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Identification of a Rust Disease of Giant Knapweed in the Czech Republic – Short Communication

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Abstract

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During the vegetation seasons 2012–2015, symptoms of severe infections by a rust disease were recorded on plants of the ornamental species *Centaurea macrocephala.* Based on morphology, the pathogen was identified as *Puccinia jaceae* or *Puccinia hieracii*, which have recently been considered as synonyms. However, substantial differences between *P. jaceae* and *P. hieracii* in nucleotide sequences of the ITS2 region provide evidence for the molecular identification of the specimen as *P. jaceae.*

Keywords: *Centaurea macrocephala*; *Grossheimia macrocephala*; ITS rDNA; *Puccinia hieracii*; *Puccinia jaceae*

Giant knapweed or Armenian basket flower [*Centaurea macrocephala* Willd., syn. *Grossheimia macrocephala* (Willd.) Sosn. et Takht.] is a robust, clump-forming perennial herb from the family Asteraceae. It is characterised by unbranched stems bearing single large yellow capitula opening from huge, artichoke type flower buds covered with metallic brown papery scales. The species is native to the Caucasus region (Gagnidze *et al.* 2002) but it has been introduced to a number of countries around the world, where it is cultivated as an ornamental perennial. However, in some countries it has become naturalised and invasive (CABI 2014). In the Czech Republic this ornamental plant is grown relatively infrequently (Štěpánek 2004), although it is currently offered by numerous internet garden shops. In the region of its origin it is considered medicinal, a source of vitamin E (Charchoglian 1998), and it contains a sesquiterpene lactone (grosheimin) effective in the regulation of body weight and treatment of obesity (Grothe *et al.* 2011). The plant is also a good source of pollen for honeybees (Wróblewska 2011). Giant knapweed may be attacked by several fungal pathogens, especially those causing powdery mildews or rusts (Farr & Rossman 2016). The objective of the present study was to identify and characterise the rust species recently found on this perspective ornamental.

MATERIAL AND METHODS

Morphological characterisation. Giant knapweed has been grown in a permanent field culture at the Crop Research Institute in Olomouc, as an

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ornamental and melliferous species. Natural infection of plants by a rust disease was observed annually within the four-year period 2012–2015. Leaves with the rust symptoms were taken from the plants and herbarised. The specimen was deposited in the Herbarium of the Department of Botany, Palacky University in Olomouc, Czech Republic, as voucher 33481 (date of collection: July 7, 2014). Urediniospores and teliospores isolated from the infected leaves were mounted in water and examined microscopically (magnification 400× and 1000×), using an Olympus CX 40 light microscope supplied with AxioCam ERc5s camera (Karl Zeiss AG, Oberkochen, Germany). Detailed morphological characteristics were measured from the photographs of preparations with accuracy 0.01 μm using the program AxioVision SE64 Rel., Version 4.9.1. The measurements are reported below as maxima and minima, and the mean plus and minus the standard deviation of 100 measurements. Average values were rounded to the nearest 0.5 μm.

DNA extraction, PCR and sequencing. Genomic DNA was extracted from urediniospores scraped from the leaf using an SDS extraction method (Edwards *et al.* 1991). Concentration of DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, USA) and kept at –80°C until used for further analysis. Two genomic regions were sequenced in this study. The small subunit nrDNA (18S) was amplified and sequenced with Rust18S-R and NS1 primers (White *et al.* 1990), and additional sequencing primers (NS3, NS4, NS5, NS6). A part of the nrDNA region containing the internal transcribed spacer region 1 (ITS-1), the 5.8S subunit, the internal transcribed spacer region 2 (ITS-2), and a part of the large subunit (28S) of nrDNA was PCR-amplified with nested PCR according to Kropp *et al.* (1997) (Rust1 and ITS1-F; ITS4 and ITS5); and primer combination Rust2inv (Aime 2006) and LR6 (Vilgalys & Hester 1990). Both primers were used for sequencing as well as internal sequencing primers LR0R (Moncalvo *et al.* 1995) and LR3 (Vilgalys & Hester 1990). PCRs were conducted in 40 μl reaction volume containing 4 μl of DNA (50 ng/μl), 1 μl of each primer (20 μM), 8 μl of 5X My Taq Red Reaction Buffer with 10 mM dNTPs, 0.22 μl of My Taq DNA Polymerase (Bioline, London, UK) and 26 μl PCR grade water. PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Germany) using the following conditions: 4 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 45–57°C and 1 min at 72°C, and a final extension (4 min at 72°C). PCR products were cleaned using a GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, USA) and sequenced (Macrogene Europe, Amsterdam, the Netherlands). Geneious 7.1.8 (Biomatters Ltd., Auckland, New Zealand) was used for contig assembly from partial reads, the editing of base calls and concatenation of partial genomic regions. Resulting alignments were deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/; accession numbers KX468973 and KX468974).

