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Peltigera neodegenii sp. nov. from Central China

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Abstract—*Peltigera neodegenii* from Central China is described and illustrated as a new species. It is similar in general appearance to *P. degenii, P. membranacea,* and *P. canina*, but is distinguished by its shiny upper surface in the central part of lobes, tomenta around the margins of upper surface of lobes, distinct and raised veins on the lower surface of lobes, and simple rhizines. Comparisons of the ITS (ITS1, 5.8S, ITS2) sequences of the nuclear ribosomal DNA repeat tandem, both in phylogenetic analysis and secondary structure models of ITS2, support the taxonomic distinctness of this species.

Key words—biodiversity, cyanolichen, *Peltigeraceae*, *Peltigerales*, Shennongjia Mountain

Introduction

Peltigera Willd. (*Peltigeraceae*), characterized by large foliose thalli and a worldwide distribution in moist habitats, currently includes more than 90 species (Kirk & al. 2008; Han & al. 2013, 2015; Manoharan-Basil & al. 2016). In modern lichen taxonomy, molecular techniques have been used widely to delimit species within the genus (Goffinet & Miądlikowska 1999, Sérusiaux & al. 2009). The nrDNA ITS region, the main molecular marker or barcode used to characterize fungal and lichen species (Schoch & al. 2012, Guo 2013),

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has been helpful in revealing phylogenetic relationships in *Peltigera* (Goffinet & al. 2003; O'Brien & al. 2009; Sérusiaux & al. 2009; Han & al. 2013, 2015; Manoharan-Basil & al. 2016).

Peltigera has been relatively well studied in the northern hemisphere and a phylogeny combining morphological and chemical characters with LSU nrDNA sequence analysis has been carried out (Miądlikowska & Lutzoni 2000). In Asia, about 40 species have been reported, and the nrDNA ITS sequence data of most species are available from GenBank (Martínez & al. 2003; Han & al. 2013, 2015). Of the 30 species recorded in China (Chen 1986; Vitikainen 1986; Wei 1991; Stenroos & al. 1994; Wu & Liu 2012; Han & al. 2013, 2015; Niu & al. 2016), 14 belong to the *P. canina* group, in *P.* sect. *Peltigera*. During our ongoing study of *Peltigera* species in China, we identified a new species based on morphological characteristics and nrDNA ITS sequence data, which we describe here as *Peltigera neodegenii*. We also compare the secondary structure of the *P. neodegenii* nrDNA ITS2 region with those of two closely related species, *P. degenii* Gyeln. and *P. membranacea* (Ach.) Nyl.

Materials & methods

Specimens & morphology

Specimens were collected from Shennongjia forest region in Central China and all examined morphologically using a Motic SMZ-140 or Zeiss-Stereo Discovery-V12 dissecting microscope and Leica DM500 compound microscope. Asci and ascospores were observed in sections of apothecia cut by hand with razor and mounted in water after staining in 0.2% toluidine blue for about 15 min. Descriptions and terminology follow Vitikainen (2004). Thin layer chromatography (TLC) was performed on all specimens using solvent systems C and G according to Orange & al. (2010). The specimens are deposited both in the Herbarium Mycologicum Academiae Sinicae-Lichenes, Beijing, China (HMAS-L) and the Lichen Section of Botanical Herbarium, Hebei Normal University, Shijiazhuang, China (HBNU).

DNA extraction, PCR amplification, sequencing

The samples of lobe tips were cut for DNA extraction from five specimens (including the type and three other *P. neodegenii* specimens and one *Peltigera praetextata* specimen). DNA was extracted using the DNAsecure Plant DNA Kit following the manufacturer's protocol. The ITS region was amplified according to Han & al. (2013, 2015). The entire nrITS region (ITS1+5.8S+ITS2) was targeted for Polymerase Chain Reaction (PCR) using primers ITS1F and ITS4 (White & al. 1990) in a 25 µL volume containing 0.75 units of TransStart Taq Polymerase, 2.5 µL of ITS buffer, 0.5 µl of a 5 µM solution of the primers, 2 µL of 2.5 mM for each dNTP solution, and 1 µL of genomic DNA. Thermal cycling conditions were 95°C for 3 min initiation, followed by 35 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min

with a final extension at 72°C for 10 min. PCR products were screened on 1% agarose gels stained with ethidium bromide and sequenced by the Genewiz Inc. (Suzhou, Jiangsu, China).

