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Loss of pathogens in threatened plant species

Amanda K. Gibson, Jorge I. Mena-Ali and Michael E. Hood

A. K. Gibson, J. I. Mena-Ali and M. E. Hood (mhood@amherst.edu), Dept of Biology, McGuire Life Sciences Building, Amherst College, Rts 9 and 116, Amherst, MA 01002-5000, USA.

Global declines in biodiversity create an urgent need to address the impact of infectious disease in the small and fragmented populations that characterize threatened species. However, the paucity of empirical data provides little ability to predict whether disease generally accelerates threatened species towards extinction or becomes less important as populations decline. This study tests whether plant species threatened with extinction exhibit lower disease frequencies and lower overall parasite species richness while also experimentally testing for the effect of physiological disease resistance. Herbarium surveys of the genus *Silene* revealed that anther-smut disease was significantly less frequent in threatened species than non-threatened species, and this effect was not constrained by the host phylogeny or by physiological resistance. Moreover, analysis across a much broader range of plants (using US Federal designations) revealed that species with endangered status had significantly lower species richness of fungal pathogens than closely-related, non-endangered species. These results support the role of host ecology, rather than physiological resistance or phylogeny, in determining overall lower incidences and diversity of diseases in plant species threatened by extinction. Low disease incidence accompanied by susceptibility in threatened species may result from selection against costly resistance genes in the absence of disease.

Ever-increasing numbers of species are threatened with extinction as environments change, habitats disappear, and populations face overexploitation (IUCN 2008). Our understanding of the unique ecological and evolutionary characteristics of these small, fragmented populations remains limited, despite their critical importance to conservation efforts. For such species, infectious disease represents a particularly relevant type of ecological interaction, due to the direct impact on host fitness and the dependence of disease spread upon host demography, including size and interconnectedness of populations (Hess 1996, Gog et al. 2002). In particular, threatened plant species have been largely ignored in the consideration of disease and host endangerment.

Some studies suggest that declining populations are more severely influenced by disease than are non-threatened species. For example, a reduction in available habitat may concentrate hosts into smaller, denser populations, facilitating disease transmission and increasing stress-related effects upon disease expression (Holmes 1996, Gillespie and Chapman 2008). Declining populations may also experience strong genetic effects, such as drift and inbreeding; the resulting loss of allelic diversity and heterozygosity has been linked to increased susceptibility and disease-induced mortality (Coltman et al. 1999, Reid et al. 2003, Spielman et al. 2004). These characteristics predict an enhanced role for infectious diseases in driving threatened species toward eventual extinction (reviewed by Smith et al. 2006, 2009). In addition, some factors that contribute to population decline, such as habitat disturbance and the introduction of exotic species, may also contribute directly to the emergence of disease in threatened species (Gilbert and Hubbell 1996).

In contrast, some studies have proposed that threatened species exhibit lowered incidence of disease relative to non-threatened species. An analogy can be drawn to 'marginal' populations (i.e. those at the boundary of a species range), which tend to be isolated and small and to harbour fewer natural enemies than populations in the center of the host's distribution (Carlsson-Granér and Thrall 2002, Galeuchet et al. 2005, Alexander et al. 2007). Indeed, such interconnectedness of a metapopulation structure has consistently been shown to impact pathogen ecology and evolution, including studies focussing on species conservation (Hess 1996, Gog et al. 2002). In one of the most pertinent empirical analyses, Altizer et al. (2007) recently quantified the parasite species richness in threatened and non-threatened primates. Controlling for sampling effort and phylogenetic relationships, they found that threatened primates had a reduction in the number of parasite species relative to non-threatened species. To our knowledge, a similar comparative study on pathogen species richness has not been conducted in other major groups of organisms.

Here, a three-part study on the role of disease in threatened plant species is presented. We focused on anther-smut disease, an infection of plants in the Caryophyllaceae caused by the fungus *Microbotryum violaceum sensu lato*. We applied phylogenetic comparative methods to determine whether threatened and non-threatened *Silene* species differed in the incidence of anther-smut disease in a survey of

herbarium specimens. Furthermore, to assess the possibility that threatened species lack disease due to higher resistance than non-threatened species, we measured physiological resistance of threatened *Silene* species via artificial inoculation. Finally, to determine whether observed patterns can be generalized to a broader range of plant species, we used available online pathogen databases to compare the diversity of fungal pathogens infecting North American plants with or without US federal status as threatened or endangered. These approaches provide novel insights into the dynamics of infectious disease and ecological phenomena that may accompany present-day population declines.

Methods

Model system

The fungal pathogen *Microbotryum violaceum* (Basidiomycetes: Microbotryales) causes anther-smut disease in plants of the Caryophyllaceae (Giraud et al. 2008). Anther smut sterilizes infected hosts by aborting female structures and replacing pollen in the anthers with powdery, dark-coloured fungal spores. The disease has been shown to have slight and inconsistent effects upon host mortality (Alexander and Antonovics 1995, Carlsson-Granér 2006). The disease is transmitted primarily by pollinators visiting infected flowers (Antonovics et al. 1994, Biere and Honders 1998). The pathogen overwinters inside the living plant and is thus restricted to populations of perennial hosts (Hood et al. 2010). Many perennial species in the Caryophyllaceae are hosts to *Microbotryum*, and within this plant family the genus *Silene* includes a particularly large number of host species (Thrall et al. 1993, Hood et al. 2010). Though traditionally referred to as a single species, the name *M. violaceum* for pathogens on the Caryophyllaceae represents a suite of divergent fungal species (Le Gac et al. 2007), with each typically being found on only a single host species (Refrégier et al. 2008). The genus name *Microbotryum* will be used hereafter to refer to this group of pathogens.