RESULTS AND DISCUSSION

Symptoms and morphology. Between 2012 and 2015, symptoms of severe infection by a rust disease were recorded on plants of *C. macrocephala.* Infected leaf blades were densely covered with tiny (0.5–1.5 mm), coalescent, yellow spots, and rusty sori. Spermogonia and uredinoid aecia were not found. Uredia were amphigenous, denser on the underside of leaves, scattered, up to 0.5 mm in diameter, round, pulverulent, rusty brown. Urediniospores (n = 100) were spherical to broadly ellipsoid or broadly obovoid, cinnamon brown, measuring 22.3–31.6 μm (mean 26.8 μm, SD ± 1.4) × 19.9–30.6 μm (mean 24.5 μm, SD ± 1.7) with the length/width (l/w) ratio of 1.0–1.2(–1.4). Spore walls, except for the base, were very thin (0.8–1.8 μm; mean 1.1 μm, SD ± 0.2) and roughly echinulate; spines were 1.3–3.7 μm (mean 2.3 μm, SD ± 0.4) apart. Germ pores were two and supra-equatorial, above pores there was a weakly developed hyaline papilla. Telia were amphigenous, dense on the underside of leaves, up to 1 mm in diameter, round, pulverulent, blackish brown. Teliospores (n = 100) were bicellular, slightly or not constricted at septum, broadly ellipsoid, oval or slightly obovoid, apex and base rounded, colour cinnamon- or chestnut-brown, size 28.7–42.6 μm (mean 34.7 μm, SD ± 2.7) × 19.4–28.2 μm (mean 23.8 μm, SD ± 1.4) with the l/w ratio of 1.2 –1.7. Walls were uniformly thick (1.2–3.4 μm; mean 2.2 μm, SD ± 0.3) and finely verrucose. Germ pores of both cells were 1/3 to 1/2 depressed, and pedicels were hyaline, short, and deciduous.

Molecular identification. The alignment of the ITS1-LSU region resulted in 1 555 bp long contig, which consisted of a part of the ITS1 (62 bp), complete 5.8S-ITS2 region (156 and 215 bp, respectively), and a part of the 26S ribosomal RNA gene (1107 bp). A BLAST search of the NCBI database (www.ncbi.nlm.nih.gov/BLAST/) revealed 99% identity to two

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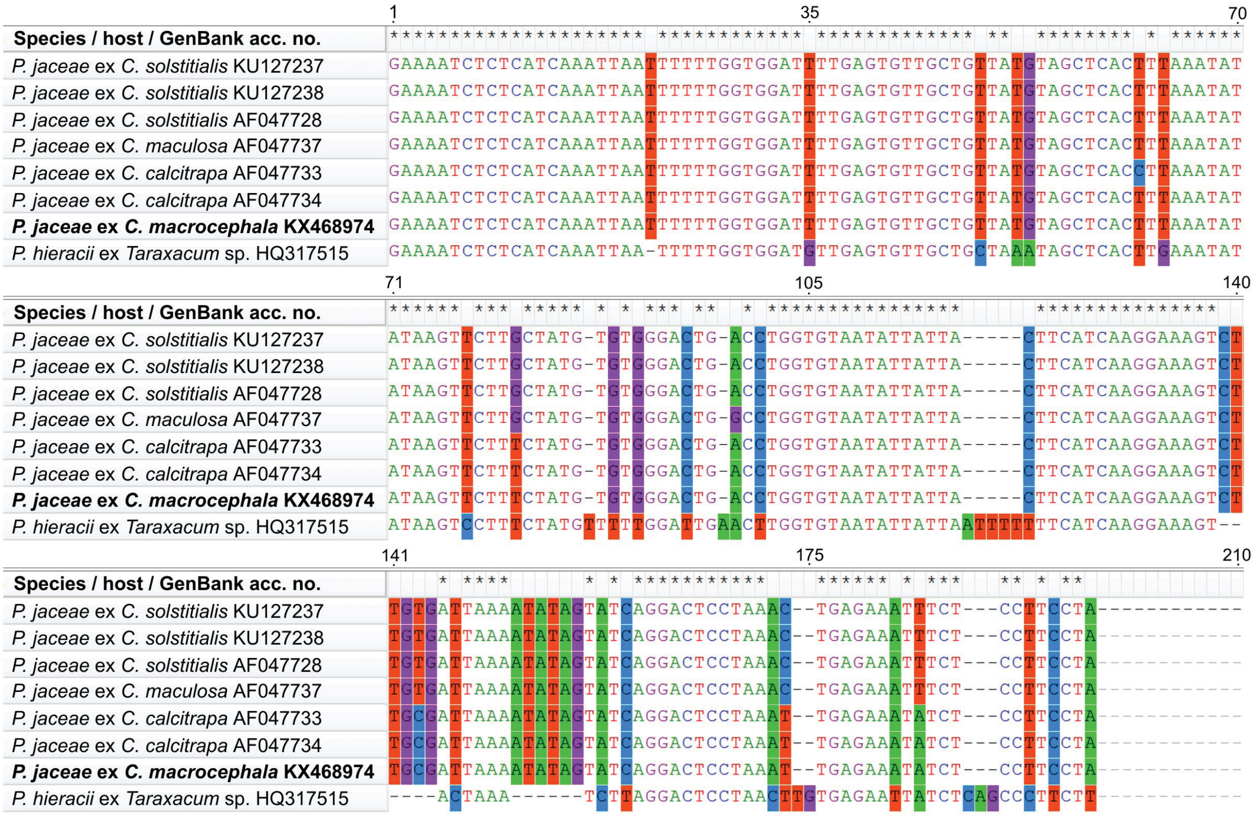

