

## ***Wojnowicia viburni*, sp. nov., from China and its phylogenetic placement**

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A new species of *Wojnowicia* was isolated from leaves of *Viburnum utile* in Guizhou Province, China. The new species, *Wojnowicia viburni*, is described, illustrated and compared with similar species. It differs from other *Wojnowicia* species in its 6–8 euseptate, smaller conidia and lack of setae. Phylogenetic analysis of LSU rDNA sequence data shows that the species groups with *W. hirta* and *Ophiosphaerella herpotricha* in the family *Phaeosphaeriaceae*. The genus *Wojnowicia* is emended to include species without setae. This is the first record of the genus from China as well as from Asia. A key is provided for the four species accepted in *Wojnowicia*.

Keywords: asexual fungi, coelomycetes, molecular phylogeny, taxonomy.

Coelomycetes are a group of asexual fungi belonging to many families within the Ascomycota, although a few genera (e.g. *Ellula* and *Fibulocoela*) are Basidiomycota (Wijayawardene *et al.* 2012 a). Many coelomycetous asexual states belong to the class Dothideomycetes (Chomnunti *et al.* 2011, Crous *et al.* 2009, Liu *et al.* 2012, Zhang *et al.* 2012) but, in general, their taxonomic placement is poorly established. Approximately 37 % of coelomycete genera are linked to sexual states or can be accommodated in ascomycete families (Wijayawardene *et al.* 2012 b) however, many cannot be accommodated in the Ascomycota taxonomic framework because they lack molecular data. Wijayawardene *et al.* (2012 c) emphasized the need for recollecting, isolating

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and sequencing these orphan coelomycete genera so that they can be placed in natural taxonomic position.

*Wojnowicia* Sacc. was introduced by Saccardo (1892) with *W. hirta* (J. Schröt.) Sacc. (= *Hendersonia hirta* J. Schröt.) as the type species. The genus was revised by Sutton (1980) and Farr & Bills (1995) accepted three species. However, this genus was not addressed by molecular studies except de Gruyter *et al.* (2009) who have shown it is belonging to Phaeosphaeriaceae. In this paper we describe a new *Wojnowicia* species and discuss its taxonomic placement in Phaeosphaeriaceae.

## Materials and methods

### Collection and isolation

Plant pathogenic and saprobic coelomycetes were collected during a field survey in Guizhou Province, China. Leaves of *Viburnum utile* with disease symptoms were returned to the laboratory where they were observed under a stereo microscope. The fungus was isolated by the single spore isolation method as described in Chomnunti *et al.* (2011). Germinating spores were transferred aseptically to potato dextrose agar (PDA) plates and grown at 25 °C. Colony colour and morphological characteristics were assessed after 2, 4 and 6 weeks. The holotype specimen is deposited in the Herbarium of the Department of Plant Pathology, Agricultural College, Guizhou University (HGUP) (HGUP500 holotype) and the isotype is deposited in Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand (isotype MFLU12-2221). Living cultures are deposited at the Culture Collection at Mae Fah Luang University (MFLUCC), culture collection at Department of Plant Pathology, Agricultural College, Guizhou University (HGUPCC) and at Landcare Research, Private Bag 92170, Auckland, New Zealand (ICMP).

Genomic DNA was extracted from fresh mycelia, following the specification of Biomiga Fungus Genomic DNA Extraction Kit (GD2416). The primers ITS5 and ITS4, NS1 and NS4 (White *et al.* 1990) and LROR and LR5 (Vilgalys & Hester 1990) were used to amplify the regions internal transcribed spacers (ITS), small subunit rDNA (SSU) and large subunit rDNA (LSU), respectively. Polymerase chain reaction (PCR) amplification was carried out the method described in Phillips *et al.* (2008). LSU sequence of *W. viburni* and sequences downloaded from GenBank were aligned using Bioedit (Hall 2004) and ClustalX (Kohli & Bachhawat 2003). Alignments were checked and manual adjustments were made wherever necessary. Phylogenetic analyses were performed by using MEGA 5 (Tamura *et al.* 2011) for maximum likelihood (ML).

