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# Karyotypic similarity identifies multiple host-shifts of a pathogenic fungus in natural populations

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**Abstract**

The detection of incipient host-shifts is important to the study of emergent diseases because it allows the examination of ecological and genetic conditions that favor novel inter-species transmission. Mixed populations of *Silene latifolia* and *Silene vulgaris* were investigated for the putative occurrence of host-shifts by the fungal plant pathogen *Microbotryum violaceum* (the cause of anther-smut disease) between *S. latifolia* (a common host for the pathogen) and *S. vulgaris* (a rare host). Samples of the fungus from mixed and pure host populations were studied for variation in their electrophoretic karyotypes. A karyotype distance matrix showed that fungal samples clustered by locality, but not by host species. Fungal samples from *S. vulgaris* were indistinguishable from sympatric samples from *S. latifolia* in multiple cases. The results indicated at least two independent host-shifts, one in the US and perhaps two in Italy. The karyotype and ecological data indicate that the direction of the host-shifts is from *S. latifolia* to *S. vulgaris*.


*Keywords:* Host-shift; Karyotype variation; Host range; Host specificity; Host race; Molecular epidemiology; DNA fingerprinting

## 1. Introduction

Host-shifts are currently of great concern as threats to wild and agricultural populations and to human health (Frank and Jeffrey, 2001; Mayer, 2000). This is partly due to the increased global movement of organisms that creates communities of novel composition, and thus new host-shift opportunities (von Broembsen, 1989; Meslin, 1995). Here we refer to novel inter-species disease transmission by the term “host-shift”, with the understanding that in many cases the sustainability of the disease on its new host remains an issue for further research (May et al., 2001; Antonovics et al., 2002).

In diseases of human and agricultural importance, immediate control measures have understandably taken priority over studying host-shifts as they occur in *statu nascendi*. Furthermore, we often are able to detect and control such diseases only after they have reached a significant prevalence in the affected population. Therefore, the important ecological and genetic conditions during the initial stages of a host-shift are only estimated retrospectively and are poorly understood. Currently, the largest body of evidence for host-shifts comes from non-congruent phylogenies of hosts and their pathogens (Carpenter et al., 1996; Nikoh and Fakatsu, 2000; Roy, 2001), but this fails to provide information about the ecological and genetic dynamics of the populations where host-shifts took place, and requires the shifts to be sufficiently persistent to produce surviving lineages on the new hosts.

Efforts to understand how and why host-shifts occur would be greatly facilitated by identifying populations in which they have happened very recently and which can be studied without pressures for disease management. The anther-smut disease of *Silene* species provides a suitable system for such research (Antonovics et al., 2002). The disease is conspicuous, producing dark spore-filled anthers rather than pollen, infected plants are sterilized but do not experience a large increase in overwintering mortality, and the pathogen, *Microbotryum violaceum*, is obligatory parasitic and known to be largely host-species specific. Also, there are no known social or economic impacts of anther-smut, and the disease can therefore be left unmanaged in natural populations.

To identify populations where host-shifts have occurred requires evidence that the pathogen on the new host is most similar to local pathogen genotypes found on the original host, as compared to strains from other localities. This would be contrasted by the case where strains are most similar by the host species on which they were found, which would be

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evidence for separate host specific lineages of the pathogen that happen to be found in the same locality. A large number of methods are available to measure genetic similarity, including DNA sequence data or DNA fingerprinting (Soll, 2000). However, it has been suggested that variation in electrophoretic karyotype may be more useful for discriminating pathogen strains of *M. violaceum* (Perlin et al., 1997), and this is also true for other fungi (Zolan, 1995). In the current study, we developed methods for comparing electrophoretic karyotypes and investigated populations where host-shifts of anther-smut disease may have occurred from *Silene latifolia* to *Silene vulgaris*.

