Genetic variation, spatial distribution, and reproductive biology of pseudoflower-forming rust fungi (Uromyces pisi and relatives) on Euphorbia cyparissias

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Genetic variation, spatial distribution, and reproductive biology of pseudoflower-forming rust fungi (Uromyces pisi and relatives) on Euphorbia cyparissias

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1 SUMMARY

Insects play a major role in many fungal life cycles, either as vectors to transmit the fungus to a new host, or as "pollinators" to transfer the gametes of fungi among mating types. Some rust fungi of the genera Puccinia and Uromyces use flower-like traits to attract insect "pollinators". Plants infected by these fungi are generally inhibited from flowering. Instead, they are induced to form pseudoflowers, rosettes of yellow leaves that are clustered on top of the stem in a flower-like shape. Not only do fungal pseudoflowers visually resemble true flowers, but just like true flowers, they present a sweet-smelling nectar that is produced by the fungus on the surface of the host leaves. The nectar contains fungal gametes (spermatia) that are transferred by nectar-feeding insects.

Rusts of the species complex Uromyces pisi (Pers.) Wint. produce pseudoflowers on cypress spurge, Euphorbia cyparissias L. Although they were suspected to be dependent on insects for their reproduction, no study has been done so far to confirm this assumption. By conducting an insect-exclusion experiment, I was able to show that fungal sexual reproduction does rely on insect vectors (chapter 4).

In the course of the insect exclusion experiment, I observed a conspicuously high number of ants on the fungal pseudoflowers, as well as on the non-infected flowering host plants. Stéphanie Schürch, a diploma student, joined with me to study the role of ants as "pollinators" of non-infected and infected Euphorbia plants. We showed that ants were able to pollinate the non-infected E. cyparissias plants, however, they did not fertilise the fungus (chapter 5).

Flying insects, especially flies and solitary bees, visited often and moved freely among non-infected host flowers and fungal pseudoflowers. Therefore, I hypothesised that pseudoflowers and true flowers might interact through these "pollinators" by either competing for insects or by facilitating each other's visits. An artificial array experiment showed that true flowers were strongly preferred over pseudoflowers, however, no influence on the visitation rates among the two species could be observed when pseudoflowers and true flowers co-occurred in mixtures (chapter 4). In a non-manipulative field study I then measured the reproductive success of pseudoflowers and true flowers in combination with the density and frequency of neighbouring pseudoflowers and true flowers. This study, in contrast to the array experiment, showed that pseudoflowers were serious competitors for true flowers, whereas pseudoflowers were not seriously influenced by non-infected hosts (chapter 6).

Although it is known that the species complex U. pisi consists of 11 described species that all induce pseudoflowers on E. cyparissias, I never distinguished among the different species in the experiments described above. The reason for this was that the fungal species are morphologically indistinguishable on E. cyparissias. Only after successful
fertilisation through insects do they switch to another host. The taxonomy of the different species is based on the choice of this alternate host, as well as on the shape and surface of teliospores produced on this host. For further studies on the ecology of these rusts it will be indispensable to be able to differentiate them while they produce pseudoflowers. I therefore used a molecular method, sequencing the internal transcribed spacer region (ITS) in the rDNA, to identify the different species. The ITS region has been found to be appropriate for phylogenetic analyses of other fungi, and also provided valuable markers for the study on Uromyces. I was able to isolate DNA from five different species in the U. pisi complex, as well as from two species in the closely related species complex Uromyces scutellatus. Phylogenetic analyses suggested that Uromyces scutellatus, a microcyclic fungus with a simple life cycle of only 2-3 spore stages, may have evolved from the macrocyclic ancestors in the U. pisi species complex (chapter 7). I further applied this molecular method to evaluate the species distribution of U. pisi within and among E. cyparissias populations in Switzerland. Although I found five different species of U. pisi on Fabaceae, only two of them were also isolated from freshly collected E. cyparissias (U. pisi s.s. and U. striatus). One further species was identified on E. cyparissias from a herbarium specimen. U. pisi s.s. and U. striatus were co-occurring within the same host population and were both found at elevations from 500 to 2000 m (chapter 7).
2 ZUSAMMENFASSUNG


3.1 ON THE HISTORY OF PHYTOPATHOLOGY

Today, all fungi world-wide are hypothesised to consist of 1.5 million species, from which only about 5%, 70'464 have been described (Hawksworth, 1997). 22'244 of the described species belong to the phylum Basidiomycota (Hawksworth, 1997). Rust fungi (Uredinales) constitute one of the largest groups in the Basidiomycota with about 5000-6000 described species (Hiratsuka and Sato, 1982) and include many economically important phytopathogenic fungi, such as the wheat stem rust *Puccinia graminis*, the white pine blister rust (*Cronartium ribicola*) or the coffee leaf rust (*Hemileia vastatrix*) (Cummings and Hiratsuka, 1983).

In the late 17th century fungi were first recognized as the source of plant diseases (Hoffmann et al., 1994). In 1755, Tillet demonstrated the infectious nature of pathogenic fungi with experiments on the wheat bunt fungus *Tilletia caries*. But the trigger for more intensive studies was the serious epidemic infection on potatoes by the potato late blight fungus *Phytophthora infestans* after an uncommonly rainy and cold season in Europe. In 1845, the famine led to the death of about one million people in Ireland. A further 2 million people emigrated from Ireland to Australia or the New World to avoid starvation (Dixon, 1998). About one year after the catastrophic epidemics in Ireland, Miles Berkeley, an amateur scientist and English priest of Northamptonshire, studied the infected potato plants and observed tiny threads within them under a microscope. He hypothesised that these threads killed the plant. Anton de Bary confirmed Berkeley’s observations in 1860 (Hoffmann et al., 1994; Dixon, 1998). De Bary was one of the pioneers in phytopathology, a branch of natural sciences that became more and more important to humans as the world population and agricultural production increased over the last 100 years. Increased agriculture led to higher pre-harvest losses, and travel to new epidemics through pathogens that were being introduced from foreign countries and continents, such as in 1949 the introduction of corn leaf rust (*Puccinia polysora*) from Central America to West Africa, or in 1970 the introduction of coffee leaf rust (*Hemileia vastatrix*) from Central Africa to Brasil (Schlösser, 1983). Despite the pest controls used today, diseases, insects and weeds still lead to the loss of up to or more than 50% of the harvest (Hoffmann et al., 1994). The pre-harvest losses in crops world wide, caused by disease only, were estimated between 15-17% in rice, 13-17% in wheat, and 10-15% in barley in the years 1988-1990 (Hoffmann et al., 1994). The lower numbers indicate losses using pest controls, the higher numbers indicate losses without the use of pest controls. In addition to these pre-harvest losses, one has to add 9-20% post-harvest losses through pests during storage.
One very important step in phytopathology, again owing to the studies of De Bary, was the realisation that *Puccinia graminis*, a rust fungus that seriously threatens wheat, needed a second host plant to be able to live through its life cycle. De Bary could combine *P. graminis* with *Aecidium Berberidis*, a fungus that up to then was treated as a separate species on barberry (*Berberis vulgaris*). He could show that the two described species belonged to the same fungus that alternates between these hosts. By doing so, he proved what many farmers already knew from their experience, that barberry around the fields led to rust infection of the wheat next to them (De Bary, 1865). The example of *P. graminis* shows that it can be very important to know the whole life cycle of phytopathogenic fungi to be able to prevent them from destroying food plants.

### 3.2 Rust fungi and their life cycles

Rust fungi (*Uredinales*) belong to the phylum Basidiomycota. Fungi in this phylum produce sexual spores called basidiospores. Basidiospores are produced outside the meiosporangium, compared to the ascospores of fungi in the phylum Ascomycota, where the sexual spores are produced within the meiosporangium. The name „rust fungi“ comes from the fact that infection by these fungi is often visible when rusty colored fungal pustules and spores are formed on the surface of host leaves.

As in the example of *P. graminis* in the former chapter, many rusts have heteroecious life cycles, meaning that they alternate between two hosts that often belong to different plant families. Other fungi spend all their lives on one host and are called autoecious (Hawksworth et al., 1995). Rust fungi that exhibit a full life cycle are termed macrocyclic. They go through five different spore stages. Macrocyclic rusts can be heteroecious or autoecious. However, many rust species have a reduced life cycle, deleting either one or several spore stages, and are termed demi- or microcyclic. All microcyclic species are autoecious (Peterson, 1974).

Most of the heteroecious fungi have been described as separate species on the two hosts, or may have only been found on one host so far. Using inoculation experiments, some of them have later been recognised as one species. Still today, however, there are thousands of rusts from which only one host is known. Moreover, today's taxonomy of the rusts still lacks phylogenetic foundation and is mostly based on host preferences.

In this thesis I worked with species in the *Uromyces pisi* complex. These are typical heteroecious, macrocyclic rust fungi, alternating between cypress spurge, *Euphorbia cyparissias*, and several different Fabaceae plants (Gäumann, 1959). I also included microcyclic fungi of the *Uromyces scutellatus* complex. The following life cycle descriptions are based on: Gäumann, 1959; Peterson, 1974; Hiratsuka and Sato, 1982; Cummings and Hiratsuka, 1983. The terminology used in this study is based on Ainsworth & Bisby’s dictionary of the fungi (Hawksworth et al., 1995).
**Macrocyclic life cycle**

Macrocyclic fungi have five spore states and alternate between two different hosts (Fig. 3.1). On the telial host, a host of the genus Fabaceae, the fungus produces teliospores. Teliospores are spores with thick walls representing an overwintering stage of the fungus. Teliospores generally show characteristic surfaces that have been used to group the rusts into species or genera (Fig. 3.2). These teliospores are at first dicaryotic. After the winter, they undergo karyogamy and form zygotes. After germination of the teliospores basidia are formed that produce haploid basidiospores through meiotic division. Basidiospores are wind dispersed and can only infect an aecial host in heteroecious fungi, and not a telial host. If they land on an aecial host, a *Euphorbia cyparissias* plant, basidiospores germinate and produce intercellular mycelium within their hosts. From the mycelium the fungus breaks through the host tissue and forms spermogonia on the surface of the host leaves (Fig. 3.3). Spermogonia could be compared to a complete flower. The male equivalent to pollen are the produced spermatia, fungal gametes that are contained in a sweet sugar-containing nectar. At the same time, adjacent to the spermatia, a second type of organ is formed, an aecial primordium, where the female equivalent to the stigma, a flexuous hypha, grows among the spermatia to the outside of the host (Fig. 3.3). A few studies have shown that some rusts depend on insects as "pollinators" to transfer spermatia from one fungus to the next and deposit spermatia on the flexuous hyphae, just as in pollination (Craigie, 1927; Craigie, 1931; Roy, 1993). When the deposition of the spermatia of the opposite mating types has been successful, the foreign spermatia fuse with the flexuous hyphae and the plasmogametic nucleus travels back through the mycelium, and aeciospores begin to develop. Aeciospores are dispersed by the wind to the telial host in spring. They can only infect the telial host, not another aecial host. On the telial host, they germinate and produce millions of dikaryotic urediniospores that cause further infections of telial hosts. By the end of the vegetation period they form the overwintering teliospores and a new cycle begins.

**Figure 3.1** Macrocyclic life cycle. Example of a heteroecious species in the complex *Uromyces pisi*. 
**Microcyclic rust fungi**

Many rusts show a life cycle that is different from the macrocyclic one. They can take shortcuts through the full life cycle, either leaving out just the uredinial stage (= demicyclic) or they lack both the telial and uredinial stages, which leaves only the telial and basidial stages (= microcyclic) (Fig. 3.4). Microcyclic fungi are autoecious, staying on one host during their entire life. In all macro-, demi- and microcyclic life cycles, the spermatial stage may be absent, but this stage is absent most often in microcyclic fungi.

**Figure 3.4** Microcyclic life cycle.

Example of an autoecious rust species of the complex *Uromyces scutellatus*.

The spermatial stage is optional.
3.3 Fungal pseudoflowers

Insects play a role in many of the fungal life cycles. Insects can either function as vectors to transmit the fungus to new hosts (Batra and Batra, 1985; Shykoff, Bucheli, and Kaltz, 1997) or they can transfer gametes among mating types for sexual reproduction (Craigie, 1927; Craigie, 1931; Roy, 1993; Bultmann et al., 1998; Pfunder and Roy, in press). In one case of insect-dependent sexual reproduction, it has been described that the fungus even uses traits that are very similar to the ones used in floral pollination to attract insects: scent, colour and shape (Roy, 1993). Roy reported that Arabis plants, when infected by the rust fungus Puccinia monoica, had a totally different appearance. Instead of forming true flowers, the plant was induced to form flower-like clusters of yellow leaves on top of the stem, from which a sweet-smelling and sugar-containing exudate was produced. Insects fed on this exudate and thereby transferred fungal gametes among mating types. Roy called these flower-like structures pseudoflowers. This thesis presents a second system of a rust fungus inducing pseudoflowers on its host, Uromyces pisi (Pers.) Wint. on Euphorbia cyparissias L.

3.4 Thesis outline

Pathogenic rusts of the species complex Uromyces pisi induce the formation of pseudoflowers on the host plant Euphorbia cyparissias. On these pseudoflowers, rosettes of yellow leaves that resemble true flowers, the fungus produces sweet-smelling nectar in which it presents fungal gametes. The purpose of this study was to find answers to the following questions concerning these rust fungi:

1) What is the role of insect visitation to the fungus? The establishment of insect-dependence is important to all further ecological and evolutionary studies on these fungi. We approached this question by using insect exclusion experiments. Chapter 4 presents a first study, testing the effect of the presence/absence of insects on fungal reproduction. Chapter 5 presents a second study, written as a diploma thesis by Stéphanie Schiüch and co-authored by B. Roy and myself, where we tested the importance of ants as pollinators of the uninfected host plant, as well as their importance for fungal fertilisation.

2) Assuming obligate insect-fertilisation of the fungus, can fungal pseudoflowers influence the insect visitation of surrounding non-infected host flowers and vice versa? Experiments should give an insight into the role of pseudoflowers in a flower community. I used two different approaches to answer this question, first, using artificial array experiments (chapter 4), and second, non-manipulative observations (chapter 6).
3) What is the phylogenetic relationship among species in the pseudoflower-forming species complex *Uromyces pisi* and the closely related, but insect-independent species complex *Uromyces scutellatus*? The results of the study based on molecular techniques should re-evaluate the morphologically based taxonomy of the rusts on *Euphorbia cyparissias* (Gäumann, 1959), and should provide a basis to clearly differentiate the different species by their genotypes for further ecological and evolutionary studies (chapter 7). I used the internal transcribed spacer region (ITS) of fungal rDNA to analyse the sequences among genotypes and used cladistic analysis to construct a phylogeny. The sequences were further used to test the distribution of different fungal species and genotypes within as well as among host populations.

### 3.5 Literature cited


4 POLLINATOR-MEDIATED INTERACTIONS BETWEEN A PATHOGENIC FUNGUS, *UROMYCES PISI* (**PUCCINIAEAE**), AND ITS HOST PLANT, *EUPHORBIA CYPARISSIAS* (**EUPHORBIACEAE**)

4.1 Abstract

The plant *Euphorbia cyparissias* is commonly infected by rust fungi of the species complex *Uromyces pisi*. When infected, *E. cyparissias* is unable to flower, but instead is induced by the fungus to form pseudoflowers. Pseudoflowers are rosettes of yellow leaves upon which the fungus presents its gametes in a sweet-smelling fungal nectar. We hypothesised that the fungi, as they are heterothallic, are dependent on insect visitation to cross-fertilise their mating types. We confirmed that insects are required with an insect exclusion experiment. We further hypothesised that pseudoflowers of *U. pisi* interact with uninfected true host flowers through insects during their period of co-“flowering” in early spring. We conducted artificial array experiments in the field to test whether the two species share insects and whether they influenced each other’s insect visitation. Insects moved between true flowers and pseudoflowers, but true flowers received more visits over all. Pseudoflowers and true flowers did not influence each other’s visitation rates in mixtures. However, shorter visits were observed on pseudoflowers in mixtures than monocultures, suggesting that true flowers might be competitors for pseudoflowers. Further experiments are needed to determine whether the similarity of pseudoflowers to true flowers is adaptive.

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4.2 Introduction

For over a hundred years studies have accumulated describing and discussing heterothallic rust fungi (fungi with more than one mating type) that present their haploid fungal gametes (spermatia) in a sweet-smelling, sugary nectar attractive to insects (Ráthay, 1882; Buller, 1950; Roy, 1994). Since the experiments of Craigie (1927), it has generally been assumed that the insects attracted by these fungi aid in their sexual reproduction. However, only a handful of studies have actually proven that insects are required as gamete vectors for any rust fungi (Craigie, 1927, 1931; Roy, 1993). In the few studies in which insect-dependent fertilisation was established, we can distinguish between nonsystemic (Craigie, 1927, 1931) and systemic (Roy, 1993) species of rust fungi. In nonsystemic infections, the spermatial lesions are very small and more than one mating type can be present on a leaf. In this case, fertilisation of the fungus can occur when insects crawl over the leaf, or even by direct contact of hyphae of opposite mating types in nearby pustules (Craigie, 1927), and gamete transfer among host plants is not necessary. In systemic infections, however, the spermatial lesions are usually generated by the same fungal genotype on the whole host plant, and these infections may contain only one mating type. In this case, insects are likely to be required to transfer gametes between infected host plants.

Systemic infections can also differ from nonsystemic infections by the degree to which they change host morphology. Systemic rusts of *Puccinia monoica* on *Arabis holboellii* inhibit the ability of their hosts to form functional flowers (Roy, 1993). Instead, the leaves of the host become pale yellow, the host is induced to form a rosette on top of the stem, and the fungus presents sweet-smelling nectar on these leaves. Because these rosettes of infected leaves resemble true flowers, they are referred to as pseudoflowers (Roy, 1993).

