Prof. dr hab. Paweł Migula
Department of Animal Physiology and Ecotoxicology
University of Silesia in Katowice
pawel.migula@us.edu.pl

Review of the PhD thesis written by Iwona Giska:
"Effect of metal pollution on genetic variation in natural populations of selected soil invertebrate species with different dispersal potential"

1. Evaluation of the formal fulfilments of the dissertation

The subject of the presented study is adequate to the title of the dissertation. Ms Iwona Giska's PhD dissertation is a fully original scientific paper. The author tested the main hypothesis that long-term environmental contamination affects the level of genetic diversity of natural populations of soil / litter dwelling invertebrate species. The scope of such an impact depends on the dispersal abilities of a given species.

The conceptual basis of the studies has come from the browsing of numerous scientific papers focused on relations between the environmental stressors and the genetic diversity of animal populations inhabiting unfavorable areas. She has checked how, and to what extent, the environmental stressors, including metal pollution in soils, may affect the genetic diversity. In fact, metal pollution is one of the main and measurable factors deciding on possible adaptations of organisms to the adverse living conditions. Eventually, she has stated that the results of the studies that have clarified that the relationship within environmental stress (pollutants) is "inconclusive or contradictory". She has also stated the lack of the large-scale genome-wide analysis of various species inhabiting the same polluted areas in the recent literature. The results of the studies, described in her thesis, have partly filled this gap.

I have assumed that Ms Iwona Giska's efficient and wise "navigation" at the junction of ecotoxicology, population genetics and evolutionary ecology, with a skillful use of techniques and tools, relevant to these areas of research, are advantageous to her dissertation.

The dissertation has been prepared in a compact form. In terms of editorial and aesthetic the typescript has been prepared professionally. The contents of the computer printout slightly exceeds 150 pages. Very carefully prepared and clearly presented visual documentation of each separate section and four chapters, which include the results of the basic research, are remarkable. The tables and graphs have been inserted among the parts of the text of each section or chapter. The text includes a three page-long English summary and a bit wider Polish summary.

The core part of the dissertation consists of four chapters, which constitute separate research tasks themselves. They are preceded by two initial sections: General introduction and General methods, containing necessary information related to all the aforementioned four chapters. Each chapter has been presented as a separate research task. These chapters are, in fact, written in English, typescripts of scientific articles. Three of them have been already published (Chapter I, II and IV). These parts of the dissertation are preceded by a well-designed general introduction to the study, showing arguments which clarify the necessity of searching the responses to three main research hypotheses. I consider important the following items: the general description of the study areas (experimental plots) and their characteristics, the presentation of the biological objects of research - three selected species with different migratory abilities, and the brief introduction to genetic methods applied to the study.

All chapters, with some exceptions, have been prepared in the form typical of a presentation of experimental results in scientific journals, maintaining a sequence of individual parts in accordance with the editorial requirements. The exceptions are: the alphabetic list of references cited in the whole Ph.D. thesis in standardized forms and layout (e.g. BMC Evolutionary Biology needs numeric citations in the text, listed in the order of their appearance in the text). Also the section "Material and methods" has been placed in a similar order in each chapter.

The bibliographic documentation has about 200 references. Most of the cited papers have been published in the 21. century (78%), and among them nearly 40% appeared within the last 5 years. This means that Ms Iwona Giska's study is in the middle of the modern research problems, at the crossroads of ecotoxicology, evolutionary biology and population genetics. The above mentioned problems still need to be solved. In this context, the self-citations of three recent papers should be positively perceived.

