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Research Article

Tolerance of Genetically Characterized *Folsomia candida* Strains to Phenmedipham Exposure

A comparison between reproduction and avoidance tests *

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Abstract

Background, Aims, and Scope. The springtail *Folsomia candida* is a commonly used model species in ecotoxicological soil testing. The species reproduces parthenogenetically and, thus, laboratories use different clonal lineages. In this study, we investigated if genetic divergence between *F. candida* strains impacts the reaction to chemical stress and may thus affect the outcome of toxicity tests.

Methods. In two exposure assays (life-cycle reproduction test and avoidance behaviour test), three laboratory strains of *F. candida* from Portugal (PTG), Spain (SPN) and Denmark (DNK) were exposed to different concentrations of the reference chemical phenmedipham. Genetic divergence among strains was estimated based on mitochondrial COI sequence data.

Results. No significant differences between tolerance towards phenmedipham exposure were observed in a reproduction test. In contrast, one strain (SPN) showed a decreased susceptibility to phenmedipham compared to the other strains (PTG and DNK) in the avoidance assay.

Discussion. A phylogenetic tree based on mitochondrial COI sequences revealed clear genetic differentiation between both 'reaction types'. Thus, we found a potential lineage dependent stress reaction in avoidance behaviour towards the pesticide.

Conclusions. Our findings have implications for the comparability of test results among laboratories. Reproduction tests seem to be more robust towards interclonal genetic differentiation than avoidance tests.

Recommendations and Perspectives. We recommend the use of molecular tools for simple and cost effective genetic characterization of *F. candida* strains used in chemical avoidance tests. Closer investigations concerning the relation between genetic relatedness and chemical response will provide a more detailed and comprehensive picture on the role of intraspecific genetic differentiation in stress tolerance.

Keywords: Avoidance behaviour; chemical tolerance; collembolans; genetic ecotoxicology; genetic variation; reproduction test

Introduction

Collembolans represent one of the the most frequently used group of invertebrates for the investigation of anthropogenic soil contamination (Ronday & Houx 1996, Achazi et al. 1997). Because they represent a large proportion of biomass in edaphic ecosystems, several species of collembolans have been applied as bioindicators in toxicity analyses (Pedersen et al. 2000, Sverdrup et al. 2001, Greenslade & Vaughan 2003). *Folsomia candida* is commonly used in those bioassays (Crommentuijn et al. 1993, Van Gestel & Hensbergen 1997, Crouau et al. 1999, Lock et al. 2004), because it is a widely distributed and frequent species. In addition, it is relatively easy to raise and to culture under laboratory conditions. This parthenogenetic species has a short generation time and becomes sexually mature within only 21 to 24 days of age (Fountain & Hopkin 2005). In addition to these characteristics, *F. candida* is highly sensitive towards the presence of contaminants, a fact which was determinant to its inclusion in the ISO guideline Nº 11269 (ISO 1999) as a bioindicator for chronic evaluations. Moreover, its locomotor ability makes them good candidates to be included in a future ISO guideline for avoidance tests (as a complement for ISO 2005). Recently, several studies have been published in order to assess the value of avoidance tests as a time and cost effective alternative for the established reproduction test (Heupel 2002, Natal da Luz et al. 2004).

Though phylogeographic assessments of the genetic population structure are still lacking for *F. candida*, a generally low amount of genetic variation has been presumed to exist within the species due to its clonal reproduction strategy (Simonsen & Christensen 2001). In contrast to this, considerable levels of genetic differentiation have recently been found between natural *F. candida* populations as well as between laboratory clones (Chenon et al. 2000, Frati et al. 2004, Tully et al. 2006). These genetic differences between test strains could affect sensitivity towards toxic stress, as has been shown in daphnids (Baird et al. 1990, Baird et al. 1991, Soares et al. 1992) or gastropods (Jacobsen & Forbes 1997, Jensen & Forbes 2001).

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In order to investigate if genetic differences between collembolan laboratory test strains influence the outcome of both reproduction and avoidance tests, we compared both life-history reactions (reproduction rate) and avoidance behaviour between three laboratory strains of *F. candida* under phenmedipham exposure.

This study focused on the following questions: (I) is there considerable variation between the three laboratory strains using mitochondrial COI sequencing? (II) can we observe significant differences in the reaction to phenmedipham stress and can these differences be explained in terms of genetic differentiation between the laboratory strains?

