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To whom it may concern

22 September 2016

Report on the PhD thesis submitted by Lukáš Drag

In his thesis, Lukáš Drag has intensively worked on the population genetics and phylogeography of longhorn beetles, but also on the genus *Osmoderma*. His main study subjects were *Cerambyx cerdo* and *Rosalia alpina*, the former mostly in a population genetic study analysing the genetic consequences of a reintroduction of the species, the latter mostly for phylogeographic analysis.

The thesis is presented in seven chapters of which six are already published in international journals listed in the ISI system. One further chapter is a manuscript ready for submission. Four of the publications are technical notes, three of them presenting new microsatellite loci and the last discussing methods for the preservation of biological material for the purpose of genetic analyses. Two papers and the so far unpublished manuscript have to be considered as full publications. These seven chapters are accompanied by a general introduction. Hereby, the thesis presented by Lukáš Drag formally fulfils the demands for a cumulative PhD thesis.

Let my start with the four technical notes as there is little that has to be criticised. The three technical notes delivering new microsatellite loci are all state-of-the-art, well written and informative. The technical note addressing the possible substitution of alcohol for the preservation of tissues for future DNA analyses by alternative solutions is a helpful contribution and might be of use for many researchers in the future.

Evaluating the remaining parts of the thesis unfortunately is a less enjoyable task to do.

Let me start with the introduction. This is not an appropriate introduction for a cumulative PhD thesis but some vast chapters addressing different aspects of importance in the thesis. For example, it is simply unacceptable starting the introduction of such a thesis with the sentence: "According to Spheight (1898), saproxylic invertebrates are organisms that ….". Hence the introduction gives a relatively superficial overview what saproxylic beetles are, followed by

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information on *Rosalia alpina* and *Cerambyx cerdo*. In the (much to short and little informative) chapter "Conservation priorities", the sentence "*Rosalia alpina* and *Cerambyx cerdo* are both considered as the two model species of saproxylic invertebrates for various reasons …" is not justified (what would have been necessary), but just supplied with references. The next chapter "Molecular markers" is a relatively superficial review of why molecular markers are useful in ecology and biogeography, but the link towards the own scientific work is insufficient. Similar applies to the chapter "Phylogeography" which above all is scattered with inaccuracies and even errors. Thus, weather is mixed up with climate. Further, there is no "south-western orientation of the main mountain chains" and the Carpathians are not acting as important expansion obstacle, different from the Alps and Pyrenees; etc.. The derivation of the aims of the thesis is much to short and not sufficiently underpinned with adequate reasoning.

The publication on *Cerambyx cerdo* is much less inventive than the candidate states and is based on a rather small data set. The results are highly trivial: There is no other possibility than that the re-established population is genetically more similar to a population it was taken from than from an independent nearby population. I wonder that this small and trivial data set was accepted for publication in *Conservation Genetics*.

The data set presented in chapter VI on the phylogeography of *Rosalia alpina* in south-eastern Europe is quite nice. However, the candidate fails to perform essential analysis (e.g. on gene-flow and molecular clock) or at least should explain why not doing so. Unfortunately, the discussion is remaining mostly superficial in the discussion of phylogeographic patterns, not comparing adequately the own data with other available information on the biogeography of SE Europe. More seriously, the interpretation of the results is wrong in several aspects. The candidate has NO indication for a single glacial refugium in NW Greece. He should have read before the papers addressing the leading edge – rear edge problematic (e.g. Hampe & Petit 2005) and papers on the evolution of genetic patterns following different dispersal modes (e.g. Ibrahim et al. 1996). It is a miracle to my how a flawed paper like this could have passed the review process in the *Biological Journal of the Linnean Society*.

The last chapter of this thesis is presenting phylogeographical data of *Rosalia alpina* all over the western Palaearctic. Unfortunately, the candidate is making exactly the same types of errors here again as in the previous chapter. The promised comparison with *Fagus* remains extremely superficial and there is no easy to follow story telling what would have been very easy in this case of such a nice data set.

All in all, this thesis offers some nice data sets but their interpretation and in depth discussion remains too superficial and hampered by errors. Regardless, I still think that this thesis fulfils the requirements for a successful PhD project, but not more.

