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Two new species of Undifilum, fungal endophytes of Astragalus (locoweeds) in the United States

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Abstract

New species of *Undifilum*, from locoweeds *Astragalus lentiginosus* Vitman and *Astragalus mollissimus* Torr., are described using morphological characteristics and molecular phylogenetic analyses as ***Undifilum fulvum*** Baucom & Creamer sp. nov. and ***Undifilum cinereum*** Baucom & Creamer sp. nov. Fungi were isolated from dried plants of A. lentiginosus var. araneosus, diphysus, lentiginosus, and *wahweapensis* collected from Arizona, Oregon, and Utah, USA, and *A. mollissimus* var. *biglovii, earleii*, and *mollissimus* collected from New Mexico, Oklahoma, and Texas, USA. Endophytic fungi from *Astragalus* locoweeds were compared to *Undifilum oxytropis* isolates obtained from dried plant material of *Oxytropis lamberteii* from New Mexico and *Oxytropis sericea* from Arizona, Colorado, New Mexico, Utah, and Wyoming. Extremely slow growth in vitro was observed for all, and conidia, if present, were ellipsoid with transverse septa. However, in vitro color, growth on four different media, and conidium size differed between fungi from *Astragalus* spp. and *U. oxytropis*. Neighbor-joining analyses of internal transcribed spacer (ITS) region and *glyceraldehyde-3-phosphate dehydrogenase (GPD)* gene sequences revealed that ***U. fulvum and U. cinereum*** formed a clade distinct from U. oxytropis. This was supported by neighbor-joining analyses of results generated from random amplified polymorphic DNA (RAPD) fragments using two different primers.

Keywords

Astragalus lentiginosus; *Astragalus mollissimus*; fungal endophyte; *Undifilum fulvum*; *Undifilum cinereum*

Introduction

Locoweeds have been problematic for ranchers in the United States for over a century (Marsh 1909). Consumption of locoweeds by grazing animals induces the neurological disease, locoism, characterized by staggered gait, lack of muscular coordination, and difficulty eating and drinking, thus resulting in significant economic losses to livestock (James and Panter 1989). Locoweeds, from the genera *Oxytropis* and *Astragalus*, are found throughout North America in arid regions, as well as in central China (Welsh et al. 2007; Yu et al. 2010). The toxic compound that causes locoism is swainsonine, an alkaloid □-mannosidase inhibitor (Tulsiani et al. 1984). Ingestion of swainsonine results in decreased mannosidase levels and dysfunctional glycoprotein processing (Tulsiani et al. 1984, 1988; Stegelmeier et al. 1995). Swainsonine was initially characterized from the plant Swainsona (Colegate et al. 1979), and is also produced by the fungi Rhizoctonia leguminicola,

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Metarhizium anisopliae, and Undifilum oxytropis(Schneider et al. 1983; Patrick et al. 1993; Pryor et al. 2009). *Undifilum oxytropis* was recently described as the toxin-producing fungal endophyte isolated from the *Oxytropis* spp. of locoweed.

Endophytic fungi isolated from species of *Oxytropis* and varieties of *Astragalus mollissimus* Torr were found to produce swainsonine in vitro, which led to subsequent investigations into the interactions of the fungus with the plant and locoism disease (Braun et al. 2003). All plant parts have been shown to contain the fungal endophyte except for the embryo (Cook et al. 2011) and, in fact, plants germinated from the embryo alone were fungus free and did not contain or produce the toxin swainsonine (Oldrup et al. 2010). Rats fed a diet of dried fungal endophyte mycelia showed identical symptoms of disease as rats fed whole ground locoweed plants, therefore implicating the fungal endophyte as disease causing (McLain-Romero et al. 2004). Correlation of low and high levels of swainsonine with the absence and presence of endophyte, respectively, were found in 16 populations of *Oxytropis lambertii* Pursh (Gardner et al. 2001). In a survey of both toxic and nontoxic major locoweed species of the United States, a clear relationship was found between swainsonine concentrations and the presence of *U. oxytropis* as determined by plant isolations and PCR (Ralphs et al. 2008). Furthermore, the quantity of *U. oxytropis* within the plant was positively correlated with swainsonine concentration in young plants (Achata Bottger et al. 2012; Cook et al. 2009) and the fungus was detected in swainsonine producing *A. mollissimus* and *Astragalus lentiginosus* Douglas ex Hook. (Ralphs et al. 2008; Cook et al. 2011). Overall, the current research shows that the fungus is responsible for the swainsonine production that was initially attributed to the plant, though this has only been indirectly shown for fungal endophytes of *A. lentiginosus*.

The toxin producing endophytes isolated from locoweeds in the United States were initially characterized as possible species of *Alternaria* or *Embellisia* (Braun et al. 2003). An isolate from *Oxytropis kansuensis* in China was described as *Embellisia oxytropis* (Wang et al. 2006). However, upon further taxonomic investigation of fungi from *Oxytropis sericea, O. lambertii*, and the isolate from *O. kansuensis*, a new genus *Undifilum* within the Pleosporaceae, was proposed (Pryor et al. 2009). The genus contains *U. oxytropis*, the fungus isolated from *Oxytropis species*, as well as *Undifilum bornmuelleri*, previously known as *Helminthosporium bornmuelleri*. Taxonomic refinement was based on both morphological and molecular characteristics. Conidia produced from locoweed fungi are ellipsoidal in shape, with few ovate spores, as compared to *Alternaria* sp., which are frequently ovate and produce a unique wavy hypha upon germination (Pryor et al. 2009). Conidia most often have thin septa that cannot be differentiated from conidial walls, a common feature in conidia of *Embellisia*. Phylogenetic analyses based on sequences from the *gpd*, internal transcribed spacer (ITS), and mtssu regions fully supported the monophyletic clade of the new genus *Undifilum* and demonstrated the genus as a sister to *Ulocladium* and some species of *Alternaria* (Pryor et al. 2009).

Recently, isolates from several species of locoweed in China were also examined for their taxonomic placement (Yu et al. 2010). Isolates were obtained from *Oxytropis glacialis*, *Oxytropis kansuensis*, *Oxytropis ochrocepala*, *Oxytropis serocopetala*, *Oxytropis glabra*, and *Oxytropis latibracteata* as well as *Astragalus variabilis* and *Astragalus strictus*. Sequences of the ITS region, as well as restriction fragment digests of the intergenic spacer (IGS) region, were used to characterize the differences found in isolates obtained from different locoweed plants. The ITS region was not sufficiently informative to distinguish different species but it did allow placement of the isolates within *U. oxytropis* in phylogenetic analyses. Therefore, all of their recovered fungal endophytes were classified as *U. oxytropis*, although no formal morphological characteristics were reported (Yu et al. 2010).

