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Ultrastructure and phylogeny of *Ustilago coicis*[*]

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Abstract: *Ustilago coicis* causes serious smut on *Coix lacryma-jobi* in Dayang Town, Jinyun County, Zhejiang Province of China. In this paper, ultrastructural assessments on fungus-host interactions and teliospore development are presented, and molecular phylogenetic analyses have been done to elucidate the phylogenetic placement of the taxon. Hyphal growth within infected tissues was both intracellular and intercellular and on the surface of fungus-host interaction, and the fungal cell wall and the invaginated host plasma membrane were separated by a sheath comprising two distinct layers between the fungal cell wall and the invaginated host plasma membrane. Ornamentation development of teliospore walls was unique as they appeared to be originated from the exosporium. In addition, internal transcribed spacer (ITS) and large subunit (LSU) sequence data showed that *U. coicis* is closely related to *Ustilago trichophora* which infects grass species of the genus *Echinochloa* (Poaceae).

Key words: Fungus-host interaction, Molecular phylogenetics, Smut, Teliospore wall development
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1 Introduction

Job's tears (*Coix lacryma-jobi*), also called as Chinese pearl barley or adley, is a grass (Poaceae) cultivated as a nourishing food eaten in the same way as rice and is believed to have medicinal value (Chang, *et al*., 2003). Job's tears smut is one of the most important diseases affecting this plant and occurs in many countries (Titatarn *et al*., 1983). The causal agent of Job's tears smut was named as *Ustilago coicis* Bref. by Small (1927). Although subsequently Mundkur (1940) named the smut fungus infectious on Job's tears as *C. lacryma-jobi* Mundkur, Chowdhury (1946) reported it as a synonym with *U. coici*s.

Smut genera *Ustilago* and *Sporisorium* (Ustilaginales) show a great diversity on grasses (Poaceae) (Stoll *et al*., 2005). Generic delimitation can be characterized by single teliospores/teliospore balls, sori with or without columella and peridium, and sterile cells between the teliospores (Vánky, 1987; Stoll *et al*., 2003). However, intermediate character combinations have made it difficult for consistent delimitation of these two closely related genera (Vánky, 1985; 1998; Stoll *et al*., 2003; 2005). Supplementary ultrastructural characters of teliospore wall development of smut fungi have been reported and an attempt is made to research their taxonomic relationships (Piepenbring *et al*., 1998; Stoll *et al*., 2003), but these studies have not included *U. coicis.* Molecular data have been used to delimit genera and species in Ustilaginaceae (Bauer *et al*., 1997; Begerow *et al*., 1997; Stoll *et al*., 2003; 2005). Such studies have led to the revisions of the taxonomic position of several species but did not involve *U. coicis*.

In this study, we systemically described interactions

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between *U. coicis* and *C. lacryma-jobi*, and the teliospore development at the ultrastructural level. In addition, we investigated the phylogenetic relationships of *U. coicis* with closely related taxa based on the internal transcribed spacer (ITS) and large subunit (LSU) region sequence analysis.

2 Materials and methods

2.1 Plants

Samples were collected from naturally infected leaves and inflorescences of Job's tears at different development stages in a field in Dayang Town (Jinyun County, Zhejiang Province, China) in August 2010.

2.2 Electron microscopy

Tissue samples (1 mm×2 mm) with mycelia of *U. coicis* and also with teliospore sori were fixed in glutaraldehyde (2.5%) in 0.1 mol/L sodium phosphate buffer (pH 7.0), overnight at 4 °C, including 5 min vacuum-infiltration. Samples were washed in the same buffer, and postfixed in osmium tetroxide (0.01 g/ml) in buffer for 2 h at room temperature. Tissues were dehydrated in a graded ethanol series and were then embedded in Spurr's epoxy resin, and polymerized at 70 °C for 9 h. Infection hyphae within or between cells at precise stages of development were identified by light microscopy and ultrathin sections were cut on a Reichert-Jung Ultracut-E ultramicrotome, collected onto Formvar-coated slot copper grids, and stained with uranyl acetate and lead citrate. Observations were made using a transmission electron microscope at 80 kV and a Gatan 832 CCD camera (Hitachi H-7650, Tokyo, Japan).

2.3 DNA extraction, amplification, and sequencing

The teliospores were collected from samples germinated at 28 °C on potato sucrose agar (PSA) with chloramphenicol and the single sporidia were separated as abundant sporidia were produced on PSA. The single-spore cultures were inoculated in 100 ml of liquid growth media (potato sucrose both (PSB)) in 250-ml flasks, and shaken at 150 r/min at 25 °C for 48 h. Fungal bodies were harvested by filtration, freeze-dried, ground to a final powder in liquid nitrogen, and then stored at −70 °C. About 50 mg of fungal powder was removed into a sterile 1.5 ml microcentrifuge tube, rehydrated in 600 μl of 2× CTAB buffer (100 mmol/L Tris (pH 8.0), 1.4 mol/L NaCl, 30 mmol/L ethylenediaminetetraacetic acid (EDTA), 2% hexadecyltrimethylammonium bromide) and incubated in a water bath at 65 °C for 30–60 min. Following a phenol/chloroform extraction, the genomic DNA was precipitated by isopropanol in the presence of sodium acetate. Genomic DNA was visualized in 1% agrose gels (0.01 g/ml) after ethidium bromide staining (Xie *et al*., 2010).

