Naturhistoriska
riksmuseet

Review of the PhD thesis of Alžběta Manukjanová: Molecular ecology of cryptic species of the fen moss *Hamatocaulis vernicosus*

Cryptic species are species that are similar to other species in all respects except that they are not distinguishable from each other by morphology. Although cryptic species are widespread, few studies compare the detailed biology of closely related cryptic species included in a single morpho-species. Such studies are problematic, because cryptic species are not possible to distinguish in the field. For the focal species of the present thesis, *Hamatocaulis vernicosus*, it is essential to know the biology of its two cryptic species because the morpho-species is included in Appendix II of the EU Habitat Directive. The PhD thesis of Alžběta Manukjanová fills an important gap in our knowledge of these two cryptic species. The thesis consists of seven chapters, including the Czech summary. Chapters 2-4 consist of papers published in reviewed SCI journals and chapter 5 is a submitted manuscript.

In the first chapter, Manukjanová introduces the study species, describes its biology, and the concept of cryptic species. She provides overviews of bryophyte reproduction and molecular methods in bryophyte population biology. She ends this chapter by presenting the aims of her thesis.

Chapter 2 is a methodological study, in which microsatellite primers and methods to barcode the two cryptic species of *H. vernicosus* are developed. This chapter is a prerequisite for the following chapters.

Chapter 3 investigates the frequency of sex expression and the expressed sex ratio both in the morpho-species and in the two included cryptic species. No difference was found between the two cryptic species in these respects, but it is correctly pointed out that a focus on the morpho-species may miss that even if both sexes are present at a locality they may actually belong to different cryptic species.

In chapter 4 several aspects are investigated. (1) Are the two species truly cryptic? (2) How are they distributed in the Czech Republic, at different geographic scales and in relation to selected environmental parameters? (3) What is the ITS ribotype diversity in the two cryptic species? Despite scoring 14 morphological features and analysing these by different methods, the two cryptic species could not be distinguished which shows that they are truly cryptic. Cryptic species 'clade 2' is much rarer than 'clade 1' and displays lower ITS ribotype diversity than 'clade 1'.

Naturhistoriska riksmuseet

Postadress:
Box 50007
104 05 Stockholm

Besöksadress:
Frescativägen 40
114 18 Stockholm

Telefon: 08-519 540 00
Telefax. 08-519 540 85
registrator@nrm.se

Naturhistoriska
riksmuseet

Both avoid the warmest parts of the Czech Republic, but no significant difference was found between the two cryptic species in the studied environmental parameters.

Chapter 5 investigates the genetic variation in the two cryptic species based on microsatellite data. Beside a higher genetic diversity in 'clade 1' than in 'clade 2', a larger proportion of the variation in the latter clade than in 'clade 1' was among populations. This is paralleled by a higher clonality at 'clade 2' localities than at 'clade 1' localities. Somatic mutations were observed at several localities.

Chapter 6 summarizes the main conclusions and suggests conservation measures, among other things that clade 2 should receive measures additional to those for the morpho-species.

The thesis provides important evidence regarding the two cryptic species of *Hamatocaulis vernicosus* in the Czech Republic. The new information provided by the presented research will no doubt be crucial for an efficient conservation of the two in the Czech Republic as well as in other portions of Europe, and conservation authorities need to consider this in their practical work. It makes clear that, despite many similarities between the cryptic species, there are also aspects that make it necessary to consider them separately, like any morphologically distinguishable species. There are a few issues, especially with the statistical analyses (see below), that Manukjanová needs to consider, and where I hope for clarifications at the defence. However, considering the overall quality of the thesis, and since I have no doubt that she will be able to resolve these issues successfully, I recommend that the thesis is defended as planned.

Stockholm, 30 September 2019,

Lars Hedenäs
Senior Curator, Docent
Department of Botany
ph. +46-(0)8-519 542 14
e-mail: lars.hedenas@nrm.se

Naturhistoriska
riksmuseet

Two issues in need of re-consideration:

Statistical analyses

An important issue that needs consideration before the actual defence is the sampling strategy and statistical analyses in chapters 3 and 4 (only regarding the environmental parameters), and I expect a clarification at the defence. I have discussed the issues with a colleague having some experience in analysis of this kind of plot-sampled data. We agree on the following major points and **strongly advice to consult a person with sound knowledge of statistical methods** for the proper analyses. The sampling unit in the investigation is obviously the patch. This implies that sampling per locality is unbalanced. I understand that the number of sampling units per locality was chosen to reflect population size (e.g., Table 1 on p. 43; chapter 5, p. 101) and can be used as a surrogate for this. With sampling unit = patch, results should be presented at the patch level, and the statistical analyses should compare (response variable) patch level data across localities (for example, using a Generalized Linear Model, or Mixed Model, probably with a log-link function, with locality as a factor, etc., depending on data structure). Pooling of individual ramets from all patches, per locality or all ramets across localities or, as far as I understand (since localities differ), patches across localities, are incorrect data treatment given the patch sampling. Some of the results presented in chapter 3 may therefore be irrelevant. When patch is the sampling unit, statistical analyses need to use patch as the unit of comparison, and should include 'locality' as a factor, to account for unstudied factors, together with the potential explanatory factors, such as clade, etc. and possibly number of patches number to account for population size.

