

Outcrossing increases infection success in the holoparasitic mistletoe *Tristerix aphyllus* (Loranthaceae)

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Abstract Most studies on the fitness advantage of outbreeding in host–parasite systems have been assessed from the host rather than the parasite perspective. Here, we performed experimental pollination treatments to evaluate the consequences of outbreeding on fitness-related traits in the holoparasitic mistletoe *Tristerix aphyllus* in a 2-year field study. Results indicate that self-pollinated plants had a lower fruit production than outcrossed plants (20.4% and 29.5% reduction in 2002 and 2003, respectively), and resulting inbred fruits were smaller than outcrossed fruits in both years. No effect was detected for seed mass. The percentage of germination of inbred seeds was 15.1% and 6.0% lower than outcrossed seeds in 2002 and 2003, respectively. Inbred seedlings had shorter radicles, which translated to a 71.6% and 60.0% reduction in infection success compared with outcrossed plants in 2002 and 2003, respectively. Overall, our results revealed significant inbreeding depression on almost every trait that was examined. Although the mean value of traits varied from a year to another, the magnitude of inbreeding depression did not change significantly between years. Our findings constitute the first evidence that outcrossing increases infection success and probably virulence in parasitic plant populations.

Keywords Host–parasite interactions · Inbreeding depression · Radicle · Selfing · Outcrossing · Chile · Cacti

Introduction

Inbreeding depression is a major evolutionary force that opposes self-pollination and promotes the maintenance of mixed mating systems in plants (Charlesworth and

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Charlesworth 1979, 1987; Lande and Schemske 1985). Several studies have shown significant inbreeding depression at different stages of the life cycle of flowering plants under laboratory and field conditions (reviewed in Husband and Schemske 1996; Crnokrak and Roff 1999; Keller and Waller 2002). In theory, the early stages of the life cycle should experience the largest decrease in fitness due to inbreeding because the negative effects of genetic load are often expressed early in ontogeny (Shields 1982; Levin 1989). For instance, most results indicate the progeny derived from selfing exhibit reduced performance in comparison with the progeny derived from outcrossing. One of the possible advantages of outcrossing is the higher potential of the offspring to respond to heterogeneous environments (Williams 1975; Maynard Smith 1978). Host–parasite interactions can be used as a model system to study potential benefits of outcrossing because any advantage from outcrossing may be important for host–parasite antagonistic coevolution (Ebert and Hamilton 1996; Christen et al. 2002). Increased genetic variation may also be beneficial for circumventing host defensive strategies. However, most studies have evaluated the fitness advantages of outcrossing from the host rather than the parasite perspective (e.g., Stevens et al. 1997; Coltman et al. 1999; Salathe and Ebert 2003; Stephenson et al., 2004). Furthermore, existing evidence is entirely from animal and fungal parasites (e.g., Kaltz and Shykoff 1999; Christen et al. 2002; Christen and Milinski 2003; Kurtz 2003), and to our knowledge, to date there are no studies involving systems such as parasitic plants, which may allow a meaningful generalization for parasite evolution. Here, we evaluate the consequences of outbreeding for fitness-related traits in the holoparasitic mistletoe *Tristerix aphyllus* (Loranthaceae) in a 2-year field study.

Parasitic plants are organisms that spend one or more stages of their life cycle associated with a host in a relationship that is beneficial to the parasite but not to the host (see Thompson 1994). Parasitic plants may depend partially or completely on their hosts for water, nutrients, and carbon depending on their ability to perform photosynthesis (Press and Graves 1995). Parasitic plants are a speciose and polyphyletic group with *ca* 3000 species, represented in most major ecosystems (Kuijt 1969; Press and Graves 1995). While the seeds of some parasitic plants germinate in response to a chemical signal of the host plant (Musselman and Press 1995), mistletoe seeds germinate under a broader range of conditions. The limiting step in the mistletoe life cycle is host establishment, which is dependent on several factors such as an appropriate vector, effective seed dispersal and deposition, and the presence of mistletoe–host compatibility (Clay et al. 1985; Norton and Reid 1997; Norton and Carpenter 1998).