Undifilum oxytropis isolates used for morphological characterization of the species were from O. sericea, *O. lambertii*, and *O. kansuensis* (Pryor et al. 2009). Isolates were shown to have slow growth, with hyaline hyphae that formed an interwoven black ball mass. Conidia were solitary with 3–8 thin septa, and the germination tube growth was wavy (Pryor et al. 2009). This was consistent with the first report of the locoweed fungal endophyte (Braun et al. 2003).

The objective of the current study was to determine the taxonomic placement of fungi recovered from different varieties of the locoweeds *Astragalus mollissimus* and *A. lentiginosus*. The varieties investigated have been shown previously to contain the endophyte through PCR and fungal isolation and indirectly through the detection of swainsonine (Braun et al. 2003; Ralphs et al. 2008; Cook et al. 2011). Growth and morphology of fungal endophytes were examined and compared with *U. oxytropis*. In addition, phylogenetic analyses were conducted based on sequences of the ITS and *glyceraldehyde-3-phosphate dehydrogenase (GPD)* regions, and on random amplified polymorphic DNA (RAPD) banding patterns of two primers. Overall, comparisons of morphological and molecular phylogenetic results were used to determine the specific taxonomic placement of these fungi.

Materials and methods

Astragalus and *Oxytropis* spp. plants were collected from Arizona, Colorado, New Mexico, Oklahoma, Oregon, Texas, Utah, and Wyoming, USA (Braun et al. 2003; Ralphs et al. 2008; Oldrup et al. 2010) (Table 1). For fungal endophyte recovery, four pieces of stem (length of 1 cm), four leaflets, and four flowers, if present, were removed from dried plant material using sterile forceps. Stem pieces, leaflets, and flowers were soaked in 70% ethanol for 30 s, 20% bleach for 3 min and sterilized water for 30 s. After drying, the pieces were transferred onto water agar (WA) plates and observed daily. Seed isolation was done following methods of Oldrup et al. (2010) with seed coats being placed on WA for endophyte isolation. All fungi that grew out of the plant material were transferred to potato dextrose agar (PDA) and examined weekly for color of culture and presence of spores. For assessment of sporulation, isolates were grown on WA and PDA at room temperature and placed in natural light for 1–3 weeks. Two fungi isolated from *A. lentiginosus* plants produced spores on WA and were further examined by scraping the margin of growth with a sterilized scalpel and viewing conidia with a Nikon Optiphot microscope (Nikon, Tokyo, Japan). The mean and mode for the number of septa was determined by recording the number of septa for 40 randomly selected spores in a particular isolate. In addition, for each different number of septa observed, the length and width of 10 randomly selected spores were recorded and averaged.

For radial growth experiments, fungi were cultured on PDA, WA, weak potato-carrot agar (PCA) (Ray et al. 2005), and alfalfa stem agar (ALF). Alfalfa stem agar was made by adding 1 g of ground alfalfa to 500 mL of WA prior to autoclaving. Four fungal isolates from *A. lentiginosus plants*, two isolates from *O. sericea plants*, and five isolates from *A. mollissimus plants* were grown on each media (four replicates per isolate) at 30 °C for 3 weeks. Statistical analyses of total radial growth comparisons were performed using a single factor ANOVA with $P < 0.05$ to compare growth of each species on each media.

DNA was extracted from fungal cultures using the DNeasy Plant mini kit (Qiagen, Valencia, Calif., USA). Cultures were grown on PDA and 2 cm^2 blocks of mycelia were removed from the plate and ground in a 1.5 mL tube with the lysis buffer from the kit. Subsequent steps were done according to the manufacturer's directions. The ITS region and gpd gene were amplified using primer pairs ITS4 and Or1 (Oldrup et al. 2010), and GPD1 and GPD2 (Berbee et al. 1999), respectively. PCR products were purified with the Qiaquick PCR

Purification kit (Qiagen, Valencia, Calif., USA). Sequencing reactions were performed on the purified PCR products using Big Dye Terminator version 3.1 (Life Technologies, Carlsbad, Calif., USA). After purification, the sequencing reactions were separated on an ABI 3100 capillary sequencer (NMSU Molecular Analysis Services Laboratory, Las Cruces, N. Mex., USA). Sequences were examined using Chromas (Technelysium Pty Ltd., Eden Prairie, Minn., USA) and manually edited and aligned using ClustalW (Larkin et al. 2007). Sequences for the ITS region were analyzed from 64 isolates, and sequences from 59 isolates were examined for *GPD*.

Primers OPR12 (5 □ACAGGTGCGT-3 □) and OPA20 (5-GTTGCGATCC-3 □) were used for DNA fingerprinting of isolates through RAPD PCR. Total reaction volume was 50 □L, which contained 2 □L of extracted DNA, 2 mmol/L $MgCl_2$, 0.2 □m single primer, 400 □m dNTPs, and the recommended amount of GoTaq and its 5× buffer (Promega, Madison, Wisc., USA). Reactions were performed with an initial denaturation at 94 °C for 7 min, followed by 55 cycles of 94 °C for 15 s, 36 °C for 30 min, and 72 °C for 1 min, with a final elongation time of 5 min at 72 °C. PCR products were separated on a 1.7% gel at 90V for 2 h and then viewed under UV light. Alignments of banding patterns were done by comparison to 1 kb and 100 bp ladders (Promega, Madison, Wisc., USA) and other samples. All possible sizes of bands for each primer were recorded, samples were given a 1 for presence of band or 0 for absence of bands by visual examination, and a matrix was created to be used for neighbor-joining analyses.

Phylogenetic analyses employing ITS and GPD sequences was performed using Geneious 5.5.3. (Drummond et al. 2011), and a concatenated neighbor-joining tree was generated. Majority greedy clustering and Tamura–Nei genetic distance model were used with 1000 bootstrap replicates. For RAPD data, the 1 and 0 matrix for each primer was used to generate neighbor-joining trees with PAUP (Swofford 2003) with 1000 replicates bootstrap support.

Results

Morphology of endophytes was observed weekly on PDA. Endophytes isolated from *Astragalus* locoweed species varied in colony color, although all grew at similar slow rates. Endophytes isolated from *Oxytropis plant* species were dark black with a hard mycelia mass upon mature growth; whereas,isolates from Astragalus were gray to tan or brown. Isolates from *Astragalus* plants most often contained more aerial mycelia with less development of the hardening mycelia mass with age. Among the endophytes isolated from *Astragalus* species, those isolated from *A. mollissimus* varieties were often gray to dark gray, whereas fungi isolated from *A. lentiginosus* were tan to brown, leading to the names *Undifilum cinereum* and *Undifilum fulvum*, respectively (Figs. 1A-1J).

