Masters of Science thesis

Effects of soil metal contamination on genetic diversity of the earthworm *Dendrobaena octaedra* (Savigny) in three metal contaminated areas in Finland

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YLINIEMI H. ILONA: Effects of soil metal contamination on genetic diversity of the earthworm *Dendrobaena octaedra* (Savigny) in three metal contaminated areas in Finland

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ABSTRACT

Metal contamination in soils is usually caused by human activities, e.g. emissions from metal industry or transportation. *Dendrobaena octaedra* (Savigny) is a parthenogenetically reproducing earthworm, which can be found in soils heavily contaminated by metals. The species is an obligatorily parthenogenetic i.e. it has apomictic parthenogenesis with only mitotic germ cell divisions. Thus, the genotypes of the earthworms are transmitted intact from mother to offspring. Metals can be toxic to organisms in high concentrations. Direct toxic and indirect effects of metals in soil could cause a strong selective force on *D. octaedra* individuals, which could lead to loss of genetic diversity in metal contaminated areas. In this study, the effects of soil metal contamination on genetic, i.e. clonal, diversity of *D. octaedra* was studied in three metal contaminated areas in Finland: Harjavalta, Imatra and Kokkola. The main contaminants in these areas were Cu and Zn. Samples of soil and earthworms at each study area were collected from contaminated sites close to the emission sources and from uncontaminated sites further from the sources. In Harjavalta, the sample sites were at three distances and two at the other areas. The species exposure and uptake of metals was evaluated by analyzing total and estimated bioavailable metal concentrations in soils and concentrations in earthworm tissues. Differences in clonal diversity of *D. octaedra* between the contaminated and uncontaminated sites were analyzed using allozyme electrophoresis and several diversity indices and measures. The results showed elevated metal concentrations in earthworms sampled from sites close to the emission sources. The study of clonal diversity of *D. octaedra* showed some differences between the sites in Harjavalta and Kokkola but not in Imatra. In Harjavalta, *D. octaedra* had very low clonal diversity and high clonal dominance at the sample site with moderate contamination of Cu and Zn. However, earthworms sampled from the most contaminated site did not suffer reduced genetic diversity compared to earthworms from the uncontaminated one. In Kokkola, genetic diversity of *D. octaedra* seemed to be slightly lower at the contaminated site compared to the uncontaminated one. However, the clonal diversity was relatively high at both sample sites and the differences were mostly in genotype composition rather than in clonal diversity. In Imatra, clonal diversity of *D. octaedra* was high at both sample sites and no reduction of genetic diversity was observed. In general, *D. octaedra* was quite diverse in clones, despite the long lasting metal soil contamination in these three areas. The results showed no clear reduction in genetic diversity of *D. octaedra* in metal contaminated sites at these three areas studied.

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TIIVISTELMÄ

Maaperään metalleja pääsee suuria määriä lähinnä ihmistoiminnassa, kuten esimerkiksi teollisuuden ja liikenteen päästöissä. Partenogeneettisesti lisääntyvä metsäliero (*Dendrobaena octaedra* Savigny) on laji, jota tavataan metallisaastuneilla maa-alueilla. Metsäliero on obligatorisesti partenogeneettinen, eli se lisääntyy apomiktisesti mitoottisen solujakautumisen kautta. Apomiksin tuloksena metsälieron jälkeläiset ovat geneettisesti emonsa kaltaisia klooneja. Metallit voivat olla suurissa pitoisuuksissa eliöille hyvin toksisia. Metallien suorat toksiset, tai niiden epäsuorat haitalliset vaikutukset aiheuttavat oletettavasti valintapaineen, mikä voi johtaa metsälieron geneettisen monimuotoisuuden vähenemiseen saastuneilla alueilla. Tässä tutkimuksessa selvitettiin pitkä-aikaisen metallialtistuksen vaikutusta metsälieron geneettiseen monimuotoisuuteen. Tutkimus toteutettiin keräämällä maa- ja lieronäytteitä saastuneilta ja puhtailta näytepaikoilta kolmelta eri metallisaastuneelta alueelta Suomesta: Harjavallasta, Imatralta ja Kokkolasta. Korkeimmat pitoisuudet maaperässä näillä alueilla olivat kuparilla ja sinkillä. Harjavallassa näytteitä kerättiin kolmelta eri etäisyydeltä päästölähteestä, muilta alueilta kahdelta. Metsälierojen altistumista metalleille arvioitiin analysoimalla maaperän kokonais- ja arvioidut biosaatavat metallipitoisuudet sekä metallien pitoisuudet lieroissa. Metsälierojen genotyypit eli kloonit selvitettiin allotsyymielektroforeesin avulla, ja eroja geneettisessä monimuotoisuudessa näytepaikkojen välillä vertailtiin diversiteetti-indeksien avulla. Metsälierojen kudosten metallipitoisuudet olivat kohonneet saastuneilla alueilla verrattuna puhtailta näytepaikoilta kerättyihin yksilöihin. Yleisesti ottaen metsälierojen klonaalinen diversiteetti oli melko korkeaa ja näytepaikkojen väliset erot vähäisiä pitkä-aikaisesta metallialtistuksesta huolimatta. Poikkeuksena erottui Harjavallasta kohtalaisesti saastuneelta alueelta kerätyt lierot, joiden klonaalinen diversiteetti oli hyvin alhainen ja yleisimmän kloonin yksilömäärä korkea. Pahimmin saastuneen alueen lierojen geneettinen monimuotoisuus ei kuitenkaan ollut vähentynyt verrattuna puhtaan alueen yksilöihin. Kokkolassa saastuneen alueen metsälierojen geneettinen diversiteetti oli hieman alhaisempi kuin puhtaan näytepaikan lierojen. Erot geneettisessä diversiteetissä eivät kuitenkaan olleet suuria ja diversiteetti oli suhteellisen korkea molemmilla näytepaikoilla. Imatralla metsälierojen geneettinen diversiteetti oli molemmilla näytepaikoilla korkea, eikä näytepaikkojen välillä havaittu eroja. Tuloksien perusteella ei saatu selvää näyttöä siitä, että metsälierojen geneettinen monimuotoisuus saastuneilla alueilla olisi vähentynyt verrattuna puhtaiden alueiden yksilöihin.

CONTENTS

1. INTRODUCTION

Dendrobaena octaedra (Oligochaeta, Lumbricidae) is a litter dwelling earthworm, which lives mostly in the upper layers of the soil profile. A total of sixteen species of earthworms are found in Finland, and *D. octaedra* is the only species of the genus *Dendrobaena*. This species is one of the most widely distributed lumbricid in Finland (Terhivuo & Valovirta 1978), and it is found all over Fennoscandia and European parts of Russia (Terhivuo 1988). The species is common in several habitats in which it is found most often in *Oxalis-Myrtillus type* forests, *forests with rich soils* and *Myrtillus type* forests. It does not produce permanent burrows or migrate vertically to avoid seasonal changes in temperature and moisture (Terhivuo & Valovirta 1978, Terhivuo 1988).

D. octaedra reproduces parthenogenetically (Terhivuo & Valovirta 1978). It is an obligatorily parthenogenetic species, i.e. it has apomictic parthenogenesis with only mitotic germ cell divisions. This type of parthenogenesis is rare among parthenogenetically reproducing earthworms; most of the other parthenogenetic species are automictic (Terhivuo & Saura 1990). Being a parthenogenetic species, *D. octaedra* lacks genetic recombination typical for sexually reproducing organisms. Furthermore, the genome of this apomictic species is transmitted intact from mother to the offspring. In other words, *D. octaedra* reproduces clonally. The inherited genotypic differences are therefore due to mutations and to a dissimilar level of polyploidy (Terhivuo 1988, Stearns & Hoekstra 2000). Hongell & Terhivuo (1989) found hexaploid specimens in southern Finland, and *D. octaedra* is assumed to be hexaploid or aneuploid with chromosomal numbers close to hexaploidy.

Metals occur naturally in soils in small amounts. They can also be released into the environment by human activities, e.g. from metal industry and transportation. Elevated concentrations of metals in soils can be detrimental to soil organisms. As a consequence of metal deposition, soil organisms may fail to survive in contaminated environments, or their function in decomposition processes may be reduced. Soft bodied animals like earthworms are exposed to metals directly through their skins as well as via their diet (Eijsackers 1998). Several studies have reported reduced species diversity, density and biomass in lumbricids due to increased metal concentrations in soil (Bengtsson & Tranvik 1989, Spurgeon & Hopkin 1999, Lukkari *et al.* 2004a). However, some earthworm species, including *D. octaedra*, can be found in metal contaminated soils (Bengtsson *et al.* 1983, Lukkari *et al.* 2004a).

Metal contamination in soil might be expected to have an effect on the genetic diversity of *D. octaedra* individuals. The selection pressure caused by metal toxicity or indirect effects of metal contamination may affect to the genotype composition of *D. octaedra* in contaminated soils. Furthermore, as a consequence of metal contamination, genetic diversity of *D. octaedra* could be reduced as some clones fail to survive in contaminated soil.

In this study my aim was to assess the effects of soil metal contamination on *D. octaedra*'s genetic diversity at three metal contaminated areas in Finland: Harjavalta, Imatra and Kokkola. The genetic, i.e. clonal, diversity of *D. octaedra* was compared at contaminated and uncontaminated sites at these three areas using allozyme electrophoresis and several diversity indices and measures. Clonal diversity was expected to be lower in *D. octaedra* sampled at contaminated site compared to uncontaminated one in each area. The aim of this study was also to evaluate the species exposure and uptake of metals by analyzing the total and estimated bioavailable metal concentrations in soils and metal concentrations in *D. octaedra* tissues. Analyses of total metal concentrations in soils can be useful in assessing the degree of contamination, but more informative analyses of metals

that are available to organisms are certain extraction methods, which estimate the amount of bioavailable metals in soils (Allen 2002). According to Streit (1984) the metal fraction extracted by acetic acid predicted most accurately the uptake of metals by lumbricids in form of total metal concentration in earthworm tissues. Therefore, the acetic extraction method (Streit 1984, Lukkari *et al.* 2004a) was used to estimate bioavailable metal fraction, and the ultrasound-assisted nitric and hydrochloric acid extraction method (Väisänen & Suontamo 2002, Lukkari *et al.* 2004a) was used for the total metal concentrations in soil.

2. MATERIAL AND METHODS

2.1. Sample sites and field methods

The samples for the study were collected during summers 2001 and 2002 from three different metal contaminated areas in Finland: Harjavalta, Kokkola and Imatra. Metals have accumulated into the soils in these areas from emissions of nearby metal industries for long periods of time. The soils in these areas are heavily contaminated by metals, especially by Cu, Zn, Fe and Al. The samples of *D. octaedra* were taken from contaminated and uncontaminated sites in each study area. Samples from the contaminated sites were taken close to the emission sources, where the levels of the soil metal concentrations were high, and samples of the uncontaminated site were taken further from the sources.

In Harjavalta, SW Finland (61°19'N 22°09'E), there is a Cu-Ni smelter which has been operating for over 50 years. The surroundings close to the smelter is heavily contaminated with Cu and Zn. The decomposition rate is very low near the smelter due to the metal contamination, and the soil is covered with a thick layer of litter. The sample sites in Harjavalta were at three locations: 1, 2 and 8 km from the smelter. The vegetation at the 1 km sample site was dominated by birch (*Betula pubescens*) and willows (*Salix* spp.). At the 2 and 8 km sample sites coniferous forests prevailed: the 2 km site was dominated by Scots pines (*Pinus sylvestris*) and the 8 km site by Norway spruces (*Picea abies*). The samples at the 2 and 8 km sites were collected in summer 2001: 54 individuals from both sites were taken. The one-kilometer site, where a total of 98 worms were collected, was sampled in summer 2002.

