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Willdenowia
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Distribution, infection rates and DNA barcoding of *Uromyces erythronii* (*Pucciniaceae*), a parasite of *Erythronium* (*Liliaceae*) in Europe

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Abstract: This paper presents the European distribution of the understudied, host-specific rust fungus, *Uromyces erythronii* (*Pucciniomycetes*, *Pucciniales*, *Pucciniaceae*). Distribution data were derived from the survey of herbarium materials of its European host plant, *Erythronium dens-canis*. We demonstrate the presence of this rust fungus in 14 countries within the distribution area of its host. The temporal trend of emergence of the two rust fungus generations (aecia and telia) is presented. Based on the study of 1700 *E. dens-canis* individuals, we conclude that the overall infection rate has not changed significantly over the last 200 years. During field surveys, *U. erythronii* infection was detectable in most of the studied *Erythronium* populations (88.5%). A high similarity in the nrITS region was detected among samples from Europe (Croatia, Romania) and Asia (Japan).

Key words: *Erythronium*, herbarium, host specificity, ITS, parasitism, *Pucciniaceae*, rust fungi, *Uromyces*

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Introduction

Despite (or just due to) their antagonistic nature, parasitic relationships play an important role in shaping ecosystems and biodiversity (Gómez & Nichols 2013). Diseases constrain hosts to evolve resistance or tolerance strategies (Roy & Kirchner 2000), thus the hosts and parasites are always in antagonistic co-evolutionary race (Stahl & Bishop 2000), unless the interaction eventually evolves towards commensalism (Miller & al. 2006).

The autoecious rust fungus *Uromyces erythronii* (DC.) Pass. parasitizes some spring geophyte taxa of the vascular plant family *Liliaceae* Juss., e.g. *Erythronium japonicum* Decne., *Amana edulis* (Mig.) Honda and *A. latifolia* (Makino) Honda (Fukuda & Nakamura 1987). In Europe, the only known host plant of this fungus is *E. dens-canis* L., a Red List species in several countries (Witkowski & al. 2003). According to the criteria of the IUCN Red List, *E. dens-canis* is categorized as Vulnerable ("VU") in the entire Carpathians and in Hungary,

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Critically Endangered ("CR") in Slovakia, and Endangered ("EN") in Ukraine. The species is present in Romania but not threatened ("+") (Witkowski & al. 2003). Even though threat assessment and conservation of parasites may be just as important as that of their hosts (Gómez & Nichols 2013), co-endangerment remains poorly investigated, currently being restricted to a few published works (e.g. Dunn & al. 2009; Mihalca & al. 2011 on parasitic insects).

The distribution and abundance of parasites are dependent on host populations, as shown in the case of *Uromyces erythronii* and *Erythronium japonicum* (Fukuda & Nakamura 1987). In Europe, the presence of *U. erythronii* in several countries was only recorded recently, e.g. in 2007 from Croatia (Miličević & al. 2008) and in 2016 in Ukraine (Tykhonenko & al. 2017). The earliest deposited specimens of *U. erythronii* in the Fungi Collection of the Royal Botanical Gardens, Kew, were collected in 1946 from France, in 1949 from Italy and in 1962 from Romania (with further items without collection date from Switzerland, Germany and Romania; Kew s.d.+). From Hungary, six samples of *U. erythronii* (collected in 1965, 1987, 1988 and 2007 from three localities) were deposited in the Fungi Collection of the Hungarian Natural History Museum (cf. Jandrasits & Fischl 2014). Outside the range of the indigenous distribution of its host, the presence of *U. erythronii* has been confirmed in England (Henderson 2000) and in Germany (Kruse & al. 2014), where it infects cultivated populations of *Erythronium*. The low number of known occurrence data, and the fact that all of these were recorded in the last c. 60 years, indicates a poor documentation of the distribution of this rust in Europe, or may refer to recent colonization and spreading.

Our aims in this study were (1) to map the distribution range and the frequency of *Uromyces erythronii* in Europe, predominantly the Carpathian Basin, and (2) to document possible temporal trends in the presence of the aecial and telial generations of the rust. We also aimed (3) to study whether the infection rate of the rust fungus has changed over time and (4) to generate ITS DNA barcodes for *U. erythronii* samples from the Carpathian Basin to facilitate future genetic comparisons of the populations of the species in different geographical areas and on different host plants.

