Ecological stress and sex evolution in soil microfungi

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The elucidation of the origin and maintenance of sex is a major unsolved problem in evolutionary biology. A number of hypotheses have been elaborated, but the scarcity of empirical data limits further progress. During recent years, the general inclination has changed towards pluralistic models of sex evolution, due partly to an increased diversity of studied organisms. Fungi are among the most promising organisms for testing sexual causation, as demonstrated in recent laboratory experiments. However, reconciling theory and evidence necessitates critical field observations. Here, we report new estimates of the distribution of morphologically sexual and asexual soil microfungi in nature, which indicate a remarkable trend towards increased sexuality with increasing climatic stress.

Keywords: ascomycetes; teleomorphic and anamorphic states; aridity/salinity stress; recombination

1. INTRODUCTION

Understanding the selection pressures responsible for the evolution of sex and recombination remains a major challenge for biology. There is a clear tendency to discriminate between the *origin* of sex and recombination and their *maintenance* over long evolutionary periods despite numerous ‘costs of sex’—some real, some illusory or relevant to very restricted groups of organisms (Birky 1999).

More than two dozen theoretical (mathematical) models have been proposed to explain the maintenance of sexual recombination (for reviews see Kondrashov 1993; Korol *et al.* 1994; Barton & Charlesworth 1998; Burt 2000; Otto & Lenormand 2002; Rice 2002). Combinative, or generative, hypotheses consider the main function of sex and recombination in shuffling genes. Recombination removes the negative correlation between favourable alleles at different loci, thereby increasing the efficiency of natural selection. Generative models can be classified according to the source of linkage disequilibrium (LD) between selected loci: stochastic, deterministic, genetic (caused by new mutations) or ecological (caused by variation of external conditions) (Kondrashov 1993). Fisher and Muller, in the 1930s, proposed that sex might be advantageous because it brings together beneficial mutations randomly appearing in different individuals (Maynard Smith 1978; Otto & Barton 1997). A complementary stochastic-mutation explanation considers the role of recombination in selection against deleterious mutations. According to Muller, deleterious mutations tend to be fixed in a finite asexual population owing to random drift, in spite of purifying selection (Muller’s ratchet), whereas recombination helps to stop this process. In the 1980s, a few deterministic models of selection against deleterious alleles were proposed, in which the advantage of recombination depends on LDs caused by synergistic interaction between harmful mutations produced at a high rate (for example, greater than one per genome per generation) (see Michod & Levin 1988;

Kondrashov 1993). A few other models of deterministic sources of LDs include: (i) adaptation to temporally or spatially varying environments; (ii) antagonistic species interaction (the ‘Red Queen’ hypothesis); and (iii) intra-specific competition (the ‘tangled bank’ hypothesis) (for reviews see Maynard Smith 1978; Kondrashov 1993; Barton & Charlesworth 1998; Otto & Lenormand 2002; Rice 2002). In most of these models, the conditions favouring increased recombination are associated with negative LD among selected loci caused by the rather constrained epistasis. However, some mechanisms, for example selection of beneficial mutations or varying selection, may favour recombination in the absence of epistasis, with LD being caused by finite population size (Otto & Barton 1997, 2001) and/or population subdivision (Pytkov *et al.* 1998; Lenormand & Otto 2000; Otto & Lenormand 2002).

The difficulty of producing critical evidence seems to be the limiting factor preventing real progress in a better understanding of sex and recombination and in defining the key mechanism(s) of the evolutionary preservation of sex in nature. Despite the high attractiveness of a universal explanation for the origin and maintenance of such a generic phenomenon as sex, the last decade can be characterized by an obvious trend toward pluralistic explanations, at least for sex maintenance (Korol *et al.* 1994, p. 193; Barton & Charlesworth 1998; West *et al.* 1999). To some extent, this tendency may be a joint result of an ever-increasing diversity of competing theoretical models and the range of organisms used in studies of sex evolution, with fungi being among the most promising taxa for laboratory testing of sexual causation (Birdsell & Wills 1996; Zeyl & Bell 1997; Greig *et al.* 1998). However, despite the large contribution of controlled laboratory experiments, the major problem remains enigmatic and needs elucidation from critical field observations. It should also be stressed that there is a certain psychological bias in favour of models that are more relevant to animals and plants, but less relevant to the majority of eukaryotic organisms, including fungi (Birky 1999).