Tristerix aphyllus is a self-compatible leafless mistletoe endemic to the arid and semiarid ecosystems of northern Chile that parasitizes only columnar cacti (Kuijt 1969, 1988). Seeds of *T. aphyllus* germinate immediately after bird deposition by elongating a red radicle that grows until contacting the host epidermis. After contact, a haustorial plate is developed which represents the morphological and physiological bridge between the host and parasite tissues (Kuijt 1969; Lamont 1983; Musselman and Press 1995). Seeds of the mistletoe exhibit an exceptionally long radicle in comparison with other species of the family Loranthaceae (Kuijt 1969, 1988), probably representing an adaptation to overcome the barrier imposed by the long spines of the cacti it parasitizes (Martínez del Río et al. 1995; Medel 2000). Here, we evaluate the advantage of outbreeding on fitness-related traits at the early stages of the life cycle of *T. aphyllus*. Because the magnitude of inbreeding

depression tends to be contingent to environmental conditions (Cheptou et al. 2000; Keller and Waller 2002), we evaluated the consequences of inbreeding in two consecutive years that differed in cumulative annual precipitation. Specifically, we evaluated whether inbreeding influences fruit production, fruit size, seed size, germination, radicle elongation, and establishment of *T. aphyllus* on the cactus *Echinopsis chilensis*.

Materials and methods

Study system

This study was conducted from March 2002 to March 2004 at Las Chinchillas National Reserve (31°30' S, 71°06' W, Aucó, IV Region, Chile). The climate of this region is of a semiarid Mediterranean type with most rainfall concentrated in the winter season. Mean annual precipitation is 167 mm (data from 1974 to 1999), with large inter-annual variation, and frequent droughts with unusual years of high precipitation (Jaksic 2001). The first year of this study (2002) was rainy with a cumulative precipitation of 267 mm, while the following year (2003) was dry with a cumulative precipitation of 56 mm. Vegetation is thorny with *Flourensia thurifera*, *Bahia ambrosoides*, and *Porlieria chilensis* as the most common shrubs species. Cacti include columnar *Echinopsis chilensis* and *Eulychnia acida*, and globular *Opuntia berteroniana* and *Erioseye sandillon*. Additional information about this study area has been previously reported (Medel 2000, 2001; Medel et al. 2004).

Tristerix aphyllus is a perennial holoparasitic mistletoe endemic to North and Central Chile that infects only species of cacti (Kuijt 1969, 1988). The vegetative portion of the mistletoe exists as an endophyte within the host tissues, where it taps into the phloematic vessels of the cacti (Mauseth et al. 1984, 1985; Mauseth 1990), and the only portion that emerges from the stems of cacti is its red inflorescences. The flowering season occurs between March and August with a peak from April to June (Medel et al. 2002). In the study area, *T. aphyllus* is a self-compatible plant pollinated only by the hummingbird *Sephanoides sephaniodes* (Smith-Ramirez 1999; Medel et al. 2002). Fruits are single-seed pseudo-berries containing 80% water that turn pale pink or translucent white when ripe. The bird *Mimus thenca* (Mimidae) is the only disperser of *T. aphyllus* (Martínez del Río et al. 1995, 1996; Medel 2000; Medel et al. 2002, 2004). After being defecated by the bird, the seeds adhere to the cuticle and cacti spines by a sticky viscid layer. Seeds germinate within a day of defecation and a bright red radicle grows for up to 8 weeks, or until making contact with the epidermis of the cactus. After that, a haustorial plate is developed, from which several filaments penetrate into the cactus tissues (Mauseth et al. 1984, 1985). The haustorium, rather than being a permanent absorptive organ, is a temporary structure that originates from the endophytic vegetative body of the plant (Mauseth et al. 1985). This perennial mistletoe exists as an endophyte for approximately 17 months (Botto-Mahan et al. 2000). After this period, *T. aphyllus* can flower multiples times. In the Reserve, *T. aphyllus* parasitizes indistinctly the columnar *Echinopsis chilensis* and *Eulychnia acida*. Our measurements focused on *T. aphyllus* parasitizing *E. chilensis*, the most common host in this area (Martínez del Río et al. 1995; Medel 2000; Medel et al. 2002, 2004).

Field procedures

During April 2002, we randomly selected 38 experimental plants of *T. aphyllus* on an area of ~20 ha. Because parasitized cacti can have several parasitized branches and more than one parasite individual per branch, we avoided confounding effects by choosing inflorescences of *T. aphyllus* from different cacti. To avoid pollinator visits and fruit removal by dispersers, inflorescences were isolated using fine and transparent mesh from the bud stage until the end of the fruiting season. We randomly assigned the experimental plants to one of the following pollination treatments: (1) pollination with pollen of the same plant (selfed), and (2) pollination with pollen of other plants (outcrossed). At least 10 pollen donor plants were used to pollinate each outcrossed plant. Manual Pollination was performed on an average of 160 flowers per plant. In the two treatments, flowers were emasculated and tagged before manual pollination. All fruits were collected in September 2002. In the second year, we replicated the field procedure using 60 experimental plants different from those used in the previous year and fruits were collected in September 2003.