The ITS and LSU regions were amplified using ITS1 and ITS4 as well as NL1 and NL4, respectively (O′Donnell, 1993). The purified polymerase chain reaction (PCR) products were inserted into pMDTM19-T vector (TaKaRa Co., Japan) and transformed into *Escherichia coli* DH5α competent cells. The positive clone was propagated and the recombinant plasmids were extracted using AxyPrep plasmid miniprep kit according to the manufacturer's instructions (Axygen, USA). In the meantime, they were identified by PCR and restriction endonuclease enzyme digestion. The sequence determination of recombinant plasmid was carried out by the Hangzhou Genomics Institute in both directions. The accession numbers of all sequences used for comparison are listed in Table 1.

2.4 Phylogenetic analyses

Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford, 2002). Phylogenetic trees were built using the maximum parsimony (MP) method. In the MP analyses, trees were inferred using the heuristic search option with tree bisection and reconnection (TBR) branch swapping and 1000 random sequence additions. Gaps were treated as missing data and characters were equally weighted. Maxtrees were unlimited, branches of zero length were collapsed, and all parsimonious trees were saved. Bootstrap analyses were based on 1000 replications, each with 10 replicates of random stepwise addition of taxa. Kishino-Hasegawa (KH) tests were performed in order to determine whether trees were significantly different (Kishino and Hasegawa, 1989). Trees were figured using TreeView (Page, 1996).

Table 1 List of species studied

Species	Host	Origin	GenBank acc. No. (ITS/LSU)[1]	Source[2]
Sporisorium aegypticum (A.A. Fisch. Waldh.) Vánky	*Schismus arabicus*	Iran	AY344970/AY740129	Ust. Exs. 756 (M)
S. andropogonis (Opiz) Vánky	*Bothriochloa saccharoides* (as *Andropogon saccharoides*)	Ecuador	AY740042/AY740095	56588 (M)
S. andropogonis (Opiz) Vánky	*Bothriochloa saccharoides*	Bolivia	AY740043/AY740096	Stoll *et al*., 2005
S. andropogonis-micranthi (L. Ling) L. Guo (as *S. capillipedii*)	*Capillipedium spicigerum*	Australia	AY740047/AY740100	56595 (M)
S. anthracoideisporum Vánky & R.G. Shivas	*Pseudoraphis spinescens*	Papua New Guinea	AY740044/AY740097	Stoll *et al*., 2005
S. apludae-aristatae (B.V. Patil & Thirum.) Vánky	*Apluda mutica*	India	AY740045/AY740098	56590 (M)
S. arthraxonis (Pat.) L. Guo	*Arthraxon lanceolatus*	China	AY740046/AY740099	56592 (M)
S. bursum (Berk.) Vánky	*Themeda quadrivalvis*	India	AY740154	Ust. Exs. 844 (M)
S. cenchri (Lagerh.) Vánky	*Cenchrus pilosus*	Nicaragua	AY344972/AF453943	MP 1974 (TUB)
S. chrysopogonis Vánky	*Chrysopogon fulvus*	Sri Lanka	AY344973/AY740131	Ust. Exs. 407 (M)
S. consanguineum (Ellis & Everhart) Vánky	*Aristida uruguayensis*	Argentina	AY740048/AY740101	H.U.V. 19145 (TUB)
S. cordobense (Spegazzini) Vánky	*Digitaria insularis*	Bolivia	AY740155	Stoll *et al*., 2005
S. cruentum (J.G. Kühn) Vánky	*Sorghum halepense*	USA	AY344974/AF453939	Ust. Exs. 687 (M)
S. cruentum (J.G. Kühn) Vánky	*Sorghum bicolor*	Nicaragua	AY740156	Stoll *et al*., 2005
S. culmiperdum (J. Schröt.) Vánky	*Andropogon gerardii*	Honduras	AY344975/AF133580	MP 2060 (TUB)
S. destruens (Schltdl.) Vánky	*Panicum miliaceum*	Romania	AY344976/AY747077	Ust. Exs. 472 (M)
S. dimeriae-ornithopodae Vánky & C. Menge	*Dimeria ornithopoda*	India	AY344977/AY740132	Ust. Exs. 848 (M)
S. elionuri (Henn. & A. Evans) Vánky	*Elionurus muticus*	Bolivia	AY740157	Stoll *et al*., 2005
S. enteromorphum (McAlpine) Vánky	*Themeda triandra*	South Africa	AY740158	56602 (M)
S. erythraeense (Syd. & P. Syd.) Vánky	*Hackelochloa granularis*	India	AY740049/AY740102	Ust. Exs. 849 (M)
S. everhartii (Ellis & Galloway) M. Piepenbr	*Andropogon virginicus*	Cuba	AY740159	Stoll *et al*., 2005
S. fastigiatum Vánky	*Andropogon angustatus*	Nicaragua	AY344978/AY740133	Stoll *et al*., 2005
S. formosanum (Sawada) Vánky	*Panicum repens*	Taiwan, China	AY344979/AY740134	Ust. Exs. 688 (M)
S. foveolati (Maire) Vánky	*Eremopogon foveolatus*	Canary Islands	AY740050/AY740103	MP 2365 (TUB)
S. holwayi (G.P. Clinton & Zundel) Vánky	*Andropogon bicornis*	Panama	AY344980/AF453941	Stoll *et al*., 2005
S. hwangense Vánky & C. Vánky	*Sporobolus panicoides*	Zimbabwe	AY740051/AY740104	56607 (M)
S. lacrymae-jobi (Mundkur) Vánky	*Coix lacryma-jobi*	India	AY740052/AY740105	56611 (M)
S. lepturi (Thüm.) Vánky	*Hemarthria uncinata*	Australia	AY344981/AY740135	Ust. Exs. 966 (M)
S. lepturi (Thüm.) Vánky	*Hemarthria uncinata*	Australia	AY740160	56613 (M)
S. loudetiae-pedicellatae Vánky & C. Vánky	*Loudetia pedicellata*	South Africa	AY740053/AY740106	56615 (M)
S. manilense (Syd. & P. Syd.) Vánky (as *S. sacciolepidis*)	*Sacciolepis indica*	India	AY740059/AY740112	Ust. Exs. 854 (M)
S. mishrae Vánky	*Apluda mutica*	India	AY344983/AY740136	Ust. Exs. 967 (M)
S. modestum (Syd.) H. Scholz	*Enneapogon avenaceus*	Australia	AY740054/AY740107	56617 (M)
S. monakai (Mishra) Vánky	*Isachne globosa*	India	AY740161	56618 (M)
S. moniliferum (Ellis & Everh.) L. Guo	*Heteropogon contortus*	Indonesia	AY344984/AF453940	Ust. Exs. 851 (M)
S. nealii (Ellis & F.W. Anderson) Vánky	*Heteropogon melanocarpus*	India	AY740055/AY740108	56621 (M)
S. neglectum (Niessl) Vánky	*Setaria pumila*	Germany	AY740056/AY740109	RB 2056 (TUB)
S. nervosum Vánky, C. Vánky & R.G. Shivas	*Sehima nervosum*	Australia	AY740057/AY740110	56622 (M)
S. occidentale (Seym.) Vánky & Snets	*Andropogon gerardii*	USA	AY344985/AY740137	Ust. Exs. 758 (M)
S. ophiuri (Henn.) Vánky	*Rottboellia cochinchinensis*	Unknown	AY740019/AJ236136	HB 20

