Biology of the Root-Parasitic Rhinanthoid Orobanchaceae

Ph.D. Thesis

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Annotation

Evolution, physiology and ecology of root-parasitic Rhinanthoid Orobanchaceae was investigated with special emphasis on understanding biology of hemiparasitism. The research was based on a wide range of methods including molecular phylogenetics, cultivation experiments, stable isotope analysis, electron microscopy, geometric morphometrics and flow-cytometry.

Declaration [in Czech]

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České Budějovice, 13.6.2011

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Contents

General Introduction

Most plants are photoautotrophic organisms fulfilling their energetic demands and acquiring carbon through photosynthesis, i.e. process based on conversion of light energy to chemical energy via a number of photochemical and biochemical processes (Taiz & Zeiger 2006). Although the organic carbon is the key resource for plants, they are also dependent on water and mineral nutrients which are acquired from the environment (either directly through plant roots or rhizoids or through symbiotic relations with other constituents of ecosystems, principally mycorrhizal fungi). Despite the prevailing autotrophic strategy, several plant lineages have evolved heterotrophic means of resource acquisition, parasitizing fungi (mycoheterotrophy; Leake 1994, Selosse & Cameron 2010) or other plants (plant parasitism; Musselman & Press 1995, Irving & Cameron 2009) for either all or a subset of resources required to support their vital processes.

Lineages displaying parasitic strategy of resource acquisition have evolved on multiple occasions in most of the lineages of land plants including liverworts, lycophytes, ferns gymnosperms and angiosperms. Except for the major part of parasitic angiosperms however, all of the parasites within these groups represent mycoheterotrophs (Merkcx & Freudenstein 2010), including also a single parasitic gymnosperm *Parasitaxus ustus* which combines both mycoheterotrophy and direct plant-to-plant parasitism via haustoria (Field & Brodribb 2005). On the other hand, angiosperm parasites uptaking resources directly from the host plant via haustoria are the most species-rich group of parasitic plants (Nickrent 2002, Heide-Jørgensen 2008) comprising almost 4000 species which accounts for ca. 1% of angiosperms. It is important to bear in mind that despite forming a specific functional group (Cornelissen et al. 2003), they do not form a monophyletic clade since the haustorial parasitism evolved independently at least eleven times during the evolutuion of the angiosperms (Barkman et al. 2007).

The haustorial parasites attach to roots or stems of their host via haustoria, specialized organs transferring nutrients from the vascular bundles of the host to the parasites (Musselman & Press 1995, Irving & Cameron 2009). The location of the attachment to the hosts underlies two functional groups of the parasitic plants – stem parasites and root parasites which are the subject of this thesis. Nonetheless, even more important functional classification of parasitic plants is defined by presence or absence of photosynthetic activity. Parasitic plants performing their own photosynthesis are called hemiparasites while those lacking this ability are termed holoparasites or full-parasites (Musselman & Press 1995, Nickrent 2002, Irving & Cameron 2009).

Of these, the hemiparasites display comparatively lower dependence on host resources attaching to host xylem withdrawing water, mineral nutrients and also xylem-mobile organic compounds. In general, all or at least major part of mineral nutrients and water used by the hemiparasite originates from the host (Ehleringer & Marshall 1995, Pate 1995, Jiang et al. 2003, 2004, 2010). Holoparasites, on the other hand, acquire all resources from their host plant including carbon and are unable of their own photosynthetic activity (Pate 1995, Irving & Cameron 2009). This functional classification of parasitic plants appears clear-cut; nonetheless host-to-parasite flow of organic carbon has been also documented for numerous hemiparasitic associations accounting for up to several per cent of hemiparasite biomass (e.g. *Paper 2*, see *Paper 3* for a review) blurring slightly the key difference between the hemi- and holoparasites. Moreover, rudimentary photosynthetic activity was demonstrated in some species of *Cuscuta* which are otherwise typical holoparasites highly dependent on host establishing phloem connections and effectively withdrawing organic carbon from the host (Hibberd et al. 1998). Therefore, some authors (e.g. Kolb 2002, Irving & Cameron 2009) suggest an alternative concept based on the type of vascular tissue to which the parasite is attached, i.e. xylem-feeders attached to xylem only (corresponding more or less to hemiparasites) and phloem feeders attached to both xylem and phloem (corresponding mainly to holoparasites). Nonetheless, existence of a xylem-feeding holoparasitic strategy (Ziegler 1955, *Paper 5*) illustrates difficulties with finding an exception-less while simple functional classification of parasitic plants.

This thesis deals with biology of the Rhinanthoid group of Orobanchaceae which represent a monophyletic clade within this family (Bennet & Mathews 2006). Most of its representatives are root-hemiparasites but it also comprises the holoparasitic genus *Lathraea* and several species that feature a holoparasitic life stage in their early ontogeny (*Paper 1, Paper 5*). The Rhinanthoid group is distributed globally but its diversification centre lies in the Western Eurasian region (Wolfe et al. 2005, *Paper 1*) making it rather easily accessible for local research community. Therefore, some of the Rhinanthoid species are largely used as models for studying root-hemiparasitic associations. This applies in particular for species of the genus *Rhinanthus* (e.g. Cameron & Seel 2007, Jiang et al. 2010, Hautier et al. 2010, *Paper 2*, *Paper 4*) but also e.g. *Euphrasia* (Lammi et al. 1999) and *Melampyrum* (Lechowski 1996). Some of the Rhinanthoid hemiparasites, have been demonstrated to play an important functional role in various ecosystems and under multiple abiotic conditions. This role is mainly connected with specific physiology of hemiparasitic associations resulting in often severe damage to the host species and accumulation of nutrients in the hemiparasites. By this means, the hemiparasites alter competitive relationships within communities and modify nutritional cycling in the environment which can result in an increase of species diversity of communities by decreasing competitive pressure from dominants (if these are preferred hosts) and hence facilitating species coexistence (Quested et al. 2003, Cameron et al. 2005, Press & Phoenix 2005, Bardget et al. 2006).

The major part of the Rhinanthoid Orobanchaceae grow in rather stable communities dominated by perennial plants (such as grasslands or forests) where stress or competition are limiting factors (although there are noticeable exceptions such as the *Odontites vernus* group members of which grow predominantly in disturbed habitats; *Paper 7*). It is therefore quite surprising that most of the temperate Rhinanthoid hemiparasites are annuals. The annual life history is probably closely related with

Fig. 1 Young plant of *Rhinanthus minor* established on a plot covered by a 5 cm thick layer of dead biomass. Restored meadows in White Carpathians, Czech Republic, 1st May 2011.

Fig. 2 *Rhynchocorys elephas* growing in extreme light deficiency under dense canopy of *Petasites* sp. leaves. Stara Planina Mts., Bulgaria, 28th June 2009.

efficient acquisition of host-derived resources (Press et al. 1988, Ehleringer & Marshall 1995) but also presents an apparent constraint by requiring successful seedling establishment in every growth season. This is even more apparent considering very slow growth and inefficient physiological processes (such as photosynthesis) of the seedlings of the hemiparasites prior to the attachment to the host (Seel et al. 1993, Lechowski 1996). Some of the hemiparasites (such as *Rhinanthus* spp. or *Melampyrum* spp.) therefore compensate for this constraint by production of large, resource-rich seeds enabling fast growth of seedlings at the start of the growing season and hence allowing them to inhabit sites where no other annuals persist (Kelly 1989, Strykstra et al. 2002). In addition, heterotrophic carbon acquisition from the hosts can significantly contribute to the energetic and carbon balance of the hemiparasites decreasing, at least to certain extent, their dependence on their own photosynthesis and consequently allowing them to survive even under severe light deficiency, especially if it is only temporary (*Paper 4*). Hence, e.g. *Rhinanthus minor* seedlings were observed to have established under complete shading on plots covered by a 5 cm thick layer of dead biomass, where seedlings of no other species were observed (Fig. 1). *Rhynchocorys elephas* growing and reproducing under very intense shading from the surrounding vegetation (Fig. 2) then presents an extreme example of a hemiparasite presumably dependent on host-derived carbon.

This thesis contributes to the knowledge of fundamental physiological processes connected with the heterotrophic carbon acquisition by hemiparasitic species (*Paper 2 and 3*) and deals with their implications for the population ecology of the hemiparasitic species (*Papers 3 and 4*). In addition, it presents a new synthesis of the phylogeny and evolution of the whole Rhinanthoid group (*Paper 1*) as well as detailed pictures of evolution of two of its species complexes in Central Europe (*Papers 6 and 7*). Finally, the *Paper 5* integrates both physiological and evolutionary view on the Rhinanthoid Orobanchaceae by presenting a synthesis of available data on leaf excretory glands supplied with their newly presented detailed ultrastructure. This paper consequently summarizes their function in physiological processes of the hemiparasites and newly points to their key role in the evolution of parasitism in the Rhinanthoid Orobanchaceae.

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General introduction

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List of papers with abstracts and author's contribution
Paper 1

Těšitel J, Říha P, Svobodová Š, Malinová T & Štech M (2010): Phylogeny, life history evolution and biogeography of the Rhinanthoid Orobanchaceae. Folia Geobotanica 45: 347–367.

The Rhinanthoid Orobanchaceae form a monophyletic lineage including the hemiparasitic genera *Euphrasia*, *Melampyrum*, *Tozzia*, *Bartsia*, *Nothobartsia*, *Odontites* (s. l.), *Rhinanthus*, *Rhynchocorys*, *Parentucellia*, *Hedbergia* and holoparasitic *Lathraea*. In this study, we aimed to reconstruct the phylogeny, evolution of life history traits (life cycle and seed size) and explain the extant biogeographic patterns in this group. For the phylogenetic reconstruction, we used molecular data obtained by sequencing of the nuclear ITS region and chloroplast *trnT-trnL* intergenic spacer and *matK+trnK* region. The genus *Melampyrum* was found to occupy the sister position to the rest of the group. The other genera were assembled in the sister *Rhinanthus-Rhynchocorys-Lathraea* and *Bartsia-Euphrasia-Odontites* subclades. The reconstruction of life cycle evolution yielded ambiguous results suggesting nonetheless substantially higher likelihood of perenniality compared to annuality in most ancestor lineages. Seed size varied across two orders of magnitude (average weight per seed: 0.02-7.22 mg) and displayed a notable pattern characterized by tendency to reduction in the *Bartsia-Euphrasia-Odontites* subclade compared to the rest of the group. Seed size evolution was found to be correlated with life history evolution in the group if the generally small-seeded *Bartsia-Euphrasia-Odontites* subclade is excluded. We formulated hypotheses relating the extant biogeographical affinities of individual genera to the geological history of the Euro-Caucasian diversity centre of the group. Notable dispersal events in *Euphrasia* and *Bartsia* were hypothesised to be allowed or at least facilitated by a specific combination of the life history traits.

Paper 2

Těšitel J, Plavcová L & Cameron DD (2010): Heterotrophic carbon gain by the root hemiparasites *Rhinanthus minor* and *Euphrasia rostkoviana* (Orobanchaceae). Planta 231: 1137–1144.

Hemiparasitic plants gain virtually all mineral nutrients and water from their host plant while organic carbon is provided, at least in part, by their own photosynthetic activity although their rates of assimilation are substantially lower than that found in non-parasitic plants. Hence, hemiparasites must gain at least some of their organic carbon heterotrophically from the host plant. Despite this, heterotrophic carbon gain by root hemiparasites has been investigated only for a few genera. We investigated heterotrophic carbon gain by two root hemiparasites *Rhinanthus minor* L. and *Euphrasia rostkoviana* Hayne (Orobanchaceae) using natural abundance stable isotope (δ^{13}C) profiles of both parasites attached to C_3 (wheat) and C_4 (maize) hosts coupled to a linear two-source isotope-mixing model to estimate the percentage of carbon in the parasite that was derived from the host. Both *R. minor* and *E. rostkoviana* attached to maize hosts were significantly more enriched in ^{13}C than those attached to wheat hosts with *R. minor* becoming more enriched in ^{13}C than *E. rostkoviana*. The natural abundance ^{13}C profiles of both parasites were not significantly different from their wheat hosts but were less enriched in ^{13}C than maize hosts. Using a linear two-source isotope-mixing model we estimated that *R. minor* and *E. rostkoviana* adult plants derive c. 50 and 25% of their carbon from their hosts respectively. In light of these results, we hypothesize that repeatedly observed negative

effect of competition for light on hemiparasites acts predominantly in early ontogenetic stages when parasites grow unattached or the abstraction of host nutrients in less effective.

Paper 3

Těšitel J, Plavcová L & Cameron DD (2010): Interactions between hemiparasitic plants and their hosts. The importance of organic carbon transfer. Plant Signaling and Behavior. 5: 1072–1076.

Author's contribution: JT wrote major part of the paper.

Hemiparasitic plants display a unique strategy of resource acquisition combining parasitism of other species and own photosynthetic activity. Despite the active photoassimilation and green habit, they acquire substantial amount of carbon from their hosts. The organic carbon transfer has a crucial influence on the nature of the interaction between hemiparasites and their hosts which can oscillate between parasitism and competition for light. In this minireview, we summarize methodical approaches and results of various studies dealing with carbon budget of hemiparasites and the ecological implications of carbon heterotrophy in hemiparasites.

Paper 4

Těšitel J, Lepš J, Vráblová M & Cameron DD (2011): The role of heterotrophic carbon acquisition by the hemiparasitic plant *Rhinanthus alectorolophus* in seedling establishment in natural communities: a physiological perspective. New Phytologist doi: 10.1111/j.1469-8137.2011.03777.x

Author's contribution: JT conceived the study, based on initial discussions with JL and DDC. JT performed the experiment and the data analysis and wrote the paper with the help of all co-authors.

Heterotrophic acquisition of substantial amounts of organic carbon by hemiparasitic plants was clearly demonstrated by numerous studies. Many hemiparasites are however also limited by competition for light preventing the establishment of their populations on highly productive sites. In a growth-chamber experiment, we investigated the effects of competition for light, simulated by shading, on growth and heterotrophic carbon acquisition by the hemiparasite Rhinanthus alectorolophus attached to C_3 and C_4 hosts using analyses of biomass production and stable isotopes of carbon. Shading had a detrimental effect on biomass production and vertical growth of the hemiparasites shaded since seedlings while shading imposed later caused only a moderate decrease of biomass production and had no effect on the height. Moreover, shading increased the proportion of host-derived carbon in hemiparasite biomass (up to 50% in shaded seedlings). These results demonstrate that host-derived carbon can play a crucial role in carbon budget of hemiparasites especially if they grow in productive environment with intense competition for light. The heterotrophic carbon acquisition can allow hemiparasite establishment in communities of moderate productivity helping well-attached hemiparasites to escape from the critical seedling stage.

Paper 5

Těšitel J & Tesařová M: Leaf excretory glands play a key role in physiology and evolution of parasitism of the Rhinanthoid Orobanchaceae. unpublished manunscript

Author contribution: JT conceived this study, performed a major part of the microscopic observations and wrote the paper.

The Rhinanthoid clade of the family Orobanchaceae comprises plants displaying hemiparasitic or holoparasitic strategy of resource acquisition. Some of the species belonging to it, such as *Rhinanthus* spp., represent intensively studied species and their associations with the hosts are often

used as models of hemiparasitic systems. Although there is a well-developed theory of physiological processes in these hemiparasites, in this paper we show that most of the recent studies have missed one important point. Based on an extensive summary of mainly older non-English literature sources and our own microscopic (both light and electron microscope) observations, we demonstrate that, the Rhinanthoid species feature physiologically highly active excretory glands that actively exudate water from their leaves (or rhizome scales of leaf origin). This active excretion of water presents a mechanism explaining results of previous gas-exchange measurements detecting high dark respiration and transpiration rates and a tight inter-correlation between these two processes. In addition, the active excretion of water allowed multiple evolutionary transitions from facultative hemiparasites to obligate hemiparasites with long-living underground stage featuring a rhizome covered by scales of leaf origin. In case of the genus *Lathraea*, this evolutionary trend led to a xylem-feeding holoparasitic strategy which is unique among parasitic plants.

Paper 6

Těšitel J, Malinová T, Štech M & Herbstová M (2009): Variation in the *Melampyrum sylvaticum* group in the Carpathian and Hercynian region: Two lineages with different evolutionary histories. Preslia 81: 1–22.