Here we present data on another pathogen-plant complex of systemic rust fungi that produce pseudoflowers on their hosts: fungi of the species complex *Uromyces pisi* (Pers.) Wint. on their host species *Euphorbia cyparissias* L. (Fig. 4.1). For over a hundred years this host-pathogen pair has been subject to much speculation concerning the function of nectar producing spermatia (Ráthay, 1882; Buller, 1950). Today, it is generally assumed that they are produced to attract insects for fungal fertilisation, but we do not know of any study that actually tests the role of insects for the fertilisation of the fungus. We used an insect exclusion experiment to test the dependence of the fungus on insect visitation for sexual reproduction.

Because pseudoflowers of *U. pisi* look, smell, and produce nectar like flowers (Fig. 4.1B), insects may have been and might still be the selection agents driving the evolution of the flower-like morphology. From studies of flower-pollinator interactions we know
that insects can cause selection on floral traits (reviewed in Shykoff, Bucheli, and Kaltz, 1997) and that the direction of selection can be influenced by the floral composition of the community because insects choose what to visit (Rathcke, 1983; Waser, 1983; Feinsinger, 1987). Insect-mediated species interactions can lead to either competition or facilitation.

![Image](image_url)

**Figure 4.1** (A) Uninfected flowering *Euphorbia cyparissias* plant, and (B) *E. cyparissias* infected by the rust fungus *Uromyces pisi*. Infected plants are sterilised and induced by the host to form pseudoflowers, rosettes of yellow leaves, upon which the fungus presents gametes in a sugary nectar.

Competition for pollinators may cause selection for differences in flower characters because competition can be ameliorated when mutations arise that allow the use of another pollinator or the same pollinator at a different time (Robertson, 1895, 1924; Levin and Anderson, 1970; Mosquin, 1971; Waser, 1978, 1983; Feinsinger, 1987; but also see Connell, 1980). However, pollinator preferences can also drive the evolution of similarities. When pollinators prefer the more common flower phenotype (or species) over a rare one, this preference will select the co-occurring rare phenotype (or species) towards similarity to the most common flower species (Mosquin, 1971; Straw, 1972; Thomson, 1978). This process is referred to as positive frequency-dependent selection and is important in the evolution of Müllerian floral mimicry.

Facilitation for pollinator visitation may cause selection for similarities in flower characters because co-flowering species can gain a selective advantage through their
combined attractiveness to pollinators (Grant and Grant, 1968; Macior, 1970; Brown and Kodric-Brown, 1979; Rathcke, 1983; Feinsinger, 1987; Gross, 1996). Under facilitation, individual visitation rates to flowers in mixed patches should be higher than in single-species patches. Whether facilitation of visitation translates into selection for similarity or differentiation depends on the degree of improper pollen transfer (Rathcke, 1983; Feinsinger, 1987). Improper transfer of pollen between species reduces male fitness as the pollen is placed on the wrong species, and it can also reduce female fitness if the improper pollen reduces the probability of the proper pollen making contact with the stigma, or if the improper pollen is toxic. For facilitation to drive the evolution of similarity among flowers, the benefits of similarity in terms of pollinator attraction must outweigh the disadvantages of improper pollen transfer, or mechanisms must be in place that allow proper pollen transfer despite the visual similarity of the species.

In this study we used artificial array experiments to test, under controlled densities and frequencies, whether pseudoflowers of *U. pisi* and true flowers of *E. cyparissias* share visitors, and whether insect behaviour can indicate competition or facilitation among the two species. If it is found that the species influence each other’s visitation through insects, either in a positive or a negative way, then further experiments would need to be done to determine the influence of visitor behaviour on fitness and selection of the two involved species.

### 4.3 Materials and methods

#### 4.3.1 The host and its pathogen

The host plant, *Euphorbia cyparissias* L. (cypress spurge), is a Eurasian perennial that is very common throughout Switzerland, found from 200 to nearly 3000 m above sea level. It occurs mainly on poor and fairly dry soils, along forest edges, in sparse woods, and in dry alpine grasslands. *Euphorbia cyparissias* has also been introduced to eastern Canada and north-eastern United States, where it occurs in similar habitats (Stahevitch, Crompton, and Wojtas, 1987; Gassmann and Schröder, 1995). It is self-compatible (Schürch, Pfunder, and Roy, in press), but due to protogyny generally needs insect visits to set seeds. The plants have a mutualistic relationship with ants. Ants pollinate the flowers while feeding on extrafloral nectaries (Schürch, Pfunder, and Roy, in press) and disperse the seeds, which bear an elaiosome, a food source for the ants.

Rust fungi in the species complex *Uromyces pisi* (Pers.) Wint. are common pathogens on *E. cyparissias* in Europe (Gäumann, 1959). They produce pseudoflowers with nectar on their host. *Uromyces pisi* species possess a heteroecious life cycle, alternating from *E. cyparissias* to another host, a member of the Fabaceae. Each species of the complex attacks only one species from the family of Fabaceae (Gäumann, 1959).
The incubation time of *Uromyces* in *Euphorbia* takes a whole year (Hartwich, 1955). The fungus passes the winter in the roots of the host plant and grows with the host as it sprouts in the spring, drastically changing host morphology. Infected plants are hypertrophied, with enlarged cells and intercellular spaces where the mycelium of the fungus is present (Hartwich, 1955). This makes infected leaves look succulent compared to the original leaf morphology of the host. Infected stems grow earlier in the season than uninfected stems, a result of enhanced concentrations of growth hormones (Pilet, 1952). On top of the infected stem, the fungus induces its host to form pseudoflowers (Fig. 4.1). These pseudoflowers bear some resemblance to *Euphorbia* flowers in that they are both yellow and produce sweet-smelling nectar that is easily reached by insects without requiring specialised mouth parts.

### 4.3.2 Insect Exclusion Experiment

The insect exclusion experiment was conducted in Vicques, Cras de la Combe, in the Swiss Jura Mountains at ~ 500 m above sea level (co-ordinates 599 000/245 940), in a species-rich calcareous grassland. The site was oriented 144° south-southeast at roughly a 30° angle. The area in which the *E. cyparissias* population was studied was 2680 m², with an average density of about one *E. cyparissias* stem/m² (with a mean of ~ 2-3 stems per individual). However, the population was spatially clustered and in places reached much higher local densities of up to 16 stems/m². About 20% of all stems were infected, but the proportion varied over space as well as time. Infected stems appeared ~ 1 mo earlier than uninfected ones, and partly dried up while some *E. cyparissias* flowers were still blooming. Nonetheless, overlap in nectar production and presentation of pseudoflowers and true flowers could be observed for ~ 1 mo.

The exclusion experiment was conducted in the early spring of 1997. Infected individuals of *E. cyparissias* were caged with small-meshed florist gauze nets (Kleen Test Products, division of Meridian Industries, Inc., Milwaukee, Wisconsin) to exclude insects. The bags allow light, wind, and water to penetrate, but exclude even the smallest insects. If an individual plant produced more than one stem, all stems were included together in the same cage.

The first infected *E. cyparissias* stem was found on 7 March 1997. For the rest of the month, the field was visited at least twice a week, and all infected individuals that could be found before the fungus produced spermatia and nectar were caged or left uncaged as controls, marked with a flag and numbered consecutively. During April, the field was visited at least once a week, but fresh stems of infected individuals were rare. One of the following four treatments was assigned systematically to each of the host individuals: (1) "self" (within plant) fertilisation of caged individuals with paintbrushes, (2) hand outcross
(among plant) fertilisation of caged individuals with paintbrushes, (3) caged controls without treatment, and (4) uncaged controls with natural insect visitation.

For each outcross fertilisation, three infected host individuals that were not included in the experiment otherwise were “visited” with a paintbrush and the sticky liquid nectar with spermatia was transferred back to the treated plant. For “self” fertilisation, spermatia were moved within a caged plant with a paintbrush. Each treatment application was repeated on two to five different days to each of the caged plants. The plants were harvested when the stems were either displaying aecia (fungal organs in which aeciospores as infective agents to the alternate hosts are produced) or were losing their leaves and drying up. The first stems were harvested on 2 May, the last on 20 May; after the harvest they were pressed and dried in a plant press. To collect data on fungal reproductive success, a binocular microscope (Wild, M5A, Heerbrugg) with 60-120x magnification was used. Fungal reproductive success was defined by the fungus’ ability to produce aeciospores. Aeciospores are heterokaryotic spores that are distributed by wind to the Fabaceae hosts. The production of aeciospores is the result of successful fertilisation and can therefore be compared to the production of seeds in plants. We counted the number of aecia, the fungal organs in which aeciospores are produced. We assume that more aecia also produce more aeciospores. \( \chi^2 \) tests were used to determine whether a) the production of aecia on all individuals, b) the mean proportions of aecia-bearing stems on the subset of aecia-bearing individuals, or c) the mean proportion of aecia-bearing leaves on the subset of aecia-bearing stems were independent of the pollination treatment.

4.3.3 ARTIFICIAL ARRAY EXPERIMENT

To determine whether pseudoflower visitation may be facilitated by co-occurrence with true flowers of uninfected Euphorbia cyparissias, an artificial array experiment was conducted from 18 to 19 April in Vieques (the same field site as for the insect-exclusion experiment) and from the 15 to 16 May in Ardez in the lower Engadine, southern Switzerland, at \( \sim 1410 \) m elevation. The Euphorbia population (140 m\(^2\)) near Ardez is located in a dry calcareous grassland (co-ordinates 811 225/184 025), oriented at an \( \sim 20^\circ \) angle and facing 190° south. The density of Euphorbia was much higher in Ardez than in Vieques. At the time of the experiment in Ardez we observed an average density of 53 E. cyparissias stems/m\(^2\), and about half (48%) of the stems were infected.

Florist pics (small plastic tubes) were used to arrange single stems of pseudoflowers and true flowers on two plots of 1 m\(^2\), located 5 m apart (Fig. 4.2A). Insect observations in the arrays were only conducted during sunny and calm weather conditions. To control density and frequency of the plots, all flowers were removed from these plots before setting out the arrays. Three kinds of artificial arrays were used: (1) a monoculture of ten
pseudoflowers in spermatial stage (*U. pisi*), (2) a mixture of five pseudoflowers in spermatial stage (*U. pisi*) and five true flowers of *E. cyparissias*, and (3) a monoculture of ten true flowers of *E. cyparissias*. In Vieques as well as in Ardez, two different plots 5 m apart (Fig. 4.2A) were chosen. Two observers were assigned randomly to the two plots, and both observers observed one array each during a time period of 20 min (t1-t4 in Fig. 4.2B). After each observation period, all tubes were removed and reordered for the next array. The experiment was analysed as a factorial, blocked design with two blocking factors, a spatial block, and a time block (Fig. 4.2B). A spatial block consisted of all arrays being observed once at one of the two plots and was designed to account for variation in visitation resulting from the observation plots being in locations 5 m apart. A time block consisted of all arrays being observed at both plots once (two spatial arrays) and was designed to account for variation in insect activity within and among the different times of observation.

**Figure 4.2** (A) Experimental design for the artificial array experiments. Observations were made two at a time for 20 min at each of two different plots of 1 m². (B) The experiment included spatial and temporal blocks in an orthogonal factorial design, each block included one monocultural array of *U. pisi* pseudoflowers (fungus), one (Vieques) or two (Ardez) mixed arrays of *U. pisi* pseudoflowers and true flowers of *E. cyparissias* (fungus-flower) in mixtures, and one monocultural array of true flowers of *E. cyparissias* (flower). Observations were made two at a time with two observers at the two plots (t1-t4).
In Vieques, the mixed array was recorded only once per spatial block; in Ardez, the mixed array was present twice in each spatial block. A problem common to most competition studies is that observations of two components in mixed arrays are not made independently (Snaydon, 1991). Recording the mixed array twice per spatial block gave us the opportunity to analyse only pseudoflowers or only true flowers in the two mixed arrays of Ardez and therefore to analyse the two species independently. The choice of which species was analysed per mixed array was randomly assigned.

For each visitor, the visit to a pseudoflower or true flower was recorded, and the amount of time spent per visit on each flower or pseudoflower was measured with a stopwatch. To test whether pseudoflowers and true flowers actually shared insects, movements by insects within the arrays were also recorded and analysed using Bateman’s constancy index, which measures the degree of fidelity for insects to a particular species (Waser, 1986). The particular insect visitors were identified as well as possible during the observations, and identifications were verified after the experiment by catching representatives. The data were separated into two categories: all insects and insects other than ants. Separating the ant data from the other insects is reasonable, because ants made up to ~40% of all insect visits in Vieques and ~10% in Ardez. From a different experiment we know that ants pollinate true flowers of *E. cyparissias*, but that they do not fertilise pseudoflowers of *U. pisi* (Schürch, Pfunder, and Roy, in press).

To determine the kind of interaction between fungal pseudoflowers and true flowers as mediated by insects (e.g., competition, facilitation, or no interaction), the visitation rates and the mean duration per visit were calculated for true flowers and pseudoflowers separately, and the effect of the array (monocultures vs. mixtures) was tested. A mixed-model analysis of variance (ANOVA) was used with time blocks and spatial blocks included as random factors. Three-way interactions were excluded from the model, and two-way interactions were included and removed from the model if they were nonsignificant at $P > 0.25$. Synthetic denominators, as calculated in JMP, Version 3.1. (SAS, 1994), were used to calculate $F$ ratios in the mixed models. When the interaction of “species” (pseudoflowers or true flowers) by “array” (mixtures or monocultures) was significant, we analysed the following a priori contrasts: *U. pisi*-mono (in monoculture) vs. *U. pisi*-mix (in mixtures) and *E. cyparissias*-mono (in monocultures) vs. *E. cyparissias*-mix (in mixtures). Visitation rates and mean duration per visit were analysed for “all insects” and for “insects excluding ants”. The model for visitation rates in Vieques excluding ants is not shown, because the whole-model test was not significant. The data were transformed only when necessary to meet the normality and homogeneity assumptions of ANOVA.
4.4 Results

4.4.1 Insect Exclusion Experiment

The proportion of host individuals bearing fungal aecia was significantly different among treatments ($\chi^2 = 46.74$, 3 df, $P < 0.0001$; Table 4.1). When the fungus was hand outcrossed or left to natural visitation, it produced many more aecia than when it was "selfed" or caged to prevent visits. Within the aecia-producing individuals we further observed the degree to which fertilisation was successful: while the mean proportions of aecia-bearing stems on the subset of aecia-bearing individuals were not significantly different among treatments ($\chi^2 = 7.29$, 3 df, $P = 0.0633$; Table 4.1), the proportions of aecia-bearing leaves that could be counted on the subset of aecia-bearing stems were significant ($\chi^2 = 14.344$, 3 df, $P = 0.0025$; Table 4.1).

Table 4.1 Insect exclusion experiment. Mean reproductive success (aecia formation) of U. pisi rust fungi under four different treatments: hand "self" (within host) fertilisation, hand outcross (among hosts) fertilisation, caged controls excluding all insects, and uncaged controls with natural fertilisation. Shown are the number of individuals used in the experiment (N), the proportions of individuals, stems, or leaves with successful fertilisation ($\pm 1$ SE), as well as the proportions of stems and leaves with successful fertilisation ($\pm 1$ SE) for the subset of aecia-bearing individuals (ind.) and stems.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>&quot;Self&quot;-fertilisation</th>
<th>Outcross-fertilisation</th>
<th>Caged control</th>
<th>Natural fertilisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of individuals (N)</td>
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<td>13</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Individuals with aecia</td>
<td>3 (0.19)</td>
<td>12 (0.92)</td>
<td>1 (0.05)</td>
<td>15 (1.00)</td>
</tr>
<tr>
<td>Proportion of stems with aecia (for subset of ind. with aecia)</td>
<td>0.14 ± 0.08</td>
<td>0.81 ± 0.09</td>
<td>0.05 ± 0.05</td>
<td>0.98 ± 0.02</td>
</tr>
<tr>
<td>Proportion of leaves with aecia (for subset of stems with aecia)</td>
<td>0.03 ± 0.02</td>
<td>0.25 ± 0.03</td>
<td>0.01 ± 0.01</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.18 ± 0.07</td>
<td>0.27 ± 0.03</td>
<td>0.16</td>
<td>0.47 ± 0.07</td>
</tr>
</tbody>
</table>
4.4.2 Artificial array experiment

In Vieques, a total of 297 visits were observed during two days of observation (40% ants, 34% other Hymenoptera (mostly halictid bees), 23% Diptera, and 3% various other insects). In Ardez, a total of 368 visits were recorded (74% Diptera, 14% Hymenoptera other than ants, 10% ants, and 2% various other insects). The same insect species visited both pseudoflowers and true flowers. In addition, the same individuals moved between the two species. In Vieques, ten out of 96 visits within mixed arrays were movements to different inflorescences in the same plot. Of these, three (or 30%) were between species, all three from flower to pseudoflower. At Ardez, 32 out of 174 visits within mixed arrays were movements to different inflorescences. Of these, 14 (or 40%) were movements between species, seven from pseudoflower to flower and seven from flower to pseudoflower. Bateman’s constancy index (Waser, 1986) could be calculated for the Ardez site where we had more than ten transitions between species. The calculated index was -0.02, a number very close to zero, indicating no preference for either pseudoflowers or true flowers by the insects flying between inflorescences in the mixtures. The interspecific movements were made by various species of Diptera (Calliphoridae and Syrphidae), and Hymenoptera of the family Halictidae, genus Lasioglossum.

True flowers received more visits than pseudoflowers at both locations (Figs. 4.4A, B). In monocultures in Vieques, the ratio of visits to true flowers vs. pseudoflowers was 5.0:1 for all insects and 5.6:1 for insects excluding ants. In Ardez, the monocultural plots were visited in a ratio of 2.5:1 for all insects and 2.3:1 for insects excluding ants, in favour of true flowers. Overall visitation to individual pseudoflowers and true flowers was not influenced by whether they occurred in a mixture or a monoculture (Table 4.2, Figs. 4.4A, B). These results did not change when ants were excluded (Table 4.3, Figs. 4.4A, B).