In Imatra, SE Finland (61°12'N 28 °48'E), a steel mill has been operating since 1915. There was also a Cu plant near the steel mill until 1944. The contaminated sample site was located 1 km from the mill and the uncontaminated sample site was at 4 km. The vegetation in Imatra at both sample sites was mixed forest with deciduous trees and Norway spruces (*P. abies*). The predominant shrub was *Vaccinium myrtillus* at both sites and several herbs and grasses were present. The soil in Imatra was rich in mull and clay. A total of 118 worms were collected in summer 2002, 40 from the 1 km site and 78 from the 4 km sample site.

In Kokkola, W Finland (63 °50'N 23 °08'E), there is a zinc production plant. The plant was founded in 1969. The contaminated sample site was located 2 km from the zinc plant and the uncontaminated one was at 4 km. Vegetation at the 2 km sample site was deciduous trees with several grasses. The 4 km site was located in coniferous woods, dominated by Norway spruces (*P. abies*). A total of 144 worms were sampled in summer 2002, 69 from the 2 km site and 75 from the 4 km site.

The earthworms were sampled from native soil by hand. *D. octaedra* was typically found in the top soil under partially decayed logs or stones and in the litter layer. Earthworms were put in plastic containers with soil from the sample site and stored in cold boxes until arrival at the laboratory in Jyväskylä. Containers were watered when needed. A

soil sample was taken from each sample site for the metal concentration analysis and for measurement of pH and amount of organic matter in soil.

2.2. Laboratory procedures

Sampled earthworms were taken to the laboratory and stored at cool temperature (1-5 ^{o}C) from one to five days. The earthworms were removed from the soil and transferred to clean jars filled with moistened filter paper. The jars were incubated at 15-20 ^{o}C for one day or until the earthworms had emptied their gut contents. To ensure that the earthworms' intestines were empty, they were also squeezed with forceps. Proper species identification was ensured and earthworm species other than *D. octaedra* were excluded from the study.

Ten randomly taken individuals from the samples were cut at the clitellum and the anteriormost segments were put in Eppendorf tubes and frozen (-24 ^{o}C) for the enzyme analysis. The rest of the body was weighed and frozen in Eppendorf tubes and stored in the freezer for the metal analysis. All the other individuals were put intact in Eppendorf tubes and stored in the freezer (-24 ^{o}C) until the enzyme analysis was conducted.

The soil samples from each study site were stored in the laboratory at cool temperature (1-5^{o}C). To measure pH, a part of each soil sample was placed in a container with distilled water in ratio of 1/5 soil, 4/5 distilled water. Soil and water were thoroughly mixed and pH was measured after two hours. Organic matter was measured from each soil sample (loss on ignition at 550 ^{o}C for 4h).

For the metal analysis the soil samples were homogenized and three separate sub samples were taken from each sample. The subsamples were dried at 70-90 ^{o}C for two days or more and then ground to fine powder with a mortar. Analyses for metal concentrations, both soil and earthworm samples, were conducted in the Department of Chemistry, University of Jyväskylä, with inductively coupled plasma atomic emission spectrometry (ICP-AES, Perkin-Elmer Optima 4300) and electrothermal atomic absorption spectrometry (ETAAS, Perkin-Elmer Model Analyst 800 with an AS-800 autosampler). Metal concentrations for Cu, Zn, Fe, Al, and Pb in soils were analyzed using two extraction methods. The total metal concentrations were measured using an ultrasound-assisted nitric and hydrochloric acid extraction method (Väisänen & Suontamo 2002, Lukkari *et al.* 2004a) and the estimated bioavailable metal concentrations were measured using an acetic acid extraction method, with 2.5% acetic acid (0.42M, pH 4.5) (Streit 1984, Lukkari *et al.* 2004a). The Harjavalta soil samples from the 2 and 8 km sites were analyzed in 2001 only for Cu and Zn.

2.3. Allozyme electrophoresis survey

Allozyme electrophoresis with cellulose acetate gels was applied to detect the electrophoretically distinct clones of *D. octaedra* sampled at different study sites. Allozymes are alternative forms of polypeptides of enzymes, which are used in basic body functions. Variation in allozymes has little impact on the organism's survival, and therefore they are considered as selectively equivalent or neutral (Murphy *et al.* 1996). Allozymes can be used in multilocus protein electrophoresis. This method is based on the fact that non-denatured proteins can be separated by their net charge under the influence of an electric current, and with application of histochemical stains the enzyme or the protein products of a particular gene can be revealed (Hillis *et al.* 1996). The net charge of the protein determines the protein's movement toward the anode (positive pole) or cathode (negative pole) in the supporting medium (Avise 1994). In this study the supporting medium used was cellulose acetate gel, and as a result the different forms of allozymes were represented as different band patterns on the stained gels. This method is an excellent

application in surveying levels of genetic variation in natural populations of small animals since it can be carried out in very small quantities of tissue homogenate (Hillis *et al.* 1996). Another advantage of using cellulose acetate gels as the electrophoretic matrix is the short run time: large sample sizes can be processed rapidly (Rowe & Hebert 2002).

Polyploid organisms, such as *D. octaedra*, have multiple gene copies in their genome. This influences the intensity of the band staining, and the band phenotypes might be represented in unexpected ways i.e. multiple banding and staining intensities (Rowe & Hebert 2002). Therefore, the electrophoretically detectable allozyme phenotypes of *D. octaedra* were represented as genotypes, i.e. clones, of *D. octaedra* and precise allele numbers present in a gene locus were not measured.

The survey was done in 1.7.-12.8.2002 and 18.11.-2.12.2002. The samples from Harjavalta 2 and 8 km sites were analyzed in summer 2001. Based on a preliminary survey with *D. octaedra* in 2001, eleven different enzymes were assayed. The enzymes used are shown in Table 1 with their Enzyme Commission (EC) numbers and which buffer system was used in electrophoresis. Two enzymes, 6PGDH and SOD, were used only in analyzes in 2002 and therefore samples collected from Harjavalta in 2001 were assayed only by nine enzymes. To achieve systematic scoring between the two sets of analyzed earthworms some of the samples were run together in fall 2002.

Three buffer systems were used for the enzymes: Citric acid- Aminopropyl Morpholine (CAAPM), Tris Glysine (TG) and Citrate Phosphate (CP) (Table 1). Recipes for the buffers are given in Appendix 1.

Table 1. Allozymes and buffer systems (Citric acid- Aminopropyl Morpholine; CAAPM, Tris Glysine; TG and Citrate Phosphate; CP) used in electroforesis survey of *D. octaedra*.

Allozyme		EC number[1]	Buffer system
ACON	Aconitate Hydratase	4.2.1.3	CAAPM
EST	Carboxylesterase	3.1.1.1	CP
GOT	Aspartate Aminotransferase	2.6.1.1	CAAPM
GPI	Glucose-6-Phosphate Isomerase	5.3.1.9	TG
G6PDH	Glucose-6-Phosphate Dehydrogenase	1.1.1.49	CAAPM
IDH	Isocitrate Dehydrogenase	1.1.1.42	CAAPM
MDH	Malate Dehydrogenase	1.1.1.37	CAAPM
ME	Malate Dehydrogenase NADP+	1.1.1.40	CAAPM
PGM	Phosphoglucomutase	5.4.2.2	TG
SOD	Superoxide Dismutase	1.15.1.1	CAAPM
6PGDH	6-Phosphogluconate Dehydrogenase	1.1.1.44	CAAPM

[1]Enzyme commission numbers based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB).

The samples were homogenized by grinding the worms in Eppendorf tubes with 45-200µl Tris-acetate grinding buffer (pH 7,5). The samples were kept on ice during the homogenization to prevent degradation of the enzymes. After homogenization the samples were centrifuged for one minute at 13 000 rpm. 8µl of the sample homogenate was added with a micropipette to the wells of the sample plate and loaded to the cellulose acetate gel with a loading applicator (Titan III gels, Helena Laboratories, USA).

A total of ten samples, a blue marker and a reference sample were applied to each gel. The marker was needed in monitoring the movement of the allozymes during electrophoresis. The band pattern of the reference sample enabled consistency in scoring several individuals. Before loading, the gels were soaked twenty minutes in an appropriate soaking buffer, which was the same as the electrode buffer used. The soaked gels were dried between two filter papers before applying the samples. The gels were placed in electrophoresis tanks cathodal to anodal and run at 200 volts for 20 minutes in room

temperature. Six gels could be run at the same time and the stains were prepared while they were running. After twenty minutes the current was switched off and the gels were placed in a dish one at a time and stained in a fume hood with an appropriate stain and an agar overlay. Certain light sensitive chemicals and agar was added just before the stain was poured on the gel. The recipes for stains and agar preparation are given in Appendices 2 and 3. The stained gels were incubated in a drying oven at 36 ^{o}C until the bands appeared sufficiently to resolve the allozyme phenotypes. The agar/stain overly was removed and the gels were soaked in water until scored.

Appeared bands were scored by their relative mobility on a gel. The fastest bands, which had moved furthest in a gel, were scored as A's, slower ones as B's and C's, respectively. Later on the bands, which moved even faster than A's, were scored as A+, Z's and Y's, respectively. Several banded enzymes were scored by the one or two bands, which were most visible. Other "shadow" bands were not scored. The scored gels were dried in a drying oven and stored in case a further comparative analysis of the bands would be needed. All individuals that were stained incorrectly or poorly were excluded from the study. Fig. 1 shows an example of cellulose acetate gel for allozyme ACON with band patterns all scored as A's.

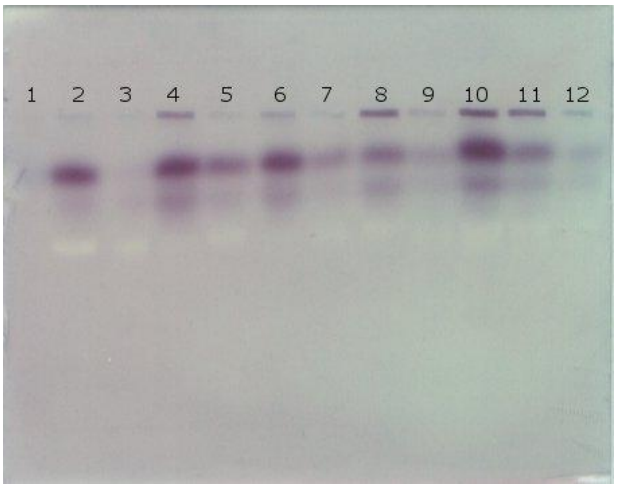

Fig. 1. An image of cellulose acetate gel for allozyme ACON with band patterns all scored as A's. Blue marker is loaded in place 1, reference sample is in place 2 and ten samples are in places 3-12. The scored bands for ACON are the uppermost ones. The bands in the middle are unscored "shadow" bands. At the lowest are the white SOD bands.

2.4. Diversity indices and statistics used

Because *D. octaedra* reproduces asexually by apomictic parthenogenesis the analyses of genetic differences were made on the level of genotypes rather than alleles. Several similarity measures and diversity indices were used to evaluate the genetic differences between the earthworms sampled at contaminated and uncontaminated study sites in the three study areas. Diversity indices are usually associated with ecological studies of species diversity. In this study the genotypes of *D. octaedra* were applied to the formulas instead of species. Diversity indices and measures used in analyses:

1. Shannon-Wiener index, H

$H = -\sum p_i \ln p_i$

where p_i is the proportion of individuals found in the *i*th genotype.