Herbarium surveys

Previous studies have shown that herbarium surveys can provide an accurate tool for determining natural distributions of the anther smut disease and other diseases (Evans 1987, Antonovics et al. 2003, Hood and Antonovics 2003, Alexander et al. 2007, Malmstrom et al. 2007). Recently, a broad herbarium survey, including $>$ 40 000 specimens in *Silene* and related genera in the tribe *Sileneae*, was used to estimate that anther-smut diseases occurs on 80% of roughly 800 perennial species (Hood et al. 2010). The presence of *Microbotryum* on herbarium specimens was determined by close examination of the anthers for the presence of the characteristic brown or purple spores (Fig. 1). As with prior studies on anther-smut disease (Antonovics et al. 2003, Hood and Antonovics 2003), there was little evidence from collectors' notes or subsequent annotations that plant specimens were recognized as diseased. As further support that the data were not biased by the appearance of anther-smut

Figure 1. Diseased herbarium specimen. Anthers of the diseased specimen are darkly-coloured and carry *Microbotryum* spores, while healthy anthers are often yellow and bear signs of pollen.

symptoms, Hood et al. (2010) showed that disease rates were not correlated with either flower size or color, which could have affected how conspicuous the disease appears to collectors. *Silene* species in the herbarium survey of Hood et al. (2010) that have been classified as threatened were analyzed for disease occurrence (Table 1). Threatened here refers also to the associated designations of endangered and imperilled depending upon the source of the classification. Threatened species were identified through review of relevant literature and various online databases, including the IUCN Red List (IUCN 2006), the Center for Plant Conservation (Center for Plant Conservation 2008), the USDA Plants Database (USDA and NRCS 2008), and NatureServe 7.1 (NatureServe 2009). Threatened sub-species of an otherwise common species were excluded because this level of classification is treated differently across herbaria and has been subject to high rates of revision. Species regarded as threatened in only limited areas of their distribution were also excluded. Data on species designated as rare or vulnerable were then combined with threatened species as a less stringent extension of the main analysis.

The herbaria surveyed in Hood et al. (2010) contained fewer specimens for threatened than for non-threatened species. In a separate survey, additional herbarium material was obtained so as to increase the sample size for threatened species beyond the prior herbarium survey: 10 and 478 specimens of the threatened species of *Silene polypetala* and *Silene tatarica*, respectively, and 32 and 54 specimens of the rare/vulnerable species *Silene ovata* and *Silene regia*, respectively. The materials examined in the expanded survey were received on loan to the Massachusetts State Herbarium (MASS) from the following institutions (alphabetically ordered by herbarium code): Butler Univ. (BUT), Univ. of

Table 1. Numbers of herbarium specimens examined for the presence of anther-smut disease among threatened *Silene* species.

	Specimens	Reference
Threatened, endangered or imperilled species		
Silene biafrae	14	IUCN Red List
Silene chlorantha	85	Ingelög et al. 1993
Silene clokeyi	1	NatureServe
Silene diclinis	29	IUCN Red List
Silene fernandezii	2	IUCN Red List
Silene hawaiiensis	9	USDA PLANTS
Silene khasiana	5	Wildlife Inst. India
Silene kumaonensis	2	Wildlife Inst. India
Silene kunawarensis	16	Wildlife Inst. India
Silene lanceolata	5	USDA PLANTS
Silene nachlingerae	10	NatureServe
Silene petersonii	52	NatureServe
Silene plankii	6	NatureServe
Silene polypetala	40	USDA PLANTS
Silene seelyi	17	USDA PLANTS
Silene sennenii	8	IUCN Red List
Silene spaldingii	17	USDA PLANTS
Silene tatarica	81	Tero et al. 2005
Silene tomentosa	3	Galán de Mera 1993
Silene vagans	1	IUCN Red List
subtotal	405	
Rare or vulnerable species		
Silene dinarica	1	Dihoru and Parvu 1987
Silene hifacensis	3	IUCN Red List
Silene invisa	9	Flora of N. America
Silene nachlingerae	10	NV Heritage Prog.
*Silene nivea**	176	USDA PLANTS
*Silene ovata**	54	USDA PLANTS
*Silene regia**	167	USDA PLANTS
Silene subciliata	19	Flora of N. America
subtotal	439	

**Silene nivea, S. ovata and S. regia* are listed as threatened in five or six US states, but are not listed in some states where they are reported to occur. References provide the sources for species classification used in this study: USDA PLANTS (plants.usda.gov); IUCN Red List (<www.iucnredlist.org>); NatureServe (<www.natureserve.org>); Flora of N. America (<www.fna.org>); Wildlife Inst. India (<www.wii.gov.in/nwdc/plants.htm>); NV Heritage Program (heritage.nv.gov/).