Additionally, differences in the outcomes between the ISO reproduction test and the avoidance behaviour assay were compared. Our findings are discussed with regard to their potential meaning for the further use of different ecotoxicological test systems with collembolans.

1 Materials and Methods

1.1 Test organisms and culture conditions

Three laboratory strains of the springtail *Folsomia candida* (Collembola: Isotomidae) were obtained from different laboratory source cultures (PTG – Coimbra University, Coimbra, Portugal; DNK – National Environmental Research Institute, Silkeborg, Denmark; SPN – University of Navarra, Pamplona, Spain). The three cultures were kept under a photoperiod of 16:8 h light:dark at a temperature of 20 ± 2°C in plastic boxes lined with a mixture of plaster of Paris and activated charcoal in a ratio of 11:1. Once a week, granulated dry yeast was added as food in small amounts in order to avoid spoilage by fungi. Ten to 12-day-old individuals from synchronized cultures were used both for reproduction and avoidance tests.

1.2 Soil contamination

For both reproduction and avoidance tests, artificial OECD soil spiked with phenmedipham (Betosip®) in different doses was prepared on the same day at which the respective assays were started. For the reproduction tests, the gradient dose consisted of 0, 5, 10, 20 and 40 mg/kg of a.i. For the avoidance tests, the same doses were used with the exception of the highest application. The artificial soil was prepared according to OECD guideline 207 (OECD 1984).

1.3 Avoidance tests

The avoidance experiments were based on the International Organization for Standardization Draft Nº 17512 (ISO 2005) for avoidance tests with *Eisenia fetida*. Each replicate consisted of a cylindrical plastic box (7x7x6 cm) divided in two halves by a transversally inserted card divider. Control soil was combined with all other doses (5, 10 and 20 mg/kg). Therefore, each replicate included a control side and a half with contaminated soil. After soil addition, the divider was removed and 20 springtails were carefully placed on the midline of each test vessel. All individuals that were dropped, injured or appeared stressed were discarded before being transferred into the containers. The experiments were performed concurrently with all three distinct cultures for all treatments. The tests ran under a photoperiod of 16:8 h light:dark, at 20 ± 2°C. During the test period, each test chamber was covered by a transparent lid in order to prevent evaporation. For each combination, five replicates and an extra container without organisms were used for pH-value and moisture determination at the end of the test. After 48 h of incubation, the card divider was reintroduced in each replicate and the soil from each side was filled into separate vessels. The card divider was removed and each soil was floated with water. After the addition of a few drops of ink and gentle stirring, animals floating on the water surface were counted. Missing organisms were considered dead. Soil pH-value and moisture was measured at the beginning and the end of the test period. As a control approach, a dual test with two sides containing control sediment was performed with five replicates performed for each strain (Yeardley et al. 1996, Hund-Rinke & Wiechering 2001).

1.4 Reproduction tests

As for the avoidance tests, the reproduction tests ran parallel with springtails of three distinct sources (PTG, DNK and SPN) under the same test conditions. Once more, OECD standard soil was used and all the procedures adopted followed the ISO guideline Nº 11267 (ISO 1999).

1.5 Statistical analyses

Fisher exact test was used in order to test for significant differences between the number of individuals found in the contaminated and control soils both in the avoidance and in dual control tests (Zar 1999). For the avoidance tests, a one-tailed hypothesis was applied and a two-tailed test was used for the dual tests. Prior to all statistical analyses, the number of individuals was corrected taking into account the mortality observed in the dual control test. EC_{50} values were calculated as Probit regression, assuming that individuals were equally distributed in the presence of control soil on both sides of the test vessel.

For the reproduction data, the software Statistica 6.0 was used to perform non-linear regressions. Two distinct models were applied depending on the different trends obtained. Therefore, the equations used were the following:

Logistic Model:
EC_{50}: Juveniles = t/(1+(dose/x)^b

Hormesis Model:
EC_{50}: Juveniles = (t*(1+h*dose))/(1+((0.5+h*dose)/0.5)*(dose/x)^b),

where t = the intercept (the control response), x = EC_{50} for the data set, dose = exposure concentration, b = a scale parameter (estimated between 0 and 4), h = the hormetic effect (estimated between 0.1 and 1)

1.6 Genetic analyses

DNA was extracted from single individuals using the Chelex 100 technique (Walsh et al. 1991). Briefly, single individuals (alive or in ethanol 70%) were transferred into 1.5 ml Eppendorf tubes containing 50 µl of Chelex 100 stock solution. Samples were incubated at 55°C for two hours and proteinase K was

denatured at 95°C for 10 minutes afterwards. After brief centrifugation tubes were stored at 4°C in the fridge.