For phylogenetic analyses, DNA sequences of the LSU region of *W. viburni* together with reference taxa of different families of Pleosporales obtained from GenBank (Tab. 1) were aligned using Bioedit (Hall 2004). A blast search was carried out to find the closest matches with taxa in the family Phaeosphaeriaceae. The whole ambiguously aligned regions within each

**Tab. 1.** Sequences representing the closest taxa to *Wojnowicia* available in GenBank.

Taxon	Accession number	GenBank number	
		LSU	SSU
<i>Ampelomyces quisqualis</i>	CBS 129.79	EU754128	EU754029
<i>Chaetosphaeronema hispidulum</i>	CBS 216.75		EU754041
<i>Cochliobolus heterostrophus</i>	AFTOL-ID 54	AY544645	
<i>Cochliobolus sativus</i>	AFTOL-ID	DQ678045	
<i>Coniothyrium palmarum</i>	CBS 400.71	EU754153	
<i>Coniothyrium palmarum</i>	CBS 758.73	EU754154	
<i>Cucurbitaria berberidis</i>	CBS 394.84	GQ387605	
<i>Didymella exigua</i>	CBS 183.55	EU754155	
<i>Didymella pisi</i>	CBS 126.54	GU237968	
<i>Dothidotthia aspera</i>	CPC 12933		EU673228
<i>Dothidotthia symphoricarpi</i>	CBS119687		EU673224
<i>Leptosphaeria doliolum</i>	CBS 541.66	JF740284	
<i>Leptosphaeria doliolum</i>	CBS 155.94	JF740282	
<i>Leptosphaeria doliolum</i>	CBS 125979	JF740283	
<i>Leptosphaeria slovacica</i>	CBS 389.80	JF740315	
<i>Leptosphaerulina australis</i>	CBS 317.83	GU301830	
<i>Ophiosphaerella herpotricha</i>	AFTOL-ID 1595	DQ767656	DQ767650
<i>Ophiosphaerella herpotricha</i>	CBS 620.86		DQ678010
<i>Phaeosphaeria avenaria</i>	DAOM 226215		AY544725
<i>Phaeosphaeria eustoma</i>	CBS 573.86		DQ678011
<i>Phaeosphaeriopsis musae</i>	CBS 120026		GU296186
<i>Phaeosphaeria nodorum</i>	CBS 110109	EU754175	
<i>Phaeosphaeria oryzae</i>	CBS 110110	GQ387591	
<i>Phoma caloplacae</i>	CBS 129338	JQ238643	
<i>Phoma cladoniicola</i>	CBS 128027	JQ238631	
<i>Phoma cladoniicola</i>	CBS 128026	JQ238628	
<i>Phoma foliaceiphila</i>	CBS 129141	JQ238640	
<i>Phoma herbarum</i>	CBS 615.75	EU754186	
<i>Paraphoma radicina</i>	CBS 102875		EU754091
<i>Phoma zae-maydis</i>	CBS 588.69	EU754192	
<i>Pleospora calvescens</i>	CBS 246.79	EU754131	
<i>Pleospora herbarum</i>	CBS 191.86	GU238160	
<i>Preussia minima</i>	AFTOL-ID 1256	DQ678056	
<i>Pyrenochaeta acicula</i>	CBS 122789	EU754204	
<i>Pyrenochaeta nobilis</i>	CBS 407.76	EU754206	
<i>Pyrenochaeta quercina</i>	CBS 115095	GQ387619	
<i>Pyrenochaetopsis decipiens</i>	CBS 343.85	GQ387624	
<i>Pyrenochaetopsis leptospora</i>	CBS 101635	GQ387627	
<i>Stagonospora foliicola</i>	CBS 343.86		EU754118
<i>Pyrenophora phaeocomes</i>	AFTOL-ID 283	DQ499596	
<i>Wojnowicia hirta</i>	CBS 160.73	EU754222	EU754123
<i>Wojnowicia hirta</i>	CBS 295.69	EU754223	EU754124
<i>Wojnowicia viburni</i>	MFLUCC 120733	KC594287	KC594288

dataset were excluded from the analyses (Begoude *et al.* 2010). In the analyses, gaps were treated as missing data, and all characters were unordered and of equal weight (Liu *et al.* 2011, 2012). Maximum-likelihood analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated from 1000 bootstrap replications (Hillis & Bull 1993).