Florida (FLAS), Univ. of Georgia (GA), Gothenburg Univ. (GB), Botanical Museum of the Univ. of Helsinki (H), Illinois Natural History Survey (ILLS), Univ. of Kentucky (KY), Miami Univ. (MU), Purdue Univ. (PUR), Univ. of Tennessee (TENN), Uppsala Univ. (UPS), and Univ. of South Carolina (USCH).

The proportion of herbarium specimens that were found to be diseased with anther-smut was compared for threatened and non-threatened species. Because the disease is restricted by host lifespan (Hood et al. 2010), only perennial threatened and non-threatened species were included in the analysis. To determine whether threatened species had fewer diseased specimens than expected, the binomial distribution probability of obtaining the observed number or fewer diseased specimens for threatened species was calculated based upon the disease rate among all perennial *Silene* specimens in the survey (i.e. overall disease rate = 0.0138, n = 28 375) and total number of threatened specimens examined. The analysis was repeated by relaxing the criterion for 'threatened' status by including additional species classified as rare or vulnerable. Threatened species and non-threatened species were also considered as independent groups for analyzing the numbers of diseased and healthy specimens by a Fisher's Exact test.

To determine whether species with threatened status belong to a phylogenetically restricted group, a phylogeny was reconstructed using chloroplast *rps16* intron sequences in GenBank NCBI (<www.ncbi.nlm.nih.gov/>; accession numbers Z83189, EF061360, DQ908814, DQ908818, DQ908819, EF061385, EF061378, DQ908822, AJ831770, DQ908825, EF061381, AY707940, AJ629915, DQ908831, DQ908833, DQ908835, EF061393, DQ908836, DQ908837, DQ908840, DQ908841, DQ908842, EF061394, DQ908846, AJ831773, DQ908847, DQ908849, AF242317, Z83173, AJ831765, DQ908852, EF674192, DQ908854 and Z83154). Threatened perennial *Silene* species were included based upon the availability of rps16 sequences. An approximately equal number of non-threatened *Silene* species, classified as having significantly high or low levels of disease in a previous study (Hood et al. 2010), were also included; to identify species that showed higher or lower disease frequencies than expected while accounting for differences in the number of specimens examined, the probabilities of deviation from random expectations (i.e. binomial distribution probabilities) were calculated for each species based on the diseased proportion of all perennial specimens. DNA sequences were aligned using ClustalW (<www.ebi.ac.uk/clustalw/>), and phylogenies were reconstructed in MEGA 4.0 software (Kumar et al. 2004) using a maximum parsimony analysis with the CNI heuristic search option, 100 random additions of sequences, and 1000 bootstrap pseudoreplicates. Bayesian posterior probabilities were determined using MrBayes ver. 3.1, with priors of state frequencies left at default settings and Markov chains initiated from a random tree, and run until the average standard deviation remained below 0.01 (i.e. 500 000 generations). These analyses based upon the *rps16* locus were intended to assess the distribution of threatened status rather than to provide systematic revisions to the group, which are underway elsewhere. *Agrostemma githago* was chosen as the outgroup to the *Silene* species based on Oxelman et al. (2001).

The correlation of disease status with threatened status was tested using a continuous Markov model in a maximum likelihood framework while controlling for the plant phylogeny, as described by Pagel (1994) and implemented in the program Mesquite 2.6 (Maddison and Maddison 2009) with 500 simulations. Evidence of phylogenetic signal for the discrete characters of threatened versus non-threatened status and presence versus absence of disease were tested in Mesquite 2.6 by comparing the observed state transition steps against a simulated distribution of state transition steps in which the character states were shuffled randomly among taxa (Maddison and Slatkin 1991). In this analysis a smaller number of observed state transition steps than expected from the randomized distribution (based upon 95% CI) would indicate that the discrete character is determined significantly by phylogenetic history; the simulated distribution of state transition steps was based on 1000 iterations.

Artificial inoculations

In order to assess whether lower levels of disease found in threatened species are the result of physiological resistance, a series of artificial inoculations was performed in the greenhouse. The following non-threatened species were used as controls: *Atocion rupestre*, *S. italica, S. latifolia, S. uniflora* and *S. vulgaris.* Control species are perennials known to maintain anther-smut in the wild and were thus expected to reflect a natural range of susceptible to disease under artificial inoculation. They were intended to control for the inoculation technique and to provide standard disease rates for comparison. *Silene sennenii* and *S. spaldingii* were chosen to represent threatened plant species for artificial inoculations, as well as the endangered subspecies *S. douglasii* var. *oraria*. Herbarium specimens of *S. douglasii* are infected by anther-smut at a rate of 0.019 (Hood et al. 2010), but the disease has not been observed in the intensively studied populations of this endangered subspecies (S. Kephart pers. comm.; Kepart and Paladino 1997, Lofflin and Kephart 2005).

Seeds of all plants were germinated in vitro and then grown to flower under greenhouse conditions. Seeds originated from natural native populations and were collected by the co-authors or by other botanists (source and locality data available upon request); there is no available information on the disease history of source populations. Seeds from each species originated from a single population, excepting *S. latifolia* which was collected from five different populations. The seed germination and inoculation procedures were as described by Hood and Antonovics (2000). Briefly, seeds were surface-sterilized prior to germination on agar media. Following expansion of the cotyledons, inoculum was applied to the plant apical meristem. After two to four days, the seedlings were transplanted to soil and maintained in the greenhouse. Upon flowering, the plants were visually assessed for symptoms of anther-smut disease and 15–250 plants per species were used in each treatment.

