AFLP-based assessment of the effects of environmental heavy metal pollution on the genetic structure of pioneer populations of *Suillus luteus*

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Summary

- The effects of environmental heavy metal pollution on the genetic structure of pioneer populations of the ectomycorrhizal basidiomycete *Suillus luteus* were assessed.
- Sporocarps were collected from nine different locations and characterized by amplified fragment length polymorphism (AFLP) markers. Six of the sampling sites were contaminated with heavy metals and were dominated by tolerant individuals. Considerable genetic diversity was found within geographic subpopulations, but no reduction of the genetic diversity of populations inhabiting contaminated soils was observed. Neither did significant clustering of subpopulations inhabiting contaminated soils occur. Overall, the genetic differentiation between subpopulations was low, but Bayesian inference indicated the presence of two genetically differentiated clusters of individuals, which may correspond to different intercompatibility groups in *S. luteus*.
- Heavy metal contamination seems to have a limited influence on the genetic structure of populations of *S. luteus*. Loss of diversity may have been prevented by sexual reproduction and rapid evolution of the tolerance trait or initial genetic bottlenecks may have been reduced by admixture and recurrent migration from surrounding populations colonizing noncontaminated soils.

Key words: AFLP (amplified fragment length polymorphism), ectomycorrhizas, genetic adaptation, heavy metal tolerance, population structure, *Suillus luteus*.

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Introduction

Soils contaminated with heavy metals can occur naturally or as a result of anthropogenic activities such as mining, agriculture, traffic and industrial processes. Although heavily polluted soils are extremely toxic, they are often colonized by a variety of plant, fungus and animal species. Since the first observation of heavy metal tolerance in plant populations originating from contaminated sites (Prat, 1934), it has been suggested that these have adaptively evolved as tolerant ecotypes and subsequently, a genetic basis to heavy metal tolerance has been presumed (Bradshaw, 1952; Macnair, 1993).

Heavy metal contaminated sites may be considered as ecological islands, surrounded by noncontaminated mainland, where a predominant environmental factor is imposing severe selection pressure on the residing populations. These islands are of various sizes (a few to several hundreds of hectares) and can be of recent origin (several decades), thus providing the opportunity to investigate the initial steps in the establishment of differentiated populations under possibly extreme selection pressure. Therefore, plant or fungus populations growing in heavy metal contaminated soils should be considered valuable models for the study of microevolution (Macnair, 1987).

Earlier studies have indicated that adaptation to elevated concentrations of heavy metals in the soil can occur at unexpectedly high speed (Wu *et al*., 1975) and that, despite the apparent founder effect, tolerant populations can maintain a high level of genetic diversity and appear to be at least

as variable as nontolerant populations (Lefèbvre & Vernet, 1990; Mengoni *et al.*, 2000). However, the number of plant species that can develop metal tolerant ecotypes seems to be small and depends on the presence of tolerant individuals in the surrounding founder populations (Al-Hiyaly *et al.*, 1993). For most plants, the anthropogenic pollution of soils has caused an unprecedented, rapid change in environmental conditions, which is likely to override their adaptive potential, especially in the case of trees with their long reproductive cycles (Schützendübel & Polle, 2002). Some trees, however, can thrive on heavy metal contaminated sites and there is growing evidence that adapted rhizosphere microorganisms may assist trees in their colonising such sites (Wilkinson & Dickinson, 1995; Meharg & Cairney, 2000; Adriaensen *et al.*, 2004). Among these soil-borne organisms, the ectomycorrhizal (ECM) fungi occupy a predominant position.

A typical root symbiont that occurs on heavy metal polluted sites is *Suillus luteus* (L.Fr.) Roussel, an ectomycorrhizal basidiomycete that associates with the roots of young pine trees that colonise disturbed sites. *S. luteus* is a typical pioneer ECM fungus that mostly colonizes pine seedlings through spores, dispersed by wind or mammals (Bruns *et al.*, 2002). The relatively short generation time (3–5 yr) and the frequent sexual reproduction with release of billions of basidiospores may favour rapid selection for genotypes adapted to specific soil conditions, which is in sharp contrast to the life cycle of their host plants (Colpaert *et al.*, 2004). Previous studies on ectomycorrhizal systems have indicated the importance of the fungal symbiont for reducing the effects of environmental heavy metal pollution in trees (Jentschke & Godbold, 2000; Adriaensen *et al.*, 2004). In the case of *S. luteus*, it has been shown *in vitro* that populations inhabiting soils contaminated with heavy metals are more tolerant to increased levels of those metals that are enriched in the soil of origin than populations from nonpolluted areas. Moreover, there is ample evidence for a genetic basis of this heavy metal tolerance and it has been suggested that the genetic diversity within such tolerant populations may be reduced significantly (Colpaert *et al.*, 2000, 2004).

