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Review of the PhD thesis
An integrative taxonomic approach to the study of trematode diversity
and life-cycles in freshwater ecosystems,
submitted by Simona Georgieva

Generally, the thesis is a nice piece of work that is comprised of eleven papers published in internationally renowned journals, and accompanied by an introductory part and concluding remarks.

First of all, a high quality of the papers needs to be underlined. They have been published in four top journals in the field of parasitology; the "International Journal for Parasitology" and the journal "Parasites and Vectors" should be mentioned as examples. Simona served four times as the first author.

Several trematode genera with complicated taxonomic relationships at the species level have been in focus - *Diplostomum*, *Tylodelphys*, *Echinostoma*, *Petasiger* and *Plagiorchis*. It should be declared that most of the papers are very complex in terms of morphological and molecular data presented together to the readers. Many of them could be mentioned in this place, so only a few examples: In case of the genus *Diplostomum*, an unrecognized genetic diversity for the European members (including Iceland) has been presented. Along with a thorough molecular analysis, clear morphological characterization of the samples (including excellent micrographs and drawings) should be appreciated. As additional examples, further description of the life cycle of *Petasiger islandicus*, or the diversity of *Plagiorchis* spp. in *Lymnaea stagnalis* and *Radix auricularia* could be mentioned. All the papers represent evidence for a broad international cooperation; the co-authors were from Germany, Iceland, Spain, United Kingdom, etc.

As for the introductory chapters, I appreciate an understandable overview of the taxonomic situation (e.g. for *Diplostomum* sp.). The importance of integrative taxonomy for adequate characterization of taxa is clearly explained. At the beginning of the text body (p. 3), necessity of a correct recognition of species for the study of evolutionary mechanisms and ecology is underlined; I would say that correct recognition is also important for other, more practical reasons - diagnostics, treatment and diverse pathogenicity linked with particular species of parasites. Later on, also these aspects are mentioned by Simona in the text, e.g., in the part describing cryptic species (p. 6) or under pathogenicity of *Diplostomum* sp. As for the aims, it should be noticed that the tasks are exacting and ambitious - integrative analyses of five trematode genera from unrelated families. Seriously to say, the results correspond to those ambitious aims - eleven papers are certainly more than it would be expected for a typical PhD thesis.

As far as the concluding remarks are concerned, the main results are nicely summarized and highlighted (e.g. global situation with the species and lineages of *Diplostomum*). As for the future prospects, I can understand authors' personal opinion with grant agencies and their willingness to provide money for e.g. ecological, morphological and molecular studies. However, I do not fully agree that (iii) description of life cycle stages is a financially unsupported area. In my view, parasitologists have an advantage: they can link their basic research with the generally accepted importance of parasites as pathogens. Speaking about the life cycle stages, this item can always be linked with host-to-host transmission of parasites and epidemiology/epizootology.

Perhaps one criticism at the end: I think that the additional three papers in the appendix 5.3 are not suitable for the thesis. The thesis itself should strictly reflect the main aims - there is no need to convince the readers about Simona's ability to write additional papers.

Taken together, I have no doubts that Simona Georgieva is a good scientist. She produced more than sufficient number of publications and proved that she is fully qualified to obtain PhD title.

Prague, 9th February 2015


Prof. Petr Horák

Mrs. Ing. Dagmar Kaftanová
Studijní Oddělení
Přírodovědecká Fakulta
Jihočeská Univerzita v Českých Budějovicích
Branišovská 1760
370 05 České Budějovice
Czech Republic

Re: PhD Thesis / Simona Georgieva; Institute of Parasitology, České Budějovice

6 February 2015

Dear Mrs Kaftanová,

Please find attached my review of Simona Georgieva's PhD thesis entitled:

An integrative taxonomic approach to the study of trematode diversity and life-cycles in freshwater ecosystems

supervised by Dr Aneta Kostadinova.

