Mixed mating in natural populations of the chestnut blight fungus, *Cryphonectria parasitica*

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As in plants, fungi exhibit wide variation in reproductive strategies and mating systems. Although most sexually reproducing fungi are either predominantly outcrossing or predominantly selfing, there are some notable exceptions. The haploid, ascomycete chestnut blight pathogen, *Cryphonectria parasitica*, has previously been shown to have a mixed mating system in one population in USA. In this report, we show that both selfing and outcrossing occur in 10 additional populations of *C. parasitica* sampled from Japan, Italy, Switzerland and USA. Progeny arrays from each population were assayed for segregation at vegetative incompatibility (*vic*) and DNA fingerprinting loci. Outcrossing rates ($\hat{t}_m$) were estimated as the proportion of progeny arrays

showing segregation at one or more loci, corrected by the probability of nondetection of outcrossing ($\hat{\alpha}$). Estimates of $\hat{t}_m$ varied from 0.74 to 0.97, with the lowest rates consistently detected in USA populations (0.74–0.78). Five populations (four in USA and one in Italy) had $\hat{t}_m$ significantly less than 1, supporting the conclusion that these populations exhibit mixed mating. The underlying causes of variation in outcrossing rates among populations of *C. parasitica* are not known, but we speculate that – as in plants – outcrossing is a function of ecological, demographic and genetic factors.

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Introduction

Mating systems have long intrigued biologists, perhaps in large part due to the interplay between mating system and life history, and the profound effects that differing modes of reproduction can have on population genetic structure. Early observations of plant breeding systems categorized species as being either predominantly selfing or predominantly outcrossing (reviewed in Schemske and Lande, 1985), and early models concerned with mating system evolution concluded that only complete selfing and complete outcrossing are evolutionarily stable, dependent on the frequency with which mutational load is purged (Jain, 1976; Lande and Schemske, 1985). Subsequent studies revealed species and populations with apparently stable intermediate levels of selfing (mixed mating systems) (Waller, 1986; Barrett and Eckert, 1990), stimulating interest in the evolutionary forces that might maintain them, such as spatial genetic structure (Holsinger, 1986; Uyenoyama *et al.*, 1993; Ronfort and Couvet, 1995), inbreeding depression (Charlesworth and Charlesworth, 1987, 1990; Charlesworth *et al.*, 1990; Uyenoyama *et al.*, 1993) and identity disequilibria, that is, associations between viability loci and mating system loci (Charlesworth and Charlesworth, 1990; Holsinger, 1991; Uyenoyama *et al.*, 1993). However, it appears that

outcrossing rates are not static properties of species, but rather that outcrossing rates can vary intraspecifically, among individuals and among populations, in response to a range of genetic and environmental factors (Barrett and Eckert, 1990; Richards, 1997).

As with early studies on plants, most fungi studied to date have been classified as being either self-compatible or self-incompatible, corresponding roughly to the terms ‘homothallic’ and ‘heterothallic’ that are used in mycology (given the parallels between plant and fungal mating systems, we prefer the former pair of terms because they are well established in the literature on plant mating systems). However, in contrast with the majority of plant mating system studies, most fungal mating system studies have been conducted in the laboratory, and have not considered the degree to which mating behavior in the laboratory may differ from mating behavior in natural populations. Of the few studies of fungal mating systems in natural populations, most have conformed more or less to laboratory results: self-compatible species predominantly selfed and self-incompatible species predominantly outcrossed (Ainsworth, 1987; Ennos and Swales, 1987; Sharland *et al.*, 1988; Sharland and Rayner, 1989a,b; Adams *et al.*, 1990; Kohn *et al.*, 1991; Kohli and Kohn, 1992; Wang, 1997; Cortesi and Milgroom, 2001; Kroken, 2001), with several exceptions (see below). However, some fungal species deviate from the strict dichotomy between self-compatibility and self-incompatibility (Mathieson, 1952; Uhm and Fujii, 1983; Perkins, 1987; Herskowitz, 1988; Harrington and McNew, 1997), supporting the need for a broader perspective on fungal mating systems.

