Opponent's assessment of diploma thesis: "Population Genetics of Cuckoo Bees as Illustrated by the Genus *Nomada*: Development of Host and Cuckoo Bee Markers for Semi-Genomic Analyses".

I had the pleasure of reviewing Kateřina Bezányiová's diploma thesis, and I am grateful for the opportunity to serve as the opponent. The literature cited, and the scope of the review part of the thesis is impressive, with nearly 40 pages of literature review. The writing, logical sequence, and coherence level can surpass that of some professional scientists who prepare a manuscript. It is evident that the student has a very good understanding of the topic and has structured the text thoughtfully. It was truly a pleasure to read.

The level of excellence in the literature review somewhat overshadows the methodological section. This is likely a natural effect of such an extensive and well-crafted review, followed by diving into the complexities of developing, validating, and testing microsatellites—a challenging topic. While the methodology section is occasionally difficult to follow, I still believe that the student has done an excellent job, and the overall outcome of the work is very positive. In most parts, the experimental section is read like a story, albeit with an open ending, leaving me eager to know how the "story" of the sequences concludes. This curiosity left me somewhat saddened, as I wished for closure, but I am excited to see "to be continued" part published and to know how the huge multiplex performed.

I have several questions and suggestions that came to my mind while reading the thesis:

1. I'm not entirely certain that I understood the process of species-specific microsatellite development. Did you perform shot-gun sequencing of multiple species, carry out de novo assembly for each separately, and then extracted microsatellite information? Or did you analyze the data for all species together at some stage? Were there any loci shared across the species sets? If not, did you test whether the final set of microsatellites amplified in other species as well?
2. From the original number of microsatellites detected, you narrowed down to 144 loci. Could you provide more details on the dropout rate at each step? I was surprised that the number was relatively low. In my experience, based on low-coverage RNA sequencing, we had thousands of loci to choose from.
3. In Figure 9, the agarose gel shows strong bands for primers and weaker for PCR products. This is also evident in the results of PCR product purification, which resulted in a considerable decrease in DNA concentration. Did you attempt to improve this? And if not, do you have an idea how to do so? Based on the gel, the best-performing sample in both sets seems to be sample 8, which is not reflected in the concentration measurements in Table 15. Are these the same samples? Could something specific about this sample explain its better performance?
4. Why did you use different concentrations of SPRI beads at various steps (i.e., 1x, 1.2x, 1.5x)?
5. You mention that it was challenging to develop primers for robust multiplexing (i.e., combining multiple samples in one reaction before adapter ligation). However, you also state that barcoded primers would address this issue. Did you consider using barcoded primers? This would allow for pooling multiple samples before SPRI cleanup after multiplex PCR, reducing the repetition of consecutive steps and the cost of consumables. Would there be an advantage over disadvantages in your case?

6. I'm curious about the estimate of the cost for Qubit measurements, which is double than my estimate. Could you validate the statement?
7. I commend the development of the UCE loci, which show great promise for transferability across species, and potentially even genera. However, there is a risk that these loci may not be polymorphic, especially at the population level. Do you have any preliminary sequencing results that could shed light on this?

With respect to all the work done, I have a few recommendations that may help streamline future steps.

- I would recommend considering a PCR-free library preparation kit to reduce the risk of chimeric products, or alternatively, using unique dual indexing. When using the same adaptor or i7 index for multiple samples with varying starting concentrations—and consequently, likely variable sequencing outputs in terms of coverage—there is a risk of failing to identify correct alleles in samples with lower concentrations.
- For adapter ligation, there are kits available that consolidate all steps into two reactions, which can be completed in about one hour. Such a kit could be used instead of the Klenow, A-tailing, and PCR adapter ligation to save precious wet lab time.
- In terms of validating the selected loci, I suggest performing individual PCR reactions with a cheaper polymerase on a single sample to test for amplification. In my experience, about 20–30% of loci fail to produce a PCR product even with primers designed from the same individual's shot-gun sequences. Having the primers of such loci present in your reactions might decrease its effectiveness.
- The computational demand could be addressed by utilizing external resources such as Metacentrum. Maybe consider that also in future work when analyzing the sequencing outputs.

In conclusion, this is an exceptionally well-written diploma thesis, with substantial work conducted both in front of the computer and in the lab. I believe the student has remarkable potential to continue in research, and I would be delighted to mentor such students. I recommend the thesis for defence and propose awarding it a grade of A.

In Brno 10. 9. 2024 Mgr. Petra Šarhanová, Ph.D.