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Stem rot and wilt on *Euonymus*

Stammfäule und Welke an *Euonymus*

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Abstract

In August 2008, stem rot and wilt symptoms of unknown origin were observed on *Euonymus japonica*. From the symptomatic stem base a fungus belonging to the genus *Calonectria* (anamorph: *Cylindrocladium*) was isolated (isolate JKI 2140). The isolate was morphologically very similar to *Calonectria colhounii* as well as to *Ca. fujianensis* and *Ca. pseudocolhounii*, except for the larger conidia. Sequence analysis of genes (ITS, BT, TEF-1 α , HIS3) showed high similarity to *Ca. colhounii*, *Ca. eucalypti*, *Ca. fujianensis* and *Ca. pseudocolhounii*. The taxonomic status of the fungal isolate from *E. japonica* is not yet clear. It belongs to the complex *Ca. colhounii* but a definitive allocation to or separation from a known *Calonectria* species is not possible on the basis of a single isolate. The fungus is provisionally named *Calonectria colhounii* compl.

The pathogenicity of the fungus was tested on *E. japonica* and *E. fortunei*. The disease symptoms originally observed on field plants of *E. japonica* were reproduced and the fungus was re-isolated. Thus the pathogenicity of isolate JKI 2140 on both *Euonymus* species is proved. Since the first occurrence of this infestation there were no further notifications of stem canker and wilt on *Euonymus* spp. in Germany. Therefore the importance of this pathogen on *Euonymus* is considered to be low.

Key words: *Calonectria*, *Cylindrocladium*, *Euonymus*, ornamental shrub, symptoms, morphology, sequence analysis

Zusammenfassung

Im August 2008 trat an *Euonymus japonica* eine Stammfäule mit Welke unbekannter Ursache auf. Aus der Triebbasis wurde ein Pilz der Gattung *Calonectria* (Anamorphe: *Cylindrocladium*) isoliert (Isolat JKI 2140). Das Isolat wies morphologisch große Ähnlichkeit mit *Ca. colhounii*, *Ca. fujianensis* sowie *Ca. pseudocolhounii* auf, allerdings waren die Konidien im Durchschnitt größer als für diese drei Arten beschrieben. Die Sequenzanalysen ergaben hohe Übereinstimmungen mit *Ca. colhounii*, *Ca. eucalypti*, *Ca. fujianensis* und *Ca. pseudocolhounii*. Der taxonomische Status des Pilzisolates aus *E. japonica* ist noch nicht eindeutig geklärt. Es gehört zum Artenkomplex *Ca. colhounii*, eine sichere Zuordnung zu oder Abtrennung von einer der bekannten Arten aus diesem Komplex lässt sich aber anhand eines einzigen Isolates nicht treffen. Der Pilz wird vorläufig als *Calonectria colhounii* compl. bezeichnet.

Die Pathogenität des Pilzes wurde an *E. japonica* und an *E. fortunei* geprüft. Die ursprünglich an *E. japonica* beobachteten Symptome ließen sich reproduzieren, der Pilz reisolieren. Die Pathogenität von Isolat JKI 2140 an beiden *Euonymus*-Arten ist damit nachgewiesen. Seit dem Erstauftreten dieses Pilzes gab es keine weiteren Meldungen über eine Stammgrundfäule mit Welkeerscheinungen an *Euonymus* in Deutschland. Die Bedeutung dieses Erregers an *Euonymus* ist deshalb als gering einzustufen.

Stichwörter: *Calonectria*, *Cylindrocladium*, *Euonymus*, Ziergehölz, Symptome, Morphologie, Sequenzierung

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Introduction

Wilt symptoms were found on one-year-old plants of *Euonymus japonica* in a nursery in the German federal state Hesse (Fig. 1a). The nursery manager had purchased rods to establish a mother stock for propagation. Approx. 15% of the stock plants showed disease symptoms. Only the cultivar *Microphyllus* was affected. The typical symptom was wilting of single shoots, pale green leaves without gloss. The shoot base was necrotic and the cortex peeled from the woody part (Fig. 1b). The wilting seemed to be a consequence of this damage. From the lower stem part a fungus was isolated which was allocated to the genus *Calonectria* because of stipe extensions with vesicles on the conidiophores. The fungal isolate was morphologically very similar to *Ca. colhounii* Peerally.