2. Simpson's index, D
$D = \sum[n_i(n_i-1)/N(N-1)]$
where n_i is the number of individuals in the *i*th genotype and N is the total number of genotypes.

3. Clonal dominance per site; the Berger-Parker index
$d = N_{max} / N$
where N_{max} is the number of individuals in the most abundant genotype and N the total number of individuals.

4. Proportion of unique genotypes per site
= number of genotypes represented only once at one site but not in the other sites / sample size

5. Clonal diversity per site
= genotypes found / sample size

6. Margalef's diversity index, D_{Mg}
$D_{Mg} = (S-1) / \ln N$
where S is the number of genotypes recorded and N the total number of individuals over all S genotypes

7. Menhinick's diversity index, D_{Mn}
$D_{Mn} = S / \sqrt{N}$
where S is the number of genotypes recorded and N the total number of individuals over all S genotypes

A randomization test was used to examine the significance of difference in Shannon-Wiener index (1.) and in Simpson's index (2.) between the contaminated and uncontaminated sites. In the randomization test the data from compared populations is fused to one pool of genotypes and populations are reformed randomly 10 000 times. The real difference value (d) from the data is compared to the distribution of the differences composed by the randomized populations (Solow 1993).

Since the sample sizes were not equal, and the number of genotypes rises with sample size, a rarefaction method was used to determine expected clonal diversity per site (5.) for a standardized sample. A computer program BIO-DAP (Gordon Thomas) was used in the rarefaction estimates. The program uses the formula corresponding to the one presented by A. E. Magurran (1988). Margalef's (6.) and Menhinick's (7.) diversity indices were calculated after rarefaction with the same computer software.

R X C test of independence using likelihood ratio test (G test) (Sokal & Rohlf 1995) was applied to assess the relationship between the sample site and particular genotypes found at that site. This test shows if there is association between genotype distributions and sampling sites.

The analysis of variance (ANOVA) and the t-test for equality of means were used in analyses of the difference in metal concentrations in the soil and the *D. octaedra* tissues between the contaminated and uncontaminated sample sites within each study area. The three sub samples derived from the original soil samples were used in statistical analyses. Tukey's HSD test was used in pair-wise comparisons. A Levene's test was used to test the homogeneity of variances, and a logarithmic transformation was used if the variances were not homogenous. If the variances were not homogenous even after logarithmic

transformation, non-parametric Mann-Whitney Test for equality of means was used instead of the t-test. SPSS 11.5 for Windows was used in all statistical analyses.

3. RESULTS

3.1. Soil characteristics

Soil pH was low in all three areas studied with pH 4.4 to 5.6. The lowest soil pH was in Harjavalta at the 2 and 8 km sample sites. Soil organic matter was high in Kokkola and Harjavalta. In Imatra, the amount of organic matter was higher at the uncontaminated sample site closer to the emission source than at the sample site further from the source. In Kokkola, the organic matter content was higher at the uncontaminated sample site compared to the contaminated one. In Harjavalta, the amount of organic matter in soil was lowest at the 2 km sample site (Table 2).

Table 2. Soil organic content (% of dry mass) and pH values measured from three metal contaminated areas in Finland, at different distances from emission sources.

	pH	Organic matter
Harjavalta		
1 km	5.4	76.1
2 km	4.5	66.0
8 km	4.4	72.0
Imatra		
1 km	5.6	74.7
4 km	5.5	51.9
Kokkola		
2 km	5.0	70.0
4 km	5.6	81.4

3.2. Metal concentrations in soil and in *D. octaedra* tissues

3.2.1. Harjavalta

In Harjavalta, the soil was heavily contaminated by Cu and Zn at the 1 and 2 km sample sites. The total and estimated bioavailable Cu and Zn concentrations (mg/kg of dry mass) in soil were highest at the sample site closest to the Cu-Ni smelter (1 km) and declined with distance (Fig.2, Fig. 3, Table 3). All sites differed from each other in total and estimated bioavailable soil Cu and Zn concentrations (Tukey's HSD, $p < 0.001$ for all pair-wise comparisons).

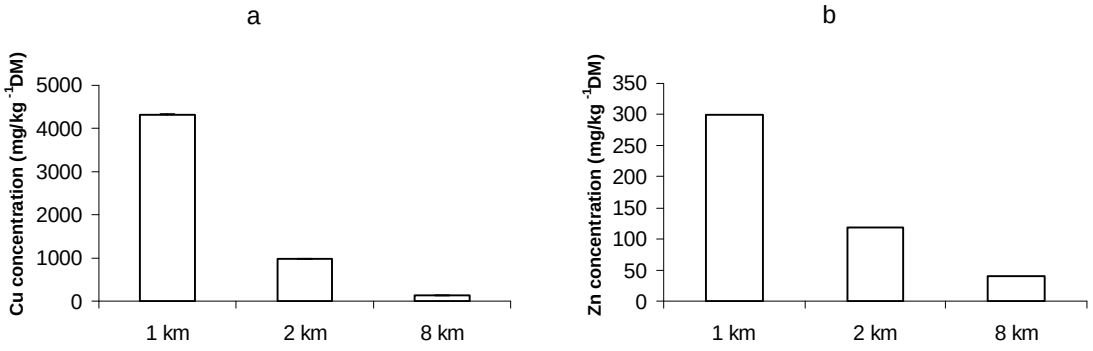

Fig. 2. Total concentrations of Cu (a) and Zn (b) in soil (mg/kg of dry mass) at three sample sites 1, 2 and 8 km from the Cu-Ni smelter in Harjavalta. The results are shown as means (±SE), n = 3.

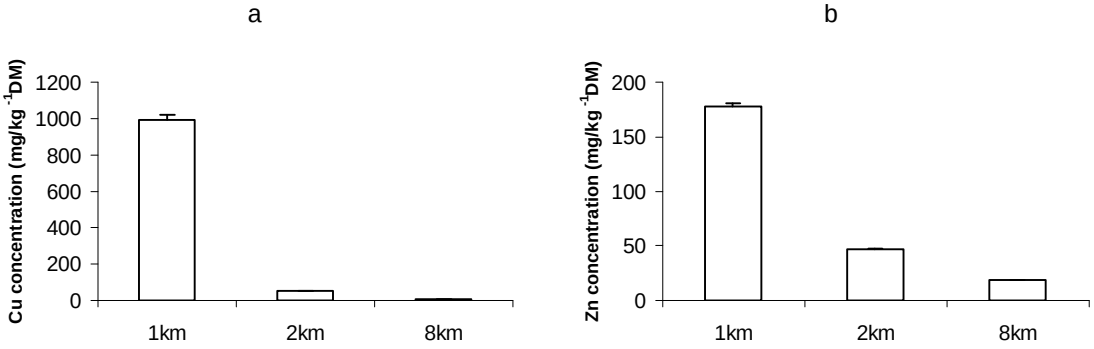

Fig. 3. Estimated bioavailable concentrations of Cu (a) and Zn (b) in soil (mg/kg of dry mass) at three sample sites 1, 2 and 8 km from the Cu-Ni smelter in Harjavalta. The results are shown as means (±SE), n = 3.

Table 3. Results of the tests for the differences (ANOVA) between the sample sites (1, 2 and 8 km) in total and estimated bioavailable soil concentrations of Cu and Zn (mg/kg of dry mass) in Harjavalta, n = 3.

	Transformation	F	p
Total metal concentrations			
Cu	log	41218.7	<0.001
Zn	-	12538.0	<0.001
Bioavailable metal concentrations			
Cu	log	3837.2	<0.001
Zn	log	4990.7	<0.001

The body burden of Cu and Zn was highest in the earthworms at the nearest study site (1 km), declining with distance (Fig. 4, Table 4). All sites differed from each other in comparisons of *D. octaedra* Cu and Zn tissue concentrations (Tukey's HSD, p = 0.001 for all pair-wise comparisons). Overall, the Zn concentration of the earthworms was higher than the Cu concentration, even though the Cu concentration in the soils was higher than Zn (Fig. 2, 3 and 4).

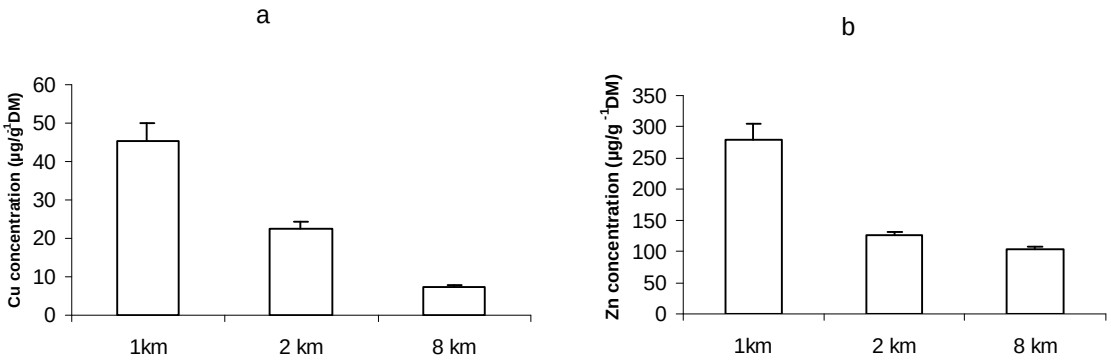

Fig. 4. Cu (a) and Zn (b) concentrations (µg/g of dry mass) of *D. octaedra* at three sample sites 1, 2 and 8 km from the Cu-Ni smelter in Harjavalta. The results are shown as means (±SE), n = 10 (1 km), n = 58 (2 km), n = 59 (8 km).

Table 4. Results of the tests for the differences (ANOVA) between the *D. octaedra* Cu and Zn tissue concentrations (µg/g of dry mass) at three sample sites 1, 2 and 8 km from the Cu-Ni smelter in Harjavalta, n = 10 (1 km), n = 58 (2 km), n = 59 (8 km).

	Transformation	F	p
Cu	log	115.03	<0.001
Zn	log	45.75	<0.001

3.2.2. Imatra

The main contaminants in the soil in Imatra were Zn, Fe and Al. Total and estimated bioavailable Zn concentrations in soil were higher at the 1 km site compared to the 4 km site (Fig. 5, Table 6). Contrary to Zn, total soil concentrations of Fe and Al were higher at the 4 km site than at the 1 km site. However, the bioavailable fractions of Fe and Al were higher at the sample site closer to the steel mill than at the sample site located at 4 km. In case of Cu and Pb, total and estimated bioavailable soil concentrations were higher at the 1 km site compared to the 4 km site, although the concentrations in soil were low at both sites (Tables 5 and 6).

