Man-made habitats – hotspots of evolutionary game between grass, fungus and fly

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Abstract. The origin and effects of an evolutionary game between species from three different kingdoms (plants, fungi and animals) are presented. We provide scientific evidence that the interaction discovered in man-made habitats leads to an early stage of coevolution. The grass *Puccinellia distans* was observed to rapidly spread in new man-made habitats, while at the same time, it was colonised by the fungus *Epichloë typhina*. The invasion of infected grasses is accompanied by alterations in life histories of both species: *P. distans* developed features promoting long-distance spreading, whereas *E. typhina* changed its life cycle by forming sexual structures for the second time, later in the vegetative season. This enables the fungus to make use of the late shoots of the grass for sexual reproduction, even though it cannot be completed because the vector of spermatia necessary for fertilisation, female *Botanophila* flies, is not present at that time. This indicates that such uncoordinated evolutionary processes had taken place before interactions between organisms became so specialised that it is difficult to presume they were the result of natural selection. Moreover, these processes could have been initiated in man-made habitats that, in particular circumstances, can become coevolutionary hotspots.

Key words: coevolutionary hotspots, human activity, *Puccinellia distans*, *Epichloë typhina*

1. Introduction

The coevolutionary processes may lead to complex interactions between partners such as in the well-documented example of the pollinating parasites of figs (Bronstein 1989; Anstett *et al.* 1997). Although the effects of coevolution are often spectacular and well recognised, processes leading to such advanced systems usually remain obscure. The geographic mosaic theory of coevolution indicates spatial differences in the power of interaction between species, which is a result of variation between habitats in the trajectories of natural selection (selection mosaics), the occurrence of reciprocal selection in only some communities (coevolutionary hotspots), and a constantly changing genetic landscape shaped by gene flow and other evolutionary forces (Thompson 1999; Nuismer *et al.* 2000). The recent results have indicated that accidental anthropogenic fragmentation of landscapes is likely to have major effect on the contri-

bution of the continuing coevolution to the persistence and dynamics of the Earth's biodiversity (Thompson & Cunnigham 2002). Here we show how interactions discovered between the three species in man-made habitats give an insight into the early stages of interspecific interaction, trying to explain how these spectacular and complex systems might have evolved. The results of our study allow to put forward another hypothesis saying that man-made habitats in certain circumstances can become typical coevolutionary hotspots, which would never have a chance to appear in the natural conditions.

2. Species of evolutionary game

The first element of the interaction is the grass species *Puccinellia distans*. It is a perennial Euro-Siberian halophyte occurring on marine and inland salines. In spring it forms shoots with inflorescences and, after wind pollination, small wind-dispersed seeds are produced.

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The grass is often grazed by herbivores and then, in autumn, it produces additional shoots with inflorescences (Lembicz 1998).

The second element of the interaction is the ascomycetous fungus *Epichloë typhina*, which infects various grass hosts as a systemic endophyte. In the asexual stage, *E. typhina* does not cause symptoms of infection (Tintjer *et al.* 2008). Its presence may even be beneficial to the host by increasing its resistance to nematode infections or to stress caused by abiotic factors, and by protecting the plant against insects and other herbivorous animals; resistance to pests is mediated by several toxic alkaloids produced by the fungus (Clay 1988; Groppe *et al.* 1999; Leuchtmann *et al.* 2000; Wilkinson *et al.* 2000; Vázquez de Aldana *et al.* 2007; Spiering *et al.* 2008). In the sexual stage, *E. typhina*, like most other species of the genus, is a pathogen causing choke disease of grasses (Sampson 1933;

Wennström 1996; Pfender & Alderman 1999; Vázquez de Aldana *et al.* 2003; Zabalgogeazcoa *et al.* 2008).

