Epitypification of *Cercospora rautensis*, the causal agent of leaf spot disease on *Securigera varia*, and its first report from Iran

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Abstract: *Cercospora* is a well-studied and important genus of plant pathogenic species responsible for leaf spots on a broad range of plant hosts. The lack of useful morphological traits and the high degree of variation therein complicate species identifications in *Cercospora*. Recent studies have revealed multi-gene DNA sequence data to be highly informative for species identification in *Cercospora*. During the present study, *Cercospora* isolates obtained from Crownvetch (*Securigera varia*) in Iran and Romania were subjected to an eight-gene (ITS, *tef1*, *actA*, *cmdA*, *his3*, *tub2*, *rpb2* and *gapdh*) analysis. By applying a polyphasic approach including morphological characteristics, host data, and molecular analyses, these isolates were identified as *C. rautensis*. To our knowledge, this is the first record of *C. rautensis* from Iran (Asia). In addition, an epitype is designated here for *C. rautensis*.

INTRODUCTION

Crownvetch (*Securigera varia* = *Coronilla varia*), is a herbaceous, perennial legume with creeping stems belonging in the family *Fabaceae* which is native to the Mediterranean region of Europe, southwest Asia, and northern Africa (Roland 1998). It is an intercropping plant in many orchards in the world, with many benefits, including controlling weeds, decreasing soil erosion, increasing soil enzyme activities, improving the soil micro-ecological environment and, like other *Fabaceae*, it is a nitrogen fixer (Qian et al. 2015, Zheng et al. 2016). However, this plant may become weedy or invasive in some regions or habitats and may displace desirable vegetation if not properly managed (Randall & Marinelli 1996, Kaufman & Kaufman 2013).

The cosmopolitan genus *Cercospora* is species-rich (2 522 legitimate species names listed in MycoBank, accessed 20 Feb. 2019) that belongs to the family *Mycosphaerellaceae* in the order *Capnodiales*. The genus comprises numerous destructive plant pathogens, for instance *C. apiic* on celery (Groe newald et al. 2006), *C. beticola* on sugar beet (Weiland & Koch 2004), *C. zonata* on faba beans (Kimber & Paull 2011), *C. zeae-maydies* and *C. zeina* on maize (Crous et al. 2006) and *C. carotae* on carrots (Kushalappa et al. 1989). *Cercospora* was established by Fuckel (Fungi Rhen. Exs., no. 117, 1863; as Fresen. ex Fuckel, see Braun & Crous 2016), and *C. apiic* was later designated as conserved type of the genus under the International Code of Nomenclature for algae, fungi, and plants, Art. 14.9 (Braun & Crous 2016). The systematics of *Cercospora* has been problematic for a long time, as there are only few distinct morphological characters useful for species discrimination and since specialised as well as plurivorous species are involved (Crous & Braun 2003, Groenewald et al. 2013, Bakhshi et al. 2015, 2018). Molecular techniques are commonly used to overcome taxonomic problems posed by the limitations of morphological characteristics. In this regard, ex-type cultures are essential for the study of *Cercospora*, because the current systematic scheme is based on multilocus phylogeny (Groenewald et al. 2013, Nguanhom et al. 2015, Soares et al. 2015, Bakhshi et al. 2015, 2018, Albu et al. 2016, Guillen et al. 2017, Guatimosim et al. 2017) and DNA can rarely be extracted from herbarium samples. Therefore, it is important to typify and epitypify species within this genus to stabilise the names for future studies, and provide connections between specimens assessed through molecular and morphological methods.

In an eight-gene molecular DNA sequence analysis employed for *Cercospora s. str.*, Bakhshi et al. (2018) revealed cryptic species within several species complexes. Therefore, besides introducing some new species, epitypes have been designated for some species which were previously regarded as synonyms of other species based on previously published five-gene phylogenies (Groenewald et al. 2013, Bakhshi et al. 2015, 2018). The objective of the present study was therefore to confirm the taxonomy and DNA phylogeny of the *Cercospora* isolates obtained from *S. varia* from Iran and Romania, which were previously synonymised under *C. armoraciae* based on a five-gene DNA dataset (Groenewald et al. 2013, Bakhshi et al. 2015).