To be continued

Table 1

Species	Host	Origin	GenBank acc. No. (ITS/LSU)[1]	Source[2]
S. ovarium (Griffiths) Vánky	*Urochloa fasciculata* (Sw.) R. Webster	Mexico	AY740020/AJ236137	Stoll *et al*., 2005
S. paspali-notati (Henn.) M. Piepenbr	*Paspalum notatum*	Cuba	AY344982/AF453944	Stoll *et al*., 2005
S. penniseti (Maire) Vánky (as *S. catharticum*)	*Pennisetum setaceum*	Canary Islands	AY344971/AY740130	MP 2367 (TUB)
S. polliniae (Magnus) Vánky	*Andropogon distachyos*	Greece	AY344987/AY740138	Ust. Exs. 690 (M)
S. provinciale (Ellis & Galloway) Vánky & Snets	*A. gerardii*	USA	AY344988/AY747076	Ust. Exs. 759 (M)
S. pseudechinolaenae Vánky & C. Menge	*Pseudechinolaena polystachya*	Indonesia	AY344989/AY740139	Ust. Exs. 853 (M)
S. puellare (Syd. & P. Syd.) G. Deml	*Hyparrhenia hirta*	Canary Islands	AY740058/AY740111	MP 2372 (TUB)
S. pulverulentum (Cooke & Massee) Vánky	*Saccharum strictum*	Yugoslavia	AY740162	56627 (M)
S. reilianum (J.G. Kühn) Langdon & Full.	*Sorghum halepense*	Greece	AY740163	Ust. Exs. 527 (M)
S. scitamineum (Syd.) M. Piepenbr., M. Stoll & Oberw (as *Ustilago scitaminea*)	*Saccharum* sp.	Cuba	AY345007/AY740147	Stoll *et al*., 2005
S. scitamineum (Syd.) M. Piepenbr., M. Stoll & Oberw (as *U. scitaminea*)	*Saccharum* sp.	Costa Rica	AY740070/AJ236138	Stoll *et al*., 2005
S. sorghi Ehrenb. ex Link	*Sorghum bicolor*	Nicaragua	AY740021/AF009872	Stoll *et al*., 2005
S. themedae-arguentis Vánky	*Themeda arguens*	Indonesia	AY344991/AY740140	Ust. Exs. 855 (M)
S. trachypogonicola Vnky & C. Vnky	*Trachypogon plumosus*	Cuba	AY344992/AY740141	Stoll *et al*., 2005
S. trachypogonis-plumosi Vánky	*T. plumosus*	Venezuela	AY740060/AY740113	56635 (M)
S. tumefaciens (McAlpine) Vánky (as *Sorosporium tumefaciens*)	*Chrysopogon aciculatus*	Sri Lanka	AY344969/AY740128	Ust. Exs. 231 (M)
S. veracruzianum (Zundel & Dunlap) M. Piepenbr	*Panicum viscidellum*	Costa Rica	Y344993/AY740114	MP 960
S. veracruzianum (Zundel & Dunlap) M. Piepenbr	*P. viscidellum*	Costa Rica	AY747075/AY740142	MP 735 (USJ)
Ustilago affinis Ellis & Everh	*Stenotaphrum secundatum*	Costa Rica	AY344995/AF133581	Stoll *et al*., 2005
U. alcornii Vánky	*Tripogon loliiformis*	Australia	AY740165	56514 (M)
U. altilis Syd.	*Triodia pungens*	Australia	AY740166	Ust. Exs. 418 (M)
U. austro-africana Vánky & C. Vánky	*Enneapogon cenchroides*	Zimbabwe	AY740061/AY740115	56516 (M)
U. avenae (Pers.) Rostr.	*Arrhenaterum elatius*	Germany	AY740063/AY740117	DB 559 (TUB)
U. avenae (Pers.) Rostr.	*A. elatius*	Germany	AY740062/AY740116	RB 3092 (TUB)
U. avenae (Pers.) Rostr.	*Avena barbata*	Canary Islands	AY344997/AF453933	MP 2362 (TUB)
U. avenae (Pers.) Rostr.	*A. barbata*	Italy	AY344996/AJ236140	F 946/GD 1292 (TUB)
U. bouriquetii Maubl. & Roger	*Stenotaphrum dimidiatum*	Réunion	AY740167	56517 (M)
U. bromivora (Tul. & C. Tul.) A.A. Fisch. Waldh	*Bromus catharticus*	Argentina	AY740064/AY740118	H.U.V. 19322
U. bullata Berk	*B. diandrus*	Canary Islands	AY344998/AF453935	MP 2363 (TUB)
U. calamagrostidis (Fuckel) G.P. Clinton	*Calamagrostis epigeios*	Bulgaria	AY740065/AY740119	56518 (M)
U. coicis Bref	*Coix lacryma-jobi*	China	JX219371/JX219374	In this study
U. crameri Körnicke	*Setaria italica*	India	AY344999/AY740143	Ust. Exs. 995 (M)
U. cynodontis (Pass.) P. Henn	*Cynodon dactylon*	Mexico	AY345000/AF009881	Stoll *et al*., 2005
U. cynodontis (Pass.) P. Henn	*C. dactylon*	Taiwan, China	AY740168	MS 1 (TUB)
U. davisii Liro	*Glyceria multiflora*	Argentina	AY740169	H.U.V. 19252
U. drakensbergiana Vánky	*Digitaria tricholaenoides*	South Africa	AY740170	56523 (M)
U. echinata J. Schröt.	*Phalaris arundinacea*	Germany	AY345001/AY740144	Ust. Exs. 540 (M)
U. esculenta P. Henn	*Zizania latifolia*	Taiwan, China	AY345002/AF453937	Ust. Exs. 590 (M)