Each species was subjected to two different inoculation treatments. In the 'combined' inoculation treatment, a pooled mixture of *a*1 and *a*2 mating type cultures from thirteen strains of *Microbotryum* isolated from the following hosts were used: *Atocion rupestre*, *Dianthus carthusianorum*, *Lychnis alpina, L. flos-cuculi, Saponaria officinalis, Silene acaulis, S. caroliniana, S. italica, S. latifolia, S. lemmonii, S. notarisii, S. nutans* and *S. paradoxa*. According to recent work by Le Gac et al. (2007), these strains represent a range of at least nine phylogenetically-independent species that are found widely among *Microbotryum* infecting the Caryophyllaceae. In the 'single' inoculum treatment, the inoculum consisted of a single pathogen species, selected from the previous list so as to minimize genetic distance between the pathogen's native host and the target host. Species of *Microbotryum* have been found to be more successful at infecting hosts that are phylogenetically close to their co-evolved host than hosts that are more distant (Sloan et al. 2008, de Vienne et al 2009). Statistical comparisons for whether the threatened status of species affected the proportion of plants that became diseased were made using the generalized linear model procedures in SPSS ver. 12 assuming a binomial logit function (SPSS Inc., Chicago, IL, USA).

Survey of North American endangered plants

To determine whether patterns observed in the *Silene–Microbotryum* system are reflective of plant–fungal disease systems in general, a broader taxonomic survey was performed using available databases on plant and fungal species. Endangered plant species were identified through the United States Department of Agriculture (USDA) PLANTS Database (<http://plants.usda.gov/>), using the following search parameters: "distribution, floristic area" = North America, and "legal status, federal T/E status" = with federal status - endangered. Plants with the status of threatened were not included, to ensure that analyses were limited to species with stringently defined federal status. Each plant species listed with federal status as endangered was paired with a non-listed species using a DNA sequence similarity search of the NCBI GenBank database (<www.ncbi.nlm.nih.gov/>). A DNA sequence for each endangered species was retrieved from GenBank, preferring the commonly used nuclear internal transcribed spacer region (ITS) of the ribosomal RNA genes when available. The DNA sequence was then used for a BLASTn search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to provide a list of closely-related plant species. A paired plant species within the same genus was then examined in the USDA PLANTS Database to ensure that it was distributed in North America and without federal or state status as endangered or threatened. If the species was listed as having endangered or threatened status, the plant species with the next most similar DNA sequence was examined until a non-threatened species was obtained. If DNA sequences of an endangered species were unavailable in GenBank, a North American member of the same genus without status as endangered or threatened was chosen at random.

The number of fungal pathogen species per endangered and non-endangered plant species was obtained from the USDA Fungal Databases (Farr and Rossman 2008, retrieved 10 March 2010 from <http://nt.ars-grin.gov/fungaldatabases/>). Pathogen species richness was chosen as the measure of disease due to the format of available data and to provide a comparable assessment to prior studies of disease and host demography (Nunn et al. 2003, Altizer et al. 2007). Data from the USDA Fungal Databases were edited to remove repeated listings of pathogen species and entries where a genus name (i.e. *Microbotryum* sp.) was listed along with identified species of the same genus; data were not partitioned according to the date or region of reported diseases. The USDA Fungal Databases was created for plants identified as hosts to the fungi, representing primarily pathogens (<http://nt.ars-grin.gov/fungaldatabases/>); a search of 100 random fungal species associated with the queried plant species did not identify putative mutualists or other symbionts. To control for the effect of sampling effort per host species upon the number of known fungal pathogens, the number of published scientific papers for each species was used as a measure of search effort using the host species name as a search term in the ISI Web of Science (WOS) search engine (<http://apps.isiknowledge.com>). This approach is the same as was used by Altizer et al. (2007) to study parasite species richness of threatened primates and by Lindenfors et al. (2007) in addressing various correlates of disease in

carnivores. Only species pairs for which at least one published citation was available for both endangered and non-endangered members were included (47 pairs; Table 3). Significance of the difference in pathogen species richness between endangered and non-endangered species was tested by a generalized linear model in SPSS using negative binomial regression with search effort (i.e. number of citations) as a covariate; a negative binomial model with log link function was used because of overdispersion in the count data, and the scale parameter was computed based on the Pearson χ^2 variance estimate of the dependent variable. In addition, to provide a paired test of pathogen species richness between endangered and non-endangered species within host genera, the number of pathogens per citation was tested using the nonparametric Wilcoxon signed-rank test in SPSS. This signed-rank test was conservative because even though non-endangered species tend to have larger numbers of citations, the overall pathogens-to-citations relationship was best fit by a linear or logarithmic model rather than an exponential model; with non-endangered species tending to have larger numbers of citation, an exponential model would overestimate their pathogens-to-citation ratios while a logarithmic or linear relationship does not.