MATERIAL AND METHODS

Specimens and isolates

Isolates used in this study (Table 1) are maintained in the collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, the working collection of Pedro Crous (CPC; housed at Westerdijk Fungal Biodiversity Institute), and the culture collection of Tabriz University (CCTU), Tabriz, Iran.
### Table 1

| Species            | Host            | Host family | Origin                   | Collector | GenBank accession numbers |
|--------------------|-----------------|-------------|--------------------------|-----------|---------------------------|
| *C. armoraciae*     | Armoracia rusticana | Brassicaceae | Romania, Fundulea          | O. Constantinescu | JX14333, JX142897, JX142807, JX142561, MH496351 |
|                    |                 |             |                          | M. Torbati | JX143531, JX143532, JX143537, JX143538, MH496360 |
|                    |                 |             |                          | M. Arzanlou | JX143533, JX143534, JX143535, JX143536, MH496361 |
|                    |                 |             |                          | M. Bakhshi | JX143537, JX143538, JX143539, JX143540, MH496362 |
|                    |                 |             |                          | M. Arzanlou | JX143541, JX143542, JX143543, JX143544, MH496363 |
|                    |                 |             |                          | M. Bakhshi | JX143545, JX143546, JX143547, JX143548, MH496364 |
|                    |                 |             |                          | M. Arzanlou | JX143550, JX143551, JX143552, JX143553, MH496365 |
|                    |                 |             |                          | M. Bakhshi | JX143554, JX143555, JX143556, JX143557, MH496366 |
|                    |                 |             |                          | M. Arzanlou | JX143558, JX143559, JX143560, JX143561, MH496367 |
|                    |                 |             |                          | M. Bakhshi | JX143562, JX143563, JX143564, JX143565, MH496368 |
| *C. bicuspidata*    | *Lepidium draba* | Brassicaceae | Romania, Cape of Good Hope | M. Bakhshi | JX143546, JX143547, JX143548, JX143549, MH496369 |
| *C. sordida*       | Sorghum halepense | Poaceae      | Iran, Guilan              | M. Bakhshi | JX143550, JX143551, JX143552, JX143553, MH496370 |
| *C. sorghicola*     | Securigera varia | Fabaceae     | Iran, Zanjan              | M. Bakhshi | JX143554, JX143555, JX143556, JX143557, MH496371 |

**DNA extraction, PCR amplification and sequencing**

DNA sequences comprised those previously extracted by Bakhshi et al. (2015) and Groenewald et al. (2013). Three additional partial nuclear genes were sequenced for each isolate. The primers Gpd1-LM and Gpd2-LM (Myllys et al. 2002) were used to amplify part of the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene. Part of the β-tubulin (*tub2*) gene was amplified using the primer set BT-1F and BT-1R (Bakhshi et al. 2018), whereas the primer set RB2p-CSF and RB2p-CR (Bakhshi et al. 2018) was used to amplify part of the DNA-directed RNA polymerase II second largest subunit (*rpb2*) gene. All amplification mixes and conditions were performed in a total volume of 12.5 μL as described by Bakhshi et al. (2018). PCR products were visualised by electrophoresis using a 1.2 % agarose gel, stained with GelRed™ (Biotium, Hayward, CA, USA) and viewed under ultraviolet light. Size estimates were made using a HyperLadder™ I molecular marker (Bioline).

Both strands of the PCR fragments were sequenced using the same primers used for amplification and the BigDye Terminator Cycle Sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer’s instructions. Sequencing amplicons were purified through Sephadex G-50 Superfine columns (SigmaAldrich, St Louis, MO, USA) and analysed with an ABI Prism 3730xl Automated DNA analyser (Life Technologies Europe BV, Applied Biosystems™, Bleiswijk, The Netherlands) as outlined by the manufacturer.

**Phylogenetic analyses**

The raw DNA sequences of *tub2*, *rpb2* and *gapdh* were edited using MEGA v. 6 (Tamura et al. 2013) and forward and reverse sequences for each isolate were assembled manually to generate consensus sequences. In addition, sequences of isolates from the *C. armoraciae* complex (Groenewald et al. 2013, Bakhshi et al. 2015, 2018) corresponding to the ITS locus (including ITS1, 5.8S, ITS2), together with parts of seven protein coding genes, viz. translation elongation factor 1-alpha (*tef1*), actin (*actA*),
calmodulin (cmdA), histone H3 (his3), tub2, rpb2 and gapdh, were retrieved from the NCBI's GenBank nucleotide database and included in the analyses (Table 1). Sequences were aligned with the MAFFT online interface using default settings (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013), and adjusted manually where necessary using MEGA v. 6. Sequences of C. sorghicola (CBS 136448 = IRAN 2672C) were used as outgroup.

