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Taiadjana M Fortuna, Alice Namias, Alodie Snirc, Antoine Branca, Michael E Hood, et al.. Multiple infections, relatedness and virulence in the anther-smut fungus castrating *Saponaria* plants. *Molecular Ecology*, 2018, pp.1 - 13. 10.1111/mec.14911 . hal-01921820

HAL Id: hal-01921820

<https://hal.science/hal-01921820>

Submitted on 14 Nov 2018

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MOLECULAR ECOLOGY

Multiple infections, relatedness and virulence in the anther-smut fungus castrating *Saponaria* plants

Journal:	<i>Molecular Ecology</i>
Manuscript ID	MEC-18-0775.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
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Keywords:	Coevolution, Disease Biology, Fungi, Host Parasite Interactions, Life History Evolution, Population Genetics - Empirical

1 **Multiple infections, relatedness and virulence in the anther-smut fungus castrating**
2 *Saponaria* plants

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14 **Keywords:** virulence, kin selection, sterilizing pathogen, hyphae, vegetative incompatibility

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Abstract

Multiple infections (co-occurrence of multiple pathogen genotypes within an individual host) can have important impacts on diseases. Relatedness among pathogens can affect the likelihood of multiple infections and their consequences through kin selection. Previous studies on the castrating anther-smut fungus *Microbotryum lychnidis-dioicae* have shown that multiple infections occur in its host plant *Silene latifolia*. Relatedness was high among fungal genotypes within plants, which could result from competitive exclusion between unrelated fungal genotypes, from population structure or from interactions between plant and fungal genotypes for infection ability. Here, we aimed at disentangling these hypotheses using *M. saponariae* and its host *Saponaria officinalis*, both experimentally tractable for these questions. By analysing populations using microsatellite markers, we also found frequent occurrence of multiple infections and high relatedness among strains within host plants. Infections resulting from experimental inoculations in the greenhouse also revealed high relatedness among strains coinfecting host plants, even in clonally-replicated plant genotypes, indicating that high relatedness within plants did not result merely from plant x fungus interactions or population structure. Furthermore, hyphal growth *in vitro* was affected by the presence of a competitor growing nearby and by its genetic similarity, although this latter effect was strain-dependent. Altogether, our results support the hypothesis that relatedness-dependent competitive exclusion occurs in *Microbotryum* fungi within plants. These micro-organisms can thus respond to competitors and to their level of relatedness.

40

41 **Introduction**

42 Multiple infections, i.e., the co-occurrence of multiple genotypes of a pathogen
43 species within a given host individual, can have important impacts on the ecology,
44 epidemiology and evolution of pathogens (Alizon *et al.* 2013; Susi *et al.* 2015; Tollenaere *et*
45 *al.* 2016). In particular, multiple infections lead to competition for resources within hosts.
46 Such competition can take the form of competitive interference between pathogen genotypes
47 via the production of inhibitory biocins (Bashey *et al.* 2012), which leads to overall slower
48 pathogen growth and thus lower virulence (Massey *et al.* 2004), virulence being defined in
49 evolutionary biology as the decrease in host fitness due to the parasite. In other cases,
50 competition may trigger a plastic response, generating faster resource uptake and faster
51 pathogen replication rates and thus lead to higher virulence (Leggett *et al.* 2013).

52 When closely-related genotypes interact within hosts they should compete less and
53 engage in less antagonistic activities, because kin selection favours cooperation between
54 related individuals (Buckling & Brockhurst 2008; Hamilton 1964a, b). Multiple infections by
55 closely related genotypes should therefore be more similar to single infections. Kin selection
56 can even directly influence the occurrence of multiple infections if a resident pathogen within
57 a host prevents the installation of unrelated genotypes but tolerates secondary infection by
58 related genotypes (Koskella *et al.* 2006).

59 The study of the anther-smut fungus *Microbotryum lychnidis-dioicae* in its host the
60 white campion, *Silene latifolia*, has provided interesting insights into within-host competition
61 by more or less related pathogens and the impact of multiple infections and relatedness on
62 virulence. *Microbotryum* anther-smut fungi castrate their host plants by aborting ovaries and
63 replacing pollen by fungal spores in flowers. Genotyping spores produced in diseased flowers
64 has revealed a high prevalence of multiple infections, with different genotypes detected in

different stems of the same *Si. latifolia* host in natural populations (Lopez-Villavicencio *et al.* 2007). The co-infecting genotypes displayed high levels of relatedness (Lopez-Villavicencio *et al.* 2007), suggesting either that (i) related strains are more likely to be able to coinfect the same hosts because they share genetic factors that bypass the defence systems of particular hosts, (ii) there is selective competitive exclusion between unrelated fungal genotypes, or (iii) pollinators deposit related strains on healthy plants because they have visited spatially-close diseased plants carrying similar genotypes due to spatial structure (Giraud 2004). In support of the competitive exclusion hypothesis, experiments have shown that sequential co-infections were more successful when the challenging strain was more closely related to the resident genotype (Koskella *et al.* 2006). Possible proximal mechanisms for competitive exclusion include vegetative incompatibility, inducing hyphal death after fusion between unrelated genotypes in fungi (Glass *et al.* 2000), or inhibition of competitor hyphal growth by toxins. However, one cannot rule out a role of host plant genotype in promoting high relatedness among *M. lychnidis-dioicae* strains within *Si. latifolia* plants. The spatial structure found in populations (Giraud 2004) would not explain the high relatedness among fungal strains within plants found in experimental inoculations (Buono *et al.* 2014; Koskella *et al.* 2006; López-Villavicencio *et al.* 2011) but could still play a role in nature in increasing further relatedness within plants (Lopez-Villavicencio *et al.* 2007).

In addition, the number of genotypes of *M. lychnidis-dioicae* strains occurring within *Si. latifolia* hosts and their relatedness were shown to affect virulence, i.e. the pathogen effect on plant fitness. Virulence was found to be higher for multiply infected plants than for singly infected ones, with more spores produced (and thus likely more host resource uptake by the pathogen), lower recovery probability and greater castration (Buono *et al.* 2014; Hood 2003; López-Villavicencio *et al.* 2011). However, higher castration rates in multiple infections were mitigated by greater relatedness (Buono *et al.* 2014; López-Villavicencio *et al.* 2011). This

90 indicated that the fungus behaved differently when competing with a close versus a distant
91 relative within the same host plant.