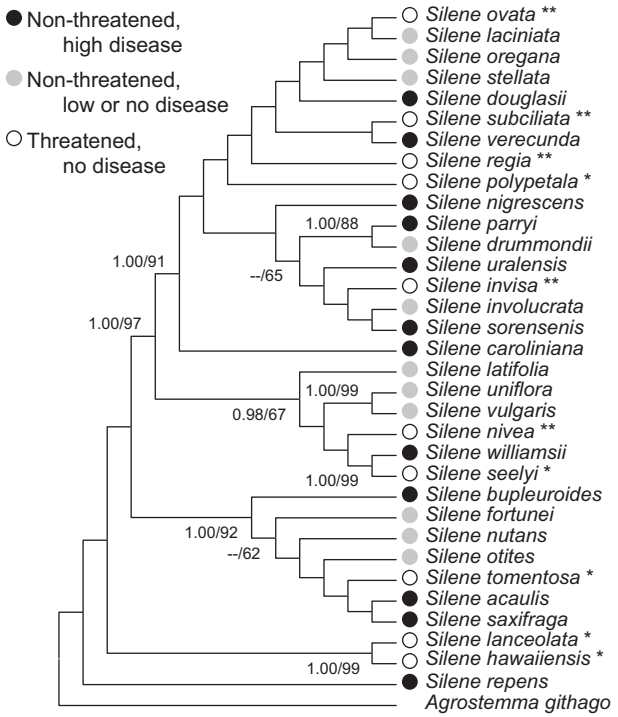

Figure 2. Phylogeny of plant species of the genus *Silene* based upon maximum parsimony analysis of *rps16* DNA sequences. Support values for tree topology are shown when they had values of Bayesian posterior probabilities/maximum parsimony bootstraps at least equal to 0.9/60, respectively. DNA sequences were obtained from GenBank NCBI for species in the categories of threatened (open circles; species classified as threatened, endangered, or imperilled are marked with asterisks, and rare or vulnerable species are marked with double asterisks), non-threatened with high disease (black closed circles), and non-threatened with low or no disease (grey closed circles). Non-threatened species were classified according to the results of Hood et al. (2009). *Agrostemma githago* was chosen as the outgroup based on Oxelman et al. (2001).

Results

Herbarium surveys

The frequency of anther-smut disease in herbarium specimens of threatened *Silene* species was significantly less than that for non-threatened species. No disease was found among 405 specimens of threatened perennial species (Table 1), where the binomial distribution probability of observing so few diseased specimens given the sample size and expected disease rate of 0.0138 (n = 28 375; Hood et al. 2010) based upon specimens of all perennial *Silene* species was a p-value = 0.0036; the disease frequency among non-threatened perennial *Silene* species was 0.0142 (n = 27 531). Furthermore, no disease was found among the 439 specimens of *Silene* species classified as rare or vulnerable, and the combination of these specimens with threatened species resulted in a binomial distribution probability of < 0.0001 of finding so few diseased specimens. Furthermore, the numbers of diseased versus healthy specimens from threatened and non-threatened species were significantly different at p = 0.0047 (Fisher's exact test), and with the inclusion of rare or vulnerable species was significant at $p < 0.0001$ (Fisher's exact test), indicating that disease frequencies differ significantly between threatened and non-threatened species.

For the four threatened or rare *Silene* species selected for the extended herbarium survey, no disease was found in these additional 574 specimens, further strengthening the statistical confidence of a lower rate of disease as compared to non-threatened species.

Analysis of the plant phylogeny revealed that *Silene* species with threatened status were found distributed across multiple well-supported clades and often together with non-threatened species having high and/or low levels of disease (Fig. 2). The negative correlation of threatened status and disease status was found to be statistically significant while controlling for the plant phylogeny (p-value from 500 simulation = 0.004; independent log likelihood = 40.317, correlated log likelihood = 35.778). There was no statistically significant evidence for a phylogenetic signal for the threatened status or disease status among the species included in the phylogeny; i.e. the estimated number of state transition steps in the host phylogeny was not less than the lower limit expected upon randomization (nine steps observed for threatened status and seven as the boundary of 95% confidence interval; 11 steps observed for disease status and eight as the boundary of 95% CI).

Artificial inoculation

Four of the five non-threatened control species were successfully infected with anther-smut disease, and infection rates were generally high (Table 2). All threatened species also demonstrated susceptibility to *Microbotryum*, displaying rates of infection between 40 and 100% (Table 2). Rates of infection did not differ between the threatened and non-threatened *Silene* (Wald's $\chi^2 = 5.8$, DF = 1, p = 0.563).

Table 2. Disease resulting from artificial inoculation of threatened and non-threatened *Silene* species. Total number of flowering individuals and rates of anther-smut disease on non-threatened and threatened experimental populations are given. Countries of origin for seeds are indicated in parentheses. All species became diseased with both single and combined sources of inoculum except *S. spaldingii*, where disease was only observed for the combined inoculum treatment. Revisions to the genus *Silene* have led to the new combination *Atocion rupestre* (Oxelman et al. 2001).