Müncheberg, 22 September 2016

Univ.-Prof. Dr. rer. nat. Thomas Schmitt (Dipl. Biol.)

Lukas Drag: Phylogeography and conservation genetics of endangered saproxylic beetles in Europe. PhD Thesis, South Bohemian University, Ceske Budejovice

Let's start with the most important thing: The evaluated thesis, consisting of a general introduction, 6 published papers and one unpublished manuscript, and a short summary and conclusions, certainly contains enough results to satisfy the requirements of the PhD degree. The work concentrates on three European beetle "conservation icons", the cerambycids Rosalia alpina and Cerambyx cerdo, and the scarabaeoid Osmoderma (two species), all being dependent on living or dead trees, and tries to analyze the populations and geographic history using molecular markers (mitochondrial COI and nuclear microsatellites). The published papers went through the reviewing process in peer-reviewed journals and are thus scientifically sound. Thus, the thesis is certainly recommended for acceptance as a basis for receiving the PhD degree. I assume that the author will introduce his work adequately himself and thus there is no need to summarize it here at length. The following comments are meant mainly as material for improvement and food for thought or perhaps discussion if time permits.

1. Model insects

Even if we accept the not very fortunately defined word "saproxylic" for Cerambyx cerdo (which is neither associated with decomposing matter nor in fact feeding on wood or at most at a very limited extent, being dependent on fresh phloem), Cerambyx and Rosalia are biologically very different. C. cerdo and related species are in fact pests if we want the trees to last or to be used for economic purposes, and indeed in some warmer regions some large Cerambyx species are increasingly considered pests of, e.g., cork oak stands. You cite Slama's book on multiple occasions, and Milan Slama clearly says that C. cerdo (and some related species) are serious pests in some regions of southern Europe, and just as elephants may, under some conditions, destroy their environment, species like C. cerdo (unlike Rosalia alpina) may, under certain conditions, heavily damage or possibly destroy the tree stands they live in. I remind the fact (certainly well known to the author) that the association of C. cerdo predominantly with old large and preferably solitary trees is specific to our region and that in other (warmer) regions, already in southern Slovakia, the supposedly conspecific beetle may develop in much younger trees, closed stands, massively attacks fresh stumps following recent forest clearings, etc. You call C. cerdo an "ecosystem engineer". I am really sometimes almost amused watching the conservation people hiding or twisting some biological characteristics of the "popular" species to avoid adverse connotations :) . "Oaks colonized by this beetle had a higher number of other beetle fauna than uncolonized trees, and offered a habitat for numerous endangered beetle species" - yes, exactly, a moribund or dying tree with galleries always has a greater diversity. But the only credit we can give to Cerambyx is really just heavily damaging the tree. Let's face it, those conservation icons were primarily selected by beetle lovers. OK, you call it "cultural preferences" :) .

2. Conservation aspects

I have no doubt that we see and speak about the same things, but I would point out the loss of continuity in the first place. The forest practice used to be done on a much smaller scale and much more selectively. Today, to be economically competitive, the forest "harvesting" (and agriculture in general) is done at a much larger scale and is not selective. Leftovers are generally removed and utilized or destroyed since the reforesting methods are also large-scale and incompatible with leaving unusable wood in place. If we cannot change that (and we

probably cannot), we cannot do much to restore diversity in production forests. Thus, we depend on some "marginal" spaces or on regions specifically managed for diversity, giving up economic production. (N.B. Now I do *not* speak about no-management protected areas - it is important to realize that central European landscape was predominantly anthropogenic and originated in times when the agriculture and forestry made it much more diversified than it would be otherwise. Thus, protected areas with no management will *not* restore the previous diversity.) The main question is: Which and how large and dense can those areas be. I am afraid that just large beetles like C. cerdo will not be able to survive in a thin mosaic of small suitable localities, and I wonder whether we will be able to give them more than that. Some such species, seemingly paradoxically, found shelters in (sub)urban areas and (for known reasons I am not going to repeat here) those old-tree refuges are also partly disappearing.

