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Molecular evidence confirms presence of anamorph of *Erysiphe diffusa* on soybean (*Glycine max*) in northeast India

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Abstract Powdery mildew on soybean was observed in October, 2014. Morphological and molecular characterisation was done for ascertaining the identity of the anamorph. Light and scanning electron microscopy revealed it to be a *Pseudoidium* anamorph. Morphological characters were examined considering the possibility of presence of *Erysiphe glycines*, *E. diffusa* and *E. pisi*. The shape and dimensions of foot cells and conidia, and wrinkling pattern on conidia were the key features for morphological identification of the pathogen. Molecular identification was done using the Internal Transcribed Spacer (ITS) region of ribosomal DNA and the primer sets ITS5 - ITS4 and ITS1 - PM6. Closely related sequences were included in the maximum likelihood analysis. Morphological and molecular identifications delineate this species as *E. diffusa*. Accurate pathogen identification is important for disease management and current breeding programs in India.

Keywords ITS · Morphology · *Glycine max* · *Erysiphe diffusa* · *E. glycines*

India is the fifth largest producer of soybean (*Glycine max*), behind the USA, Brazil, Argentina and China. Compared to the world average, productivity in India is low (Deosthali et al. 2005). This crop has great potential for enhancing food security of rural households in northeast India. Rust, powdery mildew, stem rot, Rhizoctonia web blight and pod blight are important diseases of soybean in this region causing economic loss.

Powdery mildew symptoms were observed in October 2014 in the reproductive stage of growth (R5) when seeds begin to develop in the pods. The place of collection, ICAR Research Complex for NEH Region, Umiam, Meghalaya, India (Latitude 25°30'N, Longitude 91°51'E, elevation 1000 m) is situated under mid-hills (Khasi hills). Climate in this region is sub-tropical and humid. The annual precipitation ranges from 1800 mm to 2500 mm (Patiram 2003). The pathogens *Erysiphe glycines*, *E. diffusa* and *E. pisi* are known to infect this host (Braun and Cook 2011). Morphological identification of the anamorph is difficult due to many overlaps in characters and therefore molecular characterisation is important for accurate identification of powdery mildews on pulses (Attanayake et al. 2010; McTaggart et al. 2012). In Australia, the pathogen that causes powdery mildew on soybean was identified as *E. diffusa* using molecular techniques in the absence of teleomorph stage (McTaggart et al. 2012).

Microscopic observations were done using Olympus BX 53 microscope equipped with a digital camera DP 72, Olympus. Specimens were mounted using 3 % potassium hydroxide. Image analysis was done using the Olympus cellSens platform with Standard 1.5 support software. Scanning electron microscopy was also conducted by placing a diseased portion of the leaf on double-sided adhesive transparent tape 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). The voucher

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Fig. 1 Powdery mildew symptoms on leaves of soybean

specimen has been deposited at the Agharkar Research Institute Herbarium, Pune, India (AMH- 9764).

DNA extraction was done using the QIAamp DNA Stool Mini Kit (Qiagen). Nested PCR was performed using primer sets ITS 5 - ITS 4 and then ITS 1 - PM 6 (Takamatsu and Kano 2001; White et al. 1990). Cycling conditions were initial denaturation 5 min (94 °C), denaturation 30s (94 °C), annealing 30s (54–52 °C, stepdown approach −1 °C in each cycle), extension 40s (72 °C) and final extension 5 min (72 °C). Sequencing was done using primers ITS 1 and PM 6.

Phylogenetic analysis was done using two sequences generated in this study (KR131404 and KP242024) and the GenBank reference sequences of powdery mildew species reported on legumes (Takamatsu et al. 2002). Alignment was done using Muscle implemented in MEGA 6.0 (Tamura et al. 2013). Evolutionary model was inferred using jModeltest (Posada 2008). Phylogenetic analysis was done using Maximum likelihood method with Kimura-2-parameter model (Bootstrap =1000 replicates) as implemented in MEGA 6.0 (Tamura et al. 2013). A discrete Gamma distribution was used to model evolutionary rate differences among sites. Tree optimisation was done using an extensive subtree pruning and regrafting method (SPR-5). The unpublished ITS sequence of *E. nishidana* (MUMH235) was used as outgroup (kindly provided by Dr. S. Takamatsu).