Author contribution: JT conceived the study, collected and processed most of the material with the help of TM, performed all molecular and morphometric analyses and wrote the paper with the help of MŠ.

We investigated variation in the *Melampyrum sylvaticum* group in the Carpathian and Hercynian regions using morphological and molecular tools. The aim of our study was to examine differences in the pattern of variation between the Eastern Carpathians and region of the Western Carpathians and the Hercynian Massif. We also tested correlations between putatively taxonomically important variation in corolla colour present in the *Melampyrum sylvaticum* group in the Eastern Carpathian region and other morphological and molecular traits. Samples were collected from populations of the *M. sylvaticum* group in the Hercynian Massif and the Eastern and Western Carpathians. Morphometric analyses of the size and shape of the corolla (based on thin plate spline with sliding semilandmarks), length of the anthers and especially molecular analyses based on sequencing the nuclear ITS and trnL-trnT regions of chloroplast DNA, confirmed that the populations occurring on the opposite sides of the Eastern-Western Carpathian biogeographic boundary are very different. It is likely that the eastern and western lineages have been isolated for a long time and the extant pattern of variation with character disagreement within the border zone, originated from hybridization and introgression. The differences in corolla colour did not coincide with the variation in morphological traits or molecular markers within the North-Eastern Carpathian region. In addition, the geographical distribution of the populations with contrasting corolla colours lacked any pattern and there are populations with both corolla colours as well as plants with transitional pale-yellow flowers. Therefore, it is suggested that *M. saxosum* and *M. herbichii*, microspecies delimited on the basis of corolla colour, are conspecific. The high level of molecular variation and its pattern indicate that the *M. sylvaticum* group may have survived in or near the Eastern Carpathians during the Weichselian Ice Age. This hypothesis is supported by several recent phytogeographical and palaeoecological studies, which indicate the existence of a glacial refuge in the Eastern Carpathian region. Molecular uniformity of the Western Carpathian and Hercynian populations might in contrast indicate recent (Holocene) migration from assumed perialpine refuges.

Tuleu G, Koutecký P, Štech M, Baďurová T, Košnar J, Říha P & Těšitel J: Cytotype distribution and seasonal variation in the *Odontites vernus* group in Central Europe. unpublished manuscript.

Author contribution: JT, MŠ and PK conceived the study, JT participated on field sampling and data analyses and wrote major part of the paper.

Two cytotypes were reported in the *Odontites vernus* group in Central Europe based on karyological approach. This cytotypic variation was also related to the seasonal variation, a phenomenon based on occurrence of specific phenologically and morphologically distinct ecotypes, which is also present in numerous hemiparasitic species of the Rhinanthoid clade of Orobanchaceae. In this study, we conducted a broad screening of Central European populations of the *O. vernus* group using flow-cytometry and morphological analysis of characters related to the seasonal variation (number of internodes). We confirmed the existence of a widespread late-flowering diploid ($2n = 2x = 18$) type with high number of internodes and an early-flowering tetraploid ($2n = 4x = 40$) type with low number of internodes occurring on fallows or as an agricultural weed. In contrast to previous studies, we have discovered a late-flowering tetraploid type growing mostly on steppic grasslands in regions with warm and continental climate. This type had slightly higher number of internodes than the diploid type and was clearly morphologically distinct from the early tetraploid ecotype. The pattern of the cytotypic and seasonal variation suggests an update of the current taxonomic concepts of the group. Diploids and tetraploids should thus be treated as separate species, *O. vlugaris* and *O. vernus*, respectively, the latter of which consists of two seasonal types. Due to closely similar monoploid genome size across all these types, the tetraploids are hypothesized to originated through autopolyploidy and the evolution. The pattern of the geographical distribution of populations belonging to individual types suggests their sympatric or parapatric evolution.

1 Phylogeny, life history evolution and biogeography of the Rhinanthoid Orobanchaceae

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Introduction

The family Orobanchaceae presents the largest monophyletic group comprising holoparasitic and hemiparasitic plants (Heide-Jørgensen 2008). Recent phylogenetic studies of Orobanchaceae (Wolfe at al. 2005, Bennett & Mathews 2006) have presented a solid picture of the phylogeny of the family identifying several large monophyletic lineages within Orobanchaceae. Representing one of these clades, the Rhinanthoid group consists of the hemiparasitic genera *Euphrasia* L., *Melampyrum* L., *Tozzia* L., *Bartsia* L. (incl. *Bellardia* All.), *Nothobartsia* Bolliger & Molau*)*, *Odontites* Ludw. (s. l.), *Rhinanthus* L., *Rhynchocorys* Griseb., *Parentucellia* Viv., *Hedbergia* Molau and the holoparasitic genus *Lathraea* L.

The reconstruction of Orobanchaceae phylogeny based on the phytochrome A (*PHYA*) sequence data (Bennett & Mathews 2006) presented a relatively detailed insight into the relationships among genera within the Rhinanthoid group. The sampling was however insufficient for an analysis of life history evolution, as some evolutionarily important species were missing and many genera were either missing (*Hedbergia*, *Nothobartsia*) or represented by only one or too few species (*Bartsia*, *Rhynchocorys*, *Parentucellia*). The bootstrap support for the deep topology of the Rhinanthoid group sub-tree was fairly high (60-91%), but still not absolutely convincing. In the present study, we employed a combination of sequence data of nuclear ribosomal (nrDNA; internal transcribed spacer, ITS) and two chloroplast (cpDNA) loci (*trnT-trnL* intergenic spacer, *matK+trnK* region) to reconstruct the phylogeny of the Rhinanthoid Orobanchaceae. The ITS region presents a highly variable locus that was successfully employed in several studies dealing with genetic variability at the species level in the angiosperms including Orobanchaceae (e.g. Wu et al. 2005, Těšitel et al. 2009), but was also used in one of the recent reconstructions of the whole-family phylogeny (Wolfe et al. 2005). The highly variable *trnT-trnL* region (Shaw et al. 2005) has been routinely used in studies resolving relationships among recently diverged taxa where genetic differentiation is low (e. g. Fujii et al. 1997, Albaladejo et al. 2005, Těšitel et al. 2009, Vrancken et al. 2009). However, Müller et al. (2006) have demonstrated its usefulness for reconstruction of even very high-level phylogenies (relationships among basal angiosperm lineages – whole *trnT-trnL-trnF* region). The *matK+trnK* region presents a medium-speed evolving locus that has been successfully employed in numerous studies dealing with relationships on different phylogenetic levels from subfamilial clades (e. g. Bouchenak-Khelladi et al. 2008) to the whole angiosperm clade (Müller et al. 2006).

Life cycle (annuality vs. perenniality) and seed size present the life history traits that display apparent variation and contrasts across individual genera and species of the Rhinanthoid Orobanchaceae. Both are important features for plant ecological and evolutionary strategies in a general sense (Grime et al. 1997) and have thus attracted the interest of ecologists. Tracking life cycle evolution along a phylogenetic tree has been employed in several case studies, mainly revealing evolution of annual taxa from perennial ancestors (Conti et al. 1999, Andreasen & Baldwin 2001, Fiz et al. 2002, Datson et al. 2008, Müller & Albach 2010). An analogous study dealing with hemiparasitic plants has however reconstructed a reverse trend in the Orobanchaceae

subtribe Castillejinae (Tank & Olmstead 2008). Seed size has been subject of numerous comparative and modelling-based evolutionary ecological studies (e. g. Moles et al. 2005, Rees & Venable 2007, Van der Veken et al. 2007), but investigations tracking its evolution along a particular phylogenetic tree are largely missing. Understanding the life cycle, seed size evolution and biogeography presents an appealing objective of the Rhinanthoid Orobanchaceae biology, as there is a large variation in seed size, numerous annual hemiparasites are known to grow in communities dominated by perennials (Strykstra et al. 2002) and annuality predominates in root hemiparasites in some geographical regions (e. g. temperate Europe, Tutin et al. 1972). In this study, we aim to detect trends in the life history evolution and explain correlations between biogeography and life histories. A detailed, multiple gene-based reconstruction of phylogeny that includes some evolutionarily important species that have not been studied before (e. g. genera *Hedbergia* and *Nothobartsia*, perennial species of *Rhynchocorys* and *Odontites*) serves as a solid basis for this analysis.

Material and Methods

Material and DNA sequencing

Samples of most of the species included in the phylogenetic analysis were collected in the field by the authors. Several leaves or bracts from each specimen were desiccated using silica-gel and kept at –20°C for DNA extraction. Remaining parts of each plant were processed as a standard herbarium voucher and are kept in the herbarium of the Faculty of Science, University of South Bohemia (CBFS). Voucher material was used for extraction of several specimens that were obtained from PRC and O herbaria. Most outgroup and few ingroup sequences were directly obtained from GenBank (Table 1).

Seed size data were either measured directly in species for which seeds were available or were obtained from the Seed Information Database (Liu et al. 2008). In case of *Nothobartsia aspera*, *Odontites luteus*, *Rhynchocorys odontophylla* and *R. orientalis*, seed weight data were not available but seed dimensions could be obtained from literature sources (Davis 1978, Gabrielian 1987, Castroviejo et al. 2009). A calibration regression model based on a sample set for which both seed weight dimensions were available was used to estimate the seed weight of these species. The detailed information on seed weight and dimensions in individual species and the model are summarized in Appendix 1.

DNA was extracted using Invitek Plant Extraction Kit (Invitrogen) following the standard protocol provided by the manufacturer. ITS1, 5.8S, ITS2 region of nuclear DNA, *trnT-trnL* and *matK+trnK* regions of cpDNA (see primer information in Table 2) were amplified using a Bioer XP thermal cycler under conditions summarized in Table 3. PCR was performed in a total volume of 25 µl consisting of 1X PCR Buffer, 200µM each of dNTPs, 1.25U Taq DNA polymerase (TopBio), 1 µl DNA template solution and 7.5 pmol of each primer. The primers and dNTPs were subsequently removed from the PCR product solution using Exosap-IT (USB Corp.). The sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) at the Sequencing Centre of the University of South Bohemia.

Table 1 Voucher data, descriptions of localities and GenBank DNA accession numbers of the specimens used in the phylogenetic analysis. Ingroup species are displayed in bold. N.A. in the voucher and locality columns indicates that all sequences of the respective species were obtained from the GenBank database. N.A. in the locus columns stands for sequencing failure or missing in the GenBank database.

Species	Collector, herbarium acronym and voucher code	Locality description	Locus name		
			ITS	mat+trnK	trnT-trnL
Bartsia alpina L.	J. Těšitel, CBFS, 5034	Austria, Heiligenblut: alpine tundra on N slope of the Hohe Tauern mountain ridge ca 5 km N of the village, N47°0508.28″, E012°5002.88″, 2640 m a. s. l., 3rd Jul 2007	FJ790046	FJ790106	FJ790076
Bartsia laticrenata Benth. in DC.	P. Sklenář, PRC, 9040	Ecuador, Cotopaxi: Paramo de Quispicacha, mountain ridge to the east of Cerro uchihuasi, S01°04′40″, W078°50′00″, 26th Oct 2006	FJ790054	FJ790114	FJ790084
Bartsia pedicularoides Benth. in DC.	P. Sklenář, PRC, 9076	Ecuador, Cotopaxi: Paramo de Quispicacha, eastern slope of Loma Pucyucuchu, S01°05′10″, W078°50′25″, 4300-4700 m a. s. l., 24th Oct 2006	FJ790047	FJ790107	FJ790077
Bartsia stricta (Kunth) Benth. in DC.	P. Sklenář, PRC, 9284	Ecuador, Cotopaxi: Paramo de Quispicacha, eastern slope below the summit plateau of Loma Pucyucuchu, S01°05′11″, W078°50′39″, 4500 m a.s.l., 26th Oct 2006	N.A.	FJ790121	N.A.
Bartsia trixago L.	M. Popp, O, A23102007-03	Ethiopia, Simen, Chenek: ungrazed grassland, N13°16′04″, E038°12′11″, 3748 m a. s. l., 23th Oct 2007	FJ790053	FJ790113	FJ790083
Euphrasia nemorosa (Pers.) Wallr.	Š. Svobodová, CBFS, 5090	Czech Republic, Velká Lhota: meadow ca 500 m N of the village, N49°0848′, E015°1959′, 625 m a.s.l., 20th Sep 2007	FJ790050	FJ790110	FJ790080
Euphrasia rostkoviana Hayne	Š. Svobodová, CBFS, 5092	Czech Republic, Řečice: S part of the village urban area, N49°0842′, E015°21′59″, 625 m a.s.l., 20th Sep 2007	FJ790052	FJ790112	FJ790082
Euphrasia sevanensis Juz.	J. Těšitel, CBFS, 5036	Turkey, Demirkazik: valley S of the Demirkazik peak, ca 3.5 km SE of the village, N37°4943′, E35°0916″, 2980 m a. s. l. 6th Sep 2006	FJ790049	FJ790109	FJ790079
Euphrasia stricta J.P. Wolff	Š. Svobodová, CBFS, 5091	Czech Republic, Nevcehle: unpaved road ca 300 m NNW of the village centre, N49°13′42″, E015°32′13″, 608 m a.s .l., 20th Sep 2007	FJ790051	FJ790111	FJ790081
Hedbergia abyssinica (Benth.) Molau	J. Těšitel, CBFS, 5094	Cameroon, Buea: montane grassland on SE slopes of Mt. Cameroon ca 7.3 km NW of the town, N04°11′53″, E009°11′37″, 3020 m a. s. l, 12th Nov 2008	FJ790059	FJ790119	FJ790089
Lathraea clandestina L.	N.A.	N. A.	AY911230	AF051989	N.A.

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Species	Voucher	Locality			
Lathraea squamaria L.	J. Těšitel, CBFS, 5032	Czech republic, Malčice: pathway edge in the hornbeam forest ca 0.5 km SSE of the village, N48°47'22", E014°23'34", 600 m a. s. l., 14th Apr 2007	FJ790044	HM193524	FJ790074
Melampyrum nemorosum L.	J. Těšitel, CBFS, 5026	Czech Republic, Načešice: light hornbeam forest ca 850 m WNW of the village, N49°56'34", E015°36'56", 330 m a s. l.,17th Jun 2007	FJ797592	FJ790117	FJ797593
Melampyrum pratense L.	J. Těšitel, CBFS, 5027	Czech Republic, Suchovské Mlýny: oak-hornbeam forest near the Přední louky meadows ca 1.8 km ENE of the village, N48°53'26", E017°35'59", 430 m a. s. l., 24th Jun 2007	FJ790039	FJ790099	FJ790069
Melampyrum sylvaticum L.	J. Těšitel, CBFS, 4240	Czech Republic, Ovesná: spruce forest next to the railway station, N48°48'26", 013°56'21", 740 m a .s. l., 10th Jul 2004	EU624133	FJ790104	EU653282
Nothobartsia aspera (Hoffmanns. & Link) Bolliger & Molau	J. Těšitel, CBFS, 5590	Spain, Tarifa: Atlantic heathland on the Sierra de Salaviciosa Massif, ca 10.5 km N of the town, N36°06'13",W005°38'46", 305 m a. s. l., 19th Aug 2009	HM193531	HM193527	HM193522
Odontites aucheri Boiss.	J. Těšitel, CBFS, 5041	Turkey, Gevaş, calcareous debris on the N slope of Mt. Astos 1.7 km S of the town centre, N38°16'56", E043°06'610", 1950 m a. s. l., 6th Jul 2008	FJ790038	FJ790098	FJ790068
Odontites bocconei (Guss.) Walp.	J. Těšitel, CBFS, 5593	Italy, Sicily: Cava Grande di Cassibile, shrubs on the S slope of the valley, N36°58'08", E015°05'41", 385 m a. s. l., 3rd May 2009	HM193532	HM193528	HM193523
Odontites luteus (L.) Clairv.	J. Těšitel, CBFS, 5033	Czech Republic, Pouzdřany: grassland of the Pouzdřanská steppe ca 0.9 km E of the railway station in the N part of the village, N48°56'25", E016°38'34", 230 m a. s. l., 5th Sep 2007	FJ790045	FJ790105	FJ790075
Odontites vernus Dumort.	J. Těšitel, CBFS, 5035	Turkey, Ihlara: entrance to the Ihlara valley, N38°14'23", E034°18'30", 1250 m a. s. l., 1st Sep 2006	FJ790048	FJ790108	FJ790078
Parentucellia latifolia (L.) Caruel	J. Těšitel, CBFS, 5597	Italy, Sicily, Polizzi Generosa: montane grassland of the Madonia region ca 4.5 km NE of the town, N37°50'27", E014°01'43", 1605 m a. s. l., 8th May 2009	HM193530	HM193526	HM193521
Parentucellia viscosa (L.) Caruel	J. Těšitel, CBFS, 5595	Italy, Marota-Pirilli: ruderal vegetation near petrol station ca 1.6 km W of the village, N38°26'26", E015°56'34", 50 m a s. l., 1st May 2009	HM193529	HM193525	HM193520
Rhinanthus alectorolophus (Scop.) Pollich	J. Těšitel, CBFS, 5030	Czech Republic, Podlažice: steppic meadows 1.2 km NE of the village, N49°54'02", E015°58'24", 280 m a. s. l., 17th Jun 2007	FJ790042	FJ790102	FJ790072
Rhinanthus minor L.	J. Těšitel, CBFS, 5028	Czech republic, Zubří: peat meadow ca 0.9 km WNW of the village, N49°46'47", E015°47'22", 615 m. a. s. l., 23rd Jun 2007	FJ790040	FJ790100	FJ790070