Phylogenetic analysis & sequence comparisons

The entire ITS sequences of five samples of our examined specimens and the 24 representatives selected from GenBank were aligned manually by both ClustalW and Muscle implemented in MEGA version6 (Tamura & al. 2013). We excluded the 3′ end of the 18S gene and the 5′ end of the 26S gene from the analyses. Sequences from the new species were aligned with GenBank ITS sequences from taxa sharing the most similarity with them as determined from morphological characters, Blast results of sequence data, and selected references (e.g., Miądlikowska & Lutzoni 2000, Goffinet & al. 2003, Miądlikowska & al. 2003, Sérusiaux & al. 2009).

Ambiguously aligned regions sensu Lutzoni & al. (2000) were delimited manually and excluded. The final matrix of the remaining 520 characters submitted to TreeBase with accession number TB2:S15264 may be obtained from the corresponding authors.

The evolutionary history was inferred from 29 nucleotide sequences by using both the Maximum Likelihood method based on the GTR+Γ model in MEGA6 and Bayesian inference (Huelsenbeck & Ronquist 2001) based on GTR model with rates = Invgamma. The secondary structures of ITS2 sequences for the new species and its most closely related species were predicted by The ITS2 Database III (Koetschan & al. 2010) and illustrated with PseudoViewer3 (Byun & Han 2009).

Results

Phylogenetic analysis and secondary structure of ITS2

The newly obtained sequences were submitted to GenBank. The entire ITS region, which was successfully sequenced for five samples from the collections from Mt. Shennongjia, Central China, comprises 573–574 bp (ITS1 = 216–217 bp; 5.8S = 157 bp; and ITS2 = 200 bp). We included ITS sequences from our new species and the GenBank reference sequences in the phylogenetic analyses. The data matrix of the aligned 29 sequences comprised 520 characters, of which 468 (90%) were constant and 52 (10%) were parsimony informative.

The hypervariable region in the internal transcribed spacer 1 (ITS1-HR; Miadlikowska & al. 2003) was calculated, with about 23% variable sites compared with *P. degenii*, for 86 positions in total (not shown).

The alignment dataset was analyzed using MrBayes for Bayesian inference and MEGA6 for Maximum Likelihood; similar topologies were obtained. A ML tree with bootstrap values (1000 replicats) and Bayesian posterior probabilities (BPP) at branches is shown in Fig. 1. In the phylogenetic tree, the new species formed a clade with *P. membranacea.* The different samples of the new species formed a clade with 70% ML support and 0.97 of BPP.