To be continued

Table 1

Species	Host	Origin	GenBank acc. No. (ITS/LSU)[1]	Source[2]
U. esculenta P. Henn	*Zizania latifolia*	China	JX219372/JX219375	In this study
U. esculenta P. Henn	*Zizania latifolia*	China	JX219373/JX219376	In this study
U. filiformis (Schrank) Rostr	*Glyceria fluitans*	Germany	AY740066/AY740120	RB 3011 (TUB)
U. hordei (Pers.) Lagerh (as *U. kolleri*)	*Avena sativa*	Spain	AY740068/AY740122	F 947/GD 1300
U. hordei (Pers.) Lagerh	*Hordeum vulgare*	Iran	AY345003/AF453943	Ust. Exs. 784 (M)
U. ixophori Durán	*Ixophorus unisetus*	Costa Rica	AY740067/AY740121	Stoll *et al*., 2005
U. maydis (Link) Unger	*Zea mays* L.	Germany	AY345004/AF453938	RB 3093 (TUB)
U. nuda (Jens.) Rostr	*Hordeum leporinum*	Unknown	AY740069/AJ236139	H.U.V. 17782
U. pamirica Golovin	*Bromus gracillimus*	Iran	AY345005/AY740145	Ust. Exs. 789 (M)
U. schroeteriana Henn	*Paspalum paniculatum*	Costa Rica	AY345006/AY740146	Ust. Exs. 887 (M)
U. spermophora Berk. & M.A. Curtis	*Eragrostis ferruginea*	Namibia	AY740171	F 565/H.U.V. 13634
U. striiformis (Westend.) Niessl	*Alopecurus pratensis*	Germany	AY740172	H.U.V. 18286
U. syntherismae (Schwein.) Peck	*Digitaria ternata*	India	AY740071/AY740123	Ust. Exs. 998 (M)
U. tragana Zundel	*Tragus berteronianus*	Zimbabwe	AY740072/AY740124	56562 (M)
U. trichophora (H.F. Link) F. Körnicke	*Echinochloa colona*	Cuba	AY345009/AY740148	Stoll *et al*., 2005
U. trichophora (H.F. Link) F. Körnicke	*E. colona*	Mexico	AY740023/AJ236141	Stoll *et al*., 2005
U. trichophora (H.F. Link) F. Körnicke	*E. colona*	India	AY740073/AY740125	56564 (M)
U. triodiae Vánky	*Triodia microstachya*	Australia	AY740074/AY740126	H.U.V. 17662
U. triodiae Vánky	*T. microstachya*	Australia	AY740075/AY740127	56566 (M)
U. turcomanica Tranzschel	*Eremopyrum distans*	Iran	AY345011/AF453936	F 585/H.U.V. 23
U. vetiveriae Padwick	*Vetiveria zizanioides*	Unknown	AY345011/AY740149	H.U.V. 17954
U. xerochloae Vánky & R.G. Shivas	*Xerochloa imberbis*	Australia	AY345012/AY740150	Ust. Exs. 1000 (M)

[1] ITS/LSU: ITS sequence and LSU sequence or single accession numbers with contiguous sequences (ITS and LSU). [2] GD: Günter Deml; RB: Robert Bauer; Ust. Exs.: Ustilaginales Exsiccata; H.U.V.: Herbarium Ustilaginales Vánky; M: München, Germany; TUB: Tübingen, Germany; MP: Meike Piepenbring; HB: Hansjörg Prillinger; F: Franz Oberwinkler; MS: Matthias Stoll