Figure 1. PrintScreen of the nucleotide alignment of a part of the ITS2 region. Designation of the isolate, its taxonomical identification, host species and GenBank accession number are provided. Nucleotide substitutions are highlighted with colours; gaps in the nucleotide alignment are represented by a hyphen; sequence in bold letters highlights the isolate analysed in this study

ITS1-5.8S-ITS2 sequences of strains of the full-cycle autoecious rust species *Puccinia jaceae* G.H. Otth infecting *Centaurea solstitialis* L. (KU127237, KU127238; Bruckart *et al.* 2016). Nevertheless, there are six additional *P. jaceae* records representing 188 bp long part of the ITS2 region (Yourman & Luster 2004), having 100 and 99% identity to *P. jaceae* strains 84-66, 84-62 (AF047734, AF047733; Figure 1) isolated from *Centaurea calcitrapa* L. (Yourman & Luster 2004); 98% identity to *P. jaceae* strain 84-71 (AF047728; host *C. solstitialis*) and *P. jaceae* strains FDWSRU 84-071 and 14-004 (KU127237, KU127238) analysed by Bruckart *et al.* (2016); and finally 97% identity to strain 85-192 (AF047737) isolated from *Centaurea maculosa* Lam. (Yourman & Luster 2004).

Since no *P. jaceae* sequences of small and large rRNA subunits have been deposited in the GenBank so far, a BLAST search of the 1107 bp long nucleotide alignment of 26S ribosomal RNA resulted in 99% identity to *P. balsamorrhizae* Peck, 98% identity and 100% cover of *P. acroptili* P. Syd. & Syd. strains (all published by Bruckart *et al.* 2012) and other *Puccinia* species with lower sequence cover. Finally, a BLAST search for the 1688 bp alignment of 18S rRNA resulted in 99% identity (100% cover) to various *Puccinia* species: *P. violae* (Schumach.) DC. (DQ354508; Aime 2006), *P. pelargonii-zonalis* Doidge (AY123316; Wingfield *et al.* 2004), *P. poarum* Nielsen (DQ831029; Matheny *et al.* 2006), and a number of uncultured fungal species.