I am pleased to say that each part of the Ph.D. dissertation has been written mainly in the third person (despite the general discussion), which reflects its multi-authorship. Thus, the rights of other authors have been preserved, which is often masked in many doctoral dissertations. Ms Iwona Giska is the first author in each of the main chapters. Her contribution as the leader is very high, between 75% - 85% in each of the chapters/publications. In many aspects, the form and contents of her dissertation does not differ from the doctoral theses prepared at distinguished universities and research institutions, in many Western countries. Unfortunately, by now, this is not a common form of presentation in a doctoral thesis in Poland. I hope it will be more popular and will become a standard soon. The contents of all chapters are the result of the collaboration and co-authoring with researchers representing the Department of Ecological Science, Free University, Amsterdam (The Netherlands), the Institute of Ecosystem Study, Sassari (Italy) and, of course, the Institute of Environmental Studies, Jagiellonian University (Poland).

The text has been written very clearly, with very few inclusions of inadequate terminology. The format of the dissertation is clear and reader-friendly, apart from some unnecessarily repeated fragments of the text from the earlier chapters.

One of the shortcomings in the Ph.D. dissertation is the lack of a separate table containing a list of abbreviations used in the text, both chemical and genetic terms. Such a table would help the readers, especially in the analysis of methodological correctness and verifications of the text. The Ph.D. thesis should have not only the final summary, but also the final conclusions. This part is also lacking in the evaluated text. The quality of Figs 6A and 6B (pages 81-82) demonstrating the morphological variation of *Lithobius forficatus* needs higher resolution and exact indicators drawn on the photos. I would also expect a wider general discussion. This part of the dissertation should be the right place where all the working hypotheses should be more clearly assessed, verified and critically discussed leading to the final conclusions.

In my opinion Ms Iwona Giska's dissertation fully meets the formal requirements of the doctoral work.

2. Evaluation of the scientific quality of the dissertation

The studies of genotype-environment interaction increased with the rapid development of the molecular genetics techniques. They enable more accurate analysis of the genetic variation in natural populations, including those which inhabit the environment heavily modified by human economic activity, particularly in the terms of chemical contaminants. Living conditions in such kinds of the environment are reduced, giving the chance of living and reproducing only to populations with a high potential of adaptation. Also, a significant progress in the development of techniques for the statistical analysis has been made in respect of molecular genetics, genetic quantitative traits and population genetics. Only those researchers who can skillfully combine the research in the field and in the laboratory, and are capable of using statistical tools properly, can freely move in these areas of knowledge. The author of the evaluated dissertation certainly belongs to the group. She has clearly demonstrated her positive skills: the ability to plan and conduct experiments in the field and in the laboratory, the use of novel molecular techniques which have appeared on "the scientific market" quite recently. Moreover, Ms Iwona Giska has applied her advanced theoretical knowledge to the analysis of the issues presented in the dissertation and, above all, to carrying out the field research and to the use of innovative ddRAD sequencing techniques to estimate genome-wide diversity in studying populations of three invertebrate litter dwelling species.

In my opinion scientifically new and ,to a certain extent innovative, is a wise approach applied to the study of relations between genetic population diversity and multigenerational exposure that elevated the concentration of metals in the selected three species of litter dwelling invertebrates (the earthworm, *Lumbricus rubellus*; the millipede, *Lithobius*

forficatus; and the rove beetle, *Staphylinus erythropterus*) inhabiting sites located along the metal polluted gradient, that differ in their dispersal ability, life span, development and reproduction or dietary behavior.

The studies are comprehensive, combining several areas of the expertise with the use of molecular techniques, in order to explain the significant problems allowing understanding the relationship between intensity of various environmental factors (usually adverse) and the level of genetic diversity of natural populations in three invertebrate species. In fact, in many cases the explanation of a high variability of animals collected in the field, in response to various stressing factors, is difficult. The example based on the animals used in her studies has clearly showed the differences among three species.

The main hypotheses tested by Ms Iwona Giska are clearly derived from the introductory text and are coherent with working hypotheses given in each of the four chapters. The first one: "Long term metal pollution impacts the level of genetic diversity of natural populations of soil invertebrates", seems to be the most important for the whole study and can be identified in the experimental set-up in each of the chapters, including the first one which gives valuable information on the bioavailability of metals to *L. rubellus* exposed to soils from a gradient of metal pollution and modelling of their toxicokinetics. Her study concentrated mainly on the areas rich in metal ores, along the metal pollution gradient, in the vicinity of the large zinc and lead smelter "Boleslaw" in Southern Poland.