Mean radial growth of four replicates for endophytes from each locoweed species were calculated from growth on four different media. For each media, growth was statistically different for endophytes isolated from different locoweed species using ANOVA ($P < 0.05$). Fungi isolated from *A. mollissimus* varieties (*U. cinereum*) grew only on PDA (0.8 cm at 21 days). On both the ALF and PCA media, *A. lentiginosus* endophytes had significantly higher total radial growth (1.65 and 1.61 cm) compared to *U. oxytropis* (0.76 and 1.09 cm) ($P < 0.05$). However, *U. oxytropis* had the highest mean growth on WA (2.04 cm).

Sporulation of *A. lentiginosus* endophytes (*U. fulvum*) occurred from isolates growing directly out of stem or leaf pieces on WA with natural light. For each of two isolates examined, conidia were found to be ellipsoid with thin transepta (Figs. 1K-1L). The maximum number of septa observed was six for conidia from both isolates, although most

conidia had only 1-2 septa. Mean length of conidia of the isolate from *A. lentiginosus* var. *araneosus* was from 21.8 μm for conidia with one septum to 59.4 μm for conidia with five septa on WA after 21 days. For the isolate from *A. lentiginosus* var. lentiginosus, mean conidial length was from 23.1 μm for conidia with one septum to 37.8 μm for those with three septa.

Many of the isolates yielded identical sequences of ITS and *GPD*. From all 64 sequences examined for ITS, seven unique sequences were found, and from all 59 *GPD* sequences examined, six unique sequences were found. Of the 28 ITS and 25 *GPD* individual isolate sequences examined for *U. oxytropis*, four unique sequences were found for each region. For isolates of *A. lentiginosus* endophytes (*U. fulvum*), 19 ITS sequences, and 16 *GPD* sequences resulted in two unique sequences for ITS and one for *GPD*. Astragalus mollissimus isolates (U. cinereum) had two unique sequences for each region out of 17 ITS and 18 *GPD* individual sequences examined. These unique sequences were used for phylogenetic analyses as representatives of total isolates examined (Fig. 2, total number of isolates for each unique sequence in parentheses). Because *GPD* sequences contained one less unique sequence for *A. lentiginosus* isolates, the one unique sequence was duplicated for producing the concatenated tree. Neighbor-joining analyses showed that all isolates of *U. oxytropis*, from both *O.sericea* and *O. lambertii*, formed a single clade with 64% support (Fig. 2). All isolates from *Astragalus* spp. examined formed a separate clade from *U. oxytropis* with 70% support. Furthermore, endophytic fungi of *A. lentiginosus* varieties grouped separately from endophytic fungi of *A. mollissimus* varieties with support of 99%.

Two RAPD primers provided differing fragment sizes that allowed comparison of different isolates. For the primer OPA20, two fragment patterns were observed for endophytes from *A. lentiginosus* (*U. fulvum*), three patterns for *A. mollissimus* isolates (*U. cinereum*), and only a single pattern for both *O. lambertii* and *O. sericea* isolates; *U. oxytropis* (Fig. 3, patterns labeled with number of isolates and code for plant origin and location). Neighbor-joining analysis of these fragment patterns showed that the patterns of the *Astragalus* endophytic fungi were different from that of *U. oxytropis* (Fig. 3). The second primer used, OPR12, showing that all *Astragalus* endophytes were different from *U. oxytropis* using neighbor-joining analysis with a 68% bootstrap support (Fig. 4, patterns labeled with number of isolates and code for plant origin and location). This same primer analysis showed additionally that the *A. lentiginosus* endophytes differed from all others with a bootstrap support of 57%.

Taxonomy

1. *Undifilum fulvum* Baucom and Creamer, sp. nov. (Figs. 1 (B–F), 1 (K–L))

holotype—Isolate 15–1 from *Astragalus lentiginosus* var. *wahweapensis* from Henry Mt, Utah, USA, collected in 2007; dried culture with conidia on PDA deposited in Center for Natural History Collection at New Mexico State University, Las Cruces, New Mexico 88003.

description—*Colonia in solani tuberosi glucosi agaro (PDA), colore fulvus et al.iquando cum* mycelia alba. Post triginta dies margo fuscus circa *colonia factus est. Mycelium lente crescit,* 0.83–1.7 mm in solani tuberosi glucosi agaro post quadraginta *dies 28 °C. Mycelia hyalina, septata, duae species hyphae. Hyphae in centro valde densae 2–7 μm (4 μm latae). Hyphae in margine sunt 3–5 μm (3.6 μm latae). Conidia fecunda sunt in agaro aquae et remota in solani tuberosi glucosi agaro. Conidia solitaria, septata, et cum pigmento tenue. Septa 1–6 (medio 2.8). Conidia 23–66 μm × 3–6 μm (medio 36 μm × 4.4 μm).*

Colony on PDA tan to brown color with or without white aerial mycelia. Frequent dark brown margin around colony formation after 30 or more days of growth. Mycelial growth slow, 0.83–1.7 mm, on PDA after 45 days at 28 °C. Mycelia hyaline, septate, with two types of hyphae. Hyphae at center of colony are highly condensed with width of 2–7 μm (mean 4 μm). Hyphae at colony margin and surface are less dense with surface hyphae aerial and width 3–5 μm (mean 3.6 μm). Conidia production prolific on WA and sporadic on PDA. Conidia are solitary, septate with light pigmentation. Number of septa 1–6 (mean 2.8, mode 2). Conidia 23–66 μm × 3–6 μm (mean 36 μm × 4.4 μm); L/W 8.2.

distribution—*Undifilum fulvum* was isolated from hosts *A. lentiginosus* var. *araneosus* collected from Wahwah Valley, Utah, USA, *A. lentiginosus* var. *diphysus* collected from St. Johns and Winslow, Arizona, USA, *A. lentiginosus* var. *lentiginosus* collected from Juntura, Oregon, USA, and *A. lentiginosus* var. *wahweapensis* collected from Apple Bush, Hanksville, and Henry Mt., Utah, USA.