In the present study, we aim to describe the genetic structure of recently established populations of *S. luteus* in heavy metal polluted and nonpolluted areas, using amplified fragment length polymorphism (AFLP) markers, in order to reveal whether the pattern of genetic variation is influenced by the environmental heavy metal contamination.

Materials and Methods

Samples were collected from nine different geographic subpopulations situated along a metal pollution gradient that developed in the previous century due to the activities of several zinc smelters in the province of Limburg (Belgium). Sample sizes of *c.* 30 individuals were aimed at. However, the number of sporocarps was not sufficient at several sampling sites and, consequently, sample sizes were reduced. In total, 164 sporocarps of *S. luteus* were collected, frozen in liquid nitrogen and stored at −80°C. The geographical coordinates of the sampled subpopulations and the characteristics of the corresponding forests are given in Table 1. All sampling sites are situated in the Campine phytogeographic district, which is characterised by base-poor, sandy soils of low fertility. Most forests were pioneer forests or primary plantations of mostly pine trees (*Pinus sylvestris* and *P. nigra*), sometimes mixed with birches (*Betula* sp.), and all were younger than 30 yr. Six of the sampled subpopulations were found within a radius of 2 km from the zinc smelters of Lommel (Lm, Lc, Lk and Ls) or Overpelt (N and Of) and the soils on these sites are seriously contaminated through atmospheric deposition. The remaining three subpopulations (E, Hh and P) inhabited soils with a low level of pollution and were situated, respectively, 7.6, 14.8 and 15.6 km from a zinc smelter. The subpopulations of *S. luteus* in the immediate vicinity of the smelters are strongly dominated by Zn-tolerant individuals, while the subpopulations that are more remote from the smelters consist of strictly nontolerant individuals (P) or show a combination of tolerant and nontolerant individuals (62% tolerant individuals in E and 32% in Hh; Colpaert *et al.*, 2004).

Table 1 Study sites: geographical coordinates, characteristics of the forest stands, age of the pine trees and heavy metal contamination measured as the zinc concentration in pore water and pine needles (SD in parentheses)

Site (abbreviation)	Geographical coordinates (latitude / longitude)	Forest type	Age of trees	Zn pore water (mg l^{-1})	Zn pine needles (μg g^{-1} d. wt) (yr)
Lommel Maatheide (Lm)	51°14′12″-N / 05°15′28″-E	Industrial area, mostly planted trees	27	7.7 (3.1)	272 (44)
Neerpelt (N)	51°14′01″-N / 05°24′28″-E	Industrial area, spontaneous colonisation	1–15	3.2 (1.4)	177 (58)
Overpelt fabriek (Of)	51°13′39″-N / 05°24′09″-E	Sand dunes, spontaneous colonisation	1–25	4.9 (1.5)	117 (41)
Lommel sahara (Ls)	51°14′45″-N / 05°16′23″-E	Industrial area, first rotation forest	10	3.7 (1.2)	110 (63)
Lommel containerpark (Lc)	51°14′08″-N / 05°16′26″-E	First rotation forest, previously grassland	8	4.4 (0.4)	101 (29)
Lommel kanaal (Lk)	51°14′49″-N / 05°15′42″-E	First rotation forest, disturbed soil	8	4.8 (1.1)	143 (55)
Eksel (E)	51°08′55″-N / 05°20′49″-E	Road side, spontaneous colonisation	1–15	0.54 (0.04)	43 (15)
Hechtelse heide (Hh)	51°06′33″-N / 05°22′11″-E	Sand dunes, spontaneous colonisation	1–25	0.54 (0.1)	31 (16)
Paal (P)	51°03′33″-N / 05°10′33″-E	Industrial area, first rotation forest and spontaneous colonisation	1–26	0.12 (0.03)	26 (8)

