Cryptic diversity in the *Antherospora vaillantii* complex on *Muscari* species

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**Abstract:** The anther smut fungus *Ustilago vaillantii* was described by the Tulasne brothers at the beginning of systematic mycology (Tulasne & Tulasne 1847). The specific epithet honoured the pre-Linnaean botanist Sébastien Vaillant (1669–1722), the first collector of anther-infected *Muscari comosum*. In the protologue, the Tulasne brothers included three host species of *Ustilago vaillantii* in the following order: *Muscari comosum, Scilla anthericoides*, and *Scilla maritima*. The two latter names are synonyms of the species currently named *Charysbidis maritima* (Speta 1988, Euro+Med 2006). The type host was attributed to *Muscari comosum* by Liro (1924) and Ciferri (1928), who reasonably explained this selection.

Subsequent to its description, *Ustilago vaillantii* became a catch-all for different smut specimens sporulating in the anthers of monocotyledonous plants that currently are placed in the family Asparagaceae subfam. *Scilloideae* (syn. *Hyacinthaceae*, APG III 2009, Chase et al. 2009). Some attempts to describe separate taxa within this complex, such as *Ustilago vaillantii* var. *tourneuxii* on *Bellevalia trifoliata* (Fischer von Waldheim 1880), *U. albucae* on *Albuca* sp. (Sydow & Sydow 1914a), *U. peglerae* on *Ornithogalum lacteum* (Sydow & Sydow 1914b), *U. muscari-botryoidis* on *Muscari botryoides* (Ciferri 1928), *U. scillae* on *Scilla bifolia* (Ciferri 1931) or *U. urginaeae* on *Charysbidis maritima* (Maire 1931), were based mostly on small morphological differences and/or supposed host specificity, and received moderate acceptance by smut researchers (Zundel 1953). In general, most authors recognised anther smuts on monocots as one species *Ustilago vaillantii* (Liro 1924, Vánky 1985) and such an approach was adopted until very recently (Vánky 1994).

The first classification of smut fungi based on ultrastructure and molecular phylogeny (Bauer et al. 1997, Begerow et al. 1997, Bauer et al. 2001) restricted the genus *Ustilago* to species on *Poaceae*. Consequently, *Ustilago vaillantii* was moved to the genus *Vankya*, typified by the folicolous *V. ornithogali* (Ershad 2000). Molecular analyses revealed that the anther smuts on monocots were only distantly related to the folicolous representatives of *Vankya*, and a new genus *Antherospora*, typified by *A. vaillantii*, was established for the former group (Bauer et al. 2008). The genus *Vankya* is currently restricted to host plants in *Liliaceae* subfam. *Lilioideae* (Vánky 2009). In addition to describing a new genus, Bauer et al. (2008) demonstrated that the evolution of the anther smuts on monocots is related to host taxonomy.

**Article info:** Submitted: 27 January 2013; Accepted: 28 February 2013; Published: 4 April 2013.
and consequently a narrow species concept within this group was warranted, despite limited access to fresh specimens for molecular analyses. The anther smuts on monocots are uncommon and difficult to detect because the sori are often hidden by the perianths, at least in some host genera (e.g. *Bellevalia, Muscari*). In comparison, the anther smuts on dicots (Lutz et al. 2005, 2008, Roets et al. 2008, Curran et al. 2009, Hood et al. 2010, Kemler et al. 2009, 2013, Piątek et al. 2012, 2013) are more often collected.

The species concept adopted by Bauer et al. (2008) narrowed *Antherospora vaillantii* to smuts infecting *Muscari* species (Vánky 2012). However, molecular analyses revealed genetic divergences between smut collections from different *Muscari* species (Bauer et al. 2008), which suggest that host specialization may be higher than to the genus level and that more than one species may have radiated on *Muscari* species. Indeed, in addition to *Antherospora vaillantii* on *Muscari comosum*, a second species was described for anther smuts on *Muscari*, namely *Ustilago muscari-botryoidis* on *Muscari botryoides*, although in recent monographs it was considered a synonym of the former species (Vánky 1985, 1994, 2012, Scholz & Scholz 1988, Karatygın & Azbukina 1989, Denchev 2001). The history of discovery and description of *Ustilago muscari-botryoidis* well illustrates the approach leading to the descriptions of different biological species by taxonomists working at the beginning of 20th century, prior to establishment of molecular methods. In 1921, Ciferri (1928) discovered an anther-infected population of *Muscari botryoides* in northern Italy. The co-occurring *Muscari comosum*, flowering later, was healthy. Subsequently, Ciferri (1928) made a three-year observation of a co-cultivation of infected *Muscari botryoides* with non-infected *M. comosum* and *M. neglectum* (as *M. racemosum*). After the three years, *Muscari botryoides* was still infected while *M. comosum* and *M. neglectum* remained healthy. Consequently, the anther smut on *Muscari botryoides* was described as a distinct biological species. In the study of Bauer et al. (2008), anther smut collections on *Muscari botryoides* were not available and the *Antherospora vaillantii* species complex on *Muscari*, including the identity of *Ustilago muscari-botryoidis*, remained unresolved. Misidentification of some hosts may have added further confusion.

The present study is aimed at resolving the *Antherospora vaillantii* species complex on *Muscari*, and testing whether molecular phylogenetic lineages could be defined by morphological and/or ecological characteristics. To achieve this goal molecular phylogenetic analyses using rDNA sequences were applied as well as light and scanning electron microscope examination of different anther smut specimens, including collections from type hosts of all species known to infect *Muscari*.