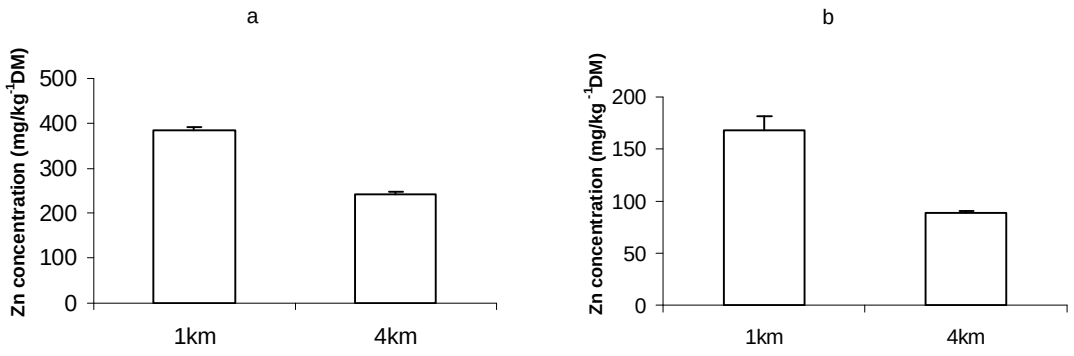

Fig. 5. Total (a) and estimated bioavailable (b) Zn concentrations (mg/kg of dry mass) in soil at Imatra sample sites 1 and 4 km from the steel mill. The results are shown as means (±SE), n = 3.

Table 5. Total and estimated bioavailable concentrations (mg/kg of dry mass) of Cu, Fe, Al and Pb in soil at Imatra sample sites 1 and 4 km from the steel mill. The results are shown as means (±SE), n = 3. LOQ = below limit of quantification.

	1 km	4 km
Total metal concentrations		
Cu	36.1 ± 0.8	13.5 ± 0.2
Fe	4500.1 ± 189.1	5458.8 ± 128.0
Al	2373.2 ± 95.0	3583.6 ± 120.5
Pb	74.6 ± 2.3	41.7 ± 0.5
Bioavailable metal concentrations		
Cu	1.9 ± 0.2	0.4 ± 0.2
Fe	47.4 ± 1.6	25.3 ± 3.1
Al	171.5 ± 4.1	133.5 ± 3.9
Pb	LOQ	LOQ

Table 6. Results of the t-test for the difference between the two sites (1 and 4 km) in total and estimated bioavailable soil concentrations of Cu, Zn, Fe, Al and Pb (mg/kg of dry mass) in Imatra, n = 3.

	transformation	t	p
Total metal concentrations			
Cu	log	38.86	<0.001
Zn	-	13.99	<0.001
Fe	-	-4.20	0.014
Al	-	-7.89	0.001
Pb	-	13.74	<0.001
Bioavailable metal concentrations			
Cu	-	5.94	0.005
Zn	log	7.59	0.002
Fe	-	6.29	0.003
Al	-	6.76	0.003
Pb	-	-	-

The Zn concentration in *D. ocatedra* tissues was higher at the 1 km sample site than at the 4 km site (Fig. 6, Table 8). Like Zn, the concentrations of Cu, Fe, Al and Pb were higher in the earthworms collected from the 1 km sample site compared to the earthworms from the 4 km site (Table 7 and 8).

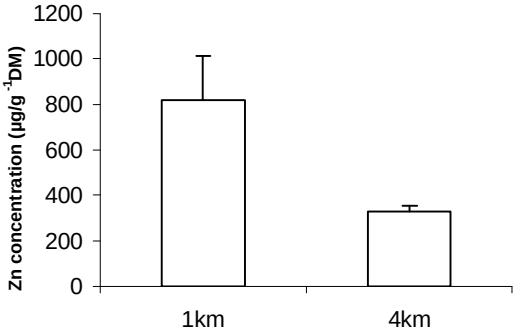

Fig. 6. Zn concentration (µg/g of dry mass) of *D. octaedra* sampled at 1 and 4 km sites from the steel mill in Imatra. The results are shown as means (±SE), n = 10.

Table 7. The Cu, Fe, Al and Pb concentrations (µg/g of dry mass) of *D. octaedra* sampled at 1 and 4 km sites from the steel mill in Imatra. The results are shown as means (±SE), n = 10.

	1 km	4 km
Cu	57.3 ± 13.7	17.2 ± 2.6
Fe	992.6 ± 241.5	273.7 ± 39.1
Al	239.5 ± 88.3	58.9 ± 24.2
Pb	26.7 ± 9.5	3.7 ± 1.3

Table 8. Results of the Mann-Whitney Tests and the t-tests for the difference between the two sites (1 and 4 km) in *D. octaedra* tissue concentrations of Cu, Fe, Al and Pb (µg/g of dry mass) in Imatra, n = 10.

	Test	Transformation	U/t	p
Fe	Mann-Whitney Test	-	11.00	0.003
Cu	T-Test	log	3.76	0.001
Zn	Mann-Whitney Test	-	10.00	0.002
Al	T-Test	log	3.33	0.004
Pb	T-Test	-	2.41	0.027

3.2.3. Kokkola

In Kokkola, the soil at the 2 km sample site was heavily contaminated by Zn. Both, total and estimated bioavailable, Zn concentrations were almost three times higher in the soil at the 2 km sample site compared to the 4 km site (Fig. 7, Table 10). In addition, the total and estimated bioavailable concentrations of Cu, Fe and Al were higher at the 2 km sample site than at the 4 km site (Table 9 and 10). The Pb concentration was low in both sample sites (Table 9).

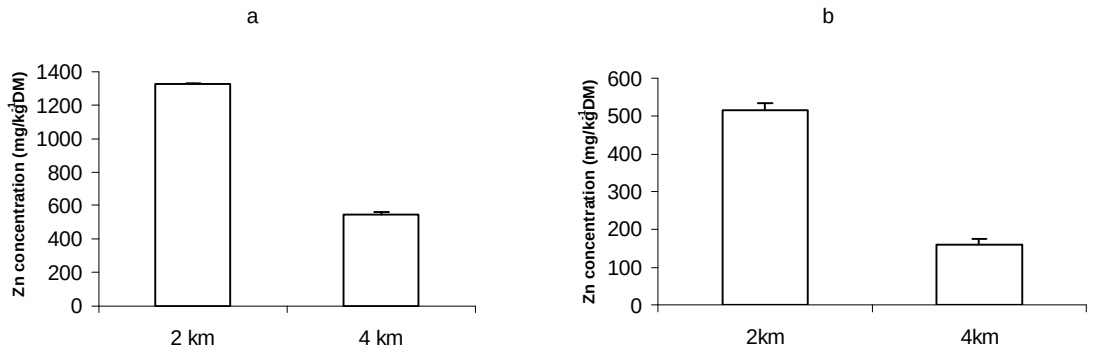

Fig. 7. Total (a) and estimated bioavailable (b) Zn concentrations (mg/kg of dry mass) in soil at Kokkola sample sites 2 and 4 km from the zinc plant. The results are shown as means (±SE), n = 3.

Table 9. Total and estimated bioavailable concentrations (mg/kg of dry mass) of Cu, Fe, Al and Pb in soil at Kokkola sample sites 2 and 4 km from the zinc plant. The results are shown as means (±SE), n = 3. LOQ = below limit of quantification.

	2 km	4 km
Total metal concentrations		
Cu	91.8 ± 0.9	24.0 ± 0.6
Fe	5152.7 ± 50.4	952.2 ± 64.5
Al	1163.3 ± 12.7	487.3 ± 36.3
Pb	133.8 ± 0.7	18.7 ± 0.7
Bioavailable metal concentrations		
Cu	3.6 ± 0.3	1.0 ± 0.2
Fe	97.2 ± 9.5	11.8 ± 3.6
Al	93.4 ± 6.4	33.1 ± 1.9
Pb	LOQ	LOQ

Table 10. Results of the t-test for the difference between the two sites (2 and 4 km) in total and estimated bioavailable soil concentrations of Cu, Zn, Fe, Al and Pb (mg/kg of dry mass) in Kokkola, n = 3.

	t	p
Total metal concentrations		
Cu	64.03	<0.001
Zn	53.59	<0.001
Fe	51.31	<0.001
Al	17.56	<0.001
Pb	112.73	<0.001
Bioavailable metal concentrations		
Cu	7.37	<0.001
Zn	15.08	<0.001
Fe	8.42	<0.001
Al	8.97	<0.001
Pb	-	-

The metal concentrations in *D. octaedra* tissues in Kokkola differed between the contaminated and uncontaminated sites only in Zn and Pb concentrations. In other words, the earthworms collected from the 1 km site had more Zn and Pb in their tissues compared to the earthworms from the 4 km sample site (Fig. 8, Table 11 and 12).

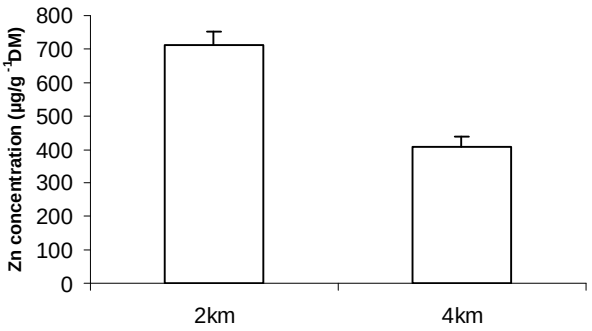

Fig. 8. Zn concentration (µg/g of dry mass) of *D. octaedra* sampled at 2 and 4 km sites from the zinc plant in Kokkola. The results are shown as means (±SE), n = 10.

Table 11. Cu, Fe, Al and Pb concentrations (µg/g of dry mass) of *D. octaedra* sampled at 2 and 4 km sites from the zinc plant in Kokkola. The results are shown as means (±SE), n = 10.

	2 km	4 km
Cu	28.9 ± 5.0	21.3 ± 3.2
Fe	252.6 ± 60.1	273.6 ± 35.5
Al	50.6 ± 10.5	46.8 ± 10.3
Pb	87.6 ± 11.6	40.8 ± 8.3

Table 12. Results of the t-tests for the difference between the two sites (2 and 4 km) in *D. octaedra* tissue concentrations of Cu, Fe, Al and Pb (µg/g of dry mass) in Kokkola, n = 10.

	t	p
Cu	1.29	0.215
Zn	5.95	<0.001
Fe	-0.30	0.767
Al	0.26	0.802
Pb	3.29	0.004

3.3. Allozymes and genetic variation

Ten allozyme loci out of eleven proved to be variable i.e. were polymorphic loci. The enzyme G6PDH was excluded from analyses since it stained poorly and was invariable throughout the samples. In addition, the band patterns of ACON, GOT, MDH and 6GPDH had little variation in alleles and one allele predominated over others throughout the samples. Since the enzymes SOD and 6GPDH were not assayed in 2001, the samples from Harjavalta at 2 and 8 km were scored only by eight enzyme loci. Therefore the analyses of genetic variation were performed by two sets: 1) including SOD and 6GPDH and 2) excluding these allozyme loci from the analyses. The results of Imatra and Kokkola presented here are the results including SOD and 6GPDH. The results of Harjavalta are from the sample set excluding these allozyme loci.

Five individuals sampled from Imatra and Kokkola had unusual banding patterns at all or most loci, which may indicate that a different species was assayed instead of *D. octaedra*. Therefore, the analyses of genetic diversity were done by both including and excluding these individuals. There was no significant difference in the results of these two data analyses (data not shown). The results shown here include these five individuals.

3.3.1. Harjavalta

A total of 98 individuals from 1 km, 36 from 2 km, and 44 from 8 km site in Harjavalta were included in the study of genetic variation. A total of 57 different genotypes were found based on variability of eight allozyme loci, even though ACON, GOT and MDH appeared to be monomorphic throughout the samples collected from Harjavalta. The 8-locus genotypes (clones) of *D. octaedra* found at the 1, 2 and 8 km sample sites are shown in Appendix 4.