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Zinc pollution was assessed by analysing Zn in soil pore water and in needles of the pine host plants. At each study site, four soil samples (up to a depth of 20 cm) of *c.* 2 kg each were collected beneath *S. luteus* sporocarps. The soils were incubated in large pots in a glasshouse and pore water was extracted 4 wk later using Rhizon soil moisture samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands) according to Knight *et al.* (1998). Zn was then analysed with atomic absorption spectroscopy (AAS). Mixed samples of first year old pine needles were collected from 10 different *Pinus sylvestris* trees at each location. Needles were washed, dried (70°C, 120 h) and milled to a fine powder for combustion in a muffle furnace (600°C, 12 h). Ashes were dissolved in acid (final concentration 0.5 M HCl) and Zn was analysed with AAS.

DNA extraction and amplified fragment length polymorphism (AFLP) analysis

Total DNA was extracted from fungal tissue taken sterile from the centre of young sporocarps using the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France). AFLP analysis was performed according to the original protocol (Vos *et al.*, 1995). In brief, *c.* 0.5 µg of genomic DNA was digested using the enzyme combination *Eco*RI/*Mse*I and synthetic adaptors were ligated to the restriction fragments using T4 DNA ligase. A preamplification PCR was performed using AFLP primers having a single selective nucleotide (*Eco*RI + A/*Mse*I + C). Final amplification of the restriction fragments used primers having three selective nucleotides of which the *Eco*RI primer was end-labeled using [γ-^{33}P]ATP and T4 polynucleotide kinase. In total, 13 different primer combinations were used to amplify different subsets of all the restriction fragments in the total digest (Table 2). PCR products were then mixed with 20 µl of formamide loading dye (98% formamide, 10 mM EDTA pH 8.0, 0.025% xylene cyanol, 0.025% bromophenol blue). These mixtures were heated for 5 min at 90°C and then quickly cooled on ice. Two microliters of each sample was loaded on a 5% denaturing polyacrylamide sequencing gel. Electrophoresis was performed at constant power (100 W) for *c.* 2.5 h. After electrophoresis the gels were transferred to Whattman paper, dried under vacuum at 80°C and exposed to Kodak Biomax film for *c.* 24 h.

Table 2 Overview of the primer sequences used in the AFLP analysis and the number of loci scored per combination

Primer combination	*Eco*RI primer +	*Mse*I primer +	# Loci scored
1	AAG	CCA	40
2	AAG	CTA	22
3	AAG	CTG	16
4	AAG	CTT	46
5	ACA	CAT	30
6	ACA	CCT	32
7	ACA	CTC	30
8	ACA	CTT	39
9	AGA	CAT	16
10	AGA	CCA	17
11	AGA	CCT	26
12	AGA	CTC	30
13	AGA	CTG	17
Total			361

Statistical analysis

The AFLP fingerprints were manually scored for the presence or absence of fragments. In order to analyze the fingerprints, bands of equal fragment size were assumed to be homologous and the relative intensity of the bands was not considered informational. Only loci with clearly amplified bands were retained for data analysis. Linkage disequilibrium between AFLPs was assessed according to Miyashita *et al.* (1999) by using a χ^2 test implemented in the program POPGENE (Version 1.32; Yeh *et al.*, 1995).

Population structure was inferred using the program STRUCTURE (Version 2.1; Pritchard *et al.*, 2000). Because it is not possible to distinguish all the genotypes with AFLP data, each genotype class was treated as being, effectively, a haploid allele and the model of no admixture was applied. This model states that each individual originates purely from one of the populations and does not implement mixed ancestry of the individual genotypes. Furthermore, allele frequencies were assumed to be correlated within populations. In order to estimate K, the number of subpopulations most appropriate for interpreting the data, a series of independent runs for each value of K between 1 and 9 was conducted. A burn-in period of 50 000 iterations was chosen and data were collected for 250 000 iterations. Three independent runs were done for each value of K and all produced highly consistent results.