**MATERIALS AND METHODS**

**Specimen sampling and documentation**

This study is based on specimens of the *Antherospora vaillantii* complex from four different *Muscari* species, originating from Germany, Israel, Slovenia, and the United Kingdom. The voucher specimens are deposited in GLM, KR, KRAM F, TUB, HAI and H.U.V. (Table 1). The latter abbreviation refers to the personal collection of Kálmán Vánky, "Herbarium Ustilaginales Vánky" currently held at his home (Gabriel-Biel-Strasse 5, D-72076 Tübingen, Germany). The following host plants previously misidentified (Bauer et al. 2008) were re-identified during the present study using *Flora Europaea* (Tutin et al. 1980): (1) the specimen from Slovenia (H.U.V. 21337) identified as *M. neglectum* is *M. botryoides*; (2) the specimen from Germany (TUB 15838) identified as *M. comosum* is *M. armeniacum*; (3) the specimen from Germany (H.U.V. 21046) identified as *M. neglectum* is *M. armeniacum*. Nomenclatural novelties were registered in MycoBank (www.MycoBank.org, Crous et al. 2004). The genotype concept follows the proposal of Chakrabarty (2010).

**Morphological examination**

Dried fungal teliospores of the investigated specimens were mounted in lactic acid, heated to boiling point, and then examined under a Nikon Eclipse 80i light microscope at a magnification of ×1000, using Nomarski optics (DIC). Spores were measured using NIS-Elements BR 3.0 imaging software. The extreme measurements were adjusted to the nearest 0.5 μm. The spore size range, mean and standard deviation of 50 spore measurements from each specimen are shown in Table 1. The spore size values are presented in a scatter diagram to show the distribution of the values. The species descriptions include combined values from all measured specimens. The specimens of the *Antherospora vaillantii* complex measured in previous work in lactophenol (Bauer et al. 2008) were measured again in lactic acid to minimize the error caused by different mounting media. LM micrographs were taken with a Nikon DS-Fi1 camera. The spores of *Antherospora on Muscari armeniacum* (KRAM F-49437), on *M. botryoides* (KR 27962), on *M. comosum* (KRAM F-49438 = HAI 2857), and on *M. tenuiflorum* (GLM 48095) were studied using scanning electron microscopy (SEM). For this purpose, dry spores were mounted on carbon tabs and fixed to an aluminium stub with double-sided transparent tape. The tabs were sputter-coated with carbon using a Cressington sputter-coater and viewed with a Hitachi S-4700 scanning electron microscope, with a working distance of ca. 12 mm. SEM micrographs were taken in the Laboratory of Field Emission Scanning Electron Microscopy and Microanalysis at the Institute of Geological Sciences, Jagiellonian University, Kraków (Poland).

**DNA extraction, PCR, and sequencing**

For methods of isolation and crushing of fungal material, DNA extraction, amplification of ITS 1 and ITS 2 regions of the rDNA including the 5.8S rDNA (ITS) and the 5´-end of the nuclear large subunit ribosomal DNA (LSU), purification of PCR products, sequencing, and processing of the raw data see Lutz et al. (2004), and Piątek et al. (2011). The DNA sequences determined for this study were deposited in GenBank (GenBank accession number are given in Table 1 and Fig. 1).

**Phylogenetic analyses**

In addition to the ITS and LSU sequences of *Antherospora* sp. on *Muscari* spp. obtained in this study, sequences from GenBank of the following species were used for molecular
phylogenetic analyses (Bauer et al. 2008, Piątek et al. 2011): *Antherospora scillae*, *A. tractemae*, *A. vaillantii*, and *A. vindobonensis* (GenBank accession numbers are included in Fig. 1).

To elucidate the phylogenetic position of the *Antherospora* specimens from *Muscari* spp. their concatenated ITS+LSU sequences were analysed within a dataset covering all *Antherospora* species available in GenBank. If present in