2. *Undifilum cinereum* Baucom and Creamer sp. nov. Figs. 1G-1J

holotype—Isolate 4–4 from *Astragalus mollissimus* var. *earleii* Alpine, Texas, USA, collected in 2007; dried culture on PDA deposited in Center for Natural History Collection at New Mexico State University, Las Cruces, New Mexico 88003.

description—*Colonia in solani tuberosi glucosi agaro (PDA), colore cinereus vel brunneus,aliquando cum margine obscuro coloniae. Mycelium lente crescit, 0.6–0.8 mm in solani tuberosi glucosi agaro post quadraginta dies 28 °C. Mycelia hyalina, septata, duae species hyphae. Hyphae in centro coloniae densae 4–5 μm (medio 4.6 μm). Hyphae in margine sunt tenues, 3–5 μm latae (medio 4.1 μm). Conidia caret.*

Colony on PDA gray to dark gray to tan color with or without dark gray to black margin of colony. Mycelial growth slow, 0.6–0.8 mm on PDA after 45 days at 28 °C. Mycelia hyaline, septate with two types of hyphae. Hyphae at center of colony condensed with width 4–5 μm (mean 4.6 μm). Hyphae at colony margin and surface less dense with some aerial hyphae width 3–5 μm (mean 4.1 μm). No conidia produced on PDA.

distribution—*Undifilum cinereum* was isolated from hosts *A. mollissimus* var. *biglovii* collected from Colfax and Jornada, New Mexico, USA, *A. mollissimus* var. *earleii* collected from Alpine and Ft. Davis, Texas, USA, and A. mollissimus var. mollissimus collected from Capulin and Farley, New Mexico, and Kenton, Oklahoma, USA.

Discussion

We described two new species of the fungal endophyte, *Undifilum*, isolated from two *Astragalus* spp. of locoweed using both morphological and molecular techniques. The new species, *U. fulvum* from *A. lentiginosus* and *U. cinereum* from *A. mollissimus*, were closely related to another species of the genus, *U. oxytropis*; however, they differ in both nucleic acid sequence and morphology. All three species are endophytes isolated from locoweed plants and have been placed in the genus *Undifilum* (Braun et al. 2003; Pryor et al. 2009).

Fungi isolated from *Astragalus* plants differed in color and in sporulation from *U. oxytropis*. *Undifilum fulvum* colonies were tan to brown in color on PDA, whereas, *U. cinereum* colonies were gray to black, both with aerial, fluffy hyphae. *Undifilum oxytropis* colonies were constantly black with a solid mass of mycelium on PDA with little to no aerial hyphae consistent with previous descriptions (Braun et al. 2003; Pryor et al. 2009). Conidia produced by *U. fulvum* were frequently ellipsoidal in shape with thin transverse septa,

consistent with described conidia of U. oxytropis and differing from conidia of both *Embellisia* and *Alternaria* (Pryor et al. 2009). In addition, longer conidia of *U. fulvum* had more septa than *U. oxytropis* (Pryor et al. 2009), but *U. fulvum* conidia had, on average, fewer septa. *Undifilum cinereum* did not produce any conidia and therefore also differed from *U. oxytropis*, which sporulates proficiently on WA in natural light. Significant differences were observed in total radial growth between the three different *Undifilum* spp. examined. This is consistent with the differences in culture morphology seen between *U. oxytropis*, *U. fulvum*, and *U. cinereum*.

Fungi isolated from *Astragalus mollissimus* species have been demonstrated previously to produce swainsonine, which is congruent with isolates of *U. oxytropis* (Braun et al. 2003; Ralphs et al. 2008). *Undifilum*, when described as a new genus, was shown to be a separate clade from *Embellisia* and *Alternaria* genera (Pryor et al. 2009). By using *Embellisia* sp. and *Alternaria* spp. as outgroups in the phylogenetic studies here, all *Undifilum* isolates examined were supported as separate from these close relatives. Further distinction between *U. fulvum* and *U. cinereum* was also observed from phylogenetic analyses of ITS and *GPD* sequences and genomic restriction patterns, specifically from RAPD primer OPR12, supporting differences seen in colony morphology and in spore production and morphology.

Fungal endophytes isolated from different species of *Astragalus* fall into different species of *Undifilum*, whereas fungi isolated from all examined *Oxytropis* species thus far have been classified as *U. oxytropis*. This is interesting on an evolutionary basis because *A. mollissimus* varieties also have higher levels of genetic variability than *Oxytropis* species based on chloroplast DNA (Kulshreshtha et al. 2004). Further research will be necessary to determine the possible existence of co-speciation between the fungal endophyte and its host.

Although fungal endophytes isolated from several different *Oxytropis* species both in the United States and China have been shown to be the same species, *U. oxytropis* (Pryor et al. 2009; Yu et al. 2010), no studies have investigated the taxonomic placement of fungal endophytes from several *Astragalus* species. Yu et al. (2010) included a swainsonine-producing endophyte isolated from *A. variabilis* that they classified on the basis of morphology and ITS sequence as *Undifilum oxytropis*. In addition, a pathogenic fungus isolated from *A. adsurgens* in China was classified as *Embellisia astragalii* (Li and Nan 2007). Since early identifications of Undifilum fungi were classified as *Embellisia*, the relationship between these fungi with the newly described species is not known. In depth characterization of fungal endophytes from Chinese *Astragalus* plants is needed to resolve this disparity with the genetic differences seen in isolates from United States Astragalus species.

Here we have characterized the fungal endophytes of *A. mollissimus* and *A. lentiginosus* varieties from the United States and confirmed their similarity to fungal endophytes isolated from *Oxytropis lambertii* and *O. sericea*. The presence of these host plant species is a common systemic problem in rangelands throughout North America. A better understanding of the toxin producing fungi that colonize these plants is essential to developing management strategies to overcome this obstacle in rangeland management.

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References

Achata Böttger JA, Creamer R, Gardner D. Seasonal changes in *Undifilum* colonization and swainsonine content of locoweeds. J. Chem. Ecol. 2012; 38(5):486–495. doi:10.1007/s10886-012-0120-z. PMID:22544335. [PubMed: 22544335]

Berbee ML, Pirseyedi M, Hubbard S. *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde 3-phosphate dehydrogenase gene sequences. Mycologia. 1999; 91(6):964–977. doi:10.2307/3761627.

Braun K, Romero J, Liddell C, Creamer R. Production of swainsonine by fungal endophytes of locoweed. Mycol. Res. 2003; 107(8):980–988. doi:10.1017/S095375620300813X. PMID:14531620. [PubMed: 14531620]

Colegate SM, Dorling PR, Huxtable CR. Spectroscopic investigation of swainsonine – alpha-mannosidase inhibitor isolated from *Swainsona-canescens*. Aust. J. Chem. 1979; 32(10):2257–2264. doi:10.1071/CH9792257.