92 The emphasis on relatedness in pathogen-pathogen interactions connects the within-
93 host dynamics to the theory of kin selection, in which relatedness is a proxy of the probability
94 of sharing an altruistic allele that is identical by descent (Hamilton 1964a, b). Relatedness is a
95 relative measure of genetic similarity, being significantly positive when two individuals are
96 genetically more similar than two randomly chosen individuals. To assess relatedness, it is
97 therefore important to determine the spatial scale at which interactions may occur, which is
98 determined by the spatial scale of dispersal. In anther-smuts fungi, some species display
99 restricted dispersal, with geographically well-delimited clusters corresponding to glacial
100 refugia, while others show less geographic subdivision, indicating larger dispersal distance
101 and more mixing among genetic clusters (Abbate *et al.* 2018; Bueker *et al.* 2016; Feurtey *et*
102 *al.* 2016; Vercken *et al.* 2010).

103 The common soapwort *Saponaria officinalis* is a multi-stemmed, large, long-lived
104 perennial plant that is parasitized by the anther-smut fungus *M. saponariae*. These features
105 make it a good system for studying multiple infections. In addition, the dikaryotic hyphal
106 stage of *M. saponariae* can be cultured, unlike *M. lychnidis-dioicae*, permitting exploration of
107 *in vitro* interactions between more or less related strains. The anther-smut fungus *M.*
108 *saponariae* also parasitizes the related plant *Sa. ocymoides* (Lutz *et al.* 2005). Here, we
109 collected *M. saponariae* across Europe. We genotyped samples using microsatellite markers
110 to determine the genetic structure and thus scale of dispersal across Europe as well as within-
111 host patterns of infection (i.e., whether multiple genotypes co-occur within plants and whether
112 they are significantly related). In addition, we performed experimental inoculations on
113 clonally-propagated *Sa. officinalis* plants to test whether preferential co-infection by related

strains still occurred without effects of plant genotype or fungal spatial structure, which would support the competitive exclusion hypothesis. We compared virulence in terms of degree of castration and diversion of host resources to parasite reproduction (spore and smutted flower production) among infections by different numbers of fungal genotypes within plants and with varying degree of relatedness. We also investigated *in vitro* hyphal growth to explore the mechanism of competition or exclusion between different fungal strains, e.g., vegetative incompatibility or growth rate inhibition in the presence of competitors, and whether variation occurred depending on genetic similarity.

Material and Methods

Sampling, microsatellite genotyping and DNA sequencing

Microbotryum saponariae was sampled at 134 sites in different geographical locations across Europe, including 121 populations on *Sa. officinalis* and 13 populations on *Sa. ocymoides* (Supporting Information Table S1). We obtained permits for collections in national parks and the Nagoya protocol has been followed for all the samples collected after November 2014 (i.e., a list of samples was sent to the French “Ministère chargé de l’environnement”). In total we sampled 210 individual plants. From all 13 *Sa. ocymoides* and 68 of the *Sa. officinalis* plants, we sampled a single stem. For the remaining 129 *Sa. officinalis* plants we sampled multiple infected stems. Multiple diseased stems per plant were sampled in 53 *Sa. officinalis* populations, with spores from 532 anthers analysed, each sampled from a different stem, from 129 plants (i.e. a mean of 4.12 anthers per plant). Because *Sa. officinalis* can grow extensive rhizomes, it can be difficult to assess whether stems a few meters apart belong to the same plant. We took a conservative approach regarding the occurrence of multiple infections, by

considering stems to belong to the same plant only when they originated from the same root, and we then sampled all diseased stems per root. Mature diseased flower buds, containing diploid teliospores, were harvested from all diseased stems in each population, and were kept in silica gel at 4°C until use.

DNA was extracted from one sporulating anther of each analysed diseased flower with the Nucleospin Soil kit (Macherey-Nagel, Germany) and ten-fold diluted for PCR amplification. We used the 22 polymorphic microsatellite markers previously developed for *M. saponariae* (Fortuna *et al.* 2016), combined into six multiplexes, to genotype the fungal strains. The markers are spread across the genome on autosomes, except the marker Msap_146, which is located on the mating-type chromosome (Fortuna *et al.* 2016).

PCR reactions were performed separately for each multiplex in 12.5 µL volume containing 3 µL of DNA, ¼ of the recommended volume of multiplex PCR Kit (Qiagen, USA) and 1.25 µL of the primer mix. The primer mix included 2 µM of unlabelled forward and reverse primer and 0.5 µM or 0.75 µM of labelled forward primer depending on the dye label. FAM and Yakima Yellow dyes were used at 0.75 µM, and ATTO 550 and ATTO 565 dyes at 0.5 µM. The PCR cycling program used was the same as described above. PCR amplicons were diluted and fractionated by capillary electrophoresis at the GENTYANE INRA platform as previously described. Alleles of each locus were scored using GENEMAPPER 5.0 (Applied Biosystems, Inc., USA).

Population structure and genetic diversity

For population structure analyses, we used only one strain per plant, i.e., 210 fungal genotypes, to avoid biasing the analyses with clusters of related individuals. We assessed population subdivision using Bayesian clustering implemented in STRUCTURE 2.3.4 (Falush *et*

161 *al.* 2003; Pritchard *et al.* 2000). We conducted independent runs with different numbers of
162 clusters ($K=2$ to 8). Each run of 500,000 iterations and 50,000 burn-in iterations was repeated
163 fifteen times, using a model allowing for admixture and correlated allele frequencies. We used
164 CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) to identify potential distinct solutions among
165 the results of independent replicate runs for each K ('Greedy' algorithm; 1000 random input
166 orders; G' statistic), and to average individual assignment probabilities (q) over independent
167 runs. The maps showing cluster assignment per locality were drawn using the packages
168 mapplots (Gerritsen 2013) and maps (Becker *et al.* 2017) in R. Only individuals with
169 membership coefficient higher than 0.75 to a cluster were plotted on the map. Individuals
170 within a square of two degrees of latitude and longitude were pooled together to avoid pie
171 chart overlap.

172 Allelic richness (A_r) was computed with the package PopGenReport in R (Adamack & Gruber
173 2014). Within-cluster observed and expected heterozygosities (H_O and H_E) and F_{IS} , as well as
174 F_{ST} among genetic clusters, were computed using Genepop on the web (Raymond & Rousset
175 1995).