Fungal fruiting structures, the stromata (Fig. 1a), form on the tillers of infected plants and restrict their flowering ability leading to partial or total sterility. Perithecia with asci containing ascospores capable of infecting other plants are formed as a result of cross-fertilisation of the fungus (Fig. 1b). This is made possible owing to the third element of the interaction – an anthomyiid fly of the genus *Botanophila*, which is specifically attracted by volatiles produced by the fungus (Leuchtmann 2007; Steinebrunner *et al.* 2008b). By visiting different fungal stromata, the fly carries and spreads spermatia, thus enabling the cross-fertilisation of the fungus (Kohlmeyer & Kohlmeyer 1974; Bultman *et al.* 1998). After fertilisation, fly eggs are laid on the fungal stromata which are later used as a food source for the larvae and adults (Fig. 1c, 1d).
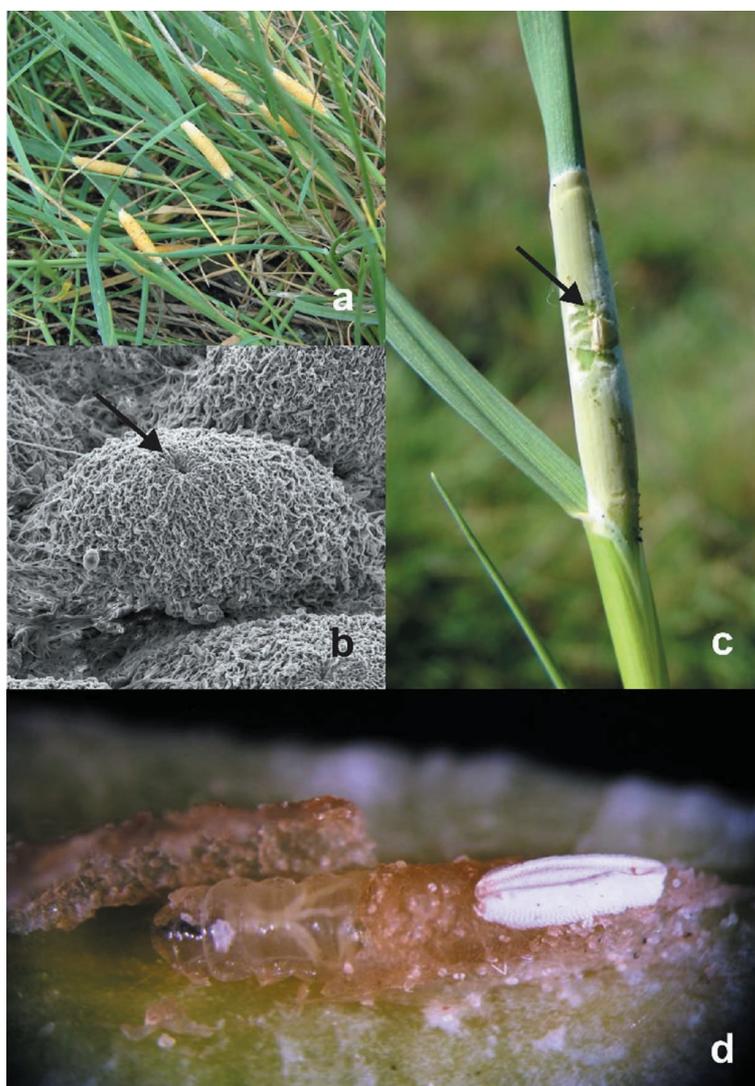
Fig. 1. Participants of the interspecific interaction: the grass *Puccinellia distans*, the fungus *Epichloë typhina* and the fly *Botanophila* sp. (fotography by Ziemowit Olszanowski, Karolina Górzyńska)
Explanations: a – tuft of the grass infected with the sexual stage of the fungus, b – stroma, the arrow points at the opening through which haploid spores are released and can infect new hosts, c – egg of the *Botanophila* fly laid on a newly formed stroma, traces of feeding are indicated (arrow), d – larva of the *Botanophila* fly emerging from the chamber.

3. Effects of evolutionary game

Since the 1960s *P. distans* has colonised anthropogenic habitats in Central Europe (Badmin 1981; Dettmar 1993; Jackowiak 1996). In the beginning, the grass was observed on municipal waste grounds, along roads and railroads, and then in the vicinity of industrial plants. The habitats it colonised were always salinated and rich in nitrogen compounds. In 1992 we observed the first symptoms of choke disease in a single population of *P. distans* in central Poland. The frequency of infected individuals forming stromata increased from 7.5% in 1992 to 67.2% in 1996 (Lembicz 1998). However, the actual frequency of the fungus may have been even higher because grass individuals with the asexual stage of *E. typhina* were indistinguishable from uninfected plants.