## 2. Materials and methods

### 2.1. Study system and sample collection

Anther-smut disease occurs on members of the plant family Caryophyllaceae, primarily in the genus *Silene* (Alexander et al., 1996). The cause of the disease is the basidiomycete fungus, *M. violaceum*. Infected plants are sterilized because the reproductive structures are converted to masses of diploid fungal spores. Insect pollinators vector these spores from diseased to healthy plants, where the fungus germinates, undergoes meiosis, mates, and infects the plant (Hood and Antonovics, 2000). The fungus has two mating types in the haploid stage (A1 and A2), which determine mating compatibility by bipolar heterothallism.

*S. latifolia* and *S. vulgaris* are common ruderal plants native to Europe and naturalized in North America. These species are often sympatric, and both produce white flowers that are moth and bee pollinated, although they do not hybridize (Marsden-Jones and Turrill, 1957; Pettersson, 1991; Altizer et al., 1998). *S. latifolia* is frequently diseased by anther-smut in its native and introduced ranges. However, *S. vulgaris* is known to be diseased only in parts of Europe but not in North America, other than the one Virginia population described in this study.

The anther-smut pathogen, *M. violaceum*, was sampled from infected *S. latifolia* and *S. vulgaris* in multiple localities (Table 1). This study was initiated by the discovery of infected *S. vulgaris* in a sympatric population with *S. latifolia* near Broadway, Rockingham County, Virginia. This is the first record of anther-smut disease on *S. vulgaris* in North America (Antonovics et al., 2002). Subsequently, we have given substantial effort to locate other such localities by censusing roadside populations in North America and Europe. The range of samples included in this study (Table 1) primarily reflects this effort, as well as contributions from collaborating researchers.

We define a "sample" as a collection of diploid spores from a single flower, which are expected to be from one dikaryotic (diploid) genotype (Baird and Garber, 1979). To avoid contamination by spores from other flowers, collections are made while infected flowers are in the bud stage. In this study, samples were kept under desiccation prior to the isolation of meiotic tetrads. Tetrads were isolated by micromanipulation after incubating the spores on potato dextrose agar for 24 h at 25 °C (Hood and Antonovics, 2000). The haploid meiotic products were maintained in culture and also placed in long-term storage (frozen under desiccation). (They are available from http://www.people.virginia.edu/~meh2s/tetradcollection.htm.) The mating type of each haploid culture was determined by mixing with tester strains of known mating type and incubation under conditions that promote conjugation (i.e. 1.5% water agar at 15 °C for 24 h).

### 2.2. Electrophoretic karyotypes

One haploid culture of A1 mating type was selected from the tetrad of each sample and subjected to pulsed field gel electrophoresis. A maximum of 13 samples were run per gel, and inclusion of some samples on multiple gels served as a methodological control across electrophoresis runs. Also, size control standards (*Saccharomyces cerevisiae*, BioRad) were included in the outside lanes of each gel.

A CHEF-DR II system (BioRad) was used to generate karyotypes by pulsed field gel electrophoresis. Haploid cell cultures were suspended into agarose plugs without generating protoplasts (McCluskey et al., 1990). Electrophoresis

Table 1
Number of anther-smut samples collected by host and locality

| | Latitude | Longitude | *S. latifolia* | *S. vulgaris* |
|---|---|---|---|---|
| Broadway, VA, US | 38°36′47″N | 78°47′57″W | 4 | 4 |
| Pembroke, VA, US | 37°19′54″N | 80°28′56″W | 1 | – |
| Aldeburgh, UK | 52°08′60″N | 1°36′00″E | 2 | – |
| Hungerford, UK | 51°25′00″N | 1°31′00″W | 2 | – |
| Ipswich, UK | 52°04′60″N | 1°10′00″W | 1 | – |
| Olomouc, CR | 49°34′60″N | 17°15′00″E | 1 | – |
| Darmstadt, DE | 49°52′14″N | 8°38′58″E | 1 | – |
| Oetwil, CH | 47°25′60″N | 8°24′00″E | 1 | – |
| Orsay, FR | 48°42′00″N | 2°10′60″E | 1 | – |
| Lamole, IT | 43°32′60″N | 11°20′60″E | 3 | 2 |
| San Gimignano, IT | 43°28′00″N | 11°01′60″E | 2 | 1 |

