Oecologia Montana 2015, **24,** 25-28

Molecular tools in alpine ecology and environmental forensics - current possibilities at Institute of High Mountain Biology

Workshop on current problems of high mountains protection in Norway and the Slovak Republic, Bø, 27.4.2015 - 1.5.2015

M. NÉMETHY
Institute of High Mountain Biology, Tatranská Javorina 7, SK-059 56 Slovak Republic, e-mail: michal.nemethy@uniza.sk

Introduction

Field of molecular biology deals with molecular basis of biological activity. Nowadays it is one of the fastest developing fields in environmental science. In ecology the present knowledge and utilization of molecular tools enable scientists to resolve complicated challenges in the process of studying species individuals and populations, environment and ecological processes. In this quest it serves as a vital extension to basic methods as a different approach of searching for answers for addressed questions within the molecular plan of large scale manifestations of classic biology. In environmental forensics this approach is applied to resolve legal cases connected to environmental crime. The Institute of High Mountain Biology (IHMB) works on studying high mountain ecosystems and addresses the questions of impact of human activities and climatic change. Institute is dealing with various tasks in applied research connected to effective management of high mountain areas. The Institute also guarantee bachelor degree "Land protection and landscape management". The laboratory of molecular biology and microbiology is a part of the Institute since the beginning of its research activities in 2005-2006. Past research in the field of molecular biology, including but not limited to PCR diagnostics, population genetics - microsatellites, analysis of single nucleotide polymorphism (SNP), identification of unknown samples for forensic purposes, analysis of microbial communities was conducted in this laboratory and could be entitled as a "basic methods". Considering the pace of development the institute has interest to keep up with the current research trends in other scientific institutions. Therefore the laboratory of molecular biology was upgraded for advanced methods in molecular biology with the help of European funding. The upgrade now allow to the staff of IHMB to perform different tasks within the different subfields of molecular biology i.e. genomics, proteomics, transcryptomics and metabolomics. It also improved educational options for students at IHMB.In favour of the educational development in science disciplines a joint master degree program (joint degree) "alpine ecology" was prepared for accreditation at the IHMB in cooperation with the Norwegian partner university Telemark University College (TUC). IHMB cooperates for several years with the Norwegian TUC in the exchange of students in the study programs. From April 27th to May 3rd the selected staff of IHMB organized and took a part at the workshop with the partners from TUC. The aim of this meeting was to enhance and discuss mutual cooperation and present current options for research and education at both partner universities in order to uplift the level of planned joined master degree. The presentation "Molecular tools in alpine ecology and forensic science" with a subtitle "Past research, current and future possibilities at Institute of High Mountain Biology" was part of the workshop. Thus the aim of this article is to summarize past and current research options at IHMB and briefly mention outcomes of discussion that followed it.

Past research at IHMB

PCR diagnostics

Method based on targeted DNA amplification in the Polymerase Chain reaction (PCR) developed by Kari Mullis in 1983 to detect the presence of pathogen organisms and diseases (Yang and Rothman 2004). PCR together with microscopic techniques was used at IHMB for identification of blood parasitoids i.e. *Leucocytozoon fringillinarum*, *Haemoproteus sp.* in *Prunella modullaris* (Haas *et al.* 2012) and *Chionomis nivalis* (Janiga *et al.* 2012). Furthermore it was identification of intestinal bacteria in *Prunella modullaris* (Kisková *et al.* 2011).

Identification of unknown species

IHMB got request from police to cooperate with them on potential crime of poaching. Forensic samples were provided as the evidence. Samples were from unknown sources. The PCR identification based on universal primers was performed. PCR

products were compared with online database. Finally the samples were identified.