We compared the fruit production, fruit mass, seed mass, seed germination, radicle elongation, and establishment success of the selfed and outcrossed progeny both in 2002 and 2003. Fruit production was measured as the probability a pollinated flower has to become into a fruit. Fruits and seeds were weighted using a microbalance with precision of 0.01 g. As with most mistletoes, seeds do not require host stimuli to germinate (Lamont 1983; Norton and Carpenter 1998); removal of the epicarp is sufficient (Mauseth et al. 1985; Kuijt 1988). We collected ripe fruits from the experimental pollination treatments and mechanically removed the epicarp to evaluate seed germination, radicle elongation, and infection success (i.e., establishment). To initiate germination, seeds were moistened two times a day during the first 2 days after epicarp removal. To evaluate establishment, 4–5 days post germination seeds were placed in contact with a cactus spine at 28 mm from the cactus surface. This distance is the average distance to the cactus epidermis of deposited seeds in natural conditions. Only one seed was located per host branch in a complete random design. To avoid potentially biased seedling mortality from differences in sun exposure and seed desiccation (Botto-Mahan et al. 2000), all artificial infections were performed on the southeast quadrant of the circular host perimeter (arc of 130° on the cactus surface). We considered successful establishment to occur when both the radicle contacted the host cuticle and the haustorial plate was developed. We confirmed the establishment status of the seedlings by monitoring them for 5 months after experimental infection, and by verifying the persistence of the haustorial plate during this period. Radicle length was measured from pictures using a scaled digital camera, and images were analyzed in UTHSCSA ImageTool for Windows, version 2.0 (University of Texas Health Science Center, San Antonio Texas, USA).

Statistical procedures

Fruit mass, seed mass, and radicle length were analyzed using a generalized linear mixed model (PROC MIXED SAS, Littell et al. 1996). Plant (nested within treatment) was included as a random effect, and treatment (selfed and outcrossed) and year were considered fixed effects. We included seed mass as covariate for radicle length. Fruit production, seed germination, and establishment were analyzed in a generalized linear modeling (GLIM) approach with the GLIMMIX macro of PROC

MIXED in SAS (SAS Institute 1997). GLIM is an extension of the traditional linear model (GLM) that is applicable to a wider range of data analysis and error structures (Crawley 1993). The GLIMMIX macro fits a GLM with random effects to the data by an iterative pseudolikelihood estimation of the model parameters. Because these traits take a dichotomous value, we modeled variation using a binomial error distribution and a logit link function. As before, the model for fruit production included plant (nested within treatment) as a random effect, and treatment and year were considered fixed effects. The model for seed germination included seed mass as covariate, and the model for establishment included seed mass and radicle length as covariates (we consider that the fact that one variable is unimportant in one stage of the plant lifecycle does not preclude it from being relevant in another stage). The significance of random effects was computed using their respective z -values tested for a significant deviation from zero (SAS Institute 1997). The significance of fixed factors was tested from type III estimable functions, with denominator degrees of freedom taken from Satterthwaite's approximation.

Inbreeding depression was estimated separately for each mistletoe trait from $\delta = 1 - w_s/w_o$, where w_s/w_o is the mean relative fitness of the selfed progeny (w_s) in relation to the outcrossed progeny (w_o). According to our design, significant inbreeding depression is revealed directly from significant treatment effects. In addition, we evaluated the inter-annual consistency of inbreeding depression in 2002 and 2003. Because inbreeding depression is computed from the ratio of the crossing treatments, the datasets for continuous variables (fruit mass, seed mass, and radicle length) were log-transformed. This transformation makes ratios additive and therefore appropriate for testing differences in the magnitude of inbreeding depression (Johnston and Schoen 1994). In the case of dichotomous variables (fruit production, seed germination, and establishment), we tested the heterogeneity in the magnitude of inbreeding depression by using the Mantel–Haenszel test for homogeneity of odd ratios (Sokal and Rohlf 1995).