conditions consisted of 0.8% chromosomal grade agarose, a run time of 96 h at 2.7 V/cm and 14 °C, with 200 s initial switch time and 1100 s final switch time in 1 × TBE. These run conditions optimize visualization of the chromosomes in the genome of *M. violaceum* by separation in the range of 0.90–3.00 million base pairs (mbp). The one largest autosome and the large sex chromosomes were not well resolved by these run conditions (Hood, 2002). Gel images (stained with ethidium bromide) were acquired under UV excitation by a digital camera and the Scion Image Software (Scion Corporation).

### 2.3. Data collection and analysis

Using the Scion Image Software and the size control standards, a region of the gel image between 0.90 and 3.00 mbp (covering a length of 289 pixels) was selected across the lanes. An electropherogram (or density profile) was calculated in this region for each lane, and the data were exported to the PeakFit v4 Software for automated baseline fitting and subtraction (Systat Software Inc.). Also, the peak height for a single chromosome band within each karyotype was determined and used to standardize variation in brightness across lanes.

A distance matrix for the electrophoretic karyotypes was constructed for the 26 samples, including two replicated runs of two samples across gels. All pair-wise comparisons were made by summing the squared differences in height at each pixel along the length of the paired electropherograms (Fig. 1). The distance matrix was analyzed using Ward' minimum-variance and the unweighted pair-group methods in the PROC CLUSTER procedures of SAS (SAS Institute Inc.). A distance matrix was constructed for geographical distances between samples. Also, two similarity matrices were constructed based upon whether they were from the same or different species, and whether they were from the same or different localities (scored as 0 or 1). These matrices were each analyzed for correlation with the karyotypic distance matrix using Mantel' randomization test with 1000 iterations (Mantel, 1967), excluding the two replicated runs.

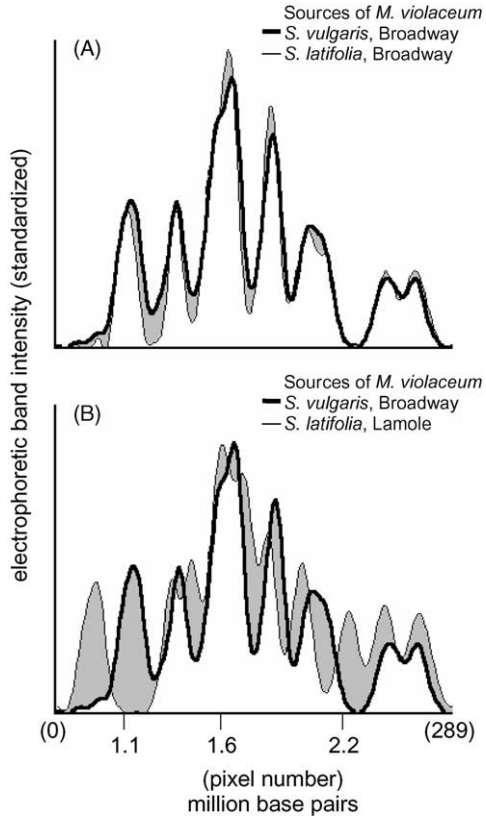


Fig. 1. Comparisons of electropherograms between *M. violaceum* from *S. vulgaris* and *M. violaceum* from either sympatric or allopatric *S. latifolia*. Peaks in the electropherograms represent the position of chromosomal bands in the electrophoretic karyotypes. The gray regions represent the differences between paired electropherograms that were summed as a measure of overall similarity. (A) Both samples are from the Broadway, Virginia. (B) The sample from *S. vulgaris* is from Broadway, Virginia, but the sample from *S. latifolia* is from Lamole, Italy.

