Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice
Czech Republic

Confidential

Review of USB FFPW PhD Thesis

First name(s), surname, titles of the PhD student: Milos Havelka, Ing.	**First name(s), surname, titles of supervisor:** Assoc. prof. Dipl.-Ing. Martin Flajshans, Dr. rer. Agr.
Title of PhD thesis: Molecular aspects of interspecific hybridization of sturgeons related to polyploidy and in situ conservation	

REVIEWER:

Surname: Arai	**Institution:** Faculty of Fisheries Sciences Graduate School of Fisheries Sciences School of Fisheries Sciences, Hokkaido University 3-1-1, Minato-cho, Hakodate, Hokkaido 041-8611, Japan
Name: Katsutoshi	
Titles: Prof.	**E-mail:** araikt@fish.hokudai.ac.jp
Please describe your professional relationship to the PhD student: International referee/Board of doctorate study	**Please describe your field of expertise:** Genetics and Developmental Biology of Fish and Shellfish

QUESTIONNAIRE

Originality, scientific importance, perspectives and impacts of results presented in the PhD thesis for basic and/or applied research

Evaluate competitiveness of the PhD thesis in the international context and compare its level with the current state of the art in the field **(extent ¼ – ½ page)**:

The Ph.D thesis submitted by Mr. Milos Havelka has focused the research aim to clarify the mechanisms of structural duplication and functional reduction of sturgeon genomes, with special references to the possible involvement of interspecific hybridization.

To evaluate genome duplication status, he had a unique approach with microsatellite data together with ploidy determination by flow cytometry. First, he disclosed that flanking regions of microsatellite arrays were highly conserved among sturgeon species belonging to different genus. Next, he showed that cross-species amplification was possible using PCR primers developed in different species of different genus within the family Acipenseridae. Finally, based on maximum numbers of alleles per locus, he successfully assigned A group sturgeons(ca 120 chromosomes, 4-5pg DNA content), B group sturgeons (ca 250 chromosomes, 8-9pg) and C group suturgen (372 chromosomes) into functional diploid, tetraploid and hexaploid status. However, presence of microsatellite loci demonstrating tetraploid, octaploid, and dodecaploid allelic band patterns in A, B, and C group sturgeons, respectively, strongly suggest the secondary on-going differentiation into lower ploidy status or functional reduction of genomes from 12n to 6n, 8n to 4n and 4n to 2n in these groups of sturgeon. These results are highly evaluated as the valuable conclusion on sturgeon genomic status based on original molecular approaches to infer contemporary genomic situation of sturgeon species.

The Ph.D candidate also showed that spontaneous genetic triploid sterlet was a true autotriploid, which was not appeared by sporadic hybridization, but by spontaneous polar body retension, based on mitochondrial DNA haplotype

and assignment test (STRUCTURE and others) of microsatellite genotypes.
In fish genetics, triploidy has been generally considered sterile or infertile because extra set of chromosomes should hinder meiotic division and subsequent gametogenesis. However, Ph.D candidate found that a spontaneous triploid Siberian sturgeon was fertile. Based on genome size, Siberian sturgeon belongs to group B (tetraploid) and thus spontaneous triploid could be considered as „hexaploid". Functional hexaploid male with even number of chromosome sets could generate fertile spermatozoa and successfully produced viable progeny with intermediate ploidy status (pentaploid) after hybridization between Siberian or Russian sturgeon females (functional tetraploid) and triploid Siberian sturgeon male (functional hexaploid). The results presented here suggest the probable involvement of spontaneous autopolyploidization and subsequent hybridization on production of new fertile allopolyploid sturgeon in the course of evolutionary history of this unique fish groups.
Unfortunalely, the candidate has not concluded inheritance manner of microsatellite alleles in the final chapter, but he obtained Mendelian segregation of microsatellite alleles in progeny between different ploidy sturgeons. In results, possible gynogenetic progeny existed and some progeny exhibited deviation from expected ratio. Thus, further studies are required on segregation manner of microstellites in sturgeon species.
Overall, Ph.D candidate successfully achieved scienticically significant and highly interesting results in his thesis and his present (J Appled Ichthyol; J Applied Genet) and future publications will provide high impacts on the field of fish genetics, especially related to basic studies on evolution via polyploidization and hybridization as well as applied aquaculture and environmental studies to conserve endangered species, such as sturgeons.