While no difference in visitation rates to mixtures and monocultural arrays was observed, some differences were observed in the time that was spent per visit at both field sites (Table 4.4, Figs. 4.4C, D). In Vieques as well as in Ardez, contrasts showed that insects spent significantly more time on pseudoflowers in monocultures than in mixtures. In Ardez, this was true for all insects as well as for insects other than ants. In Vieques, the model that did not include ants was not significant due to insufficient data and is therefore not presented here. In general, the time spent per visit was inversely related to the number of visits on pseudoflowers and true flowers in mixtures vs. monocultures (Figs. 4.4A-D).
Figure 4.4 (A) Mean visitation rates (± 1 SE) by all insects (dark bars) and insects other than ants (white bars) to pseudoflowers and true flowers in mixed arrays vs. monocultures in Vieques and (B) Ardez. (C) Mean time spent per visit (± 1 SE) by all insects (dark bars) and insects other than ants (white bars) on pseudoflowers and true flowers in mixed arrays vs. monocultures in Vieques and (D) in Ardez.
Table 4.2. The number of insect visits per individual pseudoflower or true flower in mixed arrays vs. monocultures in Vieques and Ardez for all insects. The data were not transformed. P values ≤ 0.05 are given in **boldface**.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
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<td></td>
<td></td>
</tr>
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Table 4.3 The number of visits by insects other than ants per individual pseudoflower or true flower in mixed arrays vs. monocultures in Vieques (data not transformed) and Ardez (data square-root transformed). P values ≤ 0.05 are given in **boldface**.

<table>
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Table 4.4 Time spent per insect visit on individual flowers and pseudoflowers in mixed arrays compared to monocultures in Vieques (data log transformed) and in Ardez (data not transformed) for all insects. Contrasts were made between the number of visits per individual pseudoflower of *U. pisi* and true flower of *E. cyparissias* in mixed arrays vs. monocultures. P values ≤ 0.05 are given in **boldface.**

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</tbody>
</table>

Contrast for species x array

*U. pisi-mono vs. U. pisi-mix*  **0.0233**

*E. cyp.-mono vs. E. cyp.-mix*  0.4863

<table>
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</table>

Contrast for species x array

*U. pisi-mono vs. U. pisi-mix*  **0.0003**

*E. cyp.-mono vs. E. cyp.-mix*  0.1954
Table 4.5 Time spent per visit by insects other than ants on individual flowers and pseudoflowers in mixed arrays compared to monocultures in Ardez (data square-root transformed). Contrasts were made between the number of visits per individual pseudoflower of *U. pisi* and true flower of *E. cyparissias* in mixed arrays vs. monocultures. 
P values ≤ 0.05 are given in **boldface**.

<table>
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<th>F</th>
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<td>Ardez</td>
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Contrast for species x array

*U. pisi*-mono vs. *U. pisi*-mix  **0.0016**

*E. cyp.*-mono vs. *E. cyp.*-mix  0.5218
4.5 Discussion

We addressed three questions in this study: (1) is insect visitation necessary for the fungi of *U. pisi* to reproduce, (2) do *U. pisi* and *E. cyparissias* share insect visitors, and (3) if visitors are shared, is competition or facilitation suggested by insect behaviour?

The first question was answered unambiguously with an insect exclusion experiment: insects were required for the reproduction of *U. pisi*. Other studies of nonsystemic and systemic heterothallic rust fungi have shown a similar degree of fungal reproduction when insects were excluded (0-20%), or treated by hand “self”-fertilisation (3-20%), hand outcross-fertilisation (88-96%), and naturally insect-visited controls (98-100%) (Craigie, 1927; Roy, 1993; Schürch, Pfunder, and Roy, in press). In all cases, natural insect-mediated fertilisation as well as hand outcross-fertilisation led to the most fungal reproduction. However, occasionally the fungus was able to reproduce under other treatment conditions such as self-fertilisation or no fertilisation. In our case, some degree of non-insect-mediated fertilisation may have resulted from any of three causes: a low level of self-compatibility in the fungus, infections by two fungi of opposite mating type within one host, or errors in experimental work such as accidental caging of previously fertilised stems.

In our study, we found that the formation of aecia of *U. pisi* could be restricted to single stems or even single leaves on a host individual, depending on the treatment that we applied to the fungus. This can be explained by a study of Hartwich (1955), who showed that, although all stems of one *Euphorbia* individual come out of the same root, and therefore should be infected by the same systemic fungus, the mycelium can become separated by the growth of the host plant. This separation can lead to isolation of fungal parts in single stems or even single leaves of a host. Therefore, a visit to one infected stem or leaf does not necessarily lead to a dikaryotic mycelium in the whole infected plant, and the fertilisation event can be locally restricted. If mycelial separation is common, this could mean that more insect visits to different stems or leaves on one host plant would also increase reproductive success for the fungus. Longer visitation periods on pseudoflowers could also lead to an increased fungal reproductive success if the insects wander around more and thus make more fertilisation contacts.

True flowers of the host plant *E. cyparissias* co-“flower” some of the time with fungal pseudoflowers. To test whether pseudoflowers and true flowers were visited by the same insects, we observed insect movements among single inflorescences in 50:50 mixtures. In the mixed arrays 42 insects moved between inflorescences, and of these, 17 transitions (40%) were interspecific movements between pseudoflowers and true flowers. Because the number of transitions observed was small, constancy indices are potentially unstable.
Thus we do not dwell on them here, but our observations clearly showed that insects move between the two species.

Flowering species that share pollinators can interact with each other through them. To test whether pseudoflowers and true flowers interact through insects, we conducted an artificial array experiment in the field. Artificial arrays have the advantage that they can include natural communities. We were able to include two locations with different communities into our study. But there are limitations to the generality of our results. First, the effects of visitation rates on the reproductive success of pseudoflowers and true flowers cannot easily be quantified, and interspecific movements of insects can reduce reproductive success of the opponent species (Waser, 1978; Roy, 1996). Second, it was only possible to make observations on a limited subset of frequencies, densities, sites, and days. Because pollinators are known to respond to the overall frequency and density of flowers (Thomson, 1982; Rathcke, 1983; Feinsinger, 1987), the results from the visitation experiment need to be interpreted within the context of the populations in which the arrays were set. At Vicques, the average density of Euphorbia stems in the population (infected and flowering) was one stem/m², but the distribution of stems was patchy and stems often ranged up to 16 stems/m². Thus, our density of ten inflorescences in monocultural arrays represented a reasonable compromise between average and local densities. However, true flowers were much more common than pseudoflowers in the population (4:1) than they were in our mixed arrays with five inflorescences each (1:1).

At Ardez, the average density of Euphorbia stems in the population (infected and flowering) ranged between 34 and 54 stems/m². Thus, our density of ten individuals in monocultural arrays was much smaller than average natural densities. However, it was necessary to keep the density in the arrays the same as in Vicques if we wanted to make statistical comparisons between sites. Although the density of stems in our arrays was less than the population at large, the frequency of pseudoflowers and true flowers was approximately the same as in our mixed arrays (1:1).

At both sites, true flowers were visited more often than pseudoflowers. Insects were clearly able to differentiate between Euphorbia flowers and pseudoflowers. If insects are visiting flowers based only on the frequency of a species in a population (ignoring any innate predispositions), then we would expect visitation at Vicques to be 4:1 in favour of true flowers and 1:1 at Ardez. What we actually found was visitation in favour of true flowers at both sites; the mean visitation rate per individual and hour in monocultures was 5:1 (all insects) and 5.6:1 (no ants) in Vicques and 2.5:1 (all insects) and 2.3:1 (no ants) in Ardez. The fact that visitation was higher to true flowers than their frequency alone predicts suggests that the preference for true flowers was not purely frequency dependent.

When considering interactions with insect visitors, it is important to consider not just the number of visits, but also the duration of visits. Longer visitation times have been
shown to benefit some plants through increased pollen deposition (Thomson and Plowright, 1980; Galen and Plowright, 1985; Thomson, 1986; Harder, 1990; Mitchell and Waser, 1992) and may also benefit pseudoflowers by increasing the chance of fertilising separated hyphae. Alternatively, longer visits might lower outcrossing rates by reducing the number of between-plant visits per unit time.

Our results showed that pseudoflowers in Ardez received shorter visits in mixed populations than in monocultures. If longer visits enhance the reproductive success of the fungus, our results indicate that true flowers of *E. cyparissias* might be serious competitors when visitation is a limiting resource. Further experiments are needed to determine whether pollinators are in limited supply and what the consequences of the behaviour observed here are for phenotypic selection. If pollinators are limited, then competition could select for differences between species if the differences allow the species to partition the sparse resource, but this is not the only possible outcome. For example, a preference of insects for *Euphorbia* flowers could cause selection for pseudoflowers to become more like *Euphorbia* flowers, if there is variation in pseudoflower morphology and more flower-like pseudoflowers have higher fitness than those that are less *Euphorbia*-like.

In this study we have tested some, but not all, of the conditions required to show that the pseudoflowers produced by *U. pisi* mimic the flowers of *Euphorbia*. The rust fungus pseudoflowers are somewhat similar in appearance to *Euphorbia* flowers, the rust fungus requires insect visitation for reproduction, the two taxa have some overlap in “flowering” phenology, the same insect species visit both species, and the same individual insects fly between them. Furthermore, we have shown that these species interact through their insect visitors. Competition was suggested by the fact that overall visitation to the true flowers was higher, and the duration of visits was shorter on pseudoflowers in mixtures than on those in monocultures, although this difference in duration did not affect visitation rates. Further experiments are needed to determine whether the resemblance of pseudoflowers to *Euphorbia* flowers is actually adaptive; these studies must examine fitness and not simply visitation and should examine the consequences of variation in density and frequency throughout the flowering season.

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4.6 Literature cited


5 EFFECTS OF ANTS ON THE REPRODUCTIVE SUCCESS OF EUPHORBIA CYPARISSIAS AND ASSOCIATED PATHOGENIC RUST FUNGI

5.1 Abstract

Ants are common visitors to the flowers of Euphorbia cyparissias, and also often forage on E. cyparissias stems that are infected by rust fungi of the species complex Uromyces pisi. These fungi sterilise their host, produce nectar and require insects for their sexual reproduction. Our objective was to determine whether ant visits enhance the sexual reproduction of either E. cyparissias or the rust fungi. Uromyces pisi is known to be obligatory outcrossing, whereas a breeding system experiment established that E. cyparissias can self, but sets more seeds when outcrossed. We used insect exclusion experiments to test whether ants fertilise the rust fungi and to determine whether ants are pollinators of E. cyparissias. These experiments showed that insect pollination is necessary for seed set and that ants can pollinate the flowers. However, ants do not fertilise the rust fungi.

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5.2 **Introduction**

Ants and plants are commonly associated, but they only occasionally form mutualistic relationships. For example, the seeds of only a few species are dispersed primarily by ants. Ant pollination is also rare. Only about thirty reports of ant pollination have been published during the last century (reviews by Faegri and van der Pijl, 1979; Peakall et al., 1991; Jolivet, 1996), and of these studies, there is convincing experimental evidence of ant pollination for only eleven species (Peakall, 1989; Peakall and Beattie, 1989; Gómez and Zamora, 1992; García et al., 1995; Ramsey, 1995; Gómez et al., 1996; Puterbaugh, 1998).

The best argument to explain the rarity of ant pollination is that some ant species possess metapleural glands whose secretions reduce pollen quality (Beattie et al., 1985). These glands, located on the thorax, secrete antibiotic substances that ants smear on themselves and their larvae for protection against fungal and bacterial infections (Maschwitz et al., 1970; Beattie et al., 1986). Probably due to these secretions, contact with the ant integument is harmful to pollen (Beattie et al., 1984; Gómez and Zamora, 1992). However, some pollen species are more resistant than others, not all ant species have metapleural glands, and species without these glands can also reduce pollen viability (Hull and Beattie, 1988; García et al., 1995). Nonetheless, although many pollen grains are damaged by ant contact, sometimes enough remain viable to lead to normal seed set (Ramsey, 1995).

Ants are also considered to be poor pollinators because their small size allows them to forage on flowers without touching stigmas or anthers, and pollen does not adhere well to the ants' smooth integument and it is often removed by frequent grooming. However, these hypotheses are usually not tested, and in many of these characteristics ants are not much different from bees (Beattie et al., 1984).

It has also been argued that, because ants do not fly but walk, ant visitation mostly causes self or geitonogamous pollination. This assertion is supported by the evidence collected by Ramsey (1995) and Puterbaugh (1998) but conflicts with that of García et al. (1995) and Svensson (1985). Crawling may cause self-pollination, but is also the least expensive type of movement (Heinrich, 1975) and dependence on pollinators with low energy needs may indirectly benefit the plant by allowing for reduced nectar production.

Any plant-pollinator interaction has a quality and a quantity component (Herrera, 1987). The quality term refers, for instance, to the viability of the pollen deposited on a stigma by a pollinator, while the quantity term refers to the frequency of the interaction. The contribution of a particular pollinator group to pollination is then the product of the quality and quantity component of the interaction. From this perspective, all the reasons put forward to explain the rarity of ant pollination are related to the quality component of
the interaction. However, the quantity component of the interaction must also be considered. On this point, because ants are social insects and regularly return to a foraging site as long as they are rewarded with nectar (Hölldobler and Wilson, 1990), they could surpass the classical pollinators and so compensate for their possible low quality.

*Euphorbia cyparissias* L. is a widespread species in Europe, and is commonly associated with ants, with which it has a known mutualistic relationship for seed dispersal (Dymes, 1933 cited in Stahevitch et al., 1988). Not only are ants attracted to the seeds of this species, but ants also represent a large proportion of the insect visitors to the flowers of *E. cyparissias*. In an artificial array experiment conducted in 1997 in Vicques (Switzerland), ants represented 36% of the visitors to the flowers (Pfunder and Roy, in press). In the present study, we examined whether ants actually pollinate *E. cyparissias*. We also conducted a hand pollination experiment to determine the breeding system of the plant.

*Euphorbia cyparissias* is also the host for rust fungi of the *Uromyces pisi* (Pers.) Wint. species complex. These fungi produce spores mixed with nectar on the leaves of the infected stems. Insects are required to transport these spores between the different mating types of the fungi, very much like pollination in higher plants (Pfunder and Roy, in press). In an artificial array experiment conducted in 1997, 47% of all visitors to infected stems were ants (Pfunder and Roy, in press). Because ants were frequent visitors to infected stems, and because some ants actively farm fungi for food (Mueller et al., 1998; Hölldobler and Wilson, 1990), we also asked whether ants fertilise the pathogen. If the ants do fertilise the host-specific pathogen, then they may harm the host plant indirectly through this interaction.

In this study, we addressed the three following questions: 1) What is the breeding system of *E. cyparissias*? 2) Do ants pollinate *E. cyparissias*? and 3) Do ants fertilise *Uromyces pisi*?
5.3 Materials and Methods

5.3.1 Study Site

The field site was located at Vicques (Cras de la Combe) in the Swiss Jura mountains (coordinates 599 000/245 940) at 550 m above sea level. We examined a population of *E. cyparissias* in a dry calcareous grassland that was formerly grazed and is now mown once a year in the fall.

5.3.2 The Plant

*Euphorbia cyparissias* is a common native species in Europe that has been introduced into New Zealand and Eastern North America (Stahevitch et al., 1988). It is a perennial species with vegetative reproduction from root runners. It grows in dry meadows, at ruderal sites, and forest edges.

The flowers of *E. cyparissias* are strongly reduced: the male flower is a pedicelled stamen and the female flower is simply a pistil, both without perianth parts (Stahevitch et al., 1988). One female and several male flowers are enclosed in an involucre (five bowl-shaped fused bracts) that bear four sickle-shaped nectaries and which is subtended by two bracts. This inflorescence is called a cyathium. Two to five (mostly three) cyathia are borne by one ray and the umbel formed by up to 40 rays is termed a pseudo-cyme. *E. cyparissias* was the first described example of protogyny (Sprengel, 1793 cited in Proctor and Yeo, 1973), in which the stigma is receptive before the pollen is shed. The flowers emit a sweet and strong scent. Fruits are dehiscent when dry and explode to liberate a maximum of three mature seeds each. The seeds bear an elaiosome, a food reserve for the ants that disperse the seeds (Dymes, 1933 cited in Stahevitch et al., 1988).

5.3.3 The Fungi

Rust fungi in the species complex *Uromyces pisi* (Pucciniaceae) often infect *E. cyparissias*, causing drastic changes in plant physiology and morphology (Pilet, 1952; Hartwich, 1955). Infected stems are mostly unbranched, sterile, and have thick ovate leaves instead of the normal needle-like leaves. The upper leaves on these stems are yellow and flower-like, such that the whole infected stem forms a "pseudoflower". The rust fungi produce spores mixed with sweet smelling nectar on the under side of the leaves. Insects are required to transport these spores (more accurately called spermatia) between different mating types (Pfunder and Roy, in press). After successful fertilisation, the fungi produce dispersal spores called aeciospores in structures called aecia.
5.3.4 The ants

The most commonly observed ant species during the 1998 flowering season was *Lasius alienus* ( Förster). This species undergoes an intensive trophobiotic symbiosis with aphids. In addition, it is a nectarivore, zoophage and also attends lycaenid caterpillars (Seifert, 1996). We observed this species foraging on the nectaries of healthy *E. cyparissias*, and also on infected plants. We also often observed *Formica pratensis* Retzius, and more seldom, *F. cunicularia* Latreille, on the flowers of *E. cyparissias*. All these species possess metapleural glands. Identified specimens are deposited in the insect collection of the Federal Institute of Technology (ETH) Zürich, Switzerland.

