Review of the Ph.D. thesis

Tamara Tešitelová, roz. Malinová: Ecological and evolutionary consequences of orchid dependence on mycorrhizal fungi

The orchids have always attracted the attention of the scientists; for example, I can mention the book *On the various contrivances by which British and foreign orchids are fertilized by insects* (...) written by Charles Darwin and published in 1862 or the excellent pioneer work of Noel Bernard on orchid mycorrhiza from the early 20th century. Despite this attention, there are still many major gaps in our understanding of how is the mycorrhizal symbiosis of orchids functioning. This Ph.D. thesis is based on five original papers published in highly impacted journals and it represents very nice contribution to our effort to fill some of these gaps. The exceptionally high amount of papers as well as the high impacts of the journals are very good and objective indicators of the Ph.D. thesis quality and I am not intending to discuss here any of the results published therein; the editors and the reviewers have surely done good jobs.

Instead of discussing the facts there can be found in the thesis (however, with one exception, see later), I would like to ask Tamara for what is otherwise completely or partly missing in the very compact General Introduction (10 pages) and Summary of the Results (3 pages and one line). Especially the General Introduction would, in my view, reflect all of the most important aspects of the OM symbiosis, not only the aspects more or less related to the thesis' topic and field research of OM. Answering these questions will show clearly that many important issues of OM symbiosis cannot be solved unequivocally without laboratory or greenhouse experimentation. The question will follow soon, but here I would like to remind you to the fact that majority of the most basic facts about functioning of the other mycorrhizal types, especially of arbuscular mycorrhiza, was obtained not in the field, but in lab or greenhouse strictly controlled experiments.

Tamara, would you be so kind and can you answer following simple questions and cite appropriate studies, if they exist? If there is a lack of these studies, I would like to hear your own opinion, regardless the evidence is lacking:

1. Considering the green orchids from open habitats associated with rhizoctonias, can be this symbiosis regarded as mutualistic? What characteristics should be used as a criterion for considering the symbiosis as mutualistic or parasitic?

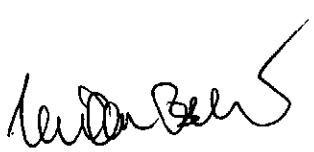
2. How do the mycorrhizal fungi contribute to the mineral nutrition of orchids? How should we arrange our experiments in order to get the evidence? Please, consider again the most abundant group of Europaean orchids, i.e. green orchids from open habitats.

3. How does the orchid mycorrhizal symbiosis respond to eutrofication? In arbuscular mycorrhiza, high supply of plants with phosphorus down regulates the formation of mycorrhiza. Is it true also for orchid mycorrhiza? Again, for simplicity, consider green orchids from open habitats associated with rhizoctonias.

4. If the OM fungi like the Sebacinales clade B or Tulasnellaceae associate not only with orchids, but also with unrelated co-occuring plants, how does it affect the growth and nutrient flows between orchids and the co-occuring plants?

Before I will summarize my review, I would like to express my opinion that from the fact, that isotopic composition of symbiotically germinated protocorms did not differ from the adult stages as well as neighboring autotrophic plants it cannot be concluded, that partial mycoheterotrophy may be much more widely distributed among orchids than hitherto assumed (Stöckel et al. 2014, cited on the p. 8). This suggestion is in my view completely confusing, because the opposite can be equally true. Simply, the method is not suitable for quantification of initial mycoheterotrophy and later partial mycoheterotrophy of green orchids and nothing more can be concluded.

If I have to summarize my review, there is no doubt that this Ph.D. thesis is exceptionally successful work finishing exceptionally successful Ph.D. study, confirming the ability of its author participate at the top scientific research, and I cordially recommend the thesis for the defense.



In Brno, October 29, 2014

RNDr. Milan Baláž, Ph.D.

Smithsonian Environmental Research Center

Review of a Ph.D. thesis entitled 'Ecological and evolutionary consequences of orchid dependence on mycorrhizal fungi' presented by an applicant Tamara Těšitelová

Referee: Dr. Melissa McCormick, PhD.

