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Giska, I.

2016

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citation for published version (APA)

Giska, I. (2016). *Effect of metal pollution on genetic variation in natural populations of selected soil invertebrate species with different dispersal potential*. AT Wydawnictwo.

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General discussion

The main hypothesis tested in this PhD thesis was that metal pollution impacts the level of genetic diversity of natural populations of soil invertebrates and the scale of this impact depends on the species dispersal abilities. However, I found no correlation between the population genetic diversity of any studied species and the level of contamination. In the less migratory species, *Lumbricus rubellus* and *Lithobius forficatus*, deep genetic structure was observed, suggesting limited gene flow, but the structuring was not associated with the concentrations of metals in the studied soils. Populations of the highly mobile rove beetle *Staphylinus erythropterus* showed very little genetic differentiation, indicating extensive gene flow, which might counteract any selective effects due to contamination.

Interestingly, the highest diversity of mtDNA and nuDNA was found among earthworms and rove beetles at the polluted site OL2, which was characterized by the highest available metal concentrations. This high genetic diversity of populations at the OL2 site may be a signature of pollution-induced processes, i.e., an increase of mutation rate or intensification of gene inflow. Below I will discuss possible explanations of the findings and their significance in relation to the existing literature. I also will discuss limitations of the performed studies and present recommendations for future research.

First of all, the exposure to metals in the Olkusz area is maybe not high enough to cause large and clearly visible effects on the population genetic level. A level of stress too low to generate changes in genetic composition was also suggested by other scientists, e.g. those studying populations of wood mouse from a pollution gradient in Belgium (Berckmoes et al. 2005). To result in changes of genetic diversity, pollution should cause drastic population size decline or constitute a strong selection pressure. This can happen if uptake of chemicals from the environment is high enough to cause adverse effects in individual organisms. These effects should include effects on fitness parameters, e.g., loss of body mass, reduced fecundity, increased mortality or developmental abnormalities. When

these fitness effects occur they will initially lead to population decline and a genetic bottleneck followed by reduction of genetic diversity. Results of the toxicokinetics study (Chapter 1) suggest that metals were available for uptake by earthworms. Both immobilization of nonessential metals (Cd, Pb) and excretion of essential ones (Cu, Zn) must have been energetically costly constituting a factor that might drive physiological or genetic changes. Such energy re-allocation, necessary in the development of metal tolerance, may reduce population fitness, consequently leading to population size decline.

Concentrations of Cd, Pb and Zn in soils at the highly polluted site OL2 exceeded the No-Observed Effect Concentrations (NOEC) for earthworm cocoon production (Cd 18 mg kg⁻¹, Pb 492 mg kg⁻¹, Zn 1879 mg kg⁻¹), but not for mortality or growth, determined in field soils contaminated with a mixture of metals (Spurgeon and Hopkin 1995). However, the NOEC values as well as the LC₅₀ and EC₅₀ values for effects on earthworm survival and reproduction, respectively, reported by different authors show large differences depending on the soil type used, duration of the experiment and the earthworm species tested (Sivakumar 2015). We cannot be sure thus whether metal pollution at the OL2 site indeed affected the earthworms' fitness to the extent that it could result in detectable genetic changes in the population. In case of the other two studied species this is even more difficult to judge. On the one hand, centipedes and rove beetles are exposed to metals because their adults feed on earthworms and other soil invertebrates that accumulate metals, they lay eggs in contaminated soil and their larvae live in soil. Therefore, for each life-stage exposure to metals seems to be high. On the other hand, metal concentrations found in tissues of centipedes and rove beetles were rather low and showed only small differences between differently polluted sites, suggesting that these animal groups developed efficient mechanisms to prevent uptake of metals from the environment or to quickly remove accumulated metal from the body (Hopkin and Martin 1983; Migula et al. 2004). In the case of centipedes and beetles this mechanism most probably involves midgut epithelium cells, which constitute the first barrier and also the place where metals are stored in intracellular vesicles of the lysosomal system, often denoted as granules (Vandenbulcke et al. 1998; Argasiński et al. 2012). Such storage granules may be expelled through merocrine, apocrine or holocrine excretion into the gut lumen. Despite such protective mechanisms, concentrations of metals in the soil of the Olkusz transect

were shown to have adverse effects on beetle populations (Stone et al. 2001; Skalski et al. 2010; Bednarska and Stachowicz 2013).