5.3.5 Breeding system of *E. cyparissias*

To determine the breeding system of *E. cyparissias*, we controlled pollination. We caged 45 plants in bud with florist gauze bags (Kleen Test Products, division of Meridian Industries, Inc., Milwaukee, Wisconsin) to exclude insects. To prevent the bags from collapsing on to the flowers, we put cylinders of chicken wire inside them. On each plant, male flowers on three rays were removed. Each ray had two female flowers. Rays were randomly assigned to one of the three following pollination treatments: (i) no further manipulation (emasculating), (ii) selfing, and (iii) outcrossing. For selfed flowers, the receptive stigmas were pollinated with pollen from freshly dehisced anthers from the same plant. Outcrossed flowers were pollinated with pollen from at least three different plants located between three and six meters away. The remaining flowers on the same stem were caged with fine-meshed bags to prevent pollen contamination of the treated flowers; the plants remained caged throughout the whole experiment.

As the fruits matured, we bagged them in dialysis tubing, so they could finish maturing without dehiscing. Seed number was recorded, and we calculated the percentage of ovules that developed into seeds (female fertility). Because each female flower contains three ovules, and two female flowers per plant received the same treatment, female fertility was calculated as the number of seeds produced by this pair of flowers divided by six. Four plants were excluded from the analysis because ants were observed in the cages.

Female fertility following outcrossing and selfing was arcsine square root transformed and analysed with an ANOVA with treatments as a fixed effect and plants as a random effect. Even after transformation, the variances were not exactly equal between treatments, but ANOVA operates well even with considerable heterogeneity of variances provided that sample sizes are similar (Zar, 1996). All statistical analyses were performed with the program JMP, version 3 (SAS, 1989-1997).
5.3.6 Ant pollination of *E. cyparissias*

We tested whether ants are pollinators by excluding particular insect groups. At the end of March, we caged 135 plants in bud and assigned them to one of the four following treatments: 1) No visitors - the plants remained caged throughout the whole experiment. 2) Ants only - only crawling insects, which were primarily ants, could visit the flowers (we used the cages described above, however, instead of tying the florist bags around the plant stems, we cut them at the edge of the wire cylinder, leaving a 1 cm gap between the ground and the cage). 3) Winged only - only flying insects could visit the flowers (to prevent crawling insects, we drove in the ground a cylinder of green hard plastic (8 cm high, cross-section of 7 cm) around the about 15 cm tall plant and smeared the top centimetre with an insect trapping adhesive (Tangle-trap from The Tanglefoot Company, Grand Rapids, Michigan). 4) All visitors - all insects could visit the flowers.

The treatments were initiated during the last week of April. We checked the plants regularly to make sure the treatments were excluding and including the proper insects. Seeds were harvested on June 23rd, 1998. For each plant, we recorded the number of seeds and the mean seed weight, and estimated the number of female flowers per plant by counting the number of rays.

For analysis we first classified the plants by whether they set seed or not, then we used a G-test of independence to determine whether the formation of seeds depended on the four treatments. To test whether pollinators were necessary for seed set, we performed a G-test between the "no visitors" and the "all visitors" plants. A second G-test was performed between the "no visitors" and the "ants only" treatments. For the treatments where insects where allowed to visit the flowers, we had continuous data and could thus use ANOVA for analysis. Female fertility for these three treatments was arcsine-square root transformed and compared with one-way ANOVA.

5.3.7 Ant-fungus interaction

To test whether ants fertilise the rust fungi, we conducted a second insect exclusion experiment, this time with infected stems. Before the fungi started producing gametes, 125 infected plants were caged and randomly assigned to one of the three following treatments already described above: 1) No visitors, 2) Ants only, and 3) Winged only. There were two kinds of control plants for this experiment: 4) All visitors - infected plants were caged as they emerged and when the treatments started, their cages were removed. Thus the "all visitors" plants act as a control for the other treatments. 5) Continuously open - these infected stems, by contrast, were never caged and received natural visitation. Treatments were initiated during the last week of April. At the end of May, we collected
all the infected plants and recorded the number of stems per plant and whether they were bearing aecia (the structures formed after successful fertilisation).

A G-test of independence was used to test whether the formation of aecia was independent of the treatments. We performed three *a priori* contrasts with G-tests: "winged only" versus "all visitors", "continuously open" vs. "all visitors", and "no visitors" vs. "ants only".

**5.4 RESULTS**

**5.4.1 BREEDING SYSTEM OF *E. cyparissias***

None of the 52 emasculated flowers set seed, indicating that *E. cyparissias* needs pollen for seed set (Fig. 5.1). Of the 20 plants that received the selfing treatment, 10 plants set seed, indicating that *E. cyparissias* is partially self-compatible. However, female fertility was significantly lower in the selfing treatment than in the outcrossing treatment (ANOVA: $F_{1,18}=15.56, P=0.0009$). On average, two selfed flowers set $1.45 \pm 0.42$ seeds versus $2.77 \pm 0.41$ for two outcrossed flowers.

![Figure 5.1 Mean female fertility (percentage of ovules that developed into seeds) ± SE for *E. cyparissias* in three pollination treatments.](image)

**5.4.2 ANT POLLINATION OF *E. cyparissias***

The exclusion of particular insect groups had a significant effect on seed set (Fig. 5.2; $G=39.72, P<0.0001, df=3$). Only 10% of the "no visitors" plants set seed ($n=20$), compared to 86% of the "all visitors" plants ($n=21$) and these plants set far fewer seeds ($3.5\pm 1.5$ versus $10.4 \pm 2.1$), indicating that the plants did not normally self-pollinate and pollination was essential for full seed set ($G=26.59, P<0.0001, df=1$). Ants were able to...
pollinate the flowers of *E. cyparissias*; the plants to which only ants had access set seed significantly more often than the ones without visitors (G-test of independence: G=3.92, P=0.048, df=1). The ANOVA showed that there were efficiency differences among the pollinator groups (F_{2.58}=8.33, P=0.0007). Contrasts showed that female fertility of the plants visited by all insects was higher than that of plants visited only by ants (F_{1.58}=6.23, P=0.015) and similar to that of plants visited only by winged insects (F_{1.58}=2.6, P=0.11). Out of the 23 plants to which only ants had access, eight plants set seed. The female fertility of these eight plants was not significantly different from that of the plants visited by all insects that set seed (Table 5.1; ANOVA: F_{1.24}=0.13, P=0.72), and their seeds tended to be larger (Table 5.1; ANOVA: F_{1.24}=3.29, P=0.08).

![Graph](image)

**Figure 5.2** Percentage of *E. cyparissias* plants that set seed ± SE and mean female fertility ± SE in an insect exclusion experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th># Plants that set seeds</th>
<th>Female fertility (%)</th>
<th>Mean seed weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Visitors</td>
<td>21</td>
<td>18</td>
<td>15.6 ± 2.9</td>
<td>2.27 ± 0.13</td>
</tr>
<tr>
<td>Ants Only</td>
<td>23</td>
<td>8</td>
<td>19.6 ± 8.7</td>
<td>2.71 ± 0.20</td>
</tr>
</tbody>
</table>

**Table 5.1** Means ± SE of reproductive success measures for *E. cyparissias* under two pollination treatments. Means and SE were calculated only for the plants that set seed under each treatment. N is the total number of plants assigned to each treatment.
5.4.3 ANT-FUNGUS INTERACTION

Ants did not contribute to the fungal reproduction, but flying insects did fertilise the fungi (Fig. 5.3). Treatment had a significant effect on the number of plants that formed accia (G=68.44, P<0.001, df=4). Accia formation did not differ significantly between the "ants only" treatment and the "no visitors" treatment (G=1.41, P<0.24, df=1), but was more frequent among the plants of the "winged only" treatment than among those that received all visitors (G=4.45, P=0.0349, df=1). The plants to which insects had continuous access bore accia more often than the plants that were caged between one and two months prior to unrestricted insect access (the "all visitors" plants), suggesting that accia formation depends on the length of exposure to insects (G=20.88, P<0.0001, df=1).

![Figure 5.3 Percentage of U. pisi infected E. cyparissias plants ± SE on which the rust fungi produced accia in an insect exclusion experiment.](image)

5.5 DISCUSSION

5.5.1 BREEDING SYSTEM OF E. CYPARISSIAS

To understand the interactions between *Euphorbia cyparissias* and its flower visitors, some knowledge about its breeding system is necessary. Our emasculation treatment established that pollen is necessary for seed set. Further, *E. cyparissias* is partially self-compatible, but does not normally self-pollinate; only 2 out of 20 of the plants from which insects were excluded set seed. These results strongly suggest that protogyny is an efficient mechanism to hinder self-pollination and indicate that *E. cyparissias* generally relies on insects for pollination. Although we did not test explicitly for pseudogamy, the fact that few of the self-fertilised flowers set any seed also suggests that they are not
pseudogamous, since self-pollen can trigger full seed set in pseudogamous apomicts (Roy, 1995).

Either self or outcross pollen can lead to seed set, but manually self-pollinated flowers set less seeds than manually outcrossed flowers did. In a primarily outcrossing species, reduced seed set with self-fertilisation is often an expression of inbreeding depression (Lee, 1988). Only about half of the ovules of the flowers outcrossed by hand produced seeds. Lethal mutations, resource limitation, or pollen limitation could have been responsible for the abortion of the other ovules. Considering that the plants examined in this study grew on a dry calcareous grassland, resource limitation is a plausible explanation. Abortion due to a lack of pollen seems unlikely, because we applied large amounts of pollen on the stigmas. However, the quality of the applied pollen was not tested. In addition, because E. cyparissias also reproduces vegetatively, some flowers that were thought to be outcrossed may have been pollinated with pollen from their own clone. Nevertheless, seed set was higher by outcrossed than by selfed plants.

Hand-outcrossed flowers had far higher female fertility than the ones exposed to all visitors (compare Figs 5.1 and 5.2), suggesting that reproduction was limited by pollinator activity (Bierzychudek, 1981). However, in our study, the number of seeds that hand-outcrossed plants could potentially produce was limited to 12 because the other flowers were not pollinated. The "all visitors" plants had far more flowers to mature, and thus, if resources were limiting, could abort some, leading to lower female fertility as this measure was calculated over all flowers present on a plant. Seed production per plant, and not per flower, should be examined to show pollen limitation (Zimmerman and Pyke, 1988). Therefore, our results cannot be interpreted as evidence for pollinator limitation, but neither can they exclude it.

5.5.2 ANT POLLINATION OF E. CYPARISSIAS

We were principally interested in knowing whether ants, which are often observed on the flowers of E. cyparissias, pollinate these flowers or whether they are nectar thieves. The insect exclusion experiment showed that ants pollinate E. cyparissias. Plants that were visited only by ants set more seeds than plants from which all insects were excluded. We also observed ants contacting both anthers and stigmas while foraging.

Concerning the extent of the ants' contribution to pollination, our results showed that the eight plants visited only by ants that set seed had a similar reproductive success as the plants visited by all types of insects. This may be explained by the behaviour of the ants. Worker ants can have a highly specialised foraging pattern and go back to the same foraging site as long as they are rewarded with food (Hölldobler and Wilson, 1990). Fowler (1983) showed how workers of Formica pallidefulva specialise on the nectaries of another Euphorbia species, E. esula, and return to the same flowers over a two week
period. He also observed that some plants are visited daily whereas others are totally ignored. We made similar observations and hypothesise that the "ants only" plants that did not set seed were simply ignored by ants, whereas the ones that set seed were regularly visited. If this hypothesis is true, then it supports the idea that ants may compensate for their low quality by high visitation frequency.

Plants visited only by flying insects had similar female fertility to the plants visited by all insects, including ants. Ants pollinated *E. cyparissias*, but their exclusion did not reduce reproductive success. If a plant is resource limited and not pollen limited, then a small decrease in pollinator visits and therefore pollen supply may not influence reproductive success. Our experiments clearly showed that ants are pollinators when they are the only visitors, but, from this result, we can only make suppositions about the ants' contribution to pollination when they co-occur with other visitors. Usually more pollinator visits bring more pollen, but when the pollinator fauna is heterogeneous, the interactions between the pollinators may influence pollen deposition (Thomson and Thomson, 1992). For instance, aggressive ants can prevent or shorten the foraging time of better pollinators (we personally observed many such interactions in 1997). In this case, their exclusion would be beneficial to plant reproduction. At our field site, *F. pratensis* workers were very bellicose, while *L. alienus* workers flee rapidly when disturbed (Seifert, 1996). Ants can also simply reduce the nectar quantity available to other pollinators, forcing the flying pollinators to visit more flowers to get the same amount of nectar and indirectly affecting the outcrossing rate (Hawkins, 1961; Heinrich and Raven, 1972).

The fact that the exclusion of ants did not affect reproductive success suggests that pollination caused by ants was not important for *E. cyparissias* in 1998 at our field site. However, ants could still be important pollinators in years and locations where flying visitors are rare and pollination would not occur otherwise. We must stress that the present study was conducted in only one population and year. A similar experiment conducted in another population or year may have a different outcome, depending on the local insect fauna or flowering phenology. Interactions between two species vary among populations and years, and range from antagonism to commensalism or even mutualism (Thompson, 1994). Additional studies are needed to clarify the ecological importance of this interaction.

### 5.5.3 Ant-fungus interaction

Finally, we were interested in the role of the ants in moving fungal spores between different mating types. Insects are usually necessary for sexual reproduction in *U. pisi*. In 1997, fungi were able to reproduce on only 5% of the infected stems from which all insects were excluded (Pfunder and Roy, in press), and in 1998 on 19% (this study).
Here, we showed that ants do not fertilise the fungi. Interestingly, ants were rarely found on infected stems in 1998 although they were almost half of all visitors in 1997. What caused this difference? First, the spring of 1998 was cooler than the previous one, and when the ants began foraging, the healthy plants were already flowering. Perhaps ants switch from pseudoflowers to flowers when these are present. Second, in the 1997 experiment, healthy and infected stems were arranged in artificial plots using florist tubes. In contrast with a natural situation, ants may not have been able to differentiate between flowering and infected stems in the tubes. It is also likely that in the 1997 experiment the location of one plot adjacent to an ant nest increased the overall number of ants observed.

The 1997 experiment also showed that ants only stayed a short time on infected stems (18s in contrast to 130s on flowers), indicating that in some way the nectar that they found on infected plants was less interesting than that in floral nectaries. Given that ants forage locally, they probably do little cross-fertilisation. Since the fungi are nearly obligate outcrossers, and usually only one mating type is found per infected plant, interplant movement is necessary for fertilisation. In this situation, ants foraging on the infected stems should probably be considered as nectar thieves. A noteworthy difference between the fungi and flowers is that, on infected stems, the nectar and the spores are mixed together, but on the true flowers, the nectar and the pollen are produced on separated structures (nectaries or anthers). Perhaps the ants prefer their nectar uncontaminated with fungal spores.

Aecia formation was significantly higher on plants visited only by winged insects than on plants visited by all insects, including ants. These results could be explained by the ants’ aggressive behaviour described above, or perhaps the metapleural gland secretions adversely affected the fungus. Another possibility is that flying insects were attracted by the plastic ring or the tanglefoot glue used to exclude the ants or by the insects accidentally trapped in this glue.

In conclusion, we showed that ants did not help the rust fungus pathogens of E. cyparissias reproduce. However, experimental evidence showed that ants pollinated E. cyparissias and that plants on which only ants foraged had the same reproductive success as plants visited by all insects. In our field site in 1998, ants were not the main pollinators, but the pollination caused by the ants may be very important in years, or locations when winged pollinators are rare.

**Acknowledgements:** We thank B. Merz for helping prepare the ants, D. Chérix for identifying them, B. Baur for providing the field site, T. Steinger for statistical advice, A. Widmer and two anonymous reviewers for comments on the manuscript, and M. Ramsey for advice. This research was supported by the Swiss National Science Foundation (NF 2-77-311-96).
5.6 Literature cited


6 EFFECTS OF FREQUENCY AND DENSITY ON THE FECUNDITY OF INSECT-POLLINATED FLOWERS AND FUNGAL PSEUDOFLowers

6.1 Abstract

*Euphorbia cyparissias* is often infected by a rust fungus of the species complex *Uromyces pisi*. Infected plants do not form true flowers, instead, the fungus induces its host to form pseudoflowers, rosettes of yellow leaves upon which the fungus presents fungal gametes in a sweet-smelling sugary nectar. Insects feed on the nectar and transfer fungal gametes between mating types, just like in pollination. Here we show that pseudoflowers and true flowers overlap in "flowering" in two populations at two elevations (550 m and 1960 m) for more than one month, although pseudoflowers start "flowering" one month earlier than true flowers. As the two species also share insect visitors, we hypothesised that they might interact through visitors by either competing for "pollinators" or by facilitating each others' insect visits. To determine whether the fecundity of pseudoflowers and true flowers was influenced by each others' presence, we recorded the mean density and frequency around individuals during their flowering periods and measured their fecundity at the end. Density and frequency changed drastically over the season, and even within the flowering periods of single pseudoflowers and true flowers. We found evidence that pseudoflowers were serious competitors for true flowers, as true flowers set less seed when they were surrounded by more pseudoflowers, reflecting both density- and frequency-dependent interactions. Pseudoflowers, on the other hand, were not obviously influenced by the presence of true flowers. Instead, a higher density of conspecific pseudoflower-neighbours decreased the reproductive success of each pseudoflower. Our findings support our hypothesis that one should include temporal variation even within flowering periods when studying the outcome of density and frequency on the fitness of a plant.

Submitted as:
M. Pfunder and B.A. Roy. Effects of frequency and density on the fecundity of insect-pollinated flowers and fungal pseudoflowers.
6.2 Introduction

Flower frequency and density, and pollinator responses to these factors, are central to theories concerning the evolution of flower forms and phenologies. Competition theory suggests that co-flowering plants should suffer reduced reproductive success because they compete for pollinator services. This theory is based on the assumption that, to optimise energy gains (Levin and Anderson, 1970; Straw, 1972), pollinators prefer flowers that grow at high densities and at high frequencies (Levin and Anderson, 1970; Waser, 1983), leaving the minority species disadvantaged. The predicted evolutionary outcomes according to competition theory are either extinction of the rarer species, or selection for temporal or morphological divergence to avoid pollinator sharing.