Population genetics

Population genetics is the study of the frequency and interaction of alleles and genes in populations (Postlethwalt 2009). IHMB is involved in research on four vertebrate species Brown bear (*Ursus arctos*), Alpine accentor (*Prunella collaris*), yellow-bellied toad (*Bombina variegata*) and Carpathian newt (*Lissotriton montadoni*). The method in use is microsatellite analysis. Microsatellite or short tandem repeats (STRs) are sequences of DNA composed of single sequence motifs two to six bases long that is continuously repeated without interruption by any base or motif. These repetitions have been detected in every organism analyzed so far. There are mostly distributed in non-coding regions of DNA. Important attribute of these sequences is that they are highly polymorphic due to slipped-strand mispairing during DNA replication (Goldstein and Schlötterer 1999). Thus multiple alleles are present in the gene pool and their length based discrimination by electrophoresis may be used to address the question of the impact of processes such as natural selection, mutations, genetic drift and gene flow on population genetics. Another tasks performed are identification of individuals and relatedness of individuals. IHMB has suitable primers for amplification of microsatellites in four mentioned species, we managed to optimize all involved reactions (DNA isolation, PCR) and we also have capillary electrophoresis at disposal to separate amplified fragments according to length in the process of genotyping.

Bacterial community analysis

Terminal fragment length polymorphism was used to assess spatial and temporal changes in composition of microbial communities in fresh water snow and peat. Method is based on isolation and amplification of metagenomic DNA from samples using fluorescently labelled primer. Restriction enzyme is used to cleave the amplified fragments, for different species there are different loci of cleavage therefore the terminal restriction fragments (T-RF's) from different species differs in length. Capillary electrophoresis or Sanger sequencing both available at IHMB are then performed to identify and quantify these T-RF's.

Single nucleotide polymorphisms (SNPs)

SNPs refers to mutations in genes in a form of change in single nucleotide. IHMB conducted research on SNPs in ALAD gene (Hrehová 2009). The gene is involved in the process of synthesis of heme (protein structure). Mutations at the gene may induce a risk of developing lead poisoning in organisms exposed to lead contamination (Onlaja and Claudio 2000). To analyze of mutations IHMB used available sequencer.

Current possibilities at IHMB

Current aim of IHMB is to implement novel techniques that are now available at IHMB with the recent upgrade of the laboratory into the environmnetal forensics science and research in molecular biology. New

Fig. 1. Next generation sequencer at IHMB.

equipment enable the IHMB staff to perform complex integrated research in different subfields or "omics" of molecular biology s.e. genomics, proteomics, transcriptomics, metabolomics. The forensic application of the knowledge and available tools for research in these paricular omics would be explained in following paragraphs. By forensics we mean the using scientific methods to explain environmental and wildlife crimes, however current aim in process of lecturing students will also touch major crimes (humans).

Genomics

The genomics is a discipline in molecular biology which study complet set of individuals DNA, genome, its structure and functions, interaction between genes and interactions between genes and environment. It works with methods of recombinant DNA, sequencing and bioinformatics (National Human Genome Research Institute 2010). The biggest asset of IHMB in the genomics is next generation sequencer (Andreassen *et al.* 2012) miSeq from Illumina© (see figure 1). This machine has various implications for research. One of the features is 16 S Amplicon sequencing. This analysis is capable to substitute t-RFLP in analysis of metagenomic DNA from microbial communities with finer resolution and capability to identify strains that are hard to find using older methods. Another feature is de novo sequencing which involves sequencing a novel genome for the first time, and requires specialized assembly of sequencing reads that might be used in identifying new species. Application in forensics comprise ability of this machine to utilize multiple assays with various markers for the purpose of DNA profiling and identification of individuals while using very little amount of sample. This comes handy in forensic cases and ecological research where both quality and quantity of samples are often very low i.e. samples collection from indirect sampling or in terms of human forensics disaster victim identification or identification of missing person from mtDNA (old cases). Further application that is vital for molecular ecology is possibility of creating new type of assays such as design of a new primer for microsatellite analysis. This way we would be able to optimize assay directly for our subject species which would provide us with more reliable data.

Valuable tool connected to genomics and transcryptomics that is available at IHMB is microarray workstation which will be introduced in the next paragraph dedicated to trascryptomics.