177 **Multiple infections and relatedness in natural populations**

178 We tested for multiple infections among the 532 single-anther samples from different stems
179 of 129 plants, i.e., we assessed whether distinct genotypes were found within individual
180 plants. We computed the relatedness among the distinct genotypes within plants. Because the
181 genetic clusters geographically largely overlapped, implying that strains from different
182 genetic clusters may interact within host populations, we calculated relatedness using the
183 whole sample of fungal strains as the reference population. Thus, we tested whether fungal
184 strains found in the same *Sa. officinalis* host individual were significantly related, i.e. were

more similar than two individuals drawn at random from among all sampled individuals. Relatedness was computed with the package ‘related’ in R (Pew *et al.* 2015) using the Lynch and Ritland estimator (Lynch & Ritland 1999).

Relatedness within each plant was computed using as reference population all fungal genotypes, including those from 99 individual plants found to harbour multiple fungal genotypes and 78 plants with a single fungal genotype (including all plants analysed, i.e., those with multiple stems or a single stem was sampled). When multiple stems of a given plant harboured the same fungal genotype, we retained only one of them in the dataset to avoid biasing the relatedness analyses by including the same fungal individual multiple times. We tested whether the mean relatedness coefficients within plants were significant by generating distributions of relatedness values by randomly shuffling individuals between plants, and calculating the average relatedness within each plant for each of 100 randomization step using the *grouprel* function in the R package ‘related’. P-values were computed as the percentage of randomized iterations where the average relatedness values of random groups were greater than or equal to the average of observed relatedness values.

Experimental inoculations under greenhouse conditions

Three different individuals of *Saponaria officinalis* grown from commercial seeds (Plant World Seeds, UK, 2015) were clonally multiplied as follows. We cultured apical buds cut from plants grown from seed *in vitro* in tubes with sterilised GH0 medium (Supporting Information Table S2). After shoot development of these plants, we cut off each node with one pair of leaves for new plantlet production. This procedure was repeated until we had 110 clonemates for each of the three plant genotypes. For inoculating plants, we prepared a spore suspension for each of four *M. saponariae* strains by suspending four anthers from a smutted

flower in 5 mL of 0.05% tween water and adjusting the concentration to 2.8×10^5 spores. μL^{-1} . We used four *M. saponariae* strains (#1047, #1206, #1207, #1228; Supporting Information Table S1) for inoculating plants, either with a single strain (single inoculation treatment, applied to 20 clonemates for each plant genotype and each fungal strain) or with a mixture of all four strains (multiple inoculation treatment, applied to 20 clonemates for each plant genotype). We soaked the whole plantlets in *Microbotryum* spore solutions for experimental inoculation treatments. For the control treatment (10 clonemates for each plant genotype), the plantlets were soaked in a 0.05% tween water solution with no fungal spores. The four inoculated *Microbotryum* strains were chosen to display a range of genetic similarity based on their shared allele distance (DAS) obtained from microsatellite markers (Supporting Information Table S3A). This range of shared allele distance allowed us to test if genetic similarity influenced the probability of co-infection and the virulence of the resulting co-infections. After inoculation (carried out on March 24th, 2016), we grew the plant clonemates *in vitro* in the GH0 medium (Supporting Information Table S2) under light and temperature controlled conditions for 19 days after which they were transplanted into soil in pots. The pots were then put directly on the greenhouse soil on May 3rd, 2016.

We observed each individual plant every two or three days over 10 weeks from late June until September 2016. We recorded the date of first flowering (number of days between fungal inoculation and first flower, diseased or not diseased) and counted the number of healthy and diseased stems and the number of healthy and smutted flowers. We genotyped smutted anthers as described above using the seven microsatellite markers (SAP_4, 27, 65, 73, 102, 107 and 109) displaying polymorphism among the fungal strains used for greenhouse inoculations. For the mixed inoculation treatment, we genotyped one anther from one diseased flower from each diseased stem for all diseased plants. In the single inoculation treatment, where we expected a single genotype per plant, we pooled three anthers, one per

diseased flower, from three diseased plants inoculated with the same strain. Genotyping the fungus of diseased plants inoculated by the same single strain showed that most were infected with the fungal genotype(s) that had been inoculated or crosses between them. Some “single strain inocula” (strain #1047, strain #1206 and strain #1228 in the plant genotype #2) appeared to have been mixes of very similar strains (e.g. differing only by being heterozygote or homozygote at one marker). However, this did not affect our analyses testing the effect of the number of genotypes and their relatedness since we analysed data based on the detected genotypes within plants. As above, we computed relatedness among fungal genotypes within plants, taking as reference population the genotypes found either across all plants or within each of the three plant genotypes.

To estimate the number of spores produced per flower, we analysed closed near-mature buds for several plants in the greenhouse, selecting balanced numbers of plants from each plant genotype and each number of fungal genotypes co-occurring within plants. We thus analysed 50 buds from different plants with a single fungal genotype, 60 buds from 30 plants with two fungal genotypes (one bud per stem in which different fungal genotypes had been detected), and 15 buds from the five plants with three fungal genotypes (one bud per stem in which different fungal genotypes had been detected). The whole dried buds were weighed, ground at 30 Hz during 20s with six glass beads per tube and re-suspended in 1.5 mL 0.05% tween water. We strained the solution twice using a 40 μ m mesh to remove all plant material while letting the fungal spores pass through the mesh; we completed the tweened spore solution to 5 mL. For each bud, we averaged the number of spores counted in four different Malassez cells under the microscope.

Statistical analyses of experimental inoculations

To assess the factors influencing infection success of our experimental inoculations, we used a generalized linear model (GLM) approach with a binomial regression and a logit link function. This model included as main factors plant genotype (with three levels), treatment (with five levels: the four strains #1206, #1207, #1228, #1047 and the mixture of four strains) and interactions between main factors. We only included data from flowering inoculated plants. An analysis of deviance based on a chi-squared test was performed to test model fit. Confidence intervals for percentages of diseased plants were computed using a binomial distribution with the `binconf` function implemented in the package `Hmisc` in R. To investigate the impact of the inoculation treatment, the identity of fungal strains, their co-occurrence within plants and their relatedness on the degree of castration of diseased plants (i.e. proportion of flowering stems that were diseased), we fitted GLMs using Poisson regressions with identity link functions. Not all factors could be tested in a single model (e.g., relatedness can only be tested in plants with at least two fungal strains), so we fitted three separate models that included as main factors, respectively: 1) plant genotype, treatment (with five levels: the four strains #1206, #1207, #1228, #1047, and the mixture of four strains) and their interactions; 2) plant genotype, number of fungal genotypes detected within the plants and their interactions; 3) plant genotype, number of genotypes detected within the plants with at least two fungal genotypes and their relatedness within plant (continuous variable) and their interactions. The first and second model included all diseased plants and the third model included only the diseased plants harbouring at least two fungal strains. An analysis of deviance was performed as above. To test whether the various strains showed different infection success under single versus multiple infections we performed a chi-squared test.

