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ENTYLOMA MAJEWSKII SP. NOV. (ENTYLOMATACEAE) ON *RANUNCULUS FICARIA* FROM IRAN*

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Abstract. Based on molecular phylogenetic analyses and spore morphology, a new smut fungus, *Entyloma majewskii* Vánky & M. Lutz, is described and illustrated, collected on *Ranunculus ficaria* L. in Iran. It is compared with *Entyloma ficariae* Thüm. *ex* A. A. Fisch. Waldh., also on *R. ficaria*. A key to *Entyloma* de Bary species on *Ranunculus* L. is given.

Key words: *Entyloma*, *Entyloma majewskii*, Iran, ITS, LSU, molecular analysis, new species, *Ranunculus*, smut fungi

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INTRODUCTION

Entyloma de Bary, in the Entylomataceae, is one of the 96 recognized genera of smut fungi (Basidiomycota). It has about 175 species, parasitizing dicotyledonous host plants belonging to 25 families. It is characterized by sori in vegetative parts of host plants, mostly in leaves and stems, usually forming spots, sometimes pustules, swellings or galls. The spores are solitary or adhering in irregular groups, permanently embedded in the host tissue, hyaline, yellow or pale yellowish brown. The spore wall is usually smooth, but often with a hyaline, gelatinous sheath. Spore germination results in holobasidia with apically produced basidiospores ('*Tilletia*-type'). Host-parasite interaction is effected via a simple interaction apparatus, and haustoria are absent. The septal pore is simple, with two membrane caps. The anamorph (*Entylomella* Höhn.) is often present.

Their simple spore morphology makes species delimitation in *Entyloma* difficult (cf. Savile 1947). Spore morphology correlated to host plant taxonomy gave good results in classification of the genus *Anthracoidea* Bref. (cf. Kukkonen 1963; Nannfeldt 1979; Vánky 1979). This procedure has also been successfully applied to some other genera of smut fungi (cf. Vánky 1994, 2002, 2011, and literature therein), but not satisfactorily for species of *Entyloma*, this due to the generally poor spore morphology and the existence of polyphagous species. A typical polyphagous species is *Entyloma microsporum* (Unger) J. Schröt., whose unmistakable morphology is an exception. The use of molecular phylogenetic analyses was the next successful step towards a natural classification of the genus *Entyloma*. Begerow *et al.* (2002) concluded that 'The phylogenetic relationships in the genus *Entyloma* are a result of joint evolution with their hosts.' Thus, several species (except for those on monocots), recognized by Liro (1938), mostly on the basis of host taxonomy, seem to be good species. On the other hand, there is evidence that several *Entyloma* species may parasitize not only members of the same host plant family or genus but even the same host plant species. Therefore it seems promising and highly desirable to study as many *Entyloma* species as possible by combined methods.

During a collecting trip to Iran in 1990, an *Entyloma* species was found on *Ranunculus ficaria* L.

* This paper is dedicated to Professor Tomasz Majewski on the occasion of his 70th birthday.

In appearance it is similar to *E. ficariae* Thüm. *ex* A. A. Fisch. Waldh. However, comparative DNA analyses of the Iranian collection and those of several European collections of *E. ficariae* revealed that they are different (a difference of 11 base pairs in the ITS nucleotide sequences and a difference of two base pairs in the LSU nucleotide sequences). A thorough comparative morphological study of them revealed significant differences, which together motivate the description of a new species for the Iranian smut fungus.

Materials and methods

The specimens examined for their morphology are the type and paratype of *Entyloma majewskii*, as well as the lectotype of *E. ficariae*, all on *Ranunculus ficaria*. The specimens analysed in this study for their DNA properties are listed in Table 1.

Morphological examination. Spore characteristics were studied from dried herbarium specimens. For light microscopy (LM), small pieces of leaf tissue with sori were cut from the host plants and placed on microscope slides in a droplet of lactophenol. Several droplets of distilled water were added and the solution with the sori was heated 2–3 times to boiling point. If needed, some additional droplets of distilled water were added. The softened sori were cut in long pieces and squashed with a lancet, covered with a cover glass, gently heated to boiling point to eliminate air bubbles from the preparation, and studied by light microscope at 1000×.

