

Contrasting phylogenetic patterns of anther smuts (Pucciniomycotina: *Microbotryum*) reflect phylogenetic patterns of their caryophyllaceous hosts

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Abstract Anther smuts in the genus *Microbotryum* often show very high host specificity toward their caryophyllaceous hosts, but some of the larger host groups such as *Dianthus* are crucially undersampled for these parasites so that the question of host specificity cannot be answered conclusively. In this study we sequenced the internal transcribed spacer (ITS) region of members of the *Microbotryum dianthorum* species complex as well as their *Dianthus* hosts. We compared phylogenetic trees of these parasites including sequences of anther smuts from other Caryophyllaceae, mainly *Silene*, with phylogenies of Caryophyllaceae that are known to harbor anther smuts. Additionally we tested whether observed patterns in parasites are due to shared ancestry or if geographic separation

is a factor that should be taken into consideration in delimitating species. Parasites on *Dianthus* showed mainly an arbitrary distribution on *Dianthus* hosts, whereas parasites on other Caryophyllaceae formed well-supported monophyletic clades that corresponded to restricted host groups. The same pattern was observed in the Caryophyllaceae studied: morphologically described *Dianthus* species did not correspond well with monophyletic clades based on molecular data, whereas other Caryophyllaceae mainly did. We suggest that these different patterns primarily result from different breeding systems and speciation times between different host groups as well as difficulties in species delimitations in the genus *Dianthus*.

Keywords Species diversification · Host specificity · Host range · *Microbotryum* · Caryophyllaceae · ITS

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Introduction

Parasitic organisms (i.e., symbionts that withdraw nutrients; sensu Hall 1974) occur in many evolutionary lineages (Hawksworth 2001; Poulin and Morand 2000) and account for much of biological diversity (Price 1980). Species richness and radiation have been mainly attributed to host shifts leading to reproductive isolation and adaption to new hosts (Zietara and Lumme 2002; Giraud et al. 2010). Even though all parasites are restricted to a certain range of hosts they infect, there are huge differences in the amount of hosts used between parasites, even in closely related organisms (De Wet et al. 2008). The range of potential hosts a parasite can use has an impact on many aspects of the parasites ecology and evolution, e.g., distribution range and fitness in a given locality. Broad host spectra enable parasite species to spread in a greater geographic range because of differences in host distributions, thereby additionally enhancing the chance for host switches (Janz and Nylin

2008). Parasites with narrow host ranges have a higher fitness on their host than multitrophic parasites occurring on the same host as they are assumed to be better adapted to their hosts (Lajeunesse and Forbes 2002).

Interestingly, phylogenetic methods have shown that host specificity seems much higher in many parasitic groups than has previously been assumed based on morphological data, but infection studies show that many parasites possess the ability to infect a multitude of hosts under artificial conditions (Poulin and Keeney 2008). This suggests that host specificity is a much more complex phenomenon than was previously assumed and involves more factors than just the co-evolution between host and parasite. One aspect of host parasite interactions that mediates this complexity is the spatial context, including geographic barriers limiting distribution of hosts and parasites. As indicated above, host specificity can influence the geographic ranges of parasite species, but geographic ranges are also shaped by life cycles and dispersal abilities of both hosts and parasites (Poulin et al. 2011). Due to differential selection of distinct populations in different areas (Thompson 2005), this complexity of factors over different spatial scales will ultimately also influence the genetic structure of the species involved. Hence, a parasites' host range and host specificity are shaped by the combination of continuously interacting spatial and evolutionary factors.

Members of the smut fungal genus *Microbotryum* Lév. are parasites on a large range of eu-dicotyledonous host plants (Vánky 1998). The different species form their spores mainly in the flowers of their hosts, but there are also species that sporulate in stems [e.g., *Microbotryum parlatoarei* (A.A. Fisch. Waldh.) Vánky] or leaves [e.g., *Microbotryum marginale* (DC.) Vánky]. Besides a great host spectrum within the genus, the most well-known *Microbotryum* species are the anther smuts of the Caryophyllaceae that infect different species of the genera *Dianthus* L., *Silene* L., *Saponaria* L., *Stellaria* L. and other members of that plant family. Many caryophyllaceous host genera, except for *Silene*, seem to have been colonized only once with subsequent radiations within these groups (Le Gac et al. 2007; Lutz et al. 2005, 2008). Traditionally, species delimitations in the caryophyllaceous anther smuts have relied on the host plant (Fig. 1a), spore morphology (Fig. 1b) and spore color, but have been debated for a long time (Liro 1924; Lindeberg 1959). Recent molecular studies have demonstrated very high host specificities in the parasites of studied *Silene* species (Le Gac et al. 2007; Lutz et al. 2005, 2008), and within this group a one-to-one relationship between parasites and hosts is a common finding, e.g., *Microbotryum chloranthae-verrucosum* M. Lutz, Göker, Piątek, Kemler, Begerow & Oberw. on *Silene chlorantha* Hort ex Fenzl (Lutz et al. 2005).

Anther smuts on species of *Dianthus* seem to be separated into several groups as well (Le Gac et al. 2007; Lutz et al.

2005, 2008), and recently new species have been proposed (Denchev et al. 2009), but these studies also indicate that host specificity of the *Dianthus* parasites may be rather low compared to other anther smuts. Thereby, the number of parasite specimens on different *Dianthus* species considered in previous studies has not been very extensive, making it difficult to assess whether this pattern of low host specificity is common in anther parasites of *Dianthus* or these observations are sampling artifacts. As low taxon sampling may have serious effects on phylogenetic errors (Hillis 1996, 1998), the intention of this study was to infer relationships between parasites on different *Dianthus* species based on a larger taxon sampling and to compare the phylogenetic pattern obtained with the pattern from other Caryophyllaceae, mainly from *Silene*, parasites. Variability of the internal transcribed spacer nrDNA (nrITS including internal transcribed spacers 1 and 2 as well as the 5.8S gene) sequences was analyzed in both parasites and hosts using phylogenetic approaches; additionally host specificity and geographical distribution were examined. This was done to answer three questions: (1) Is low host specificity in *Dianthus* parasites common or are previous observations artifacts of a sampling bias? (2) Is there a correlation between host specificity and geographic distribution in *Dianthus* and *Silene* parasites? (3) Is there a correlation between genetic variation of parasites of *Dianthus* and other Caryophyllaceae, mainly *Silene*, with host genetic variation?

Materials and methods

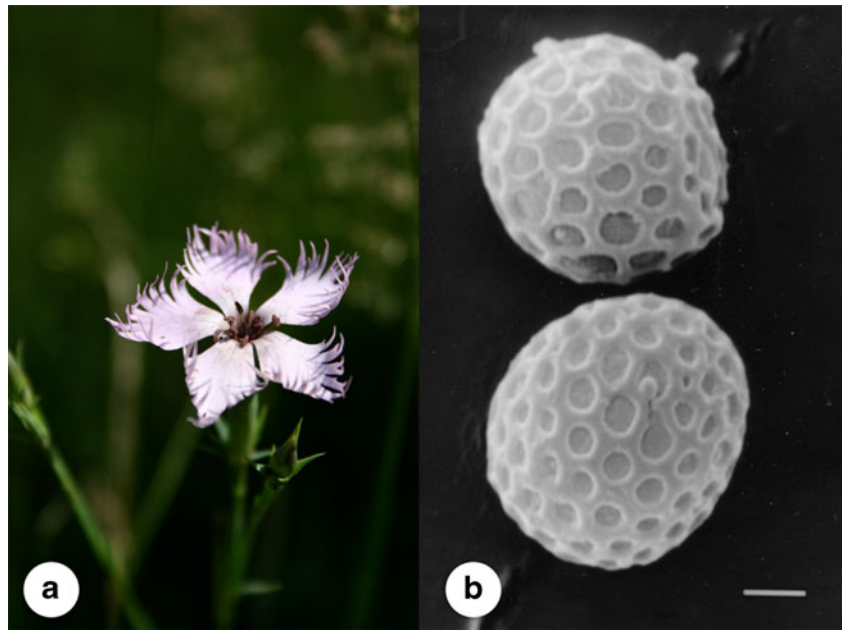
Taxonomy of *Microbotryum* and *Dianthus*

The DNA sequences from the fungal specimens of this study are listed in Table 1. The taxonomy used in the study follows Denchev (2007), Denchev et al. (2009), Vánky (1994), Lutz et al. (2005, 2008) and Piątek et al. (2012). Specimens from *Dianthus* were named *Microbotryum dianthorum* s.l. as there are no ITS sequences available for the new species recently described by Denchev et al. (2009) and the different host species can be colonized by more than one parasite species. The DNA sequences from the host plants of this study are listed in Table 2. The taxonomy used in the study follows Frajman et al. (2009a), Frajman and Oxelman (2007), Oxelman et al. (2001) and Tutin and Walters (1993).

