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Simplicillium lanosoniveum, a hyperparasite on *Aecidium elaeagni-latifoliae* in India

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Abstract A hyperparasite was observed on *Aecidium elaeagni-latifoliae* during a survey in Umiam, Meghalaya. Morphological characterization using light, scanning electron microscopy and molecular characterization by sequencing ITS region, large subunit of nuclear ribosomal DNA and phylogenetic analysis revealed the identity of the pathogen as *Simplicillium lanosoniveum*. This is the first record of hyperparasite *S. lanosoniveum* on *A. elaeagni-latifoliae* from India.

Keywords Simplicillium · Elaeagnus · ITS · LSU · Aecidium

Elaeagnus latifolia is an evergreen woody shrub, common in subtropical and temperate areas and belongs to family Elaeagnaceae. It is mostly grown in semi-wild conditions, fruits are locally known as Soh-Shang and are rich in vitamins A, C and E and contain several compounds which have anticancerous properties. It is considered as having great potential in this region because of its hardy nature and early bearing (Patel et al. 2008).

Rust of *E. latifolia* caused by *Aecidium elaeagni-latifoliae* is an emerging disease in this region. During a regular survey we came across hyperparasitised pustules of rust on the leaves of *E. latifolia* (Fig. 1). Microscopic examination was conducted using light and scanning electron microscopy. Molecular analysis was also done for confirmation. Light microscopy was conducted using an Olympus BX 53 microscope equipped with a digital camera DP 72, Olympus, and image analysis software-cellSens Standard 1.5, Olympus. Observations were made using 3 % potassium hydroxide as mounting medium. Hyperparsitised pustules were selected using a dissecting microscope and placed on double-sided cellotape then sputter-coated with gold under vacuum using Fine Coat Ion Sputter JFC–1100. Gold-coated samples were then placed on aluminium stubs for scanning electron microscopy (SEM) (JEOL JSM 6360, JEOL, Tokyo, Japan). A voucher specimen has been deposited in Ajrekar Mycological Herbarium (AMH- 9654). Microscopic analysis using light and scanning electron microscopy clearly indicated the affinity of the fungus with *Simplicillium lanosoniveum*. Phialides were solitary and conidial dimensions were 2.5 3.5×1 2 μm (Zare and Gams 2004). SEM also revealed, whole rust pustules were covered with the growth of the hyperparasite (Fig 2, 3, 4).

Nested PCR was done using NL1 and LR5 for the first round then NL1 and LR3 for second round for amplification of large subunit (LSU) of nrDNA containing D1 and D2 domains (Vilgalys and Hester 1990). The ITS region comprising ITS1-5.8S-ITS2 was amplified using primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990). PCR was done using initial denaturation for 5 min (94 °C), denaturation 30s (94 °C), annealing 40s (54-50 °C, touchdown with 1 °C decrement in every cycle), extension 1:10s or 50s (depending on the primer combination) (72 °C) and final extension 10 min (72 °C) (35 cycles). Sequencing was done using ITS1F and 4 for ITS region and NL1 and LR3 for LSU region. The sequences of ITS (KJ408447) and LSU (KJ135022) obtained in this study have been deposited in GenBank. Similarity checks were done at NCBI website. For further analysis, sequences of LSU and ITS of closely related sequences were downloaded from NCBI (Table 1, 2). Majority of the sequences included in the analysis were from Nonaka et al. (2013a) and Sung et al. (2001). Alignment was done using Clustal W implemented in MEGA 5.0 (Tamura et al. 2011 and references therein). Model

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Fig. 1 Parasitized and nonparasitized rust pustules on *Elaeagnus latifolia*

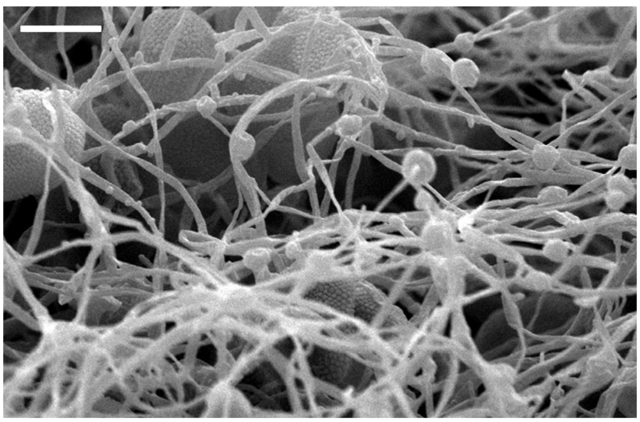

Fig. 2 Rust pustules covered with hyperparasite observed using SEM (Bar=10 μm)