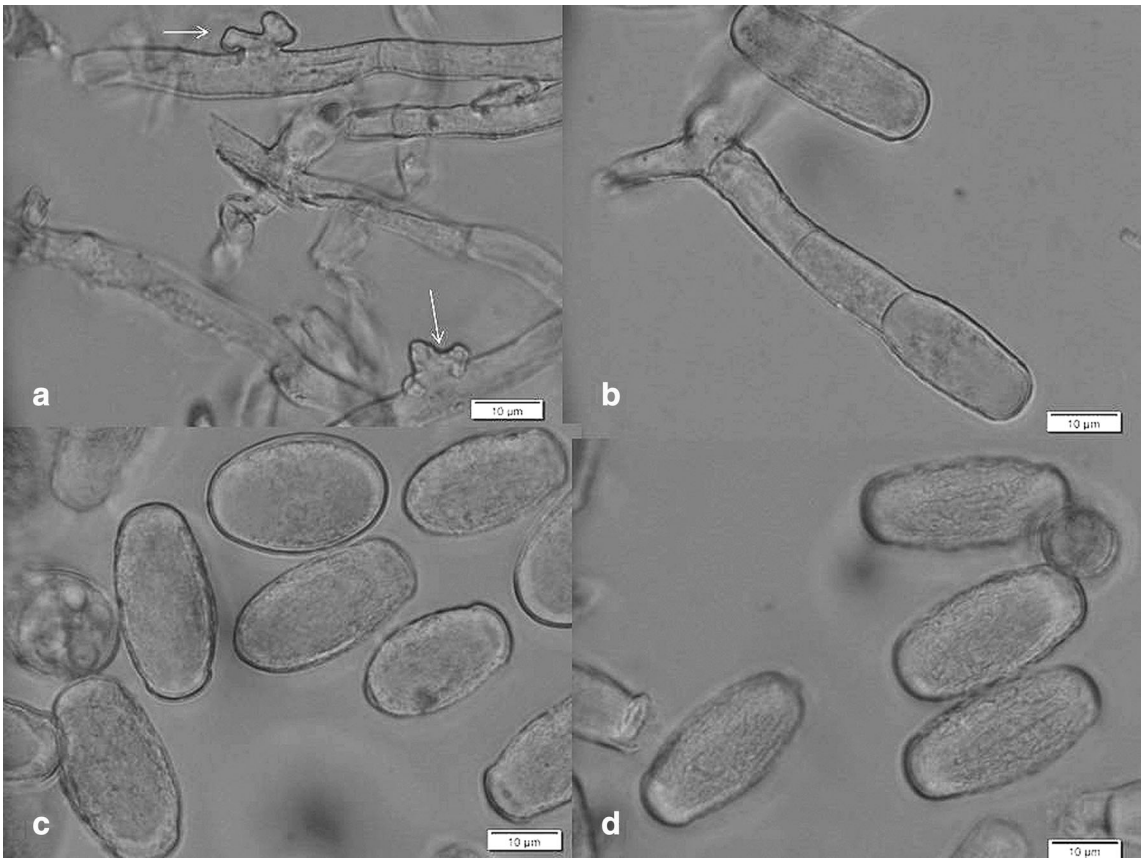

Fig. 2 **a** Lobed hyphal appressoria of *Pseudoidium* anamorph **b** Conidium and conidiophore of *Pseudoidium* anamorph **c** Conidia of *Pseudoidium* anamorph **d** Rugose longitudinal wrinkling pattern on conidia of *Pseudoidium* anamorph

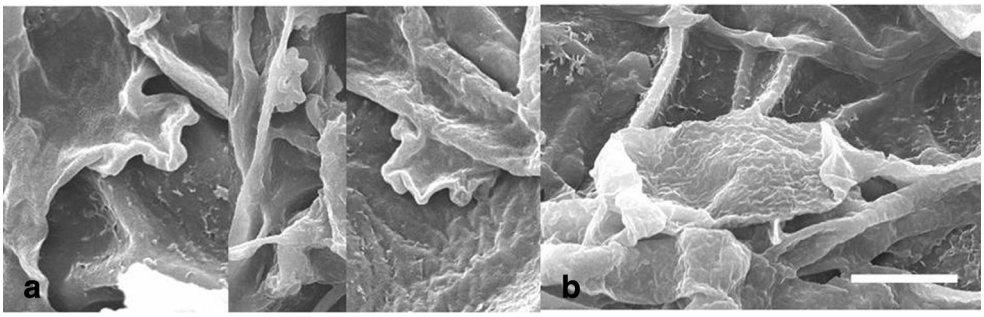

Fig. 3 **a** Lobed appressoria on hypahe of *Pseudoidium* anamorph (Bar =10 μm) **b** Longitudinal angular wrinkling pattern on conidia of *Pseudoidium* anamorph