Theories concerning facilitation of pollination are based on the assumption that a rare species can gain from nearby common plants that attract pollinators in higher numbers and thus enhance the overall visitation to the rare species (Bobisud and Neuhaus, 1975; Feinsinger, 1987). If the rare species is rewarding and derives an advantage from looking similar to the common one, it is called a Müllerian mimic (Macior, 1970; Brown and Kodric-Brown, 1979; Little, 1983; Thomson, 1983; Dafni, 1984; Barrett, 1987; Roy and Widmer, 1999). Müllerian mimicry theory is, like competition theory, based on the assumption that pollinators prefer flowers that grow at high density and high frequency. Here, however, the rarer mimic, which looks similar to the common model, benefits from the combined higher density and frequency. Selection therefore leads to convergence of the rare to the common species (Roy and Widmer, 1999). If, however, the rarer species does not reward its pollinators, but deceives the pollinators by looking like a common, rewarding model, the type of facilitation is called Batesian mimicry (Little, 1983; Barrett, 1987). In Batesian mimicry, the food deceptive species have higher fitness at low frequency, because low frequency makes it difficult for pollinators to learn to avoid non-rewarding species.

Thus, flower frequency and density can, independently or in concert, influence the evolutionary trajectories of plants interacting through shared pollinators, leading either to divergence or to convergence of species, depending on circumstances. To further complicate matters, the importance of frequency and density will vary according to pollinator species and even individual insects (Motten, 1986; Herrera, 1989; Jones, 1997), and will also vary temporally and spatially (Thompson, 1994).

Two kinds of experiments have been used to examine the relative importance of frequency- and density-dependent behaviour of pollinators for the reproductive outcome of co-flowering plants. Artificial array experiments have been used to relate density and frequency of different species to the preferences and visitation rates of pollinators (Waser and Price, 1981; Roy, 1994, 1996; Meléndez-Ackerman and Campbell, 1998; Totland
and Matthews, 1998). In some array experiments, the influence of frequency and density
on the fitness of potted plants was also examined (Waser and Price, 1981; Campbell,
1985; Armbruster and McGuire, 1991; McGuire and Armbruster, 1991). Non-manipu-
lative observations in natural communities have also been used to assess visitation rates,
pollen load or seed set. In contrast to the array experiments, these observational studies
were generally designed to include the investigation of temporal changes in the
community, studying the effects of density and frequency at different points in time, i. e.,
at intervals over the season or within one year, or across years (Schmitt, 1983a, 1983b;
Feinsinger et al., 1986; Jennersten and Kwak, 1991; Law et al., 1997; Traveset and Sáez,
1997). While array experiments are useful for separating and controlling density and
frequency, their major disadvantage is that only a few combinations of frequency and
density can be tested. On the other hand, the use of non-manipulative observations has the
advantage of including changes in the density and frequency of plants as well as
pollinators over the season, but it is difficult to disentangle the effects of density and
frequency on plant fitness.

In this paper we outline a new non-manipulative approach for studying density- and
frequency-dependent interactions. We examined the interactions between flowering host
plants of Euphorbia cyparissias L. and host plants infected by rust fungi from the species
complex Uromyces pisi (Pers.) Wint. Infected plants do not form true flowers, instead,
the pathogen induces its host to form a pseudoflower, a rosette of pale yellow leaves on
top of the stem that has the appearance of a yellow flower. Spermogonia, fungal organs in
which the fungus produces fungal gametes (spermatia) and sweet smelling nectar, are
borne on these leaves (Gaumann, 1959). Insects fertilise the rust fungus by transporting
these gametes while feeding on the nectar, just like in pollination of true flowers (Pfunder
and Roy, in press).

Because the host and its pathogen have been observed to co-"flower" in intermingled
populations, we hypothesised that they might facilitate each others’ pollinator visits.
However, for facilitation between these species to occur, they need to share insect
visitors, and the visitors need to respond in a positive frequency- and/or density-
dependent way to them and this must lead to higher fitness. In an earlier study, an
artificial array experiment, we showed that they shared insect visitors, and that the visitors
preferred the hosts’ true flowers over fungal pseudoflowers in mixtures (Pfunder and
Roy, in press). However, we found no evidence that the two species influenced each
others’ visitation rates when co-flowering or that they behaved in a frequency- or density-
dependent way (Pfunder and Roy, in press). Because the array experiment included only
a limited set of frequencies and densities, and because the experiment was only done at
one point in time, here we decided to use a non-manipulative approach that recorded
temporal changes of frequency and density over the season. We then measured fecundity
of individuals whose density and frequency of neighbours was known throughout the flowering season. We used our mapping and fecundity study to address the following questions:

1) How much do the phenologies of fungal pseudoflowers of *U. pisi* and true flowers of *E. cyparissias* overlap in "flowering" in natural communities? To address this question we examined two communities at two elevations (550 m and 1960 m) across the entire season.

2) How variable is the density and frequency around single inflorescences of pseudoflowers and true flowers within their flowering period? To assess variability within flowering periods for individuals, we measured the density and frequency around 20 pseudoflowers and 20 host inflorescences.

3) What is the temporal course of changes in density and frequency around pseudoflowers and true flowers during a season? To assess the temporal changes during a whole season, we mapped the spatial changes in flower distribution and calculated the mean weekly densities and frequencies around all pseudoflowers and true flowers over the season.

4) Does the mean frequency and density of neighbouring "flowers" over the flowering period of an individual influence its fecundity? To address this question we analysed the fecundity of true flowers and pseudoflowers as a function of the mean density and frequency over their flowering periods using regression analyses.
6.3 Materials and Methods

6.3.1 Field sites and phenology

The field work was done at two sites, one near Zermatt in the Swiss Alps (co-ordinates 624 662/095 537), and the other near Vieques in the Swiss Jura Mountains (co-ordinates 599 000/245 940). The two populations grow under similar conditions, on dry grassland, oriented south-south-east at a roughly 30° angle, but at different elevations: the Zermatt site is located at 1960 m, and the Vieques site at 550 m above sea level.

At Zermatt we chose one plot of 10 x 8 m, which enclosed almost an entire small *E. cyparissias* population. At Vieques, we observed the same total area, but we used four randomly located plots of 2 x 10 m within a large *E. cyparissias* population (> 5000 m²). We marked the position of each m² within the plots with stakes. We then laid a 1 m² aluminium frame divided into 100 cm² over each m², so we could monitor the number, and estimate the height of all *E. cyparissias* stems within each 100 cm² square. Height estimation was done by eye, controlling the estimation from time to time by actual measurements. We assessed whether stems were infected or not; if uninfected, we recorded their flowering status, if infected, we recorded the stage of the infection. We also counted and recorded all other species that were flowering in the field. At Zermatt, weekly surveys were performed from April 4th to June 24th, 1998, except April 15th and April 29th, when snow covered the site. At Vieques, weekly surveys were performed from March 2nd to May 15th, 1998.

The flowers of *E. cyparissias* are organised in pseudo-cymes on each stem. Each pseudo-cyme consists of many cyathia, and each cyathia consists of an involucre with one female and several male flowers (Stahevitch et al., 1988). For simplicity, we refer to both the pseudoflowers on one stem, as well as the pseudo-cymes on one stem, as inflorescences. Pseudoflowers were defined as "flowering" as long as the fungus produced nectar; uninfected plants were defined as "flowering" as long as we found flowers in the pseudo-cymes.

We evaluated temporal variation in flowering periods for all flowering plant species as well as for nectar producing pseudoflowers throughout the season in both communities. We excluded species that were represented by fewer than 10 flowers or inflorescences during the whole observation period. We counted single flowers for *Helianthemum alpestre* (Jacq.) DC., *Viola hirta* L., *Potentilla sterilis* (L.) and *P. neumanniana* Rchb., and *Primula veris* L. spp. veris s. str. We counted inflorescences for *E. cyparissias* (infected and uninfected), *Polygala comosa* Schkuhr, and all Fabaceae (*Hippocrepis*
comosa L.; Lotus corniculatus L. s. str.; Anthyllis vulneraria L. ssp. carpathica Nyman; Genista sagittalis L).

6.3.2 VARIATION IN DENSITY AND FREQUENCY WITHIN INDIVIDUAL FLOWERING PERIODS

To evaluate the variation in density and frequency that single flowers and pseudoflowers encountered during their flowering period, we randomly chose 20 pseudoflowers and 20 host inflorescences from the Zermatt field site that survived from the first day of flowering to seed set or accia set. Then density and frequency were evaluated for each week during the flowering period of an inflorescence. The weekly measures were averaged, and the standard error (SE) calculated. We used the program Spatial Analyst, an extension program of ArcView (ESRI, 1996), to analyse the spatial data taken at each week over the season. We divided the field site into contiguous quadrates of 100 cm² (a total of 8000 quadrates) in a map, which enabled us to sum the number of pseudoflower and E. cyparissias inflorescences around single inflorescences within specified scales. We used two different scales, circular areas with 35 cm and 85 cm radii, respectively (Figs. 6.1 and 6.2).

Small scale

A

Large scale

B

Figure 6.1 Grid system used to calculate density and frequency for two different sized areas. The dark cell in the centre of the circle represents the location of the individual for which density and frequency were estimated. The density and frequency surrounding this individual was averaged over (A) 29 cells (r=35 cm) and over (B) 197 cells (r=85 cm).
Figure 6.2 An example of how density and frequency was evaluated for single inflorescences in a given week for the whole field site. Each cell is 100 cm$^2$, giving a total of 8000 cells, and shows the number of host inflorescences at the area $r=35$ cm by which an individual in the given cell was surrounded. The darker the colours, the more neighbours there are.

We excluded the Vieques data from this part of the analysis, because although the area studied was the same (8 x 10 m), it was divided into four separate plots with many edges. We thus had too small a sample size when edge effects were considered, which was necessary in these calculations.

6.3.3 Variation in density and frequency over the season

To evaluate the variation in density and frequency during the whole season, we calculated the mean weekly frequencies and densities of neighbours around pseudoflower or host inflorescences in the way described above, again using two different scales around the individuals. To make data from the two different area measures directly comparable, the calculated density of the two scales was standardised to areas of 1 m$^2$ by multiplying
densities of the smaller area (r=35 cm; 0.385 m²) by the factor 2.60, and dividing densities of the larger area (r=85 cm; 2.270 m²) by the factor 2.27. The frequency around pseudoflowers and host inflorescences was calculated using the formula

\[
\frac{\text{pseudoflowers}}{\text{pseudoflowers} + \text{host inflorescences}}
\]

To be able to compare spatial and temporal variation in flower density and frequency between the Vieques and the Zermatt sites, we combined the four plots of 2 x 10 m from Vieques into one plot of 8 x 10 m, the size of the site studied at Zermatt, by randomly attaching the four plots onto each other along the 10 m axis. We ignored edge effects and interdependencies for this comparison. Assuming that we had measured four random samples of the whole population, the combined plots should represent the population as a whole. This assumption might be critical, especially for clustered populations, but the results still give information on variation in frequency and density. To be conservative, however, we did not test for fecundity effects at the Vieques site where actual density and frequency around a given individual was not known exactly.

### 6.3.4 Effect of density and frequency on fecundity

The effect of flowering neighbours on the reproductive success of an individual was evaluated only at the Zermatt field site, for the reasons described above. At the end of the season we measured the fecundity of pseudoflowers and host inflorescences. Fungal fecundity of pseudoflowers was measured as the proportion of infected leaves that bore aecia. Aecia are fungal organs in which aeciospores, the spores responsible for dispersal to other hosts, are produced. Aecia are usually only formed when the fungus is successfully cross-fertilised by insects (Pfunder and Roy, in press). Aecia were counted on dried samples under a dissecting microscope (Wild, M5A, Heerbrugg) at 60-120x magnification. Fecundity of *E. cyparissias* flowers was measured by absolute seed set, assessed by enclosing inflorescences at the end of the season into small gauze bags for one month, and counting the seeds afterwards.

For each inflorescence and each pseudoflower stem of known fecundity, we had the following additional information: commencement of flowering (in days after the first flower in the population), flowering duration (in days), and the mean height during flowering, as estimated weekly during the phenology census. We assumed that stems that were found within the same 100 cm² quadrates belonged to the same plant individual or fungus genotype. In this way we tried to average variability within genotypes and to average outcomes among inflorescences with the same density and frequency of neighbours. In the following, we consider these grouped stems as individuals. Pooling of
nearby stems led to an overall total of 49 flowering host individuals comprising 66 single inflorescences, and 26 fungal individuals comprising 38 single inflorescences. Each individual was assigned to its 100 cm² quadrat on the map and the density and frequency of its neighbours were again calculated as described above and in Figs. 6.1 and 6.2 (ESRI, 1996). Because many of the counted stems dried up or were trampled or eaten by animals over the season, in the end there were fewer pseudoflowers and host inflorescences with known fecundity than had been initially recorded in the field.

To estimate the density and frequency effects of neighbours, we counted flowering neighbours around each flower or pseudoflower at weekly intervals during its flowering period. While the fecundity of the individuals was pooled among single inflorescences within a grid, frequency and density around the individuals were calculated from single inflorescences. This is reasonable, because insect visitors probably respond to density and frequency of single inflorescences rather than to genotypes, but reproductive parameters should be estimated at the level of individual genotypes. However, by counting single stems, an individual’s stems were added to the density and frequency of measured neighbours, generally enhancing the density and frequency within the smaller circular area calculated compared to the larger area, and is responsible for most of the differences between the two areas.

Two stepwise removal regression analyses were used, one for the seed set of host plants, and one for the aecia set of pseudoflowers. We included the following effects into each model: (a) mean height during flowering (cm), (b) flowering period (days), (c) flowering commencement (days), (d) density of pseudoflowers at the small scale, (e) density of pseudoflowers at the large scale, (f) density of host inflorescences at the small scale, (g) density of host inflorescences at the large scale, (h) relative frequency of pseudoflowers and true flowers (number of pseudoflowers per total number or pseudoflowers and host inflorescences) at the small scale, and (i) relative frequency of pseudoflowers and true flowers at the large scale.
6.4 Results

6.4.1 Phenology

The two sites studied were located at different elevations. As a result of the altitudinal difference, phenology at the lower site was shifted one month earlier. Fungal pseudoflowers were among the first structures presenting nectar reward at both sites, and they remained "flowering" during 83 days at Zermatt and 77 days at Vieques. At both sites E. cyparissias host plants started flowering almost exactly one month after the first pseudoflowers, and ended flowering together with fungal pseudoflowers at both sites. However, when peak flowering (50% or more of the individuals in bloom) was compared, the overlap between pseudoflowers and E. cyparissias was higher at Zermatt than at Vieques. Species richness was twice as high in lower elevation Vieques (12 species, including pseudoflowers) as at Zermatt (6 species, including pseudoflowers) during the observation period (Fig. 6.3). All flowering species found at Zermatt were also flowering at Vieques, with exception of Helianthemum alpestre, a plant found only in calcareous grasslands in alpine zones.

The peak number of E. cyparissias inflorescences was similar at Zermatt and at Vieques (247 and 215 inflorescences, respectively). Differences, however, could be observed in the infection levels of the two populations, as the peak "flowering" of the pseudoflowers at Vieques reached about 20% of the hosts' peak (48 pseudoflowers), while at Zermatt, this reached about 70% (178 pseudoflowers) of the number of host inflorescences (Figs. 6.4A and B).

6.4.2 Plant height

For pseudoflowers of U. pisi as well as for inflorescences of E. cyparissias, we also included estimates on plant height during the weekly phenological recording. When the heights of all "flowering" stems were averaged over all weeks, we found that both pseudoflowers and host inflorescences were significantly smaller at Vieques than at Zermatt (Welch's t = 17.318, pooled df = 942, P < 0.001 for pseudoflowers; t = 12.743, pooled df = 1610, P < 0.001 for host inflorescences). Flowering E. cyparissias stems were larger, measuring 11.61 cm (SE = 0.22, n = 608) at Vieques, and 15.14 cm (SE = 0.17, n = 1004) at Zermatt, than fungal pseudoflower-stems that had a mean height of 8.19 cm (SE = 0.21, n = 319) at Vieques, and 13.12 cm (SE = 0.19, n = 1185) at Zermatt. Therefore, flowering pseudoflowers were 3.42 cm (Vieques) and 2.02 cm (Zermatt) shorter, on average, than true flowers. However, when we compared heights only during the weeks of flowering overlap of the two species, we found almost no differences between pseudoflowers and true flowers each week, the mean height difference over the
weeks of flowering overlap being 0.30 cm at Vieques (SE = 0.04, n = 7 weeks) and 0.64 cm at Zermatt (SE = 0.32, n = 8 weeks). We also compared only uninfected plants that set seeds and aecia-bearing pseudoflowers after successful fertilisation over all weeks. Aecia-bearing pseudoflowers showed a mean height of 13.68 cm (SE = 0.53, n = 72) at Vieques and as much as 24.63 cm (SE = 0.83, n = 80) at Zermatt, compared to seed producing true flowers, measuring 14.57 cm (SE = 0.28, n = 155) at Vieques and 14.60 cm (SE = 0.28, n = 265) at Zermatt.

6.4.3 Variation in density and frequency within individual flowering periods

The variation of density and frequency during the flowering periods of single inflorescences can be described by the standard error of the mean density and frequency during their flowering period. If the variation is low, the standard error is small. The 40 randomly chosen individuals of pseudoflowers and host inflorescences showed variation in the density and frequency of surrounding plants (Tables 6.1 and 6.2). When averaged over the 20 host individuals, the SE of the density represented 11.5-18.6% of the mean, and the SE of the frequency 13.9-17.7% (Table 6.1). The SE of the density around pseudoflowers represented 12.4-40.9% of the mean, and the SE of the frequency 8.4-16.1% (Table 6.2).