Calonectria-species are pathogenic to a wide range of plant species and may cause different symptoms. In Germany, important species on ornamental shrubs are *Ca. morganii* (Anamorph: *Cylindrocladium scoparium*) and *Ca. pseudonaviculata* (Anamorph: *Cylindrocladium pseudonaviculatum*), better known as *Cylindrocladium buxicola*. *Cy. scoparium* causes basal stem rot and wilt on *Rhododendron simsii* and *Erica gracilis* (TIMONIN and SELF, 1955; KELLING, 1981; NEUBAUER and ZINKERNAGEL, 1996). *Cy. buxicola* causes leaf and twig blight on *Buxus* sp. (HENRICOT and CULHAM, 2002; BRAND, 2005). In 1973 *Ca. colhounii* (Anamorph: *Cy. colhounii*), the causal agent of a leaf spot disease on tea plants in Mauritius, was described by Peerally as a new species. According to JEON et al. (2010) *Ca. colhounii* was established on hosts

belonging to 14 genera. The fungus may not only cause leaf blight but also basal stem rot. *Ca. colhounii* as a pathogen on blueberries is of particular importance. Reports about leaf spots on *Vaccinium corymbosum* are known from China (LUAN et al., 2006); and in Korea basal stem rot was reported on blueberry seedlings originating from USA (JEON et al., 2010). SADOWSKY et al. (2011) attributed necrotic stems and leaves on *V. corymbosum* and *V. angustifolium* also to an infection by *Ca. colhounii*. In 2008, the disease was observed for the first time in the USA. *Ca. colhounii* was also isolated from *Gaultheria* with leaf spots (EL-GOLL et al., 1997); however, in this case no pathogenicity test was performed.

The only report of *Ca. colhounii* in Europe comes from Belgium where *Ca. colhounii* was identified as the causal agent of leaf spots on *Rhododendron* (INGHELBRECHT et al., 2011). Current knowledge suggests that *Ca. colhounii* is a species complex (L. LOMBARD, pers. comm. 15.11.2013). Two species isolated recently from *Eucalyptus* infested by *Cylindrocladium* leaf blight (CLB) (CHEN et al., 2011), *Ca. fujianensis* and *Ca. pseudocolhounii*, can also be allocated to this complex.

Material and Methods

Morphological studies

The identification of the fungal isolate from *E. japonica* was done using morphological analysis according to CROUS (2002). Agar plugs of a single-spore culture of the isolate JKI 2140 were transferred to 2% malt extract agar

