



IGB • Alte Fischerhütte 2 • 16775 Stechlin • Germany

University of South Bohemia
Faculty of Science
Branišovská 31
370 05 České Budějovice
Czech Republic

www.igb-berlin.de

Leibniz-Institut für
Gewässerökologie
und Binnenfischerei

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Testimonial to the PhD Thesis submitted by

Mgr. Lenka Caisová

at the Faculty of Science, University of South Bohemia

"Molecular phylogeny and taxonomic revision
of chaetophoralean algae (Chlorophyta)"

Mrs. Caisová arranged her thesis in a cumulative manner combining two papers. The thesis is written in English and styled according to the requirements of high level scientific publications. The first paper "Polyphyly of *Chaetophora* and *Stigeoclonium* within the Chaetophorales (Chlorophyceae), revealed by sequence comparisons of nuclear-encoded SSU rRNA genes" was already published in 2011 in the Journal of Phycology 47: 164-177, by the authors Caisová, L., Marin, B., Sausen, N., Pröschold, T. and Melkonian, M. The second paper "A close-up view on ITS2 evolution and speciation – a case study in the Ulvophyceae (Chlorophyta, Viridiplantae)" is ready to submit at BMC Evolutionary Biology, and was prepared by Caisová, L., Marin, B. and Melkonian, M. Both papers are well done, excellent molecular-phylogenetic studies.

The contributions of Lenka Caisová to these two papers are clearly declared and consist mainly in sequencing work, alignment of the sequences

Dr. Lothar Krienitz

Mitarbeiter der Abteilung
Limnologie Geschichteter Seen

Alte Fischerhütte 2
16775 Stechlin
Germany

Tel: +49 (033082) 699 26
Fax: +49 (033082) 699 17

krie@igb-berlin.de

Rechnungsadresse:
Forschungsverbund Berlin e.V.
Rudower Chaussee 17
12489 Berlin, Germany

Vorstandssprecher:
Prof. Dr. Jürgen Sprekels

Geschäftsführer:
Dr. Falk Fabich

Bankverbindung:
Commerzbank AG
BLZ 100 400 00
Konto-Nr. 520430003
DE 1004 0000 0520 4300 03
COBADEFF

and phylogenetic analyses as well as in re-evaluation of traditionally used morphological, ultrastructural and ecological characters of the algae studied. Mrs. Caisová had taken over the main work load in the process of writing the manuscripts. Furthermore, she contributed by developing of ideas and methods to realize the practical and theoretical work.

The dissertation is focused on the phylogeny and species conception of some of the most difficult groups of green algae the mainly filamentous Chaetophorales and the Ulvophyceae. The results have a very high amount of originality. Lenka Caisová used modern molecular-phylogenetic methods and settled into these highly complicated subjects quickly, carefully, and creatively.

The main results of the first paper consist in sequencing of 30 strains of Chaetophorales, including three own isolates from Czech Republic, and their phylogenetic analysis. The polyphyly of the species-rich genera *Chaetophora* and *Stigeoclonium* was proved. This paper is an important contribution to the systematics of these common but neglected groups.

The second paper is of general importance in the field of the species conception of algae and contains novel findings which contradict partly to findings of other authors. On the example of 86 sequences of ITS2 and their secondary structures of Ulvales, the CBC (Compensatory Base Change) concept was applied and refined. It was shown that CBCs and hemi-CBCs originated independently; only CBCs have the potential to provide strong criteria for species delineation. The authors showed that not all CBCs in Ulvales were taxonomically relevant and indicated the way how to find out "genuine" CBC-clades by mapping all CBCs on the phylogenetic tree. These findings about the differentiation of phenetic and evolutionary approach in CBC-analyses have model character for studies in other algal groups.

The only criticism is that there are some inconsistencies between title, objectives, general introduction, and the two main parts of the thesis.

The title is focusing on Chaetophorales, however one main part of the dissertation is focused on CBC-species conception, using ITS2 in general and is concentrated in particular to the Ulvophyceae. A more balanced general introduction providing the link from Chaetophorales to the other subjects would be helpful.

Mrs. Caisová worked very purposefully and putted a lot of power into the study. The thesis is well and fluent written. The figures are complex and reflect adequately the study findings. The general format and organization of the thesis are convincing. Content and outcome of the thesis fully meet the demands of a doctoral award. This work has been done in an exemplary manner.