Transcryptomics

Microarrays provide link between the static genome and dynamic proteome in study of transcryptome the complete set of RNA molecules in the cell. It infers expression of proteins from measurement of corresponding mRNA in the process of parallel hybridization. In this process great number of oligonucleotides (probes) is bounded to the known locations on an array. Sample (target) is prepared with fluorescent tags and is added to the array. Some components of the sample bind to array. Successfully fixed targets display the fluorescence. The sequence and the positions of probes are known therefore we are able to identify components of the sample based on hybridized positions (Lesk 2012). Described technique has wide potential in the analyses of DNA and RNA for scientific and forensic purposes. Analyses of RNA could help in research of diseases, diagnoses, revealing cause of death and determination of age of wounds and post-mortem interval (Bauer 2007). DNA analysis by microarray involves analysis of SNPs for the processes of assessing relatedness of individuals when provided samples are in low quality (Divne 2005). To perform transcriptomic analysis IHMB have "Sure Scan Micro Array Scanner" from Agilent technologies©, hybridization oven and tools for samples preparation- microtome and cryo-microtome.

Proteomics

Proteomics refers to the large-scale study of proteins their functions and structure (Anderson, 1998). For the forensic research it might help in identification and analysis of biological matrices. Relevant biological matrices for an analysis for forensic purposes are blood (human or non-human), saliva, semen, vaginal fluid, and to a lesser extent nasal secretions, feces, and urine. The proteomics in this case might not only help in identification of matrix but also the species to which it belongs (Van Steendam *et al.* 2013).

In the fields of proteomics IHMB has two main tools at its disposal. Enzyme linked immunosorbent assay (ELISA) has the possibility to detect and quantify proteins in sample. It works on the principle of bonds between antigen structures and antigen specific antibodies. Based on the type of Elisa one of mentioned is immobilized to ELISA plate well surface and then it is incubated together with either antigen specific antibodies or antigen. After the following wash only analyte of interest will remain bounded to the plate. Antibodies are enzyme stained for further steps if the process. These steps include adding of solution that would start the enzymatic reaction on stained antigens what results to color response when stained antibodies are present (Reen 1994). Thus we are able to distinguish the presence of analyte in the sample and in addition be able to quantify the amount of analyte since there is a correlation between the amount of analyte and radiated color intensity. IHMB has Biotek © Synergy HTX tool that is able to perform ELISA and as part of it measure absorbance and fluorescence and thus quantify the amount of analyte (protein).

Another tool for proteomic analysis is two-dimensional electrophoresis (2D ELFO). This is the two step reaction. First step respectively first dimension separates protein according to their isoelectric point (Ip) using electrophoresis in pH gradient. This process is entitled "Isoelectric focusing"(IEF) and IHMB have the Protean i12 IEF System tool to perform this analysis. The proteins separated in IEF are passed to second step respectively dimension which will separate the proteins based on length and molecular weight in polyacrylamide gel electrophoresis. Following step is the staining and imaging and visualization of the gel. For this purpose IHMB has two machines available - Pharos FX molecular imager and Calibrated Densitometer GS-800 both from Biorad ©.

For more detailed information on involved steps and features of 2D ELFO check the review by Rabilloud *et al.* (2011). After 2D ELFO we can perform downstream analysis of separated proteins, protein blotting or mass spectrophotometry. This way we have the advantage of ability to perform complete protein profiling from sample, from isolations to separations of proteins and following downstream analysis for protein identification and quantification.

Metabolomics

Subject of analyses is the metabolome. It represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes. The research in metabolomics is concerned with assessing organism health and function at the molecular level as the impact of interactions between organisms and environment response to biota and abiotic stressors (Bundy 2009).

Two of the main tools used in metabolomics that are also available at IHMB are mass spectrophotometry (MS) and gas chromatography (GS). At IHMB we tend to study effects of anthropogenic contaminants.