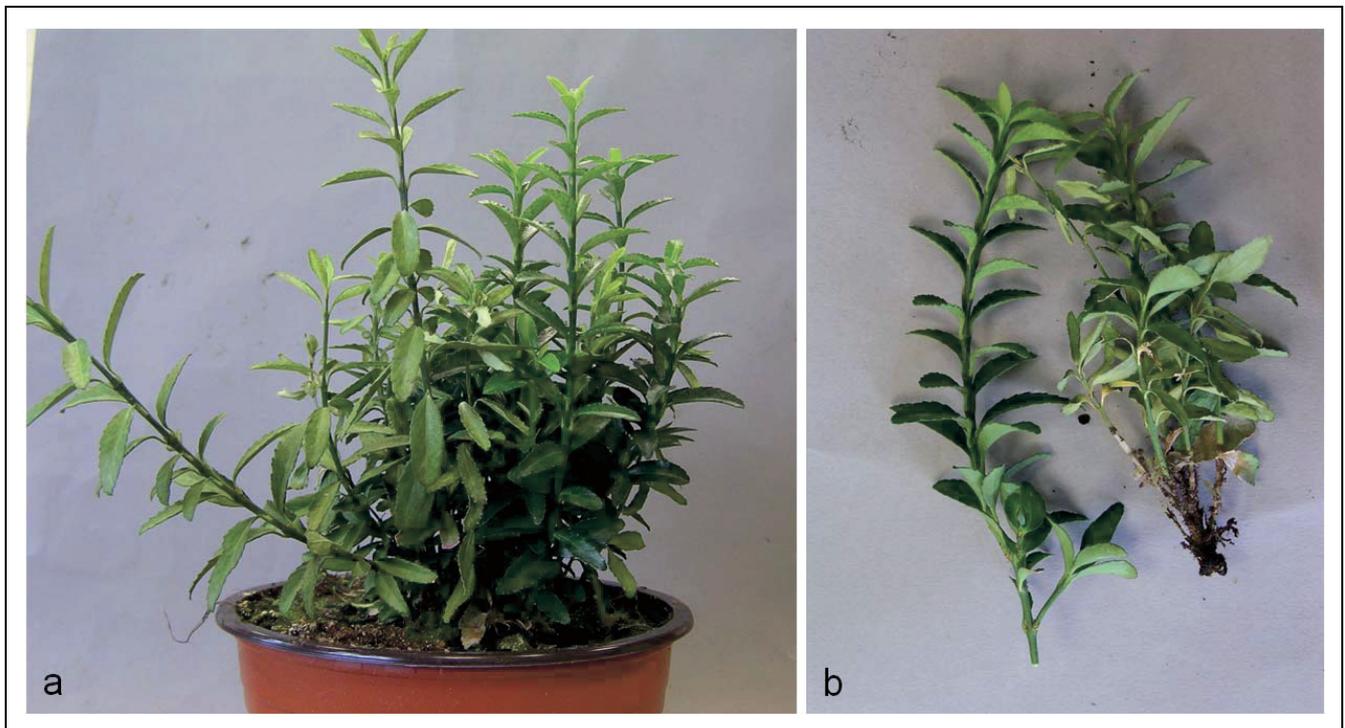


Fig. 1. Symptoms on *E. japonica* 'Microphyllus', natural infestation
a – Green wilt
b – Rot on the stem base.

(MEA) and incubated at 25°C in the dark. The anamorph was studied by light microscopy after incubation for seven days on a special nutrient-poor agar (SNA) (NIRENBERG, 1976) at 25°C under NUV-light (12 h). In two separate approaches with respectively 40 conidiospores, conidia were measured at x 1000 magnification and the 95% confidence levels were determined. The minimum and maximum ranges are given in parentheses.

For perithecia induction the isolate was cultivated at 25°C on MEA under NUV-light. Mature perithecia were studied using the Kulzer Histo-Technique ISO 7100 based on hydroxyethyl methacrylate (HEMA) according to GERRITS (1985). From the embedded perithecia thin sections each 10 µm thick were prepared with a rotary microtome.

The cardinal temperatures were determined by assessment of the radial growth on MEA after incubation for 6 days in the dark.

Sequence analysis

Four loci were amplified and sequenced: parts of the big and small subunit (LSU/SSU) as well as the 5.8S rDNA and the embedded internal transcribed spacer (ITS) regions 1 and 2, the β-tubulin gene (BT), the translation-elongation-factor TEF-1α as well as the histone H3 (HIS3). The DNA was isolated by means of the Invisorb® Spin Plant Mini Kits (STRATEC Molecular GmbH, Berlin) from mycelium of a MEA cultivated single-spore culture of the isolate JKI 2140. The following primers were used: for the amplification of the ITS-region the ITS1- and ITS4-Primer of WHITE et al. (1990), for the fragment of the β-tubulin genes the primers Bt2a and Bt2b of GLAAS and DONALDSON (1995), for the fragment of the histone H3 genes the primers H3-1a and H3-1b (GLAAS and DONALDSON, 1995) as well as a gene section of the TEF-1 α by means of the primers EF1-526F and EF1-1567R (REHNER, 2001). Successful amplification of the gene sections were cleaned (MSB® Spin PCRapace Kit, STRATEC Molecular GmbH, Berlin) and sent to the company LGC Genomics (Berlin) for sequencing in both directions. Subsequently the sequences were assembled (CLC Maim Worknech, Qiagen Aarhus) and blasted (BLAST, ALTSCHUL et al., 1997) against previously gene-bank-stored sequences (NCBI, <http://www.ncbi.nlm.nih.gov>).

