Venturia orni sp nov., a species distinct from Venturia fraxini, living in the leaves of Fraxinus ornus

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ORIGINAL ARTICLE

Venturia orni sp. nov., a species distinct from *Venturia fraxini*, living in the leaves of *Fraxinus ornus*

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Abstract *Venturia fraxini* Aderh. s.l. is an endophytic fungus in the leaves of different ash species like *Fraxinus excelsior* and *F. ornus*. This study shows that the *Venturia* species on *F. ornus* is not conspecific with *V. fraxini* s.s., but is a closely related species which is described as a new species: *Venturia orni* sp. nov. The two species distinctly differ morphologically and genetically. In contrast to *V. fraxini*, *V. orni* constantly lacks setae lining the ostiolum of the pseudothecia. In addition, the two species differ significantly and constantly in the sequences of the internal transcribed spacer (ITS) region and the genes coding for calmodulin, elongation factor EF-1α, and β-tubulin. Using the same molecular markers, another unknown endophytic *Venturia* species was discovered on *F. ornus*. However, this species never sporulated and, thus, a description of its morphology was not possible. *Venturia fraxini* and *V. orni* are host-specific and infect only *F. excelsior* and *F. ornus*, respectively, although the two hosts are sympatric at all examined sites. *Venturia orni* is abundant and exhibits high genetic and morphological variability, also in *F. ornus* planted as ornamentals outside its native range. This study indicates that *Fraxinus* spp. host an undiscovered wealth of fungal diversity.

Keywords *Venturia* · *Fusicladium* · *Fraxinus* · Fungal diversity · Ash dieback · Emerald ash borer

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Introduction

The genus *Venturia* Sacc. (Ascomycota, Venturiaceae) currently contains more than 250 species and varieties from a wide range of hosts which belong to various plant families. Most *Venturia* species are host-specific and occur only in one or a few host species, resulting from host–fungus coevolution (Beck et al. 2005; Schnabel et al. 1999). Even closely related host species can host different *Venturia* species (Tanaka and Yamamoto 1964; Ishii and Yanase 2000; Zhao et al. 2011). Most *Venturia* species colonize living leaf tissues either as endophytes or pathogens (Scholtysik et al. 2013; Ibrahim 2014; Hoffmann et al. 1985). The teleomorph is usually formed on overwintered litter from the previous year, whereas the anamorph can appear soon after leaf fall or, in the case of pathogens, already in leaf spots during the vegetation period. The teleomorphs are pseudothecia immersed in the host tissue. Many species have setae around the ostiolum (Sivanesan 1977, 1984). Several fungal genera, like *Fusicladium* Bonord., *Spilocaea* Fr., and *Pollacia* E. Bald. & Cif. have been described as anamorphs of *Venturia* (Beck et al. 2005; Schubert et al. 2013).

Only two species of *Venturia* are known to live on ash species (*Fraxinus* spp.); *Venturia fraxini* and *Fusicladium nebulosum* (Schubert et al. 2013). While *F. nebulosum* is only known from North America, *Venturia fraxini* is widely distributed in Europe, Asia, and North America. *Venturia fraxini* was first described by Aderhold (1897), and its specificity to *Fraxinus* species was recognized. Aderhold describes the teleomorph found on leaves of *F. excelsior* as mostly carrying setae around the ostiolum. Sivanesan (1977) gives a detailed description of the anamorphic and teleomorphic state of *V. fraxini*, but it is not clear if the description is based on fruit bodies on *F. excelsior*, *F. ornus*, or both. The pseudothecia are described as having setae around the

ostiolum. Schubert et al. (2013) defined *Fusicladium fraxini* on leaves of *Fraxinus ornus* as the neotype of *V. fraxini*, because the original type material of Aderhold (1897) is not preserved, and observed that the conidiogenous cells of *F. fraxini* possess both percurrent and sympodial proliferations and, consequently, considered the fungus to be intermediate between *Fusicladium* s.str. and *Spilocaea*.

Ibrahim (2014) discovered that the DNA sequence of the internal transcribed spacer (ITS) region of *Venturia* isolates from *Fraxinus ornus* differs constantly at three base pairs from that of *Venturia fraxini* deposited in GenBank. Examination of pseudothecia on old leaves showed that there are no setae around the ostiolum, increasing doubt about the conspecificity of the *Venturia* on *Fraxinus ornus* and *F. excelsior*. To test conspecificity, the morphology and sequence information at four different molecular markers (ITS region, calmodulin, elongation factor EF-1α, and β-tubulin) of the two putative species were compared.

Materials and methods

Fungal isolates

Isolates were obtained from pseudothecia on the previous year's leaf litter of *Fraxinus excelsior* and *F. ornus*, as well as from endophytic mycelia from healthy green leaves of both host species (Table 1). All isolates originate from stands with both *F. excelsior* and *F. ornus*.

Leaf litter isolates

Leaf litter was collected in spring 2015 at three sites (Table 1), screened for the presence of pseudothecia, incubated at room temperature in wet chambers to induce and/or improve sporulation, and examined every few days for mature pseudothecia and conidiophores. Single-ascospore cultures were obtained as follows. Small pieces of leaf tissue containing pseudothecia were excised from the leaflets and laid on small blocks (4 × 4 × 4 mm) of water agar. These agar blocks were placed on the inner side of the lid of a Petri dish containing 2 % (w/v) terramycine malt extract agar (TMA; 20 g L^{-1} malt extract, 15 g L^{-1} agar, 50 mg L^{-1} terramycine). The Petri dish was incubated upside down, i.e., with the lid serving as the bottom of the dish, to collect the ejected ascospores on the surface of the TMA. The lid of the Petri dish was moved twice a day for approximately 5–7 mm in the clockwise direction, so that the leaf pieces were pointing to a new spot on the agar. Single ascospores that laid far removed from other ascospores were transferred to new Petri dishes containing TMA.

Endophytic isolates

In the years 2011 to 2015, fresh leaves of *Fraxinus excelsior* and *F. ornus* from different parts of Switzerland and northern Italy were collected (Table 1). Some locations in Switzerland and the ones in northern Italy are situated south of the Alps, while all the other locations in Switzerland are situated north of the Alps. *Fraxinus excelsior* is native to all the locations, while *F. ornus* is native only to the locations south of the Alps (Info Flora 2015). The *F. ornus* trees in northern Switzerland are all planted in gardens and parks as ornamentals. The sample sites are heterogeneous and include managed and unmanaged forests, as well as parks.

For surface-sterilization, leaflets and petioles were soaked in 70 % ethanol for 1 min, followed by immersions in sodium hypochlorite (NaClO, 4 % available chlorine) for 3 min, autoclaved water for 1 min and 70 % ethanol for 30 s. Then, leaflets and petioles were left to dry for several minutes before 3-mm segments were excised from the petioles and laid out in Petri dishes (90 mm) containing TMA. Leaf disks of 7 mm diameter were punched out from the leaflets with a cork borer and transferred to TMA as well. The Petri dishes were incubated at room temperature and examined for mycelia emerging from the plant tissues every few days. Mycelia of *Venturia* species, recognized by their slow-growing heavily melanized colonies, were transferred to 2 % (w/v) malt extract agar (MEA; 20 g L^{-1} malt extract, 15 g L^{-1} agar) to obtain pure cultures.