A total of 43 genotypes were found at 1 km, 9 at 2 km and 22 at 8 km site, respectively. The clonal diversity was lowest at the 2 km site, in which 25 % of the earthworms differed in genotypes. The 1 and 8 km sites appeared to be more diverse in clones: 44% in the 1 km and 50 % in the 8 km site differed in genotypes. The proportion of unique genotypes was low at the 2 km site (only 6 % of the genotypes were unique). The proportion of unique genotypes made up 22% of the genotypes found at the 1 km site and 18% at the 8 km site. Contrary to clonal diversity values, the proportion of individuals belonging to the most common genotype was high at the 2 km site, in which the dominant

genotypes made 36 % of the individuals found at the 2 km site. The proportion of dominant genotypes was lower at the 1 and 8 km sites (11% and 14%, respectively) (Table 13).

The difference (d) in the Shannon-Wiener diversity index was significant between the 1 and 2 km sites ($d = 2.33$, $p<0.001$) and between the 2 and 8 km sites ($d = -1.79$, $p<0.001$). Differences in Simpson's diversity index showed similar kind of pattern: the difference was significant between the 1 and 2 km sites ($d = -0.14$, $p<0.001$) and between the 2 and 8 km sites ($d = 0.13$, $p<0.001$). Since low Shannon-Wiener and high Simpson's index values are associated with low diversity, the earthworms from the 2 km site seemed to be less diverse in clones than the earthworms collected from the other two sites.

Table 13. Diversity indices measured for *D. octaedra* sampled from 1 (n = 98), 2 (n = 36) and 8 km (n = 44) sample sites in Harjavalta, Finland.

	1 km	2 km	8 km
Clonal diversity	0.44	0.25	0.50
Proportion of unique genotypes	0.22	0.06	0.18
Clonal dominance; the Berger-Parker index	0.11	0.36	0.14
Shannon-Wiener index (H)	3.37	1.05	2.84
Simpson's index (D)	0.04	0.18	0.05

The R X C test of independence using G-test showed a relationship between the site and particular genotypes found at that site ($G/q = 163.43$, $df = 112$, $p<0.05$). This result suggests that the clone arrays found at each site are somehow site specific.

Because the sample sizes were not equal, a rarefaction method was used to determine the expected clonal diversity for similar sized samples. The standardized sample size used in rarefaction was 36, which was the same as the number of specimens at the 2 km site. The expected number of genotypes for similar sample sizes was 23 at the 1 km site, 19 at the 8 km site and only 9 at the 2 km site (Table 14). The Margalef's and Menhinick's diversity indices calculated for similar sized samples showed the 2 km population to be less diverse in clones than the other two populations (Table 14)

Table 14. Expected number of genotypes, Margalef's and Menhinick's diversity indices calculated for the Harjavalta sample sites after rarefaction. Standardized sample size 36.

	1 km	2 km	8 km
Expected number of genotypes	23	9	19
Margalef's diversity index	9.16	2.23	5.55
Menhinick's diversity index	4.34	1.50	3.32

3.3.2. Imatra

A total of 33 individuals from the 1 km site and 69 from the 4 km site were stained properly and used in the analyses of clonal diversity. A total of 81 electrophoretically different genotypes were found in Imatra based on variability of ten allozyme loci. The 10-locus genotypes (clones) of *D. octaedra* found at the 1 and 4 km sites are shown in Appendix 3.

A total of 30 genotypes were found at 1 km and 57 at 4 km sites. The genetic diversity in the earthworms collected from the 1 and 4 km sites seemed to be quite similar. The clonal diversity value per site was high at the both sample sites: 91% of the sampled earthworms at the 1 km site and 83% in 4 km site differed in genotypes. In addition, the proportion of unique genotypes was same in both sites (67%) and difference in the Berger-Parker index was minor between the sites. Overall, clonal diversity was high and clonal dominance was low in the earthworms sampled from Imatra (Table 15). The randomization

test did not show any difference in Shannon-Wiener diversity index and Simpson's index between the sites.

Table 15. Diversity indices measured for *D. octaedra* sampled from 1 (n = 33) and 4 km (n = 69) sites in Imatra.

	1 km	4 km
Clonal diversity	0.91	0.83
Proportion of unique genotypes	0.67	0.67
Clonal dominance; the Berger-Parker index	0.06	0.09
Shannon-Wiener index (H)	3.37	3.92
Simpson's index (D)	0.006	0.01

R X C test of independence using G-test indicated no relationship between the site and particular genotypes found at that site. The expected number of genotypes (standardized sample size 30) was 28 for the 1 km site and 27 for the 4 km site (Table 16). However, the Margalef's diversity index and Menhinick's diversity index were slightly higher at the 4 km site than at the 1 km site (Table 16). In general, the genetic variation was quite similar at the sites and no clear reduction of clonal diversity was observed at the 1 km sample site compared to the 4 km site.

Table 16. Expected number of genotypes, Margalef's and Menhinick's diversity indices calculated for the Imatra sample sites after rarefaction. Standardized sample size 30.

	1 km	4 km
Expected number of genotypes	28	27
Margalef's diversity index	8.30	13.23
Menhinick's diversity index	5.22	6.86

3.3.3. Kokkola

A total of 57 individuals from the 2 km site and 71 from the 4 km sample site were assayed successfully and used in the analyses of clonal diversity. A total of 68 electrophoretically detectable genotypes of *D. octaedra* were found in the Kokkola samples. The 10-locus genotypes (clones) of *D. octaedra* found at the 2 and 4 km sites are presented in Appendix 4.

A total of 35 genotypes were found at 2 km and 47 at the 4 km site. Both sites showed moderate clonal diversity and proportion of unique genotypes. The clonal dominance was low at both sample sites. In comparison between the two sites, clonal diversity and proportion of unique genotypes were slightly higher in the earthworms collected from the 4 km sample site than at the 2 km site, although the difference between the sites was small (Table 17).

The difference (d) in Simpson's diversity index between the sites was significant ($d = 0.01$, $p<0.0394$) suggesting the 4 km site to be more diverse in clones than the 2 km site, however the absolute difference between the sites was small. No difference was found between the sites using the Shannon-Wiener diversity index.

Table 17. Diversity indices measured for *D. octaedra* sampled from 2 (n = 57) and 4 km (n = 71) sites in Kokkola.

	2 km	4 km
Clonal diversity	0.61	0.66
Proportion of unique genotypes	0.35	0.48
Clonal dominance; the Berger-Parker index	0.09	0.11
Shannon-Wiener index (H)	3.36	3.62
Simpson's index (D)	0.03	0.02

The R X C test of independence using G-test showed a relationship between the site and particular genotypes found at that site ($G/q = 113.07$, $df = 74$, $p<0.01$). The expected number of genotypes was 32 at the 2 km site and 36 at the 4 km site (Table 18). Margalef's and Menhinick's diversity indices were higher at the 4 km population compared to the 2 km population (Table 18).

Table 18. Expected number of genotypes, Margalef's and Menhinick's diversity indices calculated for the Kokkola samples after rarefaction. Standardized sample size 50.

	2 km	4 km
Expected number of genotypes	32	36
Margalef's diversity index	8.41	10.80
Menhinick's diversity index	4.64	5.58

4. DISCUSSION

4.1. Metal concentrations in soils and in *D. octaedra* tissues

Cu and Zn are metals, that are frequently used in high amounts in metal industry. Therefore, soils close to metal industrial plants are usually severely polluted by these metals (Römbke & Moltman 1996). As expected, the results of the metal analyses showed soil metal concentrations to be highest at the sample sites closest to the emission sources. The main contaminant in soil was Cu and Zn in Harjavalta and Zn in Kokkola and Imatra. Although the emissions from the metal industry plants in all of these three study areas are nowadays strictly regulated it seems that the soils are still heavily contaminated by metals, at least in Harjavalta and Kokkola.

Metals tend to bind to soil particles, which make them very persistent pollutants. The binding of metals to soils is determined by several soil and substance characteristics, e.g. soil pH, amount of organic matter and relative amount of clay content (Römbke & Moltman 1996). These binding processes can restrict the availability of metals in soil and thus the uptake of metals by soil organisms may be reduced (Allen 2002). In this study, the estimated bioavailable metal concentrations extracted by acetic acid were much lower than the total metal concentrations in soils. In soil Cu and Zn are bound especially to organic matter and clay particles that can lower the exposure of metals to soil organisms (Yaron *et al.* 1996). Therefore, high soil clay content in Imatra and high organic matter content in Harjavalta and Kokkola apparently lower the harmful effects of metals to *D. octaedra* in those areas. On the other hand, the high soil organic matter values in Harjavalta and Kokkola might indicate lowered decomposition capacity of soil organisms, as well as the slightly higher soil organic matter in Imatra at the 1 km site compared to the 4 km site. In addition, the low pH at the sample sites may affect the bioavailable Cu since Cu is relatively immobile compared to other metals, but at low pH more of it turns into soluble Cu^{2+}-ion form (Yaron *et al.* 1996).

Metal depositions from industry are usually highest in soils close to the emission sources and in layers closest to the soil surface (Bengtson *et al.* 1983). Since the metal concentrations, e.g. Cu and Zn, in contaminated soils are usually highest in the top layers and *D. octaedra* inhabits these layers of the soil profile it is expected to be exposed to high concentrations of metals in contaminated areas. A substantial metal uptake has been reported in analyses of *D. octaedra* tissues, especially in seminal vesicles (Bengtson *et al.* 1983). The results of this study showed also increased metal concentrations in the tissues of *D. octaedra* collected from the metal contaminated sample sites in all of the three study areas. This indicates that *D. octaedra* individuals close to the emission sources are exposed

more to metals than the earthworms at less contaminated sites. In Imatra, the bioavailable concentrations Fe and Al at the closer sample site were higher as well as were the *D. octaedra* tissue concentrations compared to the corresponding values of the uncontaminated sample site. However, the total concentrations of Fe and Al in soil were higher at the sample site further from the emission source. This result implies that the estimated bioavailable soil metal concentrations predicted better the *D. octaedra* tissue concentrations compared to the total metal concentrations, at least in cases of Fe and Al in Imatra.

Trace metals, like Cu and Zn, are essential to organisms (Crosby 1989). Since Cu and Zn are used and needed in normal body functions, earthworms have evolved specific detoxifying and extraction mechanisms for these metals. Hence, earthworms can decrease the toxic effects of metals by enhanced extraction and detoxification metabolism (Edwards & Bohlen 1996, Eijsackers 1998). It has been shown that earthworms can regulate the body metal concentration at a constant level in metal contaminated soils (Morgan & Morgan 1999, Lukkari *et al.* 2005, Lukkari & Haimi 2005). In this study, the Cu concentration in *D. octaedra* tissues was lower than that of Zn, even when Cu contamination in the soil was much higher than Zn. In addition, the Cu concentration in earthworms in Harjavalta was not notably higher than in Kokkola and Imatra, although the soil Cu contamination was much higher in Harjavalta compared to the other two areas. These results indicate that *D. octaedra* has different accumulation and extraction rates for trace metals: Cu is regulated at a lower level than Zn. Similar results of Cu regulation at low body concentrations have also been reported for other earthworm species (Morgan & Morgan 1999). In the case of Zn, the soil contamination was highest in Kokkola compared to Harjavalta and Imatra. However, the body burden of Zn in *D. octaedra* in Kokkola was approximately at the same level as it was in Imatra. In addition to that, the Zn concentration in earthworms collected from Imatra was almost three times higher than in earthworms from Harjavalta, even though the Zn concentration in the soils at both sites was almost at the same level. These results might be explained by the possibility that the metals in Imatra could be more bioavailable to earthworms than in the other two study areas.