### Taxonomy

***Wojnowicia* Sacc., Syll. fung. (Abellini) 10: 328 (1892) emended**

**Habit:** Associated with leaf spots of *Viburnum utile*, conifer litter, on culms of *Triticum* spp. **Sexual state:** *Ophiosphaerella*-like. **Asexual state:** Conidiomata pycnidial, at first immersed, later appearing superficial by decay of host tissues, separate, globose, often markedly papillate or non papillate, dark brown; walls thick, composed of dark brown, thick-walled *textura angularis* becoming hyaline and thin-walled towards the inner conidiogenous region. Ostiole central or displaced to one side, papillate, circular. Setae absent or presence, when present formed around the ostiole or from the lateral pycnidial walls, straight or flexuous, unbranched, brown, septate, smooth. Conidiophores absent. Conidiogenous cells enteroblastic, phialidic, determinate, discrete, doliiform to ampulliform, hyaline, smooth, channel and collarete minute, formed from the inner pycnidial wall cells. Conidia pale brown, with several transverse eusepta, continuous, straight or curved, fusiform or cylindrical, apex and base obtuse, thin-walled, smooth, guttulate.

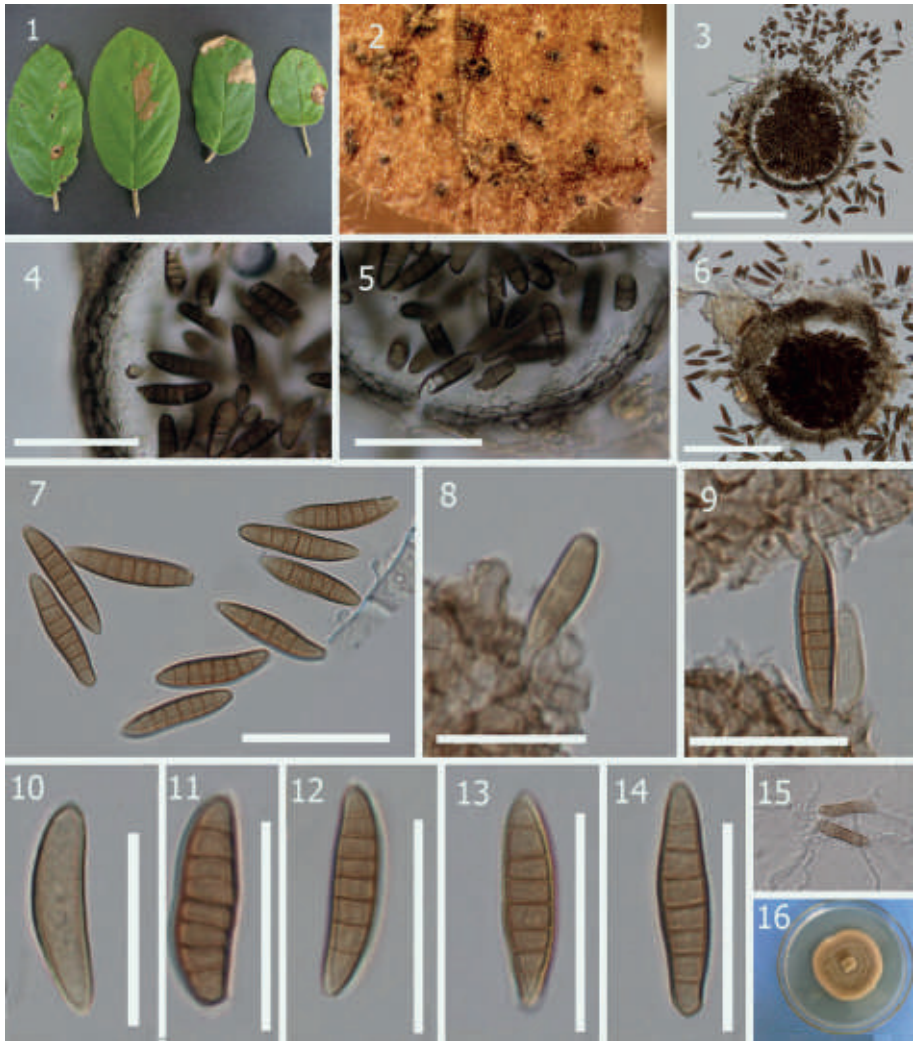
***Wojnowicia viburni* D. N. N. Wijayawardene, Yong Wang bis & K. D. Hyde, sp. nov.**

Mycobank no.: MB 803464

**Etymology.** – Named after the host genus on which the fungus occurs.

**Type.** – CHINA, Guizhou Province, Kaiyang, Longguang, on *Viburnum utile* Hemsl. leaves, 3 June 2012, *leg.* D. N. N. Wijayawardene G0603–5 (HGUP500 holotype; MFLU12–2221 isotype), ex-type living culture at MFLUCC 120733 = ICMP 19778 = HGUPCC N28.