3 Results

3.1 Interaction between *Ustilago coicis* and *Coix lacryma-jobi*

Before teliospore formation, transmission electron micrographs (TEMs) showed that hyphae of *U. coicis* were present between and within plant cells in parenchyma tissue of an ovary. The intracellular hypha growing in a cell was surrounded by a sheath comprising two distinct layers, which separated the fungal cell wall from the invaginated host plasma membrane (Fig. 1a). The plant plasma membrane was continuous but occasionally it was convoluted at some sites. The outer layer of a sheath was an electron-opaque layer surrounded by the plant plasma membrane and an inner electron-dense layer covering the hyphal wall. However, the sheaths were variable between and within parenchyma tissue. The intercellular hypha development caused intercellular space enlargement and elongation of plant cell walls but intercellular hyphal walls were occasionally only coated by an inconspicuous electron-dense layer (Fig. 1b).

In some cells with intracellular fungal development, the hyphae produced short or lateral branches (Fig. 1c). In this case the host protoplast appeared to be disintegrated and these hyphae were not surrounded by a sheath (Figs. 1c and 1d). They grew and branched extensively until individual host cells became filled with contorted and coiled hyphae, which constituted the hyphal aggregations (Fig. 1e). These hyphae were sporogenous hyphae and their hyphal aggregations were the sites of teliosporogenesis. Subsequently, the aggregations caused host cells to disintegrate, resulting in large intercellular spaces in the parenchyma tissue of an affected ovary.

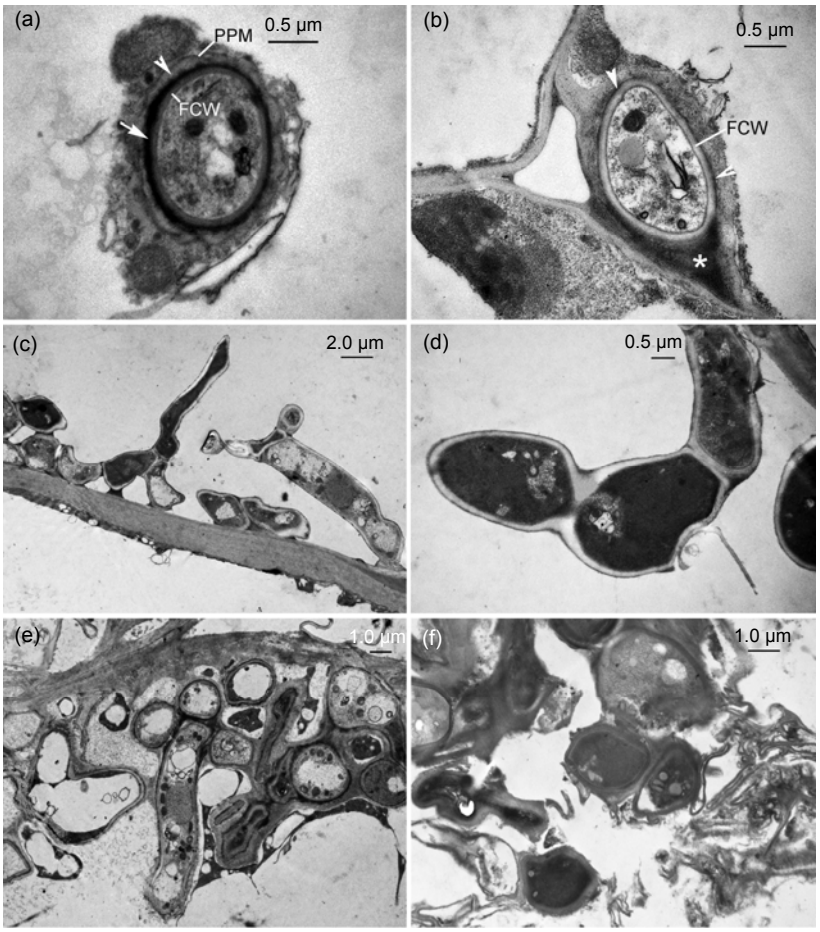

Fig. 1 Transmission electron micrographs (TEMs) of the interaction between *Ustilago coicis* and ovarian parenchyma of *Coix lacryma-jobi*
(a) Transverse section of an intracellular hypha. A hypha is surrounded by a sheath comprising electron-opaque layer (arrowhead) and electron-dense layer (arrow), which is located between the plant plasma membrane (PPM) and the fungal cell wall (FCW). (b) Transverse section of an intercellular hypha. Hyphae induce intercellular space enlargement (*) and FCW is covered by an inconspicuous electron-dense layer in some areas (arrowheads). (c) Sporogenous hyphae. (d) A magnified sporogenous hyphae. (e) Abundant hyphae in a host cell. (f) Disintegrated host tissues with fungal hyphae

3.2 Teliospore development

In a large intercellular space, abundant hyphal fragments were seen under the light microscope (Fig. 2a) and they had originated from the sporogenous hyphae. Under TEM, the hyphal fragments began to plasmolyze (Figs. 2b and 2c) and the initial exosporia of the young teliospores were produced on the plasma membranes of the plasmolyzed hyphal fragments (Fig. 2d). In this case, a young teliospore was in the completely plasmolyzed cell wall, but the fungal cell wall was still intact. With teliospore development, the electron-opaque warty ornamentation produced from the exosporium of a young teliospore (Fig. 2e) but it appears to be homogenous with the exosporium. Fungal cell walls began to disintegrate but some remnants of fungal cell wall were retained on top of warts (Fig. 2f) and young teliospores were irregular. With further development, the warty ornamentation became electron-dense and young teliospores were regularly spherical or subspherical with a few lipid globules (Figs. 2g and 2h). At this time, the exosporium also became electron-dense and was covered by spiny ornamentation, and young teliospores exhibited more lipid globules (Fig. 2i). Subsequently, an electron-opaque endosporium in a young teliospore developed beneath the exosporium (Fig. 2j). The mature teliospore wall was comprised of an endosporium of mostly constant thickness and an exosporium covered by warts (Fig. 2k).