The genetic structure of the geographic subpopulations was analysed using the Bayesian method suggested by Holsinger *et al.* (2002), which allows for the estimation of the fixation index F_{ST} (Wright, 1978) from dominant markers without prior knowledge of the degree of inbreeding within populations and without assuming Hardy–Weinberg equilibrium within populations. Some information about the level of inbreeding within populations is also obtained by using this method. The program HICKORY (Version 1.0) was used with a full model and a model that assumes no inbreeding within populations. Several runs, using noninformative priors for the coefficient of inbreeding (F_{IS}) and the fixation index, were performed and all gave consistent results (burn-in = 50 000, sample = 250 000, thin = 50). Both Bayesian methods, implemented in STRUCTURE and HICKORY, assume that the markers are unlinked within populations. However, there seems to be more linkage disequilibrium than expected by chance alone within the sampled subpopulations (see Results). Therefore, the robustness of the different fitted models was tested by running the respective programs using random subsets of the data.

Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992), as implemented in the software program ARLEQUIN (Version 2.001; Schneider *et al.*, 2000), was applied to the data in order to partition the genotypic variance into components attributable to different hierarchical levels. Two population structure models were examined. In the first model, no grouping of the geographic subpopulations was defined and AMOVA partitioned the total variance into components due to differences between subpopulations and differences between individuals within subpopulations. In the second model, the structure suggested by the Bayesian cluster analysis was subjected to AMOVA. AMOVA was based on the pairwise squared Euclidian distances among the AFLP phenotypes and the significance of the variance components at the different hierarchical levels was assessed with a permutation procedure (15 000 permutations).

In order to assess the effect of the heavy metal contamination on the pattern of genetic variation, the genetic diversity of subpopulations inhabiting polluted soils was compared with the diversity of subpopulations inhabiting nonpolluted soils and the genetic differentiation between these two groups of subpopulations was examined using nested analysis of molecular variance. Genetic diversity of the geographic subpopulations was calculated as the percentage of polymorphic loci and Nei's unbiased heterozygosity (1978), averaged over loci, using the AFLP-SURV program (Version 1.0; Vekemans *et al.*, 2002). Because AFLP loci segregate as dominant markers, Hardy–Weinberg equilibrium was assumed in order to estimate allele frequencies and subpopulation heterozygosities. The frequency of the recessive allele was estimated at each locus using a Bayesian method with a non-uniform prior distribution of the allele frequencies, resulting in fewer biased estimates of the genetic diversity (Zhivotovsky, 1999).

Results

Heavy metal contamination of the sampling sites, measured as the zinc concentration in the pore water and the pine needles, varied between 0.12 and 7.7 mg l^{-1} and between 26 and 272 µg g^{-1} d. wt, respectively (Table 1). Based on these results, a discrimination was made between polluted (Lm, Lc, Lk, Ls, N, Of) and nonpolluted (E, Hh, P) sites.

AFLP analysis was performed on the DNA extracted from 164 sampled individuals belonging to nine different subpopulations of *Suillus luteus*. Thirteen different primer combinations were used for the selective amplification (Table 2) and these revealed a total of 361 genetic markers, 347 (96%) of which were polymorphic in at least one population. Based on these fingerprints, 163 different genotypes could be distinguished, as two individuals from the Hh population shared the same AFLP phenotype. Because genetic identity among fungal isolates has been suggested as the basis for the concept of individuality (Rayner, 1991), the data of only one of the two individuals with identical AFLP profiles was retained for further analysis. The proportion of polymorphic alleles within geographic subpopulations varied between 44% and 89% (Table 3) and no private alleles were detected, nor were any of the alleles restricted to subpopulations inhabiting contaminated soils. Linkage disequilibrium was examined using a χ^2 test between all the polymorphic loci within the different subpopulations. The proportion of significant linkage disequilibria, using a significance level of 0.05, varied between 9.8% and 17.1%, which is higher than expected by chance alone. In the total sample, 22.8% of the pairwise combinations showed significant linkage disequilibrium at the 5% level. However, the linkage disequilibrium seems to be sporadic, as no specific locus pair was in linkage disequilibrium in all subpopulations.