Mrs. Caisová is very active in the field of international scientific cooperation and worked successfully in Kazakhstan, Brazil, Germany, and Austria. She presented her results during four phycological meetings in Japan, France, UK and Germany. In her home country she was involved in determination courses for limnologists and hydrobiologists. She is a very promising young phycologist.

Grade: very good



Stechlin-Neuglobsow 26.04.2011 Dr. habil. Lothar Krienitz

Lenka Caisová: Molecular phylogeny and taxonomic revision of chaetophoralean algae

This thesis examines systematics and evolution of some molecular and morphological characters in two groups of green algae, the chlorophycean order Chaetophorales and the ulvophycean order Ulvales. The thesis is subdivided in two separate parts focused on these two groups and I find that the title does not reflect completely this subdivision (basically, it refers only to the part on the Chaetophorales). Each of the two parts has produced an article in a high-profile international journal. Both articles are contributions of high scientific significance, which represent important advancements in the knowledge of the topics concerned.

The first article concerns the systematics of the green algal order Chaetophorales, with focus on the genera *Chaetophora* and *Stigeoclonium*. The Chaetophorales are among the most important green algae in freshwater environments, where they represent a group of great floristic and ecological significance; for this reason, a careful understanding of their diversity and evolution is very important. At the same time they are also a taxonomically difficult group, for which a modern assessment based on molecular data is greatly needed. The work is based on 30 new SSU rRNA sequences, which expand considerably the body of molecular data available for this group. This study is a very good piece of taxonomy, based on a state-of-the-art combination of molecular and morphological evidence. The introduction and the discussion present an interesting overview of the taxonomy of this group, which make a useful reading for people who are not familiar with it. The work is based on solid methods, which include sequencing of the SSU rRNA gene, analysis of molecular synapomorphies in the secondary structure, and cladistic analysis of morphological character evolution. The phylogenetic analyses include a combination of different phylogenetic methods and have been performed using several different softwares (which is rarely seen in taxonomic studies). The new sequences represent a great improvement in taxon sampling for this group and establish with strong support the polyphyly of the genera *Chaetophora* and *Stigeoclonium*. Overall, this article is a very significant step ahead in our understanding of the phylogeny of the Chaetophorales and will represent a fundamental reference for future work on these algae.

The second paper has a more methodological focus and concerns the evolution of the secondary structure of the ITS2 region in the green algal order Ulvales, examining its repercussions from a taxonomic point of view. The study is based on a database of 86 ITS2 sequences (12 of which were newly produced), for which the secondary structure was reconstructed; the dataset was analyzed by standard phylogenetic analyses and the diversification of ITS2 was studied following an evolutionary approach, rather than the typical phenetic one (CBCs and hCBCs were mapped on the phylogenetic tree upon reconstruction of ancestral character states). The approach used is innovative for this type of studies and provides very interesting results. In my opinion the conclusions reached in the study are very dependent on taxon sampling and on the methods used for reconstruction of ancestral character states, so they might be subjected to some revision in the future (i.e., future studies based on a wider taxon sampling and performed with different softwares might provide somewhat different results). This, however, does not affect the validity of the work carried out, which provides convincing evidence for a critical rethinking of the approach used in ITS2-based studies of algal taxonomy and phylogeny. Based on the methods used and the results presented, I find that the conclusions are fully justified and I agree with most of the points highlighted by the authors. The conclusions are very interesting and delineate a quite different scenario in the evolution of ITS2 in the Ulvales compared with other algal groups. The inapplicability of the CBCs species concept/biological species concepts is demonstrated and evidence is presented that in the Ulvales CBCs normally do not derive from the sum of two successive hCBCs. An aspect that I particularly appreciate is the hard and meticulous work carried out for the alignment of the sequences; I agree with the authors that for this type of studies a careful manual alignment is fundamental and cannot be replaced by automatised procedures. I also find that the authors did a very good job with the figures, presenting in a generally clear and visually appealing way a complex set of

evolutionary events. As general opinion, this is a very valuable study that will certainly give a direction for all studies on algal systematic based on ITS2 which will be carried out in the near future.