To assess the factors influencing diseased flowering phenology, the number of diseased flowers and the number of fungal spores produced per bud, we performed linear model

ANOVAs (type II due to unbalanced design) including the same factors as the three models fitted for plant castration analyses. Diseased flowering phenology was assessed as the difference in flowering date of the first diseased flower of each diseased plant and the mean flowering date of the uninoculated plants for the same plant genotype. In the second model for the number of diseased flowers, two additional continuous variables were added: the proportion of diseased stems and flowering onset. Tukey's HSD post hoc tests were performed to explore pairwise differences between factor levels in ANOVA or GLM models. Normality and homoscedasticity of residuals were checked for all linear models (Shapiro-Wilk test and Breusch Pagan test, respectively). In all models, non-significant interactions were sequentially removed from the models, except in cases where their inclusion improved the normality of the residuals. A student t-test was performed to assess whether the disease affected the flowering phenology compared to the healthy control plants. R commander (Fox 2005) and R 3.4.2 (R Core Team 2013) were used for all the statistical analyses.

Experimental *in vitro* hyphal growth measures

We measured *in vitro* growth of four different dikaryotic strains in pairwise competition trials (strains # 4748, 6361, AG and OQ, Table S1). In *Microbotryum*, conjugation that results in a dikaryotic hypha can only occur between haploid cells of different mating types. Haploid sporidia were isolated by micromanipulation following germination and meiosis to obtain monosporidial colonies of opposite mating types that were grown separately on potato dextrose agar (PDA) medium. We generated four different dikaryotic strains by combining specific haploid sporidia of opposite mating types. Each cross was carried out between two mating types from the same diploid parent, i.e. representing a selfing event (Supporting Information Table S1). The four *M. saponariae* strains were chosen to display a range of

genetic similarity based on their shared allele distance (DAS) obtained from microsatellite markers (Supporting Information Table S3B).

To generate the dikaryotic hyphae we mixed the haploid sporidia of opposite mating types in 0.05% tween water solution with a concentration of ca. 6.5×10^5 cells/ μ L. We used four of the six possible pairwise combinations of strains, chosen for a gradient of genetic similarities. For competition trials, 1 μ L drop of one strain was deposited in the middle of a 9cm-diameter Petri dish containing 3mL of GM96 medium (Supporting Information Table S2), which promotes *M. saponariae* hyphal growth, and its competing strain was deposited in a ring around the first central strain using a cloning cylinder of 4.7 mm diameter (Supporting Information Fig. S1). For each pair of strains, different Petri dishes were used to grow each strain either in the centre or in the outside location. Each of the four strain was also grown facing itself (Supporting Information Fig. S1). This last test compared growth with and without a competitor of a different genotype. Each combination was replicated three times. After three and ten days of growth, ten pictures were taken in different locations of each Petri dish. Six pictures of the inside strain hyphae (P1 and P2 regions, Supporting Information Fig. S1) were taken and four of the outside strain hyphae (P3 and P4 regions, Supporting Information Fig. S1) using a Nikon DSFi2 camera incorporated in the Nikon eclipse TS100 microscope. Because the physical distance between the inoculation sites of the two strains was not identical in all Petri dishes, it was measured to be included in the statistical model. For each Petri dish, hyphal density and hyphal length were measured in three locations, “inside” (hyphae from the central strain; P1 and P2 regions in Supporting Information Fig. S1), “middle” (hyphae growing towards the centre for the external strain; P1 and P2 regions in Supporting Information Fig. S1) and “outside” (hyphae growing towards the edge for the external strain; P3 and P4 regions in Supporting Information Fig. S1), using the software NIS-Elements D 4.40.00. Hyphal density was estimated as the number of hyphae divided by the

length of the arc on which hyphae had been measured and was counted on four pictures at magnification 100x (one picture in each of the regions P1 to P4, Supporting Information Fig. S1). Hyphal length was estimated as the average of three hyphal length measures on each of two pictures per Petri dish (i.e. P1 and P2 or P3 and P4, Supporting Information Fig. S1), at magnification 400x for the regions P1 and P2 and at magnification 200x for the regions P3 and P4.

Statistical analyses of *in vitro* hyphal growth measures

To assess the factors influencing the density and length of *M. saponariae* hyphae *in vitro* at day 3 and day 10, we performed a linear model ANCOVA (type II due to unbalanced design). This model included as main factors fungal strain (with four levels: #6361; #4748; #AG; #OQ), genetic similarity between strains (continuous variable), strain location (with three levels: inside, middle and outside), physical distance between the strains (continuous variable), and their interactions. R 3.4.2 (R Core Team 2013) was used for all the statistical analyses.

Results

Population structure

The STRUCTURE analysis, from K=2 to 8 clusters, revealed additional (compared to K=n-1) and well-defined clusters up to K=5 (i.e., clusters encompassing multiple genotypes with membership coefficients near the maximum; Fig. 1A and Supporting Information Fig. S2). There were fewer admixed individuals at K=5 than at lower K values and increasing K further

354 did not reveal additional well-delimited clusters (Supporting Information Fig. S2). We
355 therefore considered $K=5$ to be the biologically most relevant number of clusters. The genetic
356 diversity was quite low in all clusters (Supporting Information Table S4A), as is typical in
357 *Microbotryum* fungi. F_{ST} among clusters ranged from 0.22 to 0.63 (Supporting Information
358 Table S4B). Strains collected on *Sa. ocymoides* were assigned to clusters with those from *Sa.*
359 *officinalis*, indicating no obvious host-specialization for the fungi of these two host species
360 (Supporting Information Fig. S3). The distributions of the different clusters largely
361 overlapped geographically, indicating dispersal at large geographic scale (Supporting
362 Information Fig. 1B), and thus that strains belonging to different clusters may often interact
363 and compete. All clusters but one had significant deficits in heterozygotes, in agreement with
364 the high levels of selfing generally found in *Microbotryum* fungi (Supporting Information
365 Table S4A). The discriminant analysis of principal component (DAPC) analysis, which
366 makes no assumptions about outcrossing, generated similar general patterns (Supporting
367 Information Fig. S3).