## 3. Results

Anther-smut disease was found on *S. vulgaris* and *S. latifolia* in three localities, and these were considered the possible host-shift sites; samples were also included from eight additional localities were disease was found only on *S. latifolia* (Table 1). The first of the host-shift sites was in an abandoned hay field near Broadway, Virginia, which contained a very large and completely sympatric mixture of the two-host species (Antonovics et al., 2002). Anther-smut was commonly found in many plants of both species. The second site, in Lamole, Italy, was also a sympatric mixture of the two-host species. Here, the disease was common in *S. latifolia*, but was observed in only two plants of *S. vulgaris*. The third site, in San Gimignano, Italy, contained three diseased plants of *S. vulgaris*, but none of the sympatric *S. latifolia* was diseased. However, anther-smut disease was found and sampled from *S. latifolia* at a distance of ca. 0.5 km.

Cluster analysis of variation in electrophoretic karyotypes revealed four or five primary groups (Fig. 2). Both Ward's minimum-variance and the unweighted pair-group methods provided similar trees with regard to primary groupings, but they were not entirely identical. Multiple samples from within the same locality or same country were always found together in the primary groupings. Also, repeated runs of samples across gels, which served as methodological controls, yielded among the highest levels of karyotypic similarity.

In every case, the samples from *S. vulgaris* were most similar to those taken from *S. latifolia* in the same locality (Fig. 2). This was true even for the two possible host-shift sites within Italy, which were separated by a distance less than 30 km. The karyotypic distance matrix showed significant correlation by the Mantel' test only with the matrix of samples by locality ($r = 0.410$, $P < 0.001$). There was