As teliospores of *U. coicis* matured, sori became darker and eventually black. SEM of sori revealed masses of matured teliospores (Fig. 3a). Individual teliospores were fine spines or warts (Figs. 3b and 3c) and usually between 9 and 11 μm in diameter.

3.3 Phylogenetic analysis

Sequence analysis showed that ITS/5.8S and LSU regions from three isolates of *U. coicis* were identical and one of them, isolate UCDY01, had been submitted to the GenBank database (Table 1). The final ITS/5.8S and LSU dataset included 95 sequences with 1316 characters after alignment. Parsimony analysis resulted in 52 equally parsimonious trees. The KH test showed that these trees were not significantly different. One of these trees is shown in Fig. 4.

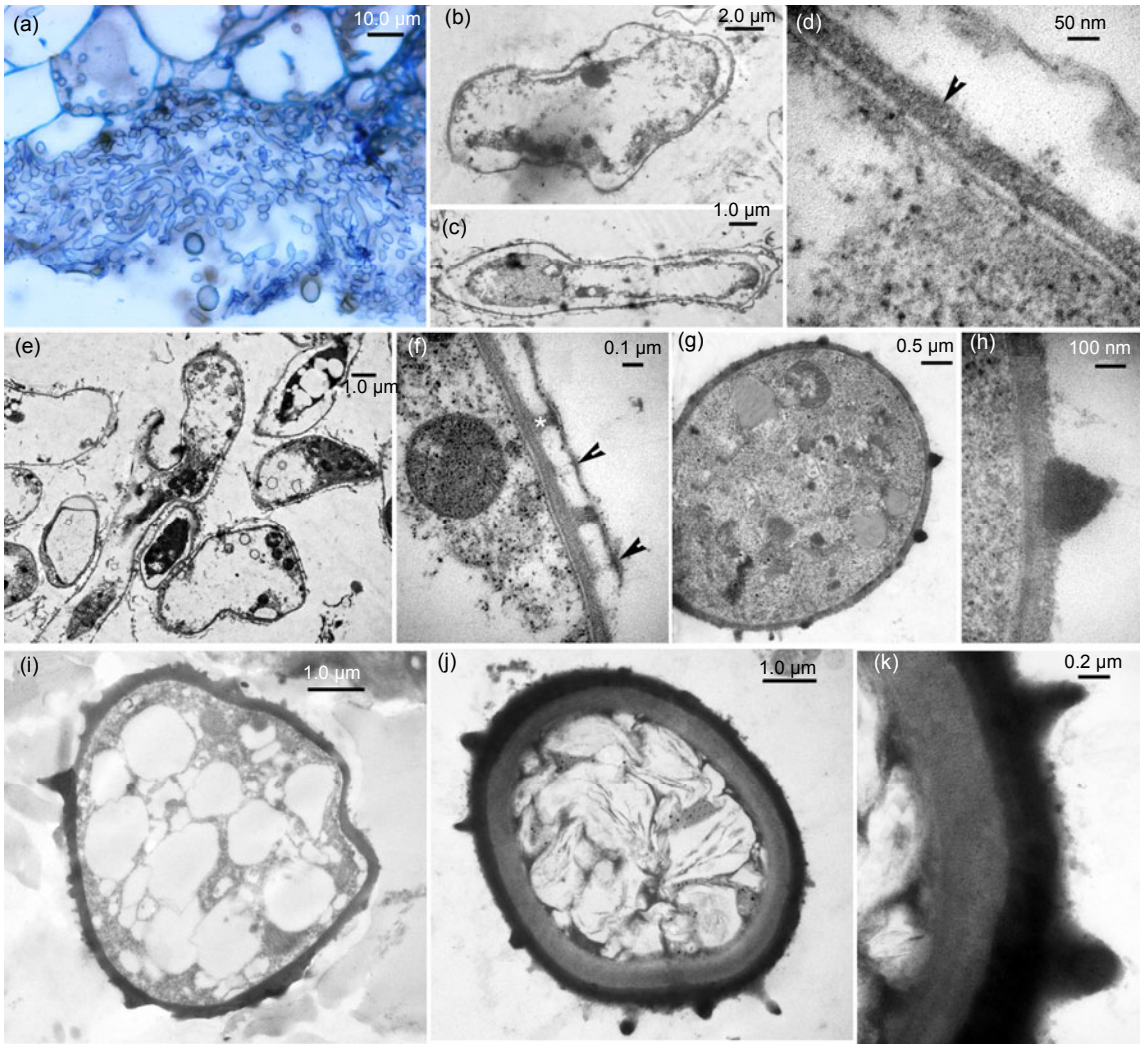

Fig. 2 Development of teliospore walls as seen by light microscopy and transmission electron micrograph (TEM)
(a) Light microscopy. Abundant hyphal segments produced from sporogenous hyphae in a large intercellular space. (b–k) TEM. (b, c) Plasmolyzed process of hyphal segments: (b) a hyphal segment being plasmolyzed; (c) a completely plasmolyzed hyphal segment. (d) Exosporium (arrowhead) of a young teliospore produced on plasma membrane in plasmolyzed process. (e) Warty ornamentation formation and fungal cell walls being degraded. (f) A magnified view of warty ornamentation (*) with the remnant (arrowheads) of fungal cell wall in (e). (g) A regularly young teliospore with electron-dense warty ornamentation. (h) Magnified view of a conical wart in (g). (i) A young teliospore with electron-dense exosporium and spines. (j) A well-developed wall of teliospore with endosporium, exosporium, and warts. (k) Magnified view of a teliospore wall in (j)