On leaves white powdery growth, circular to irregular, sometimes covering entire leaflets and symptoms were present on both the surfaces but more on upper surface were observed (Fig. 1). Hyphal appressoria were lobed (Figs. 2a and 3a); foot cells were cylindrical, measuring 23–32.5 × 7–9 μm; conidia were ellipsoid-cylindrical to doliiform, borne singly (non-catenate), measuring 29–33 × 11–17 μm (Fig. 2b and c); germ tubes were with lobed appressoria; fibrosin bodies were absent. Longitudinal angular wrinkling pattern (considered typical for *Pseudoidium* anamorphs belonging to *Erysiphe*) was evident on the surface of the conidia under light microscopy as well as SEM (Figs. 2d and 3b). Morphological descriptions of the anamorphs of the three *Erysiphe* spp. recorded on soybean (Braun and Cook 2011) were compared with those in our study and shown to fit closely with *E. diffusa* (Table 1). In particular, the foot cells were not flexuous and their smaller size as well as shape and smaller size of conidia indicated that the studied specimen did not belong to *E. pisi* or *E. glycines* (Table 1). Also, the presence of doliiform conidia did not indicate *E. pisi.*

The consensus sequences have been deposited in GenBank under accession numbers KR131404 and KP242024. Maximum likelihood analysis showed that soybean powdery mildew sequences (KR131404 and KP242024) are closely affiliated with *E. diffusa* sequences (AB078812, AB078813, FJ378880) and this separation from the remaining species is supported with a high bootstrap value (99 %) (Fig. 4). Consequently, morphological and molecular results indicate that the anamorph of the powdery mildew pathogen on *G. max* belongs to *E. diffusa*. Similar findings based on molecular and morphological characterisations of the anamorph were reported for powdery mildew on soybean in Australia (south east Queensland) (McTaggart et al. 2012).

The differentiation amongst *E. glycines*, *E. diffusa* and *E. pisi* is usually based on chasmothecia but under tropical conditions these are rare. Anamorph characters can always be used to place a species within the genus *Erysiphe* sp. on the basis of conidial surface patterns visualised using SEM, conidia borne singly, type of (lobed) appressoria on hyphae and germination pattern (Braun and Cook 2011; Cook and Braun 2009; Cook et al. 1997). Finer details such as shape and dimensions of foot cells and conidia can then be used to distinguish species on a host infected by more than one powdery mildew as in this study.

A few studies have demonstrated that some host resistance genes may be overcome by strains of the morphologically similar pathogen (can be separated on the basis of ITS sequences) in the case of powdery mildews (*er1, er2* and *Er3* are effective against *E. pisi* but *E. trifoliorum* can overcome *er1* and *Er3*) hence the identity of the pathogen is of utmost

Table 1 Anamorphic characters of different *Erysiphe* sp. on soybean

Erysiphe sp.	Footcells		Conidia	
	Shape	Dimensions	Dimensions	Shape
Erysiphe glycines	Cylindrical, straight, curved or somewhat flexuous-sinuous	20–55 × 6–11(−13) μm	25–50 × 13–25 μm	Ellipsoid-ovoid to doliiform
E. diffusa	Cylindrical	25–38 × 7.5–10 μm	25–35 × 11–17.5 μm	Ellipsoid-cylindrical (−doliiform)
E. pisi	Subcylindrical, straight or occasionally flexuous, curved-sinuous	(15–)20–50(−70) × 6–10 μm	25–55 × (10–) 13–22 μm	Ellipsoid-cylindrical
Powdery mildew on soybean in this study	Cylindrical, straight	23–32.5 × 7–9 μm	29–33 × 11–17 μm	Ellipsoid-cylindrical to doliiform

