Review on the PhD thesis "Evolution of the sex determination pathway in the Mediterranean flour moth, *Ephestia kuehniella*" by MSc. Sander Visser

The thesis by Sander Visser is composed of an introductory part, three papers and an concluding part (called Synthesis and perspectives). Only one of the papers is already published – in *PLOS Genetics*, a highly reputable journal, but I have no doubts that the other two manuscripts would be published soon in similar journals. The candidate is an author of several other papers not included into the thesis and I have no doubts that the thesis fulfils even the most demanding criteria for a successful PhD. thesis. It is well written (logically structured, language standards are high), on a highly actual topic, the studies are well designed and use a lot of various, complementary approaches (genomic, molecular genetic, cytogenetic) in a highly informative way.

The first study (the one published in *PLOS Genetics*) is a nice, very informative study carefully documenting the role of the gene *Masculinizer* in male development in *Ephestia kuehniella.* I like it a lot, the discovery of the functional Z-linked paralog of *Masc* is interesting. However, I have to say that I was absolutely excited by the second paper. It is developing in a very smart way what was learnt from the previous paper and from knowledge on sex determination in *Bombyx mori.* To me it is a true masterpiece. I admire the structure of the experiments and I am convinced that *EkFem* cluster is indeed the sex-determining gene in *Ephestia kuehniella* based on several lines of evidence, mainly according the position on W and total lack in males, the similarity with *Fem* of *Bombyx mori* (e.g. higher copy numbers), and the expression in the right time (although mothers make it more complicated by provision of *EkFem* RNA to all eggs). The clever, original way of finding the locus is astonishing! I read the story described in the paper with the greatest interest as a detective story and I was very pleasantly surprised in every paragraph of the Results! During my first reading, I kept my fingers crossed for each additional experiments – and I am still keeping them for further functional testing of the findings! The third paper is a follow up study in other species, which is equally fascinating and leads to more general ideas about the evolution of sex determination in butterflies. I was also very pleased reading the closing part of the thesis which summarizes all the findings and put them to a wider perspective. I enjoyed a lot the polemics whether the same pipeline could lead to uncovering other sex-determining loci in other lepidopteran lineages or not.

Altogether, it is clear that I evaluate the whole thesis very high, and I congratulate to it.

I have several comments and questions:

1) I would be more conservative in the generalization about the degree of conservatism in sex determination and sex differentiation in insects. In many places it is stated that these pathways are fast evolving and that the genes on the top of these cascades differ even between closely related species. We know still just a little about sex determination and sex differentiation pathways across insect megadiversity. For example, very few sex-determining genes were discovered and nearly all in dipterans, the lineage which is likely very unusual among insect lineages by a high degree of turnovers of sex chromosomes (as in many other aspects; *Drosophila melanogaster* is one of the worse "model" organism I can imagine).
2) On the other hand, the author claims in the Introduction and in several other parts of the thesis that „alternative regulation between the sexes relies on the sex-specific splicing of *dsx* pre-mRNA, resulting in sex-specific DSX proteins" in insects. But it is not generally true, see e.g. Wexler, J. *et al.* Hemimetabolous insects elucidate the origin of sexual development via alternative splicing. *Elife* **8**, e47490. https://doi.org/10.7554/eLife.47490 (2019). Again, I would be more careful about generalizations.
3) We in vertebrates are using different meaning of the same terms. I understood well what was meant, but being trained to use the terms differently, I suffered by reading the terms in different meaning. Sex-determining gene is for us the gene on the top of the cascade, i.e. the true sex determiner. For example in therian mammals, most authors would say that *Sry* is our sex-determining gene. Other genes such as *Dmrt1* (by the way an ortholog of insect *dsx*), *Sox9, Foxl2* and *Amh* are in mammals genes taking part in gonad (male or female) differentiation, but assigning them as „sex determining genes" would be a crime in my field. Therefore, I would never assign *Masc* as a locus responsible for sex determination in *Ephestia kuehniella,* this term should be to me reserved for *EkFem* (ok, it is only the candidate sex determining gene in this species). I agree that the presence of MASC protein during embryogenesis is essential for male development and dosage compensation. But similarly to *Masc*, knockdown of *Sox9* leads to feminization in mice, but *Sox9* is an autosomal gene and not sex-determining gene, although it is crucial for male development. I would never assign autosomal microRNA locus in *Bactrocera dorsalis* (p- 123) as a „locus

involved in dipteran sex determination". Also, I found confusing that what I would call simply a „sex determining locus" is assigned through the thesis in the same meaning by several terms, e.g. a „primary signal gene", „a master sex determining gene", or even „primary sex determining signal gene". I feel that we should all work together on unifying terminology in the field.