Morphological characterization

Hand sections of fruit bodies from leaf litter and from colonies on agar media (MEA and TMA) were prepared under a dissection microscope (B3, Wild, Heerbrugg, Switzerland) using a razor blade. Conidiophores were removed from leaf litter and from colonies on agar media using forceps. Hand sections and conidiophores were viewed in tap water or concentrated lactic acid. A Zeiss Axiophot Microscope (Carl Zeiss, Oberkochen, Germany) equipped with phase contrast and interference contrast optics was used to study micromorphology. Spore dimensions were measured in tap water at 1000× magnification.

The growth rates of four *Venturia* isolates each from *F. excelsior* and *F. orni* were measured in Petri dishes (90 mm) containing 24 mL of corn meal agar (CMA; Sigma-Aldrich, Steinheim, Germany, pH 6.0 at 25 °C, concentration 17 g L^{-1}). Colonized agar disks (5 mm diameter) from the margin of 20-day-old colonies on MEA were placed in the middle of the CMA dishes and incubated in darkness at 25 °C. After 17 days, the colony diameters were measured for the first time. Radial growth of the culture was measured every 3.5 days for a period of 28 days and always along the same diameter line, giving two measurements per colony at a time.

Table 1 Strains examined in this study

Strain number	CBS number[d]	*Venturia* species	Host	Geographic origin	Country	Coordinates		Elevation (m)
						N	E	
VO1[c]	140917	*V. orni*	*Fraxinus ornus*	Monte Caslano	Switzerland	45°57′40.5″	8°52′53.3″	446
VO2[ab]	140916	*V. orni*	*F. ornus*	Monte Caslano	Switzerland	45°57′41.7″	8°52′58.9″	443
VO3	140918	*V.* sp. 1	*F. ornus*	Monte Caslano	Switzerland	45°57′40.5″	8°52′53.3″	446
VO4[c]	140919	*V. orni*	*F. ornus*	Lago di Ledro	Italy	45°52′17″	10°44′24″	713
VO5[c]	140920	*V. orni*	*F. ornus*	Lago di Ledro	Italy	45°52′17″	10°44′24″	713
VO6	140921	*V. orni*	*F. ornus*	Lago di Ledro	Italy	45°52′17″	10°44′24″	713
VO8	140922	*V. orni*	*F. ornus*	Zürich	Switzerland	47°24′20″	8°31′49″	475
VO9	140923	*V. orni*	*F. ornus*	Zürich	Switzerland	47°24′20″	8°31′49″	475
VO10[abc]	140924	*V. orni*	*F. ornus*	Zürich	Switzerland	47°24′20″	8°31′49″	475
VO11	140925	*V. orni*	*F. ornus*	Bern	Switzerland	46°57′29″	7°30′05″	572
VO12	140926	*V. orni*	*F. ornus*	Thun	Switzerland	46°45′03″	7°38′13″	558
VO13	140927	*V. orni*	*F. ornus*	Thun	Switzerland	46°44′45″	7°38′14″	559
VO14	140928	*V. orni*	*F. ornus*	Bern	Switzerland	46°57′53″	7°28′49″	561
VO15	n.a.	*V.* sp. 1	*F. ornus*	Monte Caslano	Switzerland	45°57′40.5″	8°52′53.3″	446
VO16	n.a.	*V. orni*	*F. ornus*	Monte Caslano	Switzerland	45°57′40.5″	8°52′53.3″	446
VO17	n.a.	*V. orni*	*F. ornus*	Lago di Ledro	Italy	45°52′17″	10°44′24″	713
VO18	n.a.	*V. orni*	*F. ornus*	Zürich	Switzerland	47°21′54.6″	8°29′59.8″	461
VE2	140929	*V. fraxini*	*F. excelsior*	Premia	Italy	46°16′35.9″	8°21′1.2″	734
VE4	140930	*V. fraxini*	*F. excelsior*	Spiez	Switzerland	46°41′9.2″	7°39′10.6″	609
VE5	140931	*V. fraxini*	*F. excelsior*	Zürich	Switzerland	47°22′0.4″	8°28′24.9″	629
VE6[a]	140932	*V. fraxini*	*F. excelsior*	Zürich	Switzerland	47°22′0.4″	8°28′24.9″	629
VE7	140933	*V. fraxini*	*F. excelsior*	Zürich	Switzerland	47°22′0.4″	8°28′24.9″	629
VE8	140934	*V. fraxini*	*F. excelsior*	Romont	Switzerland	47°10′48″	7°18′27.5″	954
VE9	n.a.	*V.* sp. 2	*Acer pseudoplatanus*	Romont	Switzerland	47°10′48″	7°18′27.5″	954
VE10	n.a.	*V.* sp. 2	*A. pseudoplatanus*	Premia	Italy	46°16′35.9″	8°21′1.2″	734
VE11	n.a.	*V. fraxini*	*F. excelsior*	Hammerrain	Switzerland	47°16′55.5″	7°33′13.1″	632
VE12[c]	140935	*V. fraxini*	*F. excelsior*	Monte Caslano	Switzerland	45°57′40.5″	8°52′53.3″	446
VE14	n.a.	*V. fraxini*	*F. excelsior*	Lago di Ledro	Italy	45°52′41″	10°45′48″	681
VE15	n.a.	*V. fraxini*	*F. excelsior*	Monte Caslano	Switzerland	45°57′40.5″	8°52′53.3″	446
VE16	n.a.	*V. fraxini*	*F. excelsior*	Faido	Switzerland	46°28′19″	8°48′27″	680
VE17	n.a.	*V. fraxini*	*F. excelsior*	Hammerrain	Switzerland	47°16′55.5″	7°33′13.1″	632
VE18	n.a.	*V. fraxini*	*F. excelsior*	Silenen	Switzerland	46°47′41″	8°39′51″	497
VE19	n.a.	*V. fraxini*	*F. excelsior*	Faido	Switzerland	46°28′19″	8°48′27″	680
VE20	n.a.	*V. fraxini*	*F. excelsior*	Romont	Switzerland	47°10′48″	7°18′27.5″	954

Table 1 (continued)