6.4.4 Variation in density and frequency over the season

As a result of the staggered flowering of pseudoflowers and host flowers, the overall frequency of pseudoflowers changed drastically over the season (Figs. 6.4A, B), decreasing as E. cyparissias flowers increased. At both sites, the density of inflorescences of the same species, hosts around hosts and pseudoflowers around pseudoflowers, was highly variable, and depended on the size of the areas used for calculation. The smaller area shows higher density and frequency (Figs. 6.4C-6.4K), which reflects that we included the inflorescences of the individuals in question in the total counts of inflorescences. The density of the opposite species shows no such effect with pseudoflowers around hosts and hosts around pseudoflowers showing similar densities and frequencies at both scales. The seasonal course of frequencies and densities corresponds more or less to the overall phenology of the two species (Figs. 6.4A, B). The only obvious deviation from this pattern can be observed in the seasonal course of the pseudoflower density around pseudoflowers at Vieques: the density decreased while the overall number of pseudoflowers in the field was still increasing (Fig. 6.4E).
Figure 6.3 Flowering phenologies of all the blooming species in (A) Zermatt and (B) Vieques 1998. Thickened bars represent the time during which ≥ 50% of the peak number of flowers were present on the field. The black boxes represent the peak of flowering, the peak number of flowers are given behind each line.
Figure 6.4 Flowering phenology at Vieques (A) and Zermatt (B); the mean density of pseudoflowers and true flowers around single inflorescences (C, D, E, F), as well as the mean frequency around single inflorescences (G, H, I, K). Data from the small area (r=35 cm) and the large area (r=85 cm) are standardised to an area of 1 m².
Table 6.1  Variation of density and frequency around 20 randomly chosen single host inflorescences during their flowering period at Zermatt, measured at two different scales: circular areas with radii 35 cm and 85 cm. Calculated as \( \frac{pseudoflowers}{pseudoflowers + host \ inflorescences} \).

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<td>7.00±2.03</td>
<td>3.50±0.56</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>0.00±0.00</td>
<td>7.25±1.14</td>
<td>3.75±0.41</td>
</tr>
<tr>
<td>17</td>
<td>3</td>
<td>0.00±0.00</td>
<td>12.33±0.54</td>
<td>0.67±0.27</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>1.00±0.00</td>
<td>3.75±0.54</td>
<td>3.00±0.00</td>
</tr>
<tr>
<td>19</td>
<td>5</td>
<td>0.00±0.00</td>
<td>3.20±0.18</td>
<td>2.60±0.36</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>4.50±0.43</td>
</tr>
<tr>
<td>averaged over 20 ind.</td>
<td>4</td>
<td>0.97±0.18</td>
<td>6.11±0.79</td>
<td>2.70±0.31</td>
</tr>
</tbody>
</table>
Table 6.2 Variation of density and frequency around 20 randomly chosen single pseudoflowers during their flowering period at Zermatt, measured at two different scales: circular areas with radii 35 cm and 85 cm. Calculated as pseudoflowers/true flowers + host inflorescences.

<table>
<thead>
<tr>
<th>Individuals</th>
<th>flowering period in weeks (n)</th>
<th>mean density of pseudoflowers ± 1 SE</th>
<th>mean density of true flowers ± 1 SE</th>
<th>mean frequency of pseudoflowers ± 1 SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r=35 cm</td>
<td>r=85 cm</td>
<td>r=35 cm</td>
<td>r=85 cm</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>3.89±0.67</td>
<td>4.11±0.67</td>
<td>0.78±0.26</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>2.00±0.00</td>
<td>2.00±0.00</td>
<td>0.17±0.15</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>1.80±0.44</td>
<td>4.00±0.28</td>
<td>0.80±0.44</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3.33±0.27</td>
<td>8.00±1.25</td>
<td>0.33±0.27</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>2.63±0.25</td>
<td>8.75±1.41</td>
<td>1.63±0.79</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>5.00±0.53</td>
<td>6.17±0.72</td>
<td>2.17±0.96</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>4.71±0.52</td>
<td>5.71±0.75</td>
<td>2.43±0.85</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>1.00±0.00</td>
<td>3.67±0.72</td>
<td>1.00±0.82</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>2.40±0.83</td>
<td>13.20±0.87</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>4.60±0.83</td>
<td>4.80±0.95</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>4.00±0.40</td>
<td>15.00±0.98</td>
<td>2.20±0.52</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>2.57±0.19</td>
<td>7.14±0.62</td>
<td>0.86±0.31</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>5.00±0.47</td>
<td>6.00±0.47</td>
<td>0.33±0.27</td>
</tr>
<tr>
<td>14</td>
<td>9</td>
<td>1.56±0.17</td>
<td>3.00±0.42</td>
<td>0.11±0.10</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>2.75±0.41</td>
<td>3.75±0.22</td>
<td>1.00±0.61</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>2.20±0.66</td>
<td>2.60±0.67</td>
<td>3.00±0.49</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>2.67±0.30</td>
<td>6.33±1.02</td>
<td>0.67±0.19</td>
</tr>
<tr>
<td>18</td>
<td>6</td>
<td>2.67±0.30</td>
<td>6.33±1.02</td>
<td>0.67±0.19</td>
</tr>
<tr>
<td>19</td>
<td>7</td>
<td>2.00±0.35</td>
<td>3.14±0.91</td>
<td>0.29±0.17</td>
</tr>
<tr>
<td>20</td>
<td>9</td>
<td>1.44±0.17</td>
<td>3.67±0.63</td>
<td>0.11±0.10</td>
</tr>
<tr>
<td>averaged over 20 ind.</td>
<td>5.9</td>
<td>2.91±0.39</td>
<td>5.87±0.73</td>
<td>0.93±0.38</td>
</tr>
</tbody>
</table>
6.4.5 Effect of density and frequency on fecundity

The density and frequency of flowering neighbours influenced the fecundity of the individual flowers and pseudoflowers, but only at the smaller scale (r=35 cm). Flowers set more seeds when they were surrounded by a lower density and frequency of pseudoflowers (Table 6.3). Both frequency and density of surrounding pseudoflowers were highly correlated (r=0.936), but also apparently had independent effects since the analysis could separate them. Most important for the fecundity of host individuals was the mean height of the plant during its flowering period, where taller plants set significantly more seeds (Table 6.3). Neither the starting time of flowering nor the length of the flowering period had an effect on *Euphorbia* fecundity.

Pseudoflower individuals were negatively influenced by surrounding pseudoflowers and set more aecia when the density of pseudoflowers in their closer neighbourhood (r=35 cm) was lower (Table 6.3). But an even stronger influence was the starting time of pseudoflower "flowering". The earlier they started flowering, the higher was their fecundity by the end of their flowering time. This result is strongly correlated with the flowering period (r=0.907), the earlier they started, the longer they flowered. Neighbouring *Euphorbia* inflorescences did not influence the fecundity of the rust (Table 6.3).

Table 6.3 Significant results from two step-wise multiple linear regressions testing the following effects on the seed set of host plants and the aecia set of pseudoflowers: density of host inflorescences or pseudoflowers at two scales (r=35 and 85 cm), frequency of host inflorescences or pseudoflowers at two scales (r=35 and 85 cm), flowering commencement, flowering period, and mean plant height during the flowering period.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Predictor variable</th>
<th>slope</th>
<th>df</th>
<th>ms</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>seed set of host plants (n = 49)</td>
<td>Mean height during flowering</td>
<td>1.46</td>
<td>1</td>
<td>1397</td>
<td>33.06</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Density pseudoflowers (r=35 cm)</td>
<td>-2.88</td>
<td>1</td>
<td>361</td>
<td>8.36</td>
<td>0.0054</td>
</tr>
<tr>
<td></td>
<td>Frequency (r=35 cm)</td>
<td>-17.54</td>
<td>1</td>
<td>231</td>
<td>5.47</td>
<td>0.0239</td>
</tr>
<tr>
<td>aecia set of pseudoflowers (n = 26)</td>
<td>Starting time of flowering</td>
<td>-0.01</td>
<td>1</td>
<td>0.28</td>
<td>9.75</td>
<td>0.0048</td>
</tr>
<tr>
<td></td>
<td>Density pseudoflowers (r=35 cm)</td>
<td>-0.06</td>
<td>1</td>
<td>0.14</td>
<td>4.85</td>
<td>0.0379</td>
</tr>
</tbody>
</table>

\(^{1}\) Calculated as \(\frac{\text{pseudoflowers}}{\text{pseudoflowers} + \text{host inflorescences}}\)
6.5 Discussion

Differential response of pollinators to density and frequency of flowering plants is thought to be responsible for the evolution of much of the morphological and phenological variation in plant species. This study reports on the variation in density and frequency of flowers and pseudoflowers in two populations of *E. cyparissias* at different elevations, as well as on the fecundity of individuals in response to these variable densities and frequencies.

The population at the lower elevation showed higher species richness and earlier phenology than the population at the higher elevation. Besides these differences, the two phenologies were similar, with pseudoflowers starting to flower exactly one month before the first host flowers (Figs. 6.3, 6.4A and B). The early growth of pseudoflowers might be induced by the fungus, or it could be the result of host plant reaction to the infection. *Uromyces pisi* infected plants are known to show an enhanced concentration of growth-regulating hormones (auxines) and to keep this high concentration over a longer time than uninfected *E. cyparissias* host plants (Pilet, 1952). However, it is not known whether the fungus produces these hormones or whether the high concentration is a reaction of the infected plant. This study showed that the fungus clearly benefited from early "flowering". Early growth might therefore be adaptive and initiated by the fungus to avoid competition for pollinators either with flowers of *E. cyparissias* or with conspecific pseudoflowers through temporal displacement, but it is very difficult to establish whether temporal displacement is actually the result of interspecific competition (Connell, 1980).

From the enhanced growth-hormone concentrations in infected plants, one might expect that pseudoflowers will grow not only earlier but also larger than uninfected hosts. Curiously, in both populations "flowering" pseudoflower stems were smaller than flowering hosts, an observation that was already made about 100 years ago by Râthay (1882). However, the height differences were minimal during the time period when they overlapped in "flowering". Furthermore, once they finished "flowering", pseudoflowers at Zermatt continued to grow and surpassed the height of true flowers with seeds by far (24.63 cm vs. 14.60 cm), although this was not observed at Vicques (13.68 cm vs. 14.57 cm). As is known from many flowers with wind-dispersed seeds, the fungus might disperse better by exposing its wind-dispersed aeciospores above the vegetation.

Infected as well as uninfected *E. cyparissias* stems were significantly taller at Zermatt than at Vicques. This difference might be the result of differential resources at the two sites. However, it might also be the result of differential cytotaxonomy of the host plant. *Euphorbia cyparissias* is known to exist in three forms, as a fertile diploid, a sterile diploid, and a fertile tetraploid (Gassmann & Schroeder, 1995). Since polyploid individuals can generally be found at higher elevations (Bierzychudek, 1985), and at the
same time are generally larger than the diploid individuals of the same species (Stebbins, 1950), we might have surveyed a diploid population in the lower elevation at Vieques and a tetraploid population in the higher elevation at Zermatt. An analysis of the cytotaxonomic distribution of *E. cyparissias*, in combination with disease status, could be of interest. It is possible that differences may exist in infection levels or the relative abundance of the different fungal species as a result of polyploidy.

Density and frequency around individuals during their flowering periods varied strongly. So did the temporal course of changes in frequencies and densities over the season, which was, at least partially, the result of the shifted phenology of pseudoflowers and host inflorescences. Frequency and density around individuals of one species increased as the overall presence of the species in the field increased. Interestingly, pseudoflower and host frequency was almost always above 50% for the species in question, meaning that pseudoflowers and uninfected host plants were generally surrounded by more inflorescences of their own than of the other species. Even during peak flowering of the hosts, pseudoflower frequency around pseudoflowers was rarely below 50% which was especially surprising at Vieques, where overall density was much lower for pseudoflowers than for the uninfected host. This clustered distribution might be the result of the infection pattern, from spatial displacement due to competition, or just the effect from counting single inflorescences from individuals with large number of stems.

Our findings support our hypothesis that one should include temporal variation even within flowering periods when studying the outcome of density and frequency on the fitness of a plant.

In the regression model, density or frequency effects on the fecundity of pseudoflowers and their hosts were significant only within the closer area around a plant (r=35 cm). The two scales differed when density and frequency of the opposite species were measured, but not when density and frequency of the same species were measured. As we already described, this reflects the inclusion of inflorescences of the individual in question in the total counts of surrounding inflorescences, leading to a stronger effect on the smaller scale. But as our analysis led to effects on only the smaller scale, when densities of the opposite species were included, the stronger effect of the closer area can not exclusively be an effect of methodological bias. Our results suggest that, if the differences in fecundity are pollinator mediated, insect behaviour may be influenced more within relatively small areas than within larger plots.

*Euphorbia* inflorescences had lower seed set when surrounded by a higher density and frequency of infected plants, suggesting that pseudoflowers might be serious competitors for their uninfected hosts. In an earlier study using arrays, we found no significant effects of frequency and density on visitation rates nor times spent per visit on hosts (Pfunder and Roy, in press). These results suggest that the quality of insects rather than the
quantity might play the major role in determining the seed set of true flowers. Insects transferring fungal gametes to the flowers of *E. cyparissias* might decrease seed set and therefore lead to a lower seed set even if the overall visitation rate is not influenced (Waser, 1978; Feinsinger, 1987). The most significant effect on fecundity of true flowers, however, was the plant height during their flowering period. Plant height was included in the analysis to correct for the fact that we had measured the absolute seed set and not the seed/ovule ratio. Because we assumed that plant size as measured by height, and the number of ovules should be correlated, our findings are not surprising. Thus, the inclusion of height in the model improves the chances of finding a model with good fit for other variables.

Pseudoflowers mainly benefited from early and long flowering periods. They were not influenced by the height of their stems, which is not surprising either, because their aecia set was corrected by the number of leaves that they produced and therefore, aecia set was a relative measure in contrast to the absolute measure we used of seed set for the plants. While the earlier array study showed that insects preferred host flowers over pseudoflowers in mixtures (Pfunder and Roy, in press), the present study showed a positive effect of early flowering on the fecundity of pseudoflowers. These two findings support the hypothesis that early flowering of pseudoflowers might be adaptive. Pseudoflowers neither gained nor lost from co-occurrence with flowering hosts. Roy (1994) showed in array experiments that rust pseudoflowers of a *Puccinia* rust profited from co-flowering with buttercups by attracting more insects. Our array study as well as the present study suggests that the *Euphorbia - Uromyces* system is different, and that competition rather than facilitation occurs between the fungus and co-occurring flowers.

Surprisingly, we found that higher density of conspecific neighbours decreased the reproductive success of pseudoflowers (negative density-dependence). A possible explanation for these observations might be the differences in the mating system of the two species. While *E. cyparissias* plants are self-compatible (although they rarely self due to protogyny) (Schürch et al., in press), each infected plant usually has only one mating type on it, and is 80 to 95% self-incompatible (Pfunder and Roy, in press; Schürch et al., in press). As density and frequency includes the number of stems from the individual tested, we suspect that the effect seen might be the result of ineffective gamete transfer, resulting from the transfer of the same mating type from one stem to the other.

Our results suggest that the density of pseudoflowers was the most important factor determining the fecundity of pseudoflowers and true flowers in the population studied. However, the density of pseudoflowers was highly variable over the season and even within flowering periods. We therefore suggest that short-term studies of insect behaviour in arrays can give a false impression of the importance of density and frequency, as averaged over the season, and that a combination of two different approaches might
contribute more to the understanding of natural processes than each of them would per se. Additionally, the density of pseudoflowers is strongly dependent on the infection level of the population which can vary a lot among populations (Pfunder, unpublished data). One should therefore expect different outcomes of the interactions in different populations with different infection levels. It would be interesting to compare several populations with the approach used in this study to determine the effects of density and frequency on fecundity in the context of the infection patterns in different populations.

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6.6 Literature cited


Environmental Systems Research Institute (ESRI) 1996. GIS by ESRI. Arc View Spatial Analyst.


7 GENETIC VARIATION AND GEOGRAPHIC DISTRIBUTION OF PSEUDOFLOWER-FORMING RUST FUNGI (*UROMYCES PISI S. L.*)
ON *EUPHORBIA CYPARISSIAS*

7.1 ABSTRACT

To attract insects for sexual reproduction, some fungi can induce the formation of pseudoflowers on their hosts. Pseudoflowers are rosettes of yellow host leaves upon which the fungus presents gametes in sweet nectar. 11 species of the fungus complex *Uromyces pisi* can induce pseudoflowers on the host *Euphorbia cyparissias*. The taxonomy of these species is based on the choice of the alternate host, a species of the Fabaceae, as well as on spore morphology on Fabaceae. Morphological identification of the fungi on *E. cyparissias* is impossible. To identify the fungal species on infected *E. cyparissias*, we compared the sequences from the ITS region of the rDNA to the DNA from 5 identified fungal species on Fabaceae. From 41 specimens on *E. cyparissias*, collected in 1997 in Switzerland, 24 specimens could be identified as *U. pisi s.s.*, 16 specimens as *U. striatus*, and one specimen could not be identified. We therefore conclude that fungal pseudoflowers are typically induced by *U. pisi s.s.* or *U. striatus* in Switzerland, although the presence of other species is possible. We then used ITS sequences to reconstruct the phylogenetic relationship among different species in the *U. pisi* complex and two closely related microcyclic rust species of the complex *Uromyces scutellatus*. Phylogenetic analyses indicated that the microcyclic species may be descendants from macrocyclic *U. pisi* s.l. ancestors. The ITS region sequenced in this study was found to be appropriate for answering phylogenetic, as well as ecological questions, and has provided valuable markers for future studies.