Ms Iwona Giska has assessed the population genetic diversity, using population genomics techniques which analyze the responses of non-model invertebrate species to metal-contamination in their habitats. Her analyses have been based on specific mitochondrial and nuclear markers. She has applied commonly used mitochondrial DNA like genes cytochrome c oxidase subunit I, (COI) and ATP synthase F0 subunit 6 (ATP6). In case of the hermaphroditic earthworms (*L. rubellus*), the mtDNA has represented each individual used in the experiments. Moreover, she has used novel nuclear markers, such as the cost-effective identification and genotyping polymorphism applying restriction site-associated DNA (RAD) markers. The data have been obtained by the digestion of genomic material with two restriction enzymes using double digest RAD sequencing. The procedures of RAD sequencing have been presented in details in each of the chapters where this technique has been necessary (Chapters 2,3 and 4). The author benefited from the innovative laboratory protocols published in PLOs One by Peterson et al. (2012), applicable to a wide range of organisms. The method she used is flexible and can be used successfully in laboratory crosses and in pedigreed wild populations, but needs special attention in practically all steps of analyses, especially in applying proper population genetic statistics and adequate forms of visualization of the results.

I wonder whether Ms Iwona Giska expected, at the early stage of her studies, that the molecular analysis would not confirm the hypothesis and expectations that with increased

metal pollution the genetic diversity will be reduced in the order proportional to the migratory abilities of the populations of the three species of invertebrates used in her experiments. The studies have clearly demonstrated an increase of genetic diversity at the most polluted site in the most migratory rove beetle (*S. erythropterus*), and as well in *L. rubellus*, the least mobile among the three species.

The answers to the second hypothesis: "The scale of the impact of pollution on the population genetic diversity depends on the species dispersal abilities" could be seen in the analyses of the results demonstrated in chapters: 2, 3 and 4.

The populations of *L. rubellus* with the weakest possibility to migrate have been highly divergent genetically (five divergent mtDNA lineages) but not isolated reproductively. The author could only suppose that an increased mutation rate in case of L. rubellus at the most polluted site, decides on the highest genetic diversity. Slightly more mobile centipedes, *L forficatus* are rather cryptic species, with suggested reproductive isolation. The rove beetles, represented by the only flying species, *S. erythropterus*, can be the most migratory species showing only a slight genetic differentiation. Despite the increase in insects from the most polluted site, the correlation with the concentration of metals in soil has not been found. In this case an increase of immigrants was supposed to be the influencing factor. Both examples are too speculative and not proved, yet.

The third hypothesis: "In case of low-migratory species showing cryptic speciation pollution controls the distribution of the genetic lineages" is mainly addressed to the identified, the most cryptic speciation of the millipede, *L. forficatus*. Also this hypothesis has not been univocally proved. The author has finished the discussion on this subject stating" The existence of the cryptic species complicates the analysis of the population-level genetic diversity and should be taken into account in the future studies". I would like to discuss with the author how she sees the solution to the problem experimentally, taking into account the fact that *L. forficatus* are iteroparous, with a very long period of ontogenetic development.