369 **Multiple infections in natural populations and relatedness within plants**

370 A high proportion of plants carried multiple infections in natural populations, with 76.7 % of
371 the 129 plants harbouring more than one distinct fungal genotype (Fig. 2A). We detected 2.56
372 ± 0.11 (mean $\pm$ SE) genotypes per plant in the 4.27 ± 0.15 (mean $\pm$ SE) analysed diseased
373 stems per plant. Given the large geographical overlap found between clusters, fungal
374 individuals from distinct clusters may interact so that the most appropriate reference
375 population for computing relatedness pooled all clusters. The fungal genotypes sharing the
376 same individual host plant were significantly related, i.e., more similar than individuals drawn

at random from the sampled genotypes (observed mean relatedness among fungal genotypes within plants $r=0.62$, $P<0.01$; Fig. 2B).

Multiple infections, relatedness within plants and virulence measures under experimental inoculations

In order to control for potential population structure and host genotype effects favouring the establishment of related individuals within a given host plant, we performed experimental inoculations in the greenhouse on clonally-propagated *Sa. officinalis* plants. A total of 323 plants flowered between June and September, producing an average of 2.7 ± 0.1 (mean $\pm$ SE) stems per plant. All 30 of the uninoculated control plants flowered and none showed disease symptoms. A proportion of 0.78 ± 0.04 (mean $\pm$ SE) of inoculated plants produced diseased flowers and 0.77 ± 0.05 (mean $\pm$ SE) of the diseased plants bore only diseased stems. Infection success did not differ significantly between single and multiple inoculations or among plant genotypes but did among strains in single inoculations (Table 1; Fig. 3). We found no significant interaction between fungal strain and plant genotype on infection success (Table 1), indicating that there was unlikely to be a filter of fungal genotypes by the plant genotypes that could generate significant relatedness among strains within plants.

We genotyped one anther per stem per diseased plant inoculated with the mixture of four strains (one to eight stems, mean $\pm$ SE of 2.37 ± 0.18 stems per plant). Among those with multiple stems, we found a proportion of 0.72 plants carrying more than one fungal genotype per plant (1.80 ± 0.09 ; mean $\pm$ SE number of detected genotypes per plant). In multiply infected plants, fungal genotypes within plants were significantly related, i.e. were more similar than expected by chance given the fungal genotypes present in the greenhouse plants, both considering all clonemates from the three plant genotypes altogether (mean relatedness

among fungal genotypes within plants $r=0.25$, $P<0.01$), and for two of the three plant genotypes when analysing them separately (Fig. 4; plant genotype #2: mean $r=0.22$, $P=0.01$; plant genotype #3: mean $r=0.31$, $P=0.02$; plant genotype #8: mean $r=0.12$, $P=0.37$). Among the fungal genotypes found in diseased plants that corresponded to inoculated genotypes and not recombinant genotypes following multiple inoculations, the genotypes that had been found the most infectious upon single inoculations (Fig. 3) were even more represented among infecting genotypes following multiple inoculations ($\chi^2=15.77$; d.f.=6; $P=0.015$; Null hypothesis based on the percentage of infected plants by each genotype in single inoculations).

We then analysed measures of virulence (degree of castration, Fig. 5) and resource uptake by the pathogens (number of diseased flowers and spore number per flower) as functions of the number of genotypes within plants. For plants with at least two fungal genotypes, we also tested the effect of relatedness among fungal genotypes within plants on virulence and resource uptake. We found no significant effect of the number of genotypes in the plant on the degree of castration (Table 2A). For the diseased plants with at least two fungal genotypes, the degree of castration did not vary significantly with relatedness among fungal strains (Table 2B). Inoculation treatment had no effect on the degree of castration, in particular there was no significant variation among strains in their degree of castration (Supporting Information Table S5).

Disease retarded flowering such that smutted flowers were produced later than healthy flowers of uninoculated controls (mean difference in flowering date $\pm$ S.E. = 2.4 ± 0.7 ; t test, $t=3.37$, $P=0.0008$). Among diseased plants, the effect of the number of genotypes in the plant on flowering time was not significant, although the p-value was close to the 0.05 threshold (Table 3A). Among diseased plants with at least two fungal genotypes, relatedness among fungal genotypes had no significant effect on phenology of diseased flower production (Table

3B). The production of smutted flowers varied significantly among plant genotypes, increased with increasing percentage of diseased stems, and was greater for plants that flowered earlier (Supporting Information Table S6). We found no effect of the number of fungal genotypes or their relatedness within plants (Supporting Information Table S6). The number of smutted flowers did not vary significantly among fungal strains in single inoculations (Supporting Information Table S7).

We found no significant effects of inoculation treatment, plant genotype, number of fungal genotypes within plants, or relatedness among fungal genotypes on the number of spores produced per bud for the 124 diseased plants infected with one to three fungal genotypes that we analysed (Supporting Information Tables S8, S9A, S9B).

***In vitro* hyphal growth**

In order to assess whether the high within-plant relatedness resulted from relatedness-dependent hyphal growth or incompatibilities under a competition regime, we grew pairs of fungal strains *in vitro* in close interaction within Petri dishes. We measured hyphal length and density in competition situations and observed whether particular interaction behaviours occurred (Supporting Information Fig. S1, Fig. 6). The different dikaryotic strains varied in their hyphal density and length when growing *in vitro* (Tables 4 and Supporting Information Table S10; Fig. 7). We observed no sign of vegetative incompatibility or hyphal death. However, there was an effect of competition, with hyphae growing much longer and denser when on the outside, i.e., when not facing a competitor (Figs. 6 and 7; Table 4 and Supporting Information Table S10). While the genetic similarity between interacting strains had no significant effect on hyphal length, it affected hyphal density (Table 4 and Supporting

Information Table S10), although this effect was strain-dependent (Table 4, Figs. 7 and Supporting Information Fig. S4).