Phylogenetic analyses. Genomic DNA was isolated from herbarium specimens. For methods of isolation and crushing of fungal material, DNA extraction, amplification, purification of PCR products, sequencing, and processing of the raw data see Lutz *et al.* (2004). We determined base sequences of the ITS1/2 region of nuc-rDNA including 5.8S rDNA (ITS), and the 5′-end of nuc-LSU rDNA including domains D1/D2 (LSU). The ITS was amplified using the primer pair ITS1f and ITS4 (Gardes & Bruns 1993). The LSU was amplified using the primer pair NL1 and NL4 (O'Donnell 1992, 1993). For amplification of both regions the annealing temperature was adjusted to 45°C. DNA sequences prepared in the course of this study were deposited in GenBank (accession numbers given in Table 1, Figs 1 & 2).

We followed two strategies to obtain a hypothesis on the phylogenetic position of the examined *Entyloma* specimens growing on *Ranunculus ficaria*: (*i*) we analysed their ITS sequences together with a selection of *Entyloma* sequences available in GenBank, including all available sequences of *Entyloma* species growing on Ranunculaceae and the five sequences most similar to the sequence of *E. majewskii* according to a blast search (Altschul *et al.* 1997) (GenBank accession numbers given in Fig. 1); and (*ii*) we analyzed a concatenated ITS+LSU alignment sequences of a dataset reduced to the specimens growing on *Ranunculus ficaria* and their closest relatives according to the results of ITS analyses (GenBank accession numbers given in Fig. 2).

Sequences were aligned for both datasets with MAFFT 6.611 (Katoh *et al.* 2002, 2005; Katoh & Toh 2008) using the L-INS-i option. Both alignments (length: 648 bp [ITS], 1300 bp [ITS+LSU]; variable sites: 149 [ITS], 44 [ITS+LSU]) were used throughout their length. Both manipulation of the alignment by hand and manual exclusion of any positions were avoided, as recommended by Giribet and Wheeler (1999) and Gatesy *et al.* (1993) respectively.

For phylogenetic analyses both neighbor-joining analysis and a Bayesian approach were used. For neighbor-joining analysis the data were first analyzed with Modeltest 3.7 (Posada & Crandall 1998) to find the most appropriate model of DNA substitution. For both the ITS and the ITS+LSU datasets the hierarchical likelihood ratio test proposed the TrN+I+G DNA substitution model. Bootstrap values were calculated for 1000 replicates. For Bayesian analysis for both datasets we applied a Bayesian approach of phylogenetic inference using Markov chain Monte Carlo (MCMC) technique as implemented in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Four incrementally heated simultaneous Markov chains were run over 2,000,000 generations using the general time-reversible model of DNA substitution with gamma-distributed substitution rates and estimation of invariant sites, random starting trees and default starting parameters of the DNA substitution model as recommended by Huelsenbeck and Rannala (2004). Trees were sampled every 100th generation, resulting in an overall sampling of 20,001 trees. From these, the first 2001 trees were discarded (burn-in = 2001). The trees sampled after the process reached stationarity (18,000 trees) were used to compute a 50% majority rule consensus tree to obtain estimates for the *a posteriori* probabilities of groups of species. This Bayesian approach of phylogenetic analysis was repeated four times to test the independence of the results from topological priors (Huelsenbeck *et al.* 2002). Trees resulting from the ITS analyses were rooted

Table 1. List of sequenced *Entyloma* de Bary specimens with host plants, GenBank accession numbers, and reference specimens.