Table 3 Genetic diversity within nine subpopulations of *Suillus luteus* inhabiting heavy metal contaminated and non-contaminated areas in Belgium

Habitat type	Population	*N*	# individuals in cluster 1	# individuals in cluster 2	*P* (%)	*H*
Contaminated	Lm	10	6	4	73	0.352
	Ls	36	25	11	89	0.318
	Lc	11	4	7	79	0.343
	Of	6	6	0	44	0.304
	N	27	26	1	74	0.290
	Lk	20	20	0	58	0.265
Weighted average (SD)						0.306 (0.027)
Noncontaminated	Hh	8	7	1	60	0.314
	E	12	7	5	67	0.322
	P	33	31	2	75	0.285
Weighted average (SD)						0.298 (0.016)

N denotes the sample size of each subpopulation, *P* is the percentage of polymorphic loci, *H* equals the unbiased heterozygosity (Nei, 1978) and *SD* the standard deviation of the heterozygosity. The number of individuals of each subpopulation that belongs to each of the clusters derived with the program STRUCTURE (Version 2.1; Pritchard *et al*., 2000) is also given.

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Table 4 Analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) for 163 individuals of *Suillus luteus* sampled from 9 subpopulations in Belgium, employing 361 AFLP markers. (a) AMOVA without further partitioning of the sampled subpopulations; (b) AMOVA contrasting 2 differentiated groups of individuals resulting from Bayesian clustering; (c) Nested analysis contrasting subpopulations colonizing heavy metal contaminated soils (N, Of, Lm, Ls, Lc, Lk) and subpopulations inhabiting non-contaminated soils (E, Hh, P)

Source of variation	d.f.	Sum of squares	Variance component	% of total	*P*-value
(a) No partitioning of the sampled populations					
Among populations	8	485.005	1.65684	4.93	< 0.001
Within populations	154	4916.155	31.92308	95.07	< 0.001
Total	162	5401.160	33.57993		
(b) Structure based on Bayesian clustering					
Among clusters	1	395.210	7.25209	18.91	< 0.001
Within clusters	161	5005.949	31.09285	81.09	< 0.001
Total	162	5401.160	38.34494		
(c) Nested analysis – contaminated vs noncontaminated soils					
Among groups	1	48.242	−0.41037	−1.23	0.6
Among populations within groups	7	436.763	1.86866	5.60	< 0.001
Within populations	154	4916.155	31.92308	95.63	< 0.001
Total	162	5401.160	33.38137		

Data show the degrees of freedom (d.f), the sum of squared deviations, the variance component estimates, the percentage of total variance contributed by each component and the significance of the variance components (*P*-value) estimated computing 15 000 permutations.

The estimate of the posterior probability of the value of K (the number of subpopulations) more-or-less plateaus for $K = 2$. Table 3 reports the number of individuals of each sampled subpopulation that belongs to each of the derived clusters for $K = 2$, which captures the major structure in this data set. Eighty-one percent of the sampled individuals were assigned to cluster 1, which contained individuals from all subpopulations. Cluster 2 contained 19% of the samples, which mainly originated from the subpopulations Lm, Ls, Lc and E. All individuals were assigned with high probabilities to either one of the two clusters, except for sample N19, which was assigned with equal probability to each of the clusters. The highest probabilities for K using subsets of the data were similar to those obtained for the whole data set, which suggests that the result was robust and not due to the linkage disequilibria within the subpopulations.

The Bayesian analysis of the geographic population structure suggested that there was only weak evidence that F_{IS} was different from 0, as the slightly higher value of the Deviance Information Criterion (*DIC*) for the model with $F_{IS} = 0$ ($DIC = 12\,013$), in comparison with the value of *DIC* for the full model ($DIC = 12\,004$), was entirely due to model dimension (Holsinger & Wallace, 2004). As a result, the values of the posterior mean of F_{ST} were similar when using the full model and the model with $F_{IS} = 0$, being 0.036 ± 0.004 and 0.034 ± 0.003, respectively. Again, similar results were obtained when running the analysis with subsets of the whole data set.

In the first model examined, AMOVA indicated most genetic variation among individuals within geographic subpopulations (95%), although variation among subpopulations was highly significant ($P < 0.001$; Table 4a). Grouping of the individual samples according to the Bayesian clustering allowed 19% of the total variation ($P < 0.001$) to be accounted for by differences between the two clusters (Table 4b).

The genetic diversity in the total sample, estimated as Nei's unbiased heterozygosity, was 0.326. The genetic diversities within geographic subpopulations are reported in Table 3. The average diversity was 0.306 ± 0.027 for subpopulations inhabiting polluted soils and 0.298 ± 0.016 for subpopulations inhabiting nonpolluted soils. Nested AMOVA showed no significant grouping of the subpopulations inhabiting contaminated soils vs the subpopulations inhabiting non-contaminated areas (Table 4c).