| Species                  | Host               | GenBank acc. no.       | Spore size range (µm) | Mean spore size with standard deviation (µm) | Reference specimens |
|--------------------------|--------------------|------------------------|-----------------------|---------------------------------------------|---------------------|
| A. hortensis             | *M. armeniacum*    | ITS: EF653962, LSU: EF653964 | (6.0–)7.0–10.0(–13.0)× (5.5–)6.0–8.0 | 8.6 ± 1.7 × 6.8 ± 0.7 | Germany, Baden-Württemberg, Tübingen, Gabriel-Biel-Strasse 5, 17 Apr. 2005, C. & K. Vánky, H.U.V. 21046 |
| A. hortensis             | *M. armeniacum*    | ITS: KC175333, LSU: KC175326 | (6.0–)7.5–10.5(–12.5)× (5.5–)6.0–8.5(–9.0) | 8.9 ± 1.2 × 7.5 ± 0.7 | Germany, Baden-Württemberg, Tübingen, garden, Stückerstrasse 39, 22 Apr. 2011, M. Lutz, KR 27970 |
| A. hortensis             | *M. armeniacum*    | ITS: EF653997, LSU: EF653979 | (5.5–)6.0–10.5(–12.5)× 5.5–7.5(–8.0) | 7.8 ± 1.5 × 6.5 ± 0.7 | Germany, Baden-Württemberg, Kirchheim/Tack, garden Römersteinstrasse 12, 2 May 2006, N. Böhling, TUB 15838 |
| A. hortensis             | *M. armeniacum*    | ITS: KC175334, LSU: KC175327 | (6.5–)7.0–10.5(–12.5)× 6.0–8.5(–9.5) | 9.0 ± 1.4 × 7.6 ± 0.8 | UK, Wales, Ceredigion, Aberystwyth, Pengiais Road, SN-590-818 [grid reference on UK national grid], 7 Apr. 2009, A.O. Chater, KRAM F-49434 |
| A. hortensis             | *M. armeniacum*    | ITS: KC175335, LSU: KC175328 | (5.5–)6.0–10.5(–11.0)× 5.0–7.5(–8.5) | 7.9 ± 1.4 × 6.7 ± 0.7 | UK, Wales, Ceredigion, Aberystwyth, Plas Crug cemetery, SN-591-812 [grid reference on UK national grid], 16 Apr. 2010, A.O. Chater, KRAM F-49435 |
| A. hortensis             | *M. armeniacum*    | ITS: KC175336, LSU: KC175329 | 6.0–10.0(–11.0)× 5.5–6.0–8.5 | 8.1 ± 1.2 × 7.1 ± 0.7 | UK, Wales, Ceredigion, Aberystwyth, Cliff Terrace, garden of Northfield, SN-585-826 [grid reference on UK national grid], 15 Apr. 2010, A.O. Chater, KRAM F-49436 |
| A. hortensis             | *M. armeniacum*    | ITS: KC175337, LSU: KC175330 | 6.0–10.5(–12.5)× 5.5–8.5(–9.0) | 8.1 ± 1.7 × 6.9 ± 0.9 | UK, Wales, Ceredigion, Llambadarn Fawr, University campus, SN-604-811 [grid reference on UK national grid], 20 Apr. 2010, A.O. Chater, KRAM F-49437 – holotype |
| A. muscarci-botryoidis    | *M. botryoides*    | ITS: KC175332, LSU: KC175325 | (5.0–)6.0–9.5(–11.0)× 4.5–5.0–7.5(–8.0) | 7.7 ± 1.4 × 6.3 ± 0.8 | Germany, Baden-Württemberg, Hechingen, B32 in Richtung Burladingen, 22 Apr. 2011, M. Lutz, KR 27962 – neotype |
| A. muscarci-botryoidis    | *M. botryoides*    | ITS: EF653998, LSU: EF653980 | (6.5–)7.0–10.5(–11.0)× 5.5–6.0–8.5(–9.0) | 8.5 ± 1.1 × 7.2 ± 0.8 | Slovenia, Kras, 7 km ESE from Sezana, 30 Apr. 2006, C. & K. Vánky, H.U.V. 21337 |
| A. vaillantii             | *M. comosum*       | ITS: KC175338, LSU: KC175331 | (5.5–)6.0–10.5× 5.0–7.5(–8.0) | 7.9 ± 1.4 × 6.3 ± 0.8 | Israel, Haifa district, Carmel National Park, 2 Feb. 2011, K.G. Savchenko, HAI 2857, KRAM F-49438 |
| A. vaillantii             | *M. tenuiforum*    | ITS: EF653986, LSU: EF653968 | (5.5–)6.0–10.5(–13.0)× 5.0–7.5(–9.0) | 8.3 ± 1.6 × 6.9 ± 0.8 | Germany, Sachsen-Anhalt, Saalkreis, Löbjejun, Schiedsberg, 25 May 2000, H. Jage, GLM 47396 |
| A. vaillantii             | *M. tenuiforum*    | ITS: EF653987, LSU: EF653969 | 6.0–10.0(–11.0)× 5.0–7.5(–8.0) | 7.9 ± 1.2 × 6.5 ± 0.6 | Germany, Sachsen-Anhalt, Saalkreis, Löbjejun, Schiedsberg, 29 May 1999, U. Richter, GLM 48095 |
| A. vaillantii             | *M. tenuiforum*    | ITS: EF653998, LSU: EF653970 | 6.0–10.5(–12.0)× 6.0–8.5 | 8.3 ± 1.4 × 7.0 ± 0.6 | Germany, Sachsen-Anhalt, Saalkreis, Löbjejun, Schiedsberg, 24 May 1999, W. Dunka, GLM 50411 |

1. GLM – Herbarium of the Senckenberg Museum für Naturkunde Görlitz, Germany; HAI – Herbarium of the Institute of Evolution, University of Haifa, Israel; H.U.V. – Herbarium Ustilaginales Vánky, Gabriel-Biel-Str. 5, D-72076 Tübingen, Germany; KR – Herbarium of the Staatliches Museum für Naturkunde Karlsruhe, Germany; KRAM F – Mycological Herbarium of the W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków, Poland; TUB – Herbarium of the Eberhard-Karls-Universität Tübingen, Germany.
GenBank, the sequences of the respective type specimens were used.

Sequence alignment was obtained using MAFFT 6.853 (Katoh et al. 2002, 2005, Katoh & Toh 2008) using the L-INS-i option. The resulting alignment was used for both the detection of species specific autapomorphic nucleotide differences and the estimation of genetic distances within species (intraspecific) and between species (interspecific) using the software MEGA (Tamura et al. 2011). We calculated p-distances and report distances as percentage genetic distances. Gaps or different length of sequences were not used for calculations as we chose the pair-wise deletion option in MEGA.

To ensure reproducible phylogenetic analyses, manipulation of the alignment by hand as well as manual exclusion of ambiguous sites were avoided as suggested by Giribet & Wheeler (1999) and Gatesy et al. (1993), respectively. Highly divergent portions of the alignment were omitted using GBLOCKS 0.91b (Castresana 2000) with the following options: 'Minimum Number of Sequences for a Conserved Position' to 10, 'Minimum Number of Sequences for a Flank Position' to 10, 'Maximum Number of Contiguous Non-conserved Positions' to 8, 'Minimum Length of a Block' to 5 and 'Allowed Gap Positions' to 'With half'.

The resulting alignment [new number of positions: 1336 (73% of the original 1810 positions) number of variable sites: 35] was used for phylogenetic analyses using Maximum Likelihood (ML) and a Bayesian Approach (BA). ML analysis (Felsenstein 1981) was conducted with the RAXML 7.2.6 software (Stamatakis 2006), using raxmlGUI (Silvestro & Michalak 2012), invoking the GTRCAT and the rapid bootstrap option (Stamatakis et al. 2008) with 1000 replicates.

For BA a Bayesian approach to phylogenetic inference using a Markov chain Monte Carlo technique was used as implemented in the computer program MrBayes 3.2.1 (Ronquist et al. 2012). Two runs over 5,000,000 generations, each consisting of four chains, were implemented 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 & Rannala (2004). Trees were sampled every 100th generation, resulting in a sampling of 50 001 trees for each run. From these, the first 25 % of trees from each run were discarded (burnin). The remaining 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 to phylogenetic analysis was repeated five times to test the independence of the results from topological priors (Huelsenbeck et al. 2002). Trees were rooted with Antherospora scillae, A. tractemae, and A. vindobonensis.