3. Ice age biogeography

I have always had problems with the somewhat mystical "glacial refuges" concepts. Perhaps it is my ignorance, but I never quite understood what is a "differentiation center" and generally why the ice-age biogeographers automatically suppose that the species should still occur today in their glacial "refugia" (whatever that means), that they should be most diverse there, etc.? Neither bottlenecks nor quick diversification have to be associated only with settlement age. Why to postulate only range contraction with the onset of adverse conditions and range expansion during interglacials or generally warmer periods? Why to ignore a possibility of range shifts (instead of range pulsations) and thus of regional extinctions both during cooling and warming?

In the unpublished Rosalia study you write: "The basal position of the Hatay population and its high divergence from remaining populations suggest that its isolation reaches beyond the last Ice age". Please explain how and why. Let me summarize the situation: There are limited areas populated by beech in southern Turkey. There are limited populations of Rosalia in those beech growths. Both the beech and Rosalia are considered conspecific with their northern counterparts and in Rosalia the genetic difference is sometimes lower that differences within those northern populations. Is there any reason *not* to expect that the range of beech and Rosalia in what is now Turkey and some adjacent countries was much more extensive during the last glaciation? You have no Rosalia fossil record, but botanists often have at least the pollen record. There are works on beech from its current range, but has pollen analysis been done in the Turkish regions where the beech currently does not occur? I am really afraid that based on the information you have on Rosalia it is difficult to make conclusions because you do not know the Rosalia range during the glaciation or the changing of ranges and sizes of separate populations into the postglacial, and all that would have influenced the genetic diversification, bottlenecks, etc. I would tend to agree with the conclusion that most of the more northern colonization was via the Balkan peninsula, I am questioning whether it had to be primarily *from* there. And if we considered a more extensive glacial southern occurrence of Rosalia embracing the current Turkish populations, the origin of the Italian populations also opens to re-interpretation because - please note - the Italian and remaining European populations form a distinct clade! (Please remove from the MS the "five distinct clades", it is very confusing and imprecise).

4. Some comments on the molecular part (I hope that, as a non-geneticist, I did not misunderstand you on some points).

It is not quite appropriate to say that the mtDNA as a whole has a "relatively high rate of polymorphism".

If you name NUMT's as a possible difficulty in using mtDNA, then on the other side there is a low probability of duplicate genes in the mitochondria themselves. Which is an advantage because duplicate nuclear genes may obviously cause the same problems as NUMT's.

I suspect that the maternal inheritance of mitochondria might in fact bring both problems and advantages. OK, so mitochondria are clones. Of course mtDNA does not help if you need to score hetero/homozygosity; it may be a problem on a population level in species with sessile females and vagile (flying) males where mitochondria would fail to record genetic information brought in from different population(s) by incoming males and in general it would not record "unidirectional" crossings. But on the other hand it may be sometimes better than the recombination and alellic mess... And the maternal inheritance should not matter so much at higher phylogenetic levels [agreed, this is not the case here], assuming that successful crossing would occur at most between closely related taxa.

"Finally, it has been suggested that mtDNA evolution is not completely neutral." As a non-geneticist, I would ask how could you be sure that the evolution of selected nuclear sequence(s) (even microsatellites considered typically without function) was? And you yourself cite a heap of potential problems with the microsatellites. Either I misunderstand you, or this is a non-issue. Of course I agree that it is best to have representation of both mitochondrial and nuclear genome and if their results agree, it's better than when two nuclear or two mitochondrial sequences provide similar results.

5. Varia

What do you mean by "unrooted tree" (e.g., Fig. 4 in the unpublished MS)? There are no unrooted trees, there must have been some system used (by you or the software) to select a particular tree conformation. If you do not set the root manually, the software must use some default, such as midpoint rooting (but your tree does not appear to be midpoint rooted).

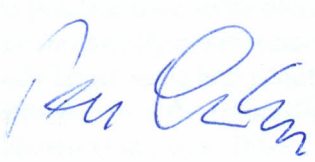
Just for completeness, Rosalia also occurs in North America (Intro).