Mitochondrial cytochrome oxidase 1 (COI) fragment was amplified in a T3 Thermocycler (Biometra). Polymerase chain reaction (PCR) reaction mix contained ~5 ng of extracted DNA, 0.2 mM dNTPs, 3 mM $MgCl_2$, 1x reaction buffer (20 mM Tris-HCl, 50 mM KCl; Invitrogen), 0.3 µM of each primer (COI Folmer primers, Folmer et al. 1994) and 1 U Taq DNA polymerase (Invitrogen) in a total volume of 15 µL. PCR reaction consisted of 5 cycles of 30 sec at 94°C, 30 sec at 52°C and 1 min 72°C, 30 cycles of 1 min at 94°C, 1 min at 52°C and 1 min and 30 sec at 72°C. Final elongation step was performed for 5 min at 72°C. After column purification (E.Z.N.A. Cycle Pure Kit, Peqlab), PCR products were sequenced in a CEQ 2000 capillary sequencer (Beckman Coulter). Sequencing reaction included 2 µl of PCR product and 5 pmol of forward primer in a total volume of 10 µl. Sequencing programme consisted of 30 cycles of 20 sec at 96°C, 20 sec at 50°C and 4 min at 60°C. Obtained sequences were aligned with the ClustalW algorhythm using the Bioedit 7.0.1 software (Hall 1999) and corrected by eye.

Phylogenetic relationship between 15 individuals of the three strains was investigated by constructing a linearized COI neighbour-joining tree in the MEGA2 programme (Kumar et al. 2001). For this, we used the Kimura 2-parameter model of sequence evolution and 1000 bootstrap replicates in order to evaluate nodal support.

2 Results

2.1 Avoidance tests

In both avoidance and dual control tests, the surviving rate was always higher or equal to 94% and statistical differences were neither found among different strains nor between treatments tested concerning this endpoint. No avoidance behaviour was detected in any of the dual control tests with the three strains (**Fig. 1**).

Phenmedipham treated soils were always avoided by PTG and DNK populations, regardless of the applied concentration. Therefore, similar EC_{50} values were calculated for these populations (4.14 mg/kg for PTG and 4.85 mg/kg for DNK, **Table 1**). SPN population showed similar behaviour with the exception of the 5 mg/kg treatment. Here, no significant differences in the number of individuals in each side of the replicates were found, which means that no avoidance behaviour was detected in this combination (see Fig. 1). Consequently, a higher EC_{50} value (8.01 mg/kg) was calculated for the SPN population (see Table 1).

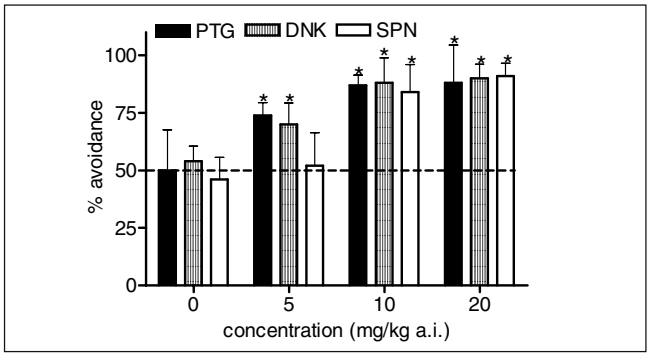

Fig. 1: Avoidance test: Percentage of *Folsomia candida* (average ± SD) in the control sides. * – indicates statistical differences ($p < 0.05$; one-tailed Fisher exact test). See Fig. 2 for abbreviations of strain origin

Table 1: EC_{50} values of three *Folsomia candida* laboratory strains for mortality, reproduction and avoidance behaviour. Shown are means and confidence ranges