Overall, I find that Dr Lenka Caisova did an excellent work, which fully deserves a Ph.D. degree. The science is strong and based on sound methods; the work is well presented and discussed with high-quality illustrations. The quality of the English is excellent and the thesis reads very well in all its parts. The hard work and dedication that animated Lenka's activity in the course of the Ph.D. transpire from the thesis. Besides the publication of the first article in the Journal of Phycology, she has presented her work in major international conferences (in which I had the opportunity to meet her and had interesting discussion about her work). Her CV shows that she was already a very active researcher with a solid background in traditional taxonomy. The work performed during the Ph.D. in a prestigious foreign laboratory gave her the opportunity to train in PCR and DNA sequencing, phylogenetic analyses and reconstruction of the secondary structure of ribosomal DNA. This experience will be of critical importance for her scientific growth; the new expertise gained in the course of the Ph.D. makes her a strong and mature scientist, with the potential to become a leader in her area in future years.

Again, I find that the quality of this thesis is very high and I don't have any criticisms. My remarks are points of reflection or requests of clarification about some matters on which I would have appreciated a bit more discussion.

1) What are the future perspectives for the taxonomy of the Chaetophorales? Based on the evidence presently available, what do you consider a "good" species in this order? What taxa do you consider a priority for future work?

2) The ITS region occurs in the nuclear genome in multiple copies. In several algae, including species of green algae, intraspecific or even intragenomic variation has been demonstrated. This is an important aspect on which I would have liked to see more discussion. At page 70 there is a brief mention of mechanisms related to intragenomic polymorphism (lines 8-10 of the second paragraph: "Alternatively, mutants may escape extinction by intragenomic rRNA homogenization .."), but otherwise this potential problem is not mentioned anywhere else in the thesis. Did you consider this matter when you produced your ITS2 sequences and reconstructed their secondary structure? Did you verify that in your strains there was no intraspecific or intragenomic variation? What are the possible implications with regard to your conclusions?

3) The species currently assigned to the order Ulvales are about 250. How do you think an expanded taxon sampling in future molecular studies might affect your conclusions? Do you think that the inclusion of new lineages in the database this might lead to modifications in the reconstruction of CBCs placement along the tree?

4) The biological species concept has been well studied and established only for relatively few algal taxa. In the case of the Ulvales, not many species have been investigated in this regard (as correctly mentioned in the discussion of the paper 2); so, it is difficult to define a biological species concept in this group. Instead, I think it would be interesting to compare the conclusions of ITS2 with conclusions based on other molecular markers. In this regard, there is a large number of *rbcL* sequences available for the Ulvales. This marker has a substitution rates suitable for the species and genus levels and in fact it is generally considered the preferred markers for species characterization in the green seaweeds. So, I think it would be interesting to compare the ITS2 results with the *rbcL* results available for this group (for which there are numerous papers available).

5) The fact that different methods and softwares may lead to different reconstructions of the secondary structure of ITS2 is at the same time one of the most intriguing and most worrying conclusions of the study. So, I will be interested to hear directly from Lenka more impressions and recommendations on this point.

6) In the materials and Methods of the paper 2 it is mentioned that sequences with obvious data errors retrieved from GenBank were not used. What type of errors did you observe?

7) Sequences labeled "*Umbraulva japonica*" and "*Blidingia minima*" in the ITS2 phylogeny. I don't think these were misidentifications; more likely they were contaminations, but I would't discard completely the possibility that at least the sequences of *Umbraulva* are in fact good.

Falso Rindl

REVIEW OF THE PH.D. THESIS OF LENKA CAISOVÁ
**MOLECULAR PHYLOGENY AND TAXONOMIC REVISION OF
CAHETOPHORALEAN ALGAE**

The Ph.D. thesis of Lenka Caisová consists of a brief introduction on 10 pages, one published manuscript and one manuscript prepared for submission. The title of the thesis itself seems to be a little inappropriate, because only one of the two papers is focused on the chaetophoralean algae, while the second and more extensive paper studies the evolution of ITS2 region in Ulvophyceae. The introduction is very brief and summarizes the history of algal classification and overviews the species concepts with a particular focus to the CBC species concept that is one of major themes of the thesis. I as non-algologist would very appreciate if the introduction briefly introduces also the organisms with some illustrations so that I had no need to look it up elsewhere especially if the author discusses quite extensively the evolution of morphological traits in some lineages in the first paper.