Discussion

Population structure in *Microbotryum saponariae*

The strains parasitizing the two species of *Saponaria*, *Sa. ocymoides* and *Sa. officinalis*, were not assigned to distinct genetic clusters, indicating that a single *Microbotryum* species parasitizes both host species as previously suggested by their identical ITS sequences (Kemler *et al.* 2009). While many *Microbotryum* species are host specific (de Vienne *et al.* 2009a; de Vienne *et al.* 2009b; Le Gac *et al.* 2007a; Le Gac *et al.* 2007b; Sloan *et al.* 2008), other more generalist species have been found, such as those parasitizing several closely related *Dianthus* host species (Le Gac *et al.* 2007a; Petit *et al.* 2017; Refrégier *et al.* 2008). We found that *M. saponariae* had high levels of homozygosity indicating a selfing mating system as in the other anther-smut fungi studied so far (Hood & Antonovics 2000; Hood & Antonovics 2004; Hood *et al.* 2005).

The anther-smut parasitizing the two *Saponaria* species showed genetic subdivision with several distinct genetic clusters. These clusters, however, showed no geographical pattern consistent with glacial refugia such as was found previously for *M. lychnidis-dioicae* across Europe (Feurtey *et al.* 2016; Gladieux *et al.* 2011; Vercken *et al.* 2010). Each cluster spanned most of the geographical range of sampling, indicating that interactions and competition may frequently occur between individuals belonging to different clusters. Thus,

the relevant scale for assessing relatedness among fungal strains within plants should be across all clusters and all sampled individuals.

Multiple infections and relatedness-dependent competition

We found frequent multiple infections by the anther-smut fungus *M. saponariae* in natural populations of the plant *Sa. officinalis* across Europe. The proportion of plants found to harbour multiple infections (>76%) is likely an underestimate, given that we used a conservative approach for delimiting individual plants (considering only stems obviously originating from the same root as one plant), while *Sa. officinalis* can grow extensive rhizomes. Multiple infections appear to be a general phenomenon in anther-smut fungi - Caryophyllaceae pathosystems, being also frequent in other anther-smut fungi, such as *M. lychnidis-dioicae* in *Si. latifolia* (Lopez-Villavicencio *et al.* 2007), *M. silenes-acaulis* in the long-lived plant *Si. acaulis* (Bueker *et al.* 2016), as well as the three *Microbotryum* species parasitizing *Dianthus* hosts (Petit *et al.* 2017).

In natural populations, we found that the anther-smut genotypes within *Sa. officinalis* individual plants were significantly related, at about the degree of full siblings. This means that fungal individuals are likely to interact with related individuals within a single host plant. Such a scenario sets the stage for kin selection to be an important evolutionary force in this system. Individuals carrying altruistic genes, for example genes that increase the prudence of the host exploitation strategy, are more likely to interact with other individuals bearing the same genes by descent. Under such conditions cooperative behaviour can evolve according to the kin selection theory (Hamilton 1964a, b).

Such high relatedness within plants in nature could be due to competitive exclusion of unrelated fungal strains but also to plant genotype effects, with more related strains being able to parasitize the same host genotypes. In addition, a fine-scale isolation by distance within populations may occur, with genetically more similar strains infecting spatially closer plants and being thus more likely to be co-deposited on healthy plants by pollinators. Regardless of the proximal mechanisms generating it, high relatedness among interacting fungal strains is expected to promote the evolution of cooperation. Nonetheless, one of our goals was to assess whether the significant relatedness among fungi within plants resulted from exclusion of unrelated competitors, plant genotype effect or mere fungal spatial structure; we therefore conducted experimental inoculations using clonally-propagated plant in the greenhouse. The finding of significant relatedness among fungal pathogens in the clonally-propagated plants showed that the similar high relatedness in natural populations was not a mere effect of fungal population structure or differential compatibility of certain fungal strains with certain host plants. The lack of significant interaction between plant and fungal genotypes in statistical models explaining infection success following experimental inoculations indeed provided no evidence that the relatedness of fungal genotypes within plants could be due to a higher ability of particular related strains to infect a given host genotype. A higher number of plants in experiments, and/or more diverse plant or fungal genotypes could reveal some genotype x genotype effects that can play a role in increasing relatedness among fungal strains within plants in nature. Similarly, isolation by distance within *Microbotryum* populations could also contribute to increase relatedness among coinfecting strains in nature. However, the high relatedness found within clonally-propagated plants in the greenhouse in our experiment without isolation by distance indicates that they are clearly not the sole effects and that competitive exclusion of unrelated strains must therefore also play an important role.

In fact, *in vitro* growth experiments suggested that competitive interactions may influence hyphal growth. While we did not observe any avoidance behaviour or vegetative incompatibility with mycelium death *in vitro*, mycelium length and density were reduced when facing a competitor. Furthermore, hyphal growth was dependent on the genetic similarity with the competitor for some strains, suggesting an active mechanism of relatedness-dependent competition among fungi. This is consistent with previous experimental inoculations showing that resident genotypes in *Si. latifolia* hosts more frequently excluded less related challenging strains (Hood 2003; Koskella *et al.* 2006). This means that an active mechanism has likely evolved in anther-smut fungi to exclude unrelated competitors for sharing host resources preferentially with related individuals, either by growing more rapidly or denser in the face of an unrelated competitor and/or by inhibiting its growth.

We found that some fungal *M. saponaria* strains were more successful than others at infecting *Sa. officinalis* plants, both in single and multiple inoculations. Interestingly, the infection advantage of the performant strains was exacerbated under multiple inoculation experiments, showing an effect of competition, which may also be mediated by mycelium growth inhibition in the face of competitors.

Our results on relatedness of fungal genotypes within plants are consistent with previous findings in the anther-smut fungus parasitizing *Si. latifolia* (Buono *et al.* 2014; López-Villavicencio *et al.* 2011), with the significant advantage here of being able to control for plant genotype effects and population structure. In contrast to previous findings in *M. lychnidis-dioicae* however, we did not detect in *M. saponariae* any impact of the number of genotypes within plants or of the relatedness among fungal strains within plants on virulence or plant resource uptake.