RESULTS

Phylogenetic analyses

Species specific autapomorphic nucleotide differences are included in the species descriptions, intraspecific and interspecific genetic distances of the ITS+LSU rDNA are given in Table 2.

The different runs of BA that were performed and the ML analyses yielded consistent topologies. To illustrate the results, the phylogenetic hypothesis resulting from the ML analysis is presented in Fig. 1. ML bootstrap support values are indicated on branches before slashes, estimates for a posteriori probabilities are indicated after slashes.

In all analyses, the Antherospora species included in previous studies (Bauer et al. 2008, Piątek et al. 2011), and Vánky et al. (2008). All Antherospora specimens from Muscari spp. clustered together forming three well supported subgroups: one contained all sampled specimens from M. botryoides, another all specimens from M. armeniacum, and the third specimens from both M. comosum and M. tenuiflorum. The former two subgroups were revealed as sister taxa.

Morphology

The morphology of the Antherospora specimens is presented in accordance with the lineages obtained in the phylogenetic analyses, namely Antherospora on Muscari armeniacum, on M. botryoides and on M. comosum+M. tenuiflorum. The specimens of Antherospora on Muscari spp. shared a similar appearance of macroscopic symptoms of the infection as well as a similar morphology of spores. In all specimens, the olivaceous sori formed in all anthers of fertile flowers, and the sori were enclosed by floral perianths. The spore ornamentation and shape were regular in all collections: the spores were finely verrucose or verruculose and usually globose, subglobose, broadly ellipsoidal, or occasionally allantoid, ovoid, pyriform, or tear-shaped. The spore sizes of Antherospora specimens on Muscari armeniacum, M. comosum and M. tenuiflorum were similar and grouped in one “cloud” on the scatter diagram, while the spore sizes of specimens on Muscari botryoides were shorter compared to the specimens from the remaining hosts (Table 1, Fig. 2).

The mean spore length was variable between collections of Antherospora on Muscari armeniacum, ranging from 7.8–9.0, less variable in specimens on M. botryoides (7.7–8.5), and quite uniform in specimens on M. comosum and M. tenuiflorum (7.9–8.3). Likewise, the mean spore width was variable between collections of Antherospora on Muscari armeniacum, ranging from 6.5–7.6, and less variable in specimens on M. botryoides (6.3–7.2), and in specimens on M. comosum and M. tenuiflorum (6.3–7.0) (Table 1). The combined mean spore length and width were similar in Antherospora on Muscari botryoides and Antherospora on M. comosum+M. tenuiflorum, while both values were somewhat larger in Antherospora on M. armeniacum. The detailed morphological characteristics of the anther smuts on Muscari spp. are included in the species descriptions and depicted in Figs 3–5.

TAXONOMY

Antherospora hortensis M. Piątek & M. Lutz, sp. nov.
MycoBank MB803427
(Fig. 3)

Etymology: In reference to the occurrence of this fungus on Muscari armeniacum in cultivated gardens, where all specimens examined in this study were collected.

Type: UK: Wales: Ceredigion, Llanbadarn Fawr, University campus, on Muscari armeniacum, 20 Apr. 2010, A.O. Chater (KRAM F-49437 – holotype)

Description: Parasitic on Muscari armeniacum. Sori in the anthers, producing a dark olive-brown, powdery mass of spores inside the pollen sacs, enclosed by the perianths. Infection systemic, all anthers in all fertile flowers in an inflorescence are infected. Spores globose, subglobose, broadly ellipsoidal, sometimes allantoid, tear-shaped or asymmetrical, (5.5–6.0–10.5–13.0) × 5.0–8.5–(9.5) µm [av. ± SD, 8.3 ± 1.5 × 7.0 ± 0.9 µm, n = 350/7], olive-brown, sometimes lighter coloured on one side; wall even, ca. 0.5–0.7 µm thick, finely, densely verruculose in LM, spore profile almost smooth or finely wavy, wall finely verruculose in SEM. Autapomorphic nucleotide differences in the ITS at the positions 192, 193, and 437 and in the LSU at the position 1311 in the alignment.

The ITS/LSU hologenotype sequences are deposited in GenBank as KC175337/KC175330, respectively.

Additional specimens examined: Germany: Baden-Württemberg: Tübingen, Gabriel-Biel-Strasse 5, on Muscari armeniacum (as M. neglectum), 17 Apr. 2005, C. & K. Vánky (H.U.V. 21046); Tübingen, Stöcklestrasse 39, on M. armeniacum, 22 Apr. 2011, M. Lutz 2338 (KR 27970); Kirchheim/Teck, garden of Römersteinstrasse 12, on M. armeniacum (as M. comosum), 2 May 2006, N. Böhling (TUB 15838). – UK: Wales: Ceredigion, Aberystwyth, Penglais Road, on M. armeniacum, 7 Apr. 2009, A.O. Chater (KRAM F-49434); Ceredigion, Aberystwyth, Plas Crug cemetery, on M. armeniacum, 16 Apr. 2010, A.O. Chater (KRAM F-49435); Ceredigion, Aberystwyth,
Host and distribution: On Muscari armeniacum (Muscari subgen. Botrysanthus, Asparagaceae), Germany, UK. – Antherospora vaillantii s. lato on Muscari armeniacum was included in Vánky (2012), but no specific details are given for the source of this record. The anther smut on Muscari schlemannii (syn. M. armeniacum) from the Berlin Botanical Garden (Magnus 1896, Scholz & Scholz 1988) and on M. cyaneoviolaceum (syn. M. armeniacum) from the Royal Botanic Gardens Kew (Kirk & Cooper 2013) most probably belongs to this species.