Material and methods

Herbarium overview

We reviewed 525 herbarium sheets (altogether more than 2000 individuals) of *Erythronium dens-canis* in eight herbaria (BP, CL, DE, GK, LJU, RRM, ZA, ZAHO – herbarium codes according to Thiers 2018+, with the exception of the unregistered RRM = Rippl-Rónai Museum, Kaposvár, Hungary) and 24 items in the fungi collection of BP (Table 1). We screened leaves and petioles of herbarium specimens for the presence of *Uromyces erythronii* with a 15× hand lens. We recorded the total number of individuals and the number of infected individuals on each sheet (with the exception of the fungi collection items, because the paper bags usually contain fragmented leaf materials instead of entire individuals, therefore we treated every item as an individual sample). Information from the herbarium labels (site, date [year, month, day] and name of collector for each collection) were registered. Number of aecial- and telial-infected individuals were quantified separately only in the materials of BP and DE. Digital photographs of all herbarium sheets were taken. We used QGIS 2.18 (Quantum GIS Development Team 2017) software for generating a distribution map.

Table 1. Collection period, sample sizes and infection rates of the screened *Erythronium dens-canis* herbarium specimens, summarized for each studied herbarium collection.

Herbarium code	**Period**	**No. herbarium sheets** (all/infected)	**No. individuals** (all/infected)
BP	1815–1987	250/113	890/205
CL	1811–2014	163/39	675/60
DE	1853–2017	33/19	120/23
GK	1963	1/0	6/0
LJU	1852–1996	25/6	117/7
RRM	1982–1983	2/0	4/0
ZA	1852–1989	40/17	191/32
ZAHO	1929–1951	11/5	41/8

Data analysis

We used a generalized linear model (GLM) with binomial error distribution to evaluate the change in infection rate of *Uromyces erythronii* over the last 200 years. Analysis was done based on 1700 *Erythronium dens-canis* individuals from 427 herbarium sheets, which were labelled with at least the year of collection. All sheets with uncertain year of collection were disregarded in the analysis. The number of infected and uninfected individuals in each year entered the model as a dependent variable, while year of collection was included as an explanatory variable. Analysis was done in the R Statistical Environment (R Core Team 2017).

Field survey

We checked the presence of *Uromyces erythronii* aecia in 26 (16 Romanian, seven Hungarian, three Croatian) populations of *Erythronium dens-canis* during flowering and fruiting phenological states. Fieldwork was carried out between 16 April and 2 May 2015, between 6 March and 9 April 2016, and between 9 and 10 March 2017. We

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Table 2. Infection rates derived from the studied *Erythronium dens-canis* herbarium sheets. Number of screened sheets, the total number of individuals on these, as well as the year of collection of the oldest infected specimen are given for each country separately.

Country	**No. herbarium sheets** (all/infected)	**No. individuals** (all/infected)	**Infection rate of herbarium sheets** (%)	**Infection rate of individuals** (%)	**Collection year of oldest infected specimen**
Romania	198/70	780/115	35.4	14.7	1834
Croatia	70/35	292/55	50.0	18.8	1833
Hungary	50/28	216/62	56.0	28.7	1925
Slovenia	35/7	162/8	20.0	4.9	1852
Austria	34/6	119/6	17.6	5.0	1880
Ukraine	28/16	114/29	57.1	25.4	1837
France	18/8	59/10	44.4	17.0	1865
Italy	18/5	59/10	27.8	17.0	1871
Czech Republic	12/3	57/3	25.0	5.3	1903
Slovakia	11/3	25/3	27.3	12.0	1857
Bulgaria	5/3	13/5	60.0	38.5	1929
Switzerland	4/1	16/4	25.0	25.0	1866
Bosnia and Herzegovina	3/1	15/4	33.3	26.7	1908
Montenegro	2/1	20/1	50.0	5.0	1908

Table 3. Main data of field survey: geographic location, elevation (alt.), date of observation, number of screened host individuals (*n*) and infection rate observed. Localities are listed alphabetically, first by country then by settlement. Abbreviations of countries studied: Cro – Croatia, Hu – Hungary, Ro – Romania.