The first paper analyses the phylogenetic relationships among green algae with particular accent to Chaetophorales. It clearly shows the polyphyly of two genera (*Chaetophora* and *Stigeoclonium*) and then revises the taxonomy in the way it was congruent with the phylogeny. I would like to stress the importance of the taxonomic revision, because many similar paper end up with stating that the taxa are polyphyletic, but do not do take the time and go on with the formal revision of the taxonomy. The paper was already accepted to a peer reviewed journal, and so it is difficult for a reviewer to reveal any more weak points. I find the conclusions well supported and have only two minor points.

- 1) The AU tests that you used for testing topologies was performed in the way that is common in many publications, i.e. on a set consisting of the best topology and a few alternative topologies. However, it is suggested that for the good performance of the AU test, the testing set of topologies should be large (hundred and more). Usually this set of topologies is created by using the top hundred (or more) topologies from maximum likelihood analysis or by using the topologies from bootstrap analysis. In to such set of very likely topologies are then included the topologies of particular interest. I have no doubt that the results of the tests would be very similar in this case, but the recommended way, how to perform the AU test is the one mentioned above.

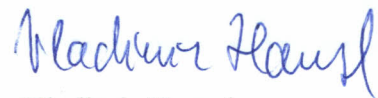
- 2) I wonder if it would be difficult to obtain the sequence of *Chaetophora globosa* that is by some authors regarded as a type species of the genus. If it appeared in the same clade as *Chaetophora lobata* that you regard as type species, there would not be any controversy in classification.

The second manuscript focuses on the evolution of CBC and hCBC in the family Ulvophyceae and draws some important conclusions regarding the CBC species concept. It is a very detailed study and after reading the manuscript it becomes absolutely clear that the authors have analysed the data very precisely and were thinking about and discussing every detail. This becomes, however, a little painful for the reader to follow the descriptions of many minutiae substitutions. In my opinion the text of the manuscript would benefit from shortening, accenting the important aspects and suppress those that are less important. I have several questions or comments regarding this manuscript:

1. I wonder whether anybody tried to apply the CBC species concept on asexually reproducing organisms?
2. I have a comment to the existence of CBC grades. The observation that organisms identical in sequences of helix 2 and helix 3 stems may not necessary form just a clade but also a grade is very important. However, this fact is quite obvious and anyone could hardly think that it would be some other way. In principle, every newly arisen CBC creates one clade formed by all descendants of the organism in which the CBC appeared but at the same time it also forms a grade formed by all remaining organism. I simply do not see any other way, how it could be.
3. Legend to the additional file 3 is insufficient as it does not explain what do the blue and red diamonds with numbers in them mean. Something is explained in the text, but the legend should be self-explaining.
4. You often mention that the CBCs in Ulvophyceae are saturated (eg. the page 68). However, the total number of CBC types in all Ulvophyceae is 12, while the maximum number of CBCs that you observe in one taxon is 4. This does not indicate a high saturation in my opinion.
5. Do you have an explanation for the fact that out of 45 "universal" base pairs in Ulvales, only 12 has been observed to undergo change?
6. In Ulvales, you observed that of all possible types of CBCs the A-U => G-C is far the most frequent. Is it a general rule that holds also to other groups of eukaryotes?

7. I find surprising the observation that hCBCs are not the intermediate steps of CBCs and that the CBCs originate rather by two substitutions with an intermediate causing a mismatch in the stem. I wonder whether it could be explained by the fact that, unlike the substitutions producing a mismatch pair, the hCBCs result in a stable state that is not disadvantageous for the organism and as such it does not put the organism under the pressure to compensate the mismatch quickly by another substitution. Because the probability of accidental substitution on the opposite strand is low, the organism remains in the state after hCBC for a long time.
8. The existence of CBC grades means that the organisms with identical base pairs in the conservative stems do not always represent a species. I wonder if the CBCs could still be used for rejecting species, i.e. if you can claim that a clade of organisms that are not identical in the CBCs do not represent a species. This itself would still be very useful in taxonomy.

At the end of my review I would like to state that the thesis demonstrates the ability of the author to collect and analyze useful scientific data, to critically think, discuss and test the scientific hypotheses and finally to write a scientific papers. By all this Lenka Caisova have proved that she deserved the title Ph.D.



Vladimir Hampl