Molecular analyses

In order to extract genomic DNA, the DNeasy™ Plant Mini Kit (QIAGEN, Germany/ USA) or the EZNA™ Fungal DNA Miniprep Kit (without adding 2-mercaptoethanol in tissue lysing; Omega Biotek, USA) was used. The ITS region of the parasites was amplified by polymerase chain reaction (PCR) using primer combinations ITS1f/ITS4 or

Fig. 1 (a) *Microbotryum dianthorum* s.l. on *Dianthus superbis*. The parasite is only visible through the formation of teliospores in the anthers of its host. (b) Spores of *Microbotryum* on *Dianthus serrulatus* (MA-Fungi-33632) displaying the characteristic reticulate ornamentation typical for this group of anther smuts



ITS1f/ITS4b with subsequent nested PCR using ITS1/ITS4 or ITS1/ITS2 and ITS3/ITS4, respectively, to obtain an approximately 650-bp-long DNA fragment. Primers were published in White et al. (1990) and Gardes and Bruns (1993). The ITS region of hosts was amplified using either ITS1/ITS4 or P17/26S-82R (Popp et al. 2005). To purify PCR products, the QIAquick™ Kit (QIAGEN, Germany/USA) was used. Samples were sequenced with the BigDye™ Terminator Cycle Sequencing Kit V3.1 (Applied Biosystems) on an automatic sequencer (ABI 3100 Genetic Analyzer). DNA sequences determined in the course of this study were deposited in GenBank; accession numbers are given in Table 1 for the fungi and Table 2 for the plants.

Phylogenetic analyses

The sequences were aligned using MAFFT 6.707b (Kato et al. 2002, 2005) using the –auto option, which resulted in the FFT-NS-i strategy. As suggested by Giribet and Wheeler (1999) and Gatesy et al. (1993), to obtain reproducible results we avoided manipulation of the alignment by hand as well as manual exclusion of ambiguous sites. Instead, GBLOCKS 0.91b (Castresana 2000) was applied to handle ambiguous alignment positions using the GBLOCKS server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), allowing for smaller final blocks as well as gap positions within the final alignment.

Parasite phylogenies were inferred using maximum likelihood and Bayesian analysis. Maximum likelihood (ML; Felsenstein 1981) analyses were conducted with the RAxML 7.0.4 software (Stamatakis 2006b) invoking the –m GTRGAMMA option. By selecting the –f a option, every fifth bootstrap tree was taken as a starting tree for the ML analysis.

ML bootstrap analysis with RAxML under the –f a option was performed with the –m GTRCAT option (Stamatakis 2006a) and 1,000 replicates. Priors for the Bayesian analysis (BA) were determined using MrModeltest 2.2 (Nylander et al. 2004), and the analysis was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). Four incrementally heated simultaneous Markov chains were run over 5,000,000 generations using random starting trees and default starting parameters of the respective DNA substitution model. Trees were sampled every 100th generation resulting in an overall sampling of 50,001. From these, the first 12,501 trees were discarded, and the rest were used to compute a 50 % majority rule consensus tree to obtain estimates for *a posteriori* probabilities.

Host phylogenies were inferred with RAxML and MrBayes (5,000,000 generations) as described above. Sequences of the genera *Atocion*, *Gypsophila*, *Heliosperma*, *Lychnis*, *Silene* and *Viscaria* taken from GenBank were only used when a parasite occurrence on the host species was reported in Vánky (1994).

Alignments, analyses details and phylogenies of the MB and ML analysis have been deposited in TreeBase (S12122).

Host and geographic data

To test whether host specificity of closely related *Microbotryum* specimens was due to shared ancestry, tip association significance testing was performed using BaTS_beta_build2 (Parker et al. 2008). BaTS estimates phylogeny-trait associations for *n* different states of a single character (i.e., host plant) using different statistics: the Fitch parsimony score (PS; Fitch 1971), the Association Index (AI; Wang et al. 2001) and the maximum exclusive single-state clade size (MC; Parker et al. 2008). To obtain significance values for phylogeny-trait

Table 1 *Microbotryum* species investigated, their host species according to Tutin & Walter 1993, host species on the herbarium label, DNA isolation number, GenBank accession number and herbarium specimen description

Species	Host (Tutin and Walter 1993)	Host (specimen label)	DNA Isolation-No.	ITS Accession-No.	Reference material
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus arenarius</i> L.	<i>Dianthus arenarius</i> L.	mk676	JQ307896	Germany, Brandenburg, Uckermark, Gartz (Oder); leg. V. Kummer; 12.06.2007; VK0345/1
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus arenarius</i> L.	<i>Dianthus arenarius</i> L.	mk677	JQ307897	Germany, Brandenburg, Uckermark, Schwedt (Oder), Kunow; leg. V. Kummer; 24.06.2007; VK0345/2
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	micf1	JQ307898	Andorra, Escaldes km 2 desde el cruce de Escaldes hacia la ermita, 1400 m; leg. T. Almaraz, I. Alvarez & M. A. García; 01.07.1996; MA-Fungi 35420
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	micf10	JQ307899	Spain, Burgos, Neila, pista hacia Villavelayo, en el cruce, 1200 m; leg. T. Almaraz, I. Alvarez, M. A. García & E. Mon; 05.07.1996; MA-Fungi 33587
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	micf45	JQ307900	Spain, Lleida, Estana, Sierra del Cadí, pista que sale del pueblo hacia la Sierra, 1400 m; leg. T. Almaraz, I. Alvarez & M. A. García; 29.06.1996; MA-Fungi 35416
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	micf46	JQ307901	Spain, Lleida, Cava, Ansovell, Sierra del Cadí, junto a la ermita, 1460 m; leg. T. Almaraz, I. Alvarez & M. A. García; 29.06.1996; MA-Fungi 35419
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	micf9	JQ307902	Spain, Burgos, Barbadillo de Herreros, en la ctra. hacia Monterrubio de Demanda, 1145 m; leg. T. Almaraz, I. Alvarez, M. A. García & E. Mon; 05.07.1995; MA-Fungi 33585
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	mk166	JQ307903	Switzerland, Valais, Simplonpass, 1800 m; leg. E., T., U. & K. Vánky; 14.08.1985; M-0066032
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	mk289	JQ307904	Germany, Brandenburg, Prignitz, near Perleberg; leg. V. Kummer & W. Fischer; 17.06.2002; B 70 0006026
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	mk290	JQ307905	Germany, Brandenburg, Prignitz, near Perleberg; leg. I. Scholz; 28.06.2002; B 70 0006025
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	mk291	JQ307906	Germany, Brandenburg, Prignitz, near Wittenberge; leg. I. Scholz; 30.06.2002; B 70 0006024
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	mk352	JQ307907	Germany, Baden-Württemberg, Tübingen, Unterjesingen, Kochertal; leg. A. Graeper; 04.06.2004; HMK71
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	mk496	JQ307908	Poland, Wyzyna Malopolska upland, Krzyzanowice Reserve, ca. 11 km from Busko Zdrój; leg. M. Piatek; 07.10.2004; exHeMP-26
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	mk510	JQ307909	Germany, Saxony-Anhalt, Thale, NSG Teufelsmauer; leg. M. Guttenberger; 14.10.2005; HMK287
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus costae</i> Willk.	<i>Dianthus costae</i> Willk.	micf65	JQ307910	Spain, Valencia, Estenas, Utiel; leg. E. García; ???; MA-Fungi 33634
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus deltooides</i> L.	<i>Dianthus deltooides</i> L.	as589	JQ307911	Russia, Karelia, Natural Reserve Kivach leg. A. Yurkov; 23.08.09; CBS12215
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus deltooides</i> L.	<i>Dianthus deltooides</i> L.	micf39	JQ307912	Spain, León, Abelgas, hacia el pico Churros, 1600 m; leg. I. Alvarez & M. A. García; 25.07.1995; MA-Fungi 37710
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus deltooides</i> L.	<i>Dianthus deltooides</i> L.	micf56	JQ307913	Spain, Palencia, Cervera de Pisuerga, valle de Tosande, ladera norte; leg. T. Almaraz; 14.07.1995; MA-Fungi 33617
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus deltooides</i> L.	<i>Dianthus deltooides</i> L.	mk495	JQ307914	Poland, Kotlina Sandomierska basin, Wola Zarczycka, ca 35 km NE from Rzeszów; leg. M. Piatek; 25.08.2005; exHeMP-35
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus furcatus</i> Balbis	<i>Dianthus benearnensis</i> Loret	micf24	JQ307915	Spain, Huesca, Panticosa, camino de Panticosa al pto. de Ordiso; 1450 m; leg. A. Herrero; 26.07.1996; MA-Fungi 37723
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus furcatus</i> Balbis	<i>Dianthus benearnensis</i> Loret	micf25	JQ307916	Spain, Huesca, Torla, valle del río Ara, 1700 m; leg. A. Herrero; 31.07.1996; MA-Fungi 37724
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus furcatus</i> Balbis	<i>Dianthus benearnensis</i> Loret	micf26	JQ307917	Spain, Huesca, Bielsa, portilla de Tella, 2070 m; leg. C. Aedo; 15.07.1994; MA-Fungi 32966
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus langeanus</i> Willk.	<i>Dianthus langeanus</i> Willk.	micf40	JQ307918	Spain, León, San Emiliano, Riologos, pico Remonta, 1950 m; leg. I. Alvarez & M. A. García; 26.07.1995; MA-Fungi 37711
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus langeanus</i> Willk.	<i>Dianthus langeanus</i> Willk.	micf41	JQ307919	Spain, León, Candín, puerto de Ancares, 1600 m; leg. I. Alvarez, M. A. García & E. Monasterio-Hue; 27.07.1995; MA-Fungi 37712