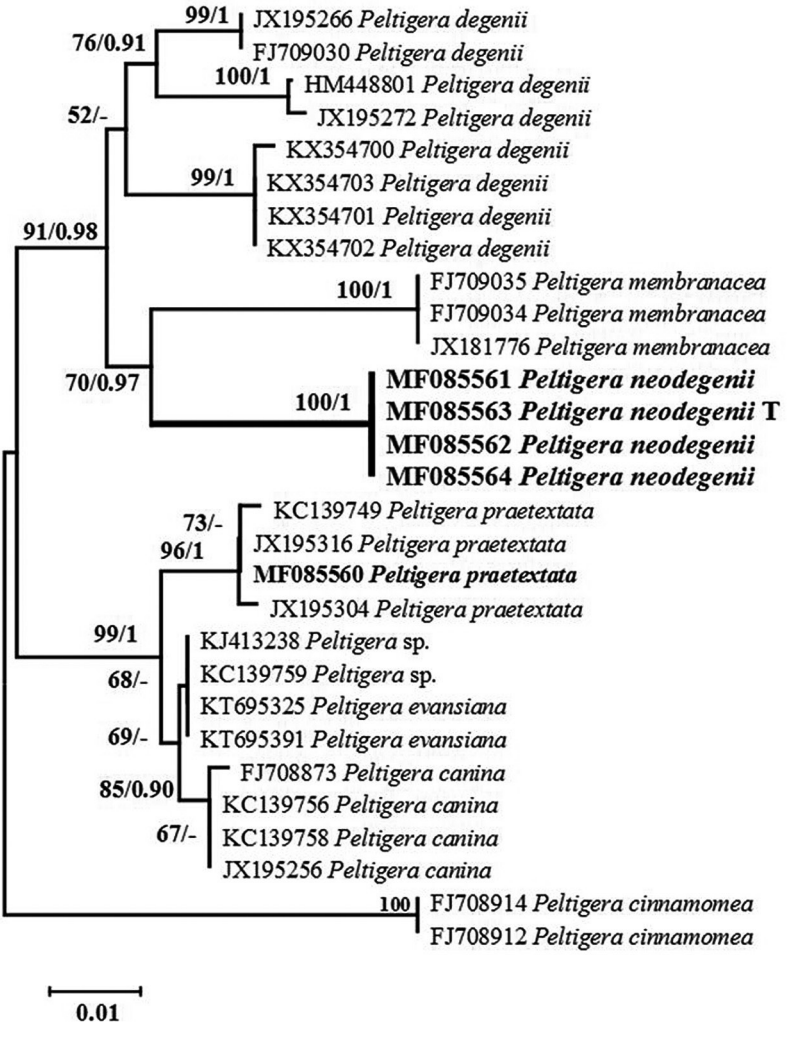

Fig. 1. Phylogenetic relationships among selected members (7 taxa, 29 sequences) closely related to *Peltigera neodegenii* based on ITS sequences. Values associated with internodes represent bootstrap support (ME-BP; before the slash) and posterior probability support (PP; after the slash). Only values ≥50% for ML-BP and ≥0·90 for PP are shown. *Peltigera cinnamomea* Goward was included as outgroup. Fonts in bold indicate newly generated sequences from specimens collected in Central China; the ex-type sequence of *P. neodegenii* (MF085563) is annotated: **T**. There was a total of 520 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 and MrBayes3.2.

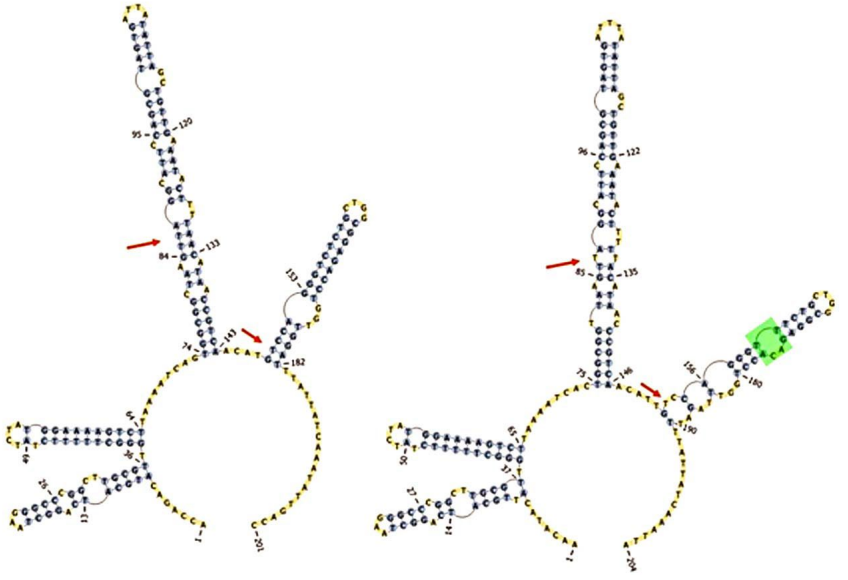

Fig. 2. The secondary structure model for the ITS2 sequence of *Peltigera neodegenii* (right, GenBank MF085563 *P. neodegenii* ex-type) and its template GI255347364 (left, GenBank FJ709030 *P. degenii*). Red arrows indicate a hemi-compensatory base change (hCBC) and the green box indicates a compensatory base change (CBC).

A high-quality secondary structure model of the ITS2 sequence representing the new species (GenBank MF085563) was derived from template GI255347364 (GenBank FJ709030, *Peltigera degenii*), the GenBank sequence closest to the new species, with 93% identity for ITS2 region (compared with only 90% identity between the new species and *P. membranacea*). The calculated free energy is –17.80 kcal/mol. Percentages of helix transfer are /100/100/96/85/ (threshold:75%; E-value: 1.2e-52) for four helices. The computed structure is shown in Fig. 2. One obvious difference between the structural models representing our new species and the closely related *P. degenii* includes one hCBC in the conserved part of helix III and one hCBC and two CBCs in the conserved part of helix IV.