Species	Host	GenBank acc. no. (ITS/LSU)	Reference specimens
Entyloma ficariae Thüm. *ex* A. A. Fisch. Waldh.	*Ranunculus ficaria* L.	AY081035/AY081013	Germany, Schleswig-Holstein, Forgan, 28 Apr. 1998, *leg. M. Lutz*, TUB 012542
Entyloma ficariae	*Ranunculus ficaria*	AY854969/HM046473	Germany, Baden-Württemberg, Glottertal, *leg. R. Berndt*, private collection M. Piepenbring (HUP 24)
Entyloma ficariae	*Ranunculus ficaria*	HM046465/HM046476	Germany, Baden-Württemberg, Gaggenau, 4 May 1998, *leg. M. Lutz*, TUB 012546
Entyloma ficariae	*Ranunculus ficaria*	HM046466/HM046474	France, Lons le Saunier, 14 May 1998, *leg. M. Lutz*, TUB 012547
Entyloma ficariae	*Ranunculus ficaria*	HM046468/HM046477	Germany, Baden-Württemberg, Tübingen, 14 Apr. 1998, *leg. M. Lutz*, TUB 012552
Entyloma ficariae	*Ranunculus ficaria*	HM046469/HM046475	France, Ganges, 16 May 1998, *leg. M. Lutz*, TUB 012545
Entyloma ficariae	*Ranunculus ficaria*	HM046472/HM046479	Slovenia, lake Bohinj, 25 May 2002, *leg. M. Lutz*, TUB 012538
Entyloma ficariae	*Ranunculus ficaria*	HM046470/HM046480	Great Britain, Wales, Cardiff, 25 May 1998, *leg. C. Lutz,* TUB 019286
Entyloma ficariae	*Ranunculus ficaria*	HM046471/HM046481	Germany, Bayern, Starnberger See, Ilkahöhe, 5 May 2002, *leg. U. Fischer & M. Lutz*, TUB 019287
Entyloma majewskii Vánky & M. Lutz	*Ranunculus ficaria*	HM046467/HM046478	Iran, Tehran Prov., 60 km E Tehran, Mts. Elburz, 'Emamzadeh-Haskei', 35°50′N, 52°02′E, alt. 2610 m., 17 May 1990, *leg. D. Ershad, T. Vánky & K. Vánky,* HUV[1] 14888 – holotype, IRAN – isotype

[1] HUV – Herbarium Ustilaginales Vánky, Tübingen, Germany

with *Entyloma atlanticum* Massenot AY081018 and *E. glaucii* P. A. Dang. AY081036; trees resulting from the ITS+LSU analyses were rooted with *E. ranunculi-repentis* Sternon AY081047/AY860052, AY854980/AY081016.

Results and discussion

Morphological results

The results of morphological studies are described below under the species names *Entyloma ficariae* and *E. majewskii*.

Molecular phylogenetic results

For both the ITS and the combined ITS+LSU datasets, the different runs of Bayesian phylogenetic analyses yielded consistent topologies congruent with the results of the neighbor-joining analysis in respect to well-supported branchings. For both datasets the consensus tree of one run of Bayesian phylogenetic analyses is presented to illustrate the results (Figs 1 & 2).

In all analyses of the ITS dataset, specimens from *Ranunculus ficaria* clustered together, being the sister taxon of *Entyloma ranunculi-repentis*. Within the specimens from *Ranunculus ficaria* the analyses revealed two distinct phylogenetic lineages: *Entyloma ficariae* and the Iranian specimen (= *E. majewskii*). However, support for the division of the species was weak for both the ITS and the combined ITS+LSU datasets. The alignments revealed a difference between the two species of 11 base pairs in the ITS nucleotide sequences, and two base pairs in the LSU nucleotide sequences.