Although most field studies of fungal mating systems have confirmed laboratory observations, several

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exceptions are worth noting. In the laboratory, the wood-rotting fungus, *Phellinus nigrolimitatus*, is self-compatible (Fischer, 1996; Kauserud and Schumacher, 2001), in contrast with a field study that showed nearly complete outcrossing (Kauserud and Schumacher, 2001). In laboratory matings, California isolates of *Fusarium circinatum*, the causal agent of pitch canker disease, predominately outcrossed, even though California populations consist almost entirely of clonally propagating lineages, and no sexual structures have been reported in nature (Wikler and Gordon, 2000; Wikler *et al*, 2000). The anther-smut pathogen, *Microbotryum violaceum*, has a genetic self-incompatibility system, yet there is little evidence for outcrossing, and most matings occur as intratetrad 'diploid selfing' between sibling haploid meiospores (Kaltz and Shykoff, 1999; Hood and Antonovics, 2000). Another example of discord between laboratory and field observations is seen in the mating system of the ascomycete chestnut blight fungus, *Cryphonectria parasitica*. In the laboratory, *C. parasitica* has a genetic self-incompatibility system similar to that of other self-incompatible ascomycetes (Marra and Milgroom, 2001). In this mating system (referred to as a bipolar mating system), a single mating-type locus (*MAT*) is biallelic, such that successful mating occurs only between individuals with opposite *MAT* alleles (Marra and Milgroom, 2001). Contrary to the observed self-incompatibility in the lab, some isolates that failed to self-fertilize in the laboratory have been shown to self-fertilize in the field (Marra and Milgroom, 2001). Furthermore, in a field population in Mountain Lake, Virginia, *C. parasitica* was estimated to have an outcrossing rate of 73%, which is significantly less than 1 (Milgroom *et al*, 1993). Thus, although *C. parasitica* is almost exclusively outcrossing in the laboratory, it appears to have the potential for mixed mating in nature.

The purpose of the research reported here was to estimate the mating system of *C. parasitica* in additional natural populations. Specifically, our objectives were to test the hypothesis that self-fertilization occurs in all *C. parasitica* populations – that is, that it is an important and relevant component of the *C. parasitica* mating system – and to assess variability in outcrossing rates among populations. Our overall strategy was to repeat the types of mating system estimates made in Virginia (Milgroom *et al*, 1993) in multiple populations from three continents. To accomplish this objective, we estimated the outcrossing rates in field populations of *C. parasitica* in USA, Europe and Japan, representing a broad geographic distribution of *C. parasitica*.

Materials and methods

Study organism

The reproductive biology of *C. parasitica* was described in detail previously (Milgroom *et al*, 1993; Marra and Milgroom, 2001). *C. parasitica* is a haploid filamentous fungus for most of its life cycle. It colonizes wounds in the bark of chestnut trees (*Castanea* spp.), invading and destroying cambial tissues, resulting in cankers on the trees, symptomatic of chestnut blight. Cankers, which consist of both fungal tissue (stroma, pl. stromata) and bark, are the site of sporogenesis of asexual spores (conidia) as well as sexual spores (ascospores). Asco-

spores are found in flask-shaped fruiting bodies (perithecia), each containing the progeny from a single mating (Milgroom *et al*, 1993).

Populations sampled

C. parasitica was sampled from a total of 10 populations from USA, Italy, Switzerland and Japan to estimate outcrossing rates (Table 1). In each population, *C. parasitica* was sampled from cankers on chestnut trees by removing small pieces of bark containing stromata. Only one canker was sampled from each single tree in most populations, although multiple cankers on the same trees were sampled from Depot Hill and Danby, New York, USA. Unless noted otherwise, we sampled only one perithecium from each bark sample, and from each perithecium we obtained at least eight single ascospore progeny, constituting the progeny array, as described previously (Milgroom *et al*, 1993). In addition, for most progeny arrays we sampled maternal genotypes by culturing from mycelium surrounding the sampled perithecium. Perithecia from two US populations (Danby and Depot Hill) were sampled from populations that had been studied intensively for spatial genetic structure (Milgroom and Lipari, 1995). West Virginia populations Parsons-1 and Parsons-2 were sampled from American chestnut, *Castanea dentata*, in September 1993 from 10-year-old clearcut plots, each of approximately 1500 m², in the Monongahela National Forest, in Parsons Township, West Virginia. We sampled perithecia from European chestnut, *Castanea sativa*, in four populations from stromata originally sampled for estimates of genetic diversity (Cortesi *et al*, 1996; Bisseger *et al*, 1997). Populations in Japan were sampled from orchards of Japanese chestnut, *Castanea crenata*, in April 1998 in Okoba, Kumamoto Prefecture in southern Japan and Chudai, Kyoto Prefecture in central Japan (Liu *et al*, 2003).