While living on *P. distans, E. typhina* does not always undergo a sexual cycle, unlike on most of its other host species. Instead, the endophyte may remain asexual and symptomless. If this is the case, it grows with the meristems of reproductive tillers into the flowers and seeds. Thus, fertility of infected hosts is no longer impaired and the endophyte may spread in the population by infecting new individuals grown from seeds.

In this study, the detection of the asexual stage of *E. typhina* in grass individuals was performed by the PCR amplification of the endophyte ß-tubulin gene fragment (*tub2-4*) (Doss *et al*. 1998). The results of sequencing of the amplified DNA fragments have confirmed their origin from the *E. typhina* genome. Using this sensitive molecular method of detection, we have found that none of 360 examined individuals sampled from 6 natural populations of *P. distans*, was infected with the fungus. Out of the 12 examined *P. distans* populations in man-made habitats the fungus was present in 5. These 5 populations grew in the habitats affected by strong anthropopressure, i.e. leaking brine and salty water, emission of calcium dust and carbon dioxide related to the production process at the nearby Soda Production Plant (Lembicz & Olejniczak 2009).

As indicated by the results of the isozyme variation study, the grass populations on man-made habitats were genetically highly monomorphous (Lembicz 1998). In
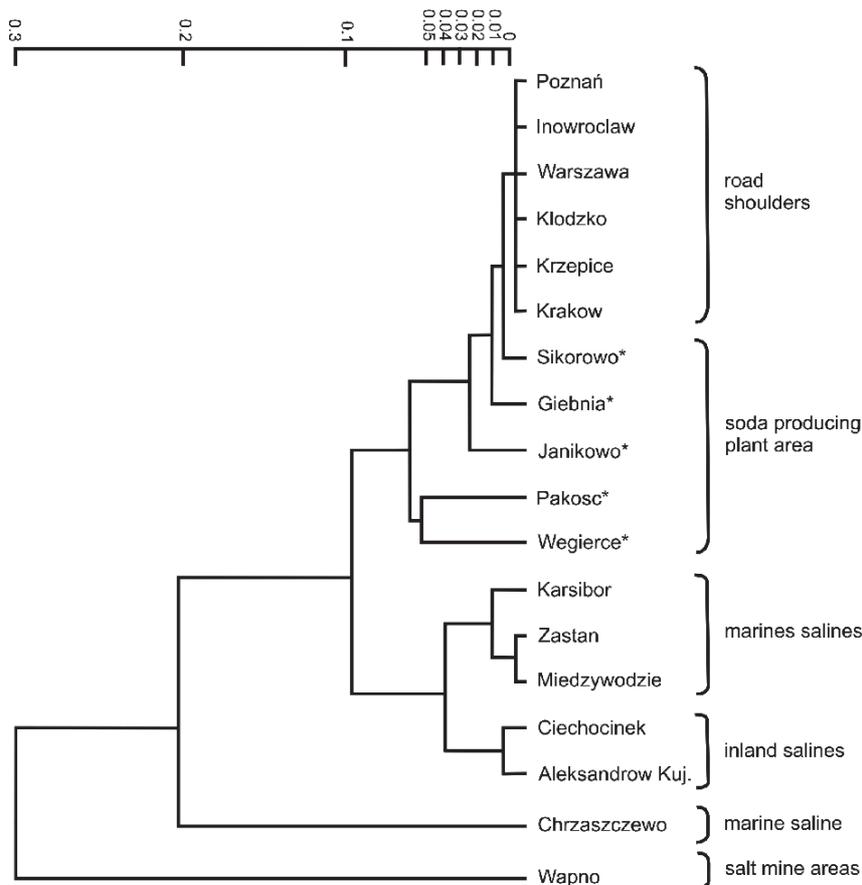
Fig. 2. The genetic distances (D_H) among the *Puccinellia distans* populations from different habitat types and geographic regions. The dendrogram was constructed on the basis of the variation in electrophoretic banding patterns of 15 enzyme systems (Hedrick 1974). Samples for enzyme electrophoresis were taken from seedlings grown on moistened filter paper in Petri dishes. Seeds for this analysis were collected from 18 populations and 60 individuals. Standard methods for starch gel electrophoresis and enzyme activity staining were employed (Wendel & Weeden 1989). Infected populations are marked with an asterisk (*). All infected populations occurr in the vicinity of the Soda Production Plant near Inowrocław.