	EC50 (mg/kg a.i.)		
Strains	**Survival**	**Reproduction**	**Avoidance behaviour**
PTG	13.62 10.68–16.55	11.31 3.93–18.69	4.14 0.80–6.48
DNK	13.30 9.22–17.37	10.91 6.69–15.13	4.85 2.76–6.39
SPN	18.45 14.71–18.78	10.82 5.72–15.93	8.01 6.35–9.51

2.2 Reproduction tests

Like in the avoidance tests, the surviving rate in the control was always higher or equal to 94% in the three springtail strains tested. The reproduction rate observed in the control vessels was always higher than 10 instars per surviving adult and the coefficient of variation of the reproduction was lower than 30% for all test strains. Thus, all the reproduction tests performed fulfilled the validity criteria defined by the ISO guideline 11267 (ISO 1999). The number of surviving adults significantly decreased at the 20 mg/kg a.i. dose in all tested strains. However, the calculated EC_{50} value was slightly higher (18.45 mg/kg a.i.) for SPN than that of the other strains (13.62 mg/kg a.i. for PTG and 13.30 mg/kg a.i. for DNK, see Table 1).

The reproduction rates of all three strains were almost zero in the 20 mg/kg a.i. treatment. Although the reproduction rate of DNK was slightly higher than the ones obtained for PTG and SPN in the 0 and 5 mg/kg a.i. doses, no significant differences were found (**Fig. 2**). Similar EC_{50} values were calculated for all three springtail populations tested (see Table 1).

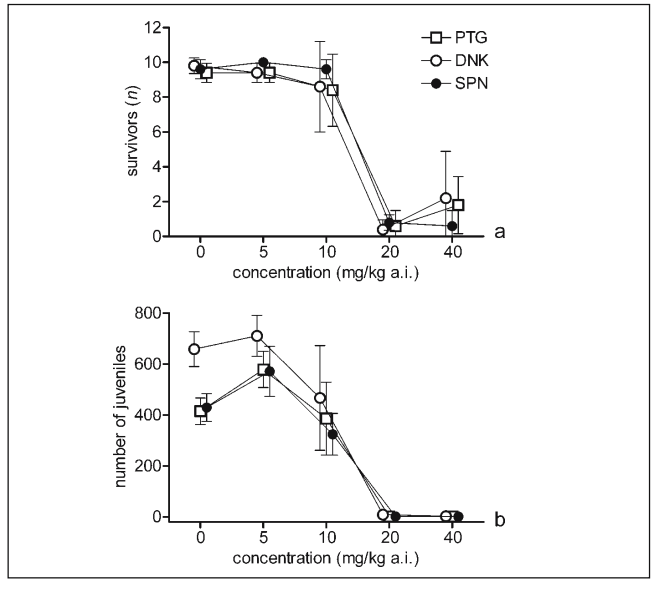

Fig. 2: Effects of phenmedipham on *Folsomia candida* adult survival (a) and reproduction (b) (average ± SD) in three different laboratory strains (PTG = Portugal, DNK = Denmark, SPN = Spain)

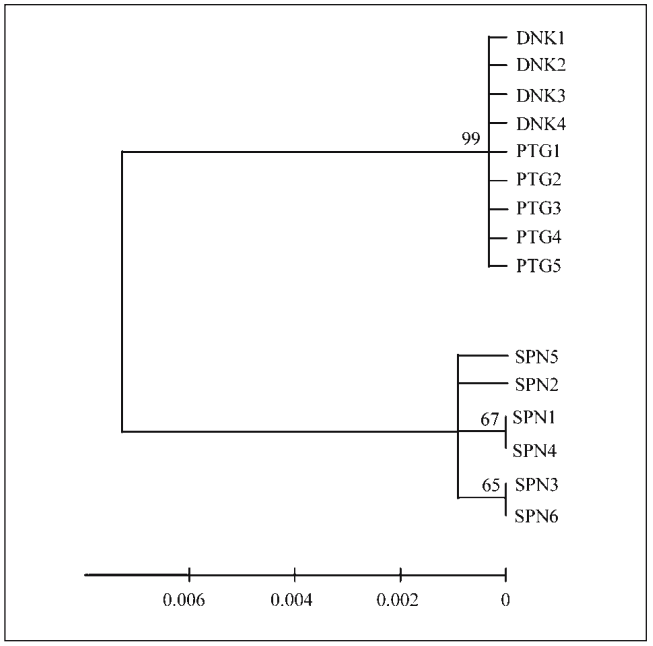

Fig. 3: Neighbour-joining tree based on COI sequences of 15 individuals from three laboratory strains (see Fig. 1 for abbreviations of strains) of *Folsomia candida*. Bootstrap values are shown at the tree nodes. The scale shows the mean substitution rate per site