Nearly all cankers sampled from USA and European populations had perithecia containing viable ascospore progeny. However, we were able to recover viable progeny from fewer than half of the cankers from the two Japanese populations, sampling progeny from 16 and 12 perithecia in Okoba and Chudai, respectively.

Distinguishing selfing from outcrossing

Selfing and outcrossing were determined for each progeny array essentially as described previously (Milgroom *et al*, 1993). For the two Japanese populations, we assayed only for segregation of vegetative incompatibility (*vic*) alleles. Vegetative incompatibility is a self/nonself recognition system controlled by at least six unlinked *vic* loci (Cortesi and Milgroom, 1998) and is assayed using methods described previously (Cortesi *et al*, 1996). For the Italian and Swiss populations, we assayed first for segregation of alleles at *vic* loci, and subsequently for segregation of alleles at DNA fingerprint (*fp*) loci. Assays for *vic* segregation were carried out first because they are relatively simple, requiring only culturing and not DNA extractions/manipulations, thereby limiting the number of fingerprinting assays that needed to be carried out subsequently. Progeny arrays for which *vic* segregation was not observed were further tested by a DNA fingerprinting method (Milgroom *et al*, 1992), which involves probing gel blots with

a transposable element (Linder-Basso *et al*, 2001) that occurs in multiple copies dispersed throughout the *C. parasitica* genome. DNA fingerprinting by this method detects the presence or absence of highly polymorphic restriction fragments at multiple independent loci (Milgroom *et al*, 1992). For the four North American populations (Danby, Depot Hill, Parsons-1 and Parsons-2), all progeny arrays were fingerprinted, but not assayed for *vic* segregation; a previous study showed that outcrossing could be detected more often by fingerprinting than by vegetative incompatibility (Milgroom *et al*, 1993). Segregation at either *vic* or fp loci in each progeny array was interpreted as evidence for outcrossing (Milgroom *et al*, 1993). As *vic* and fp allele segregation will be 1:1 among progeny from a single perithecium (Cortesi and Milgroom, 1998; Milgroom *et al*, 1992), we have a probability of >99% of detecting outcrossing with as few as eight progeny per perithecium ($1-(0.5)^8 = 0.996$).

In the Parsons-1 and Parsons-2 samples, we used an alternative DNA fingerprinting method. Instead of fingerprinting individual progeny, we cultured single progeny within an array independently, pooled tissue from these progeny, extracted DNA from the pooled sample and fingerprinted the pooled DNA. The composite fp (from the pooled sample) was then compared to the maternal fp. Outcrossing was determined by the presence of bands on autoradiographs in the composite fp that were not present in the maternal fp. We also used this method for five progeny arrays from each of the Bergamo and Crevoladossola samples. All fingerprinting was carried out using standard methods (Milgroom *et al*, 1992).