Secondly, the studied species are characterized by large population sizes. Earthworm populations can reach densities over 2000 individuals per m² (Edwards and Bohlen 1996). However in coniferous forests with acidic soil the densities are usually lower. At Olkusz transect maximum densities of ~25 ind m⁻² and patchy distribution of earthworms were reported (Tosza et al. 2010). Centipedes, although usually found individually (Kaczmarek 1979), were observed in high numbers at the Olkusz polluted sites, especially in the horse-dung traps at the sites OL1 and OL2. Similarly, the highest density of *S. erythropterus* populations was observed at the OL2 site. This is important because according to the neutral theory of molecular evolution (Kimura 1983) population genetic diversity is shaped by mutations which increase diversity and by genetic drift, which decreases diversity, especially in small populations. Any reduction of population size due to toxic effects of metals must be strong enough to result in intensified genetic drift. According to neutral theory, the heterozygosity will decrease at a rate $1/(2N_e)$ per generation (where N_e denotes effective population size). The decrease of heterozygosity also depends on the level of gene flow. The effects of genetic drift are difficult to quantify in the field, because in natural populations inhabiting contaminated sites, pollution-induced processes that reduce genetic variation occur simultaneously with processes that result in increased genetic variation. Moreover, in the field also factors different from pollution alter gene flow, genetic drift, mutations and natural selection and continuously change the genetic composition of natural populations (Medina et al. 2007). These processes may mutually mask each other's effects. If pollution causes drastic population size decline but at the same time results in intensified gene inflow from neighboring populations or increased mutation rate, a high level of population genetic diversity will be maintained. Indeed, the results of this PhD thesis suggest that metal pollution has a net positive effect on genetic diversity, possibly due to an increased mutation rate at the OL2 site.

Among the polluted sites from the Olkusz transect the highest genetic diversity of mtDNA and nuDNA in *L. rubellus* (Chapter 2) and *S. erythropterus* (Chapter 4) populations was observed at the OL2 site. As argued above, this could be caused by increased mutation rates induced by oxidative

stress. An increased mutation rate due to pollution has already been stated by several researchers, as mentioned in the introduction of this thesis and in the subsequent chapters (Eeva et al. 2006; Matson et al. 2006; Rinner et al. 2011). However, the increased mutation rate in these papers was just a suggestion and none of the mentioned researchers aimed at measuring exact values of mutation rate in relation to the level of pollution. Another explanation of highest genetic diversity in populations from the OL2 site is that this site acted as an ecological sink and high genetic diversity resulted from contamination-induced immigration from neighboring populations. This could be particularly applicable to the rove beetles, which are characterized by high dispersal abilities. However, to support this hypothesis it would be necessary to extend sampling around the smelter and estimate the direction of the gene flow. Although earthworms and centipedes are characterized by low dispersal abilities, the heterogeneity of the soil environment and the patchy distribution of metals in the soil may favor the origin of spatially defined micro-demes. Migration between demes or sampling individuals from several demes considered as one population could thus result in higher genetic diversity estimates. In this manner, the high spatial variation of metal pollution, with local hotspots of toxicity could cause a spatially variable genetic composition in soil invertebrates, even when the average level of contamination is not toxic.

Another aspect worth discussing is adaptation to pollution. However, adaptation is difficult to study without *a priori* candidate genes. Thus, I used genome scans to search for F_{ST} outlier loci that could be an indicator of metal pollution-driven selection. Such loci were found in the rove beetles (Chapter 4) but their allele frequencies were not correlated with metal concentrations in the studied soils. In the earthworms many F_{ST} outlier loci were identified (data not shown) but their pollution-related origin is difficult to prove. Dense coverage of the genome with molecular markers and known function of genes where outliers were located could help to demonstrate that a particular locus was under metal pollution-induced selection. This was the main plan in case of the *L. rubellus* experiment with RADseq. However, only a small part of the obtained RAD tags could be mapped to the reference genome, which is constructed for an individual from the B lineage present only on the British Isles. Because of this low mapping success, I have chosen *de novo* analysis of the RADseq data. In the future, when the reference genome is complete, the analysis aimed at finding signatures of local

adaptation to pollution could be attempted again. As researchers from Cardiff University, UK, are working also on the assembly of the genome of the A lineage, the percentage of mapped *L. rubellus* RAD tags obtained within this PhD project will be definitely higher than reported now based on mapping to the genome of the B lineage individual. This could enable annotation of F_{ST} outlier loci and verification whether proteins coded by outlier genes could be involved in metal detoxification processes.

A very interesting finding of this PhD thesis, although not directly related to the subject of metal pollution, is that the centipede *Lithobius forficatus* is a complex of cryptic species. This is important from the perspective of soil biodiversity estimation, which is the main component of global biodiversity. Many species of soil fauna remain undescribed, including cryptic species. Therefore, soil biodiversity is usually underestimated. This finding also puts light on the speciation processes within soil taxa. As soil is an extremely heterogeneous environment, it creates conditions favoring speciation through ecological isolation, particularly habitat isolation. Based on the findings reported in this thesis, I hypothesized that one of the identified cryptic species of *L. forficatus* could be adapted to or could prefer low pH environments. Thus the speciation within *L. forficatus* is worth further studying to prove reproductive isolation between the potential cryptic species and to reveal mechanisms of the species origin. This thesis suggests that soil factors varying at a micro-scale could be important drivers for ecological speciation and the persistence of different cryptic lineages of soil invertebrates.