Phylogenetical examinations (DNA sequence comparison)

The sequences of the single gene sections of the isolate JKI 2140 were compared phylogenetically with the *Calonectria*-species listed in CHEN et al. (2011). The sequences were aligned using the program Bioedit 7.2.5. (HALL, 1999) (CLUSTALW multiple alignment) and the nucleotide differences were analyzed. The model with the highest Bayesian information criterion (BIC) and thus the statistically most stable model for the phylogenetical analysis of the *Calonectria*-species was the time reversible algorithm according to Tamura-Nei (1993) which was used for further analyses by means of the maximum-likelihood (ML) method. These calculations were

done with the software MEGA 6 (TAMURA et al., 2013). Phylogenetic relationships were estimated by heuristic searches based on 1000 random repeats. The analyses included 57 partial gene sequences per gene (BT, HIS3, TEF-1α), representing 28 *Calonectria* species to calculate an unrooted maximum likelihood tree (CHEN et al., 2011).

Pathogenicity tests

One-year-old *Euonymus japonica* 'Microphylla' and *E. fortunei* 'Emerald'n Gold' were inoculated with a single-spore culture of *Calonectria* JKI 2140 using four different methods:

1. Spraying of the plants with a watery conidia suspension of 10⁵/ml until run-off (4.5 ml per plant).
2. Excision of the two leaves at a nodium and application of an agar plug colonized by the fungus onto the fresh wound, inoculation of three shoots per plant.
3. Wounding of the base of the main shoot by roughening with fine sandpaper type P150, subsequently application of an agar plug colonized by the fungus
4. Mixing of a homogenized *Calonectria* culture from one Petri dish with 1 L of growing medium.

The inoculation points of method 2 and 3 were wrapped with wet cellulose and parafilm for three weeks. Control plants were treated in the same way but without the fungal isolate. Twenty plants were used for each variant. The plants were incubated in a climate chamber at 21°C/16°C (day/night) with 90–100% relative humidity and a photoperiod of 12 hours. Within the first three weeks the plants were kept under a plastic tunnel and were regularly irrigated overhead. The assessment of symptoms was done three and ten weeks after the inoculation. For re-isolation samples from the edge of the lesions on the stem were surface disinfected and placed on potato-dextrose-agar (PDA). The agar plates were incubated at 20°C in the dark.

Results

Morphological characteristics

The isolate JKI 2140 developed white aerial mycelium on MEA in the dark, partly with irregular colony margins. The colony reverse turned orange (Fig. 2) due to the production of chlamydospores. The chlamydospores formed chocolate brown microsclerotia.

The conidiophores were arranged penicillately (Fig. 3a). Their stipe extensions were very long, narrow and septate, and ended in a claviform vesicle (Fig. 3b). The macroconidia were straight, cylindrical, rounded on both ends, 3-septate and were held together by hyaline slime in parallel cylindrical clusters. The average size of the conidia was 79 × 7 µm (Tab. 1).

After four to six weeks perithecia developed on MEA. Mature perithecia were dark yellow to light orange or orange-brown (Fig. 3c). They contained numerous asci