The results of the metal analyses imply that the metal contamination is somewhat site specific and it is affected by local soil characteristics. This affects to *D. octaedra*'s exposure and uptake of metals from the soil. The results of this study also support the idea that trace metals, especially Cu, are regulated on constant body concentrations. It seems that Cu concentrations in *D. octaedra* tissues are regulated on a certain level in spite of increased metal concentrations in soil. On the other hand, the rather high variances in the metal concentrations in earthworms suggest that individual earthworms may have different capacities for regulating these metals. It would be interesting to study if there is any correlation between particular clones and the metal concentrations in *D. octaedra* tissues.

4.2. Allozymes and genetic variation

4.2.1 Success in sampling

The allozyme electrophoresis method with ten different allozyme loci seemed to be sufficient to resolve the genotypes of *D. octaedra* individuals. However, the results of the analyses of genetic variation with the two sets of allozyme loci (including SOD and 6GPDH and excluding these allozyme loci from the analyses) differed significantly in the case of Simpson's diversity index between the sample sites in Kokkola (data not shown). This result may be explained by the fact that the accuracy of separating clones rises with numbers of allozymes used (Terhivuo & Saura 1990). It should be noted that the limited number of allozymes used in this study probably lumps together some of the clones and the

actual number of genotypes of *D. octaedra* are most likely higher. Furthermore, the limited number of specimens may also affect the results. It is also important to choose appropriate allozyme loci and buffer system to avoid the loss of specimens in scoring.

4.2.2 Clonal diversity

Metal contamination can create a strong selection pressure to populations of soil invertebrates (Bengtsson & Tranvik 1989). In a study of the asexually reproducing enchytraeid worm *Cognettia sphagnetorum* (Haimi *et al.* 2005), a long lasting heavy metal exposure had reduced the genetic diversity of *C. sphagnetorum* at the metal contaminated study site in Harjavalta. It was expected that metal contamination could affect genotype composition of *D. octaedra* as well: specimens from the contaminated study sites were expected to have fewer clones present and reduced clonal diversity compared to the individuals collected from uncontaminated sites.

In a survey of electrophoretically distinct clones of *D. octaedra* between the three sample sites in Harjavalta a difference was found between the sites. The most contaminated 1 km site did not suffer from reduced clonal diversity compared to the other two sites. Unexpectedly, it was the 2 km site which had fewer clones and low clonal diversity with a high clonal dominance value compared to the other sites. Since the differences in clonal diversity of *D. octaedra* are mostly due to mutations and the mutation rate should occur in equal frequencies at all sites, the high clonal dominance at the 2 km site might indicate that the dominant clone has a reproduction advantage over the other clones at this site. It is possible that there has been selection by particular environmental conditions at this site and the dominant clone is better adapted to these compared to other *D. octaedra* genotypes. However, the difference between the sites may be result of the sampling effects, particularly due to the small sample size. It also should be noted that the samples were collected in different years, which could have affected to the results. Nevertheless, the effects of metal contamination on genetic diversity of the earthworms cannot be completely excluded because the Cu concentration in the 2 km sample site was still relatively high and further, the local soil characteristics can have an effect on metal availability. On the other hand, individuals from the most contaminated site (1 km) did not differ in genetic aspect from the uncontaminated site (8 km). This finding does not support the conclusion that metal contamination has reduced the genetic diversity of *D. octaedra* in Harjavalta.

In Kokkola, the contaminated 2 km site appeared to be slightly less diverse in clones compared to the uncontaminated one. However, the clonal diversity was still relatively high at both sample sites. The result of the R X C test indicated a relationship between the site and particular genotypes found at that site. This result could be due to the fact that dominant and most frequent clones in Kokkola were not shared between the two sample sites. It seems that the genotype composition of *D. octaedra* in Kokkola differs between the sites with slight differences in genetic diversity. In a survey of electrophoretic variation in *D. octaedra* populations in eastern Fennoscandia, Terhivuo & Saura (1990) showed geographical clines and non-random distributions of *D. octaedra* clones over different biotypes and vegetation zones. In Kokkola, the vegetation at the 2 km sample site was deciduous forest with several grasses and the 4 km site was located in a coniferous forest, dominated by spruces. Thus, the results in Kokkola could also be due to differences in clone arrays of *D. octaedra* in different habitats. Furthermore, the different environmental conditions in these two habitats could have been shaping the genotype composition of *D. octaedra* leading to differences between the two sites.

In Imatra, there was only little difference, if any, between the two sample sites in study of clonal diversity of *D. octaedra*. In addition, clonal diversity was high in both sample sites. The results showed no clear reduction of clones at the site closer to the

emission source even though the Zn and Cu concentrations in earthworms at this site were elevated. Apparently, the metal contamination in Imatra has not been high enough to cause a difference in clone diversity.

D. octaedra has been observed to have high clonal diversity and a high proportion of unique genotypes (Terhivuo & Saura 1990). Based on the electrophoretic survey of six polymorphic loci, in Finland and N Norway, about every third individual of *D. octaedra* seems to represent a novel overall enzyme phenotype (Terhivuo & Saura 1990). In the present study, the clonal diversity of *D. octaedra* in the three areas studied was, in general, moderate to high. However, the clonal diversity was lowest in Harjavalta, in which 25-50 % of the earthworms differed in genotypes. In Kokkola the corresponding values of different genotypes were 61-66 % and in Imatra 83-91 %, respectively. However, it should be noted, that samples collected from Harjavalta were assayed successfully only by eight enzymes whereas the samples from Kokkola and Imatra were assayed with ten. The corresponding clonal diversity values (omitting SOD and 6GPDH) for the Kokkola samples were 42-54 % earthworms differing in genotypes and in Imatra 72-78 %, respectively. These findings suggest that genotype diversity was lowest at the most contaminated areas: Harjavalta and Kokkola. In these areas the soil contamination by Cu and Zn was high at the contaminated sample sites and metal soil concentrations were elevated even at the sample sites further from the emission sources. Therefore, it is possible that a long lasting metal contamination at these heavily contaminated areas has affected *D. octaedra'*s genetic diversity.

4.3. Strategies to survive in metal contaminated soil

To survive in a metal contaminated environment, soil invertebrate species, including earthworms, may show resistance to metals through acclimatization or genetic adaptation. Genetically inherited metal tolerance to Zn has been detected in laboratory experiments in *Eisenia fetida* (Oligochaeta) by Spurgeon & Hopkin (2000). In addition, Reinecke *et al.* (1999) showed how the same species developed Cd resistance after long-term metal exposure. Furthermore, a relationship between earlier contamination history and responses to metals has been shown in *Aporrectodea tuberculata* (Lukkari *et al.* 2005, Lukkari *et al.* 2006). It was suggested that earthworms living in contaminated environments had better capability to tolerate metals than individuals without earlier exposure. In a parthenogenetically reproducing species as *D. octaedra*, the inherited genotypic differences are due to mutations and to a dissimilar level of polyploidy. Therefore, favorable mutations must accumulate one after the other in the same lineage of one mutant individual (Crow & Kimura 1965, Terhivuo 1988). In the present study, genetic diversity of *D. octaedra* was maintained even in severely contaminated areas. It might be possible that *D. octaedra* could also be acclimatized or genetically adapted to stress caused by metals. However, this was not directly examined in this study.

Earthworms can bind metals to certain tissues or proteins, that can decrease the harmful effects of metals (Eijsackers 1998, Lukkari *et al.* 2004b). Therefore, a possible metal storage in to non-vital organs or proteins may lower the harmful effects of metals and further enable *D. octaedra* to survive in heavily contaminated soils.

The study of Morgan & Morgan (1999) showed a relationship between the diet and body metal concentrations in *Lumbricus rubellus* and *Aporrectodea caliginosa*. The litter-dwelling *L. rubellus* had lower metal burden of Cd than the endogeic *A. caliginosa*, which feeds on soil particles. Apparently the litter contains lower concentrations of metals than soil particles since metals tend to bind especially to organic matter and clay substances. Litter is usually composed of dead organic matter, e.g. dead leaves, which has not had time

to accumulate high concentrations of metals since deciduous leaves are replaced yearly (Morgan & Morgan 1999). *D. octaedra* feeds mainly on litter like *L. rubellus* and the metal accumulation via its diet is probably low since the emissions from the metal industry plants at the three areas studied are nowadays significantly decreased.

Other ecological features in addition to feeding habits of *D. octaedra* may also influence its exposure to metals in soil. Soils are heterogenous in metal distribution mainly because the soil structure and characteristics e.g. soil pore water pH varies spatially (Donker *et al.* 1994, Yaron *et al.* 1996). In fact, Salminen and Haimi (1999) showed uneven distributions of metals in Harjavalta. It has been suggested that earthworms can detect metals in soil and avoid heavily contaminated patches. By this avoidance behavior, earthworms can lower the uptake and accumulation of metals and therefore survive in metal contaminated environments (Eijsackers 1998, Lukkari *et al.* 2004b). Lukkari and Haimi (2005) demonstrated avoidance behavior of three earthworm species to Cu and Zn in experimentally contaminated soils. *D. octaedra* was the most sensitive species to these metals and *L. rubellus* the most insensitive one. Hence, it is possible that *D. octaedra* is able to avoid highly contaminated soil patches and therefore lower the uptake of metals.

One possible explanation how earthworm populations can be established in metal contaminated soils is immigration from surrounding areas. However, the possibility of immigration from less contaminated soil is unlikely in the areas studied here, since the contaminated areas around the emission sources are large and dispersal abilities of earthworms appeared to be quite limited (Edwards & Bohlen 1996).

5. CONCLUSIONS

Even though *D. octaedra* individuals from contaminated sample sites in Harjavalta, Imatra and Kokkola had elevated body metal concentrations, the study of clonal diversity showed no clear reduction of clones at metal contaminated sites compared to uncontaminated ones in the same areas. It is not clear whether the observed differences in genotype distributions and genetic diversity in Harjavalta and Kokkola are due to selection induced by metal contamination or some other environmental factors. Moreover, these differences could also be explained by sampling effects and small sample sizes. As a conclusion, the metal contamination seemed not to be high enough to have harmful effects on genetic diversity of *D. octaedra* since clonal diversity of *D. octaedra* between contaminated and uncontaminated sample sites at the three study areas (Harjavalta, Imatra and Kokkola) were not clearly reduced. However, genetic diversity of *D. octaedra* was lower in heavily contaminated areas (Harjavalta and Kokkola) than in a less contaminated one (Imatra). Survival of *D. octaedra* and maintaining genetic diversity in metal contaminated areas may be explained by behavioral and physiological characteristics of the species and furthermore, active metal regulation and metal storage to non-vital organs.

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APPENDICES

Appendix 1. Recipes for gel electrophoresis buffers used in the study.

1. TRIS-GLYCINE (TG)

30g Tris
144g Glycine
make up to 1 liter; pH = 8.5
dilute 1:9 TG:dH_2O for use

2. CITRIC ACID- AMINOPROPYL MORPHOLINE (CAAPM)

42g citric acid (anhydrous)
50g 4-(3-aminopropyl) morpholine
make up to 1 liter; pH = 7.0
dilute 1:4 CAAPM:dH_2O for use

3. 0.04M CITRATE-PHOSPHATE pH 6.4 (CP)

14.32g $Na_2HPO_4.12H_2O$
2.12g citric acid (anhydrous)
check pH and make up to 1 liter
dilute 1:3 CP:dH_20 for use as 0.01M

Appendix 2. Stain recipes.