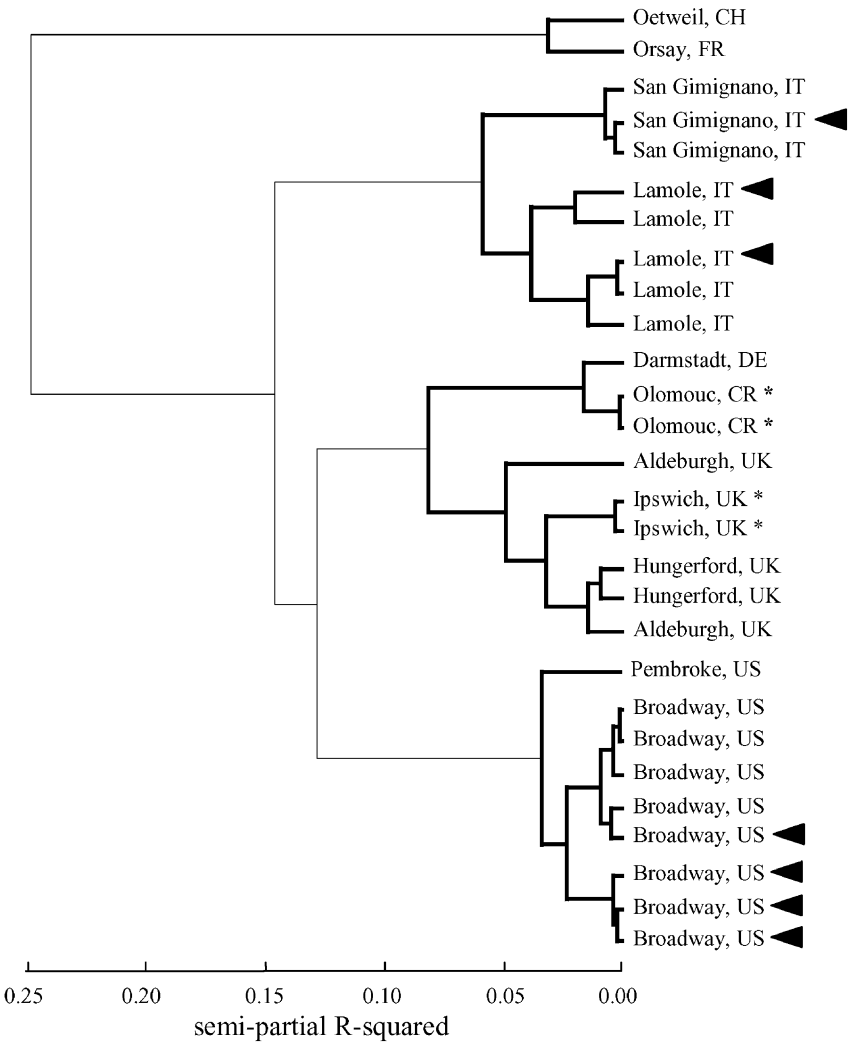


Fig. 2. A dendrogram generated from the distance matrix of electrophoretic karyotypes of *M. violaceum*. Samples of the anther-smut fungus that are marked with arrowheads were collected from *S. vulgaris*; all others were collected from *S. latifolia*. Samples marked with an asterisk (Olomouc and Ipswich) represent runs of a single sample from each of these populations that were replicated across gels as a methodological control. Semi-partial *R*-squared values indicate the proportion of variance estimated for each of the nodes. Regions of the dendrogram in bold indicates *R*-squared values >0.500.

no significant correlations with the matrices of samples by host species ($r = -0.067$, $P = 0.661$) or by geographical distance ($r = 0.068$, $P = 0.201$).

## 4. Discussion

In this study, it was predicted that host-shifts would be evidenced by pathogen strains being more similar by locality than by host species. This result is apparent from the dendrogram structure and the significance values from the Mantel's test. Thus, anther-smut transmission between *S. latifolia* and *S. vulgaris* is supported in the Broadway population, and there is at least one host-shift in Italy. There are two Italian populations where anther-smut was found on *S. vulgaris*. Within each, the most similar sample was from *S. latifolia* from the same respective population. These results would indicate two independent transmissions between *S. latifolia* and *S. vulgaris* within the Italian populations. However, because all Italian samples held together within the same primary group of the dendrogram, and because the inter-population structure is not known at this regional scale, more study is needed to confirm unequivocally that these are independent inter-species transmissions.

From the data on karyotypic variation alone we cannot determine the direction of the host-shifts; however, several additional factors support the direction of inter-species transmission being from *S. latifolia* to *S. vulgaris*. With regard to the North American collections, anther-smut on *S. vulgaris* has not been reported elsewhere in North America despite the fact that natural populations of this species have been extensively studied (Taylor et al., 2001). Also, we have observed only healthy *S. vulgaris* during the past 12 years of censusing the disease on *S. latifolia* in Virginia

(Antonovics, unpublished data). An extensive search of the local region near the Broadway population also revealed no other anther-smut on *S. vulgaris*, but quite common sympatry of this species with diseased populations of *S. latifolia*. Also, there is evidence that the fungus in the Broadway population may be somewhat maladapted to parasitize *S. vulgaris* relative to its performance on the original host, which could reflect the recent movement of *M. violaceum* into a new host environment (Antonovics et al., 2002). There is less conclusive support for the direction of transmission in the Italian populations, but again, anther-smut was very rarely seen on *S. vulgaris* in this region but was very common on *S. latifolia*.

Although questions remain unanswered regarding the age of the observed shifts and their fates, these issues are being addressed in related studies by characterizing of the ecological and genetic conditions where the host-shifts have occurred and by long-term monitoring. Also, current manipulative experiments are designed to determine the key factors for persistence of disease on the new host and its impact on the two-host community (Antonovics et al., 2002).

Recently developed electrophoretic methods that can separate megabase-sized DNA have led to a great rise in number of studies that address fungal cytology (Mills and McCluskey, 1990). Prior to this, little was know about fungal chromosomes because they are generally too small to be characterized microscopically. The most striking discovery has been the tremendous amount of karyotypic variation within and among populations. The idea that it is possible to use karyotypic information to assess similarity among fungal strains is therefore not new. Electrophoretic karyotypes were used to distinguish isolates *Microbotryum* on native North American species of *Silene* from isolates found on European species of *Silene* (Perlin et al., 1997). The karyotype patterns of several fungal pathogens, such as *Candida albicans* and *Cryptococcus neoformans*, have also been useful as an epidemiological or fingerprinting tool to identifying local sources of infection (Soll, 2000). However, we were very surprised by the sensitivity of our methods for distinguishing local populations over very short distances. It is not at all clear why *M. violaceum* is so karyotypically variable, and the phenotypic or fitness consequences of this remarkable variation remain to be investigated.