I apologise if this is somewhat later than expected, but still hopefully within the recommended time limits for completing the review, in advance of the PhD oral examination.

With kind regards,



DTJ Littlewood, PhD DSc
INDIVIDUAL MERIT RESEARCHER
HEAD OF DEPARTMENT OF LIFE SCIENCES

Simona Georgieva, PhD thesis:

An integrative taxonomic approach to the study of trematode diversity and life-cycles in freshwater ecosystems

Overview

The candidate has provided a comprehensive and outstanding body of work, tackling complex taxonomic problems of common trematode parasites of freshwater ecosystems. The selected taxa are diverse and well known, but poorly understood systematically in spite of considerable taxonomic scrutiny and effort over generations. Through the carefully executed integration of molecular data and by considering life-cycles, larval stages and ecology, the candidate has provided resolution to a number of long-standing taxonomic and nomenclatural issues, indicated useful diagnostic features and opening the way for these host-parasite systems to be better understood and utilised. This is important, since the chosen taxa (hosts and parasites) have long been selected for providing deeper insight into parasite ecology and parasite evolutionary ecology, and have great potential. Providing careful baseline data and robust phylogenies, upon which to build and test ecological and evolutionary hypotheses, is a pivotal role for systematics. Species delimitation is the first step and the candidate's choice of taxa was courageous since these taxa included many taxonomic problems known to require serious attention; additionally, the molecular prospecting undertaken revealed even more diversity thus potentially adding even more confusion.

The thesis is underpinned by a number of clearly articulated questions, aims and objectives, with each chapter reflecting a substantial component in the journey from 'taxonomic noise' to 'taxonomic signal'.

The thesis is comprised of 11 papers, of which the majority are substantial stand alone contributions, tackling a diversity of topics. Taxonomic, geographic, and host/ecological coverage is diverse and impressive, drawing upon a substantial network of collaborations, considerable new and historical fieldwork and archival samples made available by the supervisor(s). Analytical techniques undertaken are appropriate, up to date, comprehensive and expertly executed providing each chapter with an authoritative perspective. Similarly, the context underpinning each chapter is well articulated with excellent links to important and historical literature. The interpretation is closely aligned with the underlying aims and objectives and in light of modern techniques, debates and competing hypotheses/interpretations. Collectively, the papers and the contributions made to them by the candidate comprise an exemplary PhD thesis.

The introductory and concluding remarks are thorough and put the individual studies into a broader context. The synopsis of the main findings is accurate and reflects the key contributions made by each paper/study. Cumulatively, the study amounts to greater than the sum of the individual parts, reflecting a high level of intellectual understanding, perspective and professionalism.

I enjoyed the synopsis and the section on future prospects. The candidate has provided an excellent thesis.

Viewpoints and questions for the candidate to consider for discussion in the oral examination (note; it is unlikely there will be time for all of these questions as they might open up wider discussions).

Paper I – The opening paper sets the scene for how the candidate employs the predetermined methods for applying an integrative approach to taxonomy and species delimitation.

Q – in the context of an integrative approach and in light of molecular taxonomy and modern molecular methods, can the candidate explain the role of vouchers?

Q – elsewhere in the thesis, but relevant here and following on from the question above, can the candidate explain what a 'hologenophore' is and how does this concept work practically when dealing with trematode adults and larval stages?

Q – given the likely diversity of *Diplostomum* revealed through the integrative approach, what is the likely future of the markers chosen (cox1 and ITS1) in extending this work further?

Paper II – a further exploration of species delineation in the context of geographic distribution with important indicators that latitudinal differences exist in parasite diversity.

Q – the candidate used CAOS to indicate diagnostic molecular (base change) markers; looking at p.89 Supp Fig 5 and considering the 2 different genes, which of these markers/genes might be more valuable/reliable now and in the longer term?

Paper III – an elegant piece of descriptive taxonomy linking larval stages to adult stages linked to molecular lineages. No questions.