1. Aconitate Hydratase (ACON) EC 4.2.1.3 CAAPM BUFFER

16 drops cis-Aconitic acid (10mg/ml; pH adjusted to 8.0)
1.5 ml NADP (2mg/ml)
6 drops $MgCl_2$ (20mg/ml)
5 drops MTT** (10mg/ml)
5 drops PMS** (2mg/ml)
20 µl IDH** (25U/ml) (35.7mg/ml)
2 ml agar

2. Isocitrate Dehydrogenase (IDH) EC 1.1.1.42 CAAPM BUFFER

1.0 ml stock 0.09M Tris HCl, pH=7.0
1.5 ml NADP (2mg/ml)
15 drops DL-Isocitric acid (100mg/ml)
8 drops $MgCl_2$ (20mg/ml)
5 drops MTT** (10mg/ml)
5 drops PMS** (2mg/ml)
2 ml agar

3. 6-Phosphogluconate Dehydrogenase (6PGDH) EC 1.1.1.44 CAAPM BUFFER

600 µl stock 0.09M Tris HCl, pH=8.0
1.5 ml NADP (2mg/ml)
6 drops 6-Phosphogluconic acid (20mg/ml)
6 drops $MgCl_2$ (20mg/ml)
5 drops MTT** (10mg/ml)
5 drops PMS** (2mg/ml)
2 ml agar

4. Aspartate aminotransferase (GOT) EC 2.6.1. CAAPM BUFFER

2ml 0.09M Tris-HCl, pH 8.0
5 drops Fast Garnet GBC salt solution
200µl α-ketoglutarate
200µl l-asparate
2ml agar

5. Glucose-6-Phosphate Isomerase (GPI) EC 5.3.1.9 TG BUFFER

1.0 ml stock 0.09M Tris HCl, pH=8.0
1.5 ml NAD (2mg/ml)
5 drops Fructose-6-phosphate (20mg/ml)
5 drops MTT** (10mg/ml)
5 drops PMS** (2mg/ml)
3 µl G6PDH** (1U/1µl)
2 ml agar

6. Phosphoglucomutase (PGM) EC 5.4.2. TG BUFFER

1.0ml stock 0.09M Tris HCl, pH=8.0
1.5 ml NAD (2mg/ml)
5 drops $MgCl_2$ (20mg/ml)
5 drops Glucose-1-phosphate solution (50mg/ml)
5 drops MTT** (10mg/ml)
5 drops PMS** (2mg/ml)
6 µl G6PDH (1U/1µl)
2 ml agar

7. Malate Dehydrogenase (MDH) EC 1.1.1.37 CAAPM BUFFER

1.0 ml stock 0.09M Tris HCl, pH=8.0
1.5 ml NAD (2mg/ml)
13 drops Malic substrate
5 drops MTT** (10mg/ml)
5 drops PMS** (2mg/ml)
5 ml agar

8. Glucose-6-Phosphate Dehydrogenase (G6PDH) EC 1.1.1.49 CAAPM BUFFER

600 µl stock 0.09M Tris HCl, pH=8.0
1.5 ml NADP (2mg/ml)
12 drops D-Glucose-6-phosphate (20mg/ml)
6 drops $MgCl_2$ (20mg/ml)
5 drops MTT** (10mg/ml)
5 drops PMS** (2mg/ml)
2 ml agar

9. Malate Dehydrogenase $NADP^+$ (ME) EC 1.1.1.40 CAAPM BUFFER

600 µl stock 0.09M Tris HCl, pH=8.0
1.5 ml NADP (2mg/ml)
12 drops Malic substrate
5 drops $MgCl_2$ (20mg/ml)
5 drops MTT** (10mg/ml)
5 drops PMS** (2mg/ml)
2 ml agar

10. Carboxylesterase (EST) EC 3.1.1.1 CP BUFFER

1.0 ml 0.2 M Tris Maleate Buffer, pH=5.3
1.0 ml dH20
200 µl α -naphthyl acetate solution
10 drops saturated fast garnet GBC salt**
2.0 ml agar

11. Superoxide Dismutase (SOD) EC 1.15.1.1 CAAPM BUFFER

1.5 ml NADP (2mg/ml)
5 drops MTT** (10mg/ml)
5 drops PMS** (2mg/ml)
2 ml agar

Appendix 3. Recipes for stocks.

1. Agar solution **(many stains)**

2g bacterial grade agar
125ml dH_2O
heat until boiling in microwave.
store covered in a 60^o water bath before use

2. 0.09M Tris-HCl pH 7.0 stock **(many stains)**

11.1g Tris
87.5ml 1M HCl
make up to 1 liter
check and adjust pH if necessary

3. 0.09M Tris-HCl pH 8.0 stock **(many stains)**

11.1g Tris
62ml 1M HCl
make up to 1 liter
check and adjust pH if necessary

4. L-aspartate solution (50mg/ml) **(GOT)**

1g L-aspartic acid
20ml dH_2O
pH 8.0

5. α-ketoglutarate solution (50mg/ml) **(GOT)**

1g α-ketoglutaric acid
20ml dH_2O
pH 8.0

6. Malic substrate **(MDH, ME)**

18ml water
2ml stock 0.2M Tris HCl, pH=9.0
368mg L-Malic acid
adjust to pH=8.0

7. 0.2 M Tris Maleate Buffer, pH=5.3 **(EST)**

2.4g Tris
2.4g maleic acid
4.8 ml 1M NaOH
make up to 100ml in dH_2O

8. α-naphthyl acetate solution **(EST)**

10ml water
10ml acetone
0.1g α-naphthyl acetate

Appendix 4. The 8-locus clonal genotypes and their frequencies from 1 km, 2 km and 8 km sample sites at Harjavalta.

acon	idh	got	gpi	pgm	mdh	me	est	1 km	2 km	8 km	Total
A	AA	AA	AB	BA	A	AA	CA	7	5	6	18
A	AA	AA	AB	BA	A	AA	BA	3	13	1	17
A	AA	AA	AC	BA	A	AA	CA	11	1	4	16
A	AA	AA	AC	BA	A	AA	BA	2	6	4	12
A	AB	AA	AC	BA	A	AA	BA	9	0	1	10
A	AB	AA	AC	BA	A	AA	CA	9	0	0	9
A	AB	AA	AC	BA	A	AA	AA	6	0	1	7
A	AB	AA	AC	BA	A	AA	AA	1	4	1	6
A	AA	AA	AC	BD	A	AA	CA	1	0	5	6
A	AA	AA	AA+	BA	A	AA	CA	1	2	1	4
A	AA	AA	AB	BD	A	AA	CA	0	0	4	4
A	AB	AA	AB	BA	A	AA	BA	4	0	0	4
A	AA	AA	AA	BA	A	AA	BA	0	3	1	4
A	AA	AA	AB	BC	A	AA	BA	1	0	2	3
A	AA	AA	AC	BA	A	AA	AA	3	0	0	3
A	AA	AA	AC	BC	A	AA	CA	3	0	0	3
A	AA	AA	AC	BD	A	AA	BA	0	0	3	3
A	AB	AA	AC	BC	A	AA	BA	3	0	0	3
A	AA	AA	AB	BA	A	AA	DA	2	0	0	2
A	AB	AA	AB	BA	A	AA	CA	2	0	0	0
A	AB	AA	AC	BC	A	AA	AA	2	0	0	2
A	AB	AA	AC	BC	A	AA	CA	2	0	0	2
A	AB	AA	AC	BD	A	AA	BA	0	0	2	2
A	AB	AA	AC	BD	A	AA	CA	2	0	0	2
A	AB	AA	AC	BC	A	AB	CA	2	0	0	2
A	AA	AA	AA	BA	A	AA	CA	0	1	0	1
A	AA	AA	AA	BA	A	AA	CC	0	0	1	1
A	AA	AA	AA	BA	A	AB	CA	1	0	0	1
A	AA	AA	AA+	BA	A	AB	CC	1	0	0	1
A	AA	AA	AA+	BC	A	AB	BA	1	0	0	1
A	AA	AA	AA+	BD	A	AA	CA	0	0	1	1
A	AA	AA	AB	BA	A	AA	AA	0	1	0	1
A	AA	AA	AB	BA	A	AB	BA	1	0	0	1
A	AA	AA	AB	BA	A	AB	CA	1	0	0	1
A	AA	AA	AB	BC	A	AB	CA	1	0	0	1
A	AA	AA	AB	BD	A	AA	BA	0	0	1	1
A	AA	AA	AC	BC	A	AA	AA	1	0	0	1
A	AA	AA	AC	BC	A	AA	BA	1	0	0	1
A	AA	AA	AC	BD	A	AA	AA	0	0	1	1
A	AA	AA	AA+	CD	A	AA	DA	1	0	0	1
A	AA	AA	AC	BA	A	AA	BA	1	0	0	1
A	AB	AA	AA	BA	A	AA	BA	1	0	0	1
A	AB	AA	AA+	BA	A	AA	CA	1	0	0	1
A	AB	AA	AB	BA	A	AB	BA	1	0	0	1
A	AB	AA	AB	BC	A	AA	CA	0	0	1	1
A	AB	AA	AB	BC	A	AB	BA	1	0	0	1
A	AB	AA	AB	BC	A	AB	CA	1	0	0	1
A	AB	AA	AB	BC	A	AB	CA	1	0	0	1
A	AB	AA	AB	BD	A	AA	CA	0	0	1	1
A	AB	AA	AC	BA	A	AB	AA	1	0	0	1
A	AB	AA	AC	BA	A	AB	CA	1	0	0	1
A	AB	AA	AC	BC	A	AB	BA	1	0	0	1
A	AB	AA	AC	BD	A	AA	AA	0	0	1	1
A	AB	AA	AC	BD	A	AB	CA	1	0	0	1
A	AC	AA	AC	BA	A	AA	AA	1	0	0	1
A	AA	AA	AB	BA	A	AA	A+A	0	0	1	1
A	AA	AA	AC	BA	A	AA	DA	1	0	0	1
								98	36	44	176

Appendix 5. The 10-locus clonal genotypes and their frequencies from the 1 and 4 km sites at Imatra.