Species	Voucher	Locality			
Rhinanthus rumelicus Velen.	J. Těšitel, CBFS, 5031	Montenegro, Vusanje: *Pteridium aquifolium* vegetation on the southern slopes of the mountain ridge ca 0.4 km N of the eastern margin of the village, N42°31'32", E019°51'21", 1390 m a. s. l., 3rd Aug 2007	FJ790073	FJ790103	FJ790043
Rhinantus glacialis Person.	J. Těšitel, CBFS, 5029	Switzerland, Fully, Mezembroz: valley 0.8 km N of the settlement, N46°10'31", E007°08'22", 1570 m a. s. l., 8th Jul 2007	FJ790071	FJ790101	FJ790041
Rhynchocorys stricta (C. Koch) Albov	J. Těšitel, CBFS, 5047	Turkey, Barhal: meadow near the road between Naznara and Amaneskit 4.9 km WNW of the village centre (mosque), N40°59'02", E041°21'50", 1480 m a. s. l., 23rd Jun 2008	FJ790086	FJ790116	FJ790056
Rhynchocorys elephas (L.) Griseb	J. Těšitel, CBFS, 5044	Turkey, Barhal: hazel shrubs next to the road to Naznara 3 km E of the village centre (mosque), N40°58'24", E041°22'03", 1340, 23rd Jun 2008	FJ790085	FJ790115	FJ790055
Rhynchocorys kurdica Nábělek	J. Těšitel, CBFS, 5042	Turkey, Gevaş: calcareous debris on the N slope of Mt. Astos 2.3 km S of the town centre, N38°16'35", E043°06'11", 2200 m a s. l., 6th Jul 2008	FJ790067	FJ790097	FJ790037
Rhynchocorys maxima C. Richter	J. Těšitel, CBFS, 5040	Iran, Haviq: stream banks in a forest near waterfall 7.3 km WSW of the town, N38°06'52", E48°49'15", 260 m a. s. l., 4th Jul 2008	FJ790066	FJ790096	FJ790036
Rhynchocorys odontophylla Burbidge & Richardson	J. Těšitel, CBFS, 5038	Turkey, Pülümür: shrubs along a stream ca 3.3 km NNW of the village, N39°30'42", E039°52'50", 1640 m a. s. l., 21st Jun 2008	FJ790064	FJ790094	FJ790034
Rhynchocorys orientalis (L.) Bentham	J. Těšitel, CBFS, 5039	Turkey, Gölbelen: meadow ca 1.3 km SE of the village, N41°03'44", E043°07'33", 2040 m a. s. l., 27th Jun 2008	FJ790065	FJ790095	FJ790035
Tozzia alpina L.	A. Šmídová, CBFS, 5069	Ukraine, Stara Huta: small peat-bog in the Rushchina saddle below Mt. Mala Sivulja (1818 m), near red-marked tourists path, 1450 m a. s. l.	FJ790088	FJ790118	FJ790058
Alectra sessiliflora Kuntze	J. Těšitel, CBFS, 5095	Cameroon, Limbe: old lava flow on S slope of Mt. Cameroon ca 18 km NW of the town, N04°08'20", E009°06'22", 2270 m a. s. l., 13th Nov 2008	FJ790090	FJ790120	FJ790060
Castilleja lasiorhyncha (Gray) Chuang & Heckard	N.A.	N. A.	N.A.	N.A.	EF103684
Castilleja linariifolia Benth.	N.A.	N. A.	N.A.	AF051981	N.A.
Epifagus virginiana (L.) Bart.	N.A.	N. A.	N.A.	AF051982	AY209290
Lindenbergia phillipinensis Benth.	N.A.	N. A.	N.A.	AF051990	AY911231
Pedicularis densispica Franch. ex Maxim.	N.A.	N. A.	AY881108	AY949718	AY949642
Sopubia manii Skan	Š. Janeček, CBFS, 5096	Cameroon, Babanki: surrounding of the field research station ca 5 km E of the village, 2000 m a. s. l.	FJ790092	FJ790122	FJ790062

Table 2 Sequences of primers used for DNA amplification and references of the primer sequence source.

Primer name	Sequence	Reference
ITS1P	5'-CTTTATCATTTAGAGGAAGGAAG-3'	Selosse et al. 2002
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White et al. 1990
*trnT*2F	5'-CAAATGCGATGCTCTAACCT-3'	Cronn et al. 2002
trnL	5'-GAGATTTTGAGTCTCGCGTGTC-3'	Taberlet et al. 1991
*matK*5	5'-TGGGTTGCTAACTCAATGG-3'	*trn*K-3914F in Johnson and Soltis 1994
*matK*3	5'-AACTAGTCGGATGGAGTAG-3'	*trn*K-2R in Johnson & Soltis 1994
*matK*5-iF	5'-CCGAAATCAAAAGAGCGATTAG-3'	this study
*matK*3-iR	5'-TGTTTACGAGCCAAAGTT-3'	this study

Phylogenetic analyses

The E-INS-i algorithm implemented in the online version of MAFFT 6 (Katoh et al. 2002) was employed to align sequence datasets. The *matK+trnK* alignment was subsequently refined by the G-INS-i strategy after trimming the flanking ends. Further manual adjustments were conducted in BioEdit (Hall 1999) and the highly divergent regions were excluded. Gap matrices were computed in SeqState 1.4 (Müller 2005) in order to encode indel-type mutations. The modified complex indel coding (MCIC; see Simmons & Ochoterena 2000) and simple indel coding (SIC) were applied prior to the maximum parsimony and Bayesian analyses, respectively, as the dedicated software computing the Bayesian inference does not support cost matrices of MCIC.

Bayesian inference (BI) was conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Prior to the BI analyses, the appropriate substitution model was selected according to the Aikake information criterion (AIC) test implemented in the MrModeltest 2.3 software (Nylander et al. 2004). Both *matK+trnK* and *trnT-trnL* data were analyzed under the GTR+Γ model, while an additional assumption of invariable sites (the GTR+I+Γ model) was applied on the ITS dataset. Distribution of prior probability densities of the substitution rates along with the distribution of the stationary nucleotide frequencies was set to flat. The indel matrices were treated as restriction data in MrBayes and analyzed under the F81-like model; three independent indel partitions (one per locus) were submitted to the combined Bayesian analysis. Four Bayesian analyses were conducted, three of them analyzing individual locus sequence data and one combining partitioned data. Each analysis encompassed two simultaneous independent runs including six parallel Markov chains (one "cold" and five incrementally heated by a temperature step of 0.2; 10,000,000 generations, sampling every 1000). The resulting trees were used for reconstruction of a 50% majority-rule consensus tree (first 25% discarded as burn-in).

Table 3 Amplification profiles for DNA regions used in the study. *) Ramping 2°C/s

DNA region	ITS	*trnT-trnL*	*matK+trnK* (entire region)	*matK+trnK* (internal primers)
1. initial denaturation	95°C – 300s	95°C – 300s	95°C – 300s	95°C – 300s
2. denaturation	95°C – 60s	95°C – 60s	95°C – 60s	95°C – 60s
3. annealing	52°C – 90s	52°C – 90s	54°C – 60s	52°C – 60s*)
4. elongation	72°C – 90s	72°C – 90s	72°C – 120s	72°C – 120s
5. final elongation	72°C – 600s	72°C – 600s	72°C – 600s	72°C – 600s
number of cycles (steps 2-4)	32	30	35	38
primers	ITS1P + ITS4	*trnT*2F + *trnL*	*matK*5 + *matK*3	*matK*5-iF + *matK*3-iR

The same general design was applied in the maximum parsimony (MP) analysis. All characters were equally weighted in all MP analyses computed in PAUP* 4.0b4a (Swofford 2002) using a heuristic search option (TBR branch-swapping, 100 random-taxon-addition replicates, steepest descent disabled, MulTrees enabled). Statistical support of the resulting trees was obtained by a bootstrap procedure (1000 pseudoreplicates, MaxTrees = 2000).

The life cycle (annuality/perenniality) evolution in the group under study was traced using the likelihood reconstruction method (see e.g. Schluter et al. 1997) implemented in Mesquite, version 2.6 (Maddison & Maddison 2009). The life cycle was coded as a categorical character and mapped onto the rooted ingroup section of the combined-matrix BI tree. Two unequal rates of transition between character states depending on the direction of transition were assumed (asymmetrical 2-parameter Markov k-state model; Lewis 2001) and the branch lengths were considered. The likelihood-ratio test was performed as a measure of unequivocality, with the threshold set to 2.0. Hence, the reconstruction could only favour one of the competing hypotheses if the difference between log-likelihoods was greater than 2.0; the reconstructed state was otherwise considered ambiguous.

Seed size evolution was reconstructed by an ancestral state estimation maximum likelihood model (Paradis 2006) on a tree that was manually pruned not to contain missing data (i. e. taxa for which seed size was unknown). In addition, correlated evolution of seed size and life cycle was tested by phylogenetically independent contrasts (Felsenstein 1985, Paradis 2006). Seed size was entered in these analyses as the natural logarithm of the weight of one seed in micrograms. The ancestral state estimation model and phylogenetic independent contrasts with subsequent correlation analysis were calculated in R version 2.9.2 (R Development Core Team 2009) using package ape, version 2.4-1 (Paradis et al. 2004). The geographic distribution of each species was assigned to one or more of five discrete regions (Temperate Western Eurasia, Mediterranean region, Caucasus and Western Irano-Turanian region, Central Africa, South America and North America) and subdued to the dispersal-vicariance analysis in the software package DIVA (Ronquist 1997).

Results

Phylogenetic reconstruction

Sequencing of all three analyzed loci produced variable yet alignable data. Individual single-locus phylogenetic analyses revealed a more or less congruent phylogenetic signal in all analyzed loci. The trees produced by *trnT-trnL* and *matK+trnK* analyses yielded generally consistent trees, while the consistency was substantially lower in the tree based on ITS data (Table 4). The robustness of our sequence dataset was revealed by a combination of the sequence data of all three loci. The BI (Fig. 1) and MP (bootstrap support indicated in Fig. 1) analyses based on this combined dataset yielded a congruent and well resolved phylogenetic tree of the Rhinanthoid Orobanchaceae (Table 4). Both Bayesian posterior probabilities (PP) and parsimony bootstrap support (BS) mostly reached sufficiently high values providing a solid support for individual nodes. The general picture of the monophyletic (PP = 1.00, BS = 100) Rhinanthoid Orobanchaceae consisted of three main subclades. Representing one of these, the genus *Melampyrum* alone occupied the sister position to the rest of the Rhinanthoid Orobanchaceae (PP = 1.00, BS = 100). Each of the two remaining subclades comprised several genera and was also highly supported (PP = 1.00, BS = 100). Almost all reconstructed relationships achieved PP = 1.00 and BS > 90 within the subclade containing *Rhynchocorys* and the sister genera *Rhinanthus* and *Lathraea* (*Rhinanthus-Rhynchocorys-Lathraea* subclade). The second subclade (*Bartsia-Euphrasia-Odontites* subclade) comprised *Bartsia alpina* as an isolated species, the genus *Euphrasia* and an assemblage of related genera. In this assemblage *Tozzia alpina* occupies the ssiter position to the rest of the taxa. Its position was however not fully resolved, causing low statistical support (PP = 0.52, BS = 86) of the subclade comprising the remaining species. In this subclade, *Nothobartsia aspera* and *Hedbergia abyssinica* were clearly resolved as sister taxa (PP = 1.00, BS = 100), *Odontites* appears monophyletic (PP = 1.00, BS = 100), and sister (PP = 1.00, BS = 61) to the group of *Bartsia trixago*, *Parentucellia* and South American *Bartsia* species. The relationships in this last group are less well resolved compared to the

rest of the tree, mainly due to the unstable position of *Parentucellia viscosa*. Major alternative hypotheses to the BI tree proposed by some of the partial analyses are summarized in Table 4.

Life history traits and biogeography

Mapping of the life cycle onto the phylogenetic tree reconstructed perenniality as a prevailing strategy in most of the deep node ancestors of the extant lineages, although the higher likelihood of perenniality was not statistically significant in several tree nodes (Fig. 2). The overall picture of life cycle evolution can be then characterized by multiple independent transitions to annuality in several unrelated lineages (*Melampyrum*, *Rhinanthus*, *Euphrasia*, annual *Odontites* spp.) or individual species in the genus *Rhynchocorys*.

Seed size varied substantially across more than two orders of magnitude (Fig. 3; see also Appendix 1) in the Rhinanthoid Orobanchaceae. Species of the genus *Melampyrum* features the largest seeds followed by *Rhinanthus* spp., while the lightest seeds can be found in *Parentucellia*, *Hedbergia* and *Bartsia* (except *B. alpina*). The mapping on the cladogram identifies large seeds (weight around ca. 4g/1000 seeds) as a primitive feature in the Rhinanthoid Orobanchaceae, while the derived lineages tend to decrease their seed size (Fig. 3). Generally, small seed sizes are found in the whole *Bartsia-Euphrasia-Odontites* subclade. On the other hand, this trait tended to vary substantially within the *Rhinanthus-Rhynchocorys-Lathraea* subclade.

The phylogenetic independent contrast analysis of the complete data set yielded an insignificant result ($r = -0.23$, $F_{(1,25)} = 1.419$, $P > 0.05$), indicating no correlation in evolution of life cycle and seed size. Nonetheless, this insignificance was caused by opposite trends present in the small-seeded *Bartsia-Euphrasia-Odontites* subclade (non-significant positive trend, $r = 0.38$, $F_{(1,23)} = 2.224$, $P > 0.05$) and the paraphyletic rest of the whole group (significant negative relationship, $r = -0.85$, $F_{(1,9)} = 24.241$, $p < 0.001$).

The analysis of vicariance (Fig. 4) located the distribution of the ancestor of the *Rhinanthus* group in the temperate Western Eurasian region. A certain affinity to the Caucasian and Western Irano-Turanian region was reconstructed for the ancestors of the *Rhinanthus-Rhynchocorys-Lathraea* lineage. By contrast, the evolution of most of the *Bartsia-Euphrasia-Odontites* subclade is connected with the Mediterranean region. Although one *Melampyrum* and several *Rhinanthus* species immigrated North America, long distance dispersal events are mostly present in the *Bartsia-Euphrasia-Odontites* subclade. That is, *Bartsia alpina* dispersed from Europe to North America, South American *Bartsia* species dispersed from the Mediterranean region to South America, and *Euphrasia* spp dispersed worldwide, which was however not analysed by the present study.

Table 4 Summary results of individual phylogenetic analyses. The first rows show the composition of the dataset (count of the total / parsimony informative bases, coded indels for Bayesian inference BI and maximum parsimony analysis MP). The length of the best tree (L), the number of equally parsimonious trees, their consistency (CI), homoplasy indexes (HI) and retention indexes (RI) are given for the maximum parsimony analyses. Bayesian posterior probabilities (PP) and bootstrap support (BS) are shown for the nodes that are in major conflict among particular analyses.