Phylogenetic analyses were based on Bayesian inference (BI). For this purpose, the best nucleotide substitution model was selected independently for each locus using MrModeltest v. 2.3 (Nylander 2004). The individual alignments of the different loci were subsequently concatenated with Mesquite v. 2.75 (Maddison & Maddison 2011) prior to being subjected to a combined multi-gene analysis. Phylogenetic reconstruction under optimal criteria per partition was performed using Bayesian inference (BI) Markov Chain Monte Carlo (MCMC) algorithm in MrBayes v. 3.2.6 (Ronquist et al. 2012). Two simultaneous MCMC analyses, each consisting of four Markov chains, were run from random trees until the average standard deviation of split frequencies reached a value of 0.01, with trees saved every 100 generations and the heating parameter was set to 0.15. The first 25 % of saved trees were discarded as the “burn-in” phase and posterior probabilities (Rannala & Yang 1996) were calculated from the remaining trees. The resulting phylogenetic tree was printed with Geneious v. 5.6.7 (Kearse et al. 2012). Newly generated sequences in this study were deposited in NCBI’s GenBank nucleotide database (www.ncbi.nlm.nih.gov; Table 1) and alignments and respective phylogenetic trees in TreeBASE, study number 24021 (www.TreeBASE.org).

Morphology

To examine morphological characteristics, diseased leaf tissues were observed under a Nikon® SMZ1500 stereo-microscope and taxonomically informative morphological structures (stromata, conidiophores and conidia) were picked up from lesions with a sterile dissecting needle and mounted on glass slides in clear lactic acid. Structures were examined under a Nikon Eclipse 80i light microscope at ×1000, and 95 % confidence intervals were derived for the 30 measurements with extreme values given in parentheses. High-resolution photographs of microscopic fungal structures were captured with a Nikon digital sight DS-F1 high definition colour camera mounted on the above-mentioned light microscope and the layout of acquired images and photographic preparations was carried out in Adobe Photoshop CS5. Colony macro-morphology on MEA was determined after 20 d at 25 °C in the dark in duplicate and colony colour was described using the mycological colour charts of Rayner (1970).

Allele group designation

The isolates of this study along with the other isolates from the C. armoraciae species complex (Bakhshi et al. 2018), including C. armoraciae s. str. and C. bizzozeriana, were compared using the individual alignments of the eight single loci in MEGA v. 6. Allele groups were established for each locus based on sequence identity, i.e., each sequence with one or more nucleotide difference from the other sequences was regarded as a different allele.

RESULTS AND DISCUSSION

Phylogenetic analyses

The final concatenated alignment contained 12 aligned sequences of the isolates from the C. armoraciae species complex (Groenewald et al. 2013, Bakhshi et al. 2015, 2018) and 4 084 characters including alignment gaps. The gene boundaries were 1–470 bp for ITS, 475–765 bp for tef1, 770–956 bp for actA, 961–1 208 bp for cmdA, 1 213–1 568 bp for his3, 1 573–1 982 bp for tub2, 1 987–3 215 bp for rpb2, and 3 220–4 084 bp for gapdh. Four sets of four Ns were used in the alignment to separate adjacent loci and were excluded from the phylogenetic analyses.

Based on the results of MrModeltest, a SYM-gamma model with a gamma distributed rate variation for ITS, a K80-gamma model with a gamma distributed rate variation for tef1, actA and cmdA, a HKY+G with gamma-distributed rates for his3, a GTR+G model with a gamma distributed rate variation for tub2 and rpb2 were applied while gapdh required GTR+I+G with inverse gamma distributed rate variation. The ITS, tef1, actA and cmdA partitions had fixed (equal) base frequencies, whereas the remaining partitions (his3, tub2, rpb2 and gapdh) had dirichel base frequencies. From this alignment, 4 056 characters were
used for the Bayesian analysis; these contained 200 unique site patterns (7, 26, 17, 16, 32, 25, 32 and 45 for ITS, tef1, actA, cmdA, his3, tub2, rpb2 and gapdh, respectively). The Bayesian analysis lasted 565,000 generations and generated a total of 1,132 trees. After discarding the first 25% of sampled trees for burn-in the phylogenetic tree (50% majority rule consensus tree) and posterior probabilities were calculated from the remaining 850 trees (Fig. 1).

All genes were also assessed individually using Bayesian analyses under the above-mentioned substitution models, for each data partition (Fig. 2). Based on individual gene tree assessments, the isolates obtained from *Securigera varia* are supported as a clade of its own based on phylogenies derived from the *tef1*, *cmdA*, *tub2*, and *gapdh* alignments (Fig. 2).