Paper IV – another iterative step in applying integrative approaches to tackling *Diplostomum* taxonomy providing a geographic perspective on species diversity within the genus.

Q – considering Clade Q (Fig 3, p.126), what definitive hosts are likely to have these species and how might one go about testing this rapidly?

Paper V – barcodes considered in the light of exploring African diplostomid diversity with taxonomic revisions.

Q – in light of this study, what are the candidates views on when to rename/revise genera? Or, how important is it for taxonomic stability for genera to be monophyletic?

Q – are there alternative methods to completing life cycles in the lab in terms of discovering adult forms of species only known as larval forms?

Paper VI – cryptic species of *Echinostoma* 'revolutum' group. Another highly complex and important genus being given the integrative taxonomy approach.

Q – what might be the limitations of a dissection/isolation/PCR approach in establishing the true diversity of parasites in these snail hosts? I'm curious here about prospects with modern sequencing tools, eDNA and metagenomics; how might one do this with modern tools, any thoughts?

Paper VII – species complex revisited with new data. As befits a good thesis taking discrete steps, this chapter builds on the previous work to clarify taxonomic issues and species limits further.

Q – the suggestion that nad1 should be the barcode of choice is based on the comprehensive reference library accumulated so far. However, keeping in mind an integrative approach is best, at what point should this choice of marker be revisited?

Paper VIII – reassessment of species diversity; another elegant piece of work pulling out useful morphological characters for the accurate identification of both adult and cercarial stages, combined with revisionary systematics.

Q – if all the stages of a life cycle are known, how useful is this knowledge knowing also that many parasites use multiple hosts? Or to put another way, what role does life-cycle play in taxonomy and systematics.

Paper IX – life cycle of *Petasisiger islandicus*. Simple, yet well executed study providing important baseline information. Good solid systematic parasitology. No questions.

Paper X – integrative taxonomy on larval stages including a key to cercariae. Additional baseline information prepared expertly in readiness for matching to existing and as yet unidentified adult forms. No questions.

Paper XI – Species diversity fo *Plagiorchis*; another complex group. Building on authoritative previously published work/sequences, the study provides important diversity data for common freshwater snails distributed broadly across Europe and beyond. A very competent and useful study providing key taxonomic data and insight into this group. No questions.

DTJ Littlewood
NHM, February 2015

To whom it may concern

Review of the Ph.D. thesis “An integrative taxonomic approach to the study of trematode diversity and life-cycles in freshwater ecosystems” by Simona Georgieva.

The thesis focuses on taxonomic and phylogenetic studies of several genera of digeneans with freshwater life cycles occurring in Europe. The common theme of these studies is the use of integrative approach combining molecular and morphological analyses in order to obtain more robust and convincing results than these sources of data can provide when used separately. The thesis consists of 11 papers published in peer reviewed literature including some of the leading parasitological journals. They have already passed a thorough professional review which makes my job much easier. The thesis also includes an introductory section presenting the state of the knowledge on each of the concerned genera as well as the main goals of the study.

The text below contains my assessment of the thesis as well as a few remarks and questions that are mostly a matter of discussion than criticism. They are underlined for convenience.

Introduction

The beginning of this chapter describes the advent of DNA barcoding and the history of progressive merger between the molecular and morphological approaches in taxonomy, particularly in delineation of species. The discussion of advantages of the "integrative" taxonomy is somewhat excessive since it is rather a non-issue, at least for some time already. It is self-explanatory that a combination of more than one approach or source of characters is better than a single type of characters. It is also clear for a taxonomist that morphology has to be one of these types of characters if at all possible. To say the truth, I do not think that taxonomists feel any more that “their discipline is isolated from the rest of the life sciences“. In any case, the text is well written and gives a historical perspective to the research done by the candidate.