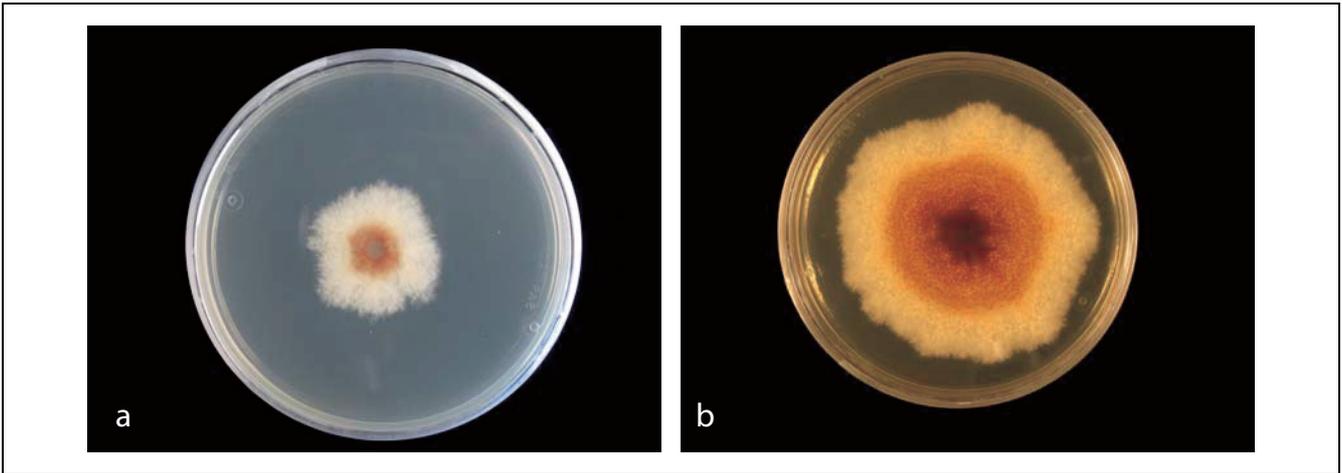


Fig. 2. Colony morphology on MEA
a – 7 days old culture
b – 14 days old culture.