Cook D, Gardner DR, Ralphs M, Pfister JA, Welch KD, Green BT. Swainsonine concentrations and endophytes amounts of *Undifilum oxytropis* in different plant parts of Oxytropis sericea. J. Chem. Ecol. 2009; 35(10):1272–1278. doi:10.1007/s10886-009-9710-9. PMID:19904570. [PubMed: 19904570]

Cook D, Gardner DR, Grum DS, Pfister JA, Ralphs MH, Welch KD, Green BT. Swainsonine and endophyte relationships in *Astragalus mollissimus* and *Astragalus lentiginosus*. J. Agric. Food Chem. 2011; 59(4):1281–1287. doi:10.1021/jf103551t. PMID:21214242. [PubMed: 21214242]

Drummond, AJ.; Ashton, B.; Buxton, S.; Cheung, M.; Cooper, A.; Duran, C.; Field, M.; Heled, J.; Kearse, M.; Markowitz, S.; Moir, R.; Stones-Havas, S.; Sturrock, S.; Thierer, T.; Wilson, A. [accessed 1 December 2011] Geneious v5.4. 2011. [Online.] Available from http://www.geneious.com

Gardner DR, Molyneux RJ, Ralphs MH. Analysis of swainsonine: extraction methods, detection, and measurement in populations of locoweeds (*Oxytropis* spp.). J. Agric. Food Chem. 2001; 49(10):4573–4580. doi:10.1021/jf010596p. PMID:11599990. [PubMed: 11599990]

James, LF.; Panter, KE. Locoweed poisoning in livestock. In: James, LF.; Elbein, AD.; Molyneux, RJ.; Warren, CD., editors. Swainsonine and related Glycosidase inhibitors. Iowa State University Press; Ames, Iowa: 1989. p. 23-38.

Kulshreshtha S, Creamer R, Sterling TM. Phylogenetic relationships among New Mexico *Astragalus mollissimus* varieties and *Oxytropis* species by restriction fragment analysis. Weed Sci. 2004; 52(6):984–988. doi:10.1614/WS-03-143R1.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics. 2007; 23(21):2947–2948. doi:10.1093/bioinformatics/btm404. PMID:17846036. [PubMed: 17846036]

Li Y, Nan Z. A new species, *Embellisia astragali* sp. nov., causing standing milk-vetch disease in China. Mycologia. 2007; 99(3):406–411. doi:10.3852/mycologia.99.3.406. PMID:17883032. [PubMed: 17883032]

Marsh, CD. The loco-weed disease of the plains. Government printing office; Washington, D.C.: 1909. p. 125

McLain-Romero J, Creamer R, Zepeda H, Strickland J, Bell G. The toxicosis of *Embellisia* fungi from locoweed (*Oxytropis lambertii*) is similar to locoweed toxicosis in rats. J. Anim. Sci. 2004; 82(7):2169–2174. PMID:15309966. [PubMed: 15309966]

Oldrup E, McLain-Romero J, Padilla A, Moya A, Gardner D, Creamer R. Localization of endophytic *Undifilum* fungi in locoweed seed and influence of environmental parameters on alocoweed in vitro culture system. Botany. 2010; 88(5):512–521. doi:10.1139/B10-026.

Patrick M, Adlard MW, Keshavarz T. Production of an indolizidine alkaloid, swainsonine by the filamentous fungus, *Metarhizium anisopliae*. Biotechnol. Lett. 1993; 15(10):997–1000. doi:10.1007/BF00129924.

Pryor BM, Creamer R, Shoemaker RA, McLain-Romero J, Hambleton S. *Undifilum*, a new genus for endophytic *Embellisia oxytropis* and parasitic *Helminthosporium bornmuelleri* on legumes. Botany. 2009; 87(2):178–194. doi:10.1139/B08-130.

Ralphs MH, Creamer R, Baucom D, Gardner DR, Welsh SL, Graham JD, Hart C, Cook D, Stegelmeier BL. Relationship between the endophyte *Embellisia* spp., and the toxic alkaloid swainsonine in major locoweed species (*Astragalus* and *Oxytropis*). J. Chem. Ecol. 2008; 34(1): 32–38. doi:10.1007/s10886-007-9399-6. PMID:18060459. [PubMed: 18060459]

Ray JD, Burgess T, Malajczuk N, Hardy GE, St J. First report of *Alternaria* blight of *Paulownia* spp. Aust. Plant Pathol. 2005; 34(1):107–109. doi:10.1071/AP04087.

Schneider M, Ungemach F, Broquist H, Harris T. (1S,2R,8R,8aR)-1,2,8-trihydroxyoctahydroindolizine (swainsonine), an -mannosidase inhibitor from *Rhizoctonia leguminicola*. Tetrahedron. 1983; 39(1):29–32. doi:10.1016/S0040-4020(01)97625-2.

Stegelmeier B, James L, Panter K, Molyneux R. Serum swainsonine concentration and -mannosidase activity in cattle and sheep ingesting *Oxytropis sericea* and *Astragalus lentiginosus*. Am. J. Vet. Res. 1995; 56(2):149–154. PMID:7717575. [PubMed: 7717575]

Swofford, DL. PAUP Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates; Sunderland, Mass: 2003.

Tulsiani DRP, Broquist HP, James LF, Touster O. The similar effects of swainsonine and locoweed on tissue glycosidases and oligosaccharides of the pig indicate that the alkaloid is the principal toxin responsible for the inductions of locoism. Arch. Biochem. Biophys. 1984; 232(1):76–85. doi: 10.1016/0003-9861(84)90522-8. PMID:6430242. [PubMed: 6430242]

Tulsiani DRP, Broquist HP, James LF, Touster O. Production of hybrid glycoproteins and accumulation of oligosaccharides in the brain of sheep and pigs administered swainsonine or locoweed. Arch. Biochem. Biophys. 1988; 264(2):607–617. doi:10.1016/0003-9861(88)90327-X. PMID:3135781. [PubMed: 3135781]

Wang Q, Nagao H, Li Y, Wang H, Kakishima M. *Embellisia oxytropis*, a new species isolated from Oxytropis kansuensis in China. Mycotaxon. 2006; 95:255–260.

Welsh, SL.; Ralphs, MH.; Panter, KE.; Pfister, JA.; James, LF. Locoweed of North America; taxonomy and toxicity. In: Panter, KE.; Pfister, JA., editors. Poisonous plants: global research and solutions. CABI; Wallingford, U.K.: 2007. p. 20-29.