Table 1 (continued)

Species	Host (Tutin and Walter 1993)	Host (specimen label)	DNA Isolation-No.	ITS Accession-No.	Reference material
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus langeanus</i> Willk.	<i>Dianthus langeanus</i> Willk.	micf42	JQ307920	Spain, León, Truchas, La Iruela, alto del Carbajal, 1345 m; leg. I. Alvarez, M. A. García & E. Monasterio-Hue; 28.07.1995; MA-Fungi 37713
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus lumutzeri</i> Wiesb.	<i>Dianthus lumutzeri</i> Wiesb.	mk298	JQ307921	Austria, Lower Austria, Hainburger Berge, Hexenberg, 400 m; leg. C. Kampicher; 19.06.1987; B 70 0007565
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus lusitanus</i> Brot.	<i>Dianthus lusitanus</i> Brot.	micf22	JQ307922	Spain, Granada, Lanjarón, barranco del río Lanjarón, al final de la pista hacia el río, 1700 m; leg. T. Almaraz & al.; 24.07.1996; MA-Fungi 37718
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus lusitanus</i> Brot.	<i>Dianthus lusitanus</i> Brot.	micf52	JQ307923	Spain, Madrid, Guadalix, 900 m; leg. M. A. García; 26.06.1996; MA-Fungi 37715
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus lusitanus</i> Brot.	<i>Dianthus lusitanus</i> Brot.	micf6	JQ307924	Spain, Almería, Bayárcal, ctra. del pto. de la Ragua al cerro del Almirez, laguna seca, 2100 m; leg. T. Almaraz & al.; 23.07.1996; MA-Fungi 37716
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus monspessulanus</i> L.	<i>Dianthus hyssopifolius</i> L. subsp. <i>hyssopifolius</i>	micf23	JQ307928	Spain, Huesca, Jaca, Peña Oroel, 1650 m; leg. B. Fernández de Betoño, P. Urrutia & J. A. Alejandro; 18.07.1986; MA-Fungi 33642
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus monspessulanus</i> L.	<i>Dianthus hyssopifolius</i> L. subsp. <i>hyssopifolius</i>	micf31	JQ307929	Spain, Huesca, Aisa, Barranco del Estarrún, 1750 m; leg. T. Almaraz & A. Cano; 06.08.1995; MA-Fungi 34531
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus monspessulanus</i> L.	<i>Dianthus hyssopifolius</i> L. subsp. <i>hyssopifolius</i>	micf32	JQ307930	Spain, Huesca, Fanlo, a 3 km del pueblo, 1150 m; leg. T. Almaraz & A. Cano; 13.08.1995; MA-Fungi 34534
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus pontederiae</i> Kerner	<i>Dianthus pontederiae</i> Kerner	mk301	JQ307931	Austria, Lower Austria, Marchtal, Marchauen near Marchegg; leg. H. Scholz; 15.09.1984; B 70 0007562
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L.	micf12	JQ307932	Spain, Burgos, Sotresgudo, Peña Amaya, 1250 m; leg. J. A. Alejandro; 11.06.1991; MA-Fungi 33661
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L.	micf55	JQ307933	Spain, Palencia, Sierra del Brezo, ermita de la Virgen del Brezo; leg. T. Almaraz; 10.07.1995; MA-Fungi 33592
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L. subsp. <i>brachyanthus</i> (Boiss.) Bernal Fern. Casas, G. López, M. Lainz & Muñoz Garm.	micf57	JQ307934	Spain, Palencia, Curavacas, vertiente nordeste; leg. M. Luceño & P. Vargas; 15.07.1985; MA-Fungi 33657
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L. subsp. <i>hispanicus</i> (Asso) O. Bolós & Vigo	micf69	JQ307935	Spain, Zaragoza, ctra. de Zaragoza a Sarinena, Lecinena y alto de Alcubierre; leg. P. Catalá, I. Aizpuru & J. Pedrol; 24.06.1986; MA-Fungi 33663
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burde	<i>Dianthus broteri</i> Boiss.	micf17	JQ307925	Spain, Castellón, El Molinar, 950 m; leg. M. Bernal, F. Muñoz & J. Pedrol; 17.07.1988; MA-Fungi 33625
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burde	<i>Dianthus broteri</i> Boiss.	micf21	JQ307926	Spain, Granada, Almegijar, 900 m; leg. T. Almaraz & al.; 24.07.1996; MA-Fungi 37717
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burde	<i>Dianthus broteri</i> Boiss.	micf5	JQ307927	Spain, Almería, Berja, Sierra de Gádor, 1100 m; leg. T. Almaraz; 24.07.1984; MA-Fungi 33632
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus</i> sp.	<i>Dianthus</i> sp.	mk117	JQ307936	France, Alpes-Maritimes, Isola, Col de la Valette; leg. M. Kemler; 02.08.2003; HMK47
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus</i> sp.	<i>Dianthus</i> sp.	mk118	JQ307937	France, Alpes-Maritimes, La Bolline, GR5, direction Rimplas; leg. M. Kemler; 04.08.2003; HMK48
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus</i> sp.	<i>Dianthus</i> sp.	mk456	JQ307938	Italy, Liguria, Moneglia; leg. M. Lutz; 02.09.2004; HMK127
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus sylvestris</i> Wulfen	<i>Dianthus sylvestris</i> Wulfen	mk313	JQ307939	Italy, Veneto, Lago di Garda, near Pisani, Monte Belpo; leg. V. Kummer; 31.05.2003; B 70 0006028
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Dianthus taiwanensis</i> Masam.	<i>Dianthus taiwanensis</i> Masam.	mk170	JQ307940	Taiwan, Nan tou Province, inter Tai chung et Hua lien, Mt. Ho Huan San, Tarako National Park, 3000 m; leg. F. Oberwinkler; 05.08.1990; M – 0066024
<i>M. dianthorum</i> (Liro) H. & I. Scholz	<i>Gypsophila repens</i> L.	<i>Gypsophila repens</i> L.	mk667	JQ307941	Germany, Bavaria, Berchtesgadener Land, Untersberg, leg. M. Guttenberger; 18.08.2006; HMK300

association, the different methods perform large numbers of randomizations (PS: Slatkin and Maddison 1989; AI: Wang et al. 2001; MC: Parker et al. 2008) for tip-character associations

to provide a null distribution to which the observed values can be compared. Thereby PS and AI calculate the statistics from a single phylogeny that is assumed to be correct, whereas MC