4) I am not totally convinced that the evaluation of the conservation of binding site sequence for *Fem* RNA in *Masc* as used in the paper 3 is a convincing proxy for the evaluation of homology of sex determination based on *Fem* piRNA. Could you please comment on it?
5) The closing paragraph of the same paper is stressing the importance of the work that it is only the second case in insects where the sex determining locus is conserved among genera. Genera are to me totally subjective categories and I would personally not use them for any similar comparison.
6) I am not convinced that in species that „have lost the W chromosome" … „the initiation of feminization cannot be controlled by a feminizing piRNA on the W chromosome." I can imagine that the *Fem* (or similar) locus/loci were translocated to an ancestral autosome forming a „cryptic" neo-W chromosomes and that ZZ/Z0 constitution is conserved due to now essential dosage compensation without direct/primary function is sex determination (similarly as in XX/X0 or X0/X0 rodents).

All these points are aimed as a contribution to discussion, at the end I want to stress again that the thesis is really great and I admire the whole work a lot!

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To Prof.
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Object: PhD Thesis review:
Evolution of the sex determination pathway in the Mediterranean flour moth, Ephestia kuehniella
Visser Sander

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The PhD candidate, Visser Sander, presented in his thesis and his publications novel findings concerning sex determination in some Lepidopteran species.

The introduction of the thesis is readable and fully covers the state of the art of the field. It also justify the use of *Ephestia kuehniella* as one of the model species for genetics of sex determination.

The outline of research focusing on *E. kuehniella* is clearly stated with three main goals; 1) Masc isolation, 2) identification of Masc regulator(s) (*Bombyx mori Masc* orthologue), such as Fem-like piRNAs, as primary sex determination signals, 3) investigation of evolutionary conservation of this primary signal in related species.
All goals were matched.
In the Visser et al., (2021) paper, the candidate and collaborators described the identification and functional analysis by RNAi of EkMasc and of one paralogue, a novelty found in this species. They confirmed that both genes seem to control Ekdsx male-specific splicing and are necessary for male vitality.

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The PhD canditate and collaborators, in the second section (paper draft) with the title: Identification of putative feminizing genes in the Mediterranean flour moth, Ephestia kuehniella, present the identification of a putative primary signal for female sex determination, corresponding to W-linked piRNA encoding genes, which seems to be analogous (or highly divergent homologous) to the one found in B. mori. A cleaver bioinformatic strategy has been used and described to reach this goal. EkFem piRNA seems to target EkMasc mRNA, similarly to the ping-pong mechanism described in B. mori.
The PhD candidate also described potential differences in the initiation of the Fem piRNA pathway between E. kuehniella and B. mori, based on original data collected while studying the embryonic expression patterns of the key genetic players.

In the third section (paper draft) with the title: "Identification of putative feminizing piRNA in the Indian meal moth, Plodia interpunctella, suggests conservation of the primary sex determining signal in pyralid moths"

The PhD candidate and colleagued used bioinformatics and available RNA sequence data to find putative piRNA sequences highly similar to the EkFem in the Indian meal moth, *Plodia interpunctella* (Pyralidae). The identified PiFem piRNA was analysed in its copy number, location, and structure on the W chromosome. Furthermore its target sequence was compare in Masculinizer orthologs of four pyralid species to investigate the level of sequence conservation of this feminizing piRNA and hence indirectly its functional conservation.

In summary; Visser performed bioinformatics analyses, BLAST comparison, RNA gene expression analysis and copy number analysis by qPCR, by Southern blot, by in situ chromosome hybridisation, by RNAi analysis, by phenotype, acquiring a wide range of technical skills. Furthermore, Visser contributed also conceptually (see author contributions for the papers) to the design of the experiments.
Visser and colleagues have shown that feminization of WZ individuals occurs via feminizing piRNA also in *E. kuehniella* and *P. interpunctella* species, as it does in B. mori. As EkFem has no similarity to BmFem a question of its evolution remains open: Have the two piRNA genes emerged independently on the W chromosome of the ancestors species to control the same target gene (Masc) or only once, but strongly diverging during time?
It is interesting that EkFem and PiFem show sequence conservation, indicating evolutionary conservation of a primary signal.
Furthermore, in contrast to B. Mori, additional copies of Masc have been found in E. kuehniella on both the Z and W chromosomes, suggesting evolutionary attempts to rewire the sex determination.