Results

Fruit production was affected by treatment and year, but we did not detect a significant interaction between these factors (Table 1). Self-pollinated plants had a 25% lower fruit production than outcrossed plants, and the mean fruit production was higher in 2002 than 2003 (Fig. 1A). The magnitude of inbreeding depression did not change between years ($\delta_{2002} = 0.207$ and $\delta_{2003} = 0.288$, Mantel–Haenszel $\chi^2 = 0.587$, $P = 0.550$). Selfed fruits were lighter than outcrossed (Table 1, Fig. 1B) and the relationship decreased marginally across years (Treatment \times Year interaction, $P = 0.089$), probably reflecting the lower inbreeding depression coefficient in the drier year (2003) ($\delta_{2002} = 0.136$ and $\delta_{2003} = 0.047$). Seed mass was not influenced by treatment and year, indicating complete absence of inbreeding depression on this trait (Table 1, Fig. 1C). The chance of seed germination was affected by treatment, year, and seed mass, but the treatment \times year interaction was not statistically significant (Table 1). Selfed seeds had a 10% lower germination than outcrossed seeds, and the mean seed germination percentage in 2002 was lower than in 2003 (Fig. 1D). Even though the magnitude of inbreeding depression for seed germination tended to decrease in the second year ($\delta_{2002} = 0.153$ and $\delta_{2003} = 0.068$), this effect was not statistically significant (Mantel–Haenszel $\chi^2 = 2.51$, $P = 0.110$). Radicle length was

Table 1 Summary of results of generalized linear mixed model analyses for traits of *Tristerix aphyllus* (see details in Statistical procedures)

Trait	Source	df	F	P
Fruit production	Treatment (T)	1, 96	13.83	0.0003
	Year (Y)	1, 15496	10.06	0.0015
	T × Y	1, 15496	0.11	0.7348
Fruit mass	Treatment (T)	1, 82	8.52	0.004
	Year (Y)	1, 2394	0.17	0.679
	T × Y	1, 2394	2.89	0.089
Seed mass	Treatment (T)	1, 82	0.65	0.424
	Year (Y)	1, 2573	0.70	0.402
	T × Y	1, 2573	2.05	0.153
Germination	Seed mass	1, 2571	12.10	<0.001
	Treatment (T)	1, 82	18.78	<0.001
	Year (Y)	1, 2571	20.52	<0.001
	T × Y	1, 2571	1.35	0.2451
Radicle length	Seed mass	1, 386	1.95	0.163
	Treatment (T)	1, 57	7.21	0.009
	Year (Y)	1, 386	27.81	<0.001
	T × Y	1, 386	1.09	0.298
Establishment	Seed mass	1, 384	0.77	0.381
	Radicle length	1, 384	29.29	<0.001
	Treatment (T)	1, 57	6.45	0.014
	Year (Y)	1, 384	16.56	<0.001
	T × Y	1, 384	0.14	0.712

Degrees of freedom, Type-III statistical *F*, and *P*-values are reported. Analyses for continuous traits were performed on log-transformed data

Note: Random effects (plants) were highly significant for every analysis that was performed ($P \leq 0.002$), indicating that an important fraction of trait variance was accounted by among-individual variation, not necessarily related with treatment, year, and their interaction. Because our focus in this paper is to evaluate the statistical significance of fixed effects and their interaction, random effects albeit important for model structure, are not depicted

affected by treatment and year, but the treatment × year interaction, and seed mass had no influence on this trait (Table 1). Selfed seedlings had shorter radicles than outcrossed ones, and mean radicle length was shorter in the year 2002 than in 2003 (Fig. 1E). We did not detect significant differences in inbreeding depression coefficients between years ($\delta_{2002} = 0.307$ and $\delta_{2003} = 0.052$; $P = 0.298$). Successful establishment was affected by treatment, year, and radicle length but the treatment × year interaction, and seed mass had no significant effects on this trait (Table 1). Selfed seedlings had a 60% lower establishment than outcrossed seedlings, and the mean establishment in 2002 was lower than in 2003 (Fig. 1F). The magnitude of inbreeding depression for establishment did not differ between years ($\delta_{2002} = 0.716$ and $\delta_{2003} = 0.608$; Mantel–Haenzel $\chi^2 = 0.01$, $P = 0.93$). In summary, our results revealed significant inbreeding depression on almost every trait that was examined. Although the mean value of some traits varied from one year to another, the magnitude of inbreeding depression did not change significantly between years.