Country: Settlement	**Latitude, longitude**	**Altitude** (m)	**Date**	***n***	**Infection rate**
Cro: Paka	46.13457°N, 16.33066°E	218	9 Mar 2017	50	52%
Cro: Presečno	46.20922°N, 16.34918°E	195	10 Mar 2017	100	9%
Cro: Veliki Lovrečan	46.38007°N, 16.07305°E	204	10 Mar 2017	100	9%
Hu: Becsehely	46.46514°N, 16.78594°E	216	6 Mar 2016	100	28%
Hu: Becsehely	46.46170°N, 16.79331°E	222	6 Mar 2016	100	20%
Hu: Csöde	46.83575°N, 16.55017°E	189	17 Apr 2015	100	31%
Hu: Lispeszentadorján	46.53072°N, 16.70973°E	216	16 Apr 2015	65	23%
Hu: Miskolc	48.13389°N, 20.71584°E	218	31 Mar 2016	116	0%
Hu: Nagybajom	46.45913°N, 17.52378°E	143	8 Mar 2016	100	1%
Hu: Tömörd	47.37599°N, 16.64622°E	269	25 Mar 2016	100	69%
Ro: Blandiana	46.00503°N, 23.36791°E	392	13 Mar 2016	50	20%
Ro: Borod	47.03083°N, 22.63694°E	432	12 Mar 2016	50	2%
Ro: Cornițel	46.97516°N, 22.68086°E	614	12 Mar 2016	50	2%
Ro: Criciova	45.63644°N, 22.09936°E	169	14 Mar 2016	50	38%
Ro: Făget	45.88340°N, 22.19166°E	248	14 Mar 2016	50	48%
Ro: Feleacu	46.71049°N, 23.58978°E	731	10 Apr 2016	100	65%
Ro: Gârbău	46.81360°N, 23.34412°E	521	12 Mar 2016	50	4%
Ro: Hațeg	45.62390°N, 22.97730°E	376	13 Mar 2016	16	0%
Ro: Huta	46.99375°N, 22.92412°E	617	2 May 2015	100	25%
Ro: Iaz	45.46088°N, 22.25210°E	226	13 Mar 2016	50	20%
Ro: Julița	46.06220°N, 22.14175°E	186	14 Mar 2016	50	0%
Ro: Mihăieşti	46.89640°N, 23.40518°E	437	10 Apr 2016	135	23%
Ro: Rimetea	46.49083°N, 23.58301°E	500	10 Apr 2016	58	55%
Ro: Săliştioara	45.98775°N, 22.83006°E	255	13 Mar 2016	50	48%
Ro: Surduc	46.96909°N, 22.18840°E	223	12 Mar 2016	50	30%
Ro: Zilah	47.14463°N, 23.08473°E	598	9 Apr 2016	50	34%

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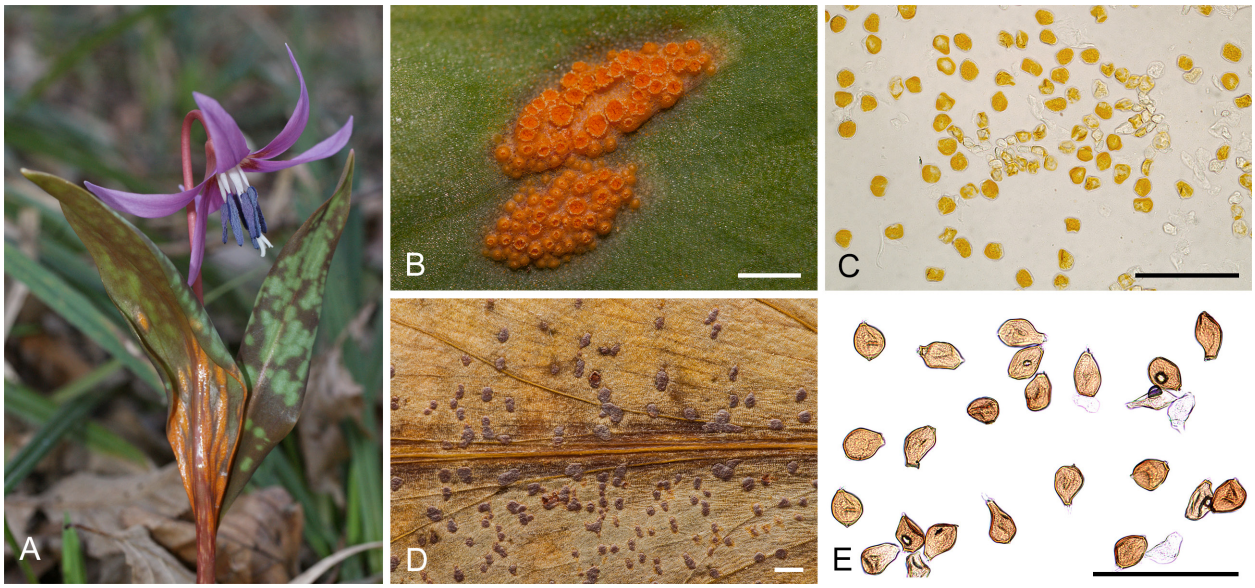