Table 2 *Dianthus* and *Petrorhagia* species studied, species name according to Tutin & Walter 1993, species name on the herbarium label, DNA isolation number, GenBank accession number and herbarium specimen description

Species (Tutin and Walters 1993)	Species (specimen label)	DNA Isolation-No.	ITS Accesion-No.	Reference material
<i>Dianthus amurensis</i> Hort. ex Jacques	<i>Dianthus amurensis</i> Hort. ex. Jacques	mk420	JQ307848	Russia, E flum. Amur, 5 kmN pag. Nizhnyaya Gavan, 50 m; leg. P. Alanko; 31.08.1976; M – 0066027
<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	DB3831	JQ307849	Germany, Baden-Württemberg, Tübingen, Spitzberg; leg. D. Begerow; 13.7.2001; DB705
<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	micp1	JQ307851	Andorra, Escaldes km 2 desde el cruce de Escaldes hacia la ermita, 1400 m; leg. T. Almaraz, I. Alvarez & M. A. García; 01.07.1996; MA-Fungi 35420
<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	micp45	JQ307852	Spain, Lleida, Estana, Sierra del Cadí, pista que sale del pueblo hacia la Sierra, 1400 m; leg. T. Almaraz, I. Alvarez & M. A. García; 29.06.1996; MA-Fungi 35416
<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	micp46	JQ307853	Spain, Lleida, Cava, Ansovell, Sierra del Cadí, junto a la ermita, 1460 m; leg. T. Almaraz, I. Alvarez & M. A. García; 29.06.1996; MA-Fungi 35419
<i>Dianthus carthusianorum</i> L.	<i>Dianthus carthusianorum</i> L.	mk415	JQ307850	Austria, Lower Austria, Eisenstadt, Reichenau a.d. Rax, Mt. Gahns, Thalhof, 580 m; leg. K. Vánky; 11.07.1982; M-0066030
<i>Dianthus costae</i> Willk.	<i>Dianthus costae</i> Willk.	micp65	JQ307854	Spain, Valencia, Estenas, Utiel; 30SXJ68, 900 m, leg. E. García; 06.1986; MA-Fungi 33634
<i>Dianthus deltoides</i> L.	<i>Dianthus deltoides</i> L.	BL79d	JQ307855	Czech Republic, Karlovy Vary, Klinovec, 1000 m; leg. W. Dietrich; 30.07.2004;
<i>Dianthus deltoides</i> L.	<i>Dianthus deltoides</i> L.	BL89	JQ307856	Germany, Berlin, Strausberg, Herrensee; leg. M. Hendrichs; 23.06.2002; HML 1387
<i>Dianthus gratianopolitanus</i> Vill.	<i>Dianthus gratianopolitanus</i> Vill.	BL121	JQ307857	Germany, Baden-Württemberg, Tübingen, Bot. Garden; leg. M. Kemler, 04.06.2003; TUB012505
<i>Dianthus langeanus</i> Willk.	<i>Dianthus langeanus</i> Willk.	micp38	JQ307858	Spain, León, Candin, puerto de Ancares, 1700 m; leg. M. Carrasco, F. Castilla, C. Martín & M. Vel; 27.06.1994; MA-Fungi 33645
<i>Dianthus lusitanus</i> Brot.	<i>Dianthus lusitanus</i> Brot.	micp22	JQ307859	Spain, Granada, Lanjarón, barranco del río Lanjarón, al final de la pista hacia el río, 1700 m; leg. T. Almaraz & al.; 24.07.1996; MA-Fungi 37718
<i>Dianthus lusitanus</i> Brot.	<i>Dianthus lusitanus</i> Brot.	micp50	JQ307860	Spain, Madrid, Cercedilla, puerto de la Fuenfria; leg. J. Fernández Casas; 06.07.1975; MA-Fungi 33656
<i>Dianthus lusitanus</i> Brot.	<i>Dianthus lusitanus</i> Brot.	micp59	JQ307861	Spain, Salamanca, Olmedo de Cameces, 7.06.1977, MA-Fungi 33652
<i>Dianthus lusitanus</i> Brot.	<i>Dianthus lusitanus</i> Brot.	micp6	JQ307862	Spain, Almería, Bayárcal, ctra. del pto. de la Ragua al cerro del Almirez, laguna seca, 2100 m; leg. T. Almaraz & al.; 23.07.1996; MA-Fungi 37716
<i>Dianthus lusitanus</i> Brot.	<i>Dianthus lusitanus</i> Brot.	micp62	JQ307863	Spain, Teruel, Orihuela del Tremedal, 30TXK18, 1700 m, 30.07.1985, MA-Fungi 33651
<i>Dianthus monspessulanus</i> L.	<i>Dianthus hyssopifolius</i> L. subsp. <i>hyssopifolius</i>	micp2	JQ307870	Spain, Alava, Lagrán, Sierra de Cantabria, Palomares, 20TWN3316, 1400 m, 23.07.1986, MA-Fungi 33641
<i>Dianthus monspessulanus</i> L.	<i>Dianthus hyssopifolius</i> L. subsp. <i>hyssopifolius</i>	micp36	JQ307872	Spain, León, Puerto de las Señales; leg. E. Rico; 26.08.1978; MA-Fungi 33639
<i>Dianthus monspessulanus</i> L.	<i>Dianthus hyssopifolius</i> L. subsp. <i>hyssopifolius</i>	micp37	JQ307873	Spain, León, Ponferrada, Montes Aquilianos, Peñalba de Santiago; leg. Lansac & Nieto; 11.07.1981; MA-Fungi 33640
<i>Dianthus monspessulanus</i> L.	<i>Dianthus hyssopifolius</i> L. subsp. <i>hyssopifolius</i>	micp47	JQ307874	Spain, Lugo, Linares de Cebrero; Casaseca 6 Fernández-Diez; 25.08.1981; MA-Fungi 33638
<i>Dianthus monspessulanus</i> L.	<i>Dianthus hyssopifolius</i> L. subsp. <i>hyssopifolius</i>	micp7	JQ307875	Spain, Asturias, Genestoso, 29TQH1171, 1200 m, 27.07.1996, MA-Fungi 37719
<i>Dianthus pontedaere</i> Kerner	<i>Dianthus pontedaere</i> Kerner	mk422	JQ307876	Austria, Lower Austria, Marchtal, Marchauen near Marchegg; leg. H. Scholz; 15.09.1984; B 70 0007562
<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L.	micp12	JQ307877	Spain, Burgos, Sotresgudo, Peña Amaya, 1250 m; leg. J. A. Alejandre; 11.06.1991; MA-Fungi 33661
<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L. subsp. <i>brachyanthus</i> (Boiss.) Bernal Fern. Casas, G. López, M. Lainz & Muñoz Gam.	micp3	JQ307878	Spain, Albacete, Lezuza, desvío Ballestero, 940 m; leg J.M. Herranz; 31.05.1986; MA-Fungi 33658
<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L. subsp. <i>hispanicus</i> (Asso) O. Bolós & Vigo	micp30	JQ307879	Spain, Huesca, San Juan de la Peña, por encima del Santuario, 1200 m; leg. R. García Adá, G. López & M. Luceño; 02.06.1987; MA-Fungi 33662

Table 2 (continued)