Submitted as:
7.2 Introduction

Rust fungi in the species complex *Uromyces pisi* (Pers.) Wint. are well known because of their conspicuous infections on *Euphorbia cyparissias* L. (cypress spurge) (Ráthay, 1882; Pilet, 1952). Infected *E. cyparissias* host plants grow earlier in the season and are inhibited from flowering. Instead, the host is induced by the fungus to form pseudo-flowers, yellow leaves that grow in a rosette on top of the stems and resemble true flowers in colour and shape (Pfunder & Roy, in press). Not only do fungal pseudo-flowers visually resemble true flowers, but, just like true flowers, they present a sweet-smelling nectar that is produced by the fungus on the surface of the yellow leaves. The nectar contains fungal gametes (spermatia) (Ráthay, 1882) that are transferred by nectar-feeding insects from one fungal mating type to the other(s) (Pfunder & Roy, in press).

Earlier studies have shown that fungal pseudoflowers can constitute a major food source for nectar-feeding insects in an early spring community, and as a result, they may significantly influence their surrounding plant and insect communities (Roy, 1994; Roy, 1996; Pfunder & Roy, in press). Gäumann (1959), in his comprehensive work about rust fungi in Middle Europe, suggests that there are as many as 11 species within the *U. pisi* species complex on *E. cyparissias* (Table 7.1). All 11 species show a complete life cycle (=macrocyclic life cycle) and are heteroecious, which means that they alternate between two hosts, here between *E. cyparissias* and one species of the family Fabaceae. The taxonomy of the different species in the *U. pisi* complex is based on the choice of the Fabaceae species, as each of the fungal species switches to a different Fabaceae host after successful fertilisation. In addition to the Fabaceae host identity, taxonomy is also based on the shape and surface of teliospores that are produced on the Fabaceae hosts. We still lack the ability to distinguish among the different species of *U. pisi* when they live and form pseudoflowers on *Euphorbia*. If several of these fungal species present their nectar at the same time within the same *Euphorbia* population, we might ask whether insects can differentiate among the single fungal species and whether different rust species can interact with each other through shared pollinators. If, however, the species do not co-flowers, they might show allopatric distribution, or they might reflect the presence or absence of Fabaceae hosts within a given population.

The main objectives of this study were twofold. First, we wanted to establish a molecular method to distinguish among the different *Uromyces* species on *E. cyparissias* to be used as a tool for future ecological and evolutionary studies on pseudoflowers. To make species identification on *Euphorbia* possible, we used the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). The ITS region in the rDNA has already been successfully used by other authors to resolve phylogenetic relationships among closely related fungal taxa (Gardes & Bruns, 1993; Zambino & Szabo, 1993; Momol &
Kimbridge, 1994; Jacobs & Rehner, 1998; Roy et al., 1998; Vogler & Bruns, 1998), or to discriminate among different fungal isolates (Pourpard et al., 1993). In this study, we compared the ITS sequences obtained from rusts on E. cyparissias with corresponding sequences of reliably identified fungi that were, with one exception, isolated from Fabaceae hosts. The genetic markers were further used to test whether multiple fungal species co-occurred within the same host population, as well as whether the different rusts on E. cyparissias differed in their geographical distribution in Switzerland.

The second objective was to re-evaluate the taxonomy of the U. pisi complex. Gäumann (1959) suggested that because his taxonomy was primarily based on host preferences, monophyletic species might have been separated into different species complexes. We tested this hypothesis by comparing the species complex U. pisi with a second closely related species complex, Uromyces scutellatus (Pers.) Lév. (Table 7.1). This species complex consists of four described species that depend on E. cyparissias as the only host (=autoecious life cycle) and show a shortened life cycle (=microcyclic). They form pseudoflowers like U. pisi, however, they rarely produce nectar and gametes (Gäumann, 1959) and can reproduce without fertilisation by insects (Pfunder, unpublished data).

Table 7.1 Rust fungi of the macrocyclic U. pisi complex and the microcyclic U. scutellatus complex that infect Euphorbia cyparissias (Gäumann, 1959). Species that were sequenced in this study are written in bold.

<table>
<thead>
<tr>
<th>Fungal taxa</th>
<th>Life cycle</th>
<th>Fabaceae hosts (examples)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uromyces pisi</strong> (Persoon) Winter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U. anthyllidis (Greville) Schroeter</td>
<td>heteroecious</td>
<td>Anthyllis spp.</td>
</tr>
<tr>
<td>U. punctatus Schroeter</td>
<td>heteroecious</td>
<td>Astragalus &amp; Oxytropis spp.</td>
</tr>
<tr>
<td>U. jordianus Bubák</td>
<td>heteroecious</td>
<td>Astragalus exscapus</td>
</tr>
<tr>
<td>U. klebahni Ed. Fischer</td>
<td>heteroecious</td>
<td>Astragalus &amp; Oxytropis spp.</td>
</tr>
<tr>
<td><strong>U. laburni</strong> (de Candolle) Fuckel</td>
<td>heteroecious</td>
<td>Genista spp.</td>
</tr>
<tr>
<td>U. hippocrepidis (Thuemen) Mayor</td>
<td>heteroecious</td>
<td>Hippocrepis spp.</td>
</tr>
<tr>
<td><strong>U. loti</strong> Blytt</td>
<td>heteroecious</td>
<td>Lotus spp.</td>
</tr>
<tr>
<td><strong>U. striatus</strong> Schroeter</td>
<td>heteroecious</td>
<td>Medicago &amp; Pisum spp.</td>
</tr>
<tr>
<td><strong>U. onobrychidis</strong> (Desmazières) Léveillé</td>
<td>heteroecious</td>
<td>Onobrychis spp.</td>
</tr>
<tr>
<td>U. fischeri Eduardi Magnus</td>
<td>heteroecious</td>
<td>Vicia spp.</td>
</tr>
<tr>
<td><strong>Uromyces scutellatus</strong> (Persoon) Léveillé</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U. scutellatus (Persoon) Léveillé</td>
<td>autoecious</td>
<td></td>
</tr>
<tr>
<td>U. kalmusii Saccardo</td>
<td>autoecious</td>
<td></td>
</tr>
<tr>
<td>U. alpestris Tranzschel</td>
<td>autoecious</td>
<td></td>
</tr>
<tr>
<td><strong>U. striolatus</strong> Tranzschel</td>
<td>autoecious</td>
<td></td>
</tr>
</tbody>
</table>

1 presumed on E. cyparissias, but not proven experimentally.
7.3 MATERIALS AND METHODS

7.3.1 FUNGAL COLLECTION AND EXTRACTION

In the spring 1997, we collected specimens of infected *E. cyparissias* plants at different locations in Switzerland. From these, 24 specimens were infected by microcyclic rusts of the species complex *U. scutellatus*. They were collected at Ausserberg, Lalden, Les Haudères, Maienfeld, Tamins and Vicques (Fig. 7.1). A further 41 samples were infected by the macrocyclic species complex *U. pisi* and were collected at Airolo, Ardez, Ausserberg, Bos-chà, Ferden, Guarda, Madrano, Pfäffikon, Vicques and Zermatt (Fig. 7.1).

![Map of Switzerland (CH) showing the locations from which infected Euphorbia and Fabaceae specimens were collected.](image)

To be able to assign the fungi from these specimens to single species within these two complexes, we compared their sequences from the ITS region to the sequences obtained from identified fungi. Seven identified fungi in the *U. pisi* s.l. complex were collected on Fabaceae in the years 1997/98 in Switzerland: *U. laburni* f.sp. *laburni* on *Genista sagittalis* (specimen no. 424), *U. loti* on *Lotus corniculatus* (specimens no. 425 and 433), *U. pisi* s.s. on *Lathyrus pratensis* (specimen no. 427), and *U. striatus* on *Medicago lupulina* (specimen no. 430) and on *Trifolium arvense* (specimens no. 456 and 457). These collections were further supplemented by herbarium specimens. From the Herbarium Zurich Z-ZT we obtained one sample from an infected *E. cyparissias* (555),
collected in Piora (Fig. 7.1) in 1983, that was suggested to be infected by *U. punctatus* due to its close appearance next to a Fabaceae infected by the same fungus. A second specimen was collected in Thayngen (Fig. 7.1) in 1963 on the Fabaceae host *Lotus corniculatus* and was identified as *U. loti*. Two more specimens were provided by L. Krieglsteiner from Germany (418 and 421), collected in 1995 (Appendix C), and were identified as *U. laburnii* f.sp. *genistiae tinctoriae*, and *U. punctatus*. For a complete list of all specimens see Appendices 7.A, 7.B and 7.C.

The freshly collected specimens were dried in Silica Gel (Merck) and stored at 4°C. DNA was extracted from dried host leaves with visible fungal organs. We followed the 2x CTAB buffer protocol of Gardes & Bruns (1993), which we modified by shaking (Retsch shaker) the dried samples together with a glass ball of 5 mm diameter in a 2 ml tube for 8-10 min before adding the CTAB. The samples were not further ground in CTAB after this treatment.

7.3.2 DNA amplification and sequencing

Double-stranded nuclear rDNA was amplified directly from diluted extractions (1/10 - 1/1000) using polymerase chain reaction (PCR). From the extracts including fungal and plant DNA, we amplified the internal transcribed spacer region (ITS1—5.8S—ITS2) of the fungal DNA only, using primers that bind specifically to the conserved regions at the 3'-end of the 18S and the 5'-end of the 28S rDNA genes of basidiomycetes. The primers used at the beginning of this study were ITS5 (White et al., 1990) and ITS4-b (Gardes & Brans, 1993). However, because amplification using these primers was rarely successful, we designed new primers with the program Oligo 4.01 Primer Analysis Software (National Biosciences, Inc.) that worked better for *Uromyces* spp. The new primers were ITS5-u [5' CAAGGTTCGTAGGGT 3'] and ITS4-u [5' GGGTTTCTCTCTCAT 3']. Sequences from PCR products amplified with the primers ITS5 and ITS4-b were subsequently confirmed with the new primers, whenever necessary. Neither primer pair bound to *Euphorbia* plant DNA. Each PCR reaction (10 -50 µl) consisted of 1/2 volume diluted extracts and 1/2 volume of a reaction mix, leading to the following final concentrations: 3 mM MgCl₂, 0.25 units Taq DNA polymerase per 10 µl reaction (Promega), 1 x polymerase buffer including 10 mM Tris-HCl and 50 mM KCl (Promega), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Promega), and 0.5 µM of each oligonucleotide primer. DNA thermal cyclers (Techne, models Genius, Cyclogene, or Progene) were used for amplification, following the cycling protocol of Gardes & Bruns (1993).

Double-stranded PCR products were purified using the QIAquick PCR purification kit following the protocol of the manufacturers (Qiagen). The purified products were dissolved in elution buffer (Qiagen) and used directly for the amplification of single-stranded DNA. Each PCR reaction (10 µl) consisted of 10 - 30 ng DNA, if necessary
diluted in dH₂O to a 5.5 μl volume, 0.16 μM primer, and 4 μl of dye terminator cycle sequencing ready reaction with AmpliTaq DNA Polymerase, FS (PE Applied Biosystems). The primers used were the same as described above, together with an additional internal primer pair in the small subunit 5.8S gene. Internal primers used were ITS2-r and ITS3-r (Vogler & Bruns, 1998), ITS2-r being replaced later by a newly designed primer ITS2-u [5' GGTGCCGTTCGAAGATTC 3'] that worked better for Uromyces. The cycle program was used as described in Roy et al. (1998). The single-stranded PCR products were purified with ethanol and dissolved in 12.5 μl template suppressor reagent (PE Applied Biosystems) before sequencing.

Sequencing was done using an ABI Prism™ 310 Genetic Analyser (PE Applied Biosystems). To combine the sequences from the different primers we used the program ABI PRISM™ Sequence Navigator 1.0.1 (PE Applied Biosystems). Different samples that showed no differences in their nucleotide sequence are in the following analysis represented by a single genotype, and only unique genotypes were used for the analysis (Appendices A, B, and C).

7.3.3 Sequence analysis
All unique genotypes were aligned in Clustal X (Thompson et al., 1997) with further visual alignment. The genotype sequences were analysed together with sequences of *Puccinia monoica* Arth. (genbank accession number U88226) and *Puccinia thlaspeos* C. Schub. (genbank accession number U88218) as outgroups. *Puccinia monoica* is a North American macrocyclic species complex that alternates between grasses and Brassicaceae and that also forms pseudoflowers on their aecial host of Brassicaceae (Roy, 1993). *Puccinia thlaspeos* is closely related to *P. monoica* (Roy et al., 1998), but is microcyclic on the Brassicaceae host.

Trees were generated by using Fitch parsimony as implemented in PAUP vers. 4.0b2a (Swofford, 1999), using accelerated transformation (ACCTRAN) to handle character-state optimisation, and collapsing branches if the maximal length of branches were zero. Gaps were handled as missing data. A separate gap matrix was constructed where gaps were coded as binary presence/absence data (0/1). One-basepair (bp), as well as multi-bp gaps were considered to represent single events. Insertions/deletions differing in length were considered as separate characters. The most parsimonious solution was searched for, both including and excluding the gap matrix, using the heuristic search algorithm with random stepwise taxon addition of 100 replicates, setting the limit of saved trees to 20'000 (MaxTrees), saving all minimal trees (MULPARS), and using the swapping option TBR (tree-bisection-reconnection). The relative nodal support was estimated by bootstrap analysis of 100 replicates using the same parameters as above, but with the MaxTrees set to 100.
7.4 Results

7.4.1 The genotypes

We sequenced the ITS region from 5 of 11 species of the *U. pisi* complex that are described on *E. cyparissias*, using samples from identified fungi on different Fabaceae and one *E. cyparissias* host: *U. pisi* s.s., *U. loti*, *U. punctatus*, *U. laburni*, and *U. striatus* (Table 7.1, Appendix 7.C). We also sequenced 41 unidentified macrocyclic fungi obtained from pseudoflowers on *E. cyparissias*, from these, 28 unique genotypes were used for all analyses (Appendix 7.A).

To test for monophyly of the *U. pisi* species complex we additionally included sequences from the species complex *U. scutellatus* in the analyses. This species complex consists of a total of 4 described miccocyche species on *E. cyparissias*. Because all fungi from the *U. scutellatus* species complex are microcyclic, they can be identified by the morphology of their teliospores on *E. cyparissias*. The 23 *U. scutellatus* s.l. samples sequenced could be identified as *U. scutellatus* s.s., represented by 20 collections showing 8 different genotypes, and *U. striolatus*, represented by 3 collections, all showing the same genotype (Fig. 7.2, Appendix 7.B).

The ITS sequences of all fungal samples could be divided into two distinct groups. While one group of sequences had an ITS1 length of 193-203 nucleotides, the second group consisted of a much shorter ITS1 with 97-119 nucleotides and a long gap of about
80 positions. Both groups of sequences were about the same length at the ITS2 with 193-
204 nucleotides.

The aligned sequences of all 49 unique genotypes (including outgroups) contained a
total length of 659 bp, divided into 18S (18 bp), ITS1 (240 bp), 5.8S (157 bp), ITS2
(227 bp), and 28S (17 bp). The gap matrix added an additional 64 characters. We
analysed ITS1 and ITS2, both including and excluding the gap matrix for the analysis.
Including the gap matrix in the analysis led to a total of 531 characters, 310 of them being
constant, 29 parsimony-uninformative and 192 informative. Excluding the gap matrix
from the analysis led to a total of 467 characters, 308 of them being constant, 29
parsimony-uninformative and 130 informative. The mean nucleotide composition of all
taxa included was biased towards a high A-T content, with 69% A-T vs. 31% G-C.

Phylogenetic analysis

Unweighted parsimony analysis of ITS1 and ITS2 including the gap matrix found ≥
20’000 equally-parsimonious trees of 344 steps, with a consistency index (CI) of 0.77, a
retention index (RI) of 0.94, and a rescaled consistency index (RC) of 0.73. The same
analysis excluding the gap matrix also yielded ≥ 20’000 equally-parsimonious trees of
223 steps, with a CI of 0.88, a RI of 0.96, and a RC of 0.85. The strict consensus trees
of 20’000 saved trees, as well as one phylogram out of the trees, including and excluding
the gap matrix, is shown in Figs. 7.3 to 7.6.

All the identified species of *U. pisi* s.l. are clearly distinguishable by their ITS
sequences, as they were in well supported monophyletic clades with bootstrap values at
or above 93 (Figs. 7.3 and 7.5). With the exception of E036 and E555, all genotypes
collected on *Euphorbia* grouped into the clades containing *U. pisi* s.s. (17 genotypes) and
*U. striatus* (7 genotypes). While E036 could not be assigned to any other identified
species, the herbarium specimen E555 was confirmed to be infected by *U. punctatus*, as it
formed a well supported clade together with the genotype F421, an identified *U.
punctatus* sample from *Astragalus glycyphylllos*.