	Species	Number flowered	Proportion diseased
Non-threatened	*A. rupestre* (France)	21	0.76
	S. italica (Italy)	12	0.67
	S. latifolia *	243	0.68
	S. uniflora (England)	94	0.00
	S. vulgaris (United States)	62	0.11
Threatened	*S. douglasii var. oraria* (United States)	7	0.43
	S. sennenii (Spain)	54	0.37
	S. spaldingii (United States)	4	1.00

**S. latifolia* seeds were obtained from populations in the United States, England, Slovakia, Hungary and Croatia.

Survey of North American endangered plants

Pathogen species richness was significantly lower for endangered species when controlling for sampling effort by using the number of citations as a covariate (endangered status likelihood ratio $\chi^2 = 44.884$, DF = 1, $p < 0.001$); search effort and the interaction term were also significant predictors of pathogen species richness ($\chi^2 = 7.844$, DF = 1, $p = 0.006$ and $\chi^2 = 6.445$, DF = 1, $p = 0.013$, respectively), with non-endangered species showing a greater increase in pathogen species richness with sampling effort than endangered species. Among the 47 intra-generic species pairs, representing 29 plant families, endangered species had significantly lower ratios of pathogen species per citation than their non-endangered relatives (Wilcoxon signed-rank test of pathogens per literature citation, $Z = -5.163$, $p < 0.001$) (Fig. 3). The overall number of fungal pathogens per literature citation was nearly fourfold greater for non-endangered species (0.371) compared to endangered species (0.093). The correlation of pathogen species richness to literature citations for the overall dataset was best predicted by a linear regression model (linear $R^2 = 0.393$, logarithmic $R^2 = 0.323$, exponential $R^2 = 0.190$).

Discussion

In this time of striking ecosystem transformations and worldwide loss of biodiversity, the relationship between host rarity and infectious diseases has become a critical element in effective conservation programs (Smith et al. 2009). Studies have not agreed on whether diseases are likely to accelerate a declining species towards extinction (Gilbert and Hubbell 1996, Holmes 1996, Gillespie and Chapman 2008) or to play a diminished role in small, fragmented populations (Altizer et al. 2007). Our results strongly support an overall lower incidence of disease in plant species threatened with extinction. Threatened *Silene* species in the herbarium surveys were found to have a significantly lower rate of anther-smut disease compared to non-threatened species, a finding that was independent of both the *Silene* phylogeny and physiological resistance. The phylogenetic comparative methods indicate that threatened status and the correlation with lower disease frequencies have evolved multiple times in the *Silene* genus. At a much broader taxonomic level, we also found that plants species with US Federal status as endangered had fewer reported diseases than closely-related common species using an analysis that controlled for variation in search effort. In investigating disease on threatened members of the plant kingdom, our results help to provide a broad generalization to the results obtained by Altizer et al. (2007) on threatened primates. Our study is unique, however, in being the first to quantify an association between threatened status and reductions in infection frequency for a particular host-pathogen system (i.e. anther-smut disease).

The importance of within- and between-population transmission for the persistence of diseases has been most thoroughly addressed in plant systems (Burdon et al. 1995, Antonovics 2004, Laine 2004), but metapopulation structure has recently been recognized as essential to the epidemiological dynamics in animal systems, including humans, with relevance to questions of conservation and public health (Hess 1996, Gog et al. 2000, Bjørnstad et al. 2002, Adams and Kaplan 2009). In the anther-smut system, prior field studies may support the basic patterns of disease loss from small and fragmented populations, and such patterns may underlie the association of endangerment and low pathogen species richness more generally among plants. Antonovics et al. (1994) and Carlsson-Granér and Thrall (2002) reported that larger or more connected populations of the hosts *S. latifolia* and *Lychnis alpina*, respectively, are more likely to harbour anther-smut disease than smaller, isolated populations. Demographic studies of *Silene* indicate that smaller populations tend to be more ephemeral than larger populations, increasing the likelihood of local disease extinction, especially for obligate and specialized pathogens like *Microbotryum* (Thrall and Antonovics 1995). Although there is variation among taxonomic groups and among studies, large proportions of pathogens and parasites have been shown to exhibit host specificity (Pedersen et al. 2005, Poulin and Keeney 2008), or at least a strong phylogenetic constraint upon their host range in experimental inoculation studies (Gilbert and Webb 2007, de Vienne et al. 2009). However, it is worth noting that Altizer et al. (2007) found no significant difference in threatened primates for the loss of generalist versus specialist pathogens. Also, the USDA database of fungal pathogens used in this study represents a variety of fungal species characterized by a diverse range of specificities

Table 3. Details of survey of North American endangered plants. Listed are the 47 plant species with federal listing as endangered and phylogenetically-paired non-threatened species included in Wilcoxon signed-rank test analysis. Parenthetical values represent the numbers of fungal pathogens and the number of literature citations per species, respectively.