Discussion

Results from this study indicate that outcrossing is advantageous for most fitness-related traits of *T. aphyllus*. Interestingly, the advantage of outcrossing for seed

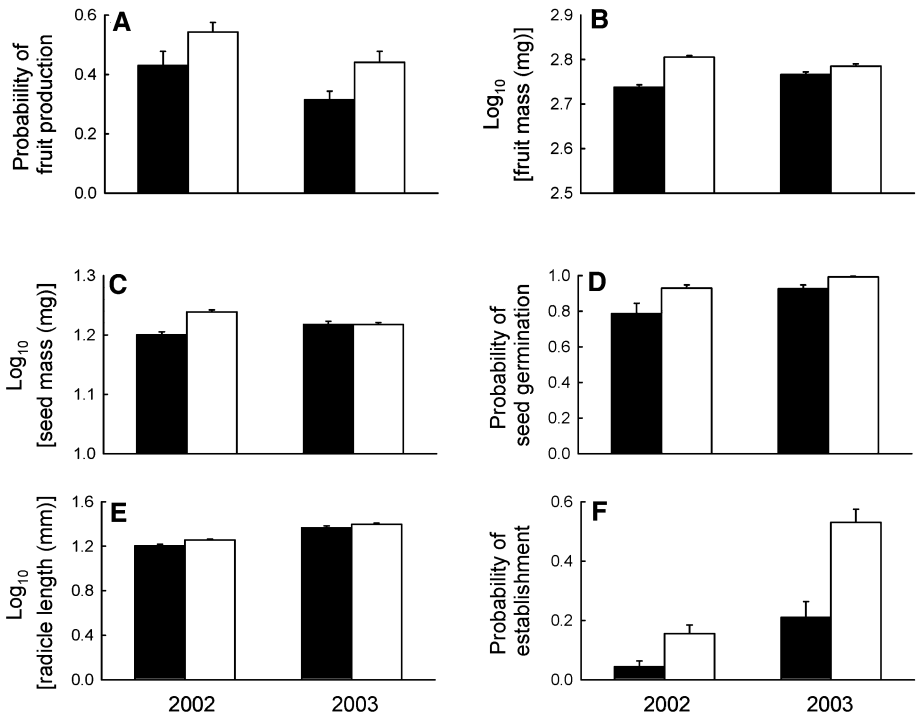


Fig. 1 Fitness-related traits of *T. aphyllus* under selfed (black) and outcrossed (white) pollination treatments in 2002 and 2003. (A) Fruit production, (B) fruit mass, (C) seed mass, (D) seed germination, (E) radicle length, (F) establishment. Means + 1 standard error are shown

germination and radicle length translated into an important increase in the chance of establishment of *T. aphyllus*, a key step for successful infection (Press and Graves 1995; Norton and Reid 1997; Norton and Carpenter 1998). This result has important implications for our understanding of the mechanisms underlying the host–parasite relationship. Because successful establishment requires the contact of the radicle apex with the cactus epidermis (Mauseth et al. 1985; Mauseth 1990; Botto-Mahan et al. 2000), the long radicles shown by the outbred progeny may provide advantage to overcome the barrier imposed by the long cacti spines that prevent infection and evolve under parasite-mediated selection (Medel 2000). In this way, outcrossed seedlings may penetrate the cactus epidermis and reach the phloematic vessels more efficiently than selfed seedlings. Because seed mass often influences germination, seedling establishment, competitive ability, and survival, among other fitness-related traits (e.g., Stanton 1984; Winn 1988; Kalisz 1989), it is possible that this trait may also contribute to the observed differences in the progeny. However, although seed mass accounted for a large fraction of variance in seed germination, we did not detect differences in seed mass between treatments, suggesting that inbreeding depression in *T. aphyllus* is not driven by seed mass variation in this system.