The choice of the taxa for the study is well justified. An extensive, detailed historical account of both traditional and molecular taxonomic and systematic studies of each concerned genus is provided and demonstrates candidate’s good knowledge of the relevant literature. I would like to emphasize the courage of Simona Georgieva as a researcher. Most taxonomists would not dare to take on genera like *Diplostomum*, *Plagiorchis* and especially *Echinostoma*. Large number of species combined with complex taxonomic histories make these genera repelling to deal with. *Echinostoma revolutum* alone gave nightmares to generations of parasitologists. Nevertheless, Simona Georgieva with co-workers tackled these groups with excellent results. They not only clarified several existing problems, but also provided baseline data that will foster future progress in the studies of these digenean groups.

The study questions are sound and challenging. Only one of them, namely “How many species globally?” is too ambitious simply because it would be unrealistic to obtain samples from the whole distribution area of these genera. I find the focus on cercariae particularly useful considering that in Europe obtaining vertebrate definitive hosts for parasitological examination (especially birds, bats, other mammals) is very difficult and in some cases nearly impossible.

Minor specific remarks:

P.4: I could not quite understand the meaning of the expression “after the establishment of a solid taxonomy”

P.5: there are also a number of more recent examples of publications on cryptic digenan species

P. 7. Members of the Diplostomidae can use amphibians, not just fish, as second intermediate (and paratenic) hosts. *Alaria* spp. have a mesocercaria stage.

P. 15, line 6: their

P. 17. I could not understand the term “cryptic life history patterns”

***Diplostomum* and other Diplostomidae (papers I-V).**

The study is unique due to the combination of thorough molecular and morphological work. Although there was a good deal of research on the genus including molecular work in both Europe and North America, none of the previous studies included quality morphological analysis of different stages of life cycle.

Among other discoveries, Simona Georgieva and co-authors have elucidated the life cycles of two *Diplostomum* species most commonly reported in Europe. They have also clearly outlined the remaining unresolved questions related to life cycles and systematics of the genus. The papers included in the thesis provide both the baseline data for future studies and an excellent example of how it should be done.

The study of subarctic *Diplostomum* in Iceland suggests that even in the high latitudes the parasite diversity can be significantly underestimated. This is in agreement with other recent and ongoing studies in the Arctic, for instance, those dealing with anoplocephalid and hymenolepidid rodents. Interestingly, the study in the south (Spain) also revealed greater than expected diversity of *Diplostomum*.

Even assuming this potentially significant “hidden” diversity I would be interested to hear the condidate’s opinion the possibility of 47 diplostomid species parasitizing fishes in a stretch of a single river in Canada as reported in the literature based on cox1 data.

Remark: I personally do not see almost any use in reporting the number of haplotypes. It only confuses a reader without informing about the actual number of species level lineages. I also do not see a good reason for the use of neighbor joining algorithm in several of the papers.

I find particularly interesting the discussion of the “barcoding gap” (or “threshold”) in case of these digeneans. It illustrates once again the difficulty of finding and use of a universal level of genetic variability (a “yardstick”) within a gene that would apply to all animals.

Question. I am interested to hear the candidate opinion on the possibility of 47 diplostomid species to be found in fishes found in a stretch of a single river in Canada as reported in the iterature.

***Echinostomatidae* (papers VI-X)**

This is an excellent series of papers which significantly advance the knowledge on the group. My only general remarks concerns somewhat excessive use of the word “cryptic” in some of the publications. For instance, it is used in a paper entirely based on cercariae (Parasites and Vectors, 2013, 6:64) where sequences of adults come from two publications that did not contain any morphological analysis at all. Simply not all necessary adults have been found and sequenced yet with proper vouchering and/or thorough morphological analysis. They will remain “cryptic” as long as researchers will only publish sequence data and will not bother to get to the morphology.