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2. THE HYPOTHESIS

We report new estimates of the distribution of morphologically sexual and asexual soil microfungi in nature, which indicate a trend towards increased sexuality with increasing climatic stress. Fungi display many reproductive patterns (Taylor *et al.* 1999). Generally, two kinds are different in principle—sexual (by means of meiospores) and asexual (by means of mitospores). Studying the distributions of fungi with these patterns in natural habitats contributes to a further understanding of the relationship between the environment and sex, revealing the mechanisms of adaptation to the environment. We analysed the distribution of soil fungi from two large groups representing micromycetes subdivided into: (i) sexual (ascomycetous) fungi; and (ii) asexual (mitosporic) fungi. Micromycetes from these groups are distributed almost everywhere (geographically as well as ecologically) and comprise a basic part of all microfungi biotas (Domsch *et al.* 1993).

Soil is considered a world of asexual microfungi. A major proportion of soil micromycetes are *ruderal*-selected fungi, which are active only in low-stress habitats, and predominate wherever readily assimilable carbon sources are available (Dix & Webster 1995). By contrast, sexual ascomycetes are believed to be mainly *stress*-selected, occupying environments with various stress levels that exclude many asexual fungi. Because of this, some selective methods were introduced for isolating soil ascomycetes, such as soil heating, special chemical treatment and various baiting techniques (e.g. Wensley 1953; Warcup & Baker 1963; Kirilenko 1978). Therefore, a low abundance of sexual ascomycetes under favourable (nutrient, temperature and moisture) soil conditions is explained by competitive exclusion by fast-reproducing ruderal-selected asexual microfungi. The question is whether the predominance of asexuals decreases with stress. We assume that the necessity to withstand permanent or variable stress imposes long-term selective pressure favouring new beneficial mutations, thereby giving an advantage to sex and recombination, which brings together various beneficial mutations (Otto & Barton 1997, 2001). Recombination also facilitates the incorporation of new beneficial mutations by making them less dependent on background selection (for a review see Rice 2002). Therefore, sex and recombination can be considered to remove or relax the limits of natural selection.

It is known that spores have two major roles in the life of the fungus—dispersal to new sites and survival in unfavourable conditions. From this point of view, sexual and asexual modes of reproduction are considered to have different ecological purposes. Mitotic spores serve mainly as dispersal propagules. The sexual stage can result in resting structures through which the organism avoids unsuitable conditions by remaining dormant for long periods (e.g. Elliot 1994; Deacon 1998; Carlile *et al.* 2001). Therefore, an additional contribution of sex in adaptation to stressful environments (unlike some other organisms; reviewed by Bell (1982)) may be related to the production of resistant (dormant) spores. Unfortunately, it would not be an easy task to separate this 'physiological' function from the generative functions of sex.

Experimentation with model organisms and recent

theoretical analysis have shown that directional selection, in combination with finite population size and/or spatial heterogeneity, may promote a selective advantage of increased recombination under much less restrictive assumptions than usually thought (Flexon & Rodell 1982; Gorodetski *et al.* 1990; Korol & Iliadi 1994; Korol *et al.* 1994; Otto & Barton 1997; Korol 1999; Lenormand & Otto 2000). Assuming that sex helps organisms to adapt to adverse conditions, one can further propose that in natural populations of fungi the abundance of sexual forms will be positively correlated with the severity of stress. To examine this, the proportion of morphologically sexual versus asexual microfungi in soil was evaluated in a few regions across a gradient of increasing aridity stress in Israel. We report new estimates indicating a remarkable trend towards increased sexuality with increasing climatic stress both regionally and locally.