Table 1. Allozyme genotypes of *Epichloë typhina* from *Puccinellia distans* and *Dactylis glomerata* (unpublished data). Values are given as migration distances (mm) adjusted to reference alleles run at standard conditions

Origin of isolate	Isozyme locus[1]											
	ACP	ACO	ALD	DIA	LAP	MDH-1	MDH-2	6PG	PGI	PGM-1	PGM-2	TPI
P. distans, Poland	5	15	29	18	22	8	13	18	28	35	10	35
P. distans, France	5	15	26	18	22	8	13	18	28	35	10	35
D. glomerata, Switzerland	6	15	26	19	22	8	13	18	28	35	16	40

Explanations: [1]ACP: acid phosphatase (EC no. 3.1.3.2); ACO: aconitase (EC no. 4.2.1.3); ALD: aldolase (EC no. 4.1.2.13); DIA: diaphorase (EC no. 1.6.4.3); LAP: leucine aminopeptidase (EC no. 3.4.11.1); MDH: malate dehydrogenase (EC no. 1.1.1.37); 6PG: 6-phosphogluconate dehydrogenase (EC no. 1.1.1.44); PGI: phosphoglucose isomerase (EC no.5.3.1.9); PGM: phosphoglucomutase (EC no. 5.4.2.2); TPI: triosephosphate isomerase (EC no, 5.3.1.1)

the road shoulder populations none of the 15 examined enzyme systems was polymorphic; in all infected populations only 2 enzymes were polymorphic. Among the 18 examined populations of *P. distans*, both the populations from salt mine areas and natural salines appeared to be distinctive in terms of relatively high values of genetic distances, whereas the ones found on the road shoulders as well as the populations infected with *E. typhina* occurring in the vicinity of the Soda Production Plant, were genetically very similar (Fig. 2).

As anticipated, these observations are consistent with the founder effect. One or few invasive plant genotypes may have reclaimed the polluted area in recent times by spreading very rapidly over the new habitat. Afterwards, the plants became infected by *Epichloë* fungi originating from neighbouring infected populations of other host plants. Alternatively, the fungus may have been already present in the founder plants but at such low frequency that it was not detected. The fungus, which proved to be genetically uniform in all infected populations based on 12 allozymes, (Table 1) could have spread in the population either by seeds or, contagiously, by ascospores. Moreover, the odour profile of the *Epichloë* stromata on *P. distans* was distinct from that of *E. typhina* on other host and therefore supporting the scenario of a pre-existing infection in the founder plants
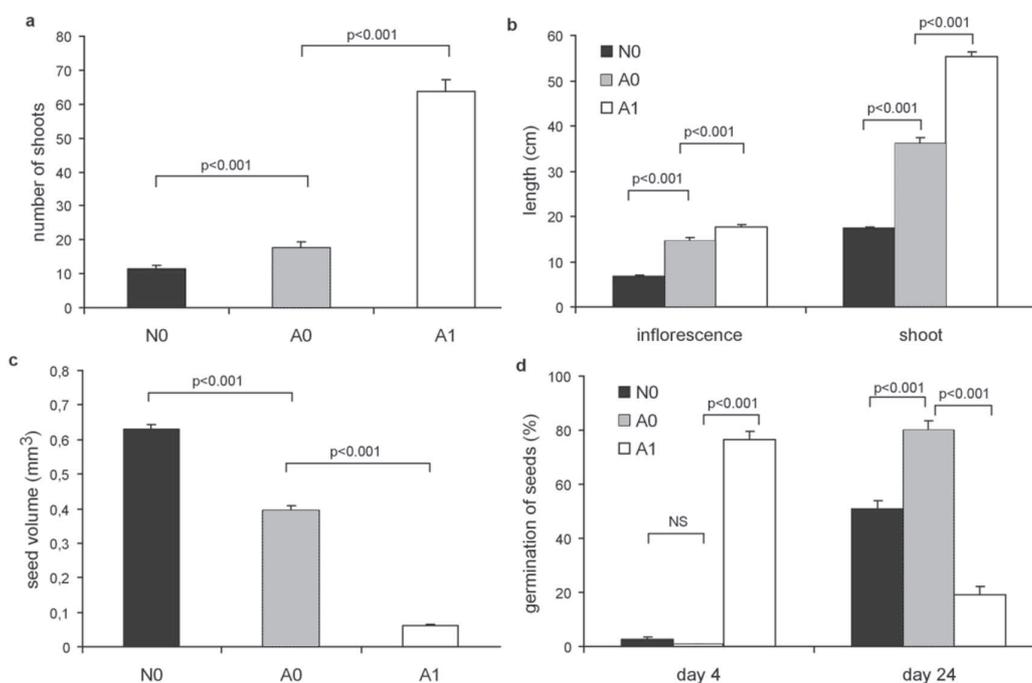
Fig. 3. Life-history parameters in *Puccinellia distans* observed in a common garden (Lembicz & Olejniczak 2007)
Explanations: the comparison is made between three groups, where N0 – individuals from natural populations, A0 – non-infected individuals from anthropogenic population, and A1 – individuals from anthropogenic population infected by the asexual stage of the fungus; a – number of generative shoots produced in spring, b – average length of shoot and length of inflorescence, c – average seed volume calculated as seed length x width x width, d – proportion of seeds germinating on the 4th and 24th day after they were placed in a germination chamber. The results of Mann-Whitney U-test are shown; the comparisons were made pair-wise with appropriate Bonferroni correction; vertical bars denote standard errors. All individuals were sampled from natural or anthropogenic populations in the season following germination. They were planted separately, at random, in the pots with homogenised soil and kept under equal conditions. The recordings of the results were made two years later.