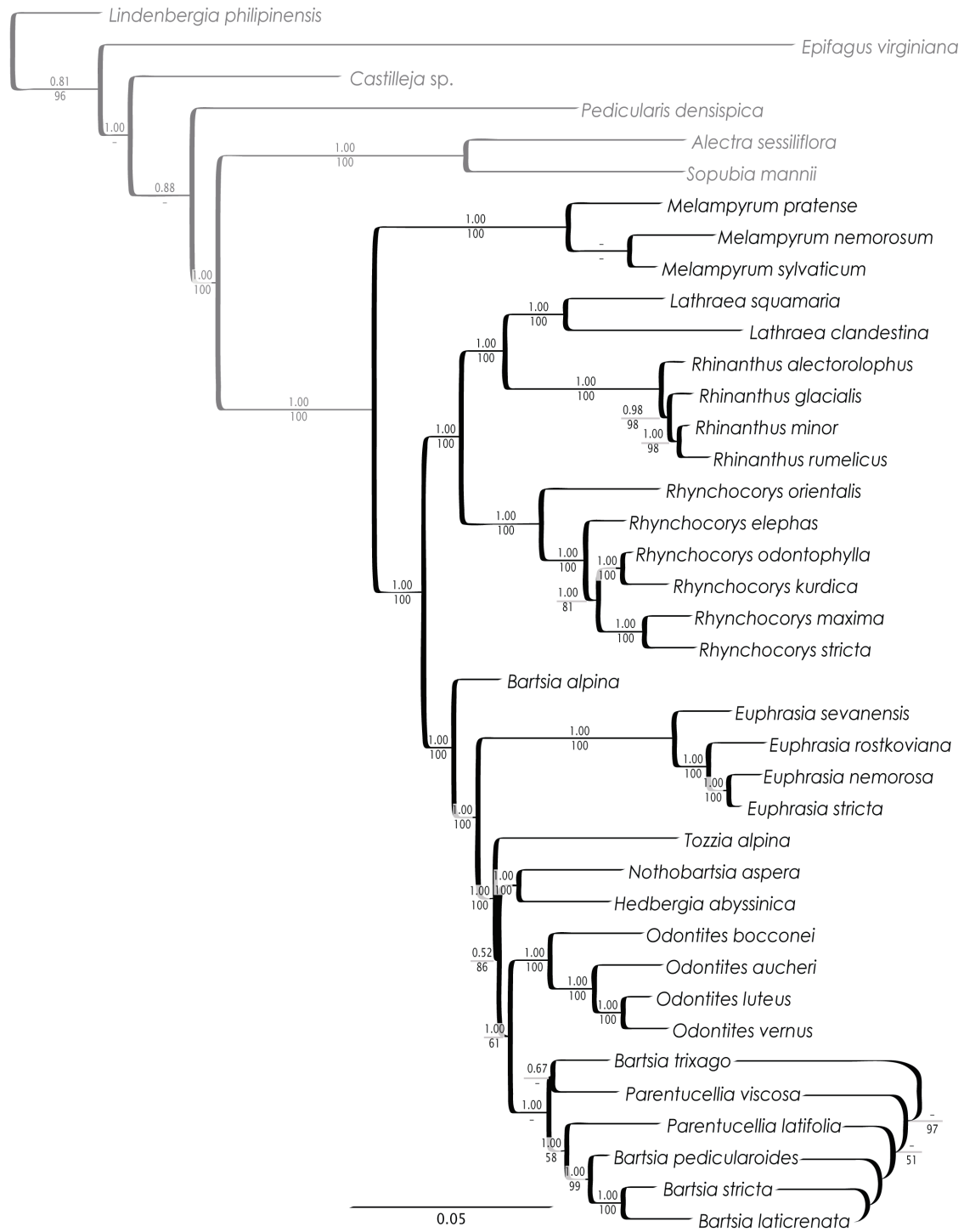|		Combined matrix		ITS		*trnT-trnL*		*matK+trnK*	
Sequence length | parsimony informative		4425	1142	765	314	1004	225	2657	603
Indel matrix length BI | MP		492	241	68	31	158	41	260	263
L (n best trees found)		4718 (1)		1547 (5)		864 (36)		2181 (16)	
CI (CI excluding uninformative)		0.63 (0.54)		0.50 (0.46)		0.75 (0.68)		0.71 (0.60)	
HI (HI excluding uninformative)		0.37 (0.45)		0.50 (0.54)		0.25 (0.32)		0.29 (0.40)	
RI		0.72		0.63		0.81		0.78	
Main conflicts among particular analyses and their statistical support		PP	BS	PP	BS	PP	BS	PP	BS
Parentucellia latifolia sister to …	*P. viscosa*						57		
	South American *Bartsia* clade	1.00	51	1.00	92	0.99	53	1.00	
Parentucellia viscosa sister to …	*Bartsia trixago*	0.67						0.88	
	South American *Bartsia* clade + *P. latifolia*		97	0.75	98				
	P. latifolia						57		
Tozzia alpina sister to …	Clade comprising species of the genus *Euphrasia*, *Odontites*, *Tozzia*, *Nothobartsia*, *Hedbergia*, *Parentucellia*, and all *Bartsia* species except *B. alpina*	1.00	100						
	Hedbergia abyssinica			0.86					
	Hedbergia abyssinica + *Nothobartsia aspera*								65
	Hedbergia abyssinica + genus *Euphrasia*					0.57			

Fig. 1 Bayesian maximum likelihood reconstruction of the Rhinanthoid Orobanchaceae phylogeny based on the combined sequence data of ITS, *trnT-trnL* spacer and *matK+trnK*. Numbers above and below nodes denote Bayesian posterior probabilities (PP) and parsimony bootstrap support (BS), respectively. The outgroups are shown in grey. Since the conflict surrounding the position of *P. viscosa* between the Bayesian inference and maximum parsimony was irresolvable by the Approximately Unbiased test of Shimodaira (AU = 0.79, *p* = 0.209; the CONSEL software by Shimodaira & Hagesawa 2001), the alternative topology proposed by MP analysis is also shown.
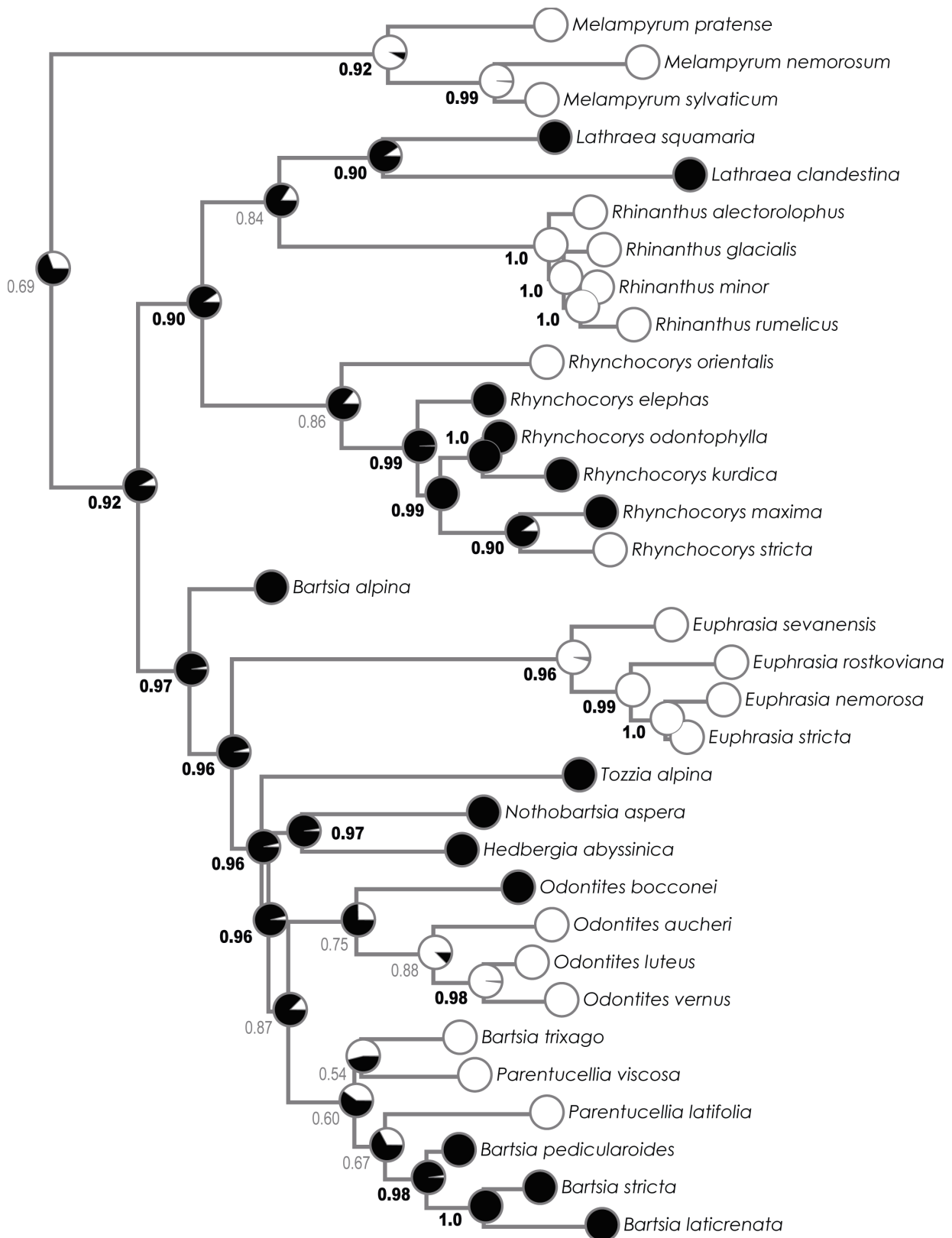
Fig. 2 Maximum likelihood reconstruction of the life cycle evolution of the analyzed species of the Rhinanthoid Orobanchaceae. *Tozzia alpina* is considered a perennial species in the analysis. The black fraction of the circle represents the likelihood of perenniality, white denotes the complementary likelihood of annuality; numbers show the proportional likelihood for the stronger hypothesis. Numbers in bold represent the unambiguous decision of reconstructed state measured by the likelihood-ratio test with a threshold of 2.00.
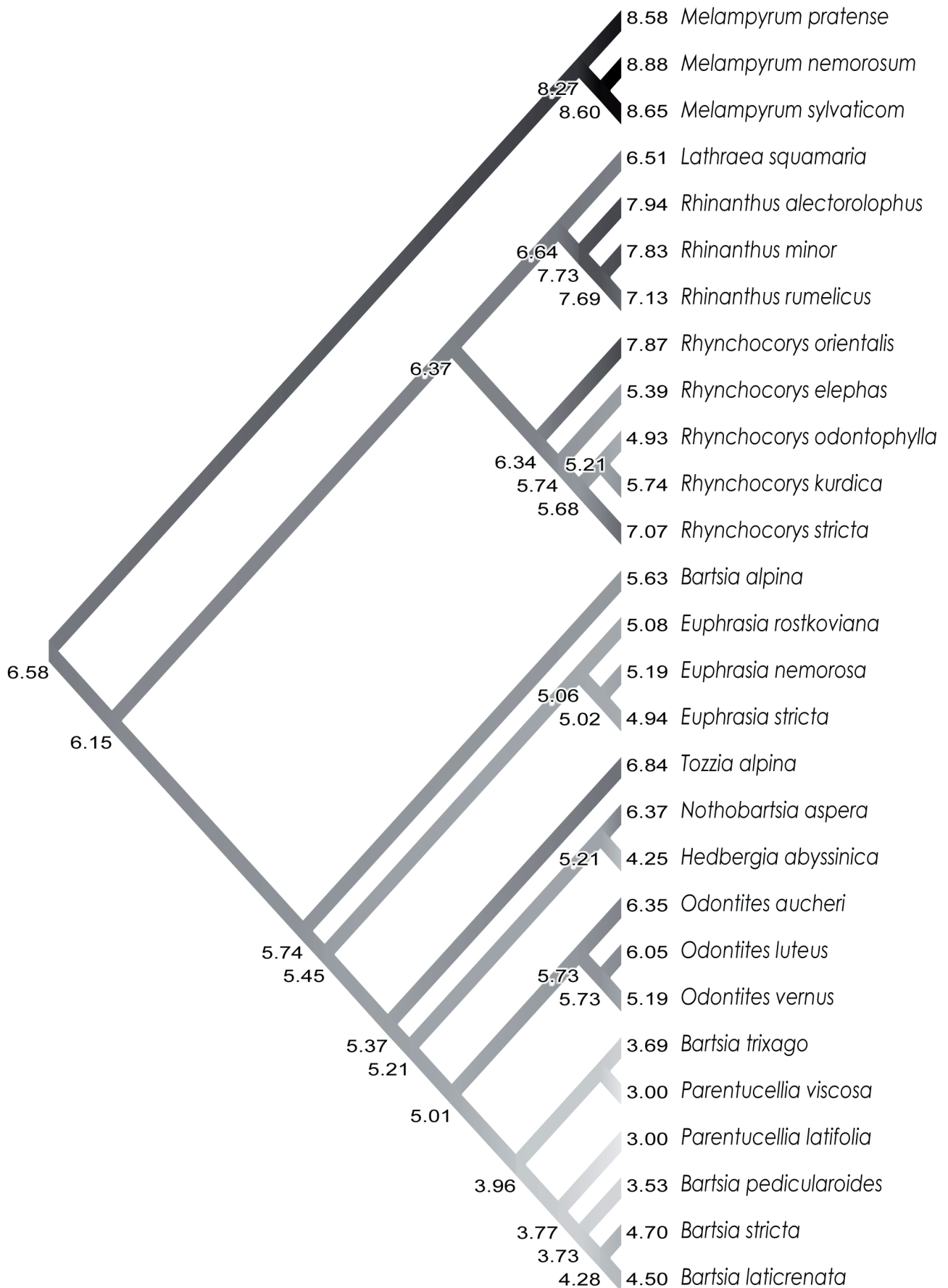
Fig. 3 Maximum likelihood reconstruction of seed size evolution in the Rhinanthoid Orobanchaceae. The natural logarithm of the weight of a single seed (in micrograms) was employed as source data in the analysis. Shading of branches approximates supposed seed size, lighter shading indicating heavier seeds.
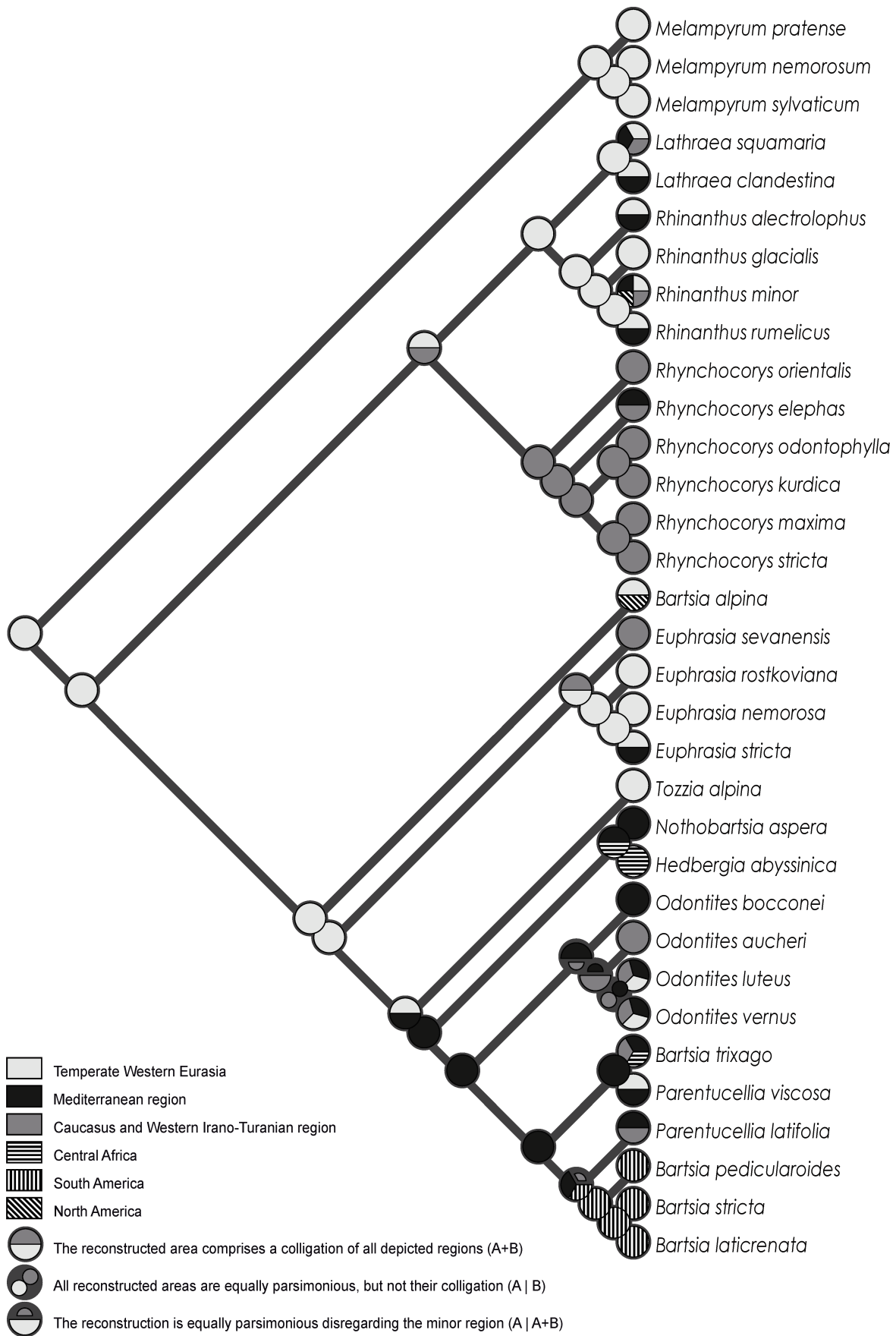
Fig. 4 Reconstruction of biogeography of the ancestors of the extant Rhinanthoid Orobanchaceae lineages computed in DIVA (dispersal-vicariance model). Optimal reconstruction required 23 dispersal events.