Fig. 1. A: infected individual of *Erythronium dens-canis*; B, C: aecia (B) and aeciospores (C) of *Uromyces erythronii* collected in Croatia (DE-soo-43285); D, E: telia (herbarium specimen, D) and teliospores (E) of *U. erythronii* collected in Hungary (DE-soo-39875). – Scale bars: B, D = 1 mm; C, E = 100 µm. – Photographs: A by A. Molnár V.; B–E by W. P. Pfliegler.

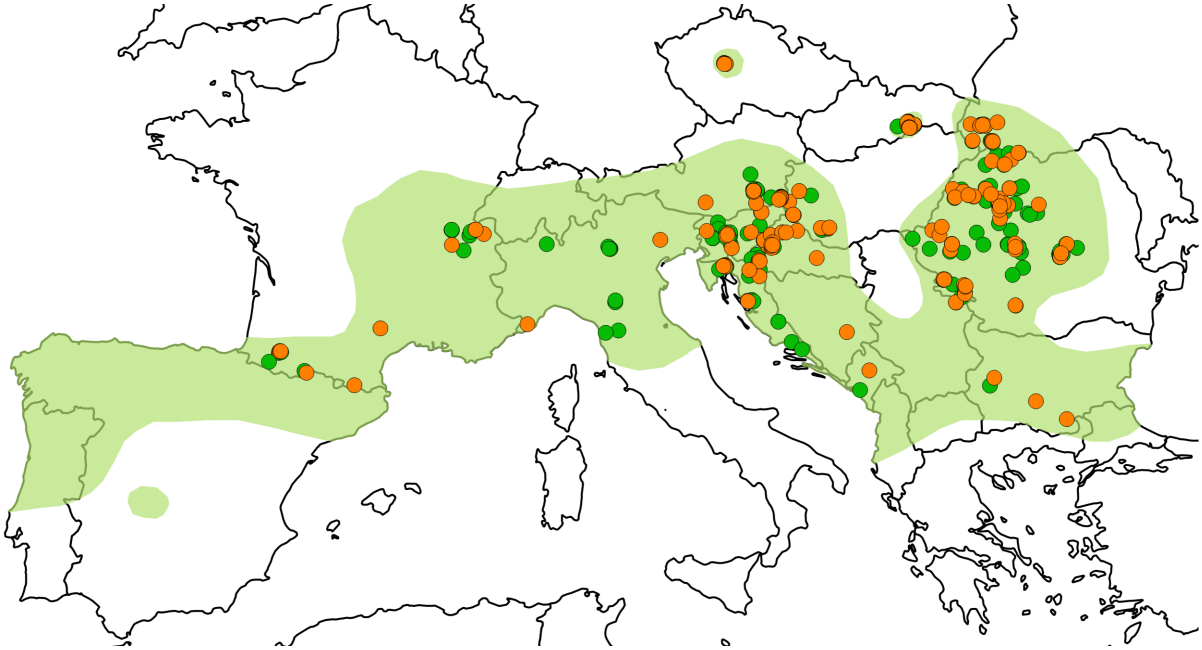


Fig. 2. European distribution of *Erythronium dens-canis* (pale green) and the origin of studied herbarium material: sheets with at least one individual infected with *Uromyces erythronii* are marked with orange points, sheets with only uninfected individuals are marked with green points.

checked 16–135 individuals (mean ± SD = 73 ± 30) in each population, depending on the local population size of the host plant (Table 3).