Discussion

Basidiomycetes often have a complex population structure because of their highly variable life histories. Their mode of dispersal can be vegetative, through mycelial propagation, or sexual, by the production of basidiospores. Most basidiomycetes have a heterothallic life cycle. In this life cycle, nuclear fusion, meiosis and spore formation occur in the basidiocarps produced by dikaryons. After spore germination, monokaryons develop and fusion between sexually compatible monokaryons, corresponding to different mating types, leads to the formation of a dikaryon. Sexual compatibility between monokaryons forms the basis for defining intercompatibility groups, which have been used to delimit biological species within basidiomycetes (Petersen, 1995; Aanen *et al*., 2000). Sexual incompatibility is often correlated with genetic divergence, but phylogenetic studies show that intercompatibility groups do not necessarily form monophyletic clusters (Aanen *et al*., 2000).

The mating system of *S. luteus* is heterothallic and it possesses a bipolar incompatibility (Fries & Neumann, 1990). In the present study, Bayesian inference of the population structure suggested a model where individual samples of *S. luteus* clustered together in two differentiated subpopulations, one of which comprised 81% of the total sample. AMOVA indicated considerable genetic differentiation between the two derived subpopulations, as 19% of the total variance was shown to be due to differences between the two clusters. This result may indicate the presence of two different mating groups in the sample. However, no intercompatibility groups have been detected in *S. luteus* populations so far. One study described the conspecificity of American and European isolates, but only five isolates were examined (Fries & Neumann, 1990). Other possible explanations for the observed population structure are asymmetric gene flow between the geographic subpopulations or admixture with more differentiated populations not included in this study.

Relatively high levels of genetic diversity were found within geographic subpopulations of *S. luteus*, but genetic differentiation between the subpopulations was limited. Most of the genetic variation in the total sample was due to differences between individuals within subpopulations (95%, Table 4a) and the estimated value of F_{ST} (0.036) was low. Moreover, Bayesian inference of the population structure grouped most of the sampled individuals (81%) into one subpopulation. These findings are most likely the result of substantial gene flow between the subpopulations and frequent sexual reproduction, which was also suggested by the high proportion of unique multilocus genotypes. Comparable estimates of genetic diversity and differentiation were reported for populations of *S. grevillei* (Zhou *et al.*, 2001) and *S. pungens* (Bonello *et al.*, 1998).

Earlier studies, using *in vitro* growth experiments, have shown a high correlation between Zn tolerance and habitat pollution for individuals of *S. luteus*, which may be the result of a causal relationship. Physiological acclimation was unlikely as a cause of the tolerance trait because frequent subculturing of the isolates on basic medium without increased metal concentration did not alter their response to elevated Zn concentrations. Therefore, a genetic basis for the tolerance trait was assumed (Colpaert *et al.*, 2004). Hence, it would be expected *a priori* that founder effects and a strong selection pressure for heavy metal tolerance force a population to pass through a bottleneck in which size and genetic variation are drastically reduced. However, no evidence was found for a consistent reduction of the genetic variation of subpopulations of *S. luteus* caused by heavy metal pollution of their environment as the average diversity was higher for subpopulations inhabiting contaminated areas than for subpopulations inhabiting noncontaminated areas (Table 3). It has to be noted though, that the variance of the genetic diversity in contaminated areas was higher than the variance in noncontaminated areas and that, in particular cases, the diversity in polluted soils was smaller. In addition, nested analysis of variance revealed no effect of the heavy metal pollution on the genetic structure of the sampled subpopulations (Table 4c). These results may seem surprising, as in a previous study, employing ISSR markers, a reduction of the genetic diversity has been recorded (Colpaert *et al.*, 2000). However, only two populations were compared and a lower number of individuals was sampled from each population, thus making it difficult to derive general conclusions.