**Description.** – Associated with leaf spots of *Viburnum utile*, on both sides, irregular, brown, leaf spots surrounded by dark brown border. **Sexual state** not observed. **Asexual state:** Conidiomata pycnidial, 175–200 µm diam., 190–220 µm high, abundant on upper surface, partly immersed in the host tissue, scattered, solitary, non-papillate, brown. Pycnidial wall 17–28 µm thick, thick-walled *textura angularis* with pigmented outer cell layer and colourless inner cell layer. Conidiophores absent. Conidiogenous cells formed on the inner layer of conidiomata, ampulliform, enteroblastic, phialidic, smooth, pale brown. Conidia 18–25 × 4–5 µm ( $\bar{x}$  =



**Figs. 1–16.** *Wojnowicia viburni* (holotype). **1.** Irregular leaf spot. **2.** Immersed conidiomata **3, 6.** Longitudinal sections of conidiomata. **4, 5.** Conidiomata wall. **7, 10–14.** Conidia. **8–9.** Developing young conidia and mature conidium attached to conidiogenous cell. **15.** Germinating conidia. **16.** Culture on PDA. Scale bars: **3, 6** 100  $\mu\text{m}$ , **4, 5, 7–9** 25  $\mu\text{m}$ , **10–14** 20  $\mu\text{m}$ .

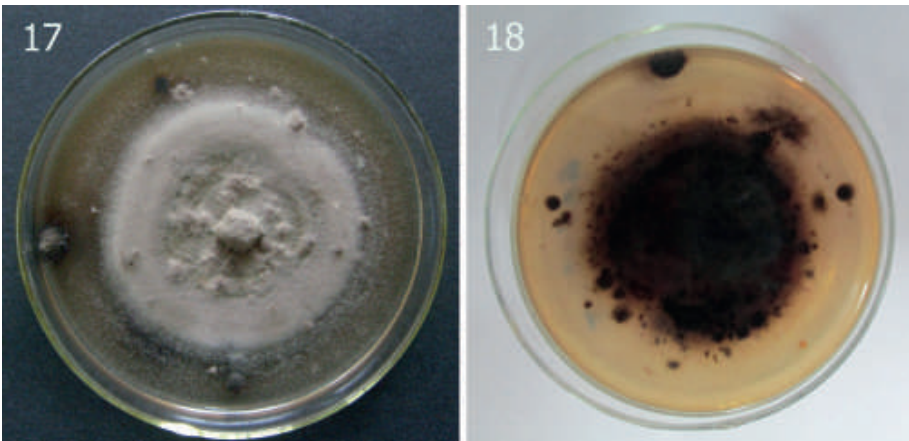
20.2  $\times$  4.3  $\mu\text{m}$ , n=20), cylindrical, straight to slightly curved, gradually tapering towards the rounded apex, with rounded apex, initially hyaline to pale brown, after maturity pale golden brown, 6–8–euseptate, thin-walled.

Colonies on PDA olive brown to greyish brown, zonate, slow growing, attaining a diam. of 5–6 cm after 14 days at 20–25  $^{\circ}\text{C}$ , later with dense mycelium, circular to irregular, with uneven margins, later comprising dense, my-

celium, after 4 weeks, greyish brown, zonate, attaining 7–8 cm diam., with thin mycelium, with conidiomata formed on the surface. Colonies after 6 weeks greyish (Fig. 3) and slightly raised.

### Phylogenetic analyses

Partial nucleotide sequences of LSU ribosomal DNA (720 bp) were obtained from the isolate. The other sequences used in the analysis were obtained from GenBank (Tab. 1). The large subunit rDNA (LSU) data comprised 35 sequences of 27 taxa including outgroup taxa. New sequences including SSU, ITS and LSU are deposited in GenBank.