Strain number	Managed (M)/unmanaged (U) forest/park (P)	North (N)/ south (S) of the Alps	GenBank accession numbers				Collection date	Collector
			ITS	β-Tubulin	Elongation factor EF-1α	Calmodulin		
VO1[c]	U	S	KT823562	KT823528	KT823596	KT823630	9/5/2013	M. Ibrahim, T. Sieber
VO2[ab]	U	S	KT823567	KT823533	KT823601	KT823635	3/8/2015	T. Sieber
VO3	U	S	KT823544	KT823510	KT823578	KT823612	9/5/2013	M. Ibrahim, T. Sieber
VO4[c]	M	S	KT823568	KT823534	KT823602	KT823636	9/3/2013	M. Ibrahim, T. Sieber
VO5[c]	M	S	KT823565	KT823531	KT823599	KT823633	9/3/2013	M. Ibrahim, T. Sieber
VO6	M	S	KT823566	KT823532	KT823600	KT823634	9/3/2013	M. Ibrahim, T. Sieber
VO8	P	N	KT823569	KT823535	KT823603	KT823637	9/13/2013	M. Ibrahim, T. Sieber
VO9	P	N	KT823561	KT823527	KT823595	KT823629	9/13/2013	M. Ibrahim, T. Sieber
VO10[abc]	P	N	KT823564	KT823530	KT823598	KT823632	5/4/2015	M. Schlegel
VO11	P	N	KT823573	KT823539	KT823607	KT823641	9/11/2013	M. Ibrahim, T. Sieber
VO12	P	N	KT823574	KT823540	KT823608	KT823642	9/15/2013	M. Ibrahim, T. Sieber
VO13	P	N	KT823563	KT823529	KT823597	KT823631	9/15/2013	M. Ibrahim, T. Sieber
VO14	P	N	KT823572	KT823538	KT823606	KT823640	9/11/2013	M. Ibrahim, T. Sieber
VO15	U	S	KT823545	KT823511	KT823579	KT823613	9/5/2015	M. Ibrahim, T. Sieber
VO16	U	S	KT823570	KT823536	KT823604	KT823638	9/5/2015	M. Ibrahim, T. Sieber
VO17	M	S	KT823575	KT823541	KT823609	KT823643	9/3/2013	M. Ibrahim, T. Sieber
VO18	P	N	KT823571	KT823537	KT823605	KT823639	9/18/2013	M. Ibrahim, T. Sieber
VE2	U	S	KT823546	KT823512	KT823580	KT823614	8/31/2013	M. Schlegel, B. Villain
VE4	M	N	KT823548	KT823514	KT823582	KT823616	8/31/2013	M. Schlegel, B. Villain
VE5	M	N	KT823555	KT823521	KT823589	KT823623	7/5/2014	M. Schlegel
VE6[a]	M	N	KT823556	KT823522	KT823590	KT823624	4/13/2015	M. Schlegel
VE7	M	N	KT823553	KT823519	KT823587	KT823621	7/5/2014	M. Schlegel
VE8	M	N	KT823558	KT823524	KT823592	KT823626	8/28/2013	V. Queloz
VE9	M	N	KT823576	KT823542	KT823610	KT823644	8/28/2013	V. Queloz
VE10	U	S	KT823577	KT823543	KT823611	KT823645	8/31/2013	M. Schlegel, B. Villain
VE11	M	N	KT823557	KT823523	KT823591	KT823625	10/9/2011	V. Queloz
VE12[c]	U	S	KT823554	KT823520	KT823588	KT823622	9/5/2015	M. Ibrahim, T. Sieber
VE14	M	S	KT823550	KT823516	KT823584	KT823618	9/3/2013	M. Ibrahim, T. Sieber
VE15	U	S	KT823549	KT823515	KT823583	KT823617	9/5/2015	M. Ibrahim, T. Sieber
VE16	M	S	KT823547	KT823513	KT823581	KT823615	8/26/2013	T. Sieber, B. Villain
VE17	M	N	KT823560	KT823526	KT823594	KT823628	10/9/2011	V. Queloz
VE18	M	N	KT823552	KT823518	KT823586	KT823620	8/26/2013	T. Sieber, B. Villain
VE19	M	S	KT823559	KT823525	KT823593	KT823627	8/26/2013	T. Sieber, B. Villain
VE20	M	N	KT823551	KT823517	KT823585	KT823619	8/28/2013	V. Queloz

[a] Monospore isolates from leaf litter; all other strains are from endophytic mycelia

[b] Herbarium specimen (leaf litter) deposited at herbarium ZT

[c] Mature pseudothecia formed in culture; dried culture deposited at herbarium ZT

[d] n.a. = not available; strain not deposited at CBS

Growth rates were determined as the mean daily mycelial growth increment.

Molecular characterization

Mycelia were transferred to fresh MEA and incubated at 20 °C for 20 days before DNA extraction. DNA was extracted using the NucleoSpin® 96 Plant II Kit (MACHEREY-NAGEL, Düren, Germany), according to the manufacturer's protocol. Polymerase chain reaction (PCR) amplification of the ITS region was done using the BITS (Bokulich and Mills 2013) and ITS4 (White et al. 1990) primers (Table 2). For the calmodulin, elongation factor EF-1α, and β-tubulin genes, new primers were designed based on sequence information from closely related species available in GenBank. For all primer pairs, amplification was done in a reaction volume of 10 μL, containing approx. 3 ng of DNA, 2 μL 5× reaction buffer, 0.2 mM dNTPs, 0.5 μM of forward and reverse primers, and 0.5 U GoTaq polymerase (Promega, Dübendorf, Switzerland). The PCR program included an initial denaturation at 95 °C for 2 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 53 °C, 80 s at 72 °C, and a final extension step of 5 min at 72 °C. Because some ITS amplifications did not work the first time, PCR was repeated with primers ITS1 (White et al. 1990) and ITS4, and a raised annealing temperature of 60 °C. The PCR products were purified using 0.5 μL Exonuclease I (Exo I, 20 U/μL) and 1 μL FastSAP (1 U/μL) from Thermo Fisher Scientific (Reinach, Switzerland). The reactions were incubated at 37 °C for 15 min and denatured at 80 °C for 15 min. Sequencing was done by Microsynth (Balgach, Switzerland). The sequences were compared with sequences deposited in GenBank using nucleotide BLAST (Altschul et al. 1990).

Phylogenetic analyses

Six multiple sequence alignments were prepared: one for each of the four sequence markers (ITS, calmodulin, elongation factor EF-1α, and β-tubulin), one using the concatenated sequences of the four markers, and one using the ITS sequences of a selection of strains from this study and ten additional sequences from closely related *Venturia* species published in GenBank (Beck et al. 2005). Multiple sequence alignments were made with MAFFT v7.017b (Katoh and Standley 2013) using the E-INS-i algorithm and examined by eye in Geneious v6.1.3 (Biomatters Ltd., Auckland, New Zealand). Minor corrections were performed manually. Ambiguously aligned sites were removed systematically with Gblocks web v0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; accessed on July 28, 2015) using the following settings: "less stringent selection", "allow smaller final blocks", "allow gap positions within final blocks", and "allow less strict flanking positions". RAxML v.7.7.7 (Stamatakis 2006) was used for maximum likelihood (ML) phylogenetic analyses and MrBayes v.3.2.2 (Ronquist et al. 2012) for phylogenies based on Bayesian inference (BI), according to Gross et al. (2015). PartitionFinder v1.1.0 (Lanfear et al. 2012) was used to determine the optimal substitution model for each sequence marker in the combined alignment. The program was run using the "greedy" search algorithm with "linked" branch lengths. The model was chosen based on the Bayesian information criterion (BIC), restricting the selection to the models accepted by RAxML and MrBayes, respectively. The same was done with the ITS phylogeny of *Venturia* spp., but without specifying any data blocks. RAxML was run using the GTR + I + G model for the combined alignment and GTR + G for the ITS dataset. It included 1000 initial independent ML inferences from the alignment file; the tree with the best likelihood score was chosen to calculate support values based on 1000 bootstrap replicates. Analyses in MrBayes comprised two parallel runs with four MCMC chains using the GTR + G model for the combined alignment and K80 + I for the ITS dataset. The burn-in period was set to 25 % of the total number of generations and trees were sampled every 500 generations. The calculation was stopped when the average standard deviation of split frequencies dropped below 0.01. Tree topologies were