540

541 **Conclusion**

542 In conclusion, our study confirms that multiple infections by highly related strains are
543 common in anther-smut fungi. Our ability to clonally propagate *Sa. officinalis* allowed us to
544 demonstrate a role of relatedness between fungal strains itself in the success of coinfection,
545 and hence to refute that observed high relatedness in coinfections be due only to population
546 structure or genotype x genotype interactions between the pathogen and its host. The ability to
547 grow *M. saponariae* mycelium *in vitro* further allowed us to obtain insights into the proximal
548 mechanisms governing competition within plants, suggesting they may occur through
549 mycelium growth inhibition in the face of competitors. Such competitive interactions in
550 pathogens can have important evolutionary consequences on the ecology, epidemiology and
551 evolution of pathogens (Alizon *et al.* 2013; Susi *et al.* 2015; Tollenaere *et al.* 2016), although
552 they have remained too little studied so far in plant fungal pathogens.

553

554 **Acknowledgements**

555 This work has been funded by the grants MULTIVIR ANR-13-BSV7-0008 and Fondation
556 Louis D. We thank all collectors who helped us gathering *M. saponariae* samples (Supporting
557 Information Table S1). We thank Amandine Dubois, Lionel Saunois, Stéphanie Le Prieur,
558 Virginie Héraudet, Ying-Chu Lo and Manuela López-Villavicencio for her help, and the
559 GENTYANE INRA platform for assistance with genotyping.

560

561 **Author contribution**

TG, MEH and JAS designed and supervised the study and obtained funding. TG, MEH and TMF obtained samples. TMF, AS, CR and TR performed lab experiments. TMF, AN, TG and AB analyzed data. TG, TMF, AN and JAS wrote the manuscript with contributions by all other authors.

Data accessibility

The genotype and virulence data are available from the Dryad Digital Repository:
<https://doi.org/10.5061/dryad.75ip4q7>

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Supporting Information

Table S1: Host plant identification and geographical location of *Microbotryum saponariae* individuals sampled from *Saponaria* plants across Europe and sample collectors.

Table S2: Composition of the media used for *in vitro* culture of *Saponaria officinalis* clonemates.

Table S3: Shared allele distance (DAS) and genetic similarity obtained from microsatellite markers between the four *Microbotryum saponariae* strains used experiments.

Table S4: Summary statistics for the *Microbotryum saponariae* natural populations in *Saponaria officinalis* and *Sa. ocymoides* plants.

Table S5: Analyse of deviance of the generalized linear model (GLM) fitted to explain the degree of castration (number of diseased stems over all stems within plant) of diseased plants.

Table S6: Analyses of deviance of generalized linear models (GLM) fitted to explain the number of diseased flowers for diseased plants depending on the plant genotype, the proportion of diseased stems, the flowering onset, the number of genotypes, their relatedness and the interactions between the main factors.

Table S7: Analyse of deviance of the generalized linear model (GLM) fitted to explain the number of diseased flowers for all diseased plants of the plant genotype, the inoculation treatment and the interactions between the main factors.

Table S8: Linear model fitted to explain the number of spores per bud for all diseased plants in the greenhouse depending on the plant genotype, the inoculation treatment and the interaction between the main factors.

Table S9: Linear model fitted to explain the number of spores per bud for diseased plants in the greenhouse.

Table S10: Analyse of deviance of the generalized linear model (GLM) fitted to explain the hyphal length after three days of growth *in vitro*.

Figures

Figure 1. Population subdivision in the anther-smut fungus *Microbotryum saponariae* on *Saponaria officinalis* and *Sa. ocymoides*. **A)** Proportions of ancestry in five clusters of *Microbotryum spp.* genotypes inferred with the STRUCTURE program at K=5. Each genotype is represented by a vertical bar, partitioned into K segments representing the amount of ancestry of its genome in K clusters. See Supplementary Figure S2 for barplots at K=2 to 8. Samples are ranked according to decreasing proportions of ancestry in their main cluster, the different clusters being separated, in an arbitrary order. **B)** Map representing the *M. saponariae* sampled populations in 134 populations of the host plants *Sa. officinalis* (pie charts circled with thin black lines) and *Sa. ocymoides* (pie charts circled with bold black lines) in Europe, and their assignment to the five main genetic clusters represented using the colours from structure clusters (A). Pie charts represent the proportion of fungal genotypes assigned to the different clusters, taking only into account those assigned to a cluster with a probability higher than 0.75, which corresponded to 76% of the analysed samples (160 samples out of 211). Individuals within a square of two degrees of latitude and longitude were pooled together to avoid pie chart overlap. Pictures of diseased plants of *Sa. officinalis* (on the right bottom) and *Sa. ocymoides* (on the left bottom) are shown.

Figure 2. Multiple infections of *Microbotryum saponariae* anther-smut fungi in natural populations of *Saponaria officinalis* with the distributions of the number of genotypes per plant (**A**) and of the relatedness among fungal genotypes within plants (**B**) with the observed values in grey and the simulated values in permutations of genotypes across plants in black. The boxplots represent the median (center line), the 25th and 75th percentiles (box bounds) and the 5th and 95th percentiles (whiskers).

Figure 3: Proportion of diseased plants resulting from greenhouse inoculation using a single strain (#1206, 1207, 1228 or 1047) or multiple strain (“all”) inocula of *Microbotryum saponariae* anther-smut fungi in *Saponaria officinalis* plants belonging to three distinct clonally-propagated plant genotypes. Plants correspond to clonemates originating from three plant genotypes: #2 (light grey), #3 (grey), #8 (dark grey). Bars correspond to 95% confidence intervals calculated from binomial probabilities.

Figure 4 - Relatedness distribution among *Microbotryum saponariae* genotypes within *Saponaria officinalis* plants in the greenhouse experiment. Plants correspond to the clonemates originating from three plant genotypes (#2 light grey, #3 grey, #8 dark grey) carrying two or more fungal genotypes. The boxplots represent the median (centre line), the 25th percentile and 75th percentiles (box bounds), and the 5th percentile and 95th percentiles (whiskers).

Figure 5 - Proportion of diseased stems (degree of castration) in greenhouse *Saponaria officinalis* plants carrying one, two or three genotypes of *Microbotryum saponariae*. Plants correspond to clonemates originating from three plant genotypes: #2 (light grey), #3 (grey), #8 (dark grey).