Several studies have suggested that inbreeding depression is more pronounced under stressful than benign conditions (e.g., Wolfe 1993; Coltman et al. 1999; Cheptou et al. 2000; see review in Armbruster and Reed 2005). Our results do not support this view. Inbreeding depression coefficients for fitness-related traits did not

differ between the two contrasting environmental conditions of 2002 and 2003 (267 and 56 mm precipitation, respectively). The lack of significant variation between years can be explained, partly, if we observe a difference between the effects of year on parental mediated versus offspring mediated traits. In parental traits, like provisioning of offspring, the effect of environment may often be expected to influence all offspring and therefore will not generally alter the cost of inbreeding because both inbred and outcrossed offspring will suffer equally. It is possible that the peculiar holoparasitic biology of the leafless *T. aphyllus* restrict the influence of the external physical environment as suggested for parasitic plants that depend entirely on the host for water and nutrients (Atsatt 1970; Atsatt and Guldberg 1978). Even though the magnitude of inbreeding depression was consistent between years, the mean values of traits showed a more complex pattern. While fruit production decreased from 2002 to 2003, germination, radicle length, and establishment increased in the drought year. These results suggest the mistletoe may compensate the low fruit production in the dry year with progeny that are more vigorous in survival, seed germination and establishment. Another explanation for greater seed establishment in the drought year may be related to changes in the host. For example, in drought years, the host may be more water stressed so the distance between the outside of the plant to the phloem vessels may be shorter.

Our results indicate that outbreeding increases radicle elongation in *T. aphyllus*, which in turn may allow the mistletoe to overcome host defenses and desiccation to achieve successful establishment and reproduction in the host species. These results suggested that changes observed at the level of infection success of the parasitic plant could propagate along reproductive development to changes in fruit production. This cascade effect is not only restricted to parasitic plants but illustrate the overall fine-tuning of plant fecundity that takes place across sequential stages of reproductive development (Lloyd 1980; Stephenson 1984; Stöcklin 1997). Several studies have documented that negative correlations among different plant fecundity components tend to be observed under resource limitation (e.g., Stephenson 1981; Medrano et al. 2000). Even though we detected significant inbreeding depression in different sequential stages of the life cycle, it is possible that the advantages of outcrossing on early stages simply propagate through subsequent stages of the life cycle rather than directly influence each stage separately. Since we measured inbreeding depression only in the early life stages of *T. aphyllus* we cannot rule out that outbreeding has a potential negative impact on fitness-related traits in other segments of the life cycle of *T. aphyllus*, such as pollination and seed dispersal stages. Further research is needed to distinguish between these two alternatives.

In the context of host–parasite relationships, one of the advantages of outcrossing for parasites relates to the increased genetic variation that allows them to overcome host defense and infect a higher diversity of the host genotypes (Gemmill et al. 1997; Agrawal and Lively 2001). For example, autogamous lineages of the cestode *Schistocephalus solidus* are less successful than outcrossed lineages at infecting the intermediary host copepod *Macrocyclus albidus* (Christen et al. 2002). Similarly, Christen and Milinski (2003) found that the fish *Gasterosteus aculeatus* was more parasitized by outcrossed than selfed parasites. Theory predicts that outcrossing parasites adapt to the most common host genotypes at a higher rate than selfed parasites. Even though parasite evolution may result in a wide variety of final conditions in the host–parasite relationship, most authors agree that parasite genotypes that use up the host resources quickly may outcompete their neighbors within a

host by reducing available resources, leading to a higher probability that parasites will kill or dramatically reduce host fitness (e.g., Bull 1994; Ebert and Herre 1996; Frank 1996). However, group or kin selection may also favor prudent exploitation of the host, favoring low virulence. Frank (1996) analyzed this balance concluding that the optimal solution involves the coefficient of relatedness among parasites. While high parasite relatedness may favor cooperation and prudent host exploitation, low relatedness favors intense within-host competition and virulence (Frank 1996). Because one of the processes influencing the level of parasite relatedness is the mixing of parasite lineages or strains, it follows that outbreeding may represent an important force promoting high parasite virulence. In the mistletoe model, mixing of lineages may occur at the pollination or seed dispersal stages, when pollinators and seed dispersal vectors pick up pollen and fruits from parasites infecting different hosts or even from different host populations. Because virulent strains can be maintained only if parasites have a high transmission capacity (Ebert 1994; Herre 1995), outcrossing parasites are expected to evolve higher transmission rates than selfed parasites. The observation that outcrossing *T. aphyllus* produced larger fruits and have higher fruit production than inbred plants suggests that outcrossing plants tend to be more attractive to the birds responsible for mistletoe transmission (Ossa 2003), which provides indirect support for this hypothesis. In summary, we have presented experimental data that outbreeding increases infection and fecundity in a parasitic plant. Our results highlight the importance of parasite outbreeding for studies of virulence, and transmission, suggesting that parasite reproductive strategies need to be considered to describe and predict the evolutionary pathway of host–parasite associations.

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