Discussion

Our reconstruction of the Rhinanthoid Orobanchaceae phylogeny generally agrees with the results of the phylogenetic analysis based on *PHYA* sequence data (Bennett & Mathews 2006). There are however important updates to this hitherto most detailed picture of the phylogeny of the group. The inclusions of the complete species spectrum of *Rhynchocorys*, species of *Hedbergia* and *Nothobartsia* and recognizing the latter two taxa as the sister genera (supported also by their similar vegetative morphology – dwarf shrubs with almost sclerophyllous leaves) present the most important contributions of this study to the knowledge of the phylogeny of the Rhinanthoid Orobanchaceae. *Bartsia alpina* was clearly shown to be a member of the clade with *Euphrasia*, *Odontites* and other *Bartsia* species, which also agrees with its morphological affinity to these taxa (e.g. Bolliger & Molau 1992). In addition, the polyphyly of the genus *Parentucellia* first suggested by Bennett & Matthews (2006) was also supported by the present study, although the exact position of *P. viscosa* on the phylogenetic tree is rather uncertain. *P. latifolia* was however clearly confirmed to be closely related to the South American *Bartsia* species. On the other hand, the genus *Odontites* s. l. was suggested as monophyletic, in contrast to the morphology-based phylogenetic and taxonomic studies (Bolliger & Wick 1990, Bolliger & Molau 1992). Although there is still substantial sampling deficiency, it seems significant that *O. aucheri*, considered as one of the most morphologically distinct taxa within *Odontites* s. l. (Bolliger & Wick 1990), was assigned to the clade consisting of *Odontites* s. str. (sensu Bolliger & Wick 1990) species in this study. In general, the conflicts between the results of molecular phylogeny (Bennett & Mathews 2006, this study) and a morphology-based phylogenetic analysis (Bolliger & Molau 1992) present in the *Bartsia-Euphrasia-Odontites* subclade can be probably attributed a combination of presence of plesiomorphies in morphological characters (such as the "*Bartsia*-like" general morphology present across the whole subclade) and autapomorphic features of some genera (typically *Euphrasia* and *Tozzia*, but in part also *Nothobartsia* and *Hedbergia*).

Our research presents so far the most complete and detailed phylogenetic reconstruction of the *Rhinanthoid* Orobanchaceae but, also leaves a number of issues requiring resolution in the future. The phylogenetic position of African *Bartsia* species remains unclear; we can only hypothesize that they are not directly related to *Hedbergia* as their floral morphology is very different (Hedberg 1957). Broader sampling of *Odontites* s. l. and South American *Bartsia* species would be also highly beneficial. Nonetheless, we believe that the solution of these issues will not cause any substantial changes of the general concept of the Rhinanthoid Orobanchaceae phylogeny presented in this study.

Life history evolution

The picture of the life cycle evolution (Fig. 2) of the Rhinanthoid Orobanchaceae indicates a reverse tendency compared to the related Castillejinae subtribe (Tank & Olmstead 2008), but otherwise corresponds to the general trend of evolution of annual species from perennial ancestors reported in many non-parasitic angiosperms (e. g. Conti et al. 1999, Andreasen & Baldwin 2001, Fiz et al. 2002, Datson et al. 2008, Müller & Albach 2010). Independent origins of annuality in the ancestors of many genera imply that various features found in annual species across different genera (such as the presence of the seasonal ecotypes; Wettstein 1895, Zopfi 1995, 1998) are not homologous but present analogies that evolved independently in different lineages. Such a pattern would also support their adaptive importance in the annual hemiparasites. Predominance of annual species in the temperate regions of the Holarctic kingdom (especially in Eurasia; Tutin et al. 1972) suggests that annuality provides an advantage in the temperate climate areas and is not just a feature inherited from a common ancestor. This advantage might be connected with more intensive and effective exploitation of the host resources in the annuals (Press et al. 1988).
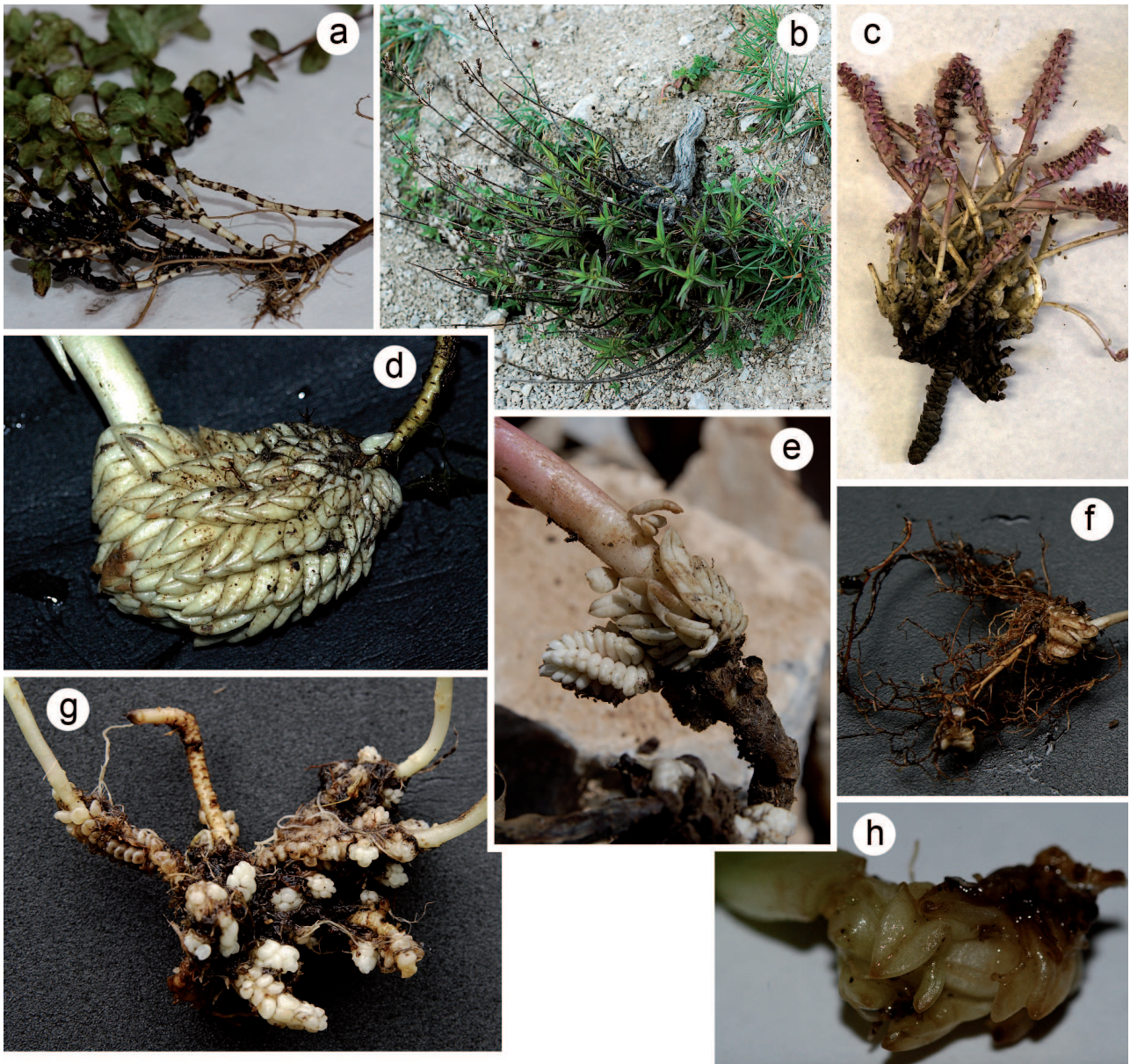
Fig. 5 Morphology of regenerative organs of selected perennial Rhinanthoid Orobanchaceae species a – rhizome of *Bartsia alpina*, b – *Odontites bocconei* (note the stem of the dwarf shrub in the upper right part of the image), c – *Lathraea squamaria* (whole plant with system of rhizomes), d – *Rhynchocorys odontophylla* (part of rhizome with scales), e – *Rhynchocorys kurdica* (system of scaly rhizomes), f – *Rhynchocorys maxima* (rhizomes wich scales), g – *Rhynchocorys elephas* (part of rhizome with scales), h– *Tozzia alpina* (short rhizome with scales).

The perennial species of the Rhinanthoid Orobanchaceae can be further divided in two functional groups underpinned by their life form (Cornelissen et al. 2003). All perennials of the *Rhinanthus-Rhynchocorys-Lathraea* subclade and *Tozzia alpina* can be classified as geophytes, i.e. species with annual shoots and below-ground rhizomes covered by fleshy scales, which are modified leaves (Weber 1975, Kubat & Weber 1987, Fig. 5). By contrast, all perennials of the *Bartsia-Euphrasia-Odontites* subclade (except *Tozzia alpina*) have perennial shoots and can be classified either as hemicryptophytes, i.e. species with regenerative organs on the ground surface (*Bartsia alpina*, South American *Bartsia* species) or as chamaephytes, i.e. dwarf shrubs (*Hedbergia*, *Nothobartsia, Odontites bocconei*; Fig. 5). This difference in life form is certainly an important aspect of the Rhinanthoid Orobanchaceae evolution, indicating a more complex evolutionary process than just the transitions between annuality and perenniality.

All species of the Rhinanthoid Orobanchaceae have relatively resource-rich seeds that sustain the plants during the initial phase of development (Heide-Jørgensen 2008), which is in direct contrast with the extremely small seeds of related obligate hemiparasites such as *Striga* or *Alectra* that have highly specialized dust-like seeds requiring an intimate contact with the host for successful germination (Irving & Cameron 2009). Large seed size appears generally advantageous for the competitive ability of seedlings (e. g. Turnbull et al. 1999, Jakobsson & Eriksson 2000). The seedling stage is a critical phase for a facultative hemiparasite, in which the plant is not yet connected to a host or the abstraction of nutrients is not very effective. Inefficient photosynthesis of the seedlings makes them extremely prone to light competition, which can be in part compensated by large resource-rich seeds. This advantage is however traded-off against lower total seed production and pronounced dispersal limitation (e. g. Bullock et al. 2003, Winkler & Heinken 2007). Extraordinarily large seeds can be in general found only in *Melampyrum* and representatives of the *Rhinanthus-Rhynchocorys-Lathraea* subclade. By contrast, all of the *Bartsia-Euphrasia-Odontites* subclade taxa seem to have adopted a strategy based on a general reduction of seed size. Therefore, they can produce a larger number of seeds that disperse more easily.

Larger seeds present in the annual species compared to the perennial species within the paraphyletic group of *Melampyrum* and the *Rhinanthus-Rhynchocorys-Lathraea* subclade can be attributed to a tendency of these species to grow in communities where competition for light is relatively intense, such as forests or meadows dominated by tall herbs (Heide-Jørgensen 2008). This requires fast development of the annual plants supported by resource-rich seeds since otherwise they would be outcompeted by surrounding perennials. On the other hand, perennial species of this group have underground holoparasitic seedlings (Weber 1975, Kubat & Weber 1987), which allows a lower rate of ontogenetic development since they are not affected by competition for light. The general reduction of seed size observed in the *Bartsia-Euphrasia-Odontites* subclade is probably one reason why no significant correlation was detected in this group. A small seed size is advantageous for dispersal, but it reduces seedling size and its competitive ability. The occurrence of most of the species of this group is therefore limited to stress-limited mountain habitats (*Bartsia alpina*, South American *Bartsia*, numerous *Euphrasia* species), ruderal or dry places (*Odontites* spp, *Parentucellia* spp., *Bartsia trixago*), where shading from the surrounding plants is not a problem due to the open character of such communities.

Biogeography

The dispersal-vicariance analysis identified temperate Western Eurasia as the origin of the Rhinanthoid Orobanchaceae, which must be however regarded with caution since *Melampyrum* species occurring in the Caucasian region are missing from the analysis. Their inclusion would probably extend the reconstructed distribution range of the ancestor by involving also this region. Such reconstruction would be also more consistent with conclusions of the biogeographic investigation of Wolfe et al. (2005), who hypothesized the origin of the group located somewhere north of the Tethys Sea. Based on the diversity centres of individual genera, the affinities of the genus *Rhynchocorys* to the Caucasian region (Fig. 4), *Rhinanthus* to the Balkans and *Melampyrum* to both of these regions (Meusel et al. 1978) further support the hypothesis on the Paratethyan origin of the Rhinanthoid Orobanchaceae (Wolfe et al. 2005). The Caucasian affinities are mostly missing in the *Bartsia-Euphrasia-Odontites* subclade and the ancestral distribution range appears located in the Mediterranean region for most of the extant lineages, which corresponds well to the distribution of diversity in most of the genera of this group (Meusel et al. 1978). The origin of predominantly alpine species of *Bartsia alpina* and *Euphrasia* spp. could be also related to the uplift of the Alps creating regions covered by favourable habitats. The minimum age of divergence between the stem lineage of *Euphrasia* was estimated as 28 Mya (Gussarova et al. 2008), which approximately corresponds with the main phase of the Alpine orogeny on the Eocene-Oligocene boundary around 34 Mya (Dezes et al. 2004). Given the very limited sampling, it is impossible to reconstruct a biogeographic origin of the genus *Euphrasia* in this study, but a recent study by Gussarova et al. (2008) placed it in Western Eurasia. Our dispersal-vicariance analysis included

samples of the likely diversification centre of this genus, which should limit the effects of insufficient sampling of this genus on the overall results of the biogeographic analysis.

The bipolar range of *Euphrasia* (Gussarova et al. 2008) and southern hemisphere distribution of *Bartsia* (excl. *B. alpina* and *B. trixago*) definitely present the most notable deviations from the predominant Holarctic-oriented distribution patterns in the Rhinanthoid Orobanchaceae. It is notable that both these genera display substantial similarities in their ecological ranges (occurrence in open alpine habitats) and life history traits (presence of both annual and perennial life cycle, small seeds albeit not the smallest in the whole group). Species with small or medium-sized seeds are in general considered better colonizers if seeds are wind dispersed and competition does not substantially affect recruitment (Eriksson & Jakobsson 1998, Coomes & Grubb 2003), which is the case for both *Euphrasia* and *Bartsia*. The effect of the presence of the perennial life cycle in some of the species is more questionable, but it is notable that in *Euphrasia*, perennial species tend to be found in isolated areas distant from the distribution centre (e. g. Azorean Islands, South-East Asian mountains, southern hemisphere regions; Gussarova et al. 2008). Therefore, we propose a hypothesis that a combination of the discussed ecological traits might be a key factor that allowed long-distance migration and establishment in the target regions resulting in the present distribution pattern.