Species (Tutin and Walters 1993)	Species (specimen label)	DNA Isolation- No.	ITS Accession- No.	Reference material
<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L.	micp52	JQ307880	Spain, Madrid, Guadalix, 900 m; leg. M.A. García; 26.06.1996; MA-Fungi 37715
<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L. subsp. <i>brachyanthus</i> (Boiss.) Bernal Fern. Casas, G. López, M. Lainz & Muñoz Garm.	micp57	JQ307881	Spain, Palencia, Curavacas, vertiente nordeste; leg. M. Luceño & P. Vargas; 15.07.1985; MA-Fungi 33657
<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L. subsp. <i>hispanicus</i> (Asso) O. Bolós & Vigo	micp58	JQ307882	Spain, Palencia, Astudillo, 30TUM9062, 13.06.1987, MA-Fungi 33664
<i>Dianthus pungens</i> L.	<i>Dianthus pungens</i> L. subsp. <i>hispanicus</i> (Asso) O. Bolós & Vigo	micp69	JQ307883	Spain, Zaragoza, ctra. de Zaragoza a Sarinena, Lecinena y alto de Alcubierre; leg. P. Catalá, I. Aizpuru & J. Pedrol; 24.06.1986; MA-Fungi 33663
<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burdet	<i>Dianthus broteri</i> Boiss.	micp15	JQ307864	Spain, Castellón, Azuébar; leg. Castroviejo & F. Quirós; 19.07.1981; MA-Fungi 33623
<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burdet	<i>Dianthus broteri</i> Boiss.	micp16	JQ307865	Spain, Castellón, de Costur a Useras, 450 m; leg. R. Figuerola & M. Palasí; 18.08.1984; MA-Fungi 33624
<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burdet	<i>Dianthus broteri</i> Boiss.	micp17	JQ307866	Spain, Castellón, El Molinar, 950 m; leg. M. Bernal, F. Muñoz & J. Pedrol; 17.07.1988; MA-Fungi 33625
<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burdet	<i>Dianthus broteri</i> Boiss.	micp18	JQ307867	Spain, Castellón, Begis, 900 m; leg. G. Mateo & A. Aguilera; ???; MA-Fungi 33626
<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burdet	<i>Dianthus broteri</i> Boiss.	micp21	JQ307868	Spain, Granada, Almegijar, 900 m; leg. T. Almaraz & al.; 24.07.1996; MA-Fungi 37717
<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burdet	<i>Dianthus broteri</i> Boiss.	micp63	JQ307869	Spain, Valencia, Alclublas, ctra. de Bodegas a Alclublas, a 2 km del pueblo, 30SXX9407, 730 m, MA-Fungi 33627
<i>Dianthus serrulatus</i> Desf. subsp. <i>barbatus</i> (Boiss.) Greuter & Burdet	<i>Dianthus broteri</i> Boiss.	micp64	JQ307870	Spain, Valencia, Sagunto, 30SYJ39, 100 m, 2.02.1986, MA-Fungi 33628
<i>Dianthus sinaicus</i> Boiss.	<i>Dianthus sinaicus</i> Boiss.	BL105	JQ307884	Israel, Jerusalem, Botanical Garden, Mt. Scopus (Har Hanegev); leg. ???; 18.12.2005; TUB014953
<i>Dianthus strictus</i> Banks & Sol. subsp. <i>strictus</i>	<i>Dianthus strictus</i> Banks & Sol. subsp. <i>strictus</i>	BL44	JQ307885	Israel, Negev, Avadad research station; leg. M. Bierkamp; 16.05.1983; TAVO 13108
<i>Dianthus strictus</i> Banks & Sol. subsp. <i>velutinus</i> (Boiss.) Greuter & Burdet	<i>Dianthus strictus</i> Banks & Sol. subsp. <i>velutinus</i> (Boiss.) Greuter & Burdet	BL36	JQ307886	Jordan, Edom mountains, Al Hishi, 10 km SSW Shobak, Palestine Grid N979/O197, 1580 m; leg. H.U. Baierle; 20.04.1986; TAVO 21792
<i>Dianthus strictus</i> Banks & Sol. subsp. <i>velutinus</i> (Boiss.) Greuter & Burdet	<i>Dianthus strictus</i> Banks & Sol. subsp. <i>velutinus</i> (Boiss.) Greuter & Burdet	BL42	JQ307885	Jordan, Edom, 1.5 km O Tafila, Wadi at-Tafila, Palestine Grid N027/O211, 1200 m; leg. H.U. Baierle; 18.05.1987; TAVO 21802
<i>Dianthus superbus</i> L. subsp. <i>alpestris</i> Kablík. ex Čelak.	<i>Dianthus superbus</i> L. subsp. <i>alpestris</i> Kablík. ex Čelak.	BL120	JQ307888	Slovenia, Triglav National Park; Seven lakes valley; leg. M. Kemler & M. Lutz; 03.08.2005; HMK104
<i>Dianthus superbus</i> L. subsp. <i>sylvestris</i> L.	<i>Dianthus superbus</i> L. subsp. <i>sylvestris</i> L.	BL68d	JQ307889	Germany, Baden-Württemberg, Tübingen-Derendingen; leg. M. Koltzenburg; 10.08.1998; HMKo989a
<i>Dianthus sylvestris</i> Wulfen	<i>Dianthus sylvestris</i> Wulfen	BL72d	JQ307890	Austria, Tirol, Ötztal, Sölden, 1900 m; leg. M. Koltzenburg; 09.07.1989; HMKo Ob983 331A
<i>Dianthus sylvestris</i> Wulfen	<i>Dianthus sylvestris</i> Wulfen	DB3971	JQ307891	Germany, Baden-Württemberg, Tübingen, Bot. Garden; leg. D. Begerow, 07.07.2002
<i>Dianthus sylvestris</i> Wulfen	<i>Dianthus sylvestris</i> subsp. <i>tergestinus</i> (Reichenbach) Hayek	DB3991	JQ307892	Germany, Baden-Württemberg, Tübingen, Bot. Garden; leg. D. Begerow, 08.07.2002
<i>Dianthus zonatus</i> Fenzl.	<i>Dianthus zonatus</i> Fenzl.	BL26	JQ307893	Turkey, Nigde, Ala Dagh Mountains, Emil Valley, 2080 m; leg. W. Frey, G. Göttel, H. Kürschner & P. Schädel; 07.07.1978; TAVO3068
<i>Petrorhagia saxifraga</i> (L.) LK	<i>Petrorhagia saxifraga</i> (L.) LK	BL66d	JQ307894	Germany, Baden-Württemberg, Tübingen, Mauerstrasse 14; leg. M. Koltzenburg; HMKo 975
<i>Petrorhagia saxifraga</i> (L.) LK	<i>Petrorhagia saxifraga</i> (L.) LK	BL85	JQ307895	Italy, Elba, Fetovia; leg. M. Hendrichs; 15.05.2000; TUB 012106

also incorporates phylogenetic error. To perform the analyses, the MrBayes tree files from both runs were used, the different hosts were coded as tip associations, and 100 randomizations were performed.

Correlation between host specificity of *Microbotryum* on Caryophyllaceae with phylogeny and geographical distribution was assessed for the members of *Dianthus* and *Silene* clades using analysis of variance (ANOVA) using nested factors implemented in the Generalized Linear Models (GLM) block of Statistica 8 (Statsoft Inc.). Therefore, results of all pair-wise comparisons between studied fungal specimens for both genetic and geographical distances were analyzed toward a set of hierarchical grouping factors (independent variables). The following categorized variables reflecting certain levels of host specificity were used as independent variables: the same host species, the same host genus and a different host genus. These factors were treated as random and nested. So, the gradation of “the same host species” was included in the next category “the same host genus,” which is embedded in the higher category “phylogenetic clade,” i.e., *Dianthus* vs. *Silene*. Effects were considered to be statistically significant at the level $p \leq 0.05$. Additionally, correlation of genetic and geographical distances was calculated using Simple Linear Correlation (Pearson r). Our data set had a large proportion of *Microbotryum* on *Dianthus* from Spain, and therefore the test was also performed using a reduced data set that included only a single specimen per species in a given geographic region.