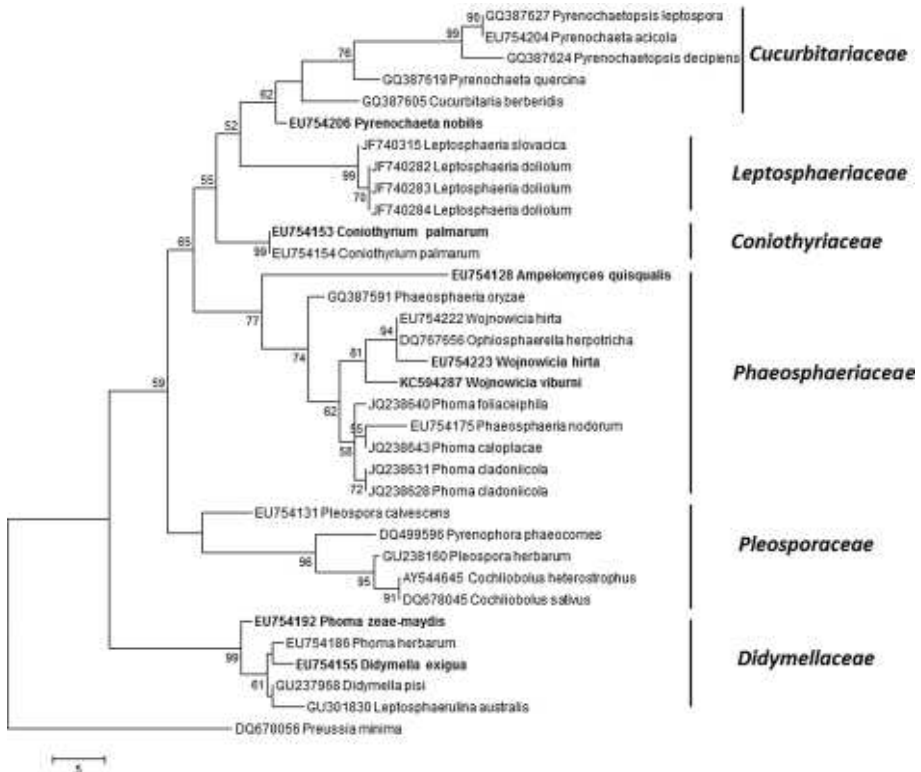
**Figs. 17–18.** Cultural characteristics of *Wojnowicia viburni* colony on PDA after six weeks. 17. Colony from upside. 18. Colony from downside.

*Wojnowicia viburni* was aligned with a set of sequences obtained from GenBank (Tab. 1) representing the closest taxa in Phaeosphaeriaceae following a blast search. The LSU alignment contained 798 characters including coded alignment gaps. Of the remaining 752, 542 were constant, while 210 were variable. Maximum likelihood analysis was carried out by MEGA 5 and the resulting tree is shown in Fig. 19.

In the analysis of SSU and LSU, data set consists of 14 taxa with *Dothidotthia aspera* and *D. symphoricarpi* as the outgroup taxa. Both sets of sequences were first analysed separately and then the individual datasets were concatenated into a combined dataset. The dataset consists of 1849 characters including coded alignment gaps. Figure 20 shows the tree generated of maximum likelihood analysis of combined gene from MEGA 5 (Tamura *et al.* 2011).

The phylogenetic tree obtained from maximum likelihood analysis showed that the new isolate grouped in Phaeosphaeriaceae with *Wojnowicia*

*hirta*, the generic type, and *Ophiosphaerella herpotricha* (Fr.) J. Walker. *Wojnowicia viburni* and *W. hirta* cluster in the same clade but are distinct. Analysis of SSU did not show this relationship clearly (data not shown). Analysis of combined LSU and SSU sequence data also clearly distinguish the species. However, the bootstrap value is slightly lower than in the LSU analysis. We have not carried out the combined gene analysis including ITS (i.e. ITS and LSU and ITS and SSU) as the sequences of *W. hirta* are not available in GenBank.



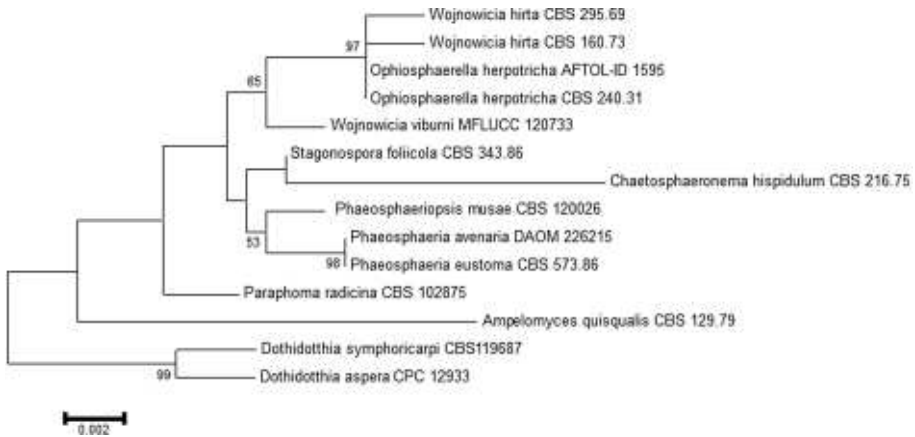
**Fig. 19.** Phylogenetic tree generated from maximum likelihood analysis from LSU rDNA sequences by using MEGA 5. Bootstrap support values >50 % from 1000 replicates are shown at nodes. The tree is rooted to *Preussia minima*. All type strains are in bold.