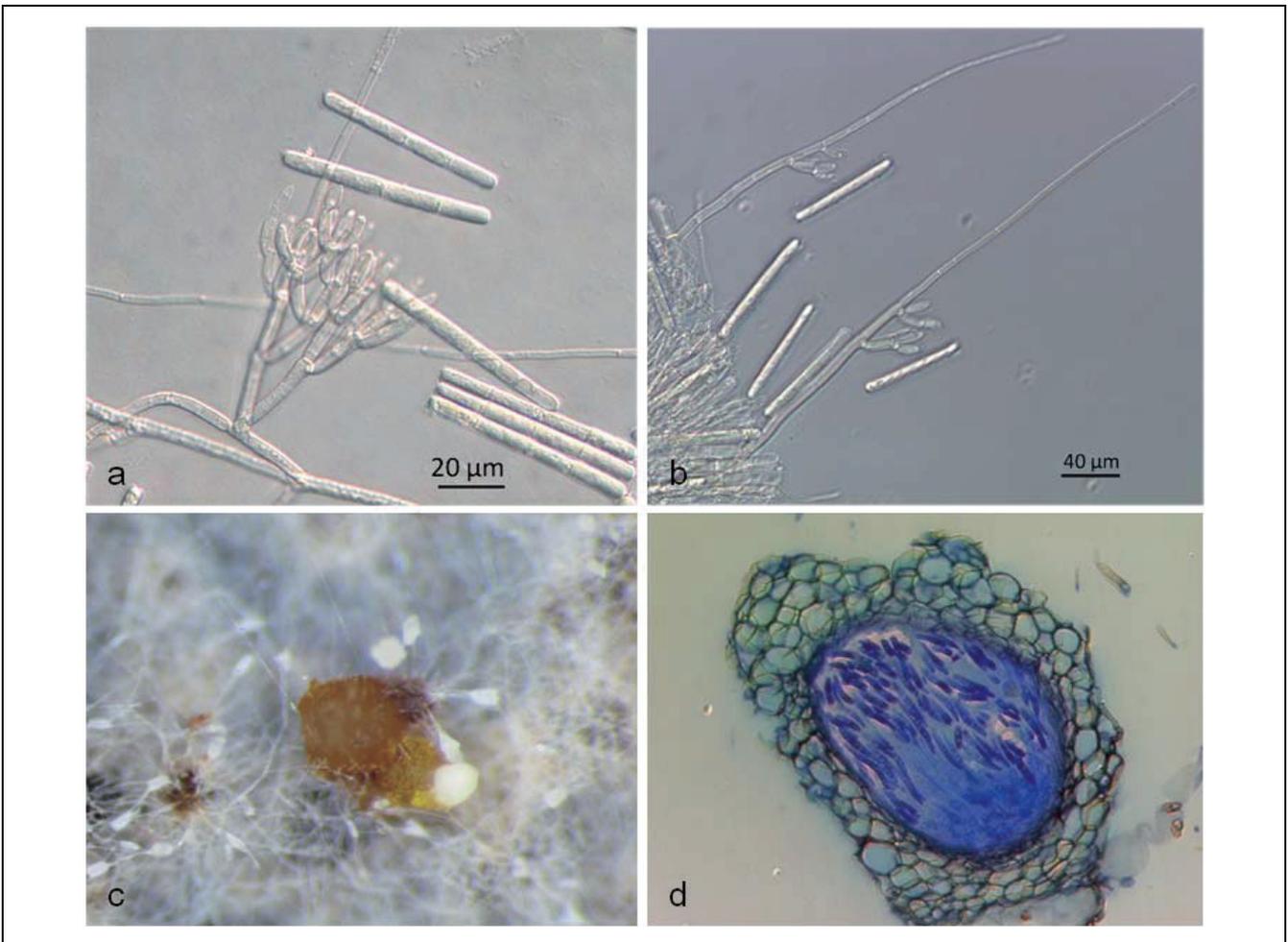


Fig. 3. Morphological characteristics of isolate JKI 2140
a – Penicillate conidiophores
b – Stipe extensions with vesicles
c – Perithecium on MEA
d – Section of perithecium with asci and ascospores.

with four ascospores each (Fig. 3d). Data on perithecia and ascospore size are listed in Tab. 1.

Cardinal temperatures for the mycelium growth: Minimum > 5°C, Maximum < 35°C, Optimum 25°C.

Tab. 1. Size of conidiospores and ascospores of five *Calonectria*-species belonging to the complex *Ca. colhounii*

Size [μm]	<i>Ca. colhounii</i> compl. (JKI 2140)	<i>Ca. colhounii</i> (CROUS, 2002)	<i>Ca. pseudocolhounii</i> sp.nov. (CHEN et al., 2011)	<i>Ca. fujianensis</i> sp. nov. (CHEN et al., 2011)	<i>Ca. eucalypti</i> sp.nov. (LOMBARD et al., 2010)
Macroconidiospores					
Length	(66-)67-82(-84)*	(30-)50-65(-80)	(49-)55-65(-74)	(48-)50-55(-60)	(66-)69-75(-80)
Width	(5-)6-7.5(-8)	(4-)5-6(-7)	(3,5-)4-5(-5.5)	(2,5-)3.5-4.5(-5)	(5-)6
Average L \times W	79 \times 7	65 \times 5	60 \times 4.5	52.5 \times 4.5	72 \times 6
Ascospores					
Length	(41-)40-69(-67)	(30-)50-65(-75)	(44-)50-62(-74)	(38-)49-62(-72) x	(25-)30-36(-56)
Width	(5-)6-7(-9)	(4-)5-6(-8)	(5-)6-7(-8)	(5-)6-7.5(-8)	(3-)5-6(-8)
Average L \times W	54 \times 7	55 \times 6	56 \times 6.5	55.5 \times 6.8	33 \times 6

* 95% confidence interval, minimum and maximum in parenthesis

Based on the morphological characteristics the fungal isolate from *E. japonica* JKI 2140 was allocated to the species complex *Calonectria colhounii*.

Sequence and phylogenetical analysis

The ITS-sequence of the isolate JKI 2140 (550 Bp) shared 100% identity with the sequence of *Ca. colhounii* isolate PDIC 660-1L (access. no. JF742647) and 99.6% with the isolate CBS 293.79 (access. no. GQ280565.1). No ITS-sequences of *Ca. pseudocolhounii* and *Ca. fujianensis* are in the GeneBank. For the other gene fragments (HIS3, BT, TEF-1 α) the sequence similarity was between 97% and 99%. (Tab. 2). *Ca. fujianensis* had the lowest differences in the number of nucleotides (SNPs) within the three genes. Phylogenetical analysis placed the isolate

JKI 2140 into the species complex *Ca. colhounii* (CHEN et al., 2011) with a very close relationship to *Ca. fujianensis* (Fig. 4).

Pathogenicity test

Three weeks after inoculation with *Calonectria*-isolate JKI 2140 all four inoculation methods resulted in disease symptoms on *E. japonica* and *E. fortunei*. The most severe damage was observed after inoculation of the wounded nodes and the wounded stem base. After inoculation of the nodes lesions on the shoots developed acropetally, followed by wilting and dieback of the shoots. After inoculation at the stem base initial symptoms were pale green leaves followed by a wilting of the plants (Fig. 5a). The stem base of these plants showed chocolate brown

Tab. 2. Sequence comparison of *Ca. colhounii* compl. JKI 2140 with closely related *Calonectria*-species. Sequence identity of the examined gene sections (%) and number of single nucleotide polymorphisms (SNPs)