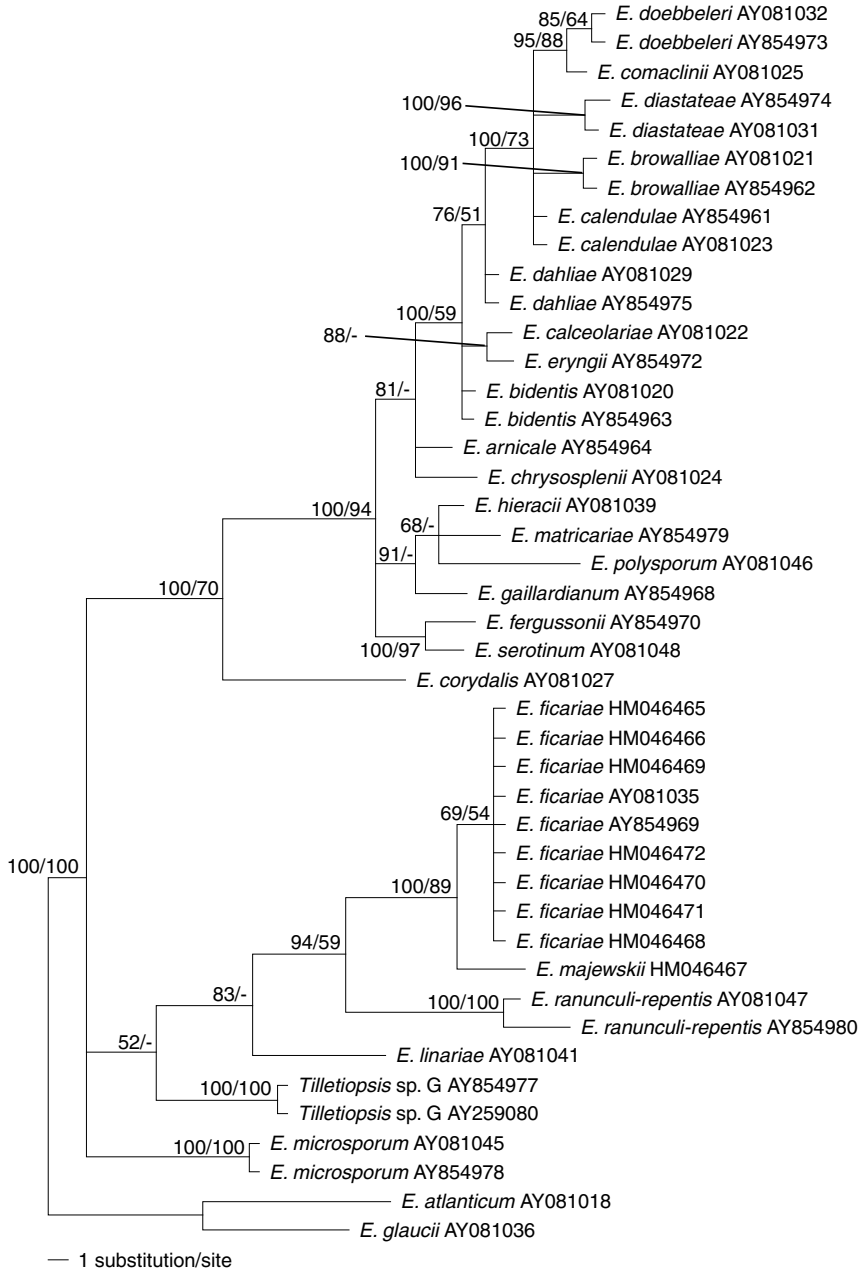


Fig. 1. Bayesian inference of phylogenetic relationships within the sampled *Entyloma* species: Markov chain Monte Carlo analysis of alignment of ITS base sequences using the GTR+I+G model of DNA substitution with gamma-distributed substitution rates and estimation of invariant sites, random starting trees and default starting parameters of the DNA substitution model. A 50% majority-rule consensus tree computed from 18,000 trees sampled after stationarity was reached is shown. The topology was rooted with *Entyloma atlanticum* Massenot AY081018 and *E. glaucii* P. A. Dang. AY081036. Numbers on branches before slashes are estimates for *a posteriori* probabilities; numbers on branches after slashes are percentage bootstrap values of 1000 replicates. Branch lengths were averaged over the sampled trees. They are scaled in terms of expected numbers of nucleotide substitutions per site. E. = *Entyloma* de Bary.