The research conducted within this thesis brings new insight into the effects of metal pollution on the genetic variation in invertebrate populations and into the issue of cryptic speciation of soil taxa. However, to make the conclusions stronger it would be necessary to confirm the obtained results with the use of more transects, preferably located at distant areas, to exclude effects like, e.g., ecosystem history. Sampling of more populations would be also beneficial for the estimation of gene flow and the direction of migration. Anyway, it also has to be realized that it is not straightforward to elucidate the effect of pollution on population genetic variation while studying natural populations which are under the influence of numerous uncontrolled environmental factors. In addition, we need better genomics information to be able to map loci responsive to pollution, to functional genes in the genome.

To sum up, the results of molecular analysis of the three studied species did not confirm the hypothesis that metal pollution decreases the genetic diversity of populations the stronger the less dispersive the species is. Indeed, in the least dispersive species, *L. rubellus*, the highest genetic diversity was found in the most polluted site, what may be caused by increased mutation rate. On the other hand, the increased genetic diversity at the most polluted site was found also in the most dispersive species, *S. erythropterus*; in this case the increased immigration rate of individuals from less polluted areas could be the reason as well. The study resulted also in discovering cryptic species in the third of the studied animals, *L. forficatus*. The existence of cryptic species complicates the analysis of the population-level genetic diversity and should be taken into account in future studies.

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Streszczenie

Spadek bioróżnorodności stanowi dziś problem na skalę ogólnoświatową. Bioróżnorodność, obok różnorodności gatunków i ekosystemów, obejmuje różnorodność genetyczną. Dlatego, aby skutecznie chronić bioróżnorodność, powinniśmy chronić również różnorodność genetyczną. Ma to szczególne znaczenie w dzisiejszych czasach, ponieważ w wyniku działalności człowieka środowisko zmienia się w niesłychanie szybkim tempie i wysoka różnorodność genetyczna może okazać się kluczowa dla możliwości adaptacji wielu gatunków do tak szybko zmieniających się, a przez to stresogennych, warunków. Jednym z czynników antropogenicznych wpływających na poziom różnorodności genetycznej w naturalnych populacjach jest zanieczyszczenie środowiska toksycznymi substancjami chemicznymi. Mimo że naukowcy od dawna próbują odpowiedzieć na pytanie, jak wielkim jest ono zagrożeniem dla różnorodności genetycznej populacji, wyniki badań przeprowadzonych do tej pory nie są jednoznaczne. Ponadto, brakuje badań wielkoskalowych obejmujących ogólnogenomowe analizy wielu gatunków z tego samego zanieczyszczonego obszaru, co pozwoliłoby głębiej spojrzeć na zmiany procesów populacyjnych wskutek ekspozycji na zanieczyszczenie.

W niniejszej rozprawie testowałam hipotezę, że długoterminowa ekspozycja na zanieczyszczenie metalami wpływa na poziom różnorodności genetycznej populacji i że skala tego wpływu zależy od zdolności gatunku do dyspersji. Badania przeprowadziłam wzdłuż gradientu zanieczyszczenia metalami w pobliżu huty cynku i ołowiu „Bolesław” niedaleko miasta Olkusz w Polsce południowej. Badaniami objęłam populacje trzech gatunków bezkręgowców różniących się zdolnością do dyspersji: dżdżownicę *Lumbricus rubellus*, wija drewniaka *Lithobius forficatus* i kusaka czerwonopokrywego *Staphylinus erythropterus*. Próby zebrałam głównie z powierzchni wyznaczonych wzdłuż olkuskiego gradientu skażenia. Różnorodność i strukturę genetyczną populacji oszacowałam na podstawie analiz RAD tagów i sekwencji mtDNA.

Jako że toksyczność metali zależy od ich biodostępności, przeprowadziłam najpierw eksperyment toksykokinetyczny mający na celu oszacowanie biodostępności metali wzdłuż badanego

gradientu skażenia (Rozdział 1). Poprzez ekspozycję dżdżownicy *L. rubellus* na glebę pochodzącą z badanego gradientu wykazałam, że mimo wysokiej zawartości materii organicznej w glebie, metale są dostępne dla organizmów glebowych. Dżdżownice intensywnie akumulowały metale niepełniące żadnej funkcji w organizmie (Cd, Pb), a w przypadku metali będących mikroelementami (Cu, Zn) wykazały efektywną regulację ich stężenia w ciele.