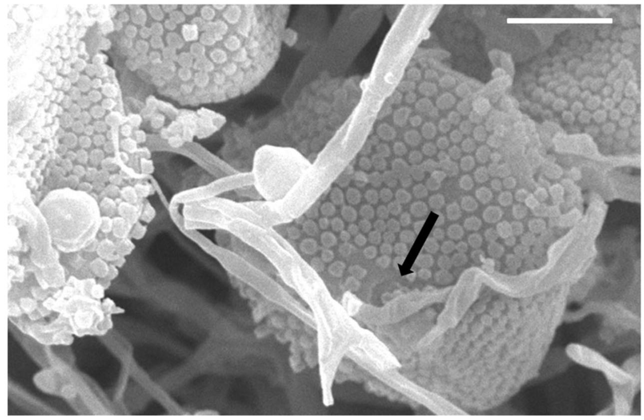

Fig. 4 Parasitized aeciospore of *Aecidium elaeagni-latifoliae* (Bar=5 μm)

Bayesian analysis with LSU sequences recovered the fungus in a well-supported (posterior probability=1.0) clade with species of *Simplicillium*. (Fig. 5). The sequence of *Verticillium dahliae* (VDU17425) was used as root. Species determination selection was done using jModeltest (Posada 2008). Phylogenetic analysis was done using MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001). Parameters used were generations=2,000,000, sampling frequency=100, nst=6, rates=gamma, no of chains set to 4 with two simultaneous runs and burnin=5,000. Tracer was used for convergence analysis (Rambaut and Drummond 2007). FigTree was used for visualising the tree (Rambaut 2012).

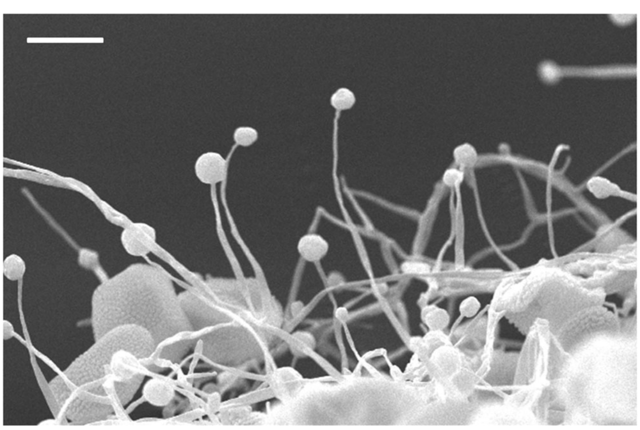

Fig. 3 Solitary phialides of *Simplicillium lanosoniveum* bearing conidia (Bar=10 μm)