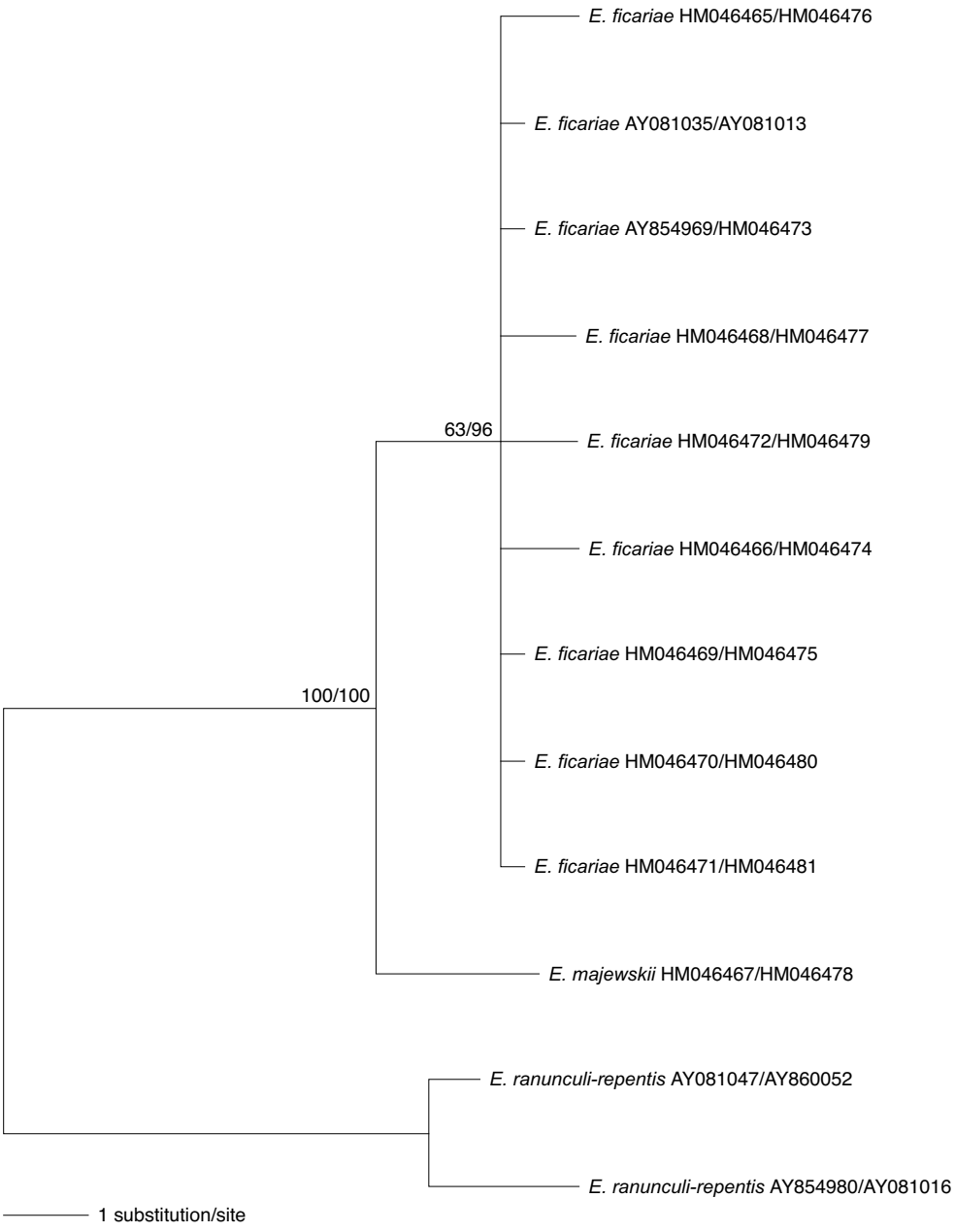


Fig. 2. Bayesian inference of phylogenetic relationships within the sampled *Entyloma* de Bary species: Markov chain Monte Carlo analysis of alignment of concatenated ITS+LSU base sequences using the GTR+I+G model of DNA substitution with gamma-distributed substitution rates and estimation of invariant sites, random starting trees and default starting parameters of the DNA substitution model. A 50% majority-rule consensus tree computed from 18,000 trees sampled after stationarity was reached is shown. The topology was rooted with *Entyloma ranunculi-repentis* Sternon AY081047/AY860052, AY854980/AY081016. Numbers on branches before slashes are estimates for *a posteriori* probabilities; numbers on branches after slashes are percentage bootstrap values of 1000 replicates. Branch lengths were averaged over the sampled trees. They are scaled in terms of expected numbers of nucleotide substitutions per site. E. = *Entyloma* de Bary.