Species	isolate no.	<i>Ca. colhounii</i> compl. JKI 2140					
		HIS3 %	453 bp SNP	BT %	360 bp SNP	TEF-1 α %	493 bp SNP
<i>Ca. colhounii</i>	CBS 293.79	98.7	6	99.6	2	97.1	13
	CBS 114704	98.7	6	98.9	5	96.9	14
<i>Ca. eucalypti</i>	CBS 125273	97.6	11	98.5	7	99.6	2
	CBS 126275	97.6	11	98.5	7	99.6	2
<i>Ca. pseudocolhounii</i>	CMW 27213	98.5	7	99.1	4	99.8	1
	CMW 27209	98.5	7	99.1	4	99.8	1
<i>Ca. fujianensis</i>	CMW 27257	99.3	3	99.8	1	99.3	3
	CMW 27263	99.3	3	99.8	1	99.3	3

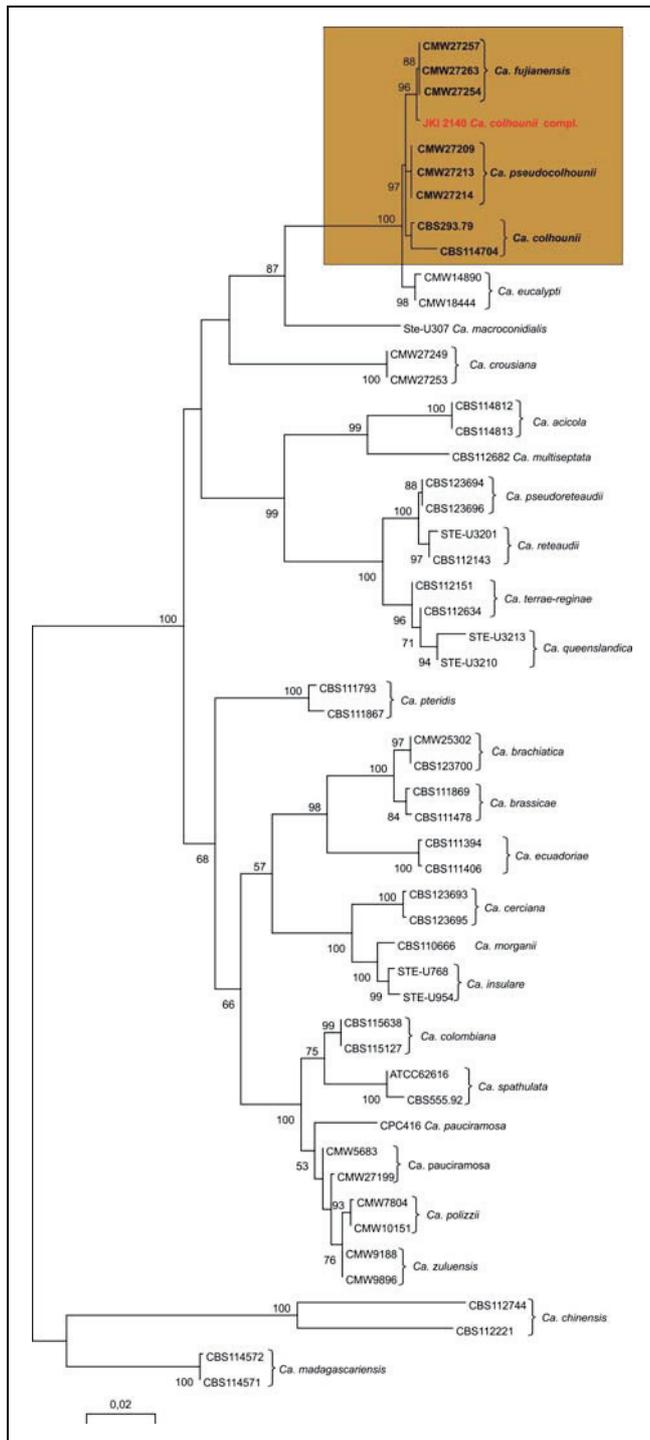


Fig. 4. Unrooted maximum likelihood-tree with *Calonectria*-isolate JKI 2140 and gene-bank reference-sequences of known *Calonectria*-species, based on the loci HIS3, BT and TEF-1 α (CHEN et al., 2011). The distances were calculated using the Tamura-Nei model TN93 + G + I. The bootstrap test was done with 1000 repeats. Only values above 50% are shown. A total 174 partial sequences of 28 *Calonectria*-species were used for phylogenetic tree construction. The analysis was done using MEGA6.