### Taxonomy

The Consolidated Species Concept (Quaedvlieg et al. 2014) accepted in recent revisions of the taxonomy of the genus *Cercospora* (e.g., Groenewald et al. 2013, Bakhshi et al. 2015, 2018) was employed in this study to distinguish the isolates of the genus *Cercospora* obtained from *Securigera varia*. These isolates were previously recognised as *C. armoraciae* based on the five-gene phylogenetic tree (ITS, *tef1*, *actA*, *cmdA*, and *his3*) (Groenewald et al. 2013, Bakhshi et al. 2015). Recently Bakhshi et al. (2018) re-assessed species of the genus *Cercospora* using a combined approach based on the evaluation of an eight-gene (ITS, *tef1*, *actA*, *cmdA*, *his3*, *tub2*, *rpb2* and *gapdh*) molecular DNA sequence dataset, host, and morphological data. The robust eight-gene phylogeny revealed several novel clades within the existing *Cercospora* species complexes, such as *C. armoraciae*, and the *C. armoraciae s. lat.* isolates were distributed over two clades, *C. armoraciae s. str.* and *C. bizzozeriana* (Bakhshi et al. 2018). In this study, the eight-gene phylogeny of the *Cercospora* isolates obtained from *Securigera varia* (as *C. armoraciae* in Groenewald et al. 2013, and Bakhshi et al. 2015) revealed that the clade comprising these two strains is completely distinct from *C. armoraciae s. str.* and *C. bizzozeriana* (*C. armoraciae s. lat.* complex) clades. Based on a literature survey and morphological similarities, the species name *C. rautensis* was assigned to this clade.

**Cercospora rautensis** C. Massal., Madonna Verona, Boll. Mus. Civico Verona 3: 19. 1909. Fig. 3.

*Synonyms:* *Cercospora coronillae-scorpioidis* Ferraris, Fl. Ital. Cryptog. I, Fungi, Hyphales: 893. 1910.
*Cercospora coronillae-variae* Lobik, Bolezni Rastenij 17: 194. 1928.

*Description in planta:* Leaf spots distinct, circular to irregular, grey-brown, without definite border, 1–5 mm diam. *Mycelium*...
internal. Coespituli amphigenous, brown. Conidiophores in moderately dense fascicles (4–25), arising from the upper cells of a well-developed, intraepidermal and substomatal, brown stroma, up to 45 µm diam; conidiophores pale brown to brown, 0–3-septate, straight to mildly geniculate, flexuous, unbranched, (30–)45–65 × 4.5–6 µm, somewhat irregular in width, becoming narrower towards the apex. Conidiogenous cells terminal or integrated, brown, smooth, proliferating sympodially, 15–40 × 3.5–5 µm, mono-local or multi-local; loci thickened, darkened, refractive, protuberant, mostly apical, sometimes lateral, 2–3.5 µm diam. Conidia solitary, subcylindrical to cylindrical, straight to mildly curved, hyaline, thin-walled, smooth, distinctly 3–9-septate, obtuse at the tip, truncate to obconically truncate at the base, (38–)65–80(–130) × (4–)4.5–6 µm; hila thickened, darkened, refractive, 2–3.5 µm diam.

**Typus**: Italy, “Nel bosco “delle Raute” presso il paesetto di Cogolo, on Securigera varia [≡ Coronilla varia] (Fabaceae), Aug., C. Massalongo (holotype VER, n.v.). Romania, Hagieni, on S. varia, 20 Jul. 1970, O. Constantinescu (epitype designated here CBS H-9861, MBT 385978), ex-epitype culture CBS 555.71 = IMI 161117 = CPC 5082.

Additional material examined: Iran, West Azerbaijan Province, Khoy, Firouragh, on Securigera varia, Jul. 2012, M. Arzanlou (IRAN 17180F, CCTU 1190 = CBS 136134).

**Culture characteristics**: Colonies on MEA reaching 60 mm diam after 20 d at 25 °C in the dark; flat with smooth, even margins and moderate aerial mycelium; surface olivaceous grey, reverse iron-grey.

**Distribution**: Italy, Hungary, Lithuania, Poland, Romania, Ukraine (Europe), Russia (European part), Georgia, Pennsylvania (USA) (Crous & Braun 2003, Farr & Rossman 2019) and Iran (Asia) (this study).