Następnie oszacowałam różnorodność genetyczną populacji wybranych gatunków bezkręgowców. Wykazałam, że najmniej mobilny gatunek, dżdżownica *L. rubellus*, jest wysoce polimorficzny i charakteryzuje się różnorodnością nukleotydów na poziomie 0.7-0.8% (Rozdział 2). Przeciwnie do przewidywań spadku różnorodności ze wzrostem skażenia, spośród populacji z olkuskiego gradientu najwyższą różnorodnością genetyczną charakteryzowała się populacja z najbardziej zanieczyszczonej powierzchni o najwyższej biodostępności metali. Ogólnie, wśród analizowanych osobników *L. rubellus* stwierdziłam pięć wysoce zróżnicowanych linii mitochondrialnych w sympatrii. Wykazałam, że linie te nie były izolowane rozrodczo, o czym świadczyło grupowanie się nuklearnych RAD tagów zgodnie z populacją pochodzenia, a nie zgodnie z liniami mitochondrialnymi. Tym samym obaliłam dodatkową hipotezę, że linie mtDNA dżdżownicy *L. rubellus* z kontynentalnej części Europy reprezentują gatunki kryptyczne.

Analizując populacje wija drewniaka *L. forficatus* (Rozdział 3) odkryłam, że stanowi on w Polsce kompleks co najmniej trzech silnie zróżnicowanych genetycznie grup reprezentujących prawdopodobnie gatunki kryptyczne. Niski poziom admiksji pomiędzy stwierdzonymi grupami, różnice w poziomie różnorodności genetycznej populacji oraz obecność utrwalonych pomiędzy grupami polimorfizmów wskazują na istnienie pomiędzy nimi izolacji rozrodczej. Nie stwierdziłam, natomiast, wpływu skażenia metalami na strukturę genetyczną wija.

W przypadku najbardziej mobilnego gatunku, *S. erythropterus*, wykazałam bardzo niskie zróżnicowanie genetyczne świadczące o intensywnym przepływie genów pomiędzy populacjami (Rozdział 4). Badane populacje charakteryzowały się zbliżonym poziomem różnorodności genetycznej genomu nuklearnego ($\pi \sim 0.5\%$) oraz niewielkimi różnicami w różnorodności mtDNA. Żadna z wykorzystanych miar różnorodności genetycznej nie była skorelowana ze stężeniem metali w glebie. Jedynie populacja kusaka z powierzchni o najwyższej biodostępności metali

charakteryzowała się różnorodnością mtDNA istotnie wyższą od różnorodności obserwowanej w innych populacjach z gradientu olkuskiego. Liczba i różnorodność haplotypów oszacowana na podstawie RAD tagów również były najwyższe w tej populacji, ale nieistotnie różne od różnorodności obserwowanej w pozostałych olkuskich populacjach.

Podsumowując, wyniki zaprezentowane w niniejszej rozprawie nie potwierdzają hipotezy, że zanieczyszczenie metalami redukuje poziom różnorodności genetycznej populacji tym bardziej im mniejsze możliwości dyspersji ma gatunek ekspozowany na zanieczyszczenie. W przypadku, zarówno najmniej mobilnego gatunku, dżdżownicy *L. rubellus*, jak również gatunku najbardziej mobilnego, t.j. kusaka *S. erythropterus*, najwyższą różnorodność genetyczną stwierdziłam w populacjach z silnie skażonej powierzchni charakteryzującej się najwyższą biodostępnością metali. Wynikać to może z tempa mutacji podwyższonego wskutek wysokiego stresu oksydacyjnego na tej powierzchni bądź też, szczególnie w przypadku kusaka, wzmożonej imigracji osobników z sąsiednich populacji do populacji spełniającej rolę „ekologicznego ujścia”. Ponadto, przeprowadzone badania zaowocowały odkryciem gatunków kryptycznych wija drewniaka *L. forficatus*, co podkreśla konieczność uwzględnienia kryptycznej różnorodności w badaniach ekotoksykologicznych.

Acknowledgements

This study was supported by the following research grants:

- 1) Foundation for Polish Science International PhD Projects Programme co-financed by the EU European Regional Development Fund within the project MPD/2009-3/5, "Environmental Stress, Population Viability and Adaptations";
- 2) Polish National Science Center Grant No. 2011/03/N/NZ8/00013; project "Searching for genomic basis of local adaptations to metal pollution in natural populations of the earthworm *Lumbricus rubellus*";
- 3) Jagiellonian University grant No. DS/MND/WBiNoZ/INoŚ/8/2011;
- 4) Jagiellonian University grant No. DS/MND/WBiNoZ/INoŚ/11/2014.