Table 2 Primers used for ITS, calmodulin, elongation factor EF-1α, and β-tubulin genes

Name	Primer sequences (5′-3′)	Target locus	Published by
BITS	F: ACCTGCGGARGGATCA	Internal transcribed spacer 1 and 2 of the rDNA	Bokulich and Mills (2013)
ITS1	F: TCCGTAGGTGAACCTGCGG	Internal transcribed spacer 1 and 2 of the rDNA	White et al. (1990)
ITS4	R: TCCTCCGCTTATTGATATGC	Internal transcribed spacer 1 and 2 of the rDNA	White et al. (1990)
Vo_Calmod_F	F: GCARGTCTCCGAGTTCAA	Calmodulin gene (partial)	Newly designed
Vo_Calmod_R	R: GAGATGAATCCGTTGTTGTC	Calmodulin gene (partial)	Newly designed
Vo_EF1_F	F: TGTTGATGGCGACGATGAGC	Elongation factor 1-α gene (partial)	Newly designed
Vo_EF1_R	R: GACAARCTMAAGGCYGAGC	Elongation factor 1-α gene (partial)	Newly designed
Vo_Beta_F	F: GGTAACCAAATTGGTGCTGCCTTC	β-Tubulin gene (partial)	Newly designed
Vo_Beta_R	R: CTGATGAACGGACAAAGTGG	β-Tubulin gene (partial)	Newly designed

visualized using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/). Alignments and trees generated in this study were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S18387).

Results

Venturia orni **M. Ibrahim, M. Schlegel & T.N. Sieber, spec. nov. (Figs. 1, 2, and 3)**

Mycobank: MB814507

Etymology: The epithet refers to the host of this fungus, *Fraxinus ornus*.

Teleomorph:

Pseudothecia scattered over the entire leaf surface, immersed, globose to subglobose 95–150 μm (average: 115 μm) high and 75–155 μm (average: 120 μm) wide, pseudoparaphysate, ostiolate, papillate but without setae (Fig. 1a). *Peridium* 15–20 μm wide, composed of (2–)3–4 layers of pigmented cells of textura angularis, cells 5–15 μm diam., cell wall 0.8–1.2 μm thick, cells surrounding ostiolum more heavily melanized (Fig. 1a). *Asci* bitunicate, oblong to obclavate, with a short pedicel, 8-spored (rarely 4-spored), in nature (31)39–47 × 9.5–10.5(13.0) μm (average: 43.4 × 10.0 μm) (Fig. 1b, c), in culture on average significantly longer 50–70(76) × 9.0–11.5 μm (average: 58.0 × 10.5 μm) (Fig. 1d, e, f). *Ascospores* uniseriate, partially overlapping to biseriate, especially at the base, ellipsoidal, with broadly rounded ends, pale brown, 1-septate, slightly constricted at the septum, the upper cell shorter than the lower one (4:5–2:3), smooth-walled, measuring 9.0–15.5 × 4.0–6.0 μm (average: 12.0 × 5.0 μm) (Fig. 1g), length but not width of ascospores variable and depending on the collection (Fig. 3).

Anamorph:

Conidiophores Fusicladium-like, arising in clusters (sporodochia) from erumpent subcuticular to intraepidermal, few-celled stromata, erect, unbranched, geniculate, septate, dark brown, smooth, walls thickened, 55–195 μm long (average length: 145 μm) and 7.0–8.0 μm wide at the base and 4.0–5.0 μm at the tip (Fig. 2a, b, d). Sporodochia interconnected