Estimation of outcrossing rates

The outcrossing rate was first estimated simply as the proportion of progeny arrays that segregated at *vic* and/or DNA fp loci within each sample. In Japan, almost every isolate of *C. parasitica* within a chestnut orchard, including 28 isolates in Chudai, was vegetatively incompatible with every other isolate (Y-C Liu and MG Milgroom, unpublished); therefore, assays for segregation at *vic* loci have a very high probability of detecting all outcrosses (Milgroom *et al*, 1993). However, for the USA and European populations, which have lower *vic* genotype diversity (Milgroom and Cortesi, 1999), we estimated the probability of not detecting an outcrossed progeny array using the method of Shaw *et al* (1981), modified for haploids (Milgroom *et al*, 1993). Briefly, the probability estimate ($\hat{\alpha}$) calculates the likelihood that outcrossing between maternal and paternal gametes will not be detected because they share the same alleles at the assayed loci; this likelihood decreases as the number of loci and the number of alleles per locus increase, and also as allelic frequencies become nearly equal to each other. For our analyses, we assumed that the maternal and paternal pools had identical allele frequencies. We base this assumption on the fact that *C. parasitica* is hermaphroditic, with both male and female structures almost always present together in a single stroma. To calculate for European populations (Bergamo, Crevoladossola, Gnosca and Lumino), we used two sets of data: estimates of *vic* allele frequencies (Milgroom and Cortesi, 1999) as well as fps of randomly sampled canker isolates (100 from Bergamo, 100 from Crevoladossola, 31 from Gnosca

and 65 from Lumino). For Danby and Depot Hill, we estimated fp allele frequencies using fp data obtained previously (Milgroom and Lipari, 1995).

To calculate $\hat{\alpha}$ in the WV populations, Parsons-1 and Parsons-2, which were assayed by pooling progeny, we used DNA fp data from 18 maternal isolates from the Parsons-2 population. In comparing the composite progeny fp to the maternal fp, segregation will only be detected at loci with a dominant allele (presence of fp band) in the paternal isolate and recessive allele (absence of fp band) in the maternal isolate; segregating loci with dominant alleles in the maternal isolate will not be detected in the pooled progeny fp because they will appear the same as the maternal fp. In order to adjust for this pooled method, we calculated the probability of nondetection for each maternal isolate based only on loci for which that maternal fp had the recessive allele, and then taking the average of these probabilities for the population.

Results

We observed both selfing and outcrossing in all 10 populations of *C. parasitica*, from three continents (Table 1). Segregation of DNA fp alleles, or DNA fp alleles in combination with *vic* alleles, was used to distinguish outcrossing from selfing in European and USA populations. In the European populations, segregation of *vic* alleles identified some, but not all, outcrossing. The estimated probabilities of not detecting outcrossing by *vic* segregation alone ($\hat{\alpha}_{vic}$) ranged from 10 to 21% (Table 1). When progeny arrays that failed to segregate for *vic* were fingerprinted, segregation was evident in an additional 15–38% of the arrays in the four European populations. Based on genotypic and allelic diversity of DNA fp loci in Europe and USA, we infer that segregation of fp alleles identifies almost all outcrosses in these samples. The probabilities of nondetection of outcrossing by fp segregation ($\hat{\alpha}_{fp}$) was ≤ 0.0063 in all populations, so that corrected outcrossing estimates ($\hat{t}_{m(fp)}$) were only negligibly greater than uncorrected estimates ($\hat{t}_{fp}$) (Table 1). Estimates of outcrossing in European and USA populations ranged from 0.74 to 0.97. All four USA populations and the Italian population Crevoladossola had outcrossing estimates significantly less than 1. Therefore, we conclude that these five populations provide definitive evidence for the occurrence of mixed mating.

To estimate outcrossing in the two Japanese populations, we used only *vic* segregation data. We found only one of 12 (8%) and two of 16 (12%) progeny arrays from Chudai and Okoba, respectively, that did not segregate at *vic* loci, resulting in uncorrected outcrossing estimates ($\hat{t}_{vic}$) of 0.92 and 0.88, respectively. We do not know the *vic* genotypes or allele frequencies in Japanese populations, as we do in Europe or USA (Milgroom and Cortesi, 1999), and therefore we could not correct estimates for nondetection of outcrossing. Neither of these estimates is significantly less than 1 (the 95% confidence interval (CI) included 1; Table 1).