The important findings from the studies dealing with toxicokinetics of metals, based on their estimated bioavailability to *L. rubellus* in soils, along the gradient of pollution are as follows:

- Completely different uptake and elimination mode of xenobiotic Pb and Cd and biogenic Zn and Cu metals (mentioned above in the assessment);
- Increase of the uptake rates with increased soil pH, however uncorrelated with other soil quality indices;
- Possibility to estimate the time necessary on the basis of kinetic parameters of the uptake and elimination rates to reach the internal concentrations of xenobiotic metals, like in earthworms inhabiting a given site along the pollution gradient;
- Elaboration of the toxicokinetic model for assessing the bioavailability of metals (xenobiotic – Cd and Pb, biogenic - Zn and Cu) for *L. rubellus* exposed to naturally

polluted soils in the sites along the pollution gradient. The author clearly stated that: "The toxicokinetics approach allows predicting the physiological fate of metals as it provides information about bioaccumulation and time to reach steady-state tissue concentration";

- Indication that soil properties had no effect on the metal uptake kinetics when the total soil concentration was compared. However, the experimental earthworms have tolerated extremely high concentrations of metals from the most polluted site (OL1), but in natural conditions the earthworms have disappeared from this site. The author hypothesizes that drought, not metal pollution, has been the main factor responsible for their absence.

- Indication, that three-week lasting experimental exposure to metals appeared too short to reach the steady state of internal Pb and Cd body concentration. At the same time both biogenic Zn and Cu have been well regulated. Thus, the uptake phase has not been seen, as well as a clear elimination phase, independent of metal concentration in the soil.

- Demonstrating through the metal toxicokinetic models for earthworms that the standard ecotoxicological tests with the earthworms as the model species, should be modified at least in the temporal scale used nowadays, and the necessity of maintaining proper soil water relations.

- Possibility of estimation the time necessary to reach an internal concentration similar to the levels measured in native earthworms collected from the analysed study sites based on the experimental design of the toxicokinetic Cd and Pb models

The bioavailability of metals and toxicokinetic models have been presented only in the case of *L. rubellus.* The impact of pollution on population structure and genetic diversity in two other species have been analysed only in relation to the soil properties, their metal concentrations and bioavailability. I am interested in Ms Iwona Giska's opinion of carrying out such experiments on *L. forficatus* and S. *erythropterus*, taking into account the results already obtained, published in her dissertation.

The indication that the selection of the "proper" species to ecotoxicological experiments needs special attention and genetic proving. The data presented in the dissertation showed clear differences among the populations of animals used. The authors have given the evidence of highly divergent mtDNA lineages of *L. rubellus* that occur in many localities in Poland and conclude that this is a highly polymorphic species, not the cryptic species, at least in Europe (Chapter 2). In case of the millipede, *L. forficatus*, the population genetic assays lead to the differentiation of at least three cryptic species. The identified cryptic clusters are highly divergent genetically, but not morphologically (Chapter 3). *S. erythropterus*, as the most dispersive species among the studied species, has been stated highly diverse genetically (in polluted sites, especially), but uncorrelated with the soil metal

pollution and other indices of the soil properties. The studies of the species have given clear evidence that migratory abilities may counteract not only metal pollution, but also other stresses related to the soil quality (Chapter 4).

The high quality of the research conducted by Ms Iwona Giska is evidenced by the fact that the majority of the study results (three articles) has been published in highly recognized journals from the JCR list, with the aims and scope adequate to the submitted publications: Environmental pollution (IF.2014: 4.143), BMC Evolutionary Biology (IF2015: 3.368) and Ecotoxicology and Environmental Safety (IF2015 : 2.762). Although the papers are of joint authorship, in all of them Ms Iwona Giska is the first author. Her contribution accounts for 75-85% of the four main chapters. The co-authors of these chapters estimated their input from 3% to 15%. A necessary declaration of each co-author has been attached to the doctoral documentation.

I suppose that at least 6 anonymous external researchers with scientific expertise related to this study have had them reviewed and at least 2 editors of each journal have determined their acceptability as the research papers. Thus, I can feel comfortable that somebody else has done the most absorbing and important work as the primary reviewers and editors. Moreover, the co-authors of each of the chapters are distinguished and well known specialists with scientific expertise in ecotoxicology, population genetics and evolutionary biology. I am also sure that the final critical reading and corrections, done on the drafted version by each of the co-authors, made the final documents smooth, readable and scientifically sound.