It is less clear from the Methods in chapter 4 whether the sampling was patch based (should be detailed in the Methods), but I assume it was. i.e., several individual shoots sampled at each sampling spot (7-49 per locality). In that case, the principal consideration outlined above applies also here. Obviously, you cannot calculate a "clade identity" per patch in case you have both clades in a patch. If you sampled multiple ramets per sampling point (patch) and want to test for effects of environmental / climate variables, you will need to randomly pick one clade-identified ramet per spot. Based on this, you can build an appropriate model with clade as response, environmental variables as (different kinds of) factors, including also locality as a factor (for variation unexplained by the selected variables), and possibly sampling spots per locality to account for population size. Population size both

Naturhistoriska riksmuseet

Postadress:
Box 50007
104 05 Stockholm

Besöksadress:
Frescativägen 40
114 18 Stockholm

Telefon: 08-519 540 00
Telefax. 08-519 540 85
registrator@nrm.se

reflects and affects a number of conditions, to which the clades may respond differently.

Linkage disequilibrium (unclear sentences)

Chapter 5, p. 107: the third last sentence from the end states that high linkage disequilibrium indicates sexual reproduction (few localities) and in the sentence after, that it shows the prevalence of asexual reproduction. So, what does high linkage disequilibrium indicate?

Naturhistoriska riksmuseet

Postadress:
Box 50007
104 05 Stockholm

Besöksadress:
Frescativägen 40
114 18 Stockholm

Telefon: 08-519 540 00
Telefax. 08-519 540 85
registrator@nrm.se

Department of Botany and Zoology, Faculty of Science
Masaryk University
Kotlářská 267/2,
611 37, Brno, Czech Republic

A review of Mgr. Alžběta Manukjanová's doctoral (PhD) thesis "Molecular ecology of cryptic species of *Hamatocaulis vernicosus.*"

Written in the University of South Bohemia in České Budějovice, Faculty of Science, School of Doctoral Studies in Biological Sciences, 2019, supervisor: doc. Jan Kucera, PhD.

Candidate Mgr. Alžběta Manukjanová submitted a dissertation "Molecular ecology of cryptic species of the fen moss *Hamatocaulis vernicosus*". The problematics of cryptic species is actual problem which starts to be explored recently with the expansion of using molecular methods for various tasks. *Hamatocaulis vernicosus* is rare non-calcareous rich fen moss enlisted in Annex 2 of the Habitats Directive (92/43/EEC). Thesis fils a gap about detail knowledge of its recently distinguished cryptic species ('clade 1' and 'clade 2'). Goals of thesis concentrate on clade's differentiation, their biology, reproduction, distribution in the Czech Republic and genetic variability. The presented work is based on three published papers in specialized journals with impact factor and one paper in the form of the submitted manuscript. Articles are supplemented by general introduction and conclusion.

Chapter one 'The general introduction' shows a closer look at the development of the taxonomy of *Hamatocaulis vernicosus*, introduce the reader to the issues of cryptic species, the reproductive biology of bryophytes and show the possibilities of using molecular methods (especially microsatellites) in bryophyte population biology.

Paper 1 is methodical paper dealing with the development of way for barcoding of both cryptic species of *Hamatocaulis vernicosus* and design of functional microsatellite primers. The team of authors developed 19 PCR primer pairs and two methods of barcoding individual clades.

Paper 2 deals with reproductive biology; the authors noted a high sex expression and found no significant difference in the expressed sex ratio of barcoded individuals between clades.

Paper 3 maps the distribution of cryptic species of Hamatocaulis vernicosus in the Czech Republic, where clade 1 is predominant in the Czech Republic. It also aims to distinguish both cryptic taxa using morphometry and ecological preferences, which did not give significant results.

Manuscript 4 study the genetic variability of both cryptic taxa studied using microsatellite markers. Results show, among other things, reproductive isolation of taxa and different evolutionary history of clades.

The last chapter 'General conclusion' summarizes and generalizes the acquired knowledge.

The structure of the work is good, and the thesis contains all standard parts; aims and questions are well formulated and are focused to up-to-date problems in bryology. The connection among papers is well-explained. The author was able to explore in detail all topics of the thesis; she used a suitable methodology and brought new pieces of information about cryptic species of Hamatocaulis vernicosus. The work represents an fundamental step in cryptic species problematics and explains some aspects of their biology, which could be generalized for bryophytes.

Because the quality of thesis complies with the dissertation requirements, I recommend the acceptance of Alžběta Manukjanová's PhD thesis for the defence.

In Brno Oct 13, 2019

Mgr. Eva Mikulášková, PhD

Mire Ecology Group
Department of Botany and Zoology
Masaryk University, Brno

FACULTY OF SCIENCE · MASARYK UNIVERSITY · BRNO
DEPARTMENT OF BOTANY AND ZOOLOGY

Tasks for the Candidate:

1) Summarize why clade 1 and 2 are considered like different species and not just a genetic line within the species and then give us a general overview of the differences (even non-significant) between clade 1 and 2.

2) Do we have other examples of morphologically indistinguishable cryptic species in bryophytes within a morphological taxon?

3) Can you compare the advantages and disadvantages of using microsatellites versus next-generation sequencing for population study?

4) How look like individual patches - in how many percents are patches non-clonal (different genotype, clade, gender)?

5) How common are sporophytes, especially in populations where both sexes are present?

6) Is there some admixed individuals between clade 1 and 2?

7) Did you have some theory why clade 1 and 2 split up?

8) Did you observe some differences between clades which was not measured (fen microtopography, water level, vegetation composition, fen edge distance, chemistry, ...)?

9) Morphometry - why you measured just a leaf? Do you think that any other parameters can be useful for morphometry?

10) How can be obtained knowledge reflected in species conservation?