The biodiversity occurrence data for *Microbotryum dianthorum* s.l. specimens from Spain were provided by the Real Jardín Botánico, Madrid (accessed through GBIF Data Portal, www.gbif.net, 2009-06-19). The other occurrence data were either obtained through the specimen label or for already published data through the publication (Kemler et al. 2006, 2009; Lutz et al. 2008; Roux et al. 1998; Sloan et al. 2008). Data were transformed into latitude and longitude data using Google Earth (<http://earth.google.com>) and subsequently converted into a distance matrix using Geographic Distance Matrix Generator, version 1.2.3 (Ersts). Genetic distances were computed using PAUP* 4b10 (Swofford 1998), whereby distance settings were the same as the ones obtained for the priors of the Bayesian analysis. Host data were coded for the same host species, the same host genus and a different host genus (Online Resource 1 and 2).

Results

Parasite phylogenies

The phylogenies of *Microbotryum* on *Dianthus*, *Silene* and other members of the caryophyllaceous anther smuts (Fig. 2) confirm previous studies in general relationships

Fig. 2 Bayesian inference of *Microbotryum* species on Caryophyllaceae based on ITS sequence data. Only branches that show a posterior probability higher than 0.9 are depicted. Bold branches indicate ML bootstrap support higher than 70. The numbers on the branches correspond to the four groups described in the Results section, and the arrow indicates anther smuts on Caryophyllaceae. The topology is rooted with *Microbotryum* species according to Kemler et al. (2006)

between the anther parasites. The anther smuts of the Caryophyllaceae form a monophyletic group that has high statistical support under both tree-inferring methods (BA: 1,00; ML: 98). Monophyletic parasite species on different Caryophyllaceae described previously have also been inferred within this study, except for *Microbotryum saponariae* and *M. violaceum* sensu stricto on *Silene nutans* in the Bayesian analysis. Disagreement between the two inference algorithms occurs mainly about the phylogenetic placement of larger groups of parasites. A sister group relationship between anther smuts from North America and Europe is only supported by the Bayesian analysis, whereas ML does not support this hypothesis. Both methods suggest at least two groups of parasites on European *Silene* species. As in previous studies (Kemler et al. 2006; Le Gac et al. 2007; Lutz et al. 2005, 2008; Refregier et al. 2008), *Microbotryum saponariae* is inferred as the sister species of *M. dianthorum* s.l. with moderate support (83) under ML, but was not supported by BA.

Within *Microbotryum dianthorum* s.l. four major monophyletic groups (Fig. 2) with strong support are inferred under both methods. The first includes only parasites on *Dianthus deltoides*, and a moderate support for a sister clade relationship of that clade with *Microbotryum* on *Gypsophila repens* is supported under ML (77), whereas this is not supported under BA. The second clade includes specimens of *Dianthus serrulatus* and *D. carthusianorum*. The third clade includes parasites on *Dianthus amurensis*, *D. carthusianorum*, *D. furcatus*, *D. jacquemontii*, *D. pontederiae* and *Petrohragia saxifraga*. The fourth and largest clade contains parasites on *Dianthus arenarius*, *D. carthusianorum*, *D. furcatus*, *D. gratianopolitanus*, *D. langeanus*, *D. humnitzeri*, *D. lusitanus*, *D. monspessulanus*, *D. serrulatus*, *D. pungens*, *D. superbus*, *D. sylvestris* and *D. taiwanensis*. Groups containing all parasite specimens from one host species are only supported for specimens of parasites on *Dianthus deltoides* and *D. amurensis* with high support for both inference methods. A paraphyletic group containing all parasites on *Dianthus sylvestris*, several specimens on *D. carthusianorum*, *D. monspessulanus* and one unidentified *Dianthus* species is only supported by BA and not by ML.

Host phylogenies

General relationships within the Caryophylloideae and between *Dianthus* species were similar to previous studies



0.3

(Fior et al. 2006; Frajman et al. 2009b; Frajman and Oxelman 2007; Oxelman et al. 2001; Valente et al. 2010), whereby our emphasis was on the patterns within the different groups and not relationships between them (Fig. 3). The host phylogenies showed different patterns between *Dianthus* and the other Caryophylloideae, respectively. The clade containing all *Dianthus* specimens was highly supported (BA: 1.00; ML: 89), but relationships between *Dianthus* and *Velezia* were not resolved. Most *Dianthus* species clustered within one group; however, this monophyly was only supported by the Bayesian analysis. Also this group showed polytomous relationships with *Velezia* and two other groups, one containing all specimens of *Dianthus strictus* and the other *D. deltoides*. In general, the resolution within the main *Dianthus* clade was rather low. Bayesian analysis highly supported a sister clade relationship between *Dianthus* specimens from Spain (clade 1 in Fig. 3) and a clade containing *Dianthus amurensis*, *D. sinaicus* and one unidentified *Dianthus* species (clade 2). A monophyletic clade containing all specimens from *Dianthus sylvestris* and one *D. carthusianorum* sample (clade 3) and a monophyletic clade containing four *D. superbus* specimens (clade 4) were highly supported by Bayesian analyses, but not by ML.

The large group containing *Atocion*, *Lychnis*, *Silene* and *Viscaria*, which will be subsequently referred to as Sileneae, showed a rather different pattern. Specimens that constituted one morphological species also clustered within one monophyletic group and were supported by high support values by at least one method. The only exceptions were the species *Silene caroliniana* and *S. virginica*, which both grouped in one clade without further resolution, and the specimens of *Viscaria vulgaris* and *S. viscosa*, which appeared polyphyletic. The specimens of *Silene otites* clustered together with a posterior probability of 0.80 and a ML bootstrap support of 65 (see phylogenies in TreeBase).

Host dependency and geographic data

Tip-association test results are displayed in Online Resource 4. AI and PS statistics showed a high significance level ($p < 0.001$) toward a host-parasite association based on common ancestry of the parasites. MC values are given for each single tip state (i.e., different hosts) and therefore give a more detailed view of host-parasite association. Host associations based on shared ancestry and rejection of a random association in parasites of *Dianthus* are supported in five instances: *Dianthus deltoides* ($p=0.009$), *D. carthusianorum* ($p=0.009$), *D. lusitanus* ($p=0.019$), *D. amurensis* ($p=0.009$) and *D. sylvestris* ($p=0.019$). In the *Silene* clade (including *Minuartia* and *Lychnis*), many host associations by shared ancestry had a high significance level.

Fig. 3 Bayesian inference of Caryophyllaceae host species based on ITS sequence data. Only branches that show a posterior probability higher than 0.9 are depicted. Bold branches indicate ML bootstrap support higher than 70. The numbers on the branches correspond to the four clades described in the Results section. The topology was rooted with *Cerastium fontanum*, *Holosteum umbelatum* and *Stellaria media*