Comments: This species is morphologically similar to Antherospora vaillantii s. str., differing by a somewhat larger (longer and wider) average size of spores and host plant in Muscari subgen. Botrysanthus. Antherospora hortensis and A. vaillantii ITS+LSU sequences differ in 32–35 positions in 16 loci. Among the sequence differences there

|                 | A. hortensis | A. muscari-botryoidis | A. vaillantii | A. vindobonensis | A. tractemae | A. scillae |
|-----------------|--------------|-----------------------|--------------|-----------------|--------------|-----------|
| A. hortensis    | 0            | 0.4-0.5               | 0.9          | 1.9             | 1            | 1.9       |
| A. muscari-botryoidis | 0.1        | 0.7                   | 1.8-1.9      | 1.1-1.2         | 1.8-1.9      |           |
| A. vaillantii   | 0            | 1.8                   | 1.1          | 1.8             |              |           |
| A. vindobonensis| 0            | 0                     | 1.7          | 0.3             |              |           |
| A. tractemae    | 0            | 0                     | 1.6          |                 |              |           |
| A. scillae      |              |                       |              |                 |              | 0         |

Fig. 2. A scatter diagram showing the spore size distribution in Antherospora hortensis (blue squares), A. muscari-botryoidis (black spots), and A. vaillantii (yellow triangles). Note that one point may include measurements of the respective length/width value from more than one spore.
Fig. 3. Antherospora hortensis sp. nov. on Muscari armeniacum. A. General habit of infected plant. B–C. Healthy (to the left) and infected (to the right) inflorescences, with fungus sporulating in the anthers. D. Close-up of the anthers with fungal spores. Note that ovary is not destroyed. E–I. Spores seen by LM, median and superficial views (KRAM F-49437). J–L. Ornamentation of spores seen by SEM (KRAM F-49437). Bars: A–C = 5 mm, D = 1 mm, E-I = 10 µm, J-L = 5 µm.
are autapomorphic nucleotide differences in the ITS at the positions 192, 193, and 437 and in the LSU at the position 1311 in the alignment for *Antherospora hortensis*.

**Antherospora muscari-botryoidis** (Cif.) M. Piątek & M. Lutz, **comb. nov.**
MycoBank MB803428
(Fig. 4)

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Fig. 4. *Antherospora muscari-botryoidis* comb. nov. on *Muscari botryoides*. A. General habit of infected plant. B–C. Infected inflorescences, with fungus sporulating in the anthers. D–G. Spores seen by LM, median and superficial views (KR 27962). H–J. Ornamentation of spores seen by SEM (KR 27962). Bars: B–C = 5 mm, D–H = 10 µm, I–J = 5 µm.
Type: **Italy**: Province of Cuneo: Alba, Moretta, towards Santa Rosalia, on *Muscari botryoides*, [year and collector unclear but probably 1921 and R. Ciferri, respectively] (type not located, probably does not exist). – **Germany**: Baden-Württemberg: Hechingen, B32 in Richtung Burdelayen, 48°20′55″N, 09°00′12″E, on *Muscari botryoides*, 22 Apr. 2011, M. Lutz (KR 27962 – neotype designated here).

Synonym: *Antherospora neglecta* Vánky, in Lutz & Vánky, Lidia 7(2–3): 35 (2009), nom. nud.

**Original material**: Slovenia: Kras, 7 km ESE from Sezana, on *Muscari botryoides* (as *M. neglectum*), 30 Apr. 2006, C. & K. Vánky (H.U.V. 21337).

Parasitic on *Muscari botryoides*. Sori in the anthers, producing a dark olive-brown, powdery mass of spores inside the pollen sacs, enclosed by the perianths. Infection systemic, all anthers in all fertile flowers in an inflorescence are infected. Spores globose, subglobose, broadly ellipsoid, sometimes tear-shaped or asymmetrical, (5.0–)6.0–10.5(–11.0) × (4.5–)5.0–8.5(–9.0) µm [av. ± SD, 8.1 ± 1.3 × 6.7 ± 0.9 µm, n = 100(2)], olive-brown; wall even, ca. 0.5–0.8 µm thick, finely, densely verruculose in LM, spore profile almost smooth or finely wavy, wall finely verrucose in SEM. Autapomorphic nucleotide difference in the LSU at the position 1078 in the alignment.

The ITS/LSU neogenotype sequences are deposited in GenBank as KC175332/KC175325, respectively.

**Host and distribution**: On *Muscari botryoides* (*Muscari* subgen. Botryanthus, Asparagaceae), Germany, Italy, and Slovenia. This species was described from Italy (Ciferri 1928), and the sequenced specimens are from Germany and Slovenia. The different anther smut records and collections on *Muscari botryoides* from Europe (Bulgaria, Germany, UK) (Scholz & Scholz 1988, Denchev 2001, Kirk & Cooper 2013) and Australasia (New Zealand) (Vánky & McKenzie 2002) probably belong to this species. It was introduced to areas outside the natural range of *Muscari botryoides*, for example to UK and New Zealand.

**Comments**: This species shares with *Antherospora hortensis* the systematic position of its host plant in *Muscari* subgen. Botryanthus, but differs by somewhat shorter spores and a different host species (*M. botryoides* vs. *M. armeniacum*). *Antherospora muscari-botryoidis* and *A. hortensis* ITS+LSU sequences differ in 11–12 positions in 8 loci. Among the sequence differences there is an autapomorphic nucleotide difference in the LSU at the position 1078 in the alignment for *Antherospora muscari-botryoidis*.

This species differs from *Antherospora vaillantii s. str.* by somewhat shorter spores and its host plant in *Muscari* subgen. Botryanthus. The host plants of *Antherospora vaillantii s. str.* belong to *Muscari* subgen. Leopoldia. *Antherospora muscari-botryoidis* and *A. vaillantii s. str.* ITS+LSU sequences differ in 22–26 positions in 11–12 loci. Among the sequence differences there is an autapomorphic nucleotide difference in the LSU at the position 1078 in the alignment for *Antherospora muscari-botryoidis*.