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Appendix 1: Calibration of seed weight using published dimension data
Weight and dimensions of seeds of species involved in the study. The data were either obtained by direct measurement ("this study" indicated as the source) or obtained from different literature sources as indicated. If a range of values was reported, mean values were used in the calibration model between seed dimensions and seed weight.

Species	Weight of 1000 seeds (mg)	Estimated weight of 1000 seeds (mg)	Source of seed weight data	Seed length (mm)	Seed width (mm)	Source of seed dimensions
Bartsia alpina	0.28		Liu et al. 2008	1.4-2.2	1.1-1.2	Castroviejo et al. 2009
Bartsia laticrenata	0.09		this study	1	0.7	this study
Bartsia pedicularoides	0.03		this study	0.8	0.3	this study
Bartsia stricta	0.11		this study	1.0	0.7	this study
Bartsia trixago	0.04		Liu et al. 2008	0.6-0.7	0.4-0.5	Castroviejo et al. 2009
Euphrasia nemorosa	0.18		this study	1.1	0.4	this study
Euphrasia rostkoviana	0.16		this study	1.4	0.5	this study
Euphrasia sevanensis	N. A.			N. A.	N. A.	
Euphrasia stricta	0.14		this study	1.3	0.5	this study
Hedbergia abyssinica	0.07		this study	1.0	0.6	this study
Lathraea clandestina	N. A.			N. A.	N. A.	
Lathraea squamaria	0.67		Liu et al. 2008	1.1	0.9	Castroviejo et al. 2009
Melampyrum nemorosum	7.22		this study	4.5	2.1	this study
Melampyrum pratense	5.3		Liu et al. 2008	5.0-6.0	2.0-2.5	Castroviejo et al. 2009
Melampyrum sylvaticum	5.7		this study	5.1	2.5	this study
Nothobartsia aspera		0.58		2.2-2.5	0,7-1	Castroviejo et al. 2009
Odontites aucheri	0.57			2.5	1.2	this study
Odontites bocconei	N. A.			N. A.	N. A.	
Odontites luteus		0.42		1.7	1	Davis 1978
				1.4-1.8	0.7-1	Castroviejo et al. 2009
Odontites vernus	0.18		this study	1.5	0.7	this study
Parentucellia latifolia	0.02		Liu et al. 2008	0.3-0.4	0.2	Castroviejo et al. 2009
Parentucellia viscosa	0.02		Liu et al. 2008	0.3-0.4	0.2	Castroviejo et al. 2009
Rhinanthus alectorolophus	2.82		Liu et al. 2008	2.9	2.1	this study
Rhinanthus glacialis	N. A.			N. A.	N. A.	
Rhinanthus minor	2.34		this study	1.9	1.7	this study
Rhinanthus rumelicus	1.24		this study	3	2.3	this study
Rhynchocorys elephas	0.22		this study	1	0.8	this study
Rhynchocorys kurdica	0.31		this study	1.1	0.8	this study
Rhynchocorys maxima	N. A.			N. A.	N. A.	
Rhynchocorys odontophylla		0.14		1	0.6	Davis 1978
Rhynchocorys orientalis		2.62		3.6	2	Davis 1978
				3.5	2	Gabrielian 1987
Rhynchocorys stricta	1.18		this study	1.8	1.5	this study
Tozzia alpina	0.93		this study	1.9	1.8	this study
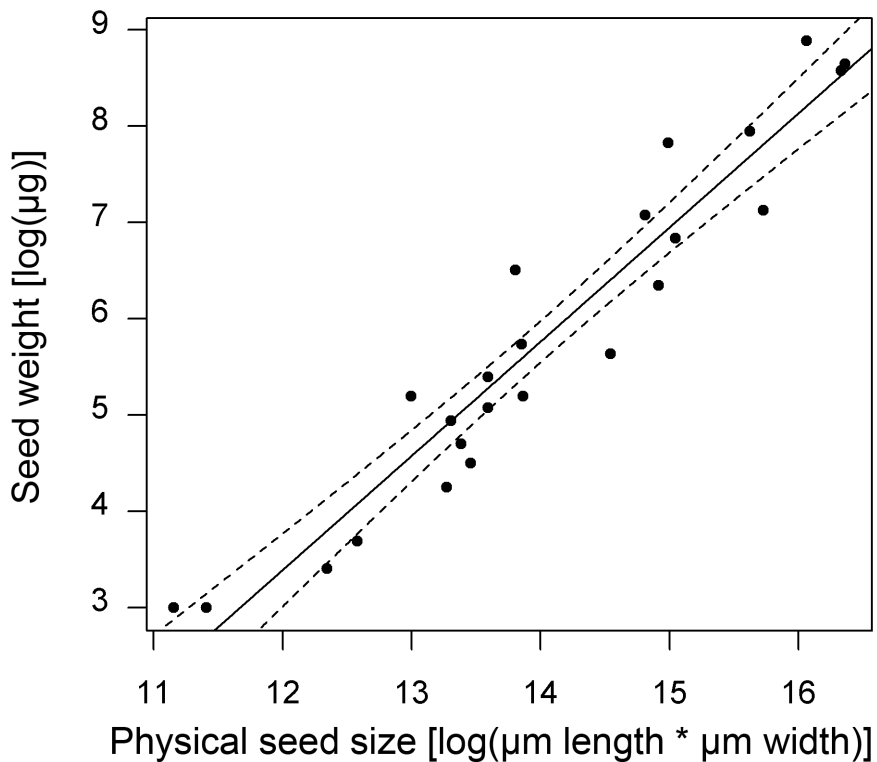
Calibration model useed to estimate seed weight of species for which only seed dimensions are available. The model is based on a linear regression in which seed weight (logarithmized weight of one seed in μg) was used as the response variable and logarithmized product of seed dimendions (in μm) as the predictor. Summary of the model: $R^2 = 0.917$, $F_{(1,22)} = 214.5$, $P < 10^{-6}$

Scatter-plot demonstrating the relationship between seed weight and dimensions. Regression line of the calibration model with 95% confidence interval is plotted on the graph.

2 Heterotrophic carbon gain by the root hemiparasites *Rhinanthus minor* and *Euphrasia rostkoviana* (Orobanchaceae)

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Introduction

Root hemiparasitic plants attack and withdraw resources from their host's vascular system through a specialised transfer organ called haustorium (Irving & Cameron 2009). Due to their often reduced root networks, hemiparasitic plants gain virtually all mineral nutrients and water from their host plant while organic carbon is provided, at least in part, by their own photosynthetic activity (Watling & Press 2001). This heterotrophic strategy has evolved on multiple occasions defined by differing physiological processes in several unrelated groups of angiosperms (Press & Graves 1995). The family Orobanchaceae is one of the most diverse plant families that that comprise exclusively root hemiparasites or holoparasites, with the exception of a single non-parasitic genus, *Lindenbergia* (Wolfe et al. 2005, Bennett & Matthews 2006). In this group, the parasitic relationship only takes place below ground while above ground, the host-parasite interaction is underpinned by competition for light (Matthies 1995). The extent to which competition for light affects hemiparasite growth and development depends on the ability of a given hemiparasite to gain assimilates from the host plant. Consequently, hemiparasites acquiring large amounts of carbon from their host plant should be able to survive even under relatively severe shading from surrounding plants. Resolving the carbon budget of the host-hemiparasite relationship thus provides valuable insights into the dependency of individual hemiparasitic plants on the heterotrophic habit and their competitive ability.

Hemiparasitic plants are capable of photosynthesis (Press et al. 1987, Press 1989, Cameron et al. 2008) which is the source of some of their organic carbon. However, the rates of assimilation are substantially lower than those found in non-parasitic plants owing to high respiratory rates in most species of hemiparasites; thus, net photosynthetic carbon gain is usually negligible (Press et al. 1988, Press 1989). Moreover, net photosynthetic carbon gain may even be below the photosynthetic compensation point in some species that respire more CO_2 than they are able to assimilate (Press et al. 1987, Press 1989). Hence, hemiparasites must gain at least certain amount of organic carbon heterotrophically from their host plant. However, since hemiparasitic plants attack host xylem and not the phloem, they only have access to xylem-mobile organic compounds, such as organic nitrogen, most likely in the form of amino acids, and virtually no carbohydrates (Irving & Cameron 2009). Using $^{14}CO_2$ labelling of host plants, the host-to-parasite flux of assimilates has been directly demonstrated in parasitic relationships between *Odontites vernus* and its hosts *Hordeum vulgare* and *Trifolium repens* (Govier et al. 1967). Although this approach was powerful in demonstrating the character of assimilates transferred via haustoria, it did not provide any quantitative assessment of the amount of organic carbon flux from the host to the parasite. Moreover, Hodgson (1973) applied similar radio-isotope tracer-based techniques to investigate the nature of host-to-parasite C

transfers between *Euphrasia officinalis* agg., *Rhinanthus minor* , *Pedicularis sylvatica*, *Orthocarpus luteus* and *Melampyrum pratense* and a range of host species but again did not provide a quantitative C budget.

In a pioneering study, Press et al. (1987) demonstrated that it is possible to quantify the proportion of host-derived carbon in hemiparasite biomass using natural abundance stable isotope values of ^{13}C of the obligate root hemiparasite *Striga hermonthica* (Del.) Benth. attached to a C_4 host. Plants undertaking C_4 photosynthesis have a significantly enriched natural abundance δ^{13}C value compared to C_3 plants owing to the differential discrimination of RUBISCO and PEPC, the first enzymes of carbon fixation in C_3 and C_4 plants respectively. Press et al. (1987) therefore harnessed these differential δ^{13}C values comparing δ^{13}C of C_3 parasite to that of its C_4 host and, by measuring the extent to which the C_3 parasite takes on C_4 δ^{13}C values, were able to estimate heterotrophic carbon gain. Using the same approach, Graves et al. (1990) found almost 50% of the leaf biomass in *Striga hermonthica* was derived from its C_4 host while Tennakoon & Pate (1996) revealed that 20-30% of the total biomass of the parasitic tree *Olax phyllanthi* (Labill.) R.Br. was host derived. However, such data for temperate hemiparasites in the Orobanchaceae family are currently absent in literature.

While using such differences in the natural abundance of stable isotopes of carbon is a powerful tool for resolving the extent of parasitic plant heterotrophy, these cultivation experiments can be potentially biased by the fact that CO_2 respired by soil (i.e. by roots and soil microorganisms) bears the isotope signature of the host whose roots dominate the cultivation pots. Thus, the CO_2 respired by the C_4 host roots and shoots and by the microorganisms decomposing dead organic matter originating from the C_4 host can be re-fixed by the parasite, which would consequently lead to an overestimation of the host-derived carbon in the hemiparasite.

This study aims to assess the extent of heterotrophic carbon gain by the temperate hemiparasites, *Rhinanthus minor* L. and *Euphrasia rostkoviana* Hayne (Orobanchaceae), from their hosts through the application of the methods of Press et al. (1987) while addressing the problem of a potential bias caused by re-fixation of soil-respired CO_2 using paired partner plants. Resolving the extent to which re-fixation of respired CO_2 has the potential to influence the δ^{13}C values of the parasite in our experimental systems is important not only for the interpretation of our own results but also has implications for previously published work (Press et al. 1987, Tennakoon & Pate 1996). Furthermore, the results presented here enhance our knowledge of heterotrophy in annual hemiparasitic forbs. This is important since a majority of studies estimating the percentage of host-derived carbon were conducted on mistletoes (e.g. Marshall et al. 1994, Bannister & Strong 2001) where the host-parasite interaction is quite different.

Material and methods
Experimental species
Rhinanthus minor and *Euphrasia rostkoviana* are hemiparasitic Orobanchaceae displaying C_3 metabolism and were used as model species in our experiments. *Rhinanthus minor* is a relatively common species occurring on meadows across Western Eurasia (Meusel et al. 1978). It is able to parasitize a wide range of host species; however, parasite performance (in terms of growth and fecundity) is greatest when attached to grasses and legumes (Cameron et al. 2006, Rümer et al. 2007). It is by far the most widely used species in recent physiological and ecophysiological studies of hemiparasitic plants (see Irving & Cameron 2009 for a review). *Euphrasia rostkoviana* grows naturally on nutrient-poor meadows and displays a similar, albeit slightly more restricted, geographical range to *R. minor*. Its suitable host range can be considered relatively broad comprising not only grasses and legumes but also certain forb species (Yeo 1964, Lammi et al. 1999). Maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) were used as host species in this study. *Plantago lanceolata* L. was introduced as a reference plant designed to capture the effect of the host identity on isotope signature value of hemiparasite assimilates caused by reassimilation of the soil-respired CO_2 (Fig. 1). Seeds of *E. rostkoviana* were collected from several hundred individuals in a wild population occurring on a meadow in the vicinity of the Horská Kvilda village

(Bohemian Forest Mts., Czech Republic). *Rhinanthus minor* seeds were collected from several hundred individuals in a wild population occurring on a mesotrophic calcareous meadow in Lathkill Dale, near Monyash (Peak District National Park, Derbyshire, UK). Seeds of both host species were obtained from the school farm of the Faculty of Agriculture, University of South Bohemia. *Plantago lanceolata* seeds were obtained from Planta Naturalis Ltd. (Markvartice, Czech Republic).

Plant cultivation

Seeds of the hosts and *P. lanceolata* were germinated on Petri dishes on moist filter paper at 18ºC. The seedlings were moved to 10 x 10 cm square pots containing substrate of Levington M3 compost and washed quartz sand (1:1, volume/volume ratio) after successful germination. This growth medium contains only minimal reserves of inorganic nutrients, which corresponds to the composition of soil at the sites where the seeds of the experimental hemiparasitic species were obtained. The pots contained a septum that partially bisected the pot diagonally (Fig. 1). This design facilitated the separation of the parasite and *P. lanceolata* roots while allowing the host roots to access the whole volume of the pots (Fig. 1). Although it has been demonstrated that *P. lanceolata* is resistant to *R. minor* (Cameron et al. 2006, Rümer et al. 2007) and the parasite is unable to abstract resources from this species (Cameron and Seel 2007), the design of the pot further decreases the probability of any interaction between *R. minor* and the reference plant. Pots were maintained in a controlled environment chamber with a cycle of 14-h light at 20°C and 10-h dark at 15°C (Conviron, Winnipeg, Canada). This diurnal light cycle corresponds to natural conditions assumed for the source populations of both hemiparasitic s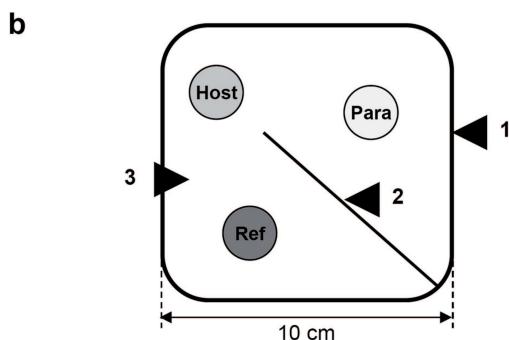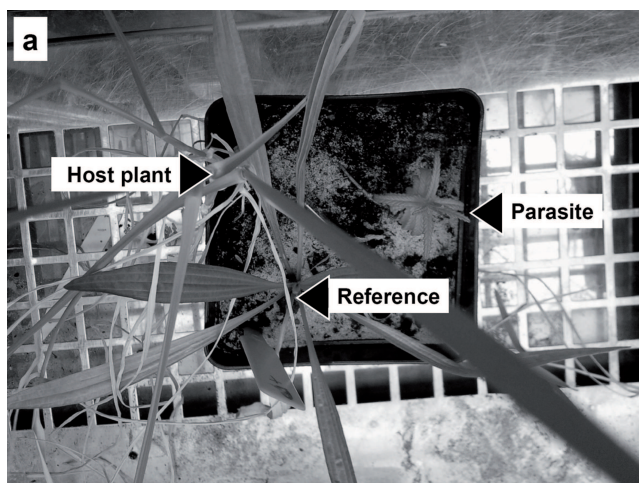pecies growing in summer season at around 50° of the northern latitude. *Rhinanthus minor* seedlings (germinated on moist filter paper at 4°C) were sown at a density of one seedling per pot ca 3-4 cm from the host plant after 8 days of host development. The replicate pots ($n = 10$ for each host-hemiparasite combination) were arranged in a randomised block design and watered every day until harvest. Hosts and the parasite *E. rostkoviana* were germinated in the same way and planted in simple, undivided pots containing a 1:1 (v/v ratio) sand compost mixture. All replicate plants ($n = 10$ for each host-hemiparasite combination) were cultivated in a growth-cabinet at the Faculty of Science, University of South Bohemia with a cycle of 14-h light and 10-h dark at constant temperature of 18°C. After 14 weeks, leaf material was harvested and dried at 80ºC for 48 h. Roots were washed and examined for the presence of haustoria to ensure attachment with the host plant. The basal leaves of the hemiparasites were excluded from the stable isotope analysis since they were produced autotrophically before the attachment to the host. The samples were homogenized separately and a 5-µg subset of each constituent part was analysed for ^{13}C content by continuous-flow mass spectrometry (PDZ Europa 2020 Isotope Ratio Mass Spectrometer (IRMS) coupled to a PDZ ANCA GSL preparation unit, SerCon Ltd, Crewe, Cheshire, UK). Data were collected as

Fig. 1 (a) Photograph of the experimental split pot showing the host plant, parasite and reference plant (*Plantago lanceolata*) and (b) a schematic diagram of the experimental split pot (1) showing host plant, parasite and reference plant, septum (2) and soil substrate (3).

atom % ^{13}C and re-expressed as delta values relative to the Pee Dee Belemnite standard (δ) using equation 1.

$$\delta^{13}C = (R_{Sample} / R_{Standard} - 1) \cdot 1000 [‰]$$ Eqn 1

where R_{Sample} = ^{13}C:^{12}C ratio in the sample and $R_{Standard}$ = ^{13}C:^{12}C ratio in the Pee Dee Belemnite standard.