3. MATERIAL AND METHODS

(a) *Sampling sites*

Soil fungi samples were collected across a regional gradient of increasing aridity starting in Mount Carmel and progressing through the Upper Galilee Mountains, the central Coastal Plain, the northern Negev Desert, the Dead Sea area, and terminating in the Dead Sea shore. Likewise, we sampled locally in two 'evolution canyons' (Nevo 2001), the first in lower Nahal Oren in Mount Carmel, and the second in lower Nahal Keziv in the Upper Galilee Mountains (table 1).

(b) *Material isolation*

Soil samples were taken from the upper layer (1–10 cm deep). Micromycetes were isolated by the soil-dilution plate method (Davet & Rouxel 2000). The following standard media were used: malt extract agar, Czapek's agar, potato dextrose agar and carboxymethylcellulose agar. Isolation of halotolerant and halophilic micromycetes from the Dead Sea area and the Dead Sea shore was conducted using a hyperosmotic medium (malt extract 5%, saccharose 3%, NaCl 8%) and Czapek's agar prepared in a mixture of 20% Dead Sea water and 80% distilled water. Streptomycin (100 g ml^{-1}) was added to the medium to suppress bacterial growth. One millilitre of the sample suspension was mixed with the agar medium at 40°C in Petri plates. The plates with inoculum were incubated at 24°C and 37°C for 2–4 weeks (two or three plates for each medium at each temperature). Colonies of each species were isolated in pure cultures for further identification and storage. Taxonomic identification of fungal isolates (including sexuals) was based on their morphological structures. Chi-squared analysis and log-linear models were employed to compare the proportions of sexual forms in the alternative microniches.

4. RESULTS

(a) *Proportion of sexuals*

To address the question of whether the advantage of sex is a function of ecological stress, we evaluated the proportion of sexual versus asexual soil microfungi in a few regions across a gradient of increasing aridity stress in Israel: the northern part (Carmel Mountains, Upper Galilee), the Mediterranean Coastal Plain, the northern Negev Desert and the Dead Sea area (table 1). The results

Table 1. Sexual species in soil mycobiota of different Israeli regions (data from Volz *et al.* (2001) and references therein, and from the present study).

region	total number of species	number of sexual species
'Evolution Canyon' I, lower Nahal Oren, Mount Carmel	166	9
'Evolution Canyon' II, lower Nahal Keziv, Upper Galilee Mountains	192	13
central Coastal Plain	243	26
northern Negev Desert	159	28
Dead Sea area	223	46
Dead Sea shore (hypersaline sand)	78	26

Figure 1. Parallelism between stress severity and proportion of sexual species in soil mycobiota of different Israeli regions: (1) 'Evolution Canyon' I, lower Nahal Oren, Mount Carmel; (2) 'Evolution Canyon' II, lower Nahal Keziv, Upper Galilee Mountains; (3) the central Coastal Plain; (4) the northern Negev Desert; (5) the Dead Sea area; and (6) the Dead Sea shore (hypersaline sand). Sites 4–6 can be characterized by increasing stress intensity. Note the nearly two-fold increase in the proportion of sexuals in the more stressful summer (s) samples at site 2 compared with the winter (w) samples. Significant differences between the various sites and the central Coastal Plain are denoted by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

obtained show a highly significant trend of an increasing proportion of sexuals with increasing aridity/salinity stress southwards, from 6%–9% in mesic northern Carmel Mountains and the Coastal Plain to 18%–33% in the southern, climatically most stressful, Negev Desert and Dead Sea area (figure 1). Among the surveyed sites, the proportion of sexuals was highest in the Dead Sea coastal-sand environment, which is characterized by a very stressful combination of long-term high temperature and continuous hypersaline and oligotrophic conditions. From this substrate, a high number of sexual ascomycetes were isolated (comprising approximately one-third of the whole microfungal community), including cellulolytic, coprophilous and keratinophilic species, and four species that were known only as phylloplane-inhabiting fungi, i.e. colonizing substrates with harsh and varying climates where sexual fungal species usually prevail (Ellis & Ellis 1997).