You write (in the unpublished article) that "Within the beech range, the beetle is absent from Great Britain..." - am I wrong or is beech considered introduced in Britain relatively recently (at most a few thousands of years) perhaps by humans?

"In Europe, the south-western orientation of the main mountain chains..." - I presume you meant to say "east-western".

Ceske Budejovice, 16 Sept 2016

Petr Svacha
Institute of Entomology



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Phylogeography and conservation genetics of endangered saproxylic beetles in Europe

Candidate: Mgr. Lukáš Drag, Faculty of Science, University of South Bohemia

Dissertation assessment

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The presented thesis is a work compiled of 6 already published scientific contributions (all of them in international journals with IF) and one manuscript "in preparation". These manuscripts are accompanied by short "*introduction*" and "*summary and conclusion*" chapters as well as a list of references three appendices. The thesis has 135 printed pages and lists 130 references (which are however listed after the Czech Alphabet).
The introductory chapter contains brief information on saproxylic beetles in Europe, on *Rosalia alpina* and *Cerambyx cerdo* as model species, followed by the chapters on conservation priorities, molecular markers and phylogeography of Europe as well as delimitations of the aims of the thesis. The conclusion section summarises the achievements of the presented papers.

What has been done?
Four, out of the seven, incorporated manuscripts are focused on particular problems of the methodology in the research of saproxylic beetles: three of them (chapter, I, II & V) deal with the identification of microsatellite loci for *R. alpina* (I), *C. cerdo* (II) and *O. eremita* and *barnabita* (V) . The microsatelite loci for the above mentioned longicorn beetles have already been used in other parts of the thesis (chapters IV,VI,VII), the microsatelites for *Osmoderma* have not been currently used /or the result of such a study is not published yet/. Anyway the fact that they are identified is very promising for further enhancing of our knowledge on this genus. The last of the methodological paper (III) is not directly dedicated to saproxylic fauna as it investigates the possibility to use two commonly accessible chemical solutions (SDS and EDTA) to conserve material for further genetic studies. The results are promising thought several limitation of the method are listed.

The non-methodological part of the thesis contains 3 scientific papers (two of them are already published in prestigious scientific journals and one manuscript is in preparation). The first manuscript (chapter IV) investigates the origin of an introduced population of great longicorn beetle *C. cerdo* found in Hluboká nad Vltavou near to České Budějovice. The paper also investigates the genetic diversity of this population. The interesting fact is, that the founder population was extremely small (only 7 females, 3 males; however the females could most probably already mate before the transfer). The current population seems to be well established, favouring the fact that a "bottle neck" in genetic diversity does not necessarily lead to population extinction (which was repeatedly shown also in population of large mammals: e.g. Asiatic Lion, Indian rhino etc...) and elsewhere.

The other two manuscripts investigate the population structure of the Rosalia longicorn beetle in Central and SE Europe (VI) and on the entire species range (VII) with emphasis to their currently observed host plant shift and the presumed expansion routes of their main host, the beech (*Fagus sylvatica*), respectively. In general the refugia of the species in Europe have been observed to lay

within the southern most current limits of the population (e.g. north-western Greece), where according to the candidate is also the presumed glacial refugium of beech. Although in chapter VI the author cannot rule out another possible refugium of Rosalia longicorn in the Eastern Alps and Adriatic coast. Equally important the polyphagous lowland populations are most likely to be linked to the local montane populations, rather than represent a distinct genetic lineage. In the second Rosalia-targeted manuscript the candidate uses a geographically broader subset of material to test the following hypotheses:

(i) Has the beach been incorporated into the diet of the beetle relatively recently, and is this incorporation facilitated the expansion of the beetle to the range of its new host? (ii) Is polyphagy the ancestral strategy of Rosalia longicorn? (iii) Do we witness broadening of host range of originally monophagous beetle?

Similarly to the previous paper also here the author concludes that the phylogeography of the Rosalia longicorn corresponds to those of its main host (*Fagus sylvatica*), with the presumed glacial refugia in the NW Greece. The ancestral strategy seem to be monophagy with *F. orientalis* as host species and thus the observed polyphagy of the beetles seems to be a recent phenomenon (however which may periodically occur in the interglacial period).