DNA barcoding

DNA was isolated from aeciospores scraped off of minute pieces of aecia removed from the leaf of a Romanian sample (collection year: 2016) and the leaf of a Croatian sample (collection year: 2017) of *Erythronium dens-canis*. Samples were deposited in the herbarium of Debrecen University (DE-soo-43284, DE-soo-43285). Following the microwave extraction protocol described in Haelewaters & al. (2015), the spores were placed in 0.5 mL PCR tubes and microwave-treated (750 W for 5 min). Subsequently 50 µL ddH_20 was added to the tube, and the spores were manually crushed with a sterile pipette tip while viewed under a dissecting microscope. The PCR tubes

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were then incubated at −20°C for 20 min. For each PCR reaction, 5 µL of the extracted material was used. PCR amplification of the internal transcribed spacer (ITS) region was carried out using GoTaq polymerase (Promega, Madison, WI, U.S.A.) and the primer pair ITS1f and ITS4 (White & al. 1990; Gardes & Bruns 1993). The PCR protocol was as follows: 95°C for 5 min, 30× (94°C 50 s, 55°C 50 s, 72°C 50 s), 72°C for 5 min. For amplification, an Applied Biosystems (Foster City, CA, U.S.A.) 2720 thermal cycler and a final volume of 50 µL were used. PCR products were loaded onto 1.2% agarose gels for electrophoresis at 100 V for 15 min and UV transillumination was used to check the product size. PCR products were cleaned with a Geneaid (New Taipei City, Taiwan) DF100 PCR cleaning kit and sequenced in both directions using the same primers (Microsynth AG, Switzerland). Sequences were trimmed of ambiguous bases at both ends in Chromas 2.6.5. (Technelysium Pty. Ltd.). Sequences compiled from the reads were deposited in ENA (European Nucleotide Archive; accession numbers: MH205916 and MH205917) and blasted in NCBI GenBank (NCBI s.d.+). Boundaries of ITS1, the 5.8S rDNA, and ITS2 were identified using ITSx (Bengtsson-Palme & al. 2013).

Microscopy

Light microscopy images were taken of material mounted in HPVA medium, with an Olympus BD40 microscope (Debrecen, Hungary) equipped with an Olympus 40× lens and digital microscope camera, using the Olympus DP Controller software. Macrophotographs were taken with a Pentax k7 DSLR camera with macrophotography setup and flash.

Results

Herbarium overview

Uromyces erythronii infection was detected on 199 herbarium sheets (335 individuals) of *Erythronium dens-canis* (Table 1). Altogether 81.2% of herbarium specimens (446 items) had exact locality data, and 62.1% of herbarium specimens (341 items) had an exact date (year, month and day). Herbarium sheets (including items from the BP fungi collection) were collected between 1811–2017 (Table 1). Among herbarium specimens, the earliest collection date across all years was 27 February, and the latest was 22 June. Additionally, an exceptionally late date, 26 October, was registered for a specimen with a vague locality description, but based on the date, a high mountain locality in the Carpathians is to be suspected.

Most of the studied herbarium sheets of *Erythronium* (198), as well as most rust-infected sheets (70) and individuals (115), originated from Romania (Table 2, Fig. 2). Numerous further infected individuals were collected in Hungary (62), Croatia (55) and Ukraine (29) (Table 2, Fig. 2). Infection rate of the specimens from the three most represented countries were 56.0% (Hungary), 50.0% (Croatia) and 35.4% (Romania). The infection rate of individuals was 28.5%, 18.8% and 14.7%, respectively (Table 2).

Aecial and telial infection were separately quantified on a total of 153 specimens (251 individuals). Aecia were found on 141 specimens (225 individuals) collected between 27 February and 28 May, and telia were found on 22 specimens (32 individuals) collected between 7 March and 22 June (Fig. 3). Simultaneous occurrence of both developmental stages of the fungus were detected only in the case of six individuals.