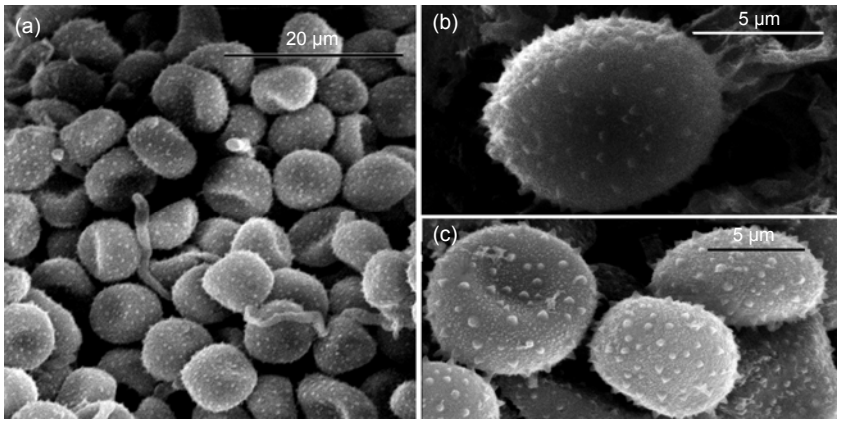

Fig. 3 Scanning electron micrographs (SEMs) of mature teliospores of *Ustilago coicis* in cupules of *Coix lacryma-jobi*
(a) Sorus. (b, c) Mature teliospores clearly showing the surface spines or warts

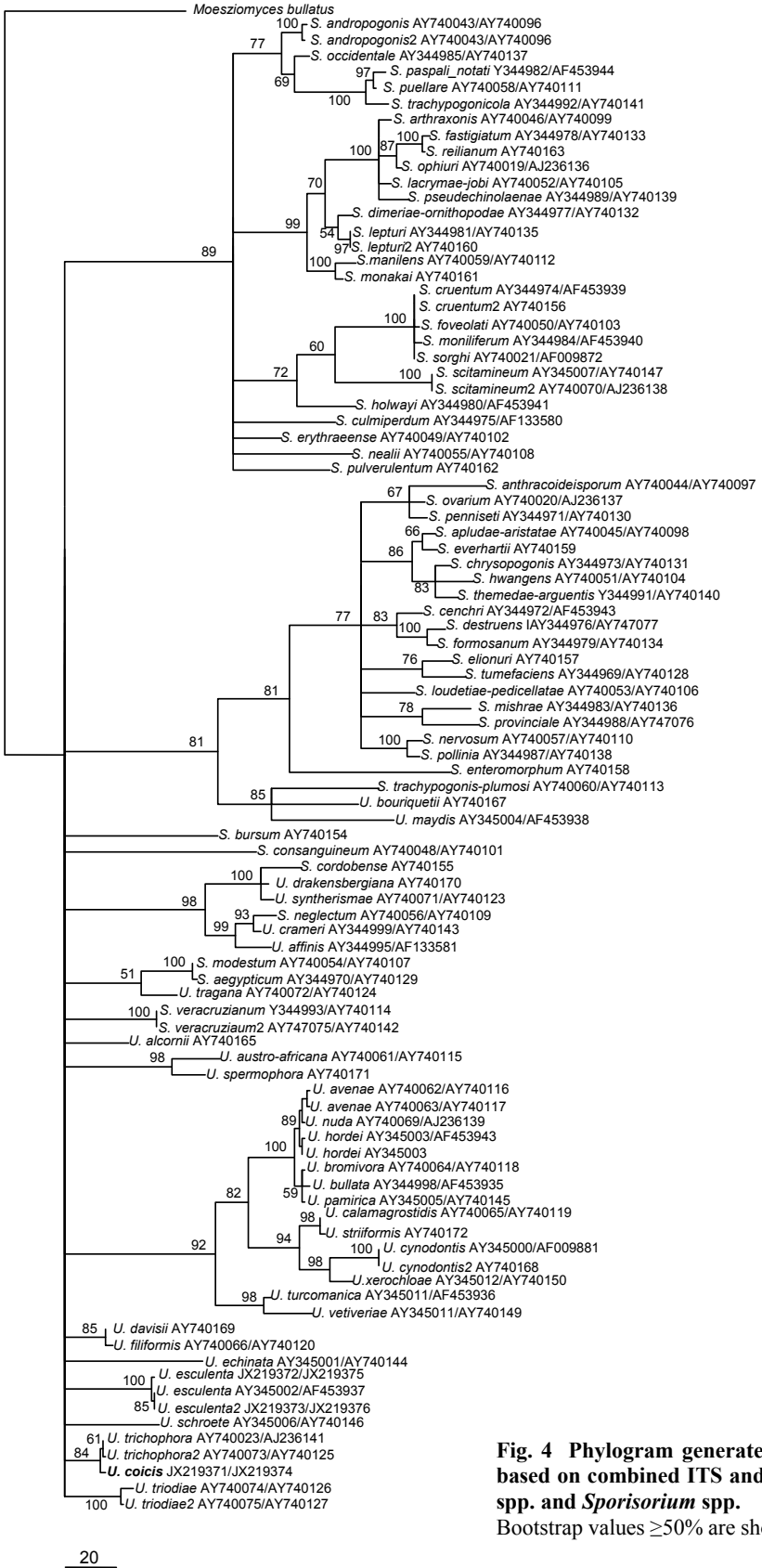

Fig. 4 Phylogram generated from parsimony analysis based on combined ITS and LSU sequences in *Ustilago* spp. and *Sporisorium* spp.