Discussion

Our study of 10 populations from three continents suggests that mixed mating is a regular feature of the

Table 1 Outcrossing estimates $\hat{t}$ for *C. parasitica* in populations from three continents

Population	N	Number of progeny arrays with no segregation	vic loci					DNA fingerprint loci				
			Number of progeny arrays segregating at vic loci	Uncorrected $\hat{t}_{vic}$	$\hat{\alpha}_{vic}$	$\hat{t}_{m(vic)}$	95% CI for $\hat{t}_{m(vic)}$ ^a	Number of progeny arrays segregating at DNA fingerprint loci	Uncorrected $\hat{t}_{fp}$	$\hat{\alpha}_{fp}$	$\hat{t}_{m(fp)}$	95% CI for $\hat{t}_{m(fp)}$
Bergamo, Italy	31	3	23	0.74	0.214	0.94	(0.86, 1.03)	5 ^{b,d,e}	0.90	0.0030	0.91	(0.80, 1.01)
Crevaladossola, Italy	25	5	17	0.68	0.198	0.85	(0.71, 0.99)*	3 ^{b,d,e}	0.80	0.0017	0.80	(0.65, 0.96)*
Gnosca, Switzerland	27	3	16	0.59	0.099	0.66	(0.48, 0.83)*	8 ^{b,d}	0.89	0.0007	0.89	(0.77, 1.01)
Lumino, Switzerland	30	1	18	0.60	0.144	0.70	(0.54, 0.87)*	11 ^{b,d}	0.97	0.0002	0.97	(0.91, 1.03)
Danby, NY, USA	20	5	—	—	—	—	—	15 ^{c,d}	0.75	0.0002	0.75	(0.56, 0.92)*
Depot Hill, NY, USA	56	13	—	—	0.057	—	—	43 ^{c,d}	0.74	0.0001	0.74	(0.63, 0.86)*
Parsons-1, WV, USA	22	5	—	—	—	—	—	17 ^{c,f}	0.77	0.0063	0.78	(0.60, 0.95)*
Parsons-2, WV, USA	22	5	—	—	—	—	—	17 ^{c,f}	0.77	0.0063	0.78	(0.60, 0.95)*
Chudai, Japan	12	1	11	0.92	—	—	(0.70, 1.07) ^g	—	—	—	—	—
Okoba, Japan	16	2	14	0.88	—	—	(0.71, 1.04) ^g	—	—	—	—	—

Estimates $\hat{t}_m$ were corrected for the probability of nondetection of outcrossing ($\hat{\alpha}$) (Shaw *et al*, 1981; Milgroom *et al*, 1993) for vic and DNA fp markers.

* $\hat{t}_m \neq 1$ at 95% level of significance.

^a95% CI = $\hat{t}_m \pm 1.96\sqrt{(\hat{t}_m)(1 - \hat{t}_m)/n}$.

^bOnly progeny arrays showing no segregation at vic loci were fingerprinted.

^cAll progeny arrays were fingerprinted.

^dBased on DNA fingerprints of at least eight individual ascospore progeny per perithecium.

^eFive progeny arrays fingerprinted by pooled method (see text).

^fAll progeny arrays fingerprinted by pooled method (see text).

^g95% CIs for Chudai and Okoba are based on uncorrected $\hat{t}_{vic}$.

C. parasitica mating system, confirming an earlier finding from a single USA population (Milgroom *et al*, 1993). We found evidence for selfing, that is, at least one progeny array lacking in segregation of highly polymorphic genetic markers, in all 10 populations. Although it would be correct to define mixed mating as any amount of selfing occurring in an otherwise outcrossing population, we believe it is appropriate to classify as mixed mating only those populations in which the frequency of selfing has the potential to significantly impact population genetic structure (notwithstanding the potential to achieve the same results through biparental inbreeding in completely outcrossing populations). Therefore, we considered mixed mating to have occurred when outcrossing rates were significantly less than 1. Five populations – all four USA populations as well as one from Italy – had outcrossing rates significantly less than 1, ranging from 0.74 (Depot Hill, NY, USA) to 0.80 (Crevoladossola, Italy), and can therefore be classified by our criteria as having undergone mixed mating. Estimates of outcrossing in USA populations were remarkably similar to each other (0.74–0.78) and to an estimate made previously in Mt Lake, VA, USA (0.73) (Milgroom *et al*, 1993). Outcrossing rates in the remaining five populations ranged from 0.88 (Okoba, Japan) to 0.97 (Lumino, Switzerland) and were not significantly less than 1. We conclude from this study that *C. parasitica* regularly undergoes selfing and that mixed mating is commonly found in natural populations.