To date, three rust species have been recorded on giant knapweed: *P. calcitrapae* DC. in Turkey (Bahcecioglu & Kabaktepe 2012), *P. hieracii* (Röhl.) H. Mart. in Turkey and Poland (Mulenko *et al.* 2008; Bahcecioglu & Kabaktepe 2012), and *P. jaceae* in Iran (Donyadoost-Chalan *et al.* 2009; Aliabadi & Abbasi 2012). However, to our knowledge, this is the first report of a rust disease on giant knapweed in the Czech Republic. Several *Puccinia* species have been reported to parasitise on *Centaurea* spp. in the Czech Republic: *P. calcitrapae* (syn. *P. centaureae* DC.), *P. cnici-oleracei* Pers. ex Desm., *P. cyani* Pass., *P. dioicae* Magnus, *P. doronici* Niessl, *P. hieracii, P. jaceae*, and *P. montana* Fuckel

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Table 1. Morphological characterisation of the rust species found on giant knapweed and comparison with literature data for *Puccinia jaceae* and *Puccinia hieracii*

Morphological character	Rust species from giant knapweed	*P. jaceae* (Bubák 1906)	*P. jaceae* (Gäumann 1959)	*P. jaceae* (Newcombe *et al.* 2009)	*P. jaceae* (Braun 1982)	*P. hieracii* (Bubák 1906)	*P. hieracii* (Gäumann 1959)	*P. hieracii* (Hennen *et al.* 2005)	*P. hieracii* (Braun 1982)
II	mainly on underside of leaves	mainly on underside of leaves	on underside of leaves			mainly on upper side of leaves	mainly on upper side of leaves	amphigenous	
II length (µm)	22–32	24–30	26–30	25–33		24–29	21–29	(21–)24–30(–35)	
II width (µm)	20–31	16–28	22–27	23–31		16–25	15–25	(17–)19–25(–29)	
II shape	spherical to broadly ellipsoid or obovoid	spherical or ovoid	spherical to ovoid	nearly spherical		spherical, ovoid to ellipsoid	spherical to ellipsoid, rarely ovoid	broadly ellipsoid or obovoid	
II surface	echinulate, no barren spots	echinulate	echinulate-verrucose	echinulate except around pores	with small barren spots	echinulate	echinulate-verrucose	echinulate except below each pore	with large barren spots
II wall thickness (µm)	0.8–1.8		1.5–2				1.5–2	1.5–2	
II germ pores	2 supra-equatorial	2 supra-equatorial	2 near apex	2	2 supra-equatorial	2 supra-equatorial	2 mostly apical	2(3) supra-equatorial	2 supra-equatorial
III	mainly on underside of leaves	mainly on underside of leaves				mainly on upper side of leaves	mainly on upper side of leaves	amphigenous	
III length (µm)	29–43	24–37	25–42	30–43	ca. 24–40	24–40	24–40	(26–)30–40(–45)	ca. 24–40
III width (µm)	19–28	16–27	21–31	21–29	ca. 20–27	16–24	16–24	(17–)20–26(–29)	ca. 20–27
III shape	broadly ellipsoid, oval, slightly ovoid	ovoid or ellipsoid	shortly ellipsoid			ellipsoid, ovoid or pyriform	ellipsoid, ovoid or pyriform	ellipsoid or oblong ellipsoid	
III apex and base	both rounded	both rounded	both rounded			apex rounded, base narrowed	both rounded or base narrowed		
III constriction at septum	slight	no	no	slight		slight or no	slight or no		
III surface	finely verrucose	finely verrucose	verrucose	verrucose	verrucose	finely verrucose	verrucose	verrucose	verrucose
III wall thickness (µm)	1.2–3.4		2		2–2.5		2	(1–)1.5–2(–3)	2–2.5
III germ pore upper cell	1/3–1/2 depressed	1/3–1/2 depressed	1/3–1/2 depressed			up to 1/2 depressed	1/3–1/2 depressed	apical or depressed	
III germ pore lower cell	1/3–1/2 depressed	1/3–1/2 depressed				up to 1/2 depressed	1/3–1/2 depressed	1/2 more depressed	