Assessment of host derived carbon in hemiparasite biomass
Rhinanthus minor, E. rostkoviana and wheat perform C$_3$ photosynthesis while maize performs C$_4$ photosynthesis. As a result of their photochemical processes, C$_4$ plants are usually significantly more enriched in ^{13}C than C$_3$ plants. Hence, it is possible to infer the extent of heterotrophic carbon gain of a hemiparasitic plant by measuring the relative change in the δ^{13}C value of the C$_3$ parasite attached to a C$_4$ host compared to when it is attached to a C$_3$ host. We used an adjusted form of a linear two-source isotope-mixing model (Marshall & Ehleringer 1990), adapted from Gebauer & Meyer (2003), to calculate proportion of host-derived carbon in hemiparasite biomass (Equation 2).

$$\%H = \left(\frac{\delta^{13}C_{P(C_4)} - \delta^{13}C_{P(C_3)}}{\delta^{13}C_{H(C_4)} - \delta^{13}C_{H(C_3)}} \right) \cdot 100 \ [\%]$$ Eqn 2

where %H = the percentage of carbon in parasite biomass that is derived from the host, $\delta^{13}C_{P(C3)}$ = δ^{13}C of the parasite growing on the C$_3$ wheat host, $\delta^{13}C_{P(C4)}$ = δ^{13}C of the parasite growing on the C$_4$ maize host, $\delta^{13}C_{H(C3)}$ = δ^{13}C of the infected wheat host and $\delta^{13}C_{H(C4)}$ = δ^{13}C of the infected maize host. For each replicate, δ^{13}C $_{P(C4)}$ and the δ^{13}C $_{H(C4)}$ of the corresponding host plant were entered into the model calculation and the average values of $\delta^{13}C_{H(C3)}$ and δ^{13}C $_{P(C3)}$ were used as the baseline reference value. The outcome of this model provides an estimate of proportion of heterotrophic carbon in hemiparasite biomass produced by an adult host-attached plant. This value integrates organic carbon flows from the host to the hemiparasite, the actual values of which can be variable in time. The model assumes that there is no fractionation during the assimilation of host-derived nutrients by the parasite or that the fractionation has the same effect on δ^{13}C in parasites cultivated with both maize and wheat. Such assumption is reasonable since direct luminal continuity exists in *R. minor* haustoria (Cameron et al. 2006). In case of *E. rostkoviana*, absence of luminal continuity cannot be excluded; nonetheless, a study on *Olax phyllanthi* which conducts nutrient transfer via contact interfacial parenchyma does not report any substantial bias connected to isotope discrimination during the transfer (Tennakoon & Pate 1996). In addition, xylem δ^{13}C is assumed to be of the same value as the bulk leaf dry matter. Information on isotopic composition of xylem sap is unfortunately missing from literature probably due to difficulty in measuring this value, caused by low organic carbon concentration. Variation in δ^{13}C across main structures of a normal autotrophic plant however tends to be fairly restricted (Bowling et al. 2008). Moreover if any significant fractionation effect occurred, it could be assumed to be equal in both host species resulting in an equal effect on both terms in the mixing model numerator.

Statistical analysis
Differences between treatment means were analysed by ANOVA followed by Fisher's multiple comparison test or using Student's *t* test using Minitab version 13 (Minitab Inc., PA, USA). Where necessary, to satisfy the test assumptions, data were arcsine square root transformed. Welch's estimation of degrees of freedom was used for *t* tests performed on data with unequal samples sizes. Untransformed means and associated standard errors are presented. The isotope-mixing model outcomes were tested for significant difference from 0 (i.e. no carbon gain from heterotrophy) using Student's *t* test.

Results

Carbon isotope composition of the host plants followed patterns expected for species displaying C_3 and C_4 metabolism (Fig. 2a,b). Experimental hemiparasitic plants attached to the wheat hosts also displayed $\delta^{13}C$ values typical for C_3 plants and were not significantly different from those of their hosts (one-way ANOVA; $P > 0.05$) (Fig. 2a,b). The $\delta^{13}C$ values of hemiparasites attached to maize were significantly enriched compared to those attached to wheat (Fig. 2a,b) indicating contribution of host carbon to hemiparasite biomass. The difference in $\delta^{13}C$ between parasites attached to maize and wheat was more pronounced in *R. minor* than in *E. rostkoviana*. However, $\delta^{13}C$ values in *R. minor* displayed higher variation.

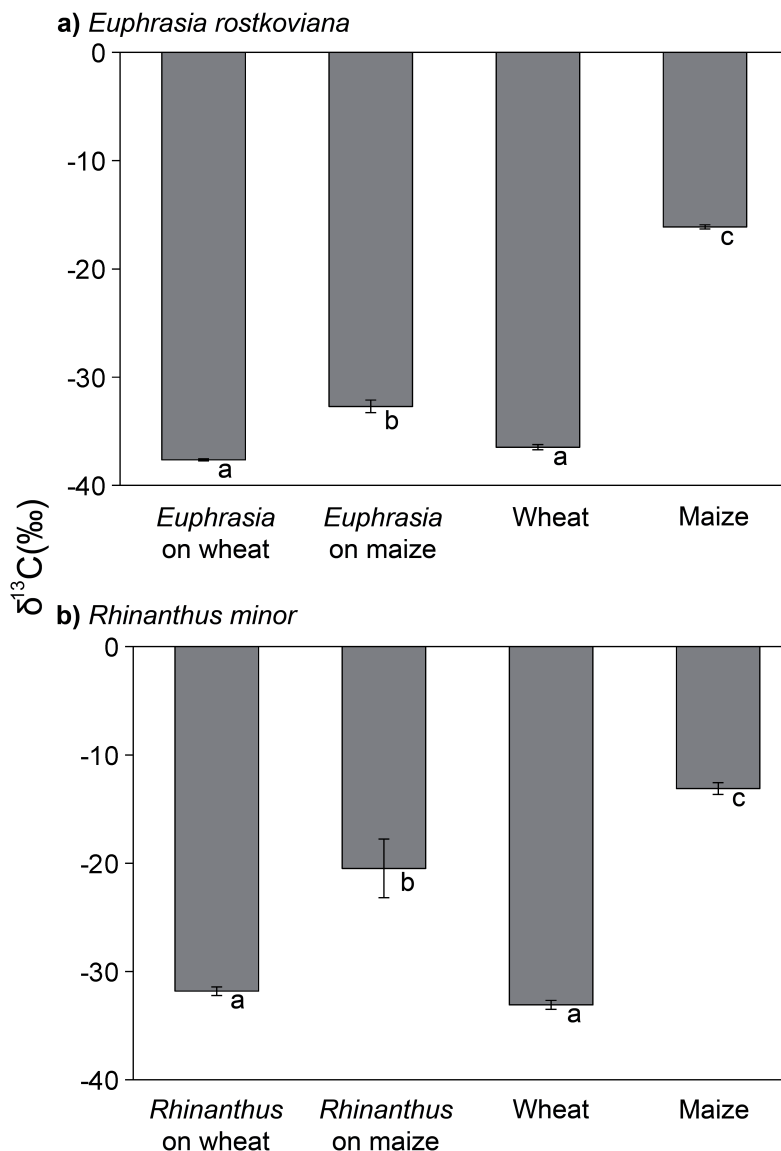**a)** *Euphrasia rostkoviana*

b) *Rhinanthus minor*

Fig. 2 (a) $\delta^{13}C$ values of wheat (C_3 photosynthesis) and maize (C_4 photosynthesis) hosts and *Euphrasia rostkoviana* (C_3 photosynthesis) or (b) *Rhinanthus minor* (C_3 photosynthesis) (b) attached to each type of host plant. Error bars represent ± 1 standard error and bars with differing letter are significantly different (1-way ANOVA – *E. rostkoviana*: *df* = 3,30, *F* = 1356, *P* < 0.001 and *R. minor*: *df* = 3,33, *F* = 94.86, *P* < 0.001).

These values of carbon isotope composition were used in the estimation of proportions of heterotrophic carbon in the biomass of the hemiparasites from the two-source linear isotope-mixing model. Mean values for the estimated percentage of heterotrophic carbon in biomass of the hemiparasites were 56% for *R. minor* (ranging from 6.2 to 89.5%) and 24% (ranging from 20.9 to 28.7%) for *E. rostkoviana* (Fig. 3). The proportions of host-derived carbon were significantly different from zero in both *R. minor* [Student's t-test (arcsine transformed data): *df* = 6, *t* = 4.70, *P* = 0.003) and *E. rostkoviana* [Student's t-test (arcsine transformed data): *df* = 2, *t* = 18.56, *P* = 0.003].

We used $\delta^{13}C$ values of the paired *P. lanceolata* reference plants to investigate the extent to which $\delta^{13}C$ values of the hemiparasites attached to the C_4 maize host could be influenced by recapturing of soil-respired CO_2. Carbon isotope ratio of the reference *P. lanceolata* plants did not significantly differ between the pots with maize and wheat (Fig. 4; Welch *t* test *df* = 8.32, *t* = 0.24,

$P = 0.82$). This implies that the recapturing of soil-respiratory CO_2 either does not occur, or occurs at such low rates that are not sufficient to bias the $\delta^{13}C$ value of the hemiparasites.
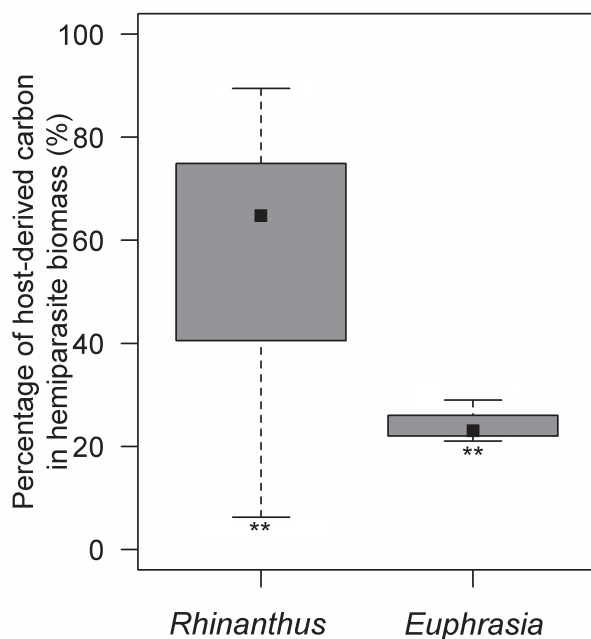

Fig. 3 Box-and-whisker plot representing the median (symbol), quartiles (box), maximum and minimum (whiskers) percentage of host derived carbon in parasite biomass for *Euphrasia rostkoviana* and *Rhinanthus minor*. Double asterisk indicates that mean is significantly different from 0 (Student's *t* test – *E. rostkoviana* (arcsine transformed data): $df = 2$, $t = 18.56$, $P = 0.003$ and *R. minor* (arcsine transformed data): $df = 6$, $t = 4.6994$, $P = 0.003$)
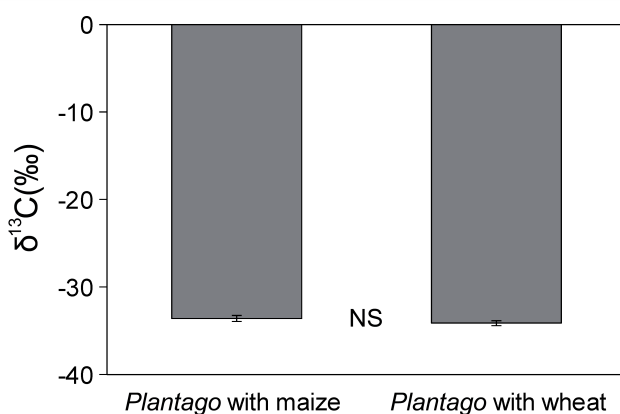

Fig. 4 $\delta^{13}C$ values of *Plantago lanceolata* (C$_3$ photosynthesis) reference plants growing with either wheat (C$_3$ photosynthesis) or maize (C$_4$ photosynthesis). Error bars represent ± 1 standard error. Treatment means were not significantly different (Welch *t* test: $df = 8$, $t = 0.24$, $P = 0.82$)

Discussion

The majority of parasitic plants display a hemiparasitic strategy for resource acquisition (Irving & Cameron 2009) and while the photosynthetic process is active in these plants, potentially significant amounts of organic carbon can also be gained from the host in the form of xylem-mobile organic compounds (Govier et al. 1967, Hodgson 1973, Jiang et al. 2008b). Direct quantitative partitioning between the two sources of assimilates to the carbon budget of hemiparasite is virtually impossible given the complexity of the metabolic interactions between host and parasite (Hibberd & Jeschke 2001). Hitherto, the most robust method for the estimation of carbon heterotrophy by parasitic plants was presented by Tennakoon & Pate (1996) based on the comparison of the natural abundance $\delta^{13}C$ value of a hemiparasite attached to either a C$_4$ or C$_3$ host and the $\delta^{13}C$ value of the C$_4$ host itself. We further refine this technique and present a novel, more straightforward approach based on a two-source isotope-mixing model, modified from Marshall and Ehleringer (1990) and Gebauer & Meyer (2003), which includes $\delta^{13}C$ data of all experimental plants (i.e. C$_3$ and C$_4$ hosts together with parasites attached to these hosts). The calculation is then based on the excess of ^{13}C in hemiparasites attached to the C$_4$ host compared to those attached to the C$_3$ host related to the difference in isotope composition between the C$_3$ and C$_4$ hosts themselves. Furthermore, by using *P. lanceolata* as a reference plant, we ruled out the possibility of the ^{13}C enrichment observed in hemiparasites when grown with C$_4$ host being an artefact due to reassimilation of respired, ^{13}C-enriched CO_2 .