The submitted thesis consists of four published papers and one submitted manuscript dealing with diverse below-ground interactions of several European orchid species and the potential consequences of mycorrhizal fungus identity for orchid ecology, distribution, and evolution. The aim of the thesis was (i) to review the factors affecting population decline in one orchid, (ii) to identify mycorrhizal fungi associated with the study orchids, (iii) to determine how those fungi change seasonally, while evaluating different methods for describing fungal diversity, (iv) to determine how mycorrhizal fungal associates change with orchid ploidy level, and (v) to begin to understand how the identity of mycorrhizal fungi has contributed to evolution of partial or total dependence on mycorrhizal fungi in one orchid genus. The scope of the thesis is well defined and includes primarily assessment of basic scientific questions but also addresses their ramifications for ecology and conservation of endangered species.

Regarding the formal aspects, the thesis has a standard structure, including a general introduction and a summary section. Individual papers are presented in a form of published journal papers. As I understand the requirement, the submitted thesis is formally correct.

Regarding the content, my general impression from the submitted thesis is excellent. The papers are very well written, with three papers in relatively high-quality scientific journals. In all papers the methods are clearly described, data are analyzed using appropriate statistical methods, results are clearly presented and well discussed, and the tables and figures are well arranged. The submitted paper addresses some of the evolutionary implications of mycorrhizal fungus identity and also has the potential to be accepted in a high-quality scientific journal. Each of these five papers presents a nice contribution to the ecology of orchid-fungus interactions, with wide-ranging implications for understanding mycorrhizal associations generally and also for orchid conservation. The first paper provided a very thorough review of the ecology of a declining alpine orchid, and included an unusually broad assessment of dependence on other species (i.e., pollinators and mycorrhizal fungi). The second paper assessed germination capabilities in four *Epipactis* species and determined that the availability of appropriate fungi was not the factor that was limiting plant distribution; rather plant distribution was likely driven by environmental factors that affected either the fungi supporting the orchid or the orchid itself. The third paper

identified diverse endophytic fungi in addition to presumably mycorrhizal fungi associated with the roots of *Pseudorchis albida*. The identity and function of non-mycorrhizal fungi in orchid roots is largely unknown and this paper provided a well-defined starting point for further research. The fourth paper is probably the strongest paper in the thesis, addressing the divergence of mycorrhizal fungal communities associated with distinct cytotypes and species. This research adds considerably to understanding of possible forces driving speciation in orchids and also in other species. The fifth paper dealt with how the identity of mycorrhizal fungal associates contributes to the evolution of mycoheterotrophy in the genus *Neottia*. This paper seemed to be overreaching a bit on the conclusions that could be drawn from a relatively small study and may require some additional work before it will be acceptable for publication (see my comments below). Overall, the quality of the thesis is excellent and the author clearly demonstrated her ability to defend her research to other scientists and produce successful publications. Therefore, I recommend this thesis to be accepted for the award of PhD.

Major remarks:

The general Introduction adds significant information beyond the introductions of individual papers and helps the thesis to stand alone. The introduction also does a good job of setting up the questions being addressed in subsequent chapters.

Paper 1: Review of the ecology of *Pseudorchis albida*

- No comments

Paper 2: Symbiotic germination in *Epipactis*

- Here and also in paper 5 (evolution of mycoheterotrophy) it seems that association with ECM fungi is taken as evidence of potential for mixotrophy or full mycoheterotrophy, yet a recent paper by Stoeckel et al. found that C and N stable isotopes do a poor job of detecting mixotrophy in orchids associated with saprotrophic fungi, especially rhizoctonias. This is a problem, because pure fungal structures of these fungi are rarely available for calculation of isotopic mixing. If rhizoctonia isotopic compositions do not follow the patterns seen for many other fungi how does this complicate interpretation of how important association with ECM fungi is in the evolution of mycoheterotrophy?
- One problem with using seed germination as indicative of appropriate conditions for orchid colonization is that seed germination only requires appropriate conditions and fungi for a short time, while establishment and development to maturity require persistently appropriate

conditions. Similarly, as noted in the paper, seed germination and the earliest stages of seedling development can occur without appropriate fungi, but development does not progress without those fungi. Please discuss what this means for designing research to accurately assess what factors govern establishment of sustainable populations.