TAXONOMY

Entyloma ficariae Thüm. *ex* A. A. Fisch. Waldh.

Bull. Soc. Nat. Moscow **52**: 309. 1877 (after June).

TYPE on *Ranunculus ficaria* L., Germany, Sachsen, Leipzig, V.1874, *G. Winter* (LECTOTYPE designated by Vánky 1985: 65, HUV 912; ISOTYPES in Thümen, Mycoth. univ. no. 219, as *E. ungerianum* de Bary forma *ficariae* Thümen, 1875; *nomen nudum*).

Sori on leaves as flat, circular or angular spots, 1–3 mm in diameter or larger by confluence, first white, later whitish green or pale yellowish brown, evident on both sides but more expressed on the abaxial side of leaves. Spores (Fig. 3) loosely crowded or scattered in host tissue, globose, subglobose, ovoid to broadly ellipsoidal, sometimes slightly irregular, 10.0–14.5(–15.0) × 11–16 µm, subhyaline to tinted pale yellowish brown; wall even, two-layered, 1.0–2.5(–3.0) µm thick. Anamorph [*Entylomella ficariae* (Berk.) Höhn.] often present.

HOSTS AND DISTRIBUTION: On Ranunculaceae: *Ranunculus ficaria* L. (*Ficaria ranunculoides* Roth; *F. verna* Huds.), *R. ficaria* subsp. *bulbifer* Lawalrée, *R. ficaria* subsp. *calthifolius* (Rchb.) Arcang. (*Ficaria calthifolia* Rchb.), *R. ficaria* L. subsp. *ficariiformis* Rouy & Foucaud; Europe, North Africa, Asia, ?North America.

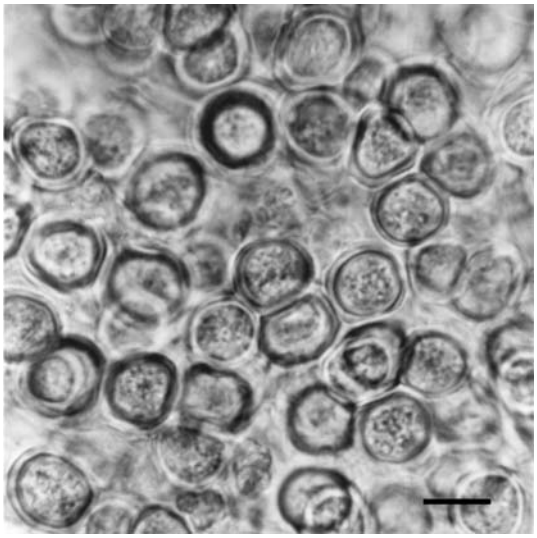


Fig. 3. Spores of *Entyloma ficariae* Thüm. *ex* A. A. Fisch. Waldh. on *Ranunculus ficaria* L. (lectotype), in LM. Scale bar = 10 µm.

Entyloma ficariae mentioned by Vánky and Ershad (1993: 2), and by Ershad (2001: 48), on *Ficaria ranunculoides* (= *Ranunculus ficaria*) from Iran, represents *E. majewskii*.