Bootstrap values ≥50% are shown above or below branches

The taxa name and GenBank number of each sequence are given on the tree. One isolate of *U. coicis* formed a monophyletic lineage and clustered together with *U. trichophora* with 84% bootstrap support when *Moesziomyces bullatus* (AB369259) was used as the outgroup taxon. Teliospores of *U. trichophora* are globose and ornamented with spines, and are similar to those of *U. coicis* (7–14 μm wide compared with 6–14 μm wide in *U. trichophora*) (Fullerton and Langdon, 1968; Titatarn *et al*., 1983). *U. trichophora* is a pathogen infecting the ovaries and vegetable parts of *Echinochloa* grass species and exhibits intermediate morphlogical characters between *Ustilago* and *Sporisorium*.

4 Discussion

U. coicis can infect the ovaries and leaves of *C. lacryma-jobi* and cause Job's tears smut. In infected tissues, hyphal distribution is both intercellular and intracellular. At the ultrastructural level, it can be seen that intracellular hypha at the interface of fungus-host interaction is encased by a sheath with an outer electron-opaque layer and an inner electron-dense layer, as shown in other species of *Ustilago*, such as *U. esculenta* (Zhang *et al*., 2012). The teliosporogenesis process in *U. coicis* is similar to those of many species of *Ustilago*-infecting members of Poaceae. Teliospores are covered by fine to coarse warts or by warts of different sizes on the same teliospore. However, ornamentation development in *U. coicis* is unique. During teliosporogenesis in almost all smut fungi, the outer layers are usually deposited first. At the beginning of ornamentation formation, the plasma membrane may be smooth or undulated, carrying the developing ornaments on its tips or in its depressions (Piepenbring *et al*., 1998). By contrast, the ornamentation of teliospores in *U. coicis* appeared to be produced on the exosporium of a young teliospore (Fig. 5), never from the plasma membrane, being different from developmental patterns of ornamentation of *Ustilago* (Piepenbring *et al*., 1998; Zhang *et al*., 2012). This shows that teliospore development in *Ustilago* is diverse.

The phylogenetic relationship between *U. coicis* and its closely related species in *Ustilago* was analyzed based on combined ITS and LSU sequence data. The phylogenetic analysis clearly indicates that *U. coicis* is closely related to *U. trichophora*.

Although peridia, columella, spore balls, and sterile cells have all been used as morphological characters to distinguish *Ustilago* from *Sporisorium*, sori of *U. trichophora* contain a columella and are packed by a peridium (Fullerton and Langdon, 1968), which are typical *Sporisorium* characters (Piepenbring, 2003), so *U. trichophora* was considered to demonstrate intermediate morphological characters (Piepenbring *et al*., 1998) between these genera. In *Ustilago* spp., *U. trichophora* and *U. coicis* have the same ornamentation with spines and could be not well differentiated by overlapping teliospore size (Fullerton and Langdon, 1968; Titatarn *et al*., 1983). Piepenbring (2004) and Stoll *et al*. (2005) found that it was because morphological characters were non-homologous and had been misused as a taxonomic standard for defining the genera, resulting in the genus complex, as shown in Fig. 4. Therefore, additional molecular loci are needed to resolve the *Ustilago-Sporisorium* complex, and should include morphological characters.

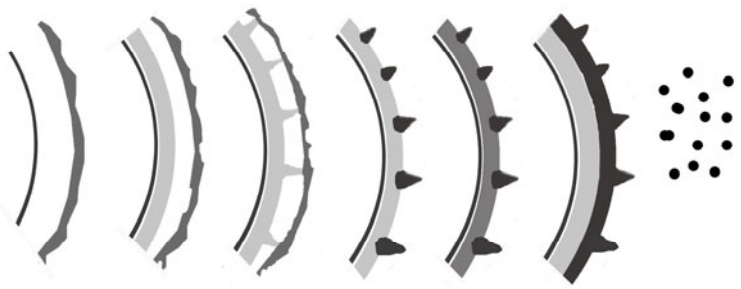

Fig. 5 Schematic drawing of teliospore wall development (from left to right) of *Ustilago coicis*
Parts of walls are shown in section, and ornamentation also in surface view. The process of a teliospore wall development is plasmolyzation of hyphal segment, exosporium deposition, formation of the ornamentation, and endosporium deposition

5 Conclusions

In this article, we showed, for the first time, that there is a unique development of teliospore walls of *U. coicis* in Ustilaginaceae. In addition, phylogeny and fungus-host interactions of *U. coicis* were ultrastructurally assessed. Therefore, this research can provide more valuable information for taxonomy and biology in Ustilaginaceae.

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Compliance with ethics guidelines

Jing-ze ZHANG, Pei-gang GUAN, Gang TAO, Mohammad Reza OJAGHIAN, and Kevin David HYDE declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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