The observation of mixed mating in populations of *C. parasitica* is of considerable interest to the fields of mycology and evolution. To date, there have been few studies of fungal mating systems as they manifest in natural populations, in contrast with the extensive body of research on plant mating systems. In a reanalysis of data on 129 plant species, Barrett and Eckert (1990) observed a bimodal distribution of outcrossing rate in plants: 23% of the species had outcrossing rates of 20% or less, 43% had outcrossing rates of 80% or more, leaving 36% of the species with intermediate levels of outcrossing, between 20% and 80%. Models concerned with the evolutionary stability of mixed mating systems in plants have not been evaluated for their relevance to fungi, which though similar to plants in some respects also differ significantly from plants in other respects. Most mixed mating system models have centered on a balance between two somewhat opposing forces, the advantages of selfing and inbreeding depression (Schemske and Lande, 1985; Barrett and Eckert, 1990). As *C. parasitica*, like most ascomycetous fungi, is haploid for most of its life cycle, the degree to which inbreeding depression plays a role in shaping *C. parasitica*'s mating system is uncertain. Interestingly, Leslie and Raju (1985) uncovered deleterious and recessive alleles at numerous loci among wild isolates of the ascomycete *Neurospora crassa*. Therefore, because the brief diploid phase of ascomycetes is essential to mating and reproduction, inbreeding depression may be an important selective force counteracting and perhaps balancing factors that promote selfing.

Estimates of outcrossing rely on the detection of segregating genetic markers among progeny. In *C. parasitica*, we used two multilocus marker systems, vegetative incompatibility (Cortesi *et al*, 1996; Cortesi and Milgroom, 1998) and DNA fingerprinting (Milgroom

et al, 1992; Milgroom *et al*, 1993). Assaying for segregation of *vic* alleles is much faster and less expensive than DNA fingerprinting because it involves only culturing the fungus (Cortesi *et al*, 1996). In Japanese populations, *vic* markers were sufficient to identify most outcrossing with high probability because of very high diversity of *vic* genotypes (Y-C Liu and MG Milgroom, unpublished data). In contrast, *vic* is not a reliable marker for detecting outcrossing in European populations because of lower *vic* allelic and genotypic diversity (Milgroom and Cortesi, 1999). In each of the four European populations, the estimated nondetection probability (Shaw *et al*, 1981; Milgroom *et al*, 1993) based on *vic* allele frequencies ($\hat{\alpha}_{vic}$) was 10–21%. In reality, *vic* segregation failed to detect 15–38% of the outcrossed progeny arrays that segregated for DNA fp markers. For Bergamo and Crevoladossola, $\hat{t}_{m(vic)}$ (0.94 and 0.85, respectively) is not significantly different than $\hat{t}_{m(fp)}$ (0.91 and 0.80, respectively). However, for both Lumino and Gnosca, $\hat{t}_{m(vic)}$ (0.70 and 0.66, respectively) is significantly lower than $\hat{t}_{m(fp)}$ (0.97 and 0.89, respectively). The consequence of the difference in estimates for these populations is that in the former two we would conclude mixed mating, whereas in the latter two we would not. Although we did not use *vic* markers in the four populations in USA, *vic* allelic and genotypic diversity are typically higher than in European populations of *C. parasitica* (Milgroom and Cortesi, 1999); for example, the estimated nondetection probability in Depot Hill was $\hat{\alpha}_{vic} \approx 6\%$. As *vic* diversity in Depot Hill is representative of other USA populations (Milgroom and Cortesi, 1999), a *vic*-based estimate corrected by α may be adequate as the sole estimator of outcrossing rates in USA.