All the presented results are highly original, highly informative and based on a modern methodology and thus (unfortunately) they remain rather unparalleled within the current beetle research. Therefore I personally see no reason why not to accept this thesis, and reward Mr. Lukáš Drag with the title PhD.

However my role here is to provide different points of view, raise questions and criticism to enable the candidate to defend himself, his thesis and work.

General questions and critical remarks

The thesis theme, as mirrored in the title, encompass a broad variety of questions and indicates that it will deal with a vast majority of saproxylic beetles fauna from Gibraltar towards Bosporus. However, in reality it deals only with two (or three) species (two longicorn beetles, one scrab).
Even though, I understand, that in the recent days it is absolutely necessary to mask, that one is working on a certain particular group of taxa, I feel that the title of the thesis is far too broad to its contentment. This could be partially fixed in the introductory chapter (pp 1-11), however the information listed here are rather superficial and the reader has to make his own review on saproxylic beetles based on the guidelines provided by the references in the text.

1) Therefore my first question is: (I) can the candidate clarify his choice of model species? Towards which subset or group(s) of saproxylic taxa may his results be extrapolated? *ii) is there any classification or subdivision of saproxylophagous beetles?, if not how would you further divide this group based on the ecological strategies or demands?*

2) On page 2 the candidate states, that *O. eremita* s.l. represents probably the most studied species of saproxylic beetle, but its ecology and life history is very different from the species listed in the thesis. While this is invariably true, can the author figure a situation where both *O. eremita* s.l. and *Rosalia alpina* can act as mutual flagships species in conservation of identical saproxylic beetle communities (e.g. in the same area).
While Osmoderma *is without any debate the most studied saproxylic beetle in the Europe, its taxonomical position remains misunderstood, leading to incorrect assumptions on the species's ecology ect. What is your opinion on this species?*

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3) A closer look on the material used in chapter VI & VII reveals, that only mountain populations of *R. alpina* from Greece have been sampled – are there any known lowland population of *R. alpina* in Greece? Or what are the southermost known limits of the lowland populations?

4) The main outcomes of the chapters VI & VII summarized by the author are:

"The phylogeographies of R. alpina and the beech seem to be tightly matched, and both species probably shared a common history. This is rather surprising since the beetle is able to sustain its populations on a phylogenetically wide range of trees. It is thus possible that although the beetle appears to be polyphagous, it is historically connected with only a single host. Its broadening of host range thus might be a recent event which periodically happens during interglacial periods, meaning that the populations currently not exploiting beech are likely to go extinct during next glaciation".

How does this statement hold in the light that:

I) In the last interglacial period (not the current one) beech is believed to be widely absent from Central Europe and the expansion of beech (along with the presence of humans) are two major characteristic of the current interglacial period

II) The main glacial refugia acting as source for *Fagus* recolonization are currently identified not to be located in SW Balkan peninsula (as postulated in the thesis) but in Southern France, Eastern Alps (Slovenia, Istria) and even from Southerm Moravia (Magri et al. 2006, Magri 2008, Kempf & Konnert 2016). The beech populations in Central Europe are most probably originating in SE Alps/Norther Dinarids? (e.g. "the Mediterranean refuges did not contribute to the colonization of central and northern Europe" Magri et al. 2006)

III) Because of ecological and biological characteristic beech is almost the "slowest" arboreal migrant (after hornbeam). Thus the incongruence between the "source" glacial refugia of the Rosalia longicorn and beech would imply that the Rosalia longicorn invaded a wast portion of its current range long after beech (only after the Ardiatic and NW Greek lines would join) Are there any population of beech in which is the species absent (except of British isles)?

IV) *Based on your current results and the known paleobiogeography of beech, would you be able to figure an alternative scenario of the postglacial recolonization of the Rosalia longicorn*

V) *With regards to your results, it would be desirable to incorporate into the thought-framework of your studies also the hypothesis of a post-glacial range-expansion (rather than recolonization) of Rosalia longicorn. Have you considered this possibility?*

In Prague,
October 3rd 2016

Mgr. Petr Šípek, PhD