Yu Y, Zhao Q, Wang J, Wang J, Wang Y, Song Y, Geng G, Li Q. Swainsonine-producing fungal endophytes from major locoweed species in China. Toxicon. 2010; 56(3):330–338. doi:10.1016/j.toxicon.2010.03.020. PMID:20380845. [PubMed: 20380845]

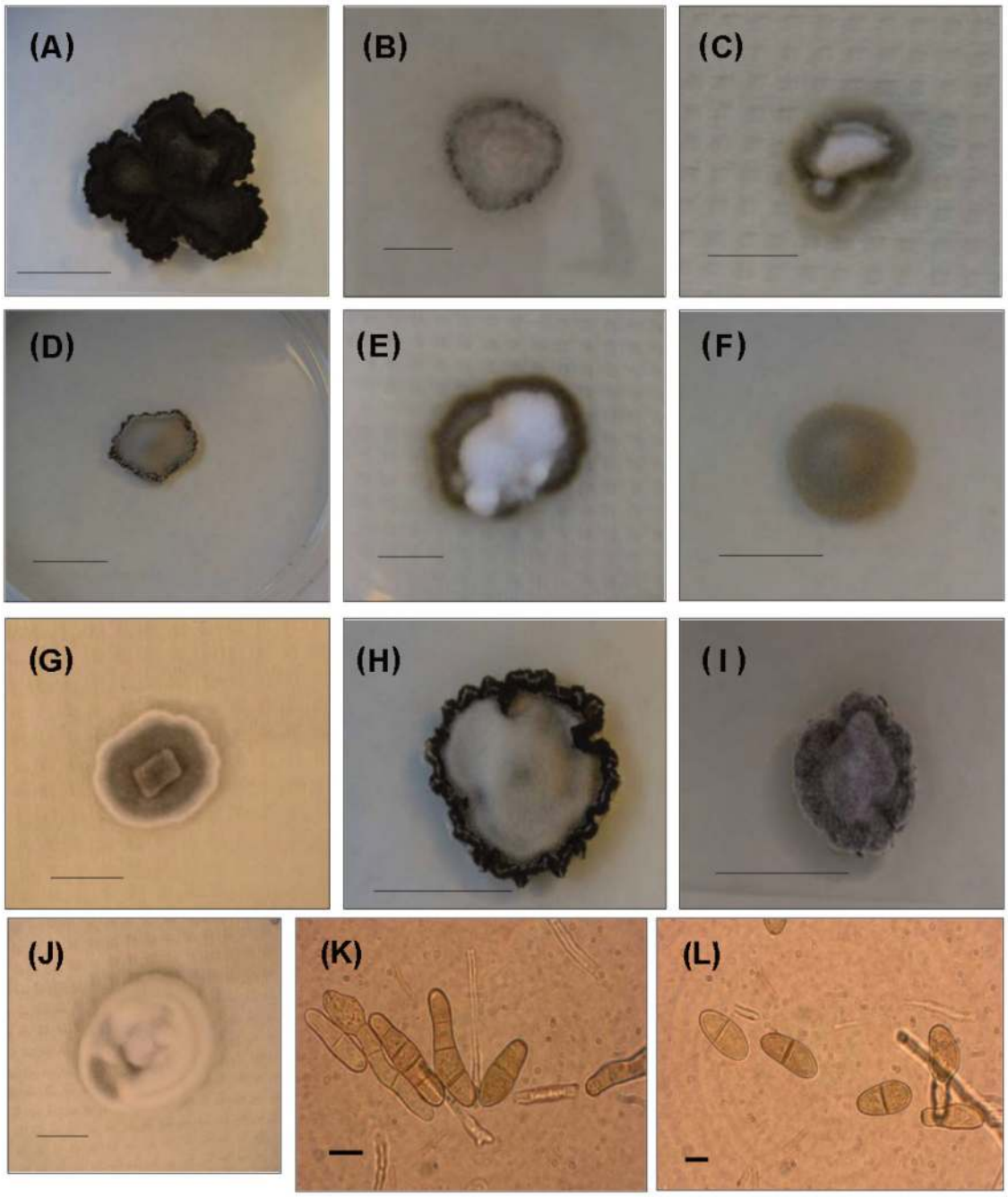

Fig. 1.

Undifilum species. (A) *Undifilum oxytropis from Oxytropis sericea*. (B–F) *Undifilum fulvum* from *Astragalus lentiginosus*. (G–J) *Undifilum cinereum* from *A. mollissimus*. (K–L) *Conidia from Undifilum fulvum*. (A–J) Cultures grown on potato dextrose agar. (K–L) Samples taken from cultures grown on water agar. Scale bars: A–J = 15 mm, K–L = 10 mm.

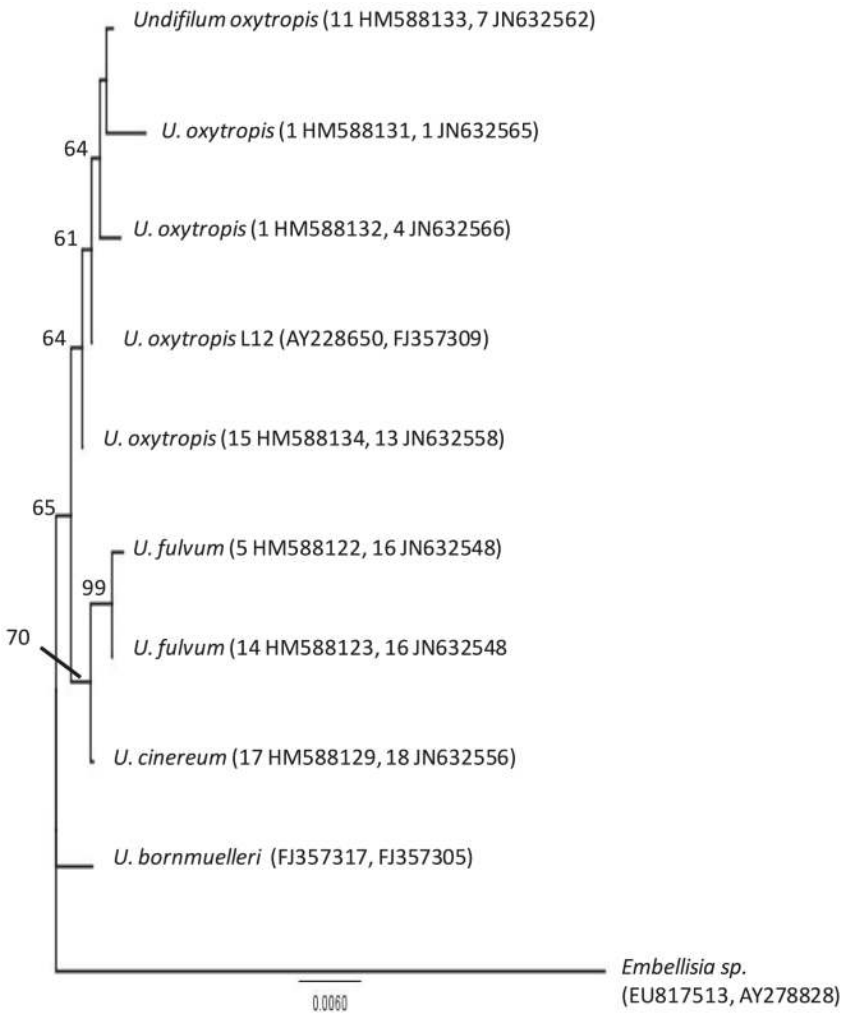

Fig. 2.