**Notes**: Based on the results of the eight-gene phylogenetic tree, the isolates obtained from S. varia from Iran and Romania, previously recognised as C. armoraciae based on five-gene phylogenetic tree (Groenewald et al. 2013, Bakhshi et al. 2015), clustered in a clade, distinct from the ex-type isolate of C. armoraciae (CBS 250.67 = CPC 5088) (Fig. 1). Therefore, this species must be regarded as a separate species and appears to be specific to S. varia. Cercospora rautensis is the only species of Cercospora known from S. varia. The collection from Iran agrees morphologically well with Chupp’s (1954) description of C. rautensis (conidiophores 20–100 × 3–5 µm, conidia acicular to cylindrical, straight to mildly curved, 35–100 × 3–5 µm, base truncate to obconically truncate, tip subobtuse). It also perfectly agrees with type material of C. coronillae-variae (LE 158151), which has been reduced to synonymy with C. rautensis (conidiophores 15–65 × 4–8 µm, conidia cylindrical, subcylindrical to slightly obclavate, 40–100 × (3.5–)4–5(–6) µm, base truncate to somewhat obconically truncate) (examined by Uwe Braun). It is unclear whether Chupp (1954) had seen and examined the type material of C. rautensis. A long time ago, U. Braun (pers. commun.) received the information that Massalongo’s types are maintained at VER, but a loan was not possible and is not possible until now. However, as long as the existence of type material of C. rautensis at VER must be assumed, neotypification is not justified to solve the issue. Since one European isolate from S. varia in Romania (CBS 555.71 =
Table 2. Results from allele group designation per locus for C. armoraciae, C. bizzozeriana and C. rautensis (C. armoraciae s. lat.) isolates.

| Species          | Culture accession number | Allele group per locus |
|------------------|--------------------------|------------------------|
| C. armoraciae    | CBS 250.67; CPC 5088 (TYPE) | I I I I I I – I |
| C. bizzozeriana  | CCTU 1013                 | I I I I I I I I |
|                  | CCTU 1022; CBS 136028     | I I I I I I I I |
|                  | CCTU 1040; CBS 136131     | I I I I I I I I |
|                  | CCTU 1107                 | I I I I I I I I |
|                  | CCTU 1117; CBS 136132     | I I I I I I I I |
|                  | CCTU 1234                 | I I I I I I I I |
|                  | CCTU 1127; CBS 136133     | I I I I I I I I |
|                  | CBS 540.71; CPC 5060      | I I I I I I I I |
|                  | CBS 258.67; CPC 5061 (TYPE) | I I I I I I I I |
| C. rautensis     | CCTU 1190; CBS 136134     | I I I I I I I I |
|                  | CBS 555.71; CPC 5082 (TYPE) | I I I I I I I I |

IMI 161117 = CPC 5082) (as C. rautensis until Jul. 2013; see Groenewald et al. 2013) also resides in this clade, I designate it here as epitype for this species, and fix the application of the name C. rautensis to this clade.

Allele group designation

The results of allele group designation of the isolates of C. rautensis and other isolates in C. armoraciae complex are summarised in Table 2. The allele group for the tef1, actA, cmdA and tuf2 sequences for both strains of C. rautensis from Iran (CBS 136134) and Romania (CBS 555.71) was similar and also different from the allele group of the C. armoraciae and C. bizzozeriana isolates. For ITS, the allele group of these two isolates was the same as C. armoraciae and C. bizzozeriana isolates, while for his3 and gapdh, these two isolates had a different allele group which was also distinct from the allele group of the C. armoraciae and C. bizzozeriana isolates (Table 2).

CONCLUSIONS

Extensive studies of Cercospora and related genera in Iran have generated records of numerous species (Hesami et al. 2012, Pirnia et al. 2012, Bakhshi et al. 2012, 2015, 2018). However, C. rautensis has not been detected in Iran before. Therefore, this is the first report of C. rautensis infection of Crownvetch in Iran. Since one European isolate was included in this study, I was able to designate an epitype here for this species as well, which was necessary to determine the application of the name C. rautensis.

In recent years, two significant advances in the understanding of Cercospora have been achieved. First, with the comprehensive molecular examination of Cercospora s. str. based on a multi-locus DNA sequence dataset of five genomic loci of the large sampling of species conducted by Groenewald et al. (2013), a backbone phylogeny was achieved for Cercospora. Second, an eight-gene molecular DNA sequence analysis of Cercospora s. str. was conducted by Bakhshi et al. (2018), which revealed cryptic species within several species complexes. One important finding of these studies is that it was not always possible to apply North American or European names to African or Asian strains and vice versa. Therefore, type specimens are essential for molecular analyses of Cercospora species for correct applications of such species names. Unfortunately, many (epi-)type cultures are lacking for a significant number of Cercospora species. These species will have to be recollected from their original hosts and continents from where they were described. These collections are necessary to stabilise the application of the names to facilitate subsequent taxonomic work on Cercospora.

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