_1	1.16 lcl	27806	28287	4582	4101	99.17
AAD34550.1_1	1.16 lcl	28356	29319	4102	3139	95.23
AAD34550.1_1	1.16 lcl	29496	30765	3141	1875	95.98
AAD34550.1_1	1.16 lcl	30811	32451	1876	299	94.88
AAD34550.1_1	1.16 lcl	32557	32854	298	1	96.31
AAD34551.1_2	1.16 lcl	33617	34603	1	987	98.28
AAD34552.1_3	1.16 lcl	36186	37467	1587	378	93.21
AAD34552.1_3	1.16 lcl	37540	37918	379	1	98.15
AAD34553.1_4	1.16 lcl	48199	48969	771	1	97.41
AAD34554.1_5	1.16 lcl	49367	50619	1	1092	85.24
AAD34555.1_6	1.16 lcl	50999	52392	1242	1	87.59
AAD34556.1_7	1.16 lcl	52757	55860	3207	239	92.99
AAD34556.1_7	1.16 lcl	56076	56256	181	1	94.48
AAD34557.1_8	1.16 lcl	57546	59057	1	1512	96.3
AAD34558.1_9	1.16 lcl	60624	60777	1	154	96.75
AAD34558.1_9	1.16 lcl	60992	61220	229	457	96.51
AAD34558.1_9	1.16 lcl	61558	62784	556	1629	84.68
AAD34559.1_10	1.16 lcl	63664	63861	1	198	98.48
AAD34559.1_10	1.16 lcl	64218	64552	343	677	97.01
AAD34559.1_10	1.16 lcl	64644	65806	677	1765	88.99
AAD34559.1_10	1.16 lcl	65870	71705	1764	7599	96.71
AAD34560.1_11	1.16 lcl	71771	71996	714	491	91.59
AAD34560.1_11	1.16 lcl	72091	72479	490	102	93.32
AAD34561.1_12	1.16 lcl	73159	74549	2229	951	89.07
AAD34561.1_12	1.16 lcl	74600	75637	952	1	88.82
AAD34562.1_13	1.16 lcl	77487	78403	869	149	77.21
AAD34562.1_13	1.16 lcl	78621	78692	72	1	98.61
AAD34563.1_14	1.16 lcl	79382	80910	1	1473	94.24
AAD34564.1_15	1.16 lcl	81870	81976	1464	1358	96.26
AAD34564.1_15	1.16 lcl	82022	82929	1364	530	89.65
AAD34564.1_15	1.16 lcl	83105	83574	470	1	97.87
AAD34565.1_16	1.16 lcl	85872	87559	1551	1	90.58
AAD34566.1_17	1.16 lcl	88864	90365	1	1445	92.48

mpileup. In the case of *D. ampelina*, out of 24,672 genes, 20,727 genes were annotated, while 3,945 genes were not annotated against NR database during functional annotation which was performed using BLASTx. The annotation resulted in having the highest number of hits on *D. ampelina*. The complete genome sequences of *A. terreus* and *D. ampelina* have been deposited at DDBJ/GenBank/EMBL under accession numbers LWBM00000000 and LWAD00000000, respectively [24, 25].

Lovastatin gene cluster (AF141924.1 and AF141925.1) comprises a total number of 17 genes, out of which 3 genes were present in AF141924.1, while the rest 14 genes were present in AF141925.1. When the consensus sequence was aligned to all 17 genes of the cluster, it was interesting to detect the entire lovastatin gene cluster in a single scaffold (1.16) (Fig. 2). This confirms that there is the presence of complete lovastatin biosynthetic gene cluster in the whole genome of *A. terreus* (Table 3). On the contrary, in the case of *D. ampelina* (an endophyte), none of the genes of lovastatin cluster was aligned on the scaffolds, confirming the complete absence of lovastatin biosynthetic gene cluster in it.

Therefore, our present findings give strong evidence on the absence of lovastatin gene cluster in endophytic fungi although a single isolate was taken for the study. The absence of lovastatin production by endophytic fungi may

be attributed to the loss of those genes responsible for lovastatin during co-evolution along with host plant [6, 14]. Other probable mechanisms that can explain the deletion of some genes and gene clusters responsible for lovastatin production are complex patterns of gene gains and losses, neofunctionalization, incomplete lineage sorting, etc. [15, 27, 32]. All of these can also lead to distinct gene-species evolutionary histories. Also, in plant system, Hydroxy methyl glutaryl CoA reductase plays a vital role in the mevalonate pathway for isoprenoid biosynthesis that are required for normal plant development and adaptation to environmental stress [18]. Mevalonate is the precursor for the synthesis of several important plant growth promoting substances and pigments such as ABA, gibberellins, ubiquinone, isoprenoids, chlorophylls, and carotenoids, if lovastatin inhibits HMG-CoA reductase (HMGR), subsequent synthesis of the above compounds would get inhibited. This prediction was supported long way back by Tatsuo, Joseph and Michael, John et al., Crowell and Salaz [11, 12, 28], that the growth of tobacco cells was inhibited by lovastatin at low concentrations and further restoration of such inhibition by cytokinin [5]. In addition to these, reports also affirm that lovastatin inhibits the biosynthesis of α-Farnesene, a major ester formed during scald development and fruitripening in 'golden supreme' apples [34]. Thus, considering the harmful effects of the fungal secondary metabolite, lovastatin, on plants, it is enticing to hypothesize that the lack of lovastatin production in endophytic fungi is not to harm the plant host system in which they are residing in.

Acknowledgements This work was supported by the SERB, Govt. of India; [Grant Number-DST/SO/FNo.SERB.SR/SO/PS/046/2011]. Authors would also like to acknowledge Prof. T. S. Suryanarayanan, Director, Vivekananda Institute of Tropical Mycology (VINSTROM), A Unit of the Ramakrishna Mission Vidyapith, Chennai 600 004, India for providing few endophytic fungi for the present research work. We also would like to thank Eurofins Genomics, India, for sequencing and bioinformatics analysis of whole genome of both fungi.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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