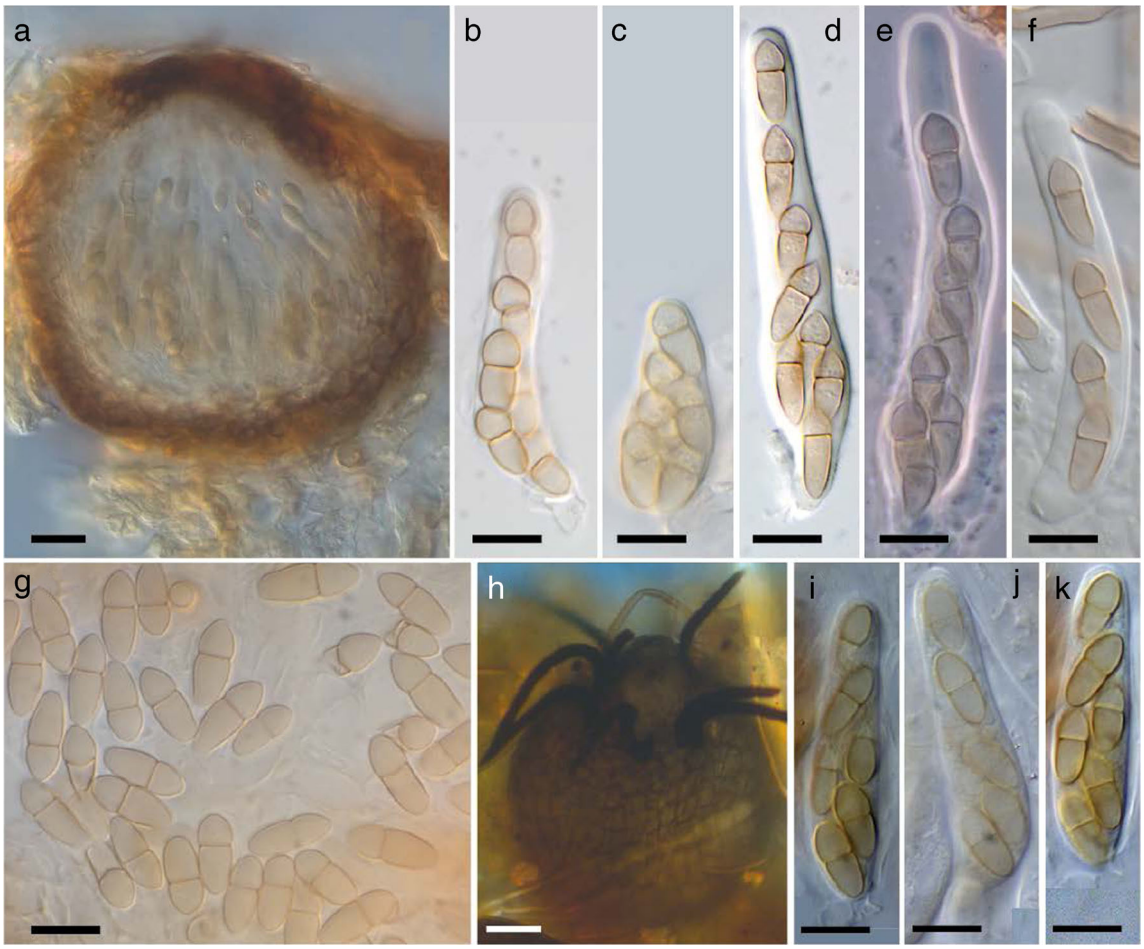

Fig. 1 Morphology of the teleomorphs of *Venturia orni* (**a**–**g**) and *V. fraxini* (**h**–**k**): **a** pseudothecium, **b**, **c** ascus and ascospores from pseudothecia in leaf litter, **d**–**f** asci with ascospores from pseudothecia formed in culture; **g** ascospores; **h** pseudothecium with setae lining the ostiolum; **i**–**k** asci with ascospores from pseudothecia formed in leaf litter. Scale bars: 20 μm in **a** and **h**, and 10 μm in **b**–**g** and **i**–**k**

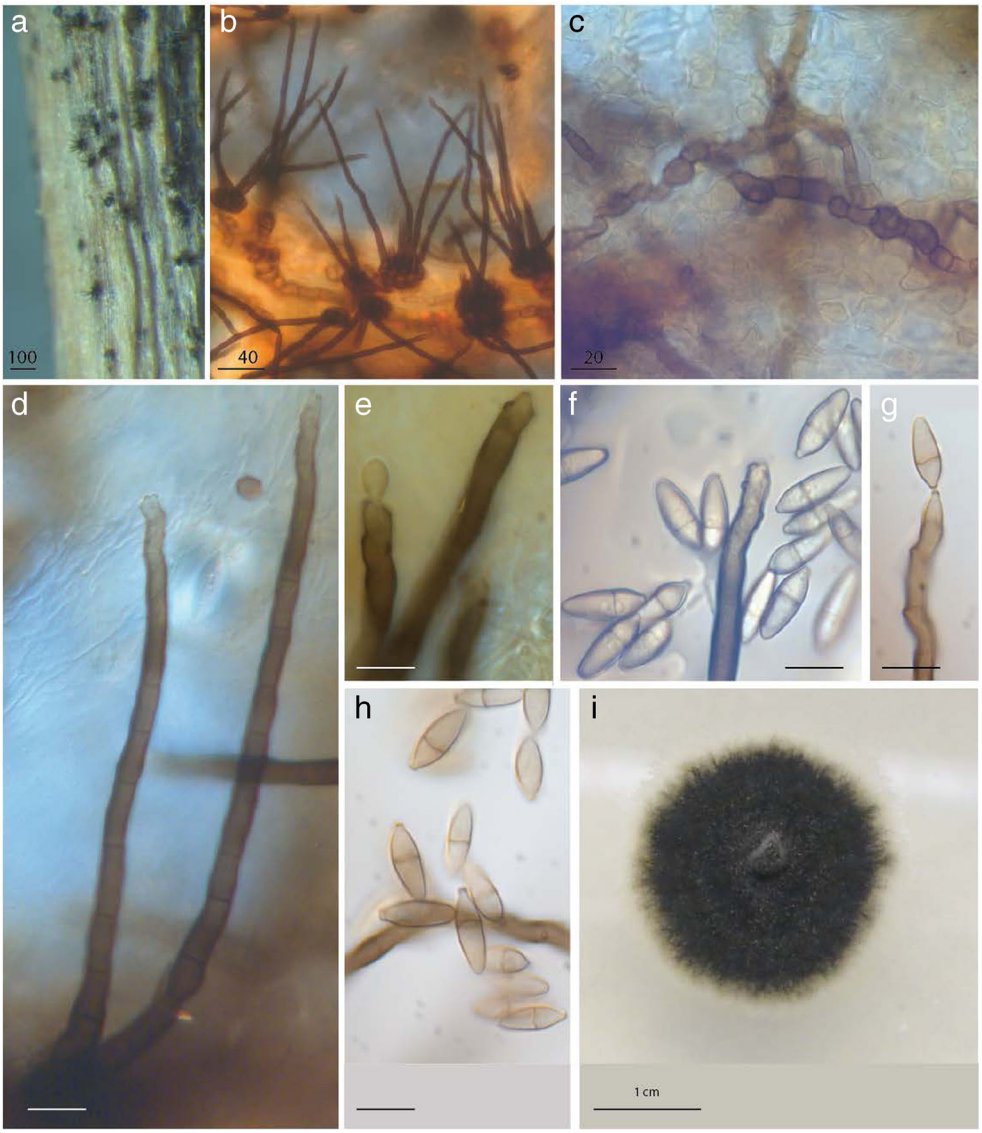

Fig. 2 Morphology of the anamorph of *Venturia orni* and vegetative structures on litter leaves: **a** sporodochia on a leaflet petiole; **b** sporodochia on a leaflet vein; **c** subcuticular to intraepidermal melanized mycelium with intercalar chlamydospores; **d** unbranched, septate conidiophores; **e** conidiogenous cells with sympodial proliferation and refractive conidiogenous loci; **f** conidiogenous cell with truncate conidiogenous loci and fusiform two-celled conidia with truncate base; **g** conidiogenous cell with dentate conidiogenous loci and detaching fusiform two-celled conidium; **h** 0-1-septate conidia; **i** colony of holotype on CMA after three weeks at 25 °C in darkness. Scale bars: 100 μm in **a**, 40 μm in **b**, 20 μm in **c**, 10 μm in **d**–**h**, and 1 cm in **i**

by subcuticular to intraepidermal mycelium of melanized, partly swollen, short, cells and intercalar chlamydospores (Fig. 2b, c). *Conidiogenous cells* terminal, geniculate, proliferation sympodial, with several truncate 1.0–1.3 μm wide loci, somewhat refractive or darkened (Fig. 2d, e, f, g). *Conidia* solitary, smooth, 11.0–14.3 × 3.7–5.6 μm (average: 12.7 × 4.6 μm), lanceolate but apical tip rounded, 0–1-septate, pale medium brown, with a truncate base (Fig. 2f, h).

Cultural characteristics on CMA: slow growing, growth rate 0.50 mm d^{-1} at 25 °C in the dark, moderate aerial mycelium and regular, but fringed margins, surface olive black, reverse dark black (Fig. 2i).

Distribution and host:

Switzerland and Italy in leaves of *Fraxinus ornus*.