Estimates of outcrossing rates varied considerably among populations. We do not yet know the mechanisms underlying these differences. However, plant mating system studies have shown that variation in outcrossing rates can be due to genetic (Lloyd, 1967; Vasek and Harding, 1976; Schoen, 1982; Schemske and Lande, 1985; Barrett and Shore, 1987; Barrett and Eckert, 1990; Lipow *et al*, 1999; Pascarella, 1997), demographic (Murawski and Hamrick, 1991; Karron *et al*, 1995) and/or environmental (Brown *et al*, 1978; Shea, 1987) factors. Among the 10 *C. parasitica* populations in our study, we observed outcrossing rates that varied from 0.74 to 0.97, with a mean of 0.84. Similar ranges in outcrossing rates among populations have been observed in several plant species. Ellstrand *et al* (1978) found outcrossing rates between 0.54 and 0.91 among 10 populations of sunflower, *Helianthus annuus*, and that outcrossing rate varied inversely with plant density. In contrast, outcrossing rates ranging from 0.62 to 0.98 of populations of buck's horn plantain, *Plantago coronopus*, correlated positively with plant density (Wolff *et al*, 1988). However, much lower outcrossing rates were reported among populations of *Lycopersicon pimpinellifolium*, ranging from 0.00 to 0.40, with outcrossing rate correlating positively with genetic variation for flower size (Rick *et al*, 1978). A negative correlation between altitude and outcrossing rates was observed in balsam fir, *Abies balsamea* (Neale and Adams, 1985).

We speculate that there are analogous genetic, demographic and/or environmental components underlying outcrossing rate variation in fungal mating systems, although given the paucity of such studies in fungi, this

remains an open and intriguing area of study. In *C. parasitica*, potential genetic factors that facilitate or inhibit selfing cannot be studied in the laboratory, where the fungus exhibits a typical ascomycete bipolar self-incompatibility system, and selfing occurs very rarely (Marra and Milgroom, 2001). Furthermore, the mechanism by which self-incompatibility is bypassed in order for selfing to occur has not yet been completely resolved. In 'homothallic' fungi, selfing typically requires both mating types, with both mating-type idiomorphs closely linked or fused on the same chromosome (Yun *et al*, 1999). However, this model for fungal self-compatibility has not been borne out in our previous studies of *C. parasitica*. *C. parasitica* appears to have two different mechanisms for selfing (McGuire *et al*, 2004). The more common of the two suggests automixis, and involves the presence of both mating-type idiomorphs in different and otherwise isogenic nuclei of the same individual, as heterokaryons. However, some selfing *C. parasitica* isolates have only one mating type or the other, and may be apomictic rather than meiotic (McGuire *et al*, 2004). The disparity in *C. parasitica*'s propensity to self in the field versus the laboratory suggests that demography and/or environment may influence variation in outcrossing rates. It is difficult to determine from the current study the demographic or environmental factors that lead to selfing because *C. parasitica* was sampled from three different *Castanea* species, on the three continents, growing in conditions varying from understory chestnut trees in various hardwood forests in the USA, to managed chestnut forests in Europe, to orchards for nut production in Japan. However, it may be possible to assess the effects of genetics, demography and environment on the mating system under field conditions, where we showed previously that isolates that would not self in the lab selfed readily in nature (Marra and Milgroom, 2001). Testing specific hypotheses will require inoculations of the same *C. parasitica* genotypes onto chestnut trees in the field under different demographic and environmental conditions. Studies to address these types of questions are currently underway in order to quantify environmental and genetic effects on mixed mating.

In conclusion, self-fertilization was observed in all 10 populations, confirming an earlier report of mixed mating ($\hat{f}_{m(fp)} = 0.73$) in a single population in USA (Milgroom *et al*, 1993). Outcrossing rates in five populations – Danby, NY, USA; Depot Hill, NY, USA; Parsons-1, WV, USA; Parsons-2, WV, USA; and Crevoladossola, Italy – were significantly less than 1, leading to a conclusion of mixed mating in these five populations. These outcrossing estimates were notably lower (0.74–0.80) than outcrossing rates observed in the remaining five populations from Italy, Switzerland and Japan (0.89–0.97). Nonetheless, selfing appears to be an important component of mating in *C. parasitica* in nature, a finding of particular interest given the robust genetic self-incompatibility system observed in the laboratory (Marra and Milgroom, 2001).

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