Taxonomy

Peltigera neodegenii L.F. Han, S.Y. Guo & Xiao M. Xu, **sp. nov.** Fig. 3

MycoBank MB821289

Differs from *Peltigera degenii* by its upper surface that is shiny at the centre but tomentose towards the margin, and by the absence of marginal phyllidia.

Type: China, Hubei Province, Shennongjia, Jinhouling, 31°28′N 110°18′E, with mosses over rock and soil, 2600 m alt., 24 April 2014, Shou-Yu Guo, Liu-Fu Han 21112. (**Holotype**, HMAS-L; GenBank MF085563; **isotype**, HBNU).

Etymology: Referring to the morphological similarity with *Peltigera degenii*.

Thallus foliose, loosely appressed, medium to large, 8–15 cm in diam.; lobes thin, somewhat brittle, 8–20 mm wide, separate or sometimes loosely overlapping, short to elongate, irregularly branching; lobe tips rounded, predominantly downturned; lobe margins mostly even or weakly crisped, occasionally with lobules; upper surface deep bluish green to blackish green (moist) or pale bluish grey to brown (dry), smooth and somewhat shiny centrally, white-gray tomentose near lobe tips, the tomentum becoming absent toward thallus centre, appressed to occasionally erect in part; soredia and isidia lacking, epruinose; lower surface distinctly veined; veins usually whitish, not appearing to overlap, narrow, often raised, usually lacking tomentum, but sometimes distinctly erect-tomentose; interstices white, lenticular to polygonal, moderately deeply set; rhizines concolourous with veins to pale brown, irregular, discrete, threadlike, occasionally branched, 4.0–8.0 mm long, lacking tomentum or sometimes distinctly erect-tomentose; cortex c. 30–55 µm thick; photobiont layer 25–40 µm thick, containing *Nostoc*; medulla white, c. 250–700 µm thick.

Apothecia growing at short lobe tips, erect, saddle-shaped, ≤5.0 mm in diam; apothecial margin smooth to crenulate; disc red brown to dark brown, smooth; paraphyses simple, septate, ± swollen at the apices and pigmented; asci clavate, *Peltigera*-type, colorless to pale, 8-spored; ascospores acicular, 3(–4)-septate, 60–95 × 2.5–5.5 µm. Pycnidia not seen.

Spot tests: all negative.

Secondary metabolites: none detected.

Ecology & distribution: On mosses and soil in subtropical mountain forest; known only from Shennongjia forest region, Hubei, China.

Additional specimens examined:

Peltigera neodegenii: **China**, **Hubei province**, **Shennongjia**, Jinhouling, 31°28′N 110°18′E, with mosses over rock and soil, 2600 m alt., 24 April 2014, Shou-Yu Guo, Liu-Fu Han 20973 (HMAS-L, GenBank MF085564); 21105 (HMAS-L, GenBank MF085561); 21111 (HMAS-L, GenBank MF085562).