Endangered species	Non-endangered species
Agave × *arizonica* (0, 1)	*Agave chrysantha* (0, 1)
Ambrosia pumila (0, 1)	*Ambrosia acanthicarpa* (2, 3)
Amsinckia grandiflora (0, 26)	*Amsinckia menziesii* (2, 2)
Arabis serotin (0, 1)	*Arabis holboellii* (15, 21)
Arctomecon humilis (0, 4)	*Argemone munita* (0, 2)
Astragalus bibullatus (0, 3)	*Astragalus bisulcatus* (11, 23)
Astragalus holmgreniorum (0, 1)	*Astragalus hamosus* (9, 9)
Astrophytum asterias (0, 3)	*Astrophytum myriostigma* (4, 3)
Blennosperma bakeri (0, 1)	*Blennosperma nanum* (0, 1)
Cercocarpus traskiae (0, 2)	*Dryas octopetala* (137, 95)
Cucurbita okeechobeensis (0, 3)	*Cucurbita pepo* (133, 1048)
Dalea foliosa (0, 4)	*Dalea formosa* (1, 1)
Deeringothamnus rugelii (0, 2)	*Asimina parviflora* (2, 3)
Dicerandra christmanii (0, 2)	*Dicerandra linearifolia* (0, 8)
Echinacea laevigata (0, 5)	*Echinacea angustifolia* (8, 112)
Echinacea tennesseensis (0, 5)	*Echinacea atrorubens* (0, 2)
Fremontodendron decumbens (0, 2)	*Chiranthodendron pentadactylon* (2, 5)
Geum radiatum (1, 3)	*Geum macrophyllum* (11, 2)
Harrisia fragrans (0, 2)	*Harrisia martinii* (1, 3)
Helianthus schweinitzii (0, 3)	*Helianthus resinosus* (3, 5)
Hymenoxys texana (0, 3)	*Hymenoxys lemmonii* (0, 1)
Hypericum cumulicola (0, 20)	*Hypericum fasciculatum* (1, 3)
Ipomopsis sancti-spiritus (0, 1)	*Ipomopsis arizonica* (0, 1)
Isoetes melanospora (0, 1)	*Isoetes howellii* (0, 5)
Isoetes tegetiformans (0, 1)	*Isoetes mattaponica* (0, 1)
Liatris ohlingerae (0, 4)	*Liatris spheroidea* (4, 4)
Lindera melissifolia (0, 10)	*Litsea cubeba* (7, 26)
Lithophragma maximum (0, 1)	*Lithophragma campanulatum* (0, 1)
Lomatium bradshawii (0, 3)	*Tauschia parishii* (1, 1)
Malacothamnus fasciculatus (3, 1)	*Modiola caroliniana* (12, 1)
Nolina brittoniana (0, 2)	*Nolina micrantha* (12, 1)
Pedicularis furbishiae (0, 6)	*Pedicularis hirsuta* (3, 3)
Penstemon haydenii (0, 3)	*Penstemon spectabilis* (6, 3)
Penstemon penlandii (0, 1)	*Penstemon pinifolius* (1, 3)
Polystichum aleuticum (0, 3)	*Polystichum munitum* (23, 9)
Potamogeton clystocarpus (0, 1)	*Potamogeton pusillus* (1, 17)
Rhus michauxii (0, 9)	*Rhus glabra* (128, 32)
Sarracenia oreophila (0, 5)	*Sarracenia alata* (1, 14)
Schwalbea americana (0, 9)	*Epifagus virginiana* (4, 22)
Silene polypetala (0, 2)	*Silene uralensis* (4, 1)
Solidago shortii (3, 21)	*Solidago petiolaris* (3, 1)
Stephanomeria malheurensis (0, 3)	*Stephanomeria exigua* (0, 7)
Torreya taxifolia (13, 11)	*Torreya californica* (5, 4)
Trifolium amoenum (0, 1)	*Trifolium macraei* (2, 1)
Trifolium stoloniferum (1, 13)	*Trifolium carolinianum* (10, 1)
Warea carteri (0, 3)	*Warea cuneifolia* (0, 1)
Zizania texana (0, 13)	*Zizania palustris* (19, 73)

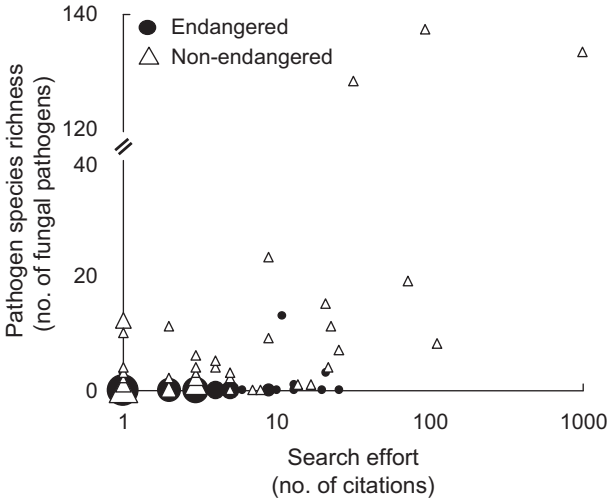

Figure 3. The relationship between search effort (literature citations) and the reported fungal pathogen species richness for endangered and non-endangered plant species. Citation numbers were obtained through the ISI Web of Science search engine, and numbers of fungal pathogens were obtained from the USDA Fungal Databases. Endangered status for each species was obtained from the USDA PLANTS Database. Endangered species are indicated by filled circles, and non-endangered species are indicated by empty triangles. Size of points corresponds to the numbers of observations with identical coordinates.

and transmission modes, and no distinctions were apparent in the patterns of loss. The loss of disease therefore appears to apply across a wide variety of pathogenic strategies. Moreover, in the case of local pathogen loss, re-introduction requires proximity to external disease sources, but such between-patch dynamics are less likely as host populations decline in size and number. Contact with closely related species may likewise decline, reducing the chances of reacquiring the disease via host-shifts; although threatened species may continue to be at risk of 'spill-over' diseases that are maintained independently on other, locally abundant hosts (Daszak et al. 2000, Pedersen et al. 2007). Thus, the loss of disease from marginal host populations is similar, but not entirely analogous to the biology of species threatened with extinction due to the general lack of connections to a larger source population of conspecifics for threatened species that could otherwise serves as source for recurrent disease introductions (Carlsson-Granér and Thrall 2002).