(b) Facultative sex: teleomorphic and anamorphic states in sexual species

Many ascomycete species are known to undergo two kinds of sporulation in their life cycle—sexual (teleomorphic state, producing ascospores) and asexual (anamorphic state, producing conidia) (figure 2). For example, *Pleospora herbarum* is one of the sexuals found in the Dead Sea shore. This species has both teleomorphic

and anamorphic states. The anamorphic (or *Stemphylium*) state is not abundant but is more or less common among soil micromycetes. It was found in almost all of the studied regions in Israel. The *Pleospora* state is well known and widely distributed among phylloplane (leaf surface) inhabiting fungi. To obtain both states of *P. herbarum* in the same culture after incubating for 3–4 weeks at 25 °C is very rare (if not unique) because the perithecia of this species only mature *in vitro* in 2–12 months at low temperatures (i.e. under stress conditions) (Domsch *et al.* 1993).

In light of the association between the proportion of sexuals and habitat hostility, it was of great interest to test whether, within the group of sexual species, the abundance of specimens with and without anamorphs is also influenced by stress-intensity characteristic of their original habitat. The results obtained clearly supported this assumption. Within the sexuals, the proportion of isolates displaying only teleomorphic states (P_{tele}), compared with those that displayed both teleomorphic and anamorphic states within the same colony ($P_{\text{tele+ana}}$), demonstrated a significant positive correlation with the severity of stress (figure 3). In particular, in the material collected from the hypersaline sand of the Dead Sea shore, 90% of sexual isolates were only teleomorphic. In the 'Evolution Canyon' II (EC II) lower Nahal Keziv soils (Nevo 2001) this number was three-fold smaller: $P_{\text{tele}} = 30\%$. Furthermore, a similar trend was revealed among seasons within the EC II: P_{tele} was 20% in the milder winter period (averaged over the two opposite slopes), whereas an estimate of $P_{\text{tele}} = 82\%$ was obtained for the summer period on the driest and sunniest locality on the south-facing slope. We presume that the aforementioned quick and successful development of the *P. herbarum* fruit bodies in pure culture derives from the microevolutionary history of the sampled population and its genetic adaptation to the highly stressful environment of the Dead Sea shore.

(c) Morphological adaptation to stress

Another interesting association with adaptation to stress was the morphological structure of the sexual sporulation of our collections. The majority of sexual micromycetes from the stressful environments (82%–91% of sexual isolates, mainly from the order Sordariales) had thick-walled dark brown or black ostiolate perithecia with asci orderly arranged in a hymenium and with large (more than 10 µm) melanin-containing ascospores that are forcibly discharged—the effect of dark pigmentation protecting fungi from the influence of solar radiation is well known (Griffin 1972; Zhdanova & Vasil'evskaya 1982). The

Figure 2. Ascomycetes from the Dead Sea shore with only teleomorphic and mixed teleomorphic and anamorphic states. (a) *Sporormiella australis* (Speg.) S.I. Ahmed et Cain with asci and ascospores. (b) *Pleospora herbarum* (Pers.) Rabenh. with sexual ascospores (upper right) and asexual conidia (lower left) remarkably revealed in the same culture.