lesions. Plants that were sprayed with a conidia suspension also developed symptoms starting with chocolate brown lesions on shoots. In addition some plants showed

leaf spots (Fig. 5b). Compared to inoculation method 1 and 2 symptoms appeared slightly later. Using inoculation methods 1, 2 and 3, 100% of the plants developed symptoms on *E. fortunei* (Fig. 6) and *E. japonica*. Planting in contaminated substrate resulted in only one symptomatic plant of each *Euonymus* species. The non-inoculated plants from the negative control did not develop disease symptoms.

The fungus was re-isolated from the inoculated *E. fortunei* and *E. japonica* plants. The characteristics of the re-isolates corresponded with those of the original isolate.

Discussion

Calonectria was isolated from symptomatic *E. japonica*. A distinct allocation of the isolate JKI 2140 to a known species was not possible. It shows high morphological similarity to *Ca. colhounii* (CROUS, 2002), but also to *Ca. fujianensis* (CHEN et al., 2011) and *Ca. pseudocolhounii* except for its larger conidia (Tab. 1). The results of the sequence analysis suggest a close relationship of isolate JKI 2140 to *Ca. fujianensis*.

Ca. fujianensis, causal agent of leaf blight on *Eucalyptus*, was defined as a new species within the *Ca. colhounii*-complex by CHEN et al. (2011). The differentiation to *Ca. colhounii* and *Ca. pseudocolhounii* is based on analysis of the gene regions β -Tubulin, histone H3 and translation elongation factor-1 alpha (TEF-1 α). The TEF-1 α gene of the isolate JKI 2140 shows a higher similarity to *Ca. pseudocolhounii*, *Ca. eucalypti* and *Ca. fujianensis* (> 99%) than to *Ca. colhounii*. The results indicate that the causal agent of the wilt on *Euonymus* is a new species within the *Ca. colhounii*-complex. As long as no further identical isolates are detected we suggest to call the isolate JKI-2140 *Calonectria colhounii* compl..

Due to cardinal temperatures min. below 5°C, max. 35°C and opt. 25°C, *Ca. colhounii* compl. is an eurythermical fungus. That indicates that the fungus is adopted to a large temperature range with a risk of infection from spring to autumn in Central Europe.

Inoculation at the stem base resulted in symptoms identical to those observed on the originally infected *Euonymus*. When the plants were sprayed with a conidia suspension stem lesions and leaf spots developed. These symptoms were similar to those on blueberry inoculated with *Ca. colhounii* (SADOWSKY et al., 2011). The results of the pathogenicity tests indicate that *Ca. colhounii* compl. JKI 2140 is highly virulent. In three out of four inoculation variants severe disease symptoms developed, including death of the plants. Wounding is not needed for infection. Thus *Ca. colhounii* compl. differs from *Cylindrocladia parva*. *Cy. parva* is described as a weak pathogen on *E. fortunei* that develops lesions all around the stem on the upper parts of the stem and extends acropetally (BRIELMAIER-LIEBETANZ et al., 2013, 2014). *Ca. colhounii* compl. seems to be only a minor pathogen of *Euonymus* spp. because no reports on stem rot and wilt on *Euonymus* were published since 2008. Nevertheless, careful



Fig. 5. Disease symptoms on *E. japonica* after inoculation with isolate JKI 2140
a – Agar plug on stem base
b – Spraying with conidia suspension.



Fig. 6. Disease symptoms on *E. fortunei* 3 weeks after inoculation with isolate JKI 2140 on the stem base.

visual monitoring of *Euonymus*-stocks for disease symptoms is recommended, especially on young plants. In case of suspicious symptoms plant samples should be diagnosed in a laboratory to avoid confusion with symptoms caused by *Cy. parva* and also *Phytophthora sp.*, which was reported to cause dieback on *E. japonica* in the USA (KEIM et al., 1981).

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