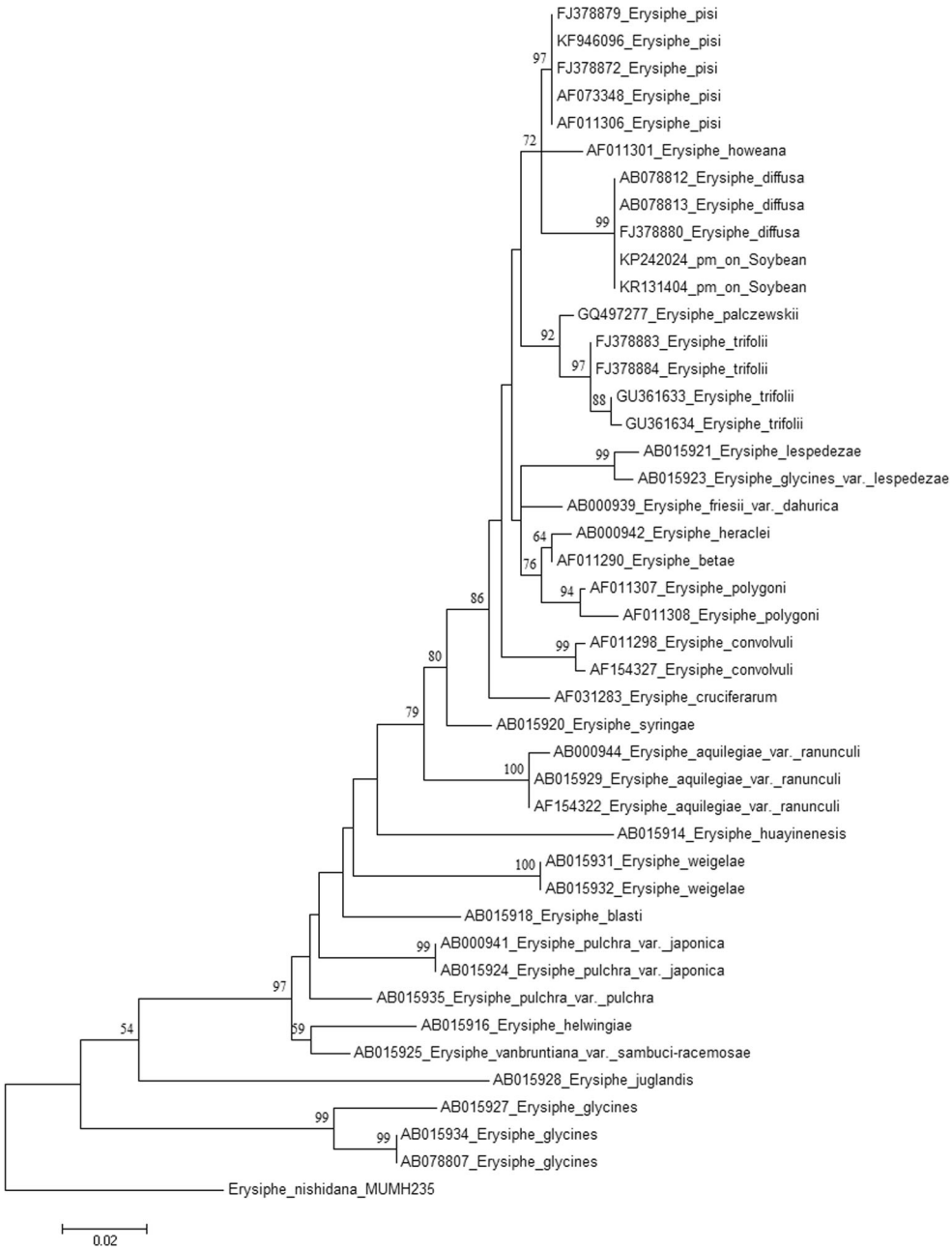

Fig. 4 Phylogenetic tree inferred using maximum likelihood method and bootstrap values are depicted above the branches

importance for resistance breeding programmes (Fondevilla et al. 2013 and references therein). Accurate pathogen identification is an important first step in breeding as well as management programs.

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Compliance with ethical standards This article does not contain any studies with human participants or animal.

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

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