Figure 3. Parallelism between stress severity and the proportion (P_{tele}) of purely teleomorphic isolates (no evidence of anamorphic state) among sampled ascomycetes. (1) 'Evolution Canyon' II (EC II), winter samples; (2) EC II, summer samples; (3) EC II, summer samples from the south-facing sunny microsites; and (4) the Dead Sea shore. The proportions of purely teleomorphic and mixed teleomorphic and anamorphic isolates are indicated by black and white, respectively. Comparisons of the alternative microniches gave the following results: for (1) versus (2): $\chi^2 = 27.2$ (d. f. = 1, $p < 10^{-6}$); for (3) versus (2): without sunny samples, $\chi^2 = 37.7$ (d. f. = 1, $p < 10^{-6}$); and for (3) versus (4): $\chi^2 = 25.2$ (d. f. = 1, $p < 10^{-6}$). Log-linear analysis gave $\chi^2 = 91.7$ (d. f. = 1, $p < 10^{-8}$) for the total trend of increasing P_{tele} : $P_{\text{tele+ana}}$ with the severity of drought/salinity.

species that were originally phylloplane inhabiting, belonged to the order Pleosporales and had multi-celled dark-coloured ascospores (greater than $20 \mu\text{m}$ in length) developed in bitunicate asci. By contrast, the basic group of ascomycetes isolated from the more favoured northern mesic localities consisted mainly (65%–72%) of species from the order Eurotiales with thin-walled light-coloured cleistothecia, unarranged asci and small (less than $5 \mu\text{m}$) pale ascospores, which are passively discharged after dissolving the ascus wall. In the stressful environments, ascospores have a combination of traits contributing mainly to resting functions, facilitating survival under stressful conditions. Even strains of the same species iso-

lated from different geographical habitats displayed morphological and developmental adaptations caused by selection in the extreme conditions. For example, the strains of *Chaetomium globosum* from the Dead Sea shore had larger ascospores ($10\text{--}12 \mu\text{m} \times 7.5\text{--}9 \mu\text{m}$) and produced mature perithecia more quickly (after 13–15 days on the medium with 20% Dead Sea water, and after 17–25 days on the standard medium) than the strains from lower Nahal Keziv (ascospores $8\text{--}10 \mu\text{m} \times 6\text{--}8 \mu\text{m}$; mature perithecia after 30–40 days on the standard medium). Variation at many loci should be involved in these patterns, but this needs direct experimental testing.

5. DISCUSSION

(a) *Reproductive morphology and 'genuine' sex*

The demonstration of a positive correlation between sex abundance and aridity/salinity stress in soil microfungi entirely depends on whether the morphological criterion is sufficiently informative. We believe that our results strongly support a positive association between ecological stress and sexual reproduction in micromycetes. An important topic to be addressed in further studies is the correspondence between sexual reproductive morphology and sexual population structure: will the populations of teleomorphic fungi display a recombining or a clonal structure? Will the characteristics of population structures parallel the described trends in reproductive morphology? Will the observed tendency of drought/salinity stresses to decrease the proportion of species with anamorphic states among sexuals be corroborated by increasing rates of mutation, recombination and gene conversion, as recently found in 'Evolution Canyon' I at lower Nahal Oren, Mount Carmel for soil microfungus *Sordaria fimicola* (Saleem *et al.* 2001)? In light of puzzling findings on recombination patterns of population structure (cryptic sex) in morphologically asexual parasitic (Burt *et al.* 1996) and saprotrophic (Geiser *et al.* 1998) fungi, these questions may also be relevant to the asexual part of the fungal communities surveyed in this study.