The name “*Antherospora neglecta* Vánky, in prep.” was assigned by Vánky to the Slovenian specimen in the checklist of smut fungi from Slovenia (Lutz & Vánky 2009). This name is a nomen nudum as it was never validated. The host plant was originally identified as *Muscari neglectum*. However, its re-identification revealed that it is *Muscari botryoides* (flowers ±globose, not elongated). Consequently, *Antherospora neglecta* is listed as a synonym of *A. muscari-botryoidis*.

**Antherospora vaillantii** (Tul. & C. Tul.) R. Bauer et al., *Mycol. Res.* **112**: 1304 (2008).

(Fig. 5)  
**Basionym**: Ustilago vaillantii Tul. & C. Tul., *Ann. Sci. Nat., Bot.*, sér. 3, 7: 90 (1847).

**Synonyms**: Yenia vaillantii (Tul. & C. Tul.) Liou, *Contrib. Inst. Bot. Natl. Acad. Peiping* **6**: 45 (1949).

**Vánky vaillantii** (Tul. & C. Tul.) Ershad, *Rostaníha* 1: 69 (2000).

**Type**: France: on *Muscari comosum*, S. Vaillant (lectotype designated by Lindeberg 1959: 141, but apparently without seeing the herbarium specimen; despite the statement of Vánky 2012: 8, who indicated that he studied the lectotype from PC, such a specimen was not located in PC in the course of the present study).

Parasitic on *Muscari comosum* and *M. tenuiflorum*. Sori in the anthers, producing a dark olive-brown, semi-powdery to powdery mass of spores inside the pollen sacs, enclosed by the perianths. Infection systemic, all anthers in all fertile flowers in an inflorescence are infected, flowers sometimes deformed. Spores globose, subglobose, broadly ellipsoid, sometimes ovoid, pyriform or asymmetrical, (5.5–)6.0–13.0 × 5.0–8.5(–9.0) µm [av. ± SD, 8.1 ± 1.4 × 6.7 ± 0.8 µm, n = 200(4)], olive-brown, sometimes lighter coloured on one side; wall even, ca. 0.5–0.7 µm thick, often darker than the rest of the spore, finely, densely verruculose in LM, spore profile almost smooth or finely wavy, wall finely verrucose in SEM. Autapomorphic nucleotide differences in the ITS at the positions 95, 1304, and 1305 and in the LSU at the positions 1292, and 1309 in the alignment.

**Specimens examined**: Germany: Sachsen-Anhalt: Saalekreis, Löbejün, Schiedsberg, on *Muscari tenuiflorum*, 25 May 2000, H. Jage (GLM 47396); Saalekreis, Löbejün, Schiedsberg, on *M. tenuiflorum*, 29 May 1999, U. Richter (GLM 48095); Saalekreis, Löbejün, Schiedsberg, on *M. tenuiflorum*, 24 May 1999, W. Durka (GLM 50411). – Israel: Hafia district: Carmel National Park, on *M. comosum*, 2 Feb. 2011, K.G. Savchenko (HAI 2857, KRAM F-49438 – representative specimens).

**Hosts and distribution**: On *Muscari comosum* and *M. tenuiflorum* (*Muscari* subgen. Leopoldia, Asparagaceae), France, Germany, and Israel. – The type material was collected in France (Tulasne & Tulasne 1847), and the sequenced specimens are from Israel (Savchenko et al. 2008).
2011) and Germany (Jage & Richter 2011). Most likely the numerous Antherospora reports on Muscari comosum and M. tenuiflorum belong to Antherospora vaillantii s. str. The anther smut reports on Muscari comosum are from Europe (Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, “Czechoslovakia”, France, Germany, Hungary, Italy, Poland, Romania, Russia – European part, Serbia, Slovenia, Switzerland, Ukraine) (e.g. Lindtner 1950, Zundel 1953, Kochman & Majewski 1973, Vánky 1985, Săvulescu 1955, Scholz & Scholz 1988, Karatygin & Azbukina 1989, Denchev 2001, Zwetko & Blanz 2004, Savchenko & Heluta 2012), from Africa (Algeria, Egypt, and Morocco).
(Zundel 1953, Zambettakis 1970), and from North America (USA – Massachusetts) (Zundel 1953). The anther smut reports on *Muscaria tenuiflorum* are from Europe (Austria, Bulgaria, “Czechoslovakia”, Germany, Poland, Romania, Serbia, Montenegro, and Ukraine) (Lindtner 1950, Zundel 1953, Sávulescu 1955, Kochman & Majewski 1973, Vánky 1985, Scholz & Scholz 1988, Karatygín & Azubukina 1989, Denchev 2001, Zwetko & Blanz 2004). The records from outside the natural range of the two *Muscaria* species, for example in the USA, Belgium (*M. comosum*) or Poland (*M. tenuiflorum*), are the result of the introduction of host plant and fungus.

Comments: The type material of *Ustilago vaillantii* could not be located, and the typification of this smut species needs some clarification. In the protologue, Tulasne & Tulasne (1847) included three host species in the following order: *Muscaria comosum*, *Scilla anthericoides*, and *Scilla maritima*, but from the text it is apparent that they directly studied only three smut specimens on *Muscaria comosum* collected by Vaillant, Delastre and Léveillé, respectively. The type host was assigned to *Muscaria comosum* by Liro (1924), and accepted as such by Ciferri (1928). Furthermore, Liro (1924) studied the Delastre specimen included in the protologue, which was then deposited in the Persoon Herbarium in Leiden (L), and confirmed that the host plant was indeed *Muscaria comosum*. That specimen should be treated as a syntype, and cannot be considered as lectotype since it was not indicated with the term “type” or an equivalent by Liro (1924), thus not meeting the criteria of Article 7.10 of the ICN. Liro (1924) only selected the type host of *Ustilago vaillantii* from the three hosts mentioned by the Tulasne brothers. Lindeberg (1959) designated Vaillant’s collection as a lectotype of *Ustilago vaillantii*, but apparently she did not see this material – the problem is that the specimen probably does not now exist.