(Steinebrunner *et al*. 2008a). The relatively short period of time since the presumed colonisation of the new habitat did not allow for any detectable genetic differentiation of the endophyte. Even though such genetic effects of colonisation have been observed in many species (Barrett & Shore 1989), they have never occurred simultaneously in both a parasite and its host.

Common garden experiments revealed substantial differences in the life history traits of the uninfected *P. distans* plants from man-made habitats compared to those from natural ones. The individuals from polluted localities produced more generative shoots, their inflorescences were larger, seeds smaller and their germination rate was higher than in those from natural habitats (Fig. 3). All these traits are often associated with successful colonisation and long-distance spreading of plants (Barrett & Richardson 1986; Van der Putten 2002). The *P. distans* individuals with asexual infections also produced more generative shoots and had larger inflorescences than the uninfected individuals, indicating that more seeds were produced. Likewise, the seeds were smaller but exhibited greater germination ability (Fig. 3). In these plants, the fungus may have contributed to the very successful invasion of the grass.

The sexual stage of *E. typhina* has been hitherto observed on *P. distans* plants only in spring. Thus, the reduction of fecundity affected only the early shoots of the grass. However, the appearance of the sexual stage increased the proportion of generative shoots produced in autumn from 3.7% (in individuals with asexual infections only) to 57.1% (Mann-Whitney U-test, $n_1 = n_2 = 30$, U = 5.5, p<0.001). In the year 2000 we noted, for the first time, a second appearance of the sexual stage in autumn, which has not been reported in literature on this species (Lembicz & Olejniczak 2007). The frequency of stromata formed on tillers of infected plants in spring did not change from the year 2000 to 2001 (Mann-Whitney U-test, $n_1 = n_2 = 30$, U = 369, NS), but the frequency of autumn stromata increased in 2001

(Mann-Whitney U-test, $n_1 = n_2 = 30$, U = 66, p<0.001). Female *Botanophila* flies, which serve as vectors of fungal spermatia, are not present at the time when *Epichloë* appears for the second time in autumn. Stromata remain unfertilised and do not form ascospores capable of infecting new plants. Therefore the production of late sexual structures of the fungus provides benefits for neither the fungus nor the plant. Moreover, stromata reduce seed production of the host and hence vertical transmission of the fungus through seeds. Disease expression in autumn clearly represents a misadaptation which could be explained by the fact that the species interactions described here have appeared relatively recently as a result of human activity and are yet to undergo further modification by means of natural selection.

The above system may serve as an example of processes taking place at early stages of coevolution. In subsequent generations the fungi producing the useless stromata may be eliminated, the period of activity of the *Botanophila* fly extended, or grass genotypes suppressing the sexual stage of the fungus favoured. The long term consequences of the coevolution may become so complex that the development of these interactions by the gradual process of natural selection may seem inconceivable. Eventually, the system is expected to gain features testifying to its advancement and approach the symbolic top of the "Mount Improbable" (Dawkins 1996).

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