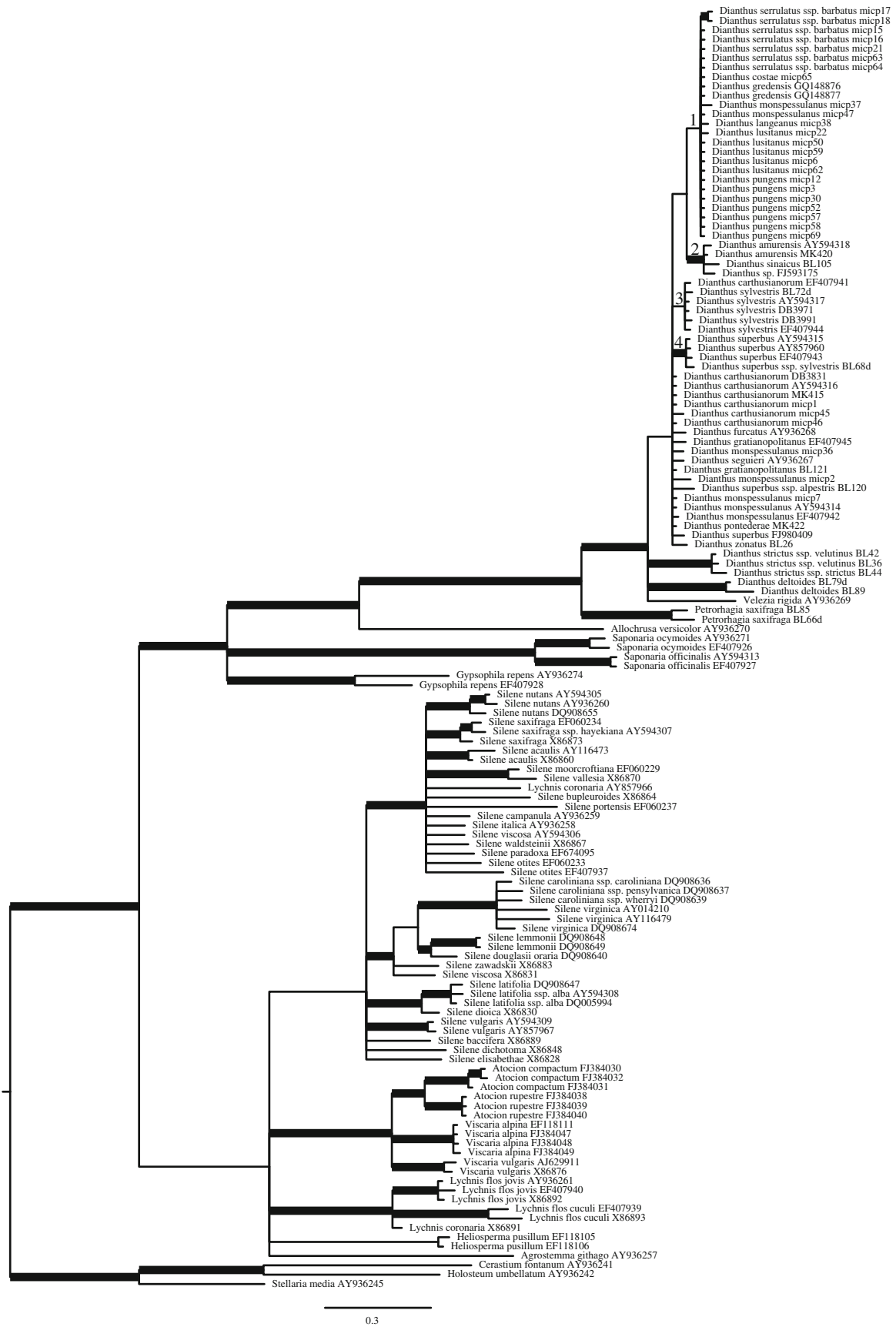
Nested ANOVA revealed two distinct patterns of genetic variability and levels of endemism of *Microbotryum* species on the two host groups *Dianthus* and *Silene*. The analyzed *Microbotryum* specimens on *Dianthus* displayed nearly the same level of inter- and intraspecific variability (expressed as pair-wise genetic distance), whereas the parasites of *Silene* were significantly less variable on the single species level and showed a greater genetic difference on the genus level (Fig. 4a). In other words, the parasites found on the same hosts of the *Dianthus* clade are in general more variable than the ones infecting the same species of *Silene* (means of 0.018 and 0.004 nucleotides per site, respectively).

The analysis of geographical distances showed very broad distribution ranges of the single parasite species on both *Dianthus* and *Silene* species (mean of ca. 800 km). In the same time, a certain level of endemism of the *Microbotryum* species on the hosts of *Dianthus* was observed (Fig. 4b). The average distance between the ranges of different species was significantly higher than the distribution range of the single species (means of 1,500 km and 800 km, respectively). In contrast, there was no statistically supported geographical association observed in the distribution of the *Microbotryum* species on *Silene*.

Discussion

Is low host specificity in *Dianthus* parasites common or are previous observations artifacts of a sampling bias?

On a broader data set, which reduces the problem of a sampling error, our analysis is consistent with previous studies (Kemler et al. 2006; Le Gac et al. 2007; Lutz et al. 2005, 2008; Refregier et al. 2008) and shows that host specificity patterns in *Dianthus* parasites are indeed different from those in the *Silene* anther smuts studied to date. Even though more than one major lineage exists within *Dianthus* parasites, host specificity is rather low. The only exceptions are the parasites on *Dianthus deltoides*, which in itself forms one of the two morphospecies within *Dianthus* that were resolved in our study. A recent multi-gene study inferred *Dianthus deltoides* as polyphyletic within the subsection Armerium, a sister clade to most other *Dianthus* species (Valente et al. 2010). As we did not have access to parasites on the other hosts in that group, it cannot be ruled out that monophyly of *Microbotryum* on *Dianthus deltoides* is an



0.3

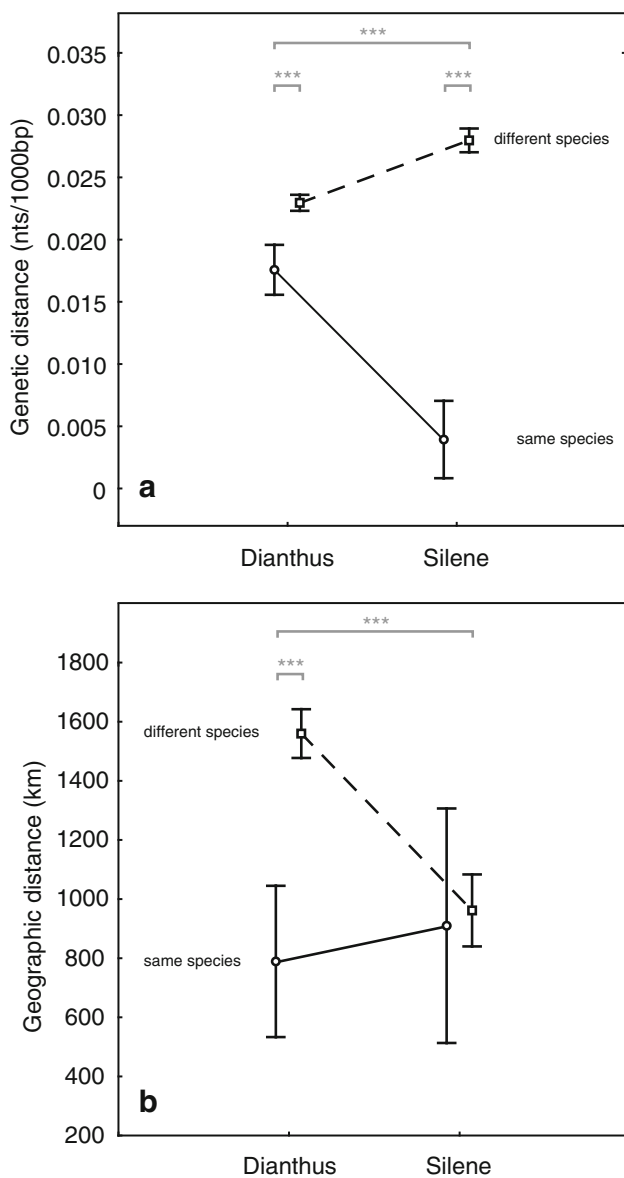


Fig. 4 Results of ANOVA using nested factors. Distribution of genetic (a) and geographic (b) distances in *Microbotryum* on *Dianthus* and *Silene* clades on inter- and intraspecific taxonomic levels, respectively. Vertical bars denote 95 % confidence intervals. Significant differences are denoted by $***p \leq 0.001$

artifact and the parasite is able to infect more than one host in the subsection Armerium. Major lineages within *Dianthus* parasites have also been detected using other genetic markers (Le Gac et al. 2007; Refregier et al. 2008), and based on these results two new species (i.e., *Microbotryum carthusianorum* and *M. shykoffianum*) have been proposed and one (i.e., *M. superbum*) has been reinstated (Denchev et al. 2009). These parasite species are able to infect several *Dianthus* species (*Microbotryum shykoffianum* and *M. superbum*) or *Gypsophila* (*M. carthusianorum*), although they seem to have a preference for certain host species. Because the parasites of single host species in our study also do not cluster together, the

results obtained using the larger sampling size are consistent with previous observations and confirm that host specificity shows different patterns in anther smuts of *Dianthus* compared to parasites of other Caryophyllaceae hosts.

Is there a correlation between host specificity and geographic distribution in *Dianthus* and *Silene* parasites?