Neighbor-joining concatenated tree based on fungal internal transcribed spacer (ITS) and *glyceraldehyde-3-phosphate dehydrogenase (GPD)* sequences of endophytes isolated from Astragalus lentiginosus (Undifilum fulvum), A. mollissimus (U. cinereum), and *Oxytropis* spp. (*U. oxytropis*). Previously described specimens for *U. oxytropis* (L12) and *U. bornmuelleri* as well as closely related species *Embellisia* sp. included for comparison and as outgroup, respectively. Numbers following names at branch-tips are the number of isolates or sequences examined that had the same sequence, and GenBank Accession numbers of reference sequences. Bootstrap values based on 1000 replicates ≥50% indicated for major nodes.

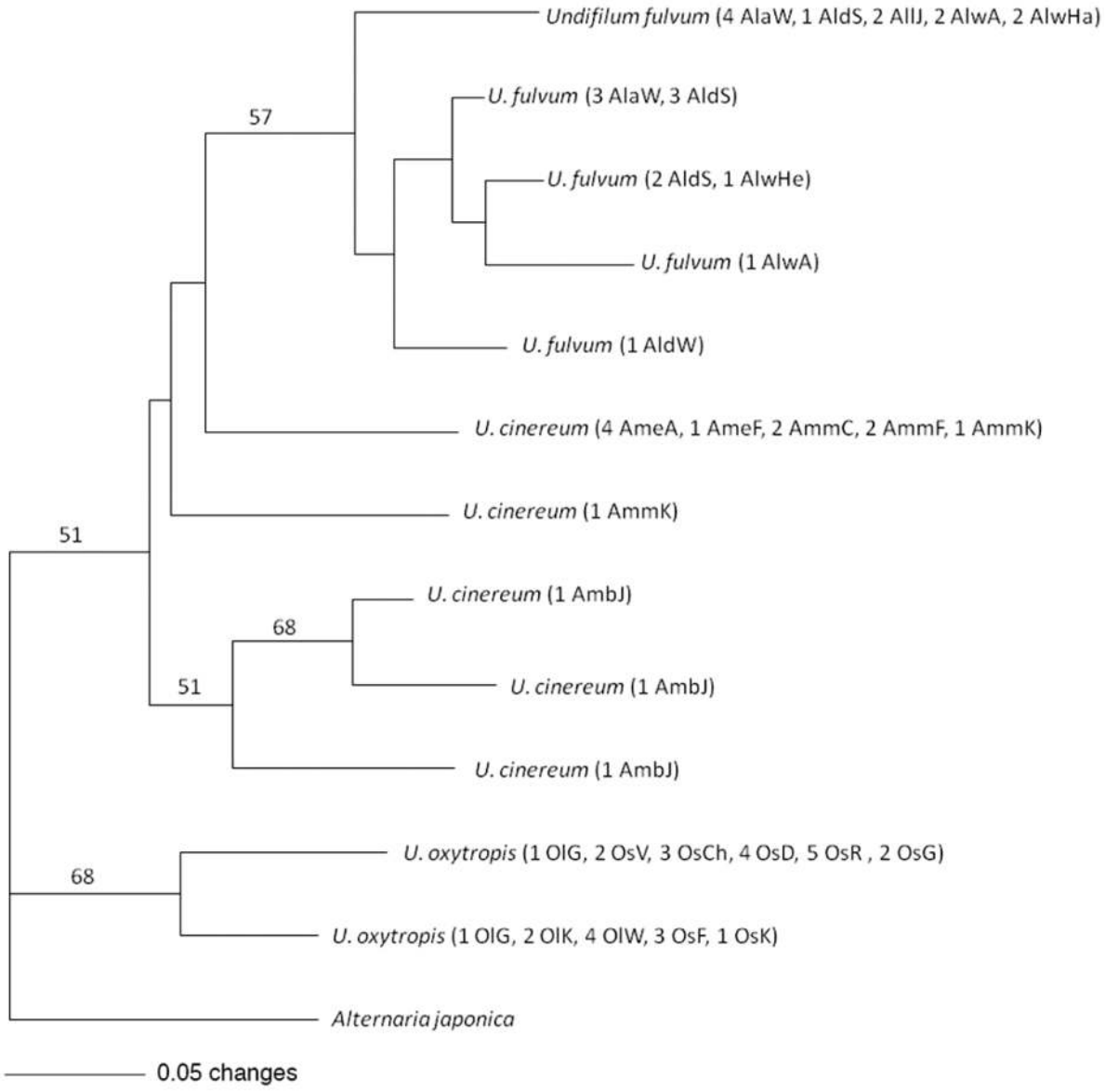

Fig. 3.

Neighbor-joining tree based on random amplified polymorphic DNA (RAPD) patterns generated from primer OPR12 for fungal endophytes isolated from *Astragalus lentiginosus (Undifilum fulvum)*, *A. mollissimus (U. cinereum)*, and *Oxytropis* spp. (*U. oxytropis*). Labels following species are number of isolates examined for host and collection site corresponding to Table 1 codes. Bootstrap values based on 1000 replicates ≥50% indicated for major nodes.