Vánky (2012) indicated that he studied the lectotype specimen from PC (he included the symbol “L” at the typification entry of *Antherospora vaillantii*) selected by Lindeberg (1959). However, the examination of all materials from PC labelled as *Ustilago vaillantii* made in the course of the present study revealed no authentic specimen of this smut fungus matching the data from the protologue (Tulasne & Tulasne 1847). Likewise, the Delastre syntype specimen mentioned in the protologue and later studied by Liro (1924) was not found in the Persoon herbarium in L (G. Thijsse, pers. comm.). It is reasonable to assume that no authentic collection of *Ustilago vaillantii* is currently preserved and that a neotype should be selected for this species. However, the neotype of *Antherospora vaillantii* based on the Israeli specimen analysed in the present study is not designated here, as in our opinion it is better to select the neotype from a fresh European collection, which is not available at present (most herbarium collections are old and not useful for molecular analyses). Thus, in line with Hawksworth (2012), the Israeli collection of *Antherospora vaillantii* s. str. on the type host *Muscaria comosum* is referred to as a “representative specimen”.

**DISCUSSION**

In this study, morphological and molecular phylogenetic analyses were performed in order to resolve the *Antherospora vaillantii* species complex on different *Muscaria* species. The specimen sampling covers four of the six *Muscaria* species reported as hosts for *Antherospora vaillantii* *s. latr* in the recent world monograph of smut fungi (Vánky 2012), including the type hosts for both species that were described for *Muscaria* anther smuts, namely *Ustilago vaillantii* (*M. comosum*) and *U. muscaria-botryoides* (*M. botryoides*).

In accordance with previous molecular phylogenetic analyses based on a smaller sampling of *Muscaria* anther smuts (Bauer et al. 2008, Piątek et al. 2011), the present analyses revealed that all anther smut specimens on *Muscaria* spp. form a monophyletic group within the *Antherospora* clade. The phylogenetic relations within this lineage agree well with the classification of the respective host species: the basal group contains anther smuts on two hosts belonging to *Muscaria* subgen. *Leopoldia* (*M. comosum*, *M. tenuiflorum*), while the derived group includes anther smuts on hosts belonging to *Muscaria* subgen. *Botryanthus* that is further subdivided into two lineages correlated with the host plant species *M. armeniacum* and *M. botryoides*, respectively. That indicates a common ancestry of the *Muscaria* anther smuts and co-evolution as a driver of their diversification. Results showing similar patterns in anther smuts on *Scilla* (Bauer et al. 2008) and *Tractema* (Piątek et al. 2011) support this hypothesis. However, confirmation of the monophyletic origin of the *Muscaria* anther smuts with a larger sampling of anther smut specimens on diverse host plants, especially on the related genus *Bellevalia*, is desirable.

The genetic divergence between three lineages of *Muscaria* anther smuts obtained does not correspond to differences in phenotypic characteristics. The morphology of all anther smut specimens was quite similar and, without support of non-morphological data, the small differences in spore size or average spore sizes observed between them could easily be treated as phenotypic variability of one single species. In contrast to the study of Bauer et al. (2008), where the spore size of one collection on *Muscaria tenuiflorum* (GLM 47396) had much larger spores and deviated from the remaining specimens on this host, the repeated measurement of the same specimen in this study did not confirm such deviation. They were similar to values obtained from the remaining specimens on *Muscaria tenuiflorum*. The different mounting media (lactophenol used by Bauer et al. 2008 vs. lactic acid used in this study) cannot explain this discrepancy because the repeated measurements of the remaining specimens used in the previous study did not reveal such differences. This discordance may have resulted from measuring spores from different sori. The examples from other studies indicate that in some smut samples, the spores measured from different sori within a collection may have a different size or morphology, for example in *Anthracoidea*

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1*M. alpinum* and *M. schliemannii* listed in this monograph are excluded here as both are synonyms of other species (Euro+Med 2006-)
hostianae (Piątek & Mulenko 2010) or Farysia ugandana (Vánky 2004a). Such anomalies between collections are also known in other groups of basidiomycetes, for example in polyopes (Niemelä et al. 2001), and in ascomycetes, for example in Calonectria (Crous et al. 2006). The large spore size values of anther smut on Muscari tenuiflorum are not included in this study. Using this example, Vánky (2008) introduced the term pseudomorphosphies for smuts with morphological differences showing no genetic differences. This concept, however, does not take into account variation of morphological characters within specimens of the same species, as well as possible anomalies in spore morphology such as discussed above.

Molecular phylogenetic analyses revealed all anther smut specimens on Muscari botryoides, on M. armeniacum, and on M. comosum and M. tenuiflorum, respectively, to be in strongly supported monophyletic groups forming independent lineages. Furthermore, each lineage had autapomorphic nucleotide differences in the ITS and/or the LSU in the alignment. In addition, pairwise genetic distances between the lineages were found sufficient to support their separation as distinct species. They ranged from 0.4–0.9 % (within the lineages pairwise genetic distances ranged from 0–0.1 %). Thus, in spite of weak morphological differences, the genetic divergence and strict correlation with host species taxonomy indicate that the three lineages of the Muscari anther smuts in fact represent three different cryptic species. The host plant species appear to be good taxonomic markers for this group of parasites, as the collections from the same host (Muscari armeniacum, M. botryoides) or closely related hosts (M. comosum+M. tenuiflorum) grouped together, irrespective of their geographic origin. This is in agreement with similar results obtained for other smut fungi (Hendrichs et al. 2005, Lutz et al. 2005, 2008, Piątek et al. 2012). Because of some uncertainties concerning the effective description of species on the basis of molecular characters (Tripp & Lendemer 2012), the descriptions of the three recognized Antherospora species on Muscari include, in addition to the host plant name and morphology, the species specific autapomorphic nucleotides of the ITS and LSU.