Elaboration of the PhD thesis, objectives of the work and deliverables
Evaluate the overall level of elaboration of the PhD thesis (structuring of the main text, comprehensibility, logicality of the chapters and their ordering) and the originality of the selected approaches to solve the objectives; evaluate publications and whether the results described correspond to objectives of the PhD thesis **(extent ¼ – ½ page)**:

In first Chapter 1, the PhD candidate、Mr. Milos Havelka, provides a comprehensive review related to 1)reasons for sturgeon population decline (over exploitation for black cavier, anthropologic habitat destruction, loss of genetic diversities), 2)evolution of sturgeon genome size estimated by DNA content and karyology, 3)natural and artificial hybridization of sturgeon by referring or citing more than 100 original or review papers and then he listed up aims of the thesis. This introduction section almost completely covered present status of sturgeon biology, genetics, cytogenetics and genomics as well as recent situation of sturgeon biodiversity, habitats, distribution, utilization and conservation.
According to the order of four major aims listed in the first introduction chapter, scientific contributions were shown as in Chapter 2 „Extensive Genome Duplication in Sturgeons: New Evidence from Microsatellite Data", Chapter 3 „First Evidence of Autotriploidization in Sterlet", Chapter 4 „Fertility of a spontaneous triploid male Siberian sturgeon", and Chapter 5 „ Investigating the segregation pattern of microsatellite alleles during experimental hybridization of polyploid sturgeon species". All these data were discussed and finalized to conclude in Chapter 6 General Discussion. Structure of PhD thesis is generally well elaborated and major text was well prepared and reasonably written, except for Chapter 5, in which, results seem to be somehow inconclusive and fragmentary. Unfortunately, the discussion section was not shown in Chapter 5. However, over all story of the thesis is very good and all the results were reasonably described corresponding to objectives raised in the introduction section. I have nothing about the comprehensibility and logicality of the thesis.

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OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

The PhD thesis submitted by Mr. Milos Havelka includes scientifically important results and will contribute not only to basic studies of sturgeon genetics, cytogenetics, genomics and evolution, but also to applied studies oriented to aquaculture, rehabilitation of wild stock by release of artificial seedlings, cavier food technology and conservation. Candidate has well studied various aspects of sturgeon issues from decline of natural populations, taxonomic confusion, human impacts on water environment in Europe, North America and China as well as present status of molecular genetics related to sturgeon evolution, with special reference to elevation of ploidy status and influence of hybridization. From both basic and applied points of view, general introduction and background of studies have been well written as the first chapter and the objectives of the thesis shown here were very clear.

Original and important contribution of this thesis was in determination of ploidy levels of group A, B and C sturgeons from microsatellite analyses using cross-amplified 11 loci (Chapter 2). PhD candidate clearly demonstrated functional diploid, tetraploiod and hexaploid status of A, B and C group sturgeons based on maximum allele number per locus in several loci, but residual tetraploid, octaploid and dodecaploid allelic band patterns were also detected in other loci. These results indicated recent scale ploidy status or functional reduction of ploidy level, but inheritance manner (disomic, tetrasomic, or octasomic allelic segregations) was not been determined in this thesis. Therefore, ploidy status or genome size of group A, B and C sturgeon species was concluded, but true inheritance manner of these sturgeon is still unknown. To solve this genetic problem, segregation studies using much larger-sample size progeny, large number of microsatellite loci and other codominant marker loci, and strict statistic approaches are required in near future. Observation of meiotic configurations (presence or absence of bivalents, multivaltens) including TEM observation of synaptonemal complex is also informative to consider inheritance manner of sturgeon species.

In next chapter 3, this thesis clearly showed that natural triploid sturgeon presumably occurred by spontaneous polar body retention and no hybridization contributed the elevation of ploidy status, based on mtDNA haplotypes and microsatellite analyses. The occurrence of spontaneous polyploids (mainly triploid) is often, especially in artificial propagation process. Thus, over ripening of ova may be related to this phenomenon.

More interesting contribution of this thesis was to provide clear evidence that genetic triploid (i.e., functional hexaploid) male of B group sturgeon (functional tetraploid) was fertile and could produce viable progeny with intermediate ploidy status when functional tetraploid and hexaploid sturgeon were crossed (Chapter 4). The results gave possible elevation of ploidy status by cross-breeding between different ploidy sturgeons. In this chapter, candidate provided high quality chromosome spreads of functional diploid, tetraploid, pentaploid and hexaploid sturgeon. Such high quality chromosome spreads were convincing and persuasive cytogenetic evidence of the present results.Hybridization between sturgeons with different ploidy status may produce new fertile polyploid which is able to be a steping stone to new polyploid species. Further investigations on the performance of inter-ploidy hybrids is interesting from the view points of reproductive potential of inter-ploidy hybrids.

In chapter 5, the candidate tried to investigate segregation pattern in microsatellite and then to conclude inheritance manner, but the results were still inconclusive and fragmentary. The data shown in tables had not been analyzed by proper statistic approaches, probably due to the shortage of sample size. Further studies to follow this thesis are required in near future. Some data in table are very interesting, because no contribution of paternal parent, i.e., gynogenetic development, was suggested.