The ANOVA analysis revealed two distinct distribution patterns in *Microbotryum* when geographic distances were considered (Fig. 4). Genetic distances in parasites on *Dianthus* are correlated to geographic distance, whereas between *Silene* parasites geographic separation is not given. Contradicting the assumption of a correlation between genetic distance and geographic distance within *Dianthus* parasites is the grouping of parasites from different geographic regions within the same monophyletic clade. On the other hand, the results of the ANOVA analysis could be influenced by the many samples from Spain (aggregated distribution). Our sampling, however, includes specimens of two species spanning a broad distribution area, namely *D. carthusianorum* and *D. deltoides* (mean distance of 800 km), and only data of about 40 % of the host species were sampled in Spain. In order to test the assumption regarding unbalanced sampling, we analyzed a reduced data set that contained a single specimen for each species of *Dianthus* parasites that occurred in the same geographical region. These results were consistent with results obtained from the large data set (Online resource 3). A possible explanation of the observed trend is that different lineages have, as has been suggested before (Le Gac et al. 2007), evolved in allopatry and came into secondary contact; therefore, some clades are still predominant in certain regions. Nevertheless, taxonomic revision including an evaluation and implementation of a species concept is urgently needed.

Is there a correlation between genetic variation of parasites on *Dianthus* and other Caryophyllaceae, mainly *Silene*, with host genetic variation?

Comparison of host and parasite phylogenies in our study shows a striking similarity between the two groups of interacting organisms. The host phylogeny shows differences in phylogenetic patterns between *Dianthus* species and other Caryophyllaceae (Fig. 3). In the non-*Dianthus* clades, except for *Lychnis coronaria*, *Silene viscosa* and *S. otites*, plants that have been delineated on a morphological base are also recognized by molecular methods. For many of the *Dianthus* species in our study this is not true, and merely *Dianthus strictus* and *D. deltoides* form monophyletic clades that only contain all members of this species. Other statistically supported monophyletic clades occur, but these either do not include all members of one morphospecies

(i.e., *Dianthus superbus*) or do not only contain members of a single morphospecies (i.e., *D. amurensis*-*D. sinicus*, *D. sylvestris*-*D. carthusianorum*).

The parasites show similar patterns. Parasites on *Dianthus*, even though they might form different species, do not show very high host correlation, whereas the parasites on *Silene* mainly form lineages that correlated well with monophyletic hosts (Fig. 2). Additionally to this manual inspection, the BaTS analysis supports that most of the *Silene* parasites that had more than one specimen sampled share parasitism on this host because of shared ancestry, whereas in *Dianthus* this was only true for a few of the parasites. The ANOVA with nested factors follows the same line, and parasites on the same *Silene* host species have a higher genetic similarity than parasites on different species (Fig. 4a), whereas in parasites of *Dianthus* genetic distances between parasites on the same host species are nearly the same as between different host species.

Possible causes for the observed differences in *Dianthus* vs. *Silene* and their parasites

The genetic structure of plant parasite populations is determined by the life-history traits of the parasite itself (i.e., host specificity, mode of reproduction, transmission and dispersal, and life cycle complexity and epidemiology), but additionally it is influenced by the life history and ecology of the host (Barrett et al. 2008). We are well aware that the correlation between host and parasite phylogenies does not imply causation, as it does not provide any mechanisms that might explain the pattern. Nevertheless, the host association is such an essential part in anther smut life history that differences in the biology of the hosts might hint at possible scenarios that result in a correlation and will help in formulating future hypotheses that can be tested explicitly.

Parasite traits that interact directly with host response (e.g., virulence factors) are under selection omitted by these host traits (e.g., defense mechanisms, physiological mechanisms). The differences in these host defense traits might therefore result in differential selection on parasite traits that in return will influence the genetic structure of parasites. Whether *Dianthus* exhibits such differences on the selection of *Microbotryum* is not known, but because of the phylogenetic proximity to the other hosts of our study, this seems rather unlikely to explain the patterns observed.

Host traits that are not directly involved in the parasite-host association (e.g., growth form, breeding system or maximal life span) can have an impact on the parasites' genetic variation through their influence on parasite features (e.g., age of reproduction or dispersal) (Barrett et al. 2008). Genetic diversity of plants will be influenced by factors involved in their reproduction and dispersal (Loveless and Hamrick 1984), and particularly breeding system and life

form seem to have a major impact (Hamrick and Godt 1996; Duminil et al. 2007). *Dianthus* species form hybrids readily, a feature that gardeners have often used to produce thousands of *Dianthus* varieties (International Dianthus Register 1983, <http://www.rhs.org.uk/Plants/RHS-Publications/Plant-registers/Dianthus>). Hybrids of *Dianthus* are also often formed in natural habitats, although they seem to be locally restricted, and parent species integrity over greater geographic areas is maintained (Tutin and Walters 1993). Nevertheless, in some instances hybridization makes it difficult to delimitate species (Bernal et al. 1990; Rohrbach 1868; Tutin and Walters 1993), and introgressive hybridization (Anderson and Hubricht 1938) could blur genetic variation between the parent species. Interfertility thereby is not equal between all *Dianthus* species, and some sub-groups hybridize more commonly (Carolin 1957), which could lead to separated species complexes with a lower within-clade genetic diversity.

In contrast, hybridization in *Silene* is not as common as in *Dianthus*, or where it is documented, introgression is limited by other factors. Interspecific crossings of eight North American *Silene* species for instance resulted in a very low pollen fertility when compared to parent plants (Kruckeberg 1955). Studies in contact zones of the closely related *Silene dioica* and *S. latifolia* showed that even though hybridization is possible between the two species, hybrids are rare (Karrenberg and Favre 2008), and species boundaries are maintained rather by habitat preferences and pollinator discrimination than by pre-zygotic barriers (Minder et al. 2007; Minder and Widmer 2008). Yet, hybridization might be a driver for speciation in the genus *Silene*. Phylogenetic analyses in a circumpolar *Silene* species complex revealed the formation of two *Silene* species by consecutive hybridization events with subsequent polyploidization (Popp et al. 2005). Thereby the parent species remained independent entities.

Along with hybridization, speciation rates might have played another crucial role in the formation of the observed patterns of *Dianthus* and the Sileneae. Speciation rates in the genus *Dianthus* have been inferred as the fastest rates observed so far for any plant genus (Valente et al. 2010). Estimates for the age of *Dianthus* ranged from 1.2–7 Ma, and the radiation in the largest group of *Dianthus*, containing the majority of species of our analyses, occurred in the last 1–2 Ma, whereas relative dating of the Sileneae puts them at an age of about 21 Ma and the *Lychnis/Silene* group to about 12 Ma (Frajman et al. 2009b). Our data show that massively elevated speciation rates in *Dianthus* cannot be the only cause of the phylogenetic patterns observed and indicates that there might actually be fewer species exhibiting an enormous morphological variability, which then might result in confusing species delimitations in *Dianthus*. Remarkably, a morphometric study on *Dianthus brachyanthus*, *D. gredensis*, *D. hispanicus*, *D. langeanus* and *D. pungens*, for instance, detected

morphological overlap between these species, and all species were merged into *D. pungens* (Crespi et al. 2007). Morphological differences of these sub-species were mainly correlated to climate or altitude. The enormous plasticity of the *Dianthus* species in our study might have led to an over-estimation of species and subspecies. The clarification of taxonomical and systematic issues in *Dianthus* therefore might additionally resolve the unusual patterns of host specificity in *Microbotryum* on this host genus.

Conclusions

Even though parasites are independent evolutionary entities, our study gives a hint that host life-history traits that lead to a specific phylogenetic pattern might also largely influence the phylogenetics of parasitic species. It also supports the recent description of new species within the anther smuts on *Dianthus*, but suggests that the host spectra of the new *Microbotryum* species have to be broadened to a large extent. A broader taxon sampling in the parasites of the Section *Armerium* should also clarify whether *Microbotryum dianthorum* s.str. is restricted to the parasite on *Dianthus deltoides*, which is the type host for that species. Additionally, our study provides some evidence that plant parasites such as *Microbotryum* are better “taxonomists” (Nannfeldt 1966) and that discrepancies in the parasite phylogenies can hint at taxonomically problematic host taxa.

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