Holotype: − Switzerland, Zürich, cemetery Nordheim, 475 m, 47°24′20″N, 8°31′49″E, leaf litter of *Fraxinus ornus* and dried culture of monospore isolate, 4–V–2015, leg. M. Schlegel. Culture VO10 (single spore isolate), CBS 140924 (MS_150504.1; ZT Myc 55333)

Other collections: − Italy, Lago di Ledro, 45°52′17″ N, 10°44′24″E, 713 m, dried culture of endophytic isolate of *Fraxinus ornus* leaf, 03–IX–2013, leg. M. Ibrahim. Culture VO4, CBS 140919 (ZT Myc 55330) – Italy, Lago di Ledro, 45°52′17″N, 10°44′24″E, 713 m, dried culture of endophytic isolate of *Fraxinus ornus* leaf, 03–IX–2013, leg. M. Ibrahim. Culture VO5, CBS 140920 (ZT Myc 55331) – Switzerland, Caslano, Monte Caslano, 45°57′41.7″N, 8°52′58.9″E, 443 m, leaf litter of *Fraxinus ornus*, 08–III–2015, leg. T. N. Sieber (TS_150308.1; ZT Myc 55328) – Switzerland, Caslano, Monte Caslano, 45°57′41.7″N, 8°52′58.9″E, 443 m, leaf litter of *Fraxinus ornus*, 08–III–2015, leg. T. N. Sieber.

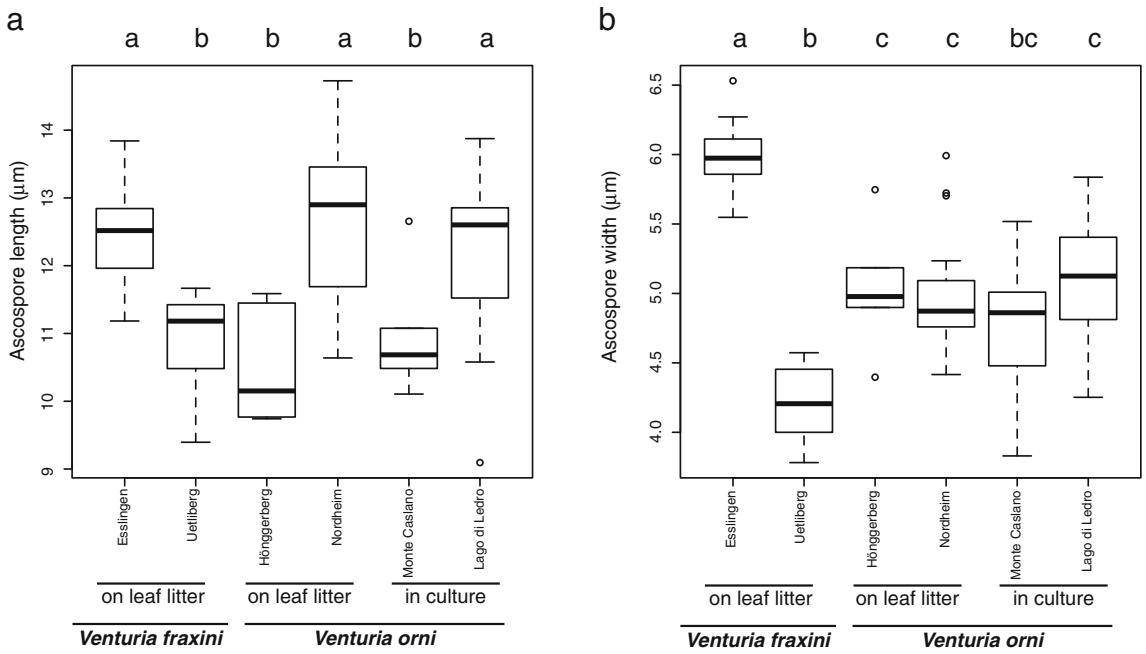

Fig. 3 Ascospore dimensions of *Venturia fraxini* and *V. orni* from various collection sites. **a** Ascospore length. **b** Ascospore width. Different letters at the top of the graphs indicate significant differences between collections (Tukey's HSD)

Culture VO2 (single spore isolate), CBS 140916 (TS_150308.2; ZT Myc 55329) – Switzerland, Caslano, Monte Caslano, 45°57′40.5″N, 8°52′53.3″E, 446 m, dried culture of endophytic isolate of *Fraxinus ornus* leaf, 05–IX–2013, leg. M. Ibrahim. Culture VO1, CBS 140917 (ZT Myc 55332) – Switzerland, Zürich, cemetery Hönggerberg, 47°24′33.5″N, 8°29′33″E, 475 m, leaf litter of *Fraxinus ornus*, 15–III–2015, leg. O. Holdenrieder. (OH_150315.1; ZT Myc 55327) – Switzerland, Zürich, cemetery Hönggerberg, 47°24′ 33.5″N, 8°29′33″E, 475 m, leaf litter of *Fraxinus ornus*, 4–V–2014, leg. O. Holdenrieder. (OH_140504.1; ZT Myc 55326) – Switzerland, Zürich, ETH Hönggerberg, near pond, 47°24′33.32″N, 8°30′37.37″E, 526 m, leaf litter of *Fraxinus ornus*, 18–V–2014, leg. O. Holdenrieder. (OH_140518.1; ZT Myc 55325), anamorph only.

Diagnosis:

Morphologically, *V. orni* differs from its closest relative *V. fraxini* by the absence of setae (length of setae: 36–80 µm) (Fig. 1h). A *Spilocaea* anamorph has never been observed in either *V. orni* or *V. fraxini*. Ascospore dimensions vary strongly among collections in both species and are, thus, not useful to distinguish the two species (Fig. 3). Likewise, ascus dimensions vary among collections within and among the two *Venturia* species (Fig. 1b–f and i–k). *Venturia orni* further differs from *V. fraxini* by its slightly slower growth on CMA at 25 °C: growth rate of *V. orni* 0.50 mmd^{-1} versus 0.54 mmd^{-1} of *V. fraxini*.

Phylogenetic analyses

The alignment lengths of the different markers were as follows (GBlocks had not been used due to no ambiguously aligned sites): ITS 449 bp; β-tubulin 621 bp, elongation factor EF-1α 257 bp; calmodulin 507 bp. The combined alignment was 1834 bp long. Bootstrap support values for the dichotomy of *Venturia fraxini* and *V. orni* on the single-gene trees were high and tree topologies congruent, clearly separating the two species. Similarly, the probability for the dichotomy of the two species was 100 % independently of whether maximum likelihood (ML) or Bayesian inference (BI) was used for phylogenetic analysis of the concatenated dataset (Fig. 4). Interestingly, two strains from *F. ornus* collected on Monte Caslano represented another unknown *Venturia* or *Fusicladium* species and were positioned basal to the *V. orni*/*V. fraxini* clade in the phylogenetic trees (Figs. 4 and 5). Similarly, another unidentified *Venturia* species was discovered on *Acer pseudoplatanus*. The ITS sequence of this species most closely matched (96.2 % similarity) sequences of *Venturia tremulae* (AY671910) and *V. inaequalis* (AF065838), whereas the similarity with *V. aceris* (EU035445) from the same host was only 93 %. In the ITS tree, the clade containing *V. orni*, *V. fraxini*, and the unknown species was positioned far removed from the other