Based on morphological and phylogenetic analyses, the following new species is proposed:

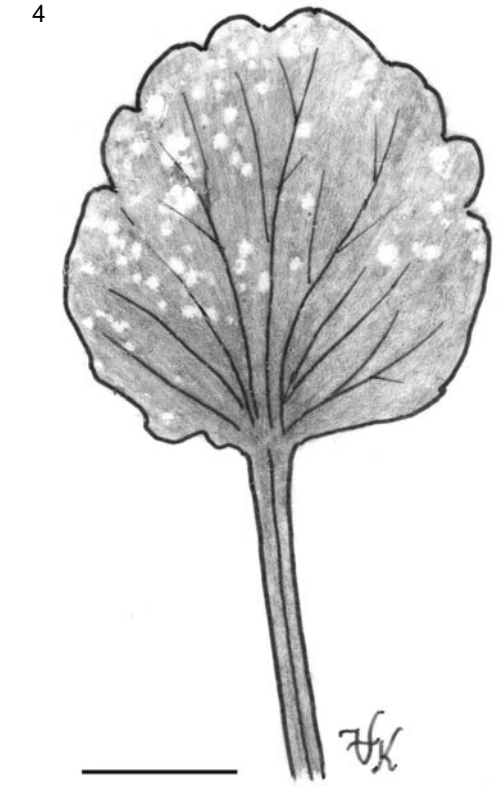
Entyloma majewskii Vánky & M. Lutz, ***sp. nov.***

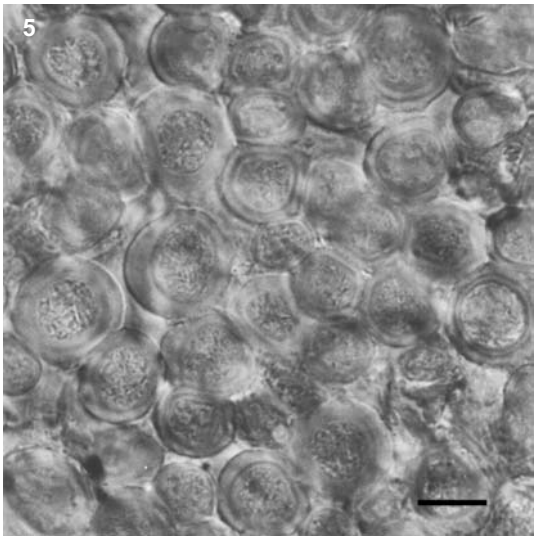
[MycoBank MB518533].

Sori in foliis sicut maculae parum bullatae, circulares vel late ellipticae, 1.0–1.5(–2.0) mm diametro vel propter confluentiam majores, primo albae, serius albido-viridis vel pallide flavidobrunneae, evidentes in utroque latere foliorum. Sporae aggregatae, globosae, subglobosae, ellipsoidales usque parum irregulares, cum latere uno vel raro lateribus duobus complanatis, 10.5–16.0(–17.5) × 10.5–17.0(–18.5) µm, subhyalinae usque flavidae tintctae; pariete aequali usque plerumque inaequaliter incrassato, bistrato, 2.5–7.0 µm crasso, endospora aequali 0.8–1.0 µm crassa inclusa, superficies sporarum levis. Anamorpha non visa. Sequentiae acidi nucleici ITS/LSU typi in collectione sequentiarum acidi nucleici NCBI (GenBank) ut HM046467/HM046478 depositae sunt.

TYPUS in matrice Ranunculus ficaria *L. (det. F. Matin, IRAN), IRAN, TEHRAN PROV., 60 km E Tehran, Mts. Elburz, 'Emamzadeh-Haskei', 35°50'N, 52°02'E, alt. 2610 m. s. m., 17.V.1990, leg. D. Ershad, T. Vánky et K. Vánky. Holotypus in Herbario Ustil. Vánky, HUV 14888, ISOTYPI in IRAN, et in Vánky, Ust. exs. no. 821* (as E. ficariae). *PARATYPI in matrice* Ranunculus ficaria, *Iran, Gorgan Prov., 35 km SW urbe Gorgan, alt. 2250 m. s. m., 16.V.1990, leg. D. Ershad, T. Vánky & K. Vánky, HUV 14887, IRAN.*