According to the GLM, the year of collection had no significant effect on the infection rate of *Erythronium dens-canis* individuals by *Uromyces erythronii* (DF = 135, E = 0.002, SE = 0.002, t-value = 0.945, p-value = 0.344).

Field survey

Uromyces erythronii infection was detectable for 88.5% of the studied *Erythronium* populations. Average infection rate of populations was mean ± SD = 25 ± 21% (range: 0–69%). The highest infection rates were documented in Tömörd, Hungary (69%) and Feleacu, Romania (65%) (Table 3). The fungus remained undetected in only three screened host populations (Hungary: Miskolc; Romania: Hațeg and Julița).

DNA barcoding

We generated ITS (ITS1-5.8S-ITS2) DNA barcodes for two samples collected during field surveys in Romania (MH205916) and Croatia (MH205917). The quick heat extraction protocol (Haelewaters & al. 2015) was successfully applied, with minute pieces of aecia being sufficient for subsequent PCR amplification. The obtained sequences were identical in their 589 bp overlapping regions (of which 517 bases corresponded to the ITS1-5.8S-ITS2). Comparison with the single available DNA barcode sequence of the species in GenBank (accession number LC203755), generated for a sample collected in Japan from the host *Erythronium japonicum* Decne., revealed a difference of only two nucleotides in the ITS1-5.8S-ITS2 region (= 99.61% similarity). Both of these, a substitution and an additional nucleotide in the European samples, were located in the ITS1.

Discussion

Our new records of *Uromyces erythronii* revealed a distribution overlapping with much of its European host, *Erythronium dens-canis* (Fig. 1). Formal reports did not

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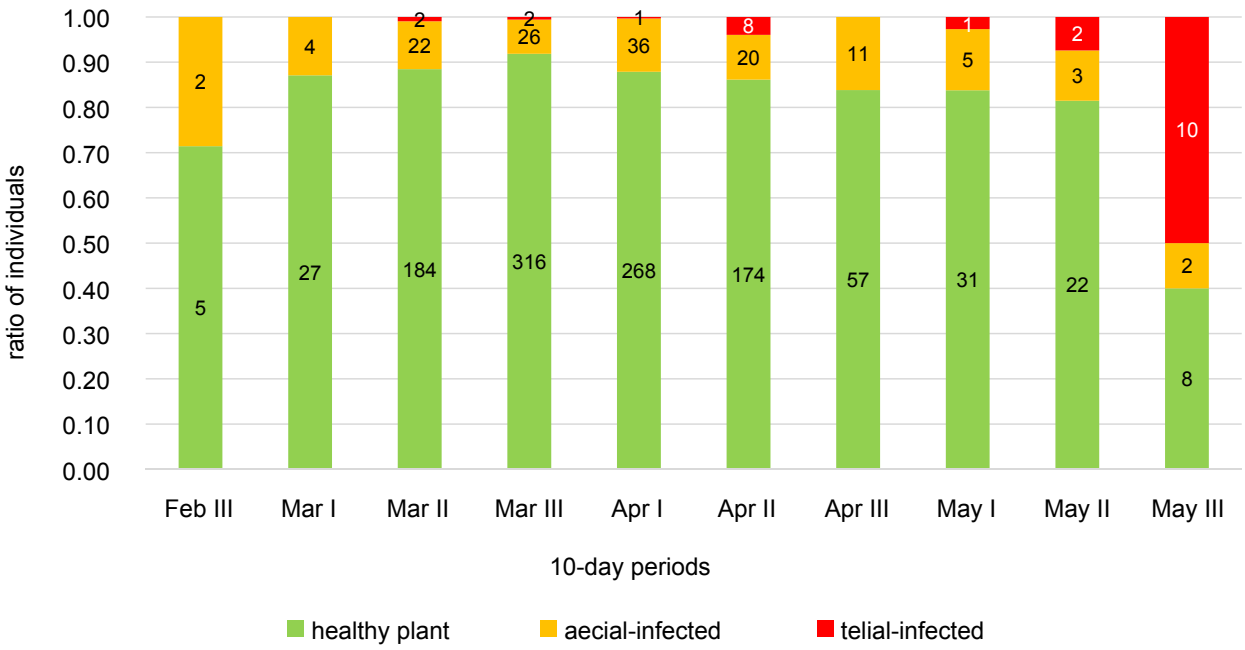