Conclusion

Current possibilities of IHMB in the field of molecular biology allows us to perform complete analysis of biological samples from the study of the genome (Genomics), its expression (Transcryptomics) and products of expression (Proteomics) and in addition the products of other cellular processes (Metabolomics). In the discussion with academics from TUC that followed after the presentation we emphasized the novel techniques and their implementation into not only our future research but also into the process of lecturing the courses biotechnology and forensic science as a part of our planned joint master degree. These techniques will be comprised in both subjects. Furthermore discussion about possibilities of cooperation in the research involved utilization of NGS for metagenomics analysis of bacterial communities since both institutions have ongoing research on this subject and project of analysis of TBE virus in mice using microarray work station. Overall outcome is thus fruitful both from educational and scientific point of view.

Acknowledgements

Activity was supported from the EEA and Norway Grants BFENV14-002.

References

Anderson, N. L. and Anderson, N. G. 1998: Proteome and proteomics: new technologies, new concepts, and new words. *Electrophoresis* **19**: 1853-1861.

Andreassen, A., Jore, S., Cuber, P., Dudman, S., Tengs, T., Isaksen, K. and Vainio, K. 2012: Prevalence of tick borne encephalitis virus in tick nymphs in relation to climatic factors on the southern coast of Norway. *Parasit Vectors*, **5**: 177.

Bauer, M. 2007: RNA in forensic science. Forensic Science International: *Genetics*, **1**: 69-74.

Bundy, J.G., Davey, M.P and Viant, M.R 2009: Environmental metabolomics: a critical review and future perspectives. *Metabolomics*, **5**: 3-21.

Divne, A.M. and Allen, M. 2005: A DNA microarray system forforensic SNP analysis. *Forensic Science International*, **154**: 111-121.

Goldstein, D.B. and Schlötterer, C. 1999: Microsatellites: Evolution and Applications. Oxford University Press, Incorporated, Oxford.

Haas, M., Lukáň, M., Kisková, J. and Hrehová, Z. 2012: Occurrence of blood parasites and intensity of infection in *Prunella modularis* in the montane and subalpine zone in the Slovak Carpathians. *Acta Parasitologica*, **57**: 221-227.

Hrehová, Z. 2009: Lead concentration in endothermic vertebrates and ALAD polymorphism. *Oecologia Montana*,**18**: 39-41.

Janiga, M., Hrehová, Z. and Kostková-Zelinová, V. 2012: Seasonal effects of lead uptake by Snow vole *Chionomys nivalis* (Martins 1842) in West Tatra Mts: Bone metal concentrations and hematological indices. *Polish Journal of Ecology*, **60**: 611-619.

Kisková, J., Hrehová, Z., Lukáň, M., Haas, M. and Jurčovičová, M. 2011: Yersinia Species in the Dunnock (*Prunella modularis*) in Sub-alpine Habitats of the Western Carpathians. *Polish Journal of Microbiology*, **60**: 79-83.

Lesk, A. 2012: Introduction to Genomics. Oxford Universty Press, Incorporated, Oxford.

National Human Genome Research Institute 2010: A brief guide to genomic. https://www.genome.gov/18016863 (retrieved 13.5.2015).

Onalaja, A.O. and Claudio L. 2000: Genetic susceptibility to lead poisoning. *Environmental Health Perspectives*, **108**: 23-28.

Postlethwalt, J.H. and Hopson, J.L. 2009: Modern Biology. Holt, Rinehart and Winston, Austin.

Reen, D. (1994). Enzyme-Linked Immunosorbent Assay (ELISA). Basic Protein and Peptide Protocols, *Humana Press*, **32**: 461-466.

Van Steendam, K., De Ceuleneer, M., Dhaenens, M., Van Hoofstat, D. and Deforce, D. 2013: Mass spectrometry-based proteomics as a tool to identify biological matrices in forensic science. *International Journal of Legal Medicine*, **127**: 287-298.

Yang, S. and Rothman, R. E. 2004: PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. *The Lancet infectious diseases* **4**: 337-348.