- The paper suggests that spreading seeds into uncolonized habitats might be used to offset some loss of existing populations. How often do you think that would be successful and why?

Paper 3: Diverse fungi with *Pseudorchis albida*

- The authors found that diverse endophytic fungal communities associated with the roots of *P. albida* in addition to mycorrhizal fungi. What do you think the possible roles are for RAF in orchid roots?
- The authors found that multiple methods were differently biased in terms of the fungal communities they revealed and they recommended a combination of culture-dependent and –independent techniques to accurately assess both the mycorrhizal and RAF communities. Can these results be used to reinterpret studies that used only one technique? Why or why not?

Paper 4: Ploidy-specific interactions

- How common is polyploization in orchids? How do you think it has contributed to the huge number of species in the Orchidaceae?
- If co-occurring species and cytotypes associate with different groups of fungi, do you think that also happens for different genotypes (see Taylor and Bruns paper)? What does this imply for how density of orchid populations (and consequently, in some cases, rarity) is determined?
- How often the results from *ex situ* experiments are consistent with host specificity *in situ*? Which factors might be causing the inconsistencies?

Paper 5: Evolution of mycoheterotrophy

- How much of the conclusion about switching to MH being associated with switching to ECM is a result of not being able to tell when pMH species that associate with rhizoctonias are getting C from their fungi? Is anything known about the isotopic discrimination of *Sebacina* spp? Could it be that the saprotrophic *Sebacina* species do not provide a distinct

enough signal to identify fungal input to orchids? There seems to be somewhat circular reasoning in this chapter. It seems circular that if an orchid (e.g., *N. camtschatea*) associated with ECM fungi it is assumed to be pMH or MH, but then only orchids that associate with ECM fungi can isotopically be considered pMH.

- If we examined all orchids and looked at their above-ground N concentration rather than just ^{13}C as a signal for pMH, what pattern do you think we would see? Would the switch to ECM precede or follow?
- While utilizing one of the few genera with MH, pMH, and presumably strictly autotrophic species is a strong way to analyze evolution of mycoheterotrophy, there are also limitations to this approach, as the genus may have characteristics that make it predisposed to mycoheterotrophy, so conclusions drawn from such a genus might not be applicable to understanding evolution in other genera. Please discuss this.
- There are also some major problems with trying to draw conclusions about evolution from only four species, one of which is represented by a single sample. Can you relate this study more firmly to studies in other genera with MH and pMH species (perhaps *Cephalanthera*?). With so few species, that also makes it particularly important to have a well-defined phylogeny. Do you know why the phylogenetic position of *Neottia ovata* was so unstable in this analysis and the relationships seem different from those in other published phylogenies of the genus?

Melissa McCormick 3 Nov 2017

Reviewer's report of the Ph.D. thesis entitled:

Ecological and evolutionary consequences of orchid dependence on mycorrhizal fungi

(by Tamara Těšitelová, University of South Bohemia in České Budějovice, Faculty of Science)

The reviewed study is focused on the theme that is important not only because it brings new information on a specific symbiosis of orchids but also because it provides a contribution to understanding the coexistence of organisms in general.

Besides the „General introduction" and final „Summary of the Resuts", the thesis is composed of 5 original publications - research reports. One publication is at the stage of submission and other 4 works have been published in highly reputed journals such as New Phytologist, Journal of Ecology, American Journal of Botany and Fungal Ecology. T. Těšitelová stands as first author on 3 works and also as corresponding author on 3 works, which indicates her deep involvement in the reported research.

The thesis conforms to the high graphical standards, contains few typescript errors and is well written. This applies not only for already published parts but also for the unpublished text portions contained only in the thesis.

„General introduction" (13 pages) represents a comprehensive synthesis of relevant literary knowledge. It contains general information on orchideoid mycorrhizal symbiosis, its phylogenetic aspects, identity of fungal symbionts and physiology of the symbiosis. Is well written and reveals complexity of the subject as well as the passion of the author. It also contains the section defining objectives of the thesis and its content. At this place, I would expect more extensive explanation of specific idea behind the definition of goals of different publications constituting the thesis. I agree, however, that the statements found in the second paragraph, pg. 12, could be taken as indications of such explanation in a primordial form which should be further expanded. For example, this chapter should state why the information about the possible limitation of distribution of orchids in different habitats by the mycorrhizal fungi is important. Further, the question appears how the effect of the ploidy of the host plant on the identity of mycorrhizal fungi is related to co-evolution of both partners.