ACON	IDH	6GPDH	GOT	GPI	PGM	MDH	ME	EST	SOD	1 km	4 km	Total
A	AB	A	AA	AC	BA	A	AA	CA	A	0	6	6
A	AA	A	AA	AB	BA	A	AA	CA	A	0	3	3
A	AA	A	AA	AB	BA	A	AA	CA	B	0	3	3
A	AB	A	AA	AC	BA	A	AA	BA	A	1	2	3
A	AB	A	AA	AC	BD	A	AA	CA	A	2	1	3
A	AA	A	AA	AA	BA	A	AA	CC	Z	0	2	2
A	AA	A	AA	AA+	BA	A	AA	CA	A	1	1	2
A	AA	A	AA	AB	BC	A	AA	CA	A	1	1	2
A	AA	A	AA	AB	BD	A	AA	CA	A	1	1	2
A	AA	A	AA	AC	BC	A	AA	BA	A	1	1	2
A	AA	A	AA	AC	BD	A	AA	AA	A	2	0	2
A	AA	A	AA	AC	BD	A	AA	CA	A	2	0	2
A	AB	A	AA	AB	BD	A	AB	BA	A	0	2	2
A	AA	A	AA	AA	BA	A	AA	CA	A	1	0	1
A	AA	A	AA	AA	BA	A	AA	CA	Z	0	1	1
A	AA	A	AA	AA	BC	A	AA	BA	B	0	1	1
A	AA	A	AA	AA	BC	A	AA	BB	A	1	0	1
A	AA	A	AA	AA	BC	A	AA	CC	A	0	1	1
A	AA	A	AA	AA	BC	A	AA	DA	Z	0	1	1
A	AA	A	AA	AA	BC	A	AB	CA	A	0	1	1
A	AA	A	AA	AA+	BA	A	AA	BA	A	0	1	1
A	AA	A	AA	AA+	BA	A	AA	CA	B	0	1	1
A	AA	A	AA	AA+	BA	A	AB	CA	A	0	1	1
A	AA	A	AA	AA+	BC	A	AA	BA	A	0	1	1
A	AA	A	AA	AA+	BC	A	AA	BA	B	1	0	1
A	AA	A	AA	AA+	BC	A	AA	CA	B	0	1	1
A	AA	A	AA	AA+	BD	A	AA	BA	A	1	0	1
A	AA	A	AA	AA+	BD	A	AA	BA	Z	0	1	1
A	AA	A	AA	AA+	BD	A	AA	CA	A	0	1	1
A	AA	A	AA	AA+	BD	A	AB	CA	B	0	1	1
A	AA	A	AA	AB	BA	A	AA	AA	A	1	0	1
A	AA	A	AA	AB	BC	A	AA	BA	A	1	0	1
A	AA	A	AA	AB	BC	A	AA	BA	B	0	1	1
A	AA	A	AA	AB	BD	A	AA	BA	A	1	0	1
A	AA	A	AA	AB	BD	A	AA	CA	B	1	0	1
A	AA	A	AA	AB	BD	A	AA	CA	Z	1	0	1
A	AA	A	AA	AB	BD	A	AA	DA	B	1	0	1
A	AA	A	AA	AC	AB	A	AA	BA	A	0	1	1
A	AA	A	AA	AC	BA	A	AA	AA	A	1	0	1
A	AA	A	AA	AC	BA	A	AA	AA	B	0	1	1
A	AA	A	AA	AC	BA	A	AA	AA	Z	0	1	1
A	AA	A	AA	AC	BA	A	AA	BA	A	1	0	1
A	AA	A	AA	AC	BA	A	AA	BA	Z	1	0	1
A	AA	A	AA	AC	BC	A	AA	AA	A	1	0	1
A	AA	A	AA	AC	BC	A	AA	CA	B	0	1	1
A	AA	A	AA	AC	BD	A	AA	BA	A	0	1	1
A	AA	A	AA	AC	BD	A	AA	BA	B	1	0	1
A	AA	A	AA	AC	BD	A	AA	BA	Z	0	1	1
A	AA	A	AA	AC	BD	A	AA	DA	A	1	0	1
A	AA	A	AA	AC	BD	A	AA	DA	Z	0	1	1
A	AA	A	AA	AC	CD	A	AA	AA	A	1	0	1
A	AA	A	AA	AC	CD	A	AA	BA	A	0	1	1
A	AA	Z	AA	AC	BA	A	AA	BA	A	1	0	1
A	AB	A	AA	AA	BC	A	AA	CA	Z	1	0	1
A	AB	A	AA	AA	BD	A	AB	CA	B	0	1	1
A	AB	A	AA	AA	BD	A	AA	CA	A	0	1	1
A	AB	A	AA	AA+	BC	A	AA	CA	B	0	1	1
A	AB	A	AA	AA+	BD	A	AA	AA	A	0	1	1
A	AB	A	AA	AB	BA	A	AA	AA	B	0	1	1
A	AB	A	AA	AB	BA	A	AA	BA	A	0	1	1
A	AB	A	AA	AB	BA	A	AA	CA	A	0	1	1
A	AB	A	AA	AB	BC	A	AA	CA	A	0	1	1
A	AB	A	AA	AB	BD	A	AA	BA	A	0	1	1
A	AB	A	AA	AB	BD	A	AA	DA	A	0	1	1
A	AB	A	AA	AC	BA	A	AA	AA	A	0	1	1
A	AB	A	AA	AC	BA	A	AA	CA	Z	1	0	1
A	AB	A	AA	AC	BA	A	AA	DA	A	0	1	1
A	AB	A	AA	AC	BA	A	AB	BA	A	0	1	1
A	AB	A	AA	AC	BA	A	AB	CA	A	0	1	1
A	AB	A	AA	AC	BC	A	AA	AA	A	0	1	1
A	AB	A	AA	AC	BC	A	AA	CA	A	0	1	1
A	AB	A	AA	AC	BC	A	AA	CA	B	0	1	1
A	AB	A	AA	AC	BC	A	AB	BA	Z	0	1	1
A	AB	A	AA	AC	BD	A	AA	AA	A	1	0	1
A	AB	A	AA	AC	BD	A	AA	BA	A	1	0	1
A	AB	A	AA	AC	BD	A	AA	CA	B	0	1	1
A	AB	A	AA	AC	BD	A	AA	DA	A	0	1	1
A	AC	A	AA	AC	BA	A	AA	BA	A	0	1	1
B	BA	A	ZZ	AA	AZ	B	AZ	A+Z	Y	0	1	1
B	BA	A	ZZ	AA+	ZZ	B	AZ	A+Z	Y	0	1	1
B	BA	B	ZZ	BD	AZ	C	AB	A+Z	Y	0	1	1
										33	69	102

Appendix 6. The 10-locus clonal genotypes and their frequencies from the 2 and 4 km sites at Kokkola.

acon	idh	6gpdh	got	gpi	pgm	mdh	me	est	sod	2 km	4 km	Total
A	AA	A	AA	AB	BC	A	AA	CA	A	0	8	8
A	AA	A	AA	AA	BC	A	AA	CA	A	0	5	5
A	AA	A	AA	AA	BC	A	AB	CA	A	1	4	5
A	AA	A	AA	AA+	AB	A	AA	CA	A	5	0	5
A	AA	A	AA	AB	AB	A	AA	CA	A	5	0	5
A	AA	A	AA	AA	AB	A	AA	CA	A	4	0	4
A	AA	A	AA	AA+	BC	A	AA	CA	A	2	2	4
A	AA	A	AA	AC	AB	A	AA	CA	A	4	0	4
A	AA	A	AA	AC	BD	A	AA	CA	A	2	2	4
A	AA	A	AA	AA	BD	A	AA	CA	A	0	3	3
A	AA	A	AA	AA+	BD	A	AA	CA	A	2	1	3
A	AA	A	AA	AB	BC	A	AA	CA	B	0	3	3
A	AA	A	AA	AB	BD	A	AA	CA	A	0	3	3
A	AA	A	AA	AC	BC	A	AA	CA	A	1	2	3
A	AB	A	AA	AC	AB	A	AA	CA	A	3	0	3
A	AA	A	AA	AA	AB	A	AB	CA	A	2	0	2
A	AA	A	AA	AA+	BC	A	AB	CA	A	0	2	2
A	AA	A	AA	AA+	BD	A	AA	BA	A	2	0	2
A	AA	A	AA	AB	BA	A	AA	CA	B	1	1	2
A	AA	A	AA	AB	BD	A	AA	CA	B	1	1	2
A	AB	A	AA	AC	BD	A	AA	BA	A	2	0	2
A	AA	A	AA	AA	AB	A	AA	BA	A	0	1	1
A	AA	A	AA	AA	AB	A	AA	BA	B	1	0	1
A	AA	A	AA	AA	AB	A	AA	CA	B	1	0	1
A	AA	A	AA	AA	AB	A	AA	CC	A	1	0	1
A	AA	A	AA	AA	BA	A	AA	CA	A	0	1	1
A	AA	A	AA	AA	BA	A	AB	CA	A	0	1	1
A	AA	A	AA	AA	BC	A	AA	CA	B	0	1	1
A	AA	A	AA	AA	BC	A	AA	CA	Z	0	1	1
A	AA	A	AA	AA	BC	A	AB	BA	A	0	1	1
A	AA	A	AA	AA	BC	A	AB	CA	B	0	1	1
A	AA	A	AA	AA	BC	A	AB	CA	Z	0	1	1
A	AA	A	AA	AA	BD	A	AA	CC	A	0	1	1
A	AA	A	AA	AA	BD	A+	AA	CC	Z	1	0	1
A	AA	A	AA	AA+	AB	A	AB	CA	B	1	0	1
A	AA	A	AA	AA+	BA	A	AA	BA	A	0	1	1
A	AA	A	AA	AA+	BC	A	AA	CA	B	0	1	1
A	AA	A	AA	AA+	BC	A	AB	BA	A	0	1	1
A	AA	A	AA	AA+	BC	A	AB	CA	B	0	1	1
A	AA	A	AA	AA+	BC	A	AB	DA	B	0	1	1
A	AA	A	AA	AA+	BD	A	AA	CA	B	0	1	1
A	AA	A	AA	AA+	BD	A	AB	CA	A	0	1	1
A	AA	A	AA	AA+	BD	A	AB	DA	A	1	0	1
A	AA	A	AA	AA+	BD	A	AB	DA	B	0	1	1
A	AA	A	AA	AB	AB	A	AA	BA	A	1	0	1
A	AA	A	AA	AB	AB	A	AA	BA	B	1	0	1
A	AA	A	AA	AB	AB	A	AA	CA	B	1	0	1
A	AA	A	AA	AB	AC	A	AA	CA	B	0	1	1
A	AA	A	AA	AB	BA	A	AA	BA	B	0	1	1
A	AA	A	AA	AB	BA	A	AA	CA	Z	0	1	1
A	AA	A	AA	AB	BA	A	AB	CA	Z	0	1	1
A	AA	A	AA	AB	BC	A	AA	DA	B	0	1	1
A	AA	A	AA	AB	BC	A	AZ	BA	A	0	1	1
A	AA	A	AA	AB	BD	A	AA	DA	B	0	1	1
A	AA	A	AA	AC	BA	A	AA	CA	A	0	1	1
A	AA	A	AA	AC	BA	A	AA	CA	Z	0	1	1
A	AA	A	AA	AC	BD	A	AA	A+A	A	1	0	1
A	AA	A	AA	AC	BD	A	AA	AA	A	1	0	1
A	AA	A	AA	AC	BD	A	AA	AA	B	1	0	1
A	AA	A	AA	AC	BD	A	AA	DA	A	1	0	1
A	AA	A	AA	AC	BD	A	AB	AA	A	1	0	1
A	AB	A	AA	AB	AB	A	AA	CA	A	1	0	1
A	AB	A	AA	AB	BA	A	AA	CA	A	0	1	1
A	AB	A	AA	AB	BA	A	AA	CA	Z	0	1	1
A	AB	A	AA	AB	BC	A	AA	CA	A	0	1	1
A	AB	A	AA	AB	BC	A	AZ	CA	A	0	1	1
A	AB	A	AA	AB	BD	A	AA	CA	A	0	1	1
A	AB	A	AA	AC	AB	A	AA	AA	A	1	0	1
A	AB	A	AA	AC	BA	A	AA	A+A	A	1	0	1
A	AB	A	AA	AC	BA	A	AA	BA	A	1	0	1
A	AB	A	AA	AC	BC	A	AA	BA	A	1	0	1
A	AB	A	AA	AC	BD	A	AA	CA	A	0	1	1
A	BA	A	AA	BD	AZ	B	AZ	A+Z	Z	1	0	1
B	BA	A	ZZ	BA	ZZ	B	AZ	A+Z	Y	0	1	1
B	BA	A	ZZ	AA	ZA	B	AZ	A+Z	Z	0	1	1
										57	71	128