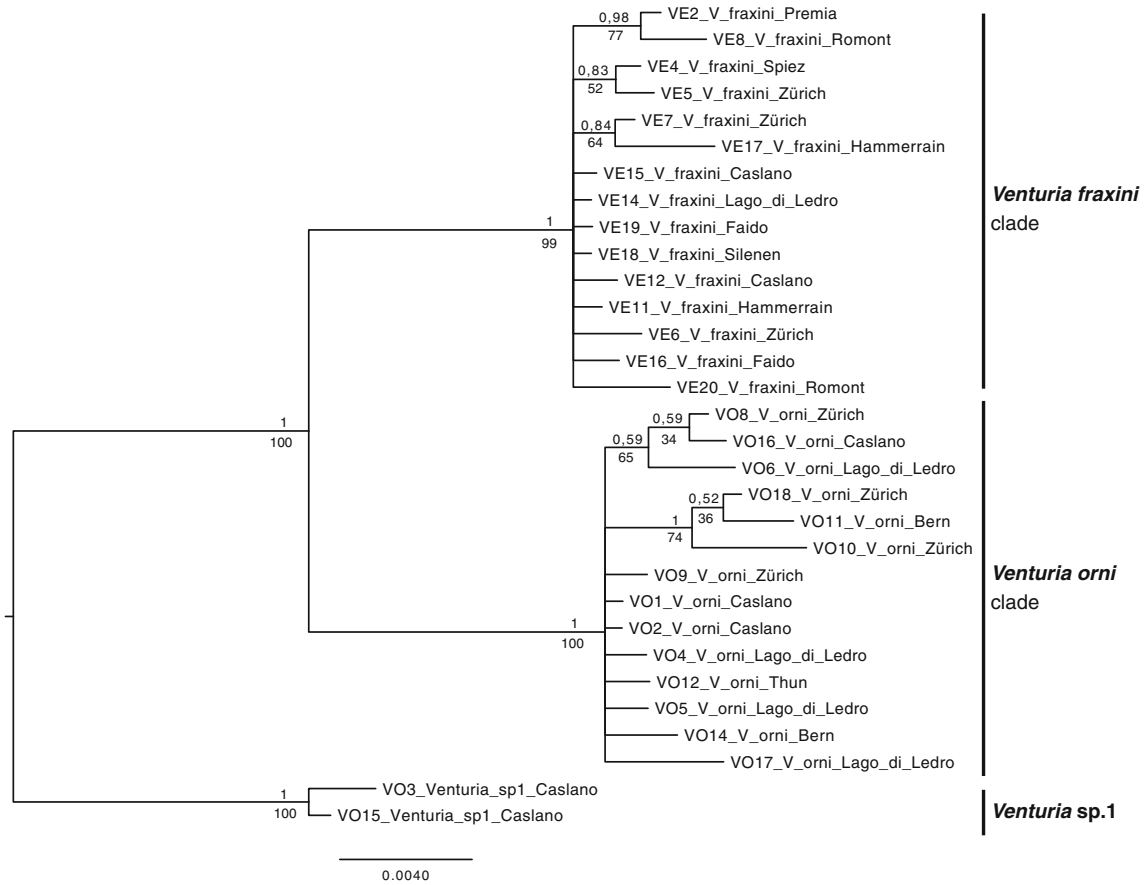

Fig. 4 Consensus phylogram (50 % majority rule) resulting from a Bayesian analysis (BI) of the alignment of the concatenated sequences of the four genes ITS rDNA, β-tubulin, EF1-α, and calmodulin using MrBayes v.3.2.2. Bayesian posterior probabilities of the BI analysis are indicated above branches, whereas bootstrap percentages ($n = 1000$) of the maximum likelihood (ML) phylogenetic analyses using RAxML v.7.7.7 are given below branches. The scale bar represents the number of substitutions per site

Venturiaceae (Fig. 5). *Venturia pyrina* and *V. nashicola*, both from pear (*Pyrus*), *V. populina* and *V. inopina*, both from *Populus*, *V. cerasi* and *V. asperata*, both from rosaceous fruit trees (*Prunus* and *Malus*, respectively), and the unknown species from *Acer pseudoplatanus* formed distinct clades supported by high bootstrap values. In contrast, bootstrap support was very low for all the other taxa (Fig. 5).

Discussion

While all investigated pseudothecia of *Venturia fraxini* had setae lining the ostiolum, pseudothecia of *V. orni* always lacked them. The absence of setae made it difficult to detect pseudothecia of *V. orni* among the fruit bodies of other leaf-colonizing fungi (e.g., *Mycosphaerella* spp.), whereas pseudothecia of *V. fraxini* were easier to spot due to the presence of setae. The majority of *Venturia* species have setae, but there are also species which sometimes or always lack them (Sivanesan 1977). Aderhold (1897) describes *V. fraxini* as mostly carrying setae. Sivanesan (1977) just states that it has setae. The lack of setae seems to be the only, but quite clear, morphological characteristic to distinguish *V. orni* from *V. fraxini*. Both species possess a *Fusicladium* anamorph which did not, however, provide any useful features to differentiate the two species neither on host material nor in culture. According to Schubert et al. (2013), the conidiogeneous cells of *V. fraxini* can proliferate percurrently (*Fusicladium* state) and sympodially (*Spilocaea* state). However, we never observed the *Spilocaea* conidial state in either species.

Venturia orni is clearly distinct from *V. fraxini* in regards to the four sequence markers examined in this study, further confirming the species status of *V. orni*. *Fraxinus excelsior* is a very common native tree in Switzerland and northern Italy, whereas *F. ornus* is native to southern Switzerland, Italy, and the eastern Mediterranean region. In northern Switzerland, *F. ornus* is planted as an ornamental tree. As a consequence of global warming, natural regeneration of *F. ornus* north of the Alps has already been observed (Bomble 2015). All the samples examined in this study