High levels of morphological, enzymatic or genetic variation within populations inhabiting polluted soils, which were at least as high as for populations inhabiting nonpolluted soils, were reported previously for several plant species such as *Silene vulgaris* (Baker & Dalby, 1980), *Silene paradoxa* (Mengoni *et al.*, 2000), *Agrostis stolonifera* (Wu *et al.*, 1975), *Armeria maritima* (Lefèbvre & Kokes, 1981; Vekemans & Lefèbvre, 1997) and *Arrhenatherum elatius* (Ducousso *et al.*, 1990). Several nonexclusive processes have been proposed to explain this surprisingly high level of variation in tolerant populations. Successive colonization events or a high frequency of tolerance in natural populations could have reversed the effects of an initial genetic bottleneck. Migration from the neighbouring populations could also be a cause of variation as well as environmental heterogeneity (Hedrick *et al.*, 1976) and human disturbance (Gouyon *et al.*, 1983). The results of this study, however, suggest that a bottleneck might never have occurred in populations of *S. luteus* inhabiting polluted soils. Multiple genotypes could have been introduced when the plantations were initially established, followed by sexual reproduction on the site and rapid evolution of the tolerance trait, which would result in genetic diversity levels being higher than expected. In addition, recurrent migration of tolerant genotypes that originate due to admixture in nonpolluted areas, may attribute to the high level of genetic diversity of populations inhabiting contaminated soils. Admixture between the tolerant populations and surrounding populations inhabiting nonpolluted soils is very likely, as indicated by the low level of population differentiation, and would explain the high frequency of tolerance observed in some of the populations surrounding polluted areas.

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References

Aanen DK, Kuyper TW, Mes THM, Hoekstra RF. 2000. The evolution of reproductive isolation in the ectomycorrhizal *Hebeloma crustuliniforme*

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aggregate (Basidiomycetes) in northwestern Europe: a phylogenetic approach. *Evolution* **54**: 1192–1206.

Adriaensen K, Van der Lelie D, Van Laere A, Vangronsveld J, Colpaert JV. 2004. A zinc-adapted fungus protects pines from zinc stress. *New Phytologist* **161**: 549–555.

Al-Hiyaly SAK, McNeilly T, Bradshaw AD, Mortimer AM. 1993. The effect of zinc contamination from electricity pylons. Genetic constraints on selection for zinc tolerance. *Heredity* **70**: 22–32.

Baker AJM, Dalby DH. 1980. Morphological variation between some isolated populations of *Silene maritima* With. in the British Isles with particular reference to inland populations on metalliferous soils. *New Phytologist* **84**: 123–138.

Bonello P, Bruns TD, Gardes M. 1998. Genetic structure of a natural population of the ectomycorrhizal fungus *Suillus pungens*. *New Phytologist* **138**: 533–542.

Bradshaw AD. 1952. Populations of *Agrostis tenuis* resistant to lead and zinc poisoning. *Nature* **169**: 1098.

Bruns TD, Tan J, Bidartando M, Szaro TM, Redecker D. 2002. Survival of *Suillus pungens* and *Amanita francheti* ectomycorrhizal genets was rare or absent after a stand-replacing fire. *New Phytologist* **155**: 517–523.

Colpaert JV, Muller LAH, Lambaerts M, Adriaensen K, Vangronsveld J. 2004. Evolutionary adaptation to Zn toxicity in populations of Suilloid fungi. *New Phytologist* **162**: 549–560.

Colpaert JV, Vandenkoornhuyse P, Adriaensen K, Vangronsveld J. 2000. Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus*. *New Phytologist* **147**: 367–379.

Ducousso A, Petit D, Valero M, Vernet P. 1990. Genetic variation between and within populations of a perennial grass: *Arrhenatherum elatius*. *Heredity* **65**: 179–188.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* **131**: 479–491.

Fries N, Neumann W. 1990. Sexual incompatibility in *Suillus luteus* and *S. granulatus*. *Mycological Research* **94**: 64–70.

Gouyon PH, Lumaret R, Valdeyron G, Vernet PH. 1983. Reproductive strategies and disturbance by man. In: Mooney HA, Godron M, eds. *Disturbance and ecosystems. Component of response. Ecological studies 44.* London, UK: Springer Verlag, 214–225.

Hedrick PW, Ginevan ME, Exing EP. 1976. Genetic polymorphism in heterogeneous environment. *Annual Review of Ecology and Systematics* **7**: 1–32.

Holsinger KE, Lewis PO, Dipak KD. 2002. A Bayesian approach to inferring population structure from dominant markers. *Molecular Ecology* **11**: 1157–1164.

Holsinger KE, Wallace LE. 2004. Bayesian approaches for the analysis of population genetic structure: an example from *Platanthera leucophaea* (Orchidaceae). *Molecular Ecology* **13**: 887–894.