This present PhD thesis will pass at my home institution and based on the novel findings (including those still to be published) it will be ranked with the top 5%.

Questions for the PhD candidate:

Evolution of Masc and Fem

1)
Masc and Fem are quickly evolving genes: what hypothesis do you prefer, if any, and why, among the following ones: 1) Masc is quickly evolving because targeted during evolution in different regions by newly evolving Fem piRNAs; 2) Fem is quickly evolving because targets a quickly evolving Masc gene.

2)
What role can play the duplication of Masc and of Fem in this evolutionary dynamic and what "evolutionary limitations to this gene-gene interactions do you imagine"?

3) group of related questions on the primary signal.

The primary signal of Medfly male sex determination, MoY seems to be conserved in distantly related Tephritidae species, including Bactrocera ones and sharing a commonage ancestor at least 80 mya (million of years ago). Furthermore, also XSE signal of *Drosophila melanogaster* seems to be conserved in Drosophilidae species covering at least 60 my.

See:

https://pubmed.ncbi.nlm.nih.gov/12739140/

https://www.genetics.org/content/genetics/186/4/1321.full.pdf

https://journals.biologists.com/dev/article/122/3/971/39033/Sex-specific-control-of-Sex-lethal-is-a-conserved

3A) What is the phylogenetic distance among E. kuehniella and P. interpunctella species in million of years?

3B) Do you think that also in B. mori close species (sharing a common ancestor in a range of some dozens mya) we will find BmFem sequence conservation?

3C) So do you think that the primary signal higher evolvability is between families rather than species? And why?

In the section: 4.1 Conservation of genes in the lepidopteran sex determination pathway.

4) related to the section: 4.1 Conservation of genes in the lepidopteran sex determination pathway.

Visser wrote:
"Our analysis of the functionally conserved Masc proteins in Chapter 3.1 showed that the C-terminus of all Masc proteins is enriched for proline, an amino acid associated with interactions with transcription factors (Gerber et al., 1994; Williamson, 1994). A proline-rich domain is also found in TRA protein, which is a direct regulator of dsx splicing in Hymenoptera, Coleoptera, and Diptera (Verhulst et al., 2010). Therefore, this proline-rich domain at the C-terminus of Masc proteins may play an important role by interacting with transcription factors in addition to acting as a stabilizer of the protein (Kiuchi et al., 2019)."

It is not clear to me the mentioned parallel and link between TRA splicing factor having a proline-rich region and Masc transcriptional factor having also a proline-rich region. The third sentence, begins with "Therefore…".
The candidate is invited to explain his speculations and reasoning on this point.

5)

How the candidate envision the use of Masc in biotech approaches to design strategies to be applied at an industrial level for example for the increase of eggs production ?

6) Bioinformatics, homology by sequence similarity, by regulation, by synteny and by function:

A group of specific questions on the state of the art and on nomenclature of genes; considering his bioinformatics skills, I invite the PhD candidate to search by BLAST

1) for Drosophila transformer orthologue outside of Drosophilidae using Dmtra DNA sequence or DmTRA amino acid sequence;
2) 2) to search by BLAST Drosophila genome and proteome using either Ceratitis, Musca or Apis TRA orthologues;
3) 3) using CcTRA protein to search for TRA orthologues in Musca, Apis or Coleoptera databased.

a) If a 2BLASTp analysis of DmTRA and CcTRA proteins cannot conclude that the two proteins are related and they share a common ancestry, what are the other features leading to this conclusion that Dmtra is functionally conserved as a female-determining transducer gene in the Medfly?

b) Cctra, with the respect of Dmtra, has the additional master function of auto regulating and maintaining the female sex choice epigenetically. Furthermore, TRA orthologues found in many other insect species, share similarity to CcTRA but not to DmTRA and autoregulate as Cctra but not Dmtra does. So, do you think it is correct to indicate all the *tra* genes are orthologues of Dmtra, or rather it would be necessary to indicate them as Cctra orthologues;

c) Finally, is PiFem an orthologue not only of EkFem but also of BmFem? If not, why you have chosen to name it Fem as in B. mori? Is this nomenclature opening the road to confusion?

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Best regards
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