Sori (Fig. 4) on leaves as slightly bullate, circular or broadly elliptic spots, 1.0–1.5(–2.0) mm in diameter or larger by confluence, first white, later whitish green or pale yellowish brown, evident on both sides of the leaves. Spores (Fig. 5) crowded, globose, subglobose, ellipsoidal to slightly irregular, with one, rarely two flattened sides, 10.5–16.0(–17.5) × 10.5–17.0(–18.5) µm, subhyaline to tinted pale yellowish; wall evenly to usually unevenly thickened, two-layered, 2.5–7.0 µm wide,





Figs 4–5. *Entyloma majewskii* Vánky & M. Lutz *sp. nov.* on *Ranunculus ficaria* L. (type). 4 – Sori on leaf of host plant, producing spots; scale bar = 1 cm. 5 – Spores in LM; scale bar = 10 μm.

including the even, 0.8–1.0 μm thick endospore, spore surface smooth. Anamorph not seen. The ITS/LSU type sequences are deposited in GenBank as HM046467/HM046478 respectively.

HOSTS AND DISTRIBUTION. On Ranunculaceae: *Ranunculus ficaria* L.; Asia (Iran). Known only from the type and paratype collections.

ETYMOLOGY. The species name honors the outstanding Polish mycologist, phytopathologist, botanist, teacher and friend, Professor Tomasz Majewski, one of the world's leading specialists on Laboulbeniales, who has made an abiding contribution with his publications on Polish smut fungi, Micromycetes in Poland, and many others listed in the volume of *Polish Botanical Journal* published in his honor.

COMMENTS. On species of *Ranunculus* L. five *Entyloma* species are known, some of them host-specific, others polyphagous. The five species are *E. ficariae*, *E. majewskii, E. microsporum*, *E. ranunculi-repentis* and *E. verruculosum* Pass.

Entyloma majewskii and *E. ficariae* occur only on *Ranunculus ficaria* (including its three subspecies in the case of the latter smut). The main morphological differences between these two species are that the sori of *E. majewskii* appear as slightly bullate, circular or broadly elliptic spots on the leaves, evident on both sides of the leaves, while in *E. ficariae* these are flat, circular or angular, evident on both sides but more expressed on the abaxial side of the leaves. The spores of *E. majewskii* are crowded, globose, subglobose, ellipsoidal to slightly irregular, with one, rarely two flattened sides, 10.5–16.0(–17.5) × 10.5–17.0(–18.5) μm, with an evenly to usually unevenly thickened wall which is two-layered, 2.5–7.0 μm wide, including the even, 0.8–1.0 μm thick endospore. The spores in *E. ficariae* are loosely crowded or scattered in the host tissue, globose, subglobose, ovoid to broadly ellipsoidal, sometimes slightly irregular, 10.0–14.5(–15.0) × 11–16 μm, with an evenly 1.0–2.5(–3.0) μm thick, two-layered wall. The anamorph in *E. majewskii* is absent, but in *E. ficariae* is usually present [*Entylomella ficariae* (Berk.) Höhnel].

KEY TO THE *ENTYLOMA* SPECIES ON *RANUNCULUS*

1. Sori as hard, swollen, wart-like pustules or swellings on leaves; spore wall 1–9 μm thick, smooth . . . . . ***E. microsporum***
1.* Sori as flat or slightly swollen leaf spots; spore wall thinner, 1–7 μm thick, smooth or verrucose. 2
   2. Sori as slightly swollen leaf spots; spore wall 2.5–7.0 μm thick. . . . . ***E. majewskii***
   2.* Sori as flat leaf spots; spore wall thinner, 1–3 μm thick 3
3. Sori indistinct, 5–30 mm long; spores with 1.0–2.5 μm high warts . . . . . ***E. verruculosum***
3.* Sori evident, 1–5 mm long; spores smooth 4
   4. Spores 11–16 μm long, wall 1.0–2.5(–3.0) μm thick . . . . . ***E. ficariae***
   4.* Spores 9–14 μm long, wall 1–2 μm thick . . . . . ***E. ranunculi-repentis***

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