Even when the pathogen is introduced, the low density of hosts within marginal populations may limit transmission opportunities for many diseases, potentially below the level required for sustained infection ($R_o < 1$) (Antonovics et al. 2001). Similar expectations would exist for small population sizes for threatened species and may contribute to the result of lower pathogen species richness across the broad range of endangered plants. Interestingly, at least at small spatial scales, anther-smut disease exhibits frequency-dependent transmission; spore dispersal is accomplished through insect vectors that adjust flight distances in accordance with plant density (Antonovics and Alexander 1992). Even extremely small host populations can, according to theory, maintain a disease characterized by frequency-dependent transmission (Anderson and May 1991, Antonovics 2005). Therefore, this study's findings provide further evidence that, in natural populations, the observed association of species decline and disease loss is more likely to be caused by larger-scale between-patch dynamics involving rates of local extinction and re-colonization. Similarly, Altizer et al. (2007) found that the mode of disease transmission had no discernible effect on the association between the threatened status of primates and pathogen diversity, further suggesting the importance of regional or metapopulation effects. Given the distinctions between species endangerment and marginal populations mentioned above, assessing the importance of between-patch dynamics would benefit from further empirical studies on pathogen species richness over a range of spatial scales and including variation across host population size and fragmentation.

Importantly, an expectation when host ecology prevents the maintenance of disease is that costly physiological mechanism for resistance should be selected against (Simms 1992). Previous studies have demonstrated a cost of resistance to anther-smut disease (Biere and Antonovics 1996), and the selective loss of resistance genes in marginal populations is suggested by Carlsson-Granér and Thrall (2002); their study found that the highest prevalence of anther smut (proportion of local individuals infected) in the few marginal populations of *L. alpina* where disease was present, and the simulation model could capture this distribution of disease only when resistant individuals were selected against in healthy populations. Although drift-like processes in small, disease-free populations are likely to limit the efficiency of selection against costly resistance genes, Laine (2006) recently described "coevolutionary coldspots" in the *Plantago*-powdery mildew system where the absence of a pathogen at very fine spatial and temporal scales may drive the maintenance or loss of resistance. Moreover, because the current inoculation experiment necessarily used pathogen strains that were not endemic and likely were maladapted to the threatened hosts (Sloan et al. 2008), these plant species may be even more devoid of resistance than our results have initially suggested. Further studies are needed to address the evolutionary dynamics of disease resistance due to the combinations of selective and non-selective forces during species declines and the loss of associated pathogens.

Though all the threatened *Silene* species included here have perennial life-histories compatible with maintaining anther-smut disease (Hood et al. 2010), even species that are the subject of active investigations for purposes of conservation biology have not been found to be infected in the field (e.g. *S. tatarica*; Tero et al. 2005; J. Aspi pers. comm.). Similarly, *Silene douglasii*, a common North American species, is frequently diseased (Hood et al. 2010), yet disease is absent in intensively-studied populations of the endangered subspecies *S. douglasii var. oraria* (S. Kephart pers comm.; Kepart and Paladino 1997, Lofflin and Kephart 2005).

The threatened status of a species could potentially bias the collection of material from natural populations, but the lack of reports of disease in even these closely-monitored populations and the general absence of collectors' notes or annotations indicating recognition of the symptoms in infected plants helps to support the utility of herbarium surveys for accurately representing disease distribution in the field. It is not the case that all threatened *Silene* species are necessarily disease-free, but rather that the average frequency of anther-smut across threatened species is less than for non-threatened species. For example, there is at least one report in the literature of anther-smut on a threatened species, specifically *S. chlorantha* (Lutz et al. 2005), although this particular species was represented by too few herbarium samples to conclude that the level of disease was statistically different from non-threatened species. Field studies specifically to investigate the presence of disease in threatened species should be undertaken to confirm these results.

Conclusions from this study run contrary to previous research where threatened species experienced increased disease-related harm in association with habitat loss causing a greater concentration of individuals (Holmes 1996, Gillespie and Chapman 2008). Mobile animal species may thus represent a distinct category of threatened hosts, differentiated by their ability to move and form denser, more geographically-constrained populations. The loss of disease may apply more generally, encompassing species, such as plants and other non-motile organisms, which do not actively respond to habitat loss. Interestingly, the threatened status of even highly mobile primate species was associated with the loss of pathogen diversity (Altizer et al. 2007), suggesting that the ecological processes investigated here are of importance to a broad diversity of organisms. Future studies should therefore consider disease loss and the potential consequences for resistance variation during restoration of threatened species to larger numbers and occupancy of ancestral geographic distributions.

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