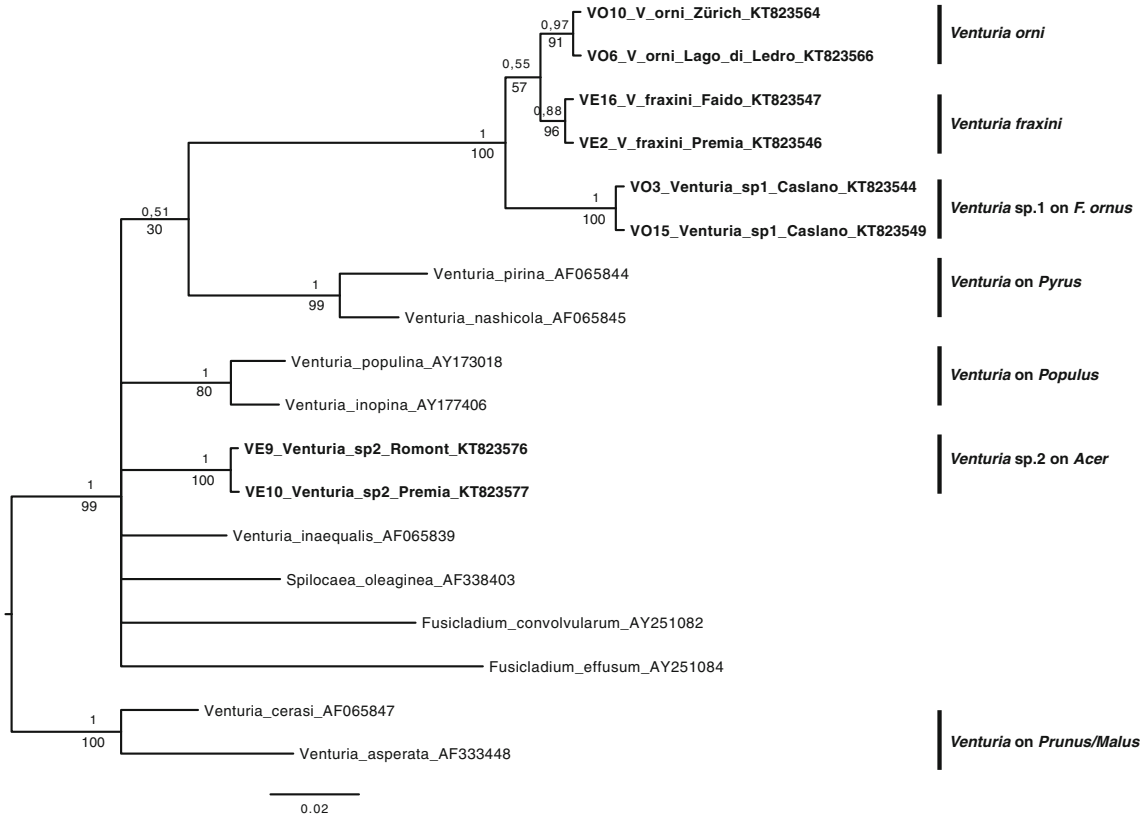

Fig. 5 Consensus phylogram (50 % majority rule) resulting from a Bayesian analysis (BI) of the ITS sequence alignment of the *Venturia orni* group and other members of the Venturiaceae using MrBayes v.3.2.2. Bayesian posterior probabilities of the BI analysis are indicated above branches, whereas bootstrap percentages ($n = 1000$) of the maximum likelihood (ML) phylogenetic analyses using RAxML v.7.7.7 are given below branches. *Venturia cerasi* and *V. asperata* served as outgroup. Taxa printed in bold were sequenced during this study. The scale bar represents the number of substitutions per site

originated from sites with both *F. ornus* and *F. excelsior*. No cross-infections were observed. *Venturia orni* was found only on *F. ornus* and *V. fraxini* only on *F. excelsior*, i.e., the two species seem to be strictly host-specific. Similarly, preliminary cross-infection experiments in a climate chamber confirmed host specificity (M. Schlegel, unpublished). Since *F. ornus* is not native to the northern side of the Alps, it is surprising to find *V. orni* so abundantly at all sampled sites north of the Alps. These locations lie in the cities and agglomerations of Zürich, Bern, and Thun. The non-native trees are obviously capable of maintaining a population of a host-specific fungus. It is not clear how *V. orni* crossed the Alps. Maybe it was introduced together with its host. Similarly, *V. fraxini* had been introduced together with *F. excelsior* into New Zealand (Chen 2012), and is now able to maintain itself in this new habitat. Another possibility is that *V. orni* migrated by wind to the new habitat, since both conidia and ascospores are airborne. Molecular data indicated that all eight strains examined in this study from north of the Alps represent different genotypes, i.e., they could be the result of multiple introductions. From other *Venturia* species, it is known that gene flow between populations is high, even over wide geographical distances and barriers (Kasanen et al. 2004; Tenzer et al. 1999). Alternatively, rates of recombination in *V. orni* might have been high since the fungus was first introduced on the northern side of the Alps together with its host.

Two *Venturia* isolates from *F. ornus* were closely related but clearly different from both *V. orni* and *V. fraxini* at the four examined sequence markers (Figs. 4 and 5). No closely matching sequence was found in GenBank. These strains probably represent another undescribed taxon. Since neither of the two isolates sporulated in culture, a morphological description could not be provided. The same is true for the two *Venturia* isolates from *Acer pseudoplatanus* (Fig. 5).

Venturia orni and *V. fraxini* are very closely related species, living in the leaf tissue of closely related hosts. The two *Venturia* species cannot been distinguished based on anamorph morphology. This is probably the reason why these two species had never been recognized as distinct. The literature mentions *V. fraxini* occurring on *F. excelsior*, *F. ornus*, and other *Fraxinus* species (Schubert et al. 2013; Sivanesan 1977). It is not clear if Sivanesan's (1977) morphological

description of pseudothecia is based only on the ones on *F. excelsior* or if it also includes those on *F. ornus*. It is likely that all existing descriptions of the teleomorph of *V. fraxini* are only based on pseudothecia found on leaves of *F. excelsior*. The neotype of *V. fraxini* defined by Schubert et al. (2013) on *F. ornus* very likely is not *V. fraxini*, but one of the species discovered during this study: *V. orni* or *Venturia* sp. 1. To our knowledge, the only two *Venturia*/*Fusicladium* species described from *Fraxinus* spp. are *V. fraxini* and *F. nebulosum*, the latter being known only from North America. Our study indicates that *Venturia* diversity on *Fraxinus* spp. is probably much higher than previously believed. Because all *Venturia* species on *Fraxinus* spp. were considered to be *V. fraxini* in the past, we do not know the area of distribution of *V. fraxini* and *V. orni*, although we can assume that the areas of distribution correspond with those of their hosts due to host specificity. Besides *F. excelsior* and *F. ornus*, *F. angustifolia* is another widely distributed ash tree in Europe. It will be interesting to study the *Venturia* species colonizing the leaf tissues of this host. Hashemi et al. (2014) found *V. fraxini* in Iran on *Fraxinus* sp. Conidiophores of this Iranian *V. fraxini* were described as being "conspicuously lobed at the base", indicating that they could represent another undescribed species. Some *Venturia* species on *Fraxinus* spp. may become threatened by ash dieback in Europe caused by the introduced pathogen *Hymenoscyphus fraxineus* (Gross et al. 2014; Baral et al. 2014). *Hymenoscyphus fraxineus* causes severe dieback on *F. excelsior* and *F. angustifolia*, but *F. ornus* seems to be resistant (Kirisits and Schwanda 2015). However, there are other *Fraxinus* diseases either "waiting" at the borders of Europe or already invading the continent. For example, the emerald ash borer (*Agrilus planipennis*) which is currently devastating *Fraxinus* spp. in North America was recently discovered in eastern Europe (Orlova-Bienkowskaja 2014). Thus, it is a race against time to discover the hidden diversity of mycobiota in *Fraxinus* species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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