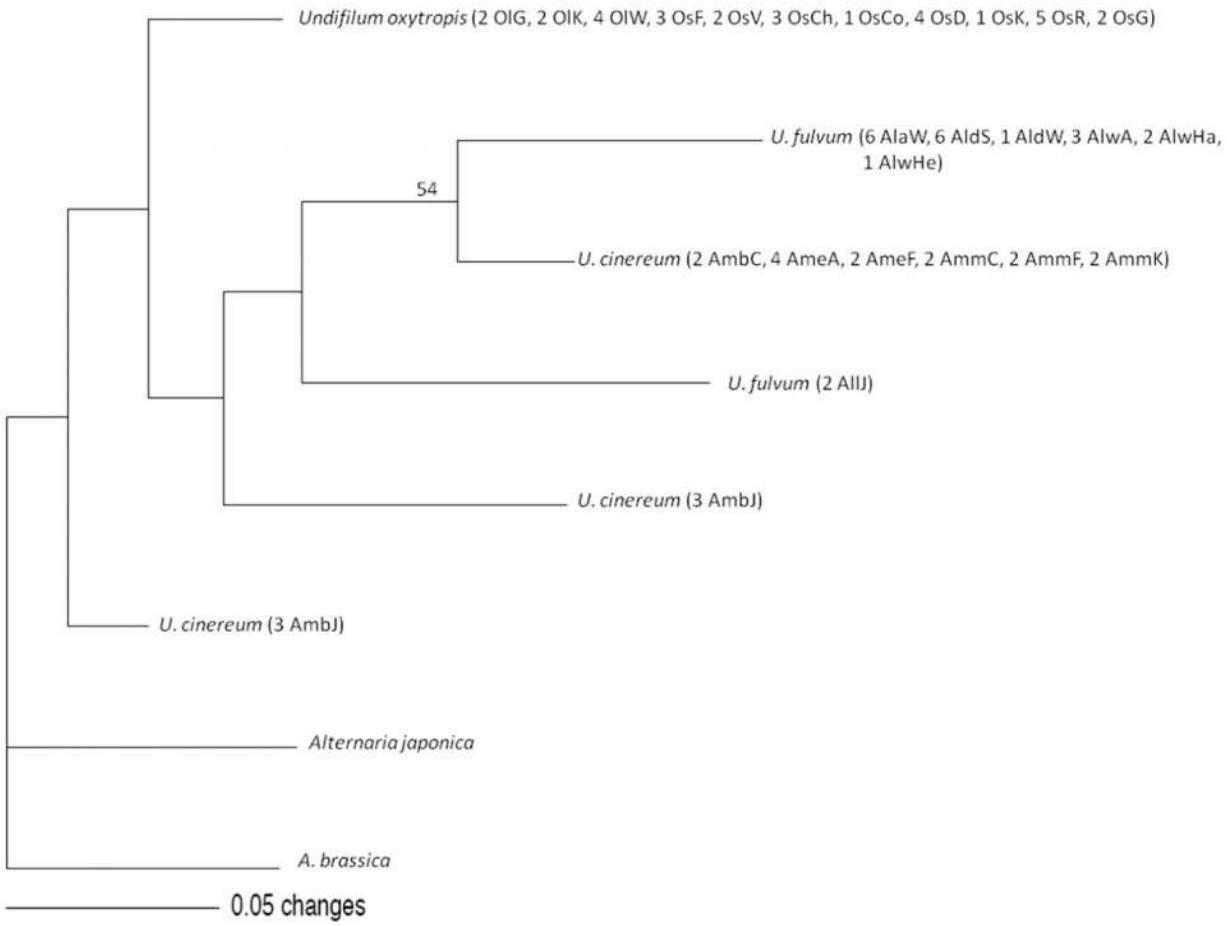

Fig. 4.

Neighbor-joining tree based on random amplified polymorphic DNA (RAPD) patterns generated from primer OPA20 for fungal endophytes isolated from *Astragalus lentiginosus (Undifilum fulvum)*, *A. mollissimus (U. cinereum)*, and *Oxytropis* spp. (*U. oxytropis*). Labels following species are number of isolates examined for host and collection site corresponding to Table 1 codes. Bootstrap values based on 1000 replicates ≥50% indicated for major nodes.

Table 1

Plant source and collection location, internal transcribed spacer (ITS) and *glyceraldehyde-3-phosphate dehydrogenase (GPD)* GenBank Accession numbers, and random amplified polymorphic DNA (RAPD) codes for fungal isolates.

	Plant origin				ITS		GPD		RAPD		
Fungus	**Genus**	**Species**	**Variety**	**Locations**	**No.***	**Rep.†**	**No.***	**Rep.†**	**Code‡**	**OPR12***	**OPA20***
Undifilum fulvum	*Astragalus*	*lentiginosus*	araneosus	Wahwah Valley, UT	4	HM588122	5	JN632548	AlaW	7	6
					2	HM588123					
			diphysus	St. Johns, AZ	5	HM588123	4	JN632548	AldS	6	6
				Winslow, AZ	1	HM588123	1	JN632548	AldW	1	1
			lentiginosus	Juntura, OR	2	HM588123	2	JN632548	AllJ	2	2
			wahweapensis	Apple Bush, UT	3	HM588123	2	JN632548	AlwA	3	3
				Hanksville, UT	1	HM588122	1	JN632548	AlwHa	2	2
					1	HM588123		JN632548			
				Henry Mts, UT			1	JN632548	AlwHe	1	1
Undifilum cinereum	*Astragalus*	*mollissimus*	biglovii	Colfax, NM	2	HM588129	2	JN632556	AmbC		2
				Jornada, NM	4	HM588129	5	JN632556	AmbJ	3	6
			earleii	Alpine, TX	2	HM588129	1	JN632556	AmeA	4	4
				Ft. Davis, TX	4	HM588129	4	JN632556	AmeF	1	2
			mollissimus	Capulin, NM	1	HM588129	2	JN632556	AmmC	2	2
				Farley, NM	2	HM588129	2	JN632556	AmmF	2	2
				Kenton, OK	2	HM588129	2	JN632556	AmmK	2	2
Undifilum oxytropis	*Oxytropis*	*lambertii*		Grant, NM	3	HM588133	2	JN632558	OlG	2	2
				Kingston, NM	1	HM588131	1	JN632566	OlK	2	2
					2	HM588133	1	JN632562			
				Winston, NM	1	HM588132	2	JN632558	OlW	4	4
					4	HM588133	1	JN632566			
	Oxytropis	*sericea*		Flagstaff, AZ	1	HM588134	2	JN632558	OsF	3	3
				Virgindale, CO	2	HM588134	1	JN632566	OsV	2	2
							1	JN632558			
				Chico, NM	4	HM588134	2	JN632562	OsCh	3	3
							1	JN632558			

Fungus	Plant origin				ITS		GPD		RAPD		
	Genus	Species	Variety	Locations	No.*	Rep.†	No.*	Rep.†	Code‡	OPR12*	OPA20*
				Colfax, NM					OsCo		1
				Des Moines, NM	4	HM588134	2	JN632562	OsD	4	4
							1	JN632558			
				Kanub, UT	1	HM588133	1	JN632566	OsK	1	1
				Raft River, UT	1	HM588133	1	JN632562	OsR	5	5
					2	HM588134	4	JN632558			
				Green River, WY	2	HM588134	1	JN632565	OsG	2	2
							1	JN632562			
Undifilum oxytropis §						AY228650		FJ357309			
Undifilum bornmuelleri §						FJ357317		FJ357305			
Embellisia sp.§						EU817513		AY278828			

*Number of isolates examined.

†GenBank Accession numbers for representative sequence for isolates examined.

‡Code for RAPD patterns (Figs. 3 and 4) for plant host and collection location.

§Fungi used for phylogenetic comparisons.