Over all results and conclusions provided by this PhD thesis are highly evaluated, because the candidate provided ploidy status of different groups of sturgeon based on new estimation methodology based on microsatellite data as well as clear relationship between fertility and functional hexaploidy. All these results have provided new insights to sturgeon genetics and genomics. The quality of this PhD thesis is concluded to be more than the international standard.

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FINAL RECOMMENDATION

- [x] **PhD Thesis can be recommended for defence**
- [ ] **PhD Thesis can be recommended with reservations for defence**
- [ ] **PhD Thesis can not be recommended for defence**

June 17, 2013 Hakodate
Date and place

Katsutoshi Arai
Name and signature

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First name(s), surname, titles of the PhD student: Miloš Havelka, Ing.	**First name(s), surname, titles of supervisor:** doc. Ing. Martin Flajšhans, Dr. rer. Agr.
Title of PhD thesis: Molecular aspects of interspecific hybridization of sturgeons related to polyploidy and in situ conservation	

REVIEWER:

Surname: Kalous	**Institution:** Česká zemědělská univerzita v Praze Kamýcká 961/129 Praha 6 - Suchdol
Name: Lukáš	
Titles: doc. Ing., Ph.D.	**E-mail:** kalous@af.czu.cz
Please describe your professional relationship to the PhD student: I do not have any.	**Please describe your field of expertise:** Ichthyology, Aquaculture, Human impact on aquatic ecosystems

QUESTIONNAIRE

Originality, scientific importance, perspectives and impacts of results presented in the PhD thesis for basic and/or applied research

Evaluate competitiveness of the PhD thesis in the international context and compare its level with the current state of the art in the field **(extent ¼ – ½ page)**:

Today there are approximately 3500 records in the database "Web of Science" for the term "sturgeon" with more than 200 papers published last year. The topic "sturgeon aquaculture" gained 5250 hits in Google in June 2013. There are no doubts that sturgeons are actual topic for both basic and applied research. The topic sturgeon draws together basic and applied research in many aspects whether for the conservation purposes or for aquaculture. One of these aspects is *e.g.* an urgent need of proper fish identification and understanding of evolutionary ploidy level mechanisms.

The PhD thesis of Miloš Havelka composes of overall review of the sturgeon problematic and presents two published papers, one in Journal of Applied Ichthyology and the second in the Journal of Applied Genetics. Furthermore the PhD thesis includes one manuscript ready for submission and "draft" of the results from the experimental hybridization of polyploid sturgeon species. Presented results are valuable contribution for progress in solving aforementioned needs. The publications in peer review journals (from which one is the platform for sturgeon research) I see as prima facie evidence of an adequate high standard of the presented work in terms of international context and the current state of the art in the field.

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Structuring of the text as well as comprehensibility and ordering of the chapters is well done. I was able to read the text without difficulty and the level of English is very good. There is logical and rational links between the component parts of the thesis. The selected approaches to solve the objectives I found conventional but the matter on which they are applied is very unconventional. Such situation needs more analytic work for understanding the complexity of sturgeon story when explaining data from the conventional analysis. I appreciate involvement of cytogenetics namely karyology which by my opinion is necessary for better understanding of the data from molecular genetics and ploidy level analysis.
Presented publication passed lengthy and often painful process of peer review, by probably better expert in the field than me. Anyway after careful reading I could not find anything I had to criticize and I may say that the results described correspond to objectives of the PhD thesis.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

The sturgeons are enigmatic fish attracting attention not only of biologist; however the more biologist is fascinated when penetrating into interesting combinations of ploidy levels produced by hybridization or autopolyploidy mechanisms. I welcomed the recommendation of author to discriminate terminologically between chromosomal ploidy and DNA ploidy of sturgeon species. This obvious difference in genetic and cytognetic ploidy led to many misunderstandings in the scientific community.
While reading the work, I could not help myself to feel certain scepticism with regard to the complexity of the problem and common practices in aquaculture or in the management of aquatic ecosystems. Escapes from aquaculture, migration obstacles in rivers or in well-intended reintroduction without sufficient knowledge of fish stocks are likely to lead to further hybridization and outbreeding. Here I come to one question, which I would like to ask. Perhaps the evolutionary plasticity of sturgeon in relation to ploidy and functional reductions events were the reasons why they were able to survive 200 million years to the present. How the author will respond to the rather bold claim that the processes pursued in his work with sturgeon are just normal strategies of their genes and genomes how to survive. How is it with the ancestors of contemporary sturgeon species and their hybridization in the past? Are there any information or could we get some?

Presented work does not solve the overall complexity of the sturgeon story but it is valuable contribution to do so.

After successful defence I recommend to award Miloš Havelka the title PhD.

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FINAL RECOMMENDATION

☒ **PhD Thesis can be recommended for defence**
☐ **PhD Thesis can be recommended with reservations for defence**
☐ **PhD Thesis can not be recommended for defence**

27. 6. 2013 Únětice

.....
Date and place

Lukáš Kalous

Name and signature