## Discussion

Saccardo (1892) established *Wojnowicia*, with *W. hirta* as the type species. The genus was characterized by Sutton (1975) as having black, setose, pycnidial conidiomata which are often papillate, with well-defined ostioles, and having enteroblastic and phialidic conidiogenous cells and brown, transversely euseptate conidia. Sutton (1975, 1980) accepted only two spe-

cies, *W. hirta* and *W. ephedrae* Hollós. *Wojnowicia tenella* Pat. and *W. graminis* (McAlpine) Sacc. & D. Sacc. were considered synonyms of *W. hirta* by Sutton (1980). Farr & Bills (1995) followed Sutton (1975, 1980) in accepting two species and also described *W. colluvium* D. F. Farr & Bills. *Wojnowicia buxi* Bertault & Malençon, described by Malençon & Bertault (1976), was considered a younger synonym of *W. ephedrae* by Farr & Bills (1995). Index Fungorum (2013) lists *W. bryophila* Racov. and *W. exilis* (Corda) Sacc. & Traverso as epithets of *Wojnowicia*. However Sutton (1980) and Farr & Bills (1995) have not accepted these two epithets in *Wojnowicia*.

Sutton (1980) listed the genus from Australia, Canada, France, Hungary, Ireland, Italy, Tunisia, Turkey and the United Kingdom. Farr & Bills (1995) described their species from the United States of America. Therefore, this is the first record of the genus from China and Asia.



**Fig. 20.** Phylogenetic tree generated from maximum likelihood analysis of combined dataset of LSU and SSU sequences by using MEGA 5. Bootstrap support values for maximum likelihood >50 % from 1000 replicates are shown at nodes. The tree is rooted to *Dothidotthia aspera* and *D. symphoricarpi*.

The 5–8-euseptate conidia found in *W. hirta* are quite similar to those in *W. viburni*, however, conidia of *W. hirta* are longer (35–45  $\mu\text{m}$ ). The conidiomata of both species are subepidermal to epidermal though in *W. viburni* conidiomata are not papillate. The most characteristic difference is the lack of setae in *W. viburni*; setae are found in all other species of *Wojnowicia* (Figs. 1–16).

Besides the study of de Gruyter *et al.* (2009), *Wojnowicia* has not been included in any molecular study. De Gruyter *et al.* (2009) showed that *W. hirta* grouped in Phaeosphaeriaceae with *Ophiosphaerella herpotricha* and this is confirmed in our study. Zhang *et al.* (2012) also placed *Wojnowicia* in Phaeosphaeriaceae (Pleosporales). This indicates that *Wojnowicia* may be



the asexual state of *Ophiosphaerella*, however, *O. herpotricha* is not the type species of *Ophiosphaerella* (Spegazzini 1909). Therefore, before *Ophiosphaerella* is synonymized under the older name *Wojnowicia*, it is necessary to confirm that *O. herpotricha* and *O. graminicola* are congeneric using cultural techniques or molecular data.

However we can conclude that *Wojnowicia* has *Ophiosphaerella*-like sexual morphs, as *Wojnowicia hirta*, the generic type, groups with *Ophiosphaerella herpotricha*.

### Key to species of *Wojnowicia*

1. Conidia 5–8 euseptate..... 2
1. Conidia 1–4 euseptate..... 3
2. Conidia 5–7 euseptate, 35–45 µm long, pycnidia superficial at maturity ..... *W. hirta*
2. Conidia 5–8 euseptate, 17–28 µm, pycnidia immersed at maturity ..... *W. viburni*
3. Conidia primarily 3 euseptate, 19–36 µm long, pycnidia superficial, papillate ..... *W. colluvium*
3. Conidia primarily 2 euseptate 20–29 µm long, pycnidia immersed, non-papillate ..... *W. ephedrae*

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