Table 1 Large subunit sequences obtained from NCBI

Organism	LSU sequence	Strain	References
Simplicillium chinense	JQ410322	LC1345	Liu and Cai 2012
Simplicillium chinense	JQ410321	LC1342	Liu and Cai 2012
Simplicillium lanosoniveum	KC009174	41559-3	Rajkumar and Chaturvedi 2012 (Unpublished)
Simplicillium obclavatum	JX081385	BAB519_asp18	Raol et al. 2012 (Unpublished)
Simplicillium obclavatum	HQ232175	CBS 311.74	Summerbell et al. 2011
Simplicillium lanosoniveum	HQ232006	CBS 321.72	Summerbell et al. 2011
Simplicillium lanosoniveum	AF339553	CBS 704.86	Sung et al. 2001
Simplicillium lanosoniveum	AF339554	IMI 317442	Sung et al. 2001
Simplicillium lamellicola	AF339552	CBS 116.25	Sung et al. 2001
Simplicillium obclavatum	AF339517	CBS 311.74	Sung et al. 2001
Pochonia chlamydosporia	AF339544	CBS 504.66	Sung et al. 2001
Lecanicillium lecanii	AF339556	CBS 126.27	Sung et al. 2001
Rotiferophthora angustispora	AF339535	CBS 101437	Sung et al. 2001
Pochonia rubescens	AB709833	FKI-5829	Nonaka et al. 2013b
Pochonia rubescens	AB709832	FKI-1083	Nonaka et al. 2013b
Pochonia bulbillosa	AB709809	FKI-4395	Nonaka et al. 2013b
Haptocillium sinense	AF339546	CBS 131.95	Sung et al. 2001
Haptocillium sinense	AF339545	CBS 567.95	Sung et al. 2001
Haptocillium sinense	AF339543	IMI 356051	Sung et al. 2001
Haptocillium sphaerosporum	AF339541	CBS 522.80	Sung et al. 2001
Lecanicillium primulinum	AB712265	JCM 18527	Kaifuchi et al. 2013
Lecanicillium primulinum	AB712264	JCM 18526	Kaifuchi et al. 2013
Lecanicillium attenuatum	AF339565	CBS 402.78	Sung et al. 2001
Lecanicillium psalliotae	AF339560	CBS 532.81	Sung et al. 2001
Verticillium dahliae	VDU17425	ATCC 16535	Rehner and Samuels 1995
Verticillium nigrescens	EF543841	CBS 577.50	Zare et al. 2007
Simplicillium lanosoniveum	HQ232006	CBS 321.72	Summerbell et al. 2011
Simplicillium lanosoniveum	AF339554	IMI 317442	Sung et al. 2001
Simplicillium obclavatum	HQ232175	CBS 311.74	Summerbell et al. 2011
Simplicillium lamellicola	AF339552	CBS 116.25	Sung et al. 2001

Table 2 ITS sequences obtained from NCBI

Organism	ITS sequence	Strain	References	Source
Simplicillium lanosoniveum	EF641862	CBS 962.72	Zare and Gams 2008	-
Simplicillium cylindrosporum	AB604006	FKI-5985	Nonaka et al. 2013a	soil
Simplicillium lanosoniveum	AJ292396	CBS 704.86	Zare et al. 2000	-
Simplicillium subtropicum	AB604001	JCM 18183	Nonaka et al. 2013a	soil
Simplicillium minatense	AB603991	JCM 18177	Nonaka et al. 2013a	soil
Simplicillium obclavatum	AB604000	JCM 18179	Nonaka et al. 2013a	soil
Simplicillium lanosoniveum	EU939525	Cs0701	Chen et al. 2008	*Salvinia molesta* (causing brown spot)
Simplicillium lanosoniveum	EF513003	IMI 303103b	Kouvelis et al. 2008	-
Acremonium obclavatum	AJ292394	CBS 311.74	Zare et al. 2000	-
Simplicillium minatense	AB603992	Type strain	Nonaka et al. 2013a	Soil
Torrubiella luteorostrata	AY624174	CBS 398.86	Luangsaard et al. 2005	-
Verticillium chlamydosporium var. chlamydosporium	AJ292397	CBS 103.65	Zare et al. 2000	-
Simplicillium subtropicum	AB603995	JCM 18181	Nonaka et al. 2013a	Soil
Simplicillium subtropicum	AB603990	JCM 18180	Nonaka et al. 2013a	Soil
Simplicillium aogashimaense	AB604004	JCM 18168	Nonaka et al. 2013a	Soil
Simplicillium aogashimaense	AB604002	JCM 18167	Nonaka et al. 2013a	Soil
Simplicillium sympodiophorum	AB604003	JCM 18184	Nonaka et al. 2013a	Soil
Simplicillium lamellicola	AB378533	KYK00006	Ilyas et al. 2008 (Unpublished)	Coccidae, Hemiptera
Simplicillium lamellicola	AJ292393	CBS 116.25	Zare et al. 2000	-
Simplicillium chinense	JQ410324	LC1345	Liu and Cai 2012	-
Simplicillium chinense	JQ410323	LC1342	Liu and Cai 2012	-
Simplicillium wallacei	EF513022	IMI 331549	Kouvelis et al. 2008	-
Simplicillium wallacei	EF641891	CBS 101237	Zare and Gams 2008	Lepidopteran larva on palm leaf