In this study, a primary concern was to develop an objective method for comparing electrophoretic karyotypes from a broad range of samples. Most previous studies were not based on measuring a degree of similarity but rather used qualitative assessments of karyotype variation or an absolute identity among strains (Perfect et al., 1993; Soll, 2000). Because this study included samples from a wide geographical range, the karyotypes are expected to vary between localities, and a more quantitative approach was needed. We, therefore, used a computer based subtractive method to compare electropherograms. This method was remarkably accurate, as judged by the concordance with replicate runs using the same haploid genotype.

Established methods for measuring similarity among other forms of DNA fingerprints assume homology of equally sized electrophoretic bands and then use a presence/absence classification across samples. In our case, this was not possible because of the subjectivity associated with deciding the number of adjacent pixels within which two peaks would be considered to represent homologous chromosomes, and the problem of chromosomes being hidden within tight groups of co-migrating bands.

The major drawback of using karyotypic variation to measure genetic similarity is that we have a very poor understanding of how karyotypes evolve, and consequently a generally accepted phylogenetic model is currently lacking. Changes in electrophoretic karyotypes can arise by a variety of mechanism, including translocations, deletions and duplications, or chromosomal fusion and breakage. Each of these will have potential consequences at the levels of both gene expression and the mechanics of chromosomal pairing during cell division. The studies that have incorporated chromosomal variation into phylogeny reconstruction have been largely based on microscopically visible karyotypes, including work on primates (Nagamachi et al., 1999), rodents (Brittondavidian et al., 1995), Drosophila (Brehm et al., 1991), and plants (Darlington, 1956, Tagashira and Kondo, 2001). In each of these cases, the phylogenetic comparisons have been largely between species, where karyotypic evolution is sufficiently slow that each species still contains recognizably similar chromosomes, yet where banding patterns and size characteristics indicate variation between species.

The tremendous genomic plasticity in fungi makes it possible to compare samples within a species by their overall karyotypic patterns. Most often observed in asexual fungi, karyotypic variation is thought to be limited in sexual species because non-homology between the chromosomes will likely cause meiotic abnormalities (Kistler and Miao, 1992). *M. violaceum* is an obligately sexual species, and thus we might have expected little chromosomal variation. However, we have shown that it frequently undergoes a system of intra-tetrad mating, which is analogous to meiotic restitution in organisms with automictic parthenogenesis (Hood and Antonovics, 2000). This may have previously unanticipated consequences for the maintenance of chromosomal as well as allelic variation. Much more understanding of the basic principles of karyotype evolution in fungi is still needed before the issues of inferring descent from such variation are resolved.

Great caution should therefore be taken before drawing direct phylogenetic conclusions from electrophoretic karyotypes, especially with regard to the relative rates of change in different regions of the tree. However, it is encouraging that the dendrogram we obtained was highly reflective of spatial structure. Indeed, the method appeared to be successful at clustering moderately related strains (from different populations in a common region). Samples generally clustered within countries, within localities, and within-populations. An interesting exception was the

Aldeburgh population. Samples from this population were in the same cluster with other UK samples, but were otherwise quite distant from each other in the dendrogram.

The use of replicated samples across gels provides a measure for the amount of resolution inherent in the method. From this, it appears that some of the within-population samples were essentially identical karyotypes because their level of similarity was at least equal to that between the replicated controls. In two of the populations where host-shifts were observed, samples from *S. vulgaris* were indistinguishable from sympatric samples from *S. latifolia*, suggesting a recent or continuing movement of the fungus onto the new host. It remains to be determined under what conditions such host-shifts become permanent and lead to the kind of large karyotypic differences that are found between different and presumably long-established host races.

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