In my opinion the scientific quality of the PhD dissertation is very high.

3. Final conclusions

All the analyzed elements of the doctoral thesis, presented by Ms Iwona Giska, are highly appreciated. The project is well thought, of outstanding scientific value, skillfully combining three research areas using the common methodology: ecotoxicology, evolutionary biology and population genetics. Ms Iwona Giska, as the PhD student, has presented herself as a person very well and thoroughly prepared for laboratory and field work, able to use modern and sophisticated analytical techniques. She has mastered very well the intricacies of preparing and smooth writing scientific papers, evidenced by her already published significant part of the results, presented in the dissertation in three articles, in journals from the JCR indexed list, reaching the total IF score = 10.293, and a total MNSiW score = 110 points.

Since I highly appreciate the merit of her work, its performance and interpretation, I confidently say that Ms Iwona Giska's dissertation meets the requirements set for the doctorate hearings by applicable law referred to Article 13 of the Act of 14 March 2003. Degrees and the Academic Title and Degrees and Title in the field of art, as amended Act

(Dz. U. of 2003., No. 65, item. 595, no. 164, item. 1365 to 2010. No. 96, item. 620, No. 182 item. 1228 to 2011. No. 84, item 455), and also contained in the Regulation of the Minister of Science and Higher Education of 22.09. 2011. Therefore I ask the High Council of the Faculty of Biology and Earth Sciences of the Jagiellonian University for Ms Iwona Giska's admission to further stages of the doctoral degree, designed to give the doctoral degree in biological sciences and the discipline of biology.

4. Wnioski końcowe

Wszystkie analizowane elementy rozprawy doktorskiej przedstawionej przez mgr Iwonę Giskę oceniłem wysoko. Jest to projekt dobrze przemyślany o wyróżniającej się wartości naukowej umiejętnie łączącej trzy obszary badawcze korzystające ze wspólnych metodologii, lecz odpowiadających na różne w zakresie i kierunkach pytania naukowe: ekotoksykologii, biologii ewolucyjnej i genetyki populacji.

Doktorantka jest bardzo dobrze i wszechstronnie przygotowana do pracy laboratoryjnej; potrafi posługiwać się nowoczesnymi technikami analitycznymi. Potrafi również prowadzić badania terenowe. Opanowała bardzo dobrze zawiłości przygotowywania i sprawnego pisania prac naukowych, czego dowodem jest to, że zdążyła opublikować już znaczną część wyników swoich badań w formie trzech prac w czasopismach z listy A MNSiW, o łącznym IF = 10,293, i przypisanej im łącznej punktacji MNSiW = 110.

Ponieważ wysoko oceniam wartość merytoryczną pracy, jej wykonanie i sposób interpretacji z przekonaniem stwierdzam, że rozprawa mgr Iwony Giski spełnia z naddatkiem warunki stawiane przed rozprawami doktorskimi przez obowiązujące prawo określone w artykule 13 ustawy z dnia 14 marca 2003 r. o stopniach i tytule naukowym oraz o stopniach i tytule w zakresie sztuki wraz z późniejszymi zmianami Ustawy (Dz. U. z 2003 r., Nr 65, poz. 595; Nr. 164 , poz. 1365, z 2010 r. Nr 96, poz. 620, Nr 182, poz. 1228, z 2011 r. Nr 84, poz 455), a także zawarte w Rozporządzeniu Ministra Nauki i Szkolnictwa Wyższego z dnia 22.09. 2011r. Dlatego proszę Wysoką Radę Wydziału Biologii i Nauk o Ziemi Uniwersytetu Jagiellońskiego o dopuszczenie mgr Iwony Giski do dalszych etapów przewodu doktorskiego zmierzające do nadania stopnia naukowego doktora w dziedzinie nauk biologicznych w dyscyplinie biologia.

Paweł Migula

Katowice, 22.02.2016.