Within the clades of *U. pisi* s.s. and *U. striatus*, genetic differences existed, but they
were generally restricted to 1-3 changes (Figs. 7.4 and 7.6). However, a few genotypes
still showed differentiation supported by bootstrap values ranging from 63 to 76 (Figs.
7.3 and 7.5). Interestingly, the samples that had the large deletion of over 80 bp in the
ITS1 region never formed a single clade, but instead they appeared in two different clades
and the deletion event appeared to have occurred twice. While the microcyclic species *U.
scutellatus* s.s. and *U. striolatus* had the full length ITS1, the species in the macrocyclic
species complex *U. pisi* s.s. mostly had the deletion, with the exception of the species *U.
Figure 7.3 Strict consensus of 20,000 equally-parsimonious trees based on the ITS1 and ITS2 sequences (including gaps). Each terminal taxon represents a unique genotype, genotypes starting with E were collected on *E. cyparissias*, genotypes starting with F were collected on their Fabaceae host. Genotypes of identified species are written in bold. Genotypes marked with ‘Vi’ were all found in Vieques and indicate the high variability of genotypes within one host population. Bootstrap values based on 100 replicates are given above the branches.
Figure 7.4 Phylogram showing one randomly chosen tree out of 20'000 found in a heuristic search from one tree of the heuristic analysis based on the ITS1 and ITS2 sequences (including gaps). The number of changes are given above the branches. The branch connecting the ingroup with the outgroup has not been drawn to scale.
Figure 7.5 Strict consensus of 20'000 equally-parsimonious trees based on the ITS1 and ITS2 sequences (excluding gaps). Each terminal taxon represents a unique genotype, genotypes starting with E were collected on *E. cyparissias*, genotypes starting with F were collected on their Fabaceae host. Genotypes of identified species are written in **bold**. Bootstrap values based on 100 replicates are given above the branches.
Figure 7.6 Phylogram showing one randomly chosen tree out of 20'000 found in a heuristic search from one tree of the heuristic analysis based on the ITS1 and ITS2 sequences (excluding gaps). The number of changes are given above the branches. The branch connecting the ingroup with the outgroup has not been drawn to scale.
7.4.2 GENOTYPE VARIATION WITHIN AND AMONG HOST POPULATIONS

We collected infected Euphorbia hosts from 14 host populations. In 8 populations we collected only macrocyclic, in 4 populations only microcyclic, and in 2 populations both macro- and microcyclic rust species (Table 7.2). From one population, Zermatt, only one sample was sequenced. From all other populations we sequenced at least 2, but up to 7 samples per population, and between 2 and 5 samples per species. In 9 of the 10 host populations that were infected by macrocyclic species, we found either *U. pisi* s.s. (6 populations) or *U. striatus* (3 populations). One further population was infected by both species (Vicques). In all host populations that were infected by microcyclic species, we found either *U. striolatus* (1 population) or *U. scutellatus* s.s. (5 populations), but never both species co-occurring.

<table>
<thead>
<tr>
<th>Euphorbia population</th>
<th>Uromyces species</th>
<th># Specimens</th>
<th># Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Airolo</td>
<td><em>U. pisi</em> (ma)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Ardez</td>
<td><em>U. striatus</em> (ma)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Ausserberg</td>
<td><em>U. striatus</em> (ma)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>U. scutellatus</em> (mi)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Bos-cha</td>
<td><em>U. pisi</em> (ma)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Ferden</td>
<td><em>U. pisi</em> (ma)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Guarda</td>
<td><em>U. striatus</em> (ma)</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Lalden</td>
<td><em>U. scutellatus</em> (mi)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Les Haudères</td>
<td><em>U. striolatus</em> (mi)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Madrano</td>
<td><em>U. pisi</em> (ma)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Maienfeld</td>
<td><em>U. scutellatus</em> (mi)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Pfäffikon</td>
<td><em>U. pisi</em> (ma)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Tamins</td>
<td><em>U. scutellatus</em> (mi)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Vicques</td>
<td><em>U. pisi</em> (ma)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><em>U. striatus</em> (ma)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>U. scutellatus</em> (mi)</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Zermatt</td>
<td><em>U. pisi</em> (ma)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

(ma) = macrocyclic species, (mi) = microcyclic species.
Within the fungal species we found high genotypic variation (Table 7.2). We always found more than one (2 - 7) genotypes per population (2 - 5 per species), except in the populations Guarda, Tamins and Les Haudères. Some of the genotypes were represented in more than one population. In Vieques, where the most specimens were collected (17) we found as many as 3 different species with 13 different genotypes: 5 unique genotypes within the macrocyclic species U. pisi s.s., two unique genotypes within the macrocyclic species U. striatus, and 3 unique genotypes within the microcyclic species U. scutellatus s.s. The specimens that were collected on Fabaceae in Vieques were identified as U. pisi, U. striatus, U. laburni, and U. loti. In the consensus tree in Fig. 7.3 all genotypes that were found at least once in Vieques are marked with a 'Vi'.

To test for altitudinal distribution of the different genotypes or species, we mapped the elevations of the genotypes as they were grouped in the consensus tree from Fig. 7.3 (Fig. 7.7). We could observe no pattern in the altitudinal distribution of the macrocyclic species clades U. pisi s.s. and U. striatus. Both species were found from 500 m to above 1500 m, although only U. pisi was found at an elevation as high as 1960 m in Zermatt. The microcyclic species U. scutellatus was only found below 1000 m (8 genotypes from 20 samples), while the only location where the species U. striolatus was found was above 1500 m (one genotype from 3 samples).

![Figure 7.7](image_url)  
**Figure 7.7** The elevation of locations where the single genotypes were found (order of genotypes as in consensus tree Fig. 7.3). Genotypes with more than one collection at a given location are indicated by numbers below the stars. The elevations from the unidentified genotype E036 and the genotypes from German specimens are not included.
7.5 Discussion

7.5.1 Species identification and taxonomy

Molecular methods allowed us to identify the different species of the pseudoflower-forming *Uromyces pisi* s.l. rusts on *Euphorbia cyparissias*. On *E. cyparissias*, it is impossible to identify these rusts morphologically, but they can be identified on their alternate hosts, different species of Fabaceae. We used the ITS sequence from 5 identified *U. pisi* s.l. species on Fabaceae as genetic references and compared their sequences to the sequences obtained from pseudoflower-forming rusts on *E. cyparissias*. Phylogenetic analyses showed that all 5 identified species from the Fabaceae were resolved into single monophyletic clades with high nodal support, as measured by bootstrapped analyses. Unique genotypes obtained from pseudoflower-forming rusts on *E. cyparissias* were analysed together with these identified species, and all genotypes except E036 grouped together with 2 of the 5 identified species: *U. pisi* s.s. and *U. striatus*. As the herbarium specimen E555, infected by *U. punctatus*, was also collected on *E. cyparissias* in Switzerland, we conclude that fungal pseudoflowers are usually induced by the two species *U. pisi* and *U. striatus* in Switzerland, but that the presence of other species such as *U. punctatus* is also likely.

The ITS sequences used in this study were appropriate for the identification of fungal species during life stages when identification is otherwise impossible. While sequencing might be the best approach for a positive identification of a species, faster and less expensive methods might be sufficient for the rapid differentiation among several species that are known to occur. For example, the two species *U. pisi* s.s. and *U. striatus* can be distinguished by sequencing the ITS region and comparing the length of ITS fragments digested with restriction enzymes (i.e., HinfI or Alul; Pfunder, unpublished data). These specific species can even be differentiated by just comparing the length of the ITS1 region, because *U. pisi* s.s. has an 80 bp deletion that does not occur in *U. striatus*.

The sequences of the ITS region were further used to re-evaluate the taxonomy of the different fungal species in the *U. pisi* complex. The phylogenetic analyses were done in two ways, either including or excluding insertion/deletion events as characters. The two resulting consensus trees showed similar separations of the single *Uromyces* species into different clades (Figs. 7.3 and 7.5). However, the topology of the two trees differed at the base of the separate species clades and the relationship among species could not be unequivocally resolved in either one of the trees. This unresolved relationship is also reflected in the fact that both consensus trees suggested that a deletion event, resulting in the loss of more than 80 nucleotides in the ITS1 region, has happened at two independent times (Figs. 7.3 and 7.5). The sequences of the species *U. laburni*, *U. punctatus*, *U. loti*,
and *U. pisi* s.s. all showed this deletion, but the topologies of both trees separated these species into two clades that did not share a common ancestor having the deletion. Because large deletions like these are generally assumed to be caused by unequal crossing-over (Li & Graur, 1991), such an event is very unlikely to happen twice independently. To test whether this duplication in our trees might have resulted from an inappropriate outgroup, we repeated the analyses without outgroups, as well as with the species *Melampsora euphorbiae* as an outgroup (Pfunder, unpublished data). The new analyses showed the same pattern as the previous ones, and dismissed the possibility of an inappropriate outgroup. We then used topologically constrained trees to force monophyly for the species with the deletion, but with all other conditions as in the consensus tree in Fig. 7.3. The resolved most-parsimonious constrained trees were two steps longer than the consensus tree of Fig. 7.3, the CI and RI being the same as in the tree of Fig. 7.3, the RC being slightly different at 0.72 (compared to 0.73). The topology of the consensus tree of 20'000 saved equally-parsimonious trees showed the same main species clades as before, however, the clade including the species *U. laburni, U. punctatus,* and *U. loti* now grouped together with the clade of *U. pisi* s.s., instead of laying at the base of all other *Uromyces* species (Fig. 7.8).

To test the hypothesis that microcyclic species in the *Uromyces scutellatus* complex evolved from macrocyclic species in the *U. pisi* complex (Gaumann, 1959), we included two species from the *U. scutellatus* complex in these analyses, *U. scutellatus* s.s. and *U. striolatus*. In general, the hypothesis was supported by our results, however, the interpretation from the different analyses differed. In the consensus tree from the analysis including gaps as characters, both microcyclic species, *U. striolatus* and *U. scutellatus*, appeared to have evolved from macrocyclic ancestors. However, the topology of this tree does not resolve whether they evolved independently or not (Fig. 7.3). When gaps were excluded from the analyses, the ancestral state of *U. scutellatus* could not be resolved, as the node of this clade laid at the base of the tree. The topology of *U. striolatus*, however, again supported our hypothesis in this analysis, showing that the species derived from a macrocyclic ancestor (Fig. 7.5). Finally, the consensus tree from the topologically constrained tree (Fig. 7.8) showed the opposite topology from the analysis without gaps: while the ancestor of *U. striolatus* could not be identified, the clade of *U. scutellatus* supported the hypothesis.

The results suggesting that microcyclic species might have evolved from macrocyclic ancestors would also support Tranzschel's law. This concept was first advanced by Tranzschel (1904) and predicts that microcyclic species should always produce teliospores on the same hosts where the ancestral macrocyclic species produce their aeciospores. The two microcyclic species *U. scutellatus* s.s. and *U. striolatus* form
teliospores on E. cyparissias, the host where U. pisi s.l. species produce their aeciospores.

All molecular analyses done in this study support Gäumann’s (1959) morphology based taxonomy of the different species in the U. pisi complex. The relationship among the different species could not be evaluated unequivocally. We believe that the consensus tree from topologically constrained trees shows the most reliable solution to this relationships, however, we point out that more work needs to be done to solve the interspecific phylogeny, and that it will be necessary to include more species into the analysis.
Figure 7.8 Strict consensus of 20,000 topological constrained trees to force monophyly among species with a 80 bp deletion (vertical bar) on the ITS1 and ITS2 sequences (including gaps). Each terminal taxon represents a unique genotype, genotypes starting with E collected on E. cyparissias, genotypes starting with F collected on their Fabaceae host. Genotypes of identified species are written in **bold**. Bootstrap values based on 100 replicates are given above the branches.
7.5.2 Geographic distribution of species

The genetic variation in the ITS region was appropriate for species identification and phylogenetic analysis. But this molecular tool can also be useful for answering ecological questions. In this study we used the results from the genetic species identification to study the species composition within populations, as well as the geographic distribution of the different species. The two common pseudoflower-forming species U. pisi s.s. and U. striatus were both found within the same Euphorbia population at Vieques, or at locations that are very close to each other, as in Ardez vs. Bos-cha (Fig. 7.1, Table 7.2). In contrast, the two microcyclic species U. striolatus and U. scutellatus s.s. were never found within the same host population. In two host populations we found microcyclic and macrocyclic species co-occurring (Table 7.2).

The results from the molecular species identification were also used to test whether the different species and genotypes showed any altitudinal pattern depending on their collection site. The two macrocyclic species, U. pisi s.s. and U. striatus, shared the altitudinal distribution from 500 - 1500 m, while the two microcyclic species showed differences in the altitudes of their respective host populations: Uromyces scutellatus s.s. was only found below 1000 m, whereas U. striolatus was found only once at Les Haudères at 1580 m. Our collection methods were not adequate to allow for generalisations to be made. However, our expectation that microcyclic species might be restricted to, or at least more common at higher elevations, were not fulfilled. Our expectations were based on the hypothesis of Savile (1971) who found that microcyclic species were much more common in arctic regions than in temperate zones, due to the better adaptation of microcyclic fungi to the short summer under arctic conditions. Savile's hypothesis was also supported by Müller & Magnuson (1987) who reported that microcyclic rust fungi are more common in alpine zones in Switzerland than in non-alpine regions. However, the highest elevation where we collected Uromyces fungi was at 1960 m in Zermatt. The climate at this elevation would not be defined as arctic or alpine in either one of the two studies discussed. But E. cyparissias hosts do occur in alpine zones in Switzerland and extensive sampling at elevations above the tree line might reveal a preponderance of microcyclic species. However, shorter seasons might be of lesser constraint for the macrocyclic Uromyces species on E. cyparissias, as all are systemic perennials which allows for more than one year to complete the life cycle (Gäumann, 1959; Savile, 1971).
7.5.3 **Genotypic variation and speciation**

Within many of the resolved species clades we found an extraordinarily high number of different genotypes. We suggest that these subgroups might represent cryptic species. The variation in the species complex *U. pisi* was already mentioned to be exceptional by Gäumann (1959). The whole species complex, including species that infect other hosts than *E. cyparissias*, consists of 51 species on 31 plant genera. What caused this speciation is not known. Fungal speciation might be the consequence of divergence with the Fabaceae hosts or of jumps to new Fabaceae hosts, as each of the described species infects a different species in the family of Fabaceae. Alternatively, the evolution of pseudoflowers on the *Euphorbia* host and the sexual reproduction through insects might have been a key event, leading to an adaptive radiation of the fungal species. However, this hypothesis requires that insects can differentiate among the different fungal species. We know from this study that several pseudoflower-forming *Uromyces* species can co-‘flower’ within the same host population. We are currently testing whether the different rust species produce different insect-attracting fragrances. If experimental studies show that insects can differentiate among species, by scent or by any other cues, then insects might also be able to induce ‘pollinator’-mediated selection, just as in true flowers (Rathcke, 1983; Waser, 1983; Feinsinger, 1987; Shykoff, Bucheli & Kaltz, 1997).

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7.6 Literature cited


### Appendix 7.A

Sequenced macrocyclic rusts of the species complex *Uromyces pisi* collected on *Euphorbia cyparissias*.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Specimen</th>
<th>Genbank #</th>
<th>Leg.</th>
<th>Location</th>
<th>Elevation (m)</th>
<th>ITSl</th>
<th>5.8S</th>
<th>ITS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>E028</td>
<td>28</td>
<td>AF180165</td>
<td>M. Pfunder</td>
<td>Vicques (CH)</td>
<td>590</td>
<td>116</td>
<td>156</td>
<td>202</td>
</tr>
<tr>
<td>E029</td>
<td>29</td>
<td>AF180166</td>
<td>M. Pfunder</td>
<td>Vicques (CH)</td>
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### Appendix 7.B

Sequenced microcyclic rusts of the species complex *Uromyces scutellatus* s.l. on *Euphorbia cyparissias*.

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<th>ITS2</th>
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<td><em>U. puncatus</em></td>
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† With exception of E555 all genotypes were obtained from Fabaceae hosts.
8 ACKNOWLEDGEMENTS

I want to express my warmest thanks to my thesis advisor BITTY ROY. She gave me as much independence as I wished for, but was always there for me with her everlasting enthusiasm to give inspiring advice, support my ideas, and to bring me back to the path whenever I was lost. I also want to thank her for the great times we had in the field and for many cordial chats along this scientific path.

Special thanks to my co-advisor ADRIAN LEUCHTMANN for his many useful comments and suggestions on the manuscripts, as well as for his engagement in organising or joining discussion groups, and for advice in numerous doctoral workshops.

I am grateful to JACQUI SHYKOFF, who was ready at any time, either in person or over the web, to give advice and comments on the manuscripts, and to discuss various aspects of evolutionary biology with me.

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Many thanks to the ZOC people DOMINIK BREM, ELKE KARASU, MARCO SOLIVA, ALEX WIDMER, and DORIS ZUBER for interesting discussion groups and for standing by me through lab- and paperwork. Thanks also to E. HORAK and Hr. JAUCH for the splendid pictures from the Scanning Electron Microscope.

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Of course, all these years would have been not half the fun without my colleagues from the institute who shared with me hours and hours of coffee and lunch breaks, laughing, discussing and: .....hackysack playing!

Last but not least, I want to thank my family and OTTO HOLZGANG for their trust in my doing, for their support, and for pulling me back to reality whenever I was stuck in scientific drift-sand.
## 9 Curriculum Vitae

Monika Pfunder, born in Schaffhausen, 27 July 1970

<table>
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<tr>
<td>Spring 1986 - Autumn 1990</td>
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<tr>
<td>Autumn 1990 - Autumn 1994</td>
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<td>Ecological and systematical biology (XA)</td>
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<tr>
<td>Autumn 1994</td>
<td>Diploma: Thesis: &quot;Lake of Brienz; species composition and trophic structure of plankton in a particle rich oligotrophic lake&quot;;</td>
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<td>Advisors: H. R. Bürgi, EAWAG Dübendorf and the Laboratory of Water and Soil in Bern</td>
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<td>Summer 1995 - Summer 1996</td>
<td>Practical work</td>
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<td>University of Princeton, NJ, USA</td>
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<tr>
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<td>Department of ecology and evolutionary biology</td>
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<tr>
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<td>Research: evolution of RNA; homeobox genes</td>
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<td>Geobotanical Institute, Stiftung Rübel</td>
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<tr>
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<td>Dr. B. A. Roy</td>
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<td></td>
<td>Study of the genetic variation, spatial distribution, and reproductive biology of pseudoflower-forming rust fungi of the species complex <em>U. pisi</em> and related species on the host plant <em>E. cyparissias</em>.</td>
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<tr>
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<td>Advisors: B. A. Roy and A. Leuchtmann from the Geobotanical Institute, Swiss Federal Institute of Technology Zürich, and J. A. Shykoff from Laboratoire d’Évolution et Systématique, Université Paris-Sud</td>
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Publications


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