Simona Georgieva's publications is a good example of how to do it right and reduce the number of cryptic species. In that sense the paper by Georgieva et al. (2014) revisiting the *Echinostoma revolutum* species complex was a significant step forward in this direction. The only minor remark here: unless a description is provided in the same work, one should not write "n. sp.", e.g., *Echinostoma* n. sp. It remains an *Echinostoma* sp. (1, 2, 3...) until described. Besides, "n. sp." attached to a generic name without a specific epithet does not make much sense.

The paper by Faltynkova et al. (2014) is a logical continuation of the work on cercariae. It links the discovered cercarial genetic lineages with adult digeneans thus closing some of the "cryptic" species problems. The authors assess the diversity of *Echinostoma* in Europe and provide a comprehensive description of *Echinostoma nasincovae* n. sp. that includes both larval and adult stages. As a cherry on the cake, the authors also provided long overdue quality morphological re-descriptions of *E. paraulum* and *E. revolutum* which also contain all stages of life cycle including adults from experimental infections. Keys to identification of both cercariae and adults are provided. This work not only clarifies ambiguities regarding these species in Europe, but helps resolving a similar situation in North America. The depth and quality of these studies are impressive and set a standard for future studies of echinostomatids and other digenean taxa.

I commend the candidate for making a substantiated choice of nad1 for "barcoding" in this particular digenean group. Due to differences in mutation rates between genes and across animal taxa attempts to institute a single "barcoding" gene or gene region are not really viable, although the approach is very useful. The optimal "barcoding" targets may vary from taxon to taxon.

I thoroughly enjoyed reading the candidate's publications on *Petasiger*, partly because *Petasiger* cercariae are my personal favorites of all freshwater cercariae due to their peculiar morphology and mode of locomotion. These studies use cercarial morphology, hosts and molecular data to describe life cycles of some already known species and reveal the presence of additional *Petasiger* spp. that remain on the "things to do" list for the future.

Plagiorchis (paper XI)

The large work on *Plagiorchis* cercariae in central Europe is a very important step towards better understanding of the diversity of this genus in Europe and elsewhere that I hope will result in a thorough revision of the genus. It is as exciting as it is difficult due to the very large number of described species, their morphological uniformity and complex taxonomic history. Many years ago a need to distinguish among species of *Plagiorchis* parasitic in bats forced me to look for a different source of characters and DNA sequencing has become a tool of choice. The authors of this paper did an excellent job collecting samples, identifying monophyletic lineages and providing a key to the identification of cercariae. I am only a little bit skeptical regarding the utility of some of the morphological features used in the key to the identification of *Plagiorchis* cercariae. I see myself having a hard time counting every "refractile granule" and trying to distinguish cercariae with 50 or 70 such granules. The same concerns a minuscule (1 micron) difference in the size of the stylet thickening. Sequencing of cercariae matched with those of adult digeneans are clearly the most practical way of identifying these parasites. I anticipate further expansion of the geographic and taxonomic coverage of this important study.

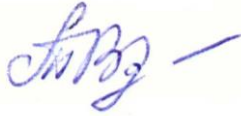
The main findings of the published studies are well summarized in the synopsis of the main finding and conclusions. They are somewhat repetitive, but I assume this is a required format. The results are formidable and characterized by a high level of novelty. The presented series of

publications makes a significant contribution to our knowledge of the involved groups of digeneans and our field of science in general.

The candidate has also expanded her knowledge and skill set by participating in several side projects that resulted in quality publications.

By conducting and successfully completing these difficult and at times very intricate studies, Simona Georgieva has convincingly demonstrated that she has become an excellent professional possessing broad and deep knowledge of the discipline as well as diverse methodological toolkit allowing to address and resolve complex scientific questions. She is already a valued member of the international parasitological community and I have no doubt that she has a bright future as a researcher. I recommend awarding Ms. Simona Georgieva a sought scientific degree of Doctor of Philosophy without reservations.

Sincerely,

A handwritten signature in blue ink, appearing to read 'V. Tkach', followed by a horizontal line.

Vasyl V. Tkach, Ph.D., D.Sc.
Professor