Jentschke G, Godbold DL. 2000. Metal toxicity and ectomycorrhizas. *Physologia Plantarum* **109**: 107–116.

Knight BP, Chaudri AM, McGrath SP, Giller KE. 1998. Determination of chemical availability of cadmium and zinc in soils using inert soil moisture samplers. *Environmental Pollution* **99**: 293–298.

Lefèbvre C, Kokes P. 1981. Variation électrophorétique des estérases des feuilles d'*Armeria maritima* (Mill.) Willd: quelques aspects taxonomiques et évolutifs. *Bulletin de la Societé Royale de Botanique Du Belgique* **177**: 114–131.

Lefèbvre C, Vernet P. 1990. Microevolutionary processes on contaminated deposits. In: Shaw J, ed. *Heavy metal tolerance in plants: evolutionary aspects*. Boca Raton, FL, USA: CRC Press, 286–297.

Macnair MR. 1987. Heavy metal tolerance in plants: a model evolutionary system. *Trends in Ecology and Evolution* **2**: 354–359.

Macnair MR. 1993. The genetics of metal tolerance in vascular plants. Tansley Review, 49. *New Phytologist* **124**: 541–559.

Meharg AA, Cairney JWG. 2000. Co-evolution of mycorrhizal symbionts and their hosts to metal-contaminated environments. *Advances in Ecological Research* **30**: 69–112.

Mengoni A, Gonnelli C, Galardi F, Gabbrielli R, Bazzicalupo M. 2000. Genetic diversity and heavy metal tolerance in populations of *Silene paradoxa* L. (Caryophyllaceae): a random amplified polymorphic DNA analysis. *Molecular Ecology* **9**: 1319–1324.

Miyashita NT, Kawabe A, Innan H. 1999. DNA variation in the wild plant *Arabidopsis thaliana* revealed by amplified fragment length polymorphism analysis. *Genetics* **152**: 1723–1731.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**: 583–590.

Petersen RH. 1995. There's more to a mushroom than meets the eye: mating studies in the Agaricales. *Mycologia* **87**: 1–17.

Prat S. 1934. Die Erblichkeit der Resistenz gene Kupfer. *Berichte der Deutschen Botanischen Gesellschaft* **52**: 65–78.

Pritchard JK, Stephans M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Rayner ADM. 1991. The challenge of the individualistic mycelium. *Mycologia* **83**: 48–71.

Schneider S, Roessli D, Excoffier L. 2000. *ARLEQUIN: a software for population genetic data*. Geneva, Switzerland: Genetics and Biometry Laboratory, University of Geneva.

Schützendübel A, Polle A. 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany* **53**: 1351–1365.

Vekemans X, Beauwens T, Lemaire M, Roldan-Ruiz I. 2002. Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. *Molecular Ecology* **11**: 139–151.

Vekemans X, Lefèbvre C. 1997. On the evolution of heavy-metal tolerant populations in *Armeria maritima*: Evidence from allozyme variation and reproductive barriers. *Journal of Evolutionary Biology* **10**: 175–191.

Vos P, Hogers R, Bleeker M, Reijans M, Van Delee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

Wilkinson DM, Dickinson NM. 1995. Metal resistance in trees: The role of mycorrhizae. *Oikos* **72**: 298–300.

Wright S. 1978. *Evolution and the genetics of populations: variability within and among natural populations*, Vol. 4. Chicago, IL, USA: University of Chicago Press.

Wu L, Bradshaw AD, Thurman DA. 1975. The potential for evolution of heavy metal tolerance in plants. III. The rapid evolution of copper tolerance in *Agrostis stolonifera*. *Heredity* **34**: 165–178.

Yeh F, Yang RC, Boyle T. 1995. *POPGENE, the user-friendly shareware for population genetic analysis*. Edmonton, AB, Canada: Molecular Biology and Biotechnology Center, University of Alberta.

Zhivotovsky LA. 1999. Estimating population structure in diploids with multilocus dominant DNA markers. *Molecular Ecology* **8**: 907–913.

Zhou Z, Miwa M, Hogetsu T. 2001. Polymorphism of simple sequence repeats reveals gene flow within and between ectomycorrhizal *Suillus grevillei* populations. *New Phytologist* **149**: 339–348.

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