(b) *Competitive versus complementary explanations*

Based on long-term experiments with *Drosophila*, we have proposed that selection for any trait might promote evolution towards increased recombination rates (Korol & Iliadi 1994; Korol *et al.* 1994; Korol 1999, and references therein). In fact, these expectations were later justified in a series of theoretical models of recombination evolution driven by (directional) selection of beneficial mutation (Otto & Barton 1997, 2001). Selection regimes invoked by adaptation to extreme stressful environments seem to fit this model. Indeed, besides artificial selection (for example, domestication), stressful environments experienced by marginal populations are probably the best rationale for this model, because stabilizing selection here cannot outweigh directional selection; the latter continuously improves the adaptability to the key limiting factor(s) by recruiting new beneficial mutations (Korol 1999). Likewise, a fluctuating selection regime is another feature of such habitats (Nevo & Beiles 1988; Nevo *et al.* 1996). This combination of varying selection and a permanent predominance of directional over stabilizing selection seems to explain the remarkable trend of a sharply increasing abundance of morphologically sexual microfungi with increasing drought/salinity stress. However, the contribution of other factors cannot be excluded, reflecting the necessity of a 'pluralistic' approach (Korol *et al.* 1994, p. 193; West *et al.* 1999) to explain the factors maintaining sex and recombination: (i) finite population size; (ii) spatial variation in selection regime; (iii) temporal variation in selection regime; and (iv) selection against deleterious mutations. Some of these (namely, i–iii) complement the ecological hypothesis of selection of beneficial mutations (Otto & Lenormand 2002), whereas selection against deleterious mutations is traditionally considered as competitive rather than complementary to ecological hypotheses.

In our view, the mutation hypothesis is an oversimplification when the problem of the maintenance of sex is considered in the context of evolution under extreme stressful conditions. Indeed, stressful conditions are known to simultaneously affect the rates of recombination, mutation and transposition of mobile elements (Plough 1917; Korol *et al.* 1994; Kidwell & Lisch 1997). An increase in recombination in response to stress ensures a higher genetic variability in the progeny, and hence more opportunities for genotypic adaptation. Concurrently, this should generate a larger per-chromosome variance in the frequency of (spontaneous and stress-induced) harmful mutations and, consequently, a higher efficiency of purifying selection (Korol *et al.* 1994; Korol 1999). Therefore, a putative increase in the mutation rate may be compensated by a higher rate of recombination, while nothing can be said about the change in the mode of selection regime (with respect to the level of synergistic epistasis) with increasing severity of stress. All this leaves us uncertain as to whether a mutation hypothesis is applicable as a contributing mechanism to explain the sharp increase in the abundance of sexuals with increasing severity of stress revealed in this study.

The evidence presented clearly shows the increasing advantage of sexual reproduction in soil microfungi with increasing stress, which is accompanied by the production

of adaptive morphological structures, which allow fungi to remain dormant and survive unfavourable conditions. To discriminate between a 'physiological' and a generative interpretation of the putative benefit of sexual reproduction under stress, it would be helpful to measure the resistance of meiospores and mitospores in stressful conditions (high salinity, desiccation). Such an assay could be easily conducted on ascomycetous and zygomycetous species by producing both kinds of spores in culture, with their strains collected from contrasting (saline versus non-saline, wet versus dry) environments.

We believe that our results corroborate the findings of theoretical models and previous experiments that adaptation to varying and/or stressful conditions creates selective advantages to sex and recombination (Gorodetski *et al.* 1990; Korol & Iliadi 1994; Korol *et al.* 1994; Birdsell & Wills 1996; Otto & Barton 1997; Zeyl & Bell 1997; Greig *et al.* 1998; Lamb *et al.* 1998; Lenormand & Otto 2000; Korol 2001; Saleem *et al.* 2001). Notably, the long-term research into genetic diversity in natural populations of plants and animals at both the protein (Nevo *et al.* 1984) and the DNA (Nevo *et al.* 1996) levels, conducted during the last three decades in the Institute of Evolution, University of Haifa, at global, regional and local scales showed adaptively similar patterns. The results indicate that genetic diversity in nature is non-random, and positively correlated with abiotic and biotic environmental heterogeneity and stress (Nevo 2001). Our theory is that genetic diversity and sex levels may be coupled, reflecting adaptive patterns, driven primarily by natural selection. However, much work is needed to discriminate between different explanatory models of the evolutionary advantage of sex invoked by the adaptation to a stressful environment for any organism, and for fungi in particular (Taylor *et al.* 1999).

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