The three lineages of anther smuts on Muscari correspond to Antherospora vaillantii s. str., Ustilago muscari-botryoidis and an undescribed species. The basal lineage containing the collection on Muscari comosum from Israel is assigned to Antherospora vaillantii s. str. The molecular results indicate that this species is additionally able to infect the closely related Muscari tenuiflorum. The clade represented by two specimens on Muscari botryoides is referred to a second species, for which the name Ustilago muscari-botryoidis is available (Ciferri 1928). Because this is a species of Antherospora, a new combination in this genus is introduced. In contrast to other monographers who synonymized it with Antherospora vaillantii (e.g. Vánky 1985, 1994, 2012, Scholz & Scholz 1988, Karatygin & Azbukina 1989, Denchef 2001), the present study confirms that Ciferri (1928) was correct in describing Ustilago muscari-botryoidis. Compared to the remaining anther smuts on Muscari, Antherospora muscari-botryoidis has somewhat smaller spores. However, more specimens should be analysed both morphologically and genetically to confirm this feature as stable and taxonomically informative for this smut.

The third lineage, containing specimens on Muscari armeniacum, represents a previously undescribed species. The discovery of this species is unexpected as all anther-infected Muscari armeniacum plants were found in gardens outside the natural range of the host species. This special habitat occurrence is reflected in the proposed epithet for this species, Antherospora hortensis. Except for the brief information in Vánky (2012), Muscari armeniacum was not reported in any publication as a host for anther smuts, at least under this species name. However, the old records of Ustilago vaillantii on Muscari schieliannii from Berlin Botanical Garden (Magnus 1896), where it was observed during the years 1892–1897 (Scholz & Scholz 1988), most probably belong to the same pathogen. Muscari schieliannii is a synonym of M. armeniacum (Euro+Med 2006-). Likewise, the 1931 collection of Ustilago vaillantii on Muscari cyanoviolaceum from Kew Gardens (Kirk & Cooper 2013) probably also belongs to Antherospora hortensis. Muscari cyanoviolaceum is a synonym of M. armeniacum (Euro+Med 2006-). This indicates that Antherospora hortensis persisted on cultivated plants in Germany and UK for a long time and the recent findings in Baden-Württemberg and Wales are probably only the result of special collecting activity directed to smut fungi. The concentration of localities around Tübingen in Germany as well as around Aberystwyth in UK may also reflect local transmission of infected plants between gardens by gardeners. The anther smuts on monocots may propagate by bulbs or even by infected seeds (Massee 1914, Kochman 1936). The current data suggest that the distribution of Antherospora hortensis is as yet localized. This could be also supported by the observation that Antherospora hortensis was not found in Kraków in Poland despite that Muscari armeniacum is commonly cultivated in gardens and has been examined by one of us (MP) for anther smuts every year since 2007. At present Antherospora hortensis does not pose a significant disease threat, although this may change since ongoing climate change allows many pathogens to spread to new geographical areas.

The origin of Antherospora hortensis is unclear. The native occurrence of Muscari armeniacum is in the Balkan Peninsula, but no smutted specimens have been reported from this area. In this region there are reports of anther smuts on Muscari neglectum (Lindtner 1950, Zündel 1953, Denchef 2001). These two Muscari species were often misidentified, and according to Tutin et al. (1980), many records of M. neglectum from the Balkan Peninsula in fact represent M. armeniacum. The occurrence of anther smuts on Muscari neglectum should be re-examined, preferably with fresh collections as drying often affects flower colour, which is a diagnostic character of M. neglectum.

Besides the host plants included in the current study or discussed above, two further species have been reported for Antherospora vaillantii s. lato in the recent world monograph (Vánky 2012), namely Muscari alpinum and M. moschatum. The identity of Muscari alpinum is uncertain since there are two names with the same epithet: M. alpinum J. Gay ex Baker and M. alpinum Szafer ex Racz., which are synonyms of M. tenuiflorum and M. botryoides, respectively (Euro+Med 2006-). Although Vánky (2012) attributed this name to “J. Gay”, in the original report the species name is given without
any authorities (Bubák 1916). The anther smut on Muscari moschatum that was reported from Georgia (Karatygin & Azbukina 1989), is a putative fourth distinct species within the Antherospora vaillantii complex, a hypothesis based on the isolated position of the host in Muscari subgen. Muscari. Fresh materials for molecular and morphological analyses are desirable to confirm this hypothesis.

In addition to resolving the species within the Antherospora vaillantii complex on Muscari, this study gives further evidence that host specificity may reveal cryptic diversity in some smut fungi, to a greater degree than morphology. Past assumptions were that host specificity was restricted to the host family (Fischer & Shaw 1953), subtribe (Vánky 2004b) or, mostly, genus level (Vánky 1994, 2012). Recent molecular studies indicate that host specialization is, in most cases, at the species level (Kemler et al. 2009, Kemler et al. 2013). This is not an absolute rule since some smut species may infect two or more host species from the same genus (Castlebury & Carris 1999, Lutz et al. 2005, Curran et al. 2009, Kemler et al. 2013), or two or more species from different but usually phylogenetically closely related genera (Carris et al. 2007, Vánky & Lutz 2007). Thus, the level of host specificity in different smut species reported from multiple host species and genera, including the other members of the genus Antherospora, remains to be tested and is a challenge for future studies.

ACKNOWLEDGEMENTS

We thank Michael Weiß, Sigisfredo Garnica, and Robert Bauer (Tübingen, Germany) for providing facilities for molecular analyses, Kyryl G. Savchenko (Haifa, Israel) for sending a material, the Curators of herbaria: Herbert Boyle (GLM), Bart Buyck (PC), Markus Scholler (KR), and Kálmán Vánky (H.U.V.) for loan of specimens, and Anna Łatkiewicz (Kraków, Poland) for her help with the SEM pictures. Gerard Thijssse (Leiden, Netherlands) tried to locate the Delastre collection of Ustilago vaillantii in the Persoon herbarium (L) and we greatly appreciate his endeavours.

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