Figure 6: *In vitro* mycelium growth experiments. Pictures of *Microbotryum saponariae* hyphae growing *in vitro* using an inverted microscope. See Figure S1 for a schema of the experimental design and definitions of outside and inside strains. **A)** Mycelia at the confrontation zone for the strain #6361 situated as both the internal and external strains after three days of growth. Inverted microscope x100. Scale bar = 100 μ m; **B)** Mycelia at the confrontation zone for the strain #6361 situated as the internal strain and the strain OQ situated as the external strain after three days of growth. Inverted microscope x100. Scale bar

= 100 μ m; **C)** Strain OQ growing inside as the external strain facing itself after three days of growth. Inverted microscope x200. Scale bar = 100 μ m; **D)** Strain OQ growing inside as the internal strain facing itself after three days of growth. Inverted microscope x400. Scale bar = 60 μ m; **E)** Strain AG growing inside as the internal strain facing the strain OQ growing as the external strain, after ten 10 days of growth. The black arrow indicates a hypha septum. Inverted microscope x400. Scale bar = 60 μ m; **F)** Mycelia at the confrontation zone of the strain #6361 growing inside as the internal strain and the strain OQ situated as the external strain after ten days of growth. Inverted microscope x100. Scale bar = 100 μ m; **G)** Mycelia at the confrontation zone of the strain #6361 growing inside as the internal strain and the strain #4748 situated as the external strain after ten days of growth. Inverted microscope x200. Scale bar = 100 μ m; **H)** Mycelia at the confrontation zone of the strain #4748 situated as the internal strain and the strain #6361 situated as the external strain after ten days of growth. Inverted microscope x400. Scale bar = 60 μ m;

Figure 7: *Microbotryum saponariae* hyphal density after ten days of *in vitro* growth, for the four strains (AG, OQ, #4748, #6361), and in three locations, inside (light grey), middle (grey) and outside (dark grey). For more details about experimental set up see Figure S1.

Table 1: Analyse of deviance of the generalized linear model (GLM) fitted to explain the probability to become diseased for plants in the greenhouse depending on the plant genotype (three levels: genotypes #2, 4 or 8), the inoculation treatment (five levels: strains #1206, 1207, 1228, 1047 and all strains in a mixture) and the interaction between the main factors. The GLM approach used a binomial regression with logit link function. Tukey HSD post-hoc tests for significant effects are given in footnotes.

	χ^2	D.f.	P-value
Plant genotype	0.993	2	0.6087
Inoculation treatment ¹	53.873	4	<0.001***
Plant genotype x Inoculation treatment	6.987	8	0.5380

¹The strain #1047 infected significantly fewer plants than the other strains (Tukey HSD, 1047 vs 1206: q=-0.53, P<0.001; 1047 vs 1207: q=-0.35, P<0.001; 1047 vs 1228: q=-0.33, P<0.001; 1047 vs all: q=0.38, P<0.001).

¹The strain #1206 infected significantly more plants than the strain #1228 (q=-0.20, P=0.05).

¹Similar proportions of plants became diseased when inoculated with the mixture of all strains and with single inocula (strains #1206, 1228 and 1207).

Table 2: Analyse of deviance of generalized linear models (GLM) fitted to explain the degree of castration *i.e.* the proportion of diseased stems of all diseased plants A) depending on the plant genotype (three levels: genotypes #2, 4 or 8), the number of fungal strains within plants (three levels: 1, 2 or more) and the interaction between the main factors; B) depending on the plant genotype (three levels: genotypes #2, 4 or 8), the number of fungal genotypes within plants (two levels: two or more), their relatedness and the interactions between the main factors. The GLM approach used a Poisson regression with identity link function and the total number of stems within plant as offset. Non-significant interaction was removed from the model.

(A)

	χ^2	D.f.	P-value
Plant genotype	0.0315	2	0.9844
Nb. genotypes	5.1089	2	0.0777

(B)

	χ^2	D.f.	P-value
Plant genotype	0.1262	2	0.9388
Nb. genotypes	2.7829	1	0.09528
Relatedness	0.2127	1	0.6447

Table 3: Analyses of variance of the linear model fitted to explain the onset of flowering A) for the first diseased flower of greenhouse plants (inoculated with a single strain or multiple strains) depending on the plant genotype (three levels: genotypes #2, 4 or 8), the number of fungal genotypes (three levels: 1, 2 or more) and the interaction between the main factors; B) for the first diseased flower of greenhouse plants infected by at least two fungal genotypes depending on the plant genotype (three levels: genotypes #2, 4 or 8), the number of genotypes (two levels: two or more), their relatedness and the interaction between the main factors. Data was cubic root transformed to better meet the requirements of normality and homoscedasticity of errors. Non-significant interaction was removed from the model. Tukey HSD post-hoc tests for significant effects are given as footnotes.

(A)

	Sum of squares	D.f.	F-ratio	P-value
Plant genotype	12.82	2	1.5190	0.2212
Nb. genotypes ¹	24.84	2	2.9439	0.0547

¹ Plants diseased by three or more fungal genotypes tended to flower later than those diseased by a single genotype (Tukey HSD: $q=1.996$, $P=0.052$).

(B)

	Sum of squares	D.f.	F-ratio	P-value
Plant genotype	12.111	2	1.7196	0.1948
Nb. genotypes	10.223	1	2.9029	0.0978
Relatedness	7.057	1	2.0039	0.1663

Table 4: Analyse of deviance of the generalized linear model (GLM) fitted to explain the hyphal density after three days of growth *in vitro* depending on the fungal strain identity (three levels: #AG, #OQ, #6361, #4748), the physical distance between strains on the medium, the genetic similarity between strains, the location of the measure (three levels: inside strain, outside strain, outside strain-inside, see Fig. S1) and the interactions between the main factors. The GLM approached used a Gaussian regression with identity link function. The non-significant interactions were sequentially removed from the models. The analysis of hyphal density after ten days of growth gave similar results.

	χ^2	D.f.	P-value
Strain	0.098	3	<0.001***
Physical distance	0.004	1	<0.001***
Genetic similarity	3.5×10^{-5}	1	0.578
Location	0.072	2	<0.001***
Strain * Location	0.008	6	<0.001***
Strain * Location* Genetic similarity	0.003	11	0.001**

Figure 1

175x202mm (100 x 100 DPI)

(A)

Number of genotypes
within plant

Molecular Ecology

(B)

Frequency of plants

Page 38 of 43

Figure 4

138x123mm (100 x 100 DPI)

Figure 7

261x172mm (100 x 100 DPI)