2.3 Genetic analyses

COI sequences of 408 bp length were obtained from 15 *F. candida* individuals (DNK = 4, PTG = 5, SPN = 6 sequences, GenBank accession numbers). The resulting neighbour-joining tree is shown in **Fig. 3**. Two major clades were obtained. One clade consisted of individuals from DNK and PTG. No sequence variation was found within this clade. The second clade included all six individuals from the SPN strain. Mean pairwise sequence divergence within this clade was 0.04%. Mean sequence divergence between the clades was 1.39%.

3 Discussion

The use of clonal lineages from different origins has been shown to influence tolerance to chemical exposure and reproducibility of toxicological bioassays critically (Baird et al. 1990, 1991). Chenon et al. (2000) and Tully et al. (2006) showed that considerable genetic variation exists between laboratory clones of *Folsomia candida* though its parthenogenetic reproduction and cultivation regime. We found considerable genetic variation between the lineages analysed. Thus, different laboratories performing ecotoxicological tests are indeed working with genetically differentiated strains. Significant but minor differences were observed between different clones of *F. candida* in interclonal comparisons of chemical tolerance (Crommentuijn et al. 1995, Aldaya et al. 2006). However, these studies lack genotyping of clonal lineages and, thus, the origin of interclonal variation in stress response remains speculative here. In this study, we show that differences in pesticide tolerance can indeed be explained by the amount of genetic divergence between *F. candida* strains. While just minor differences in pesticide tolerance were observed in the reproduction test (see Fig. 2), significant variation was found in phenmedipham avoidance behaviour between the laboratory strains (see Fig. 1). In this test, the population obtained from Spain (SPN) showed an increased level of tolerance to the stressor compared to the strains from Denmark and Portugal, respectively. According to this, the Spanish strain is clearly genetically differentiated from the other strains at the mitochondrial COI locus (see Fig. 3). Thus, there might be a genetic basis for the variation in chemical avoidance which is reflected by the level of genetic relationship between the strains. No genetic differences were observed between the DNK and PTG individuals and both strains showed a highly similar avoidance behaviour.

According to our findings, we propose that genetic variation between laboratory strains used for ecotoxicological tests can influence chemical tolerance and, thus, the outcome of toxicity tests with *F. candida*. Tolerance differences between the three strains were relatively low in our test. However, it has been shown recently that high genetic divergence exists between *F. candida* clones at the COI locus, which led to the hypothesis that *F. candida* might consist of at least two genetically highly distinct cryptic lineages (Frati et al. 2004). In addition, Tully et al. (2006) revealed that there is considerable intraspecific variation at the more conserved 18S rDNA locus within *F. candida*. Sequencing of this marker gene of two individuals from each strain investigated showed that no variation exists at this locus between DNK, PTG and SPN (João Barateiro Diogo, unpublished data). Consequently, our study covers just a small proportion of genetic variation that exists within this species and, thus, most likely between different laboratory strains. Exposure tests with *F. candida* could be chronically biased due to genetically fixed tolerance differences, as e.g. found in *Daphnia magna* (Soares et al. 1992, Münziger & Monicelli 1991). Comparisons of variation in cadmium tolerance between clonal lineages of this species showed differences of two orders of magnitude (Baird et al. 1991). Whether similar interclonal variation exists for *F. candida* remains speculative here. However, our findings indicate that comparisons between outcomes of tests performed with different collembolan laboratory strains should be made with caution.

4 Conclusions

We showed that tolerance differences exist between laboratory strains of *F. candida* in avoidance behaviour towards pesticide exposure. Avoidance tests with *F. candida* and other collembolans may be highly influenced by the existence of highly differentiated intraspecific lineages. In order to maintain comparability of test results, we recommend COI sequencing of at least a few individuals from all test strains used in ecotoxicological tests. This procedure is relatively simple and cost effective, and will help to resolve discrepancies between test outcomes. Strict regulations of test design and laboratory conditions are provided by international guidelines for ecotoxicological tests. Unfortunately, no such rigorous recommendations exist for test organisms.

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