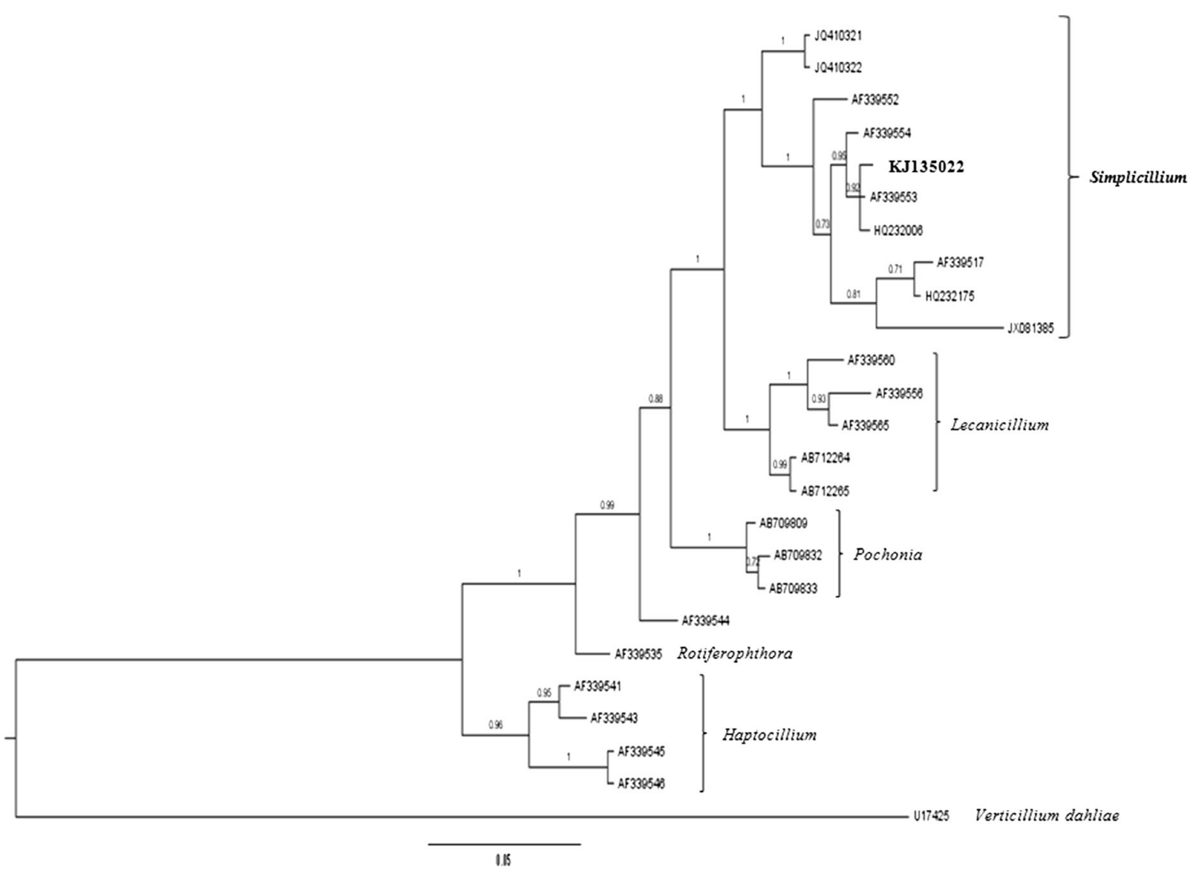

Fig. 5 Phylogenetic analysis based on large subunit sequences

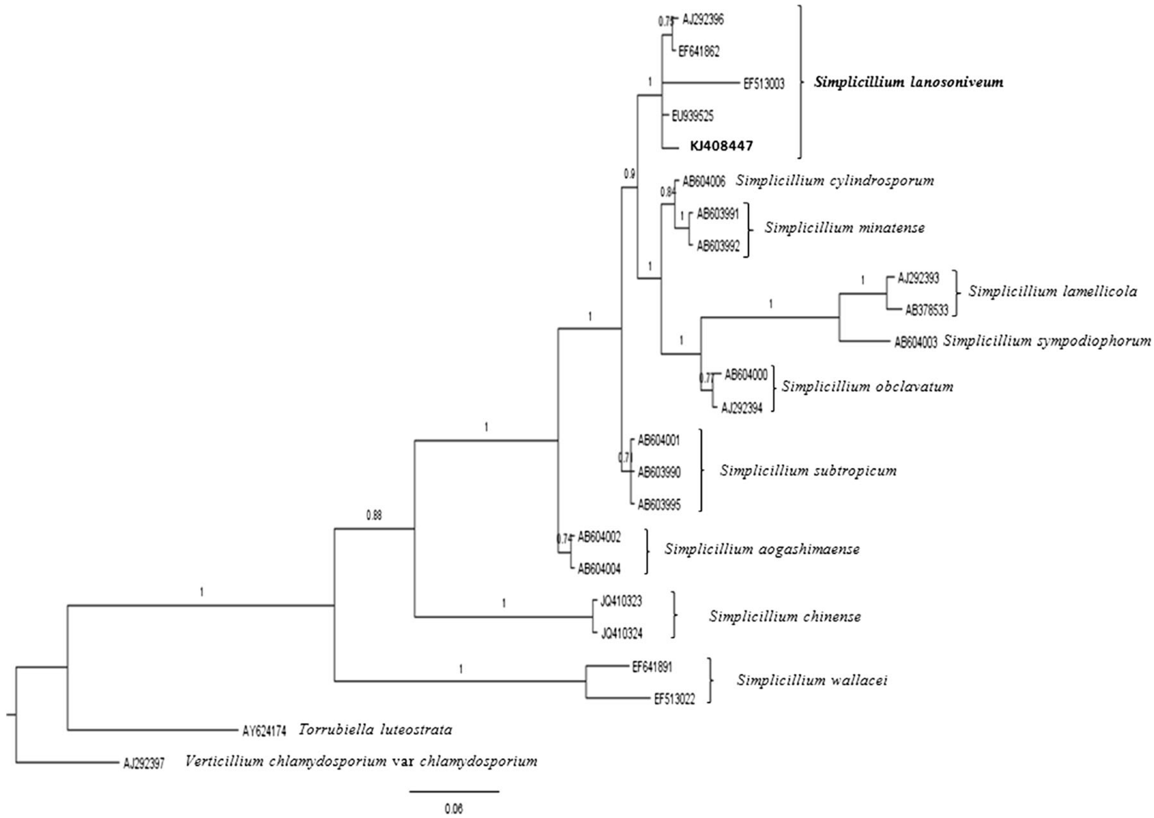

Fig. 6 Phylogenetic analysis based on ITS region

was done by conducting phylogenetic analysis using ITS region and *Verticillium chlamydosporium* var. *chlamydosporium* (=*Pochonia chlamydosporia* var. *chlamydosporia*) (AJ292397) sequence was chosen as outgroup. Results clearly indicated that the fungus belongs to *S. lanosonieveum* since it clustered with *S. lanosonieveum* with posterior probability value of 1.0 (Fig. 6). For ITS region minimum evolution method as implemented in MEGA 5.0 (Tamura et al. 2011 and references therein) was also used and it also clustered our sequence with *S. lanosoniveum* with bootstrap support value of 98 %.

Verticillium was regarded as heterogeneous group having phytopathogenic, fungicolous, entomopathogenic and saprophytic species. The genus was divided into sections *Verticillium*, *Nigrescentia*, *Prostrata* and *Albo-erecta*. Fungicolous and enomopathogenic species of *Verticilillium* were placed in the genera *Simplicillium* and *Lecanicillium* based on a systematic study by Zare and Gams (2004). A phylogenetic concordance method is used for species delimitation in these genera.

So, to our knowledge this is the first record of *S. lanosoniveum* on *A. elaeagni-latifoliae* causing rust of *E. latifolia* in India. *Simplicillium lanosoniveum* has a potential to be used for managing this rust as a sustainable and ecofriendly alternative.

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