Chapter II represents several aspects of biology and inventory of the distribution of *Pseudorchis albida* and its dynamics. Mycorrhizal symbiosis is mentioned marginally.

Chapter III describes the seed germination of *Epipactis* spp. under the influence of environmental factors and mycorrhizal fungi and is extremely interesting. The main finding of

this chapter is that the incidence of the mycorrhizal fungi colonizing orchids and supporting their germination does not depend dramatically on the environmental factors (habitat conditions) and that suitable mycorrhizal fungi can be found in (and recruited from) practically any forest soil. This is called „broad germination potential" (pg. 74). If this is the case, it may indicate relatively loose relationship between the plant partner and symbiotic fungi. How the affinity of *Epipactis* spp. adults and seedlings to the „pezizalean" (Pezizales?, pg. 75) fungi can be explained? Is it reasonable to take this association as a result of co-evolution of these two groups of organisms (i. e. the genus *Epipactis* on one side and „pezizalean" fungi on the other)?

Chapter IV is interesting as the study of methodology suitable for evaluation of the diversity of orchideoid mycorrhizal fungi in an orchid species. Unfortunately, the data presented on pg. 113 (Fig. 1) indicate that the „functional" diversity obtained using isolation-dependent approach (from pelotons) has negligible overlap with the „molecular" diversity and no overlap with diversity obtained using isolation dependent approach from root segments! This is serious finding which may disqualify the nonspecific molecular methods (PCR with primers ITS1 and ITS4, pg. 104) from detecting orchideoid mycorrhizal fungi in root samples. I am very interested in the opinion of the author. Taking the diversity of isolates derived from pelotons as the diversity of true mycorrhizal fungi, we should reject any methodology which omits the fungi forming pelotons if we need to describe the community of the mycorrhizal fungi. Other fungi (not detected by isolation from pelotons) must be probably taken as accompanying opportunists. Is it possible to use more specific modification of PCR which would provide higher sensitivity to *Tulasnella* spp. forming pelotons (primer ITS4Tul, pg. 149)?

Chapter V is dealing with effects of host ploidy on the community of orchideoid mycorrhizal fungi („mycorrhizal associations"). The data indicate that cytotype of the plant strongly affects the proportions of different *Tulasnella* OTUs (pgs. 147-148). An attempt to speculatively explain this finding is given on pg. 151. As the genome duplication is common event in the evolution of Orchidaceae (pg. 135), the above finding provides the direct evidence of selection of mycorrhizal fungi as a result of plant genome evolution.

Chapter VI attempts to elucidate possible shifts of the community of orchideoid mycorrhizal fungi as a result of mycoheterotrophy development. The authors studied phylogenetic relationships between different phototrophic, partially mycoheterotrophic and mycoheterotrophic *Neottia* species. Their study resulted in well supported idea of evolution of this plant group with phototrophy as ancestral life strategy. They further tried to find the

differences in mycorrhizal symbionts that would correspond to different life strategies of hosts. However, in the cladogram on pg. 179 (Fig. 4), there are only two OTUs that fall into the clade A of Sebacinales that correspond to fungi from hosts with partially mycoheterotrophic or mycoheterotrophic life strategy. I am not sure whether it is possible to build strong statements about the evolutionary shifts of the community of mycorrhizal fungi on the basis of such reduced dataset.

„Summary of the results" is detailed and provides a synthesis of results of all the 5 publications. The thesis is characterized here as a „set of research pilot studies in little investigated directions, and further research should evaluate its general value" (pg. 207). Some criticism and limitations of the reported research are provided at the same place.

Taking into account the facts mentioned above as well as the opinion attained by the reading the content of the thesis, I state that the reviewed work meets the criteria established for PhD thesis. T. Těšitelová proved to be able to collect and evalute experimental data as well as to interpret them in the context of existing scientific knowledge. I thus propose to award her a PhD degree.

Underlined sections/questions would merit discussion during the thesis defense.

In Prague, Oct 28, 2014

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