Fig. 3. Seasonal trend of presence of aecia and telia structures of *Uromyces erythronii* on *Erythronium dens-canis* herbarium individuals. Arabic numbers on the columns refer to the number of individuals. Roman numbers refer to the first (1–10), second (11–20) and third (21–31) 10-day periods of each month.

exist on the presence of the rust in Bosnia and Herzegovina, Bulgaria, Montenegro, Slovakia and Slovenia, although possible presence was suggested (e.g. Kruse & al. 2014; Tykhonenko & al. 2017). Our study confirms the presence of the rust in these countries (Table 2) based on frequently unintentional collections of the fungus in the form of *Erythronium* herbarium specimens. These specimens occasionally predate the formal description of the fungus (Table 2, Fig. 2).

The often remarkably high infection rate in herbarium material was reflected in the results of the field surveys conducted within the framework of this study. An overwhelming majority of studied Croatian, Hungarian and Romanian *Erythronium* populations were found to be infected. Based on our experiences, examining relatively few *Erythronium* individuals during the flowering and fruiting seasons may be sufficient to detect *Uromyces erythronii* in the field. On the other hand, infection rates vary widely across populations (0–69%; Table 3). Variable infection rate is a known phenomenon in case of rust infections (Ericson & al. 1999), and it has to be noted that the highest infection rate was detected in a population (Tömörd, Hungary) that most probably originated from a deliberate planting (Molnár V., ined.). Such inbred populations are generally more susceptible to pathogens than outcrossed populations (e.g. Burdon & al. 1999).

The connected life cycles of *Uromyces erythronii* and *Erythronium japonicum* were illustrated and detailed by Fukuda & Nakamura (1987). They found that the growing period of *E. japonicum* is restricted to one or two months (from late February to early May). However, we documented a somewhat longer period for *E. dens-canis*: 10% of the infected individuals were collected in the second and third 10-day periods of May. Fukuda & Nakamura highlighted that the early-emerging individuals of *E. japonicum* are more rusty than later-growing ones. It seems that this phenomenon is also true in the case of *E. dens-canis*, because individuals collected in late February bear aecia in higher frequency (29%) than individuals collected from March to May (8–14%) (Fig. 3). Fukuda & Nakamura also stated that aecia may develop only on the larger leaves in case of two-leaved individuals of *Erythronium*. Contrary to this, we observed aecial infection on both leaves in some cases, which may be caused by independent infection events by basidiospores. Otherwise, the less tightly rolled leaves of *E. dens-canis* in the emerging stage may also explain the higher infection rate of these. Secondary infection by aeciospores generating the telial generation affected only a small proportion of individuals (Fig. 3) in line with the observations of Fukuda & Nakamura.

Invasions and emerging infectious diseases caused by representatives of various fungus families have been extensively discussed (e.g. Anderson & al. 2004; Parker & Gilbert 2004; Desprez-Loustau & al. 2007). Fungal invasions often originate from host shift, even across continents (Palm & Rossman 2003). According to our results, the infection rate of *Uromyces erythronii* on *Erythronium dens-canis* did not change during the documented last two centuries (GLM, DF= 135, E = 0.002, SE = 0.002, t-value = 0.945, p-value = 0.344). Consequently, it is apparently not a novel host-pathogen relationship and the current wide distribution of the host emerged much earlier.

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DNA barcode sequencing revealed a high similarity in the ITS region among samples from Croatia, Romania and Japan. The 100% identity of the two European sequences and their 99.61% similarity to a Japanese sample from a different host species, *Erythronium japonicum*, in the ITS region suggests a low intraspecific geographic or host-related diversity and no indication of cryptic speciation in *Uromyces erythronii*.

In conclusion, *Uromyces erythronii* is more widespread and abundant in Europe than previously reported. The species potentially occurs in the whole distribution area of its host. The apparent expansion of *U. erythronii* is not a consequence of its recent spread, because its earlier distribution is well documented but previously unstudied by herbarium specimens collected decades or even centuries earlier. Our results also signify the importance of incorporating vascular plant collections in the study of microscopic fungi (see also Denchev & Denchev 2016).

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