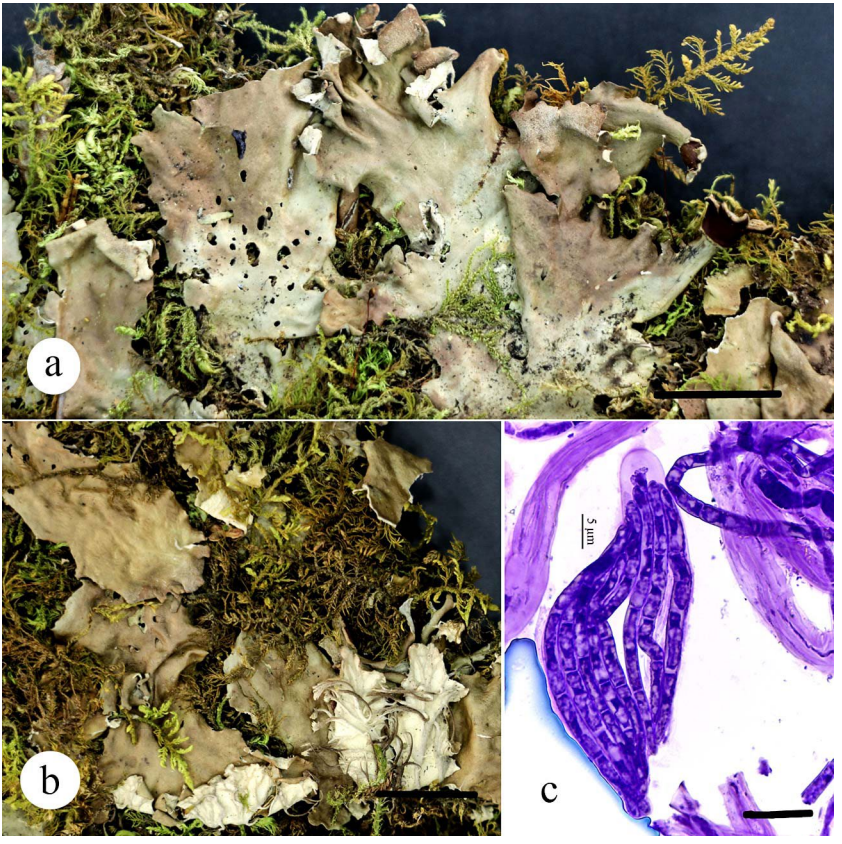

Fig. 3. *Peltigera neodegenii* (holotype, HMAS-L Guo & Han 21112). a: upper surface and apothecia (dry); b: upper surface, veins, and rhizines (dry); c: section through apothecium (stained by 0.2% toluidine blue for c. 15 min) showing asci and ascospores. Scale bars: a, b = 10 mm; c = 20 µm.

Peltigera praetextata (Flörke ex Sommerf.) Zopf: **CHINA**, **Hubei province**, **Shennongjia**, Laojunshan, 31°49′N 110°32′E, Shou-Yu Guo, Liu-Fu Han 20953 (HMAS-L, GenBank MF085560).

Discussion

The new species shows closer phylogenetic relationships with *Peltigera membranacea* and *P. degenii* than with other species in the ITS sequence analyses (Fig. 1), while the secondary structure models for ITS2 sequences suggest a greater with *P. degenii* (Fig. 2). (In contrast, there was very little similarity with the secondary structure model for the ITS2 sequence of *P. membranacea*, GenBank JX181776 [not shown].)

Morphologically *Peltigera neodegenii* is characterized by the presence of a cyanobacterial photobiont (*Nostoc* sp.) combined with a centrally shiny but marginally tomentose upper surface, predominantly downturned lobe tips, mostly even lobe margins, simple rhizines, and saddle-shaped apothecia. *Peltigera neodegenii* may be confused with *P. membranacea,* which also has a shiny central upper surface shiny and tomentose lobe ends, but which differs by sometimes wider (<4.0 cm) lobes, tomentose veins, and rope-like, tomentose rhizines (Wu & Liu 2012).

Peltigera canina (L.) Willd. and *P. degenii* also resemble *P. neodegenii* in morphology, but *P. canina* differs in its penicillate to confluent rhizines, while *P. degenii* differs in having glossy, glabrous upper surface and distinct marginal phyllidia (Goward & al. 1995; Wu & Liu 2012). *Peltigera evansiana* Gyeln. and *P. praetextata* are readily distinguished from *P. neodegenii: P. evansiana* by having granular isidia covering the lobe surfaces, and *P. praetextata* by having phyllidia produced at the margin and along cracks.

Previous studies that have used DNA sequence data for species recognition in *Peltigera* required monophyly both in single-locus ITS phylogenies and diagnosable morphological differences (Goffinet & Miądlikowska 1999; Goward & Goffinet 2000; Goffinet & al. 2003; Miądlikowska & al. 2003; Han & al. 2013, 2015). Here we add secondary structure models for ITS2 sequences, which are also useful characters to distinguish lichen species (Liu & Guo 2009, Cao & al. 2011, Guo 2013, Han & al. 2015). Coleman (2007) considered two organisms as representing different species if the conserved parts of their ITS2 sequences differed by more than one compensatory base change (CBC) or more than four hemi-compensatory base changes (hCBC). The threshold range between intraspecific and interspecific taxa for at least one CBC or four hCBCs in the four helices (I-IV) (Schultz & al. 2005, Guo 2013, Han & al. 2015). For *Peltigera neodegenii* and its template *P. degenii,* there are two hCBCs and two CBCs in the conserved part of helix III and helix IV, further supporting *P. neodegenii* as an independent species.

In conclusion, both distinctive morphological and molecular characteristics of *Peltigera neodegenii* confirm it as new to science.

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