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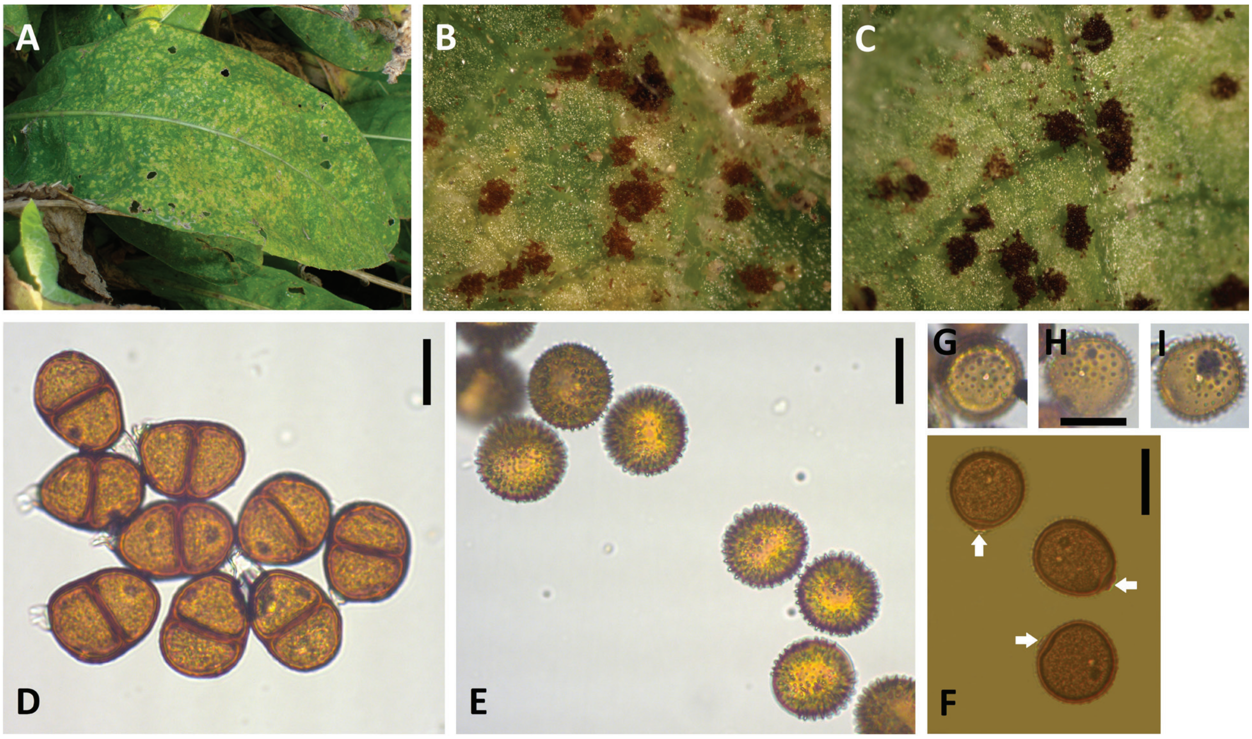

Figure 2. Rust disease of giant knapweed caused by *Puccinia jaceae* (bars = 20 μm): (**A**) – severely infected leaf; (**B**) – uredia; (**C**) – telia; (**D**) – teliospores; (**E**) – urediniospores focused on the echinulate surface; (**F**) – two supra-equatorial pores (arrows indicate the location of the hilum); (**G**)–(**I**) – area around pores

(Urban & Marková 2009). Of these, *P. cnici-oleracei, P. doronici* and *P. dioicae* do not form either uredia or telia on *Centaurea* spp. (Urban & Marková 2009). Further, urediniospores of *P. calcitrapae* have three germ pores (Bubák 1906; Gäumann 1959; Braun 1982) compared to the specimen found on giant knapweed. From the remaining species, only *P. hieracii* and *P. jaceae* come into consideration, as they have two supra-equatorial pores (Braun 1982), just as our specimen. According to Braun (1982), the main features distinguishing these two species are the mean width of teliospores and the size of barren spots on urediniospores. The mean width of teliospores from the giant knapweed did not exceed 25 μm, which is typical of *P. hieracii* (Braun 1982). Otherwise, measurements for the rust in the present study were in the range of values published by various authors both for *P. hieracii* and *P. jaceae* (Table 1). However, the wall of urediniospores from the giant knapweed was echinulate over the entire surface without any visible barren spots (Figure 2) and the wall of urediniospores was mostly much thinner than that of both *P. hieracii* and *P. jaceae*. These morphological features did not provide any clear determination of our specimen as either of those species. That basically corresponds to the inconsistent classification of both species in the current literature, treating *P. jaceae* either as a distinct species (Aliabadi & Abbasi 2012; Bruckart *et al.* 2016) or as a synonym of *P. hieracii* (Farr & Rossman 2016; Index Fungorum 2016). However, most authors do not take into account the molecular data. When a part of the ITS2 region of all *P. jaceae* sequences deposited in GenBank was aligned and compared with *P. hieracii* record HQ317515 (Liu *et al.* 2015), we found substantial differences between *P. jaceae* and *P. hieracii* represented by several single nucleotide positions, gaps and insertions/deletions (Figure 1). These differences provide evidence for considering *P. jaceae* and *P. hieracii* as distinct species, and for the molecular identification of the specimen from giant knapweed as *P. jaceae*.

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