This refined natural abundance stable isotope method allows a reliable estimation of heterotrophic carbon acquisition in two temperate species, *R. minor* and *E. rostkoviana*. We demonstrate that *R. minor* has a potential to acquire an extensive amount of organic carbon from its host. The mixing model calculations suggest that on average over 50 % of leaf biomass of the experimental individuals consists of host-derived carbon although the model outputs of are highly variable. By contrast, the proportion of heterotrophic carbon in *E. rostkoviana* reached only a maximum of 30%. Such a difference between the two species studied is not to be unexpected. *Rhinanthus minor* is known to be very efficient in the extraction of solutes of the host xylem (Jiang et al. 2003, Cameron & Seel 2007) due to an advanced haustorial structure (Cameron and Seel 2007, Rümer et al. 2007) Moreover, infection by *R. minor* is certainly more detrimental (in terms of host growth and fecundity reduction) than most other temperate hemiparasites (Gibson & Watkinson 1992, Matthies 1995). As a result of the extensive host damage, *Rhinanthus* species have been shown to exert significant effects on the structure and function of the communities they inhabit (Ameloot et al. 2005, Cameron et al. 2005, Cameron et al. 2009). The proportion of heterotrophic carbon in *Euphrasia rostkoviana* reached only up to 30% corresponding to a relatively conservative hemiparasitic strategy (i.e. comparatively low dependence on resource supply from the host) reportedreported, e.g. for *Olax phyllanthi*, which displays similar degree of carbon heterotrophy (Tennakoon & Pate 1996).

Our estimates of heterotrophic carbon gain by *R. minor* and *E. rostkoviana* are based on a cultivation experiment conducted under controlled conditions using host plants that are not native hosts for the two hemiparasitic species although maize and wheat are highly susceptible to both hemiparasite species Therefore, these results must be taken with caution when inferring the consequences to plants growing in a natural environment. Nevertheless, this study is the first to provide a quantitative assessment of the potential for *R. minor* and *E. rostkoviana* to abstract and utilise heterotrophically derived carbon. Moreover, the extent of carbon acquisition in mature plants of *R. minor* recorded in our study is comparable to the values reported for *Striga* species (Press et al. 1987, Graves et al. 1990, 1992). Species of both genera, *Striga* and *Rhinanthus*, are also known to cause substantial damage to their hosts leading to significant reductions in host biomass and disruption of host metabolic processes (Watling & Press 2001, Cameron et al. 2008). Both *Rhinanthus* (Cameron et al. 2008) and *Striga* (Watling & Press 2001, Rank et al. 2004) suppress host growth and photosynthesis, a potential consequence of which is a reduction in host competition pressure. Nonetheless, this is much more prominent in *Striga* which has been postulated to produce cytotoxic metabolites interfering with photosynthetic metabolic pathways in some host species (Musselman 1980). There are however also other substantial differences in their biology; *Striga* is an obligate hemiparasite requiring host-root induction to germinate. Its seedling produces a primary haustorium and is completely dependent on host carbon supply in the first stage of its development (Irving and Cameron 2009). In contrast, *Rhinanthus* is a facultative hemiparasite producing large seeds that germinate independently of the presence of a host plant. *Rhinanthus* seedlings are fully autotrophic and emerge from the soil prior to attachment to the host plant. After emergence, *Rhinanthus* seedlings produce roots that forage for a suitable host to which they attach via secondary haustoria (Irving & Cameron 2009). The early stage of development is evidently a critical part of facultative hemiparasite life cycle as the seedlings are highly sensitive to competition for light due to slow development and inefficient photosynthesis, This may explain the observation that competition for light with the host has negative effect on *Rhinanthus* (Matthies 1995, Hejcman et al. 2007).

Both *R. minor* and *E. rostkoviana* are xylem-feeding hemiparasites. Direct luminal continuity between host and parasite vessel elements has been reported in *R. minor* haustoria (Cameron et al. 2006, Cameron & Seel 2007, Rümer et al. 2007). The uptake of xylem solutes is driven by mass flow because of the lower water potential of the parasite induced through the accumulation of osmotically active sugar alcohols (Jiang et al. 2008a) and elevated parasite transpiration rates facilitated by permanently open stomata that are insensitive to ABA-induced closure (Jiang et al. 2003). As a consequence of xylem-xylem continuity, the relative uptake of

individual nutrients is directly related to their concentration in host xylem; the composition of mineral nutrients xylem sap of *R. minor* and its hosts is indeed very similar (Jiang et al. 2008b, Irving & Cameron 2009). This is also supported by the observation that the $\delta^{13}C$ values recorded in hemiparasites growing on a C_3 host are not significantly different from the $\delta^{13}C$ values recorded for the host itself (Fig. 2). Xylem sap is generally assumed to contain only a minimal amount of organic carbon but a recent investigation has revealed that organic compounds can constitute ca 50% of xylem solutes (Alvarez et al. 2008) suggesting that the xylem is in fact a rich source of organic carbon for the parasite. Hence, the high rate of carbon heterotrophy in *R. minor* reported in our study is not surprising given its highly efficient abstraction of xylem solutes when attached to a suitable host (Jiang et al. 2003, Cameron & Seel 2007). Substantial variability in the degree of heterotrophy observed among individual plants of *R. minor* might be in part explained by a positive feedback relationship between hemiparasite growth and the amount of xylem sap it is able to abstract from the host's vascular system. The mass flow of xylem sap from host to parasite, driven by a low parasite water potential, should increase with the size of a parasite and its leaf area because large parasites represent greater sink strengths due to an increase in the total transpiration rate per individual plant. Such a positive feedback loop is likely to be more prominent in *R. minor* because of its larger leaf area. This would be in an agreement with the present experimental data. This hypothesis however requires further investigation.

Clearly, both *R. minor* and *E. rostkoviana* can abstract substantial amounts of organic carbon from their hosts; however, the nature of the mechanisms underlying the interplay between hemiparasite and host individuals in determining the degree of heterotrophy, hemiparasite growth and its effect on the host remain unclear and warrant further investigation.

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3 Interactions between hemiparasitic plants and their hosts – the importance of organic carbon transfer

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Abstract

Hemiparasitic plants display a unique strategy of resource acquisition combining parasitism of other species and own photosynthetic activity. Despite the active photoassimilation and green habit, they acquire substantial amount of carbon from their hosts. The organic carbon transfer has a crucial influence on the nature of the interaction between hemiparasites and their hosts which can oscillate between parasitism and competition for light. In this minireview, we summarize methodical approaches and results of various studies dealing with carbon budget of hemiparasites and the ecological implications of carbon heterotrophy in hemiparasites.

Introduction

Hemiparasitic plants withdraw resources from the vascular system of their hosts through a specialised transfer organ called haustorium (Irving & Cameron 2009). Hemiparasites attack the host's xylem, in contrast to the holoparasites that infect both host phloem and xylem, and as a consequence, hemiparasitic plants have access to water and mineral nutrients but little carbon (Irving & Cameron 2009). Due to their reduced or non-existing root networks, hemiparasitic plants acquire virtually all mineral nutrients and water from the host while organic carbon is provided, at least in part, by their own photosynthetic activity (Watling & Press 2001, Nickrent 2002). This is in contrast to holoparasitic plants which rely on the host for the supply of both organic and inorganic nutrients. The location of the attachment to the host and the degree of host dependency represent the most important characters defining the three basic functional types within hemiparasitic plants. Root hemiparasites attack host roots but their above-ground appearance is usually not substantially different from that of a non-parasitic plant. This group can be further divided in two – facultative and obligate hemiparasites consisting of plants that are able (at least sometimes) or unable to complete their life cycle without an attachment to the host respectively. Stem hemiparasites are attached to the host stem (usually trunk or branches) and are all obligate parasites, unable to survive without a host.

Hemiparasitic plants have an ambiguous relationship with their hosts which, on the one hand, represent exclusive sources of inorganic nutrients but on the other hand, the co-occurrence of these host plants in the hemiparasite vicinity imposes competition for light. The nature and intensity of this competitive relationship varies across different groups and species of hemiparasites. The ability of hemiparasites to acquire organic carbon (largely in the form of xylem-mobile organic and amino acids) is certainly the key factor affecting this interaction since hemiparasites that are capable of efficient organic carbon abstraction should be minimally affected by shading from their host. The fact that hemiparasites can exhibit substantial carbon heterotrophy is now supported by a large number of studies, although a traditional point of view on hemiparasites that highlights the

importance of inorganic resources (mainly nitrogen) acquisition is still prevailing. Therefore, we decided to summarize available information on hemiparasite heterotrophy, outline techniques for assessing the proportion of heterotrophy and estimating the overall carbon budget, and discuss possible implications of this phenomenon on hemiparasite ecology.

Mechanisms of resource acquisition in different functional types of hemiparasites

The anatomy of haustoria and the mechanisms of resource acquisition by root hemiparasites as well as abstraction efficiency have been shown to differ between species in and is link to their dependence on their host and life history. Annual hemiparasites such as *Striga* spp. or *Rhinanthus* spp. are capable of highly efficient abstraction of solutes from the host xylem which is underpinned by direct luminal continuity between host and parasite vessels (Dörr 1997, Hibberd & Jeschke 2001, Cameron et al. 2006, Irving & Cameron 2009). The transfer of solutes occurs as a passive mass flow driven by a water potential gradient between the host and the parasite. The hemiparasite maintains more negative water potential than its host, which is generated by substantially elevated transpiration rates and abnormal behaviour of stomata which do not close even in dark or under water stress conditions (Press et al. 1988, Press 1989, Jiang et al. 2003) as well as the accumulation of osmotically active compounds such as sugar alcohols, especially mannitol (Irving & Cameron 2009). In contrast, perennial species such as *Bartsia alpina*, *Olax phyllanthi* and *Santalum album* tend to display a rather conservative strategy of parasitic resource acquisition (Press 1989, Ehleringer & Marshall 1995). Solute transfer in haustoria formed by these hemiparasites proceeds across cell walls or contact parenchyma (Tennakoon & Pate 1997, Hibberd & Jeschke 2001, Tennakoon & Cameron 2006). Although the exact mechanism is unknown, this process is probably based on an active transmembrane transport as it does not appear to require a water potential difference (Tennakoon & Cameron 2006). Transpiration rates of the perennial hemiparasites therefore tend to be similar to those of a non-parasitic plant and often do not exceed those of their host (Tennakoon & Pate 1997, Tennakoon & Cameron 2006).

Stem hemiparasites usually parasitize tree or shrubby species. Not only do they gain nutrients and water from the host xylem, but are also provided with a favourable position in the canopy in which they can receive a large amount of photosythetically active radiation. The solute transfer in their haustoria is usually based on a combination of all possible mechanisms (i.e. luminal continuity, across a cell wall, across parenchyma cells) relative proportion of which differs across species, individuals and individual haustoria (Finneran & Calvin 1999, Heide-Jørgensen 2008). Mistletoes usually keep more negative water potential then their host; nonetheless its dynamics is usually coupled to that of the host plant and the stomata behave not as abnormally as in annual root hemiparasites (Glatzel 1983, Ulmann et al. 1985, Ehleringer et al. 1986). However, little is known about the physiology of resource acquisition in other stem hemiparasites such as species of the genus *Cassytha*.

Heterotrophic carbon gain

Hemiparasitic species are connected to the host xylem suggesting that water and mineral nutrients are the primary resources that are abstracted from the host. This is especially assumed in facultative root hemiparasites that are less dependent on the host resources compared to other parasitic species (Irving & Cameron 2009). Many root-hemiparasitic species nonetheless display inefficient photosynthesis and high respiratory rates (Press 1989). Mistletoes usually exhibit photosynthetic characteristics more typical of shade plants, i.e. low mean CO_2 assimilation rates, low electron transport rate (ΦPS_{II}), low light saturation point and low chlorophyll a/b ratio (Strong et al. 2000) despite experiencing favourable light conditions high in the canopy. In addition, seedlings of obligate root hemiparasites (e.g. *Striga* spp.) are achlorophyllous and hence completely dependent on the host. All these facts suggest that substantial transfer of organic carbon from the host must occur to support growth of many hemiparasitic species despite the lack of a connection to the phloem (Dörr 1997). The existence of a host-to-hemiparasite flux of organic carbon in a hemiparasitic relationship was demonstrated in several root-hemiparasitic associations using radioactive $^{14}CO_2$ labelling (Gov-

ier et al. 1967, Hodgson 1973). Neither of these studies however presented a quantitative estimation of the carbon budget. Quantification of host-derived carbon in hemiparasites can be estimated using the stable isotopes of carbon in a hemiparasitic relationship with a host species that performs C_4 photosynthesis in contrast to the C_3 hemiparasites. Its photoassimilates are therefore significantly enriched in ^{13}C compared to those of C_3 plants due to the differential isotope discrimination of RUBISCO and PEPC, primary CO_2-fixing enzymes in C_3 and C_4 plants respectively. The proportion of host derived carbon in the hemiparasite biomass can be then calculated from the ^{13}C value of biomass of a hemiparasite connected to the C_4 host using an isotope mixing model comparing it to a C_3 reference. This reference can be obtained either as measured isotopic composition of biomass of a hemiparasite attached to the C_3 host or can be calculated as an expected carbon isotope composition of the hemiparasite based on a gas exchange measurement that provide the c_i/c_a ratio (ratio between CO_2 concentration within leaf and in ambient air) using the formula of Farquhar et al.:

$$\delta^{13}C_p = \delta^{13}C_{atm} - a\,(b-a)\,c_i/c_a$$

where, $\delta^{13}C_p$ is the isotopic ratio in plant biomass, $\delta^{13}C_{atm}$ the isotopic ratio of atmospheric CO_2, a is the fractionation during diffusion of CO_2 in the air through stomatal pore and b is the net fractionation of RUBISCO. Such quantification of host-derived carbon in hemiparasites was first conducted for the hemiparasitic relationships of *Striga hermonthica* and *S. asiatica* attached to *Sorghum bicolor*. Elevated $\delta^{13}C$ values detected in the hemiparasites suggested that ca 28% and 35% of carbon in mature leaves of *S. hermonthica* and *S. asiatica* biomass is host-derived (Press et al. 1987). A similar, approach was then used to estimate carbon budget of numerous hemiparasitic species demonstrating that ca 20%-80% of hemiparasite biomass is derived from the host assimilates differing across species and developmental stages (Graves et al. 1990, Tennakoon & Pate 1996, Těšitel et al. 2010). Early developmental stages of obligatory hemiparasites (*Striga* spp.) were demonstrated to be highly dependent on host-derived carbon as expected from their belowground growth-habit (Fig. 1; Press et al. 1987, Graves et al. 1990). Heterotrophic carbon acquisition appears to be particularly high in

Fig 1. Mean proportion of heterotrophic carbon in bimass od variious species and life stages of root-hemiparasitic plants. In some cases, only isotopic composition was reported in the original studies. Proportion of heterotrophic carbon was then calculated using an isotope mixing model as in Těšitel et al. (2010). [1]Press et al. (1987), [2]Graves et al. (1990), [3]Tennakoon & Pate (1996), [4]Ducharme & Ehleringer (1996), [5]Těšitel et al. (2010)

hemiparasites that display direct luminal continuity with the host vessels in haustoria such as *Striga* spp. or *Rhinanthus minor* (Dörr 1997, Cameron et al. 2006). The results are however very variable across and within different studies which might be affected by different nature of individual host-hemiparasite genotype interplay.

Estimation of the carbon budget of hemiparasites parasitizing C_3 plants is also possible. Sufficient difference in water use efficiency (WUE) must however exist between the C_3 host and the hemiparasite in order to provide contrast between $\delta^{13}C$ values of their assimilates. This approach was used in a study dealing with root-hemiparasitic *Castilleja linariifolia* (Fig. 1; Ducharme & Ehleringer 1996) and is the most common method for estimating the carbon budget in stem hemiparasites which rarely parasitize C_4 hosts. Numerous studies conducted in arid or semiarid conditions have thus demonstrated 50-80% proportion of host derived carbon in biomass of mistletoes (Fig. 2; Marshall & Ehleringer 1990, Schulze et al. 1991, Richter et al. 1995, Wang et al. 2008). This extent of heterotrophy was also confirmed by an analysis of species parasitizing CAM hosts that have a similar carbon isotope composition to C_4 plants (Schulze et al. 1991, Richter et al. 1995). Another study dealing with New Zealand mistletoes however showed limitation of such approach for mistletoes occurring in temperate climate regions. The difference in WUE between hosts and hemiparasites was too small in this case resulting in unrealistic (sometimes more than 100% of host-derived carbon) and highly variable outputs of carbon isotope mixing models (Bannister & Strong 2001).

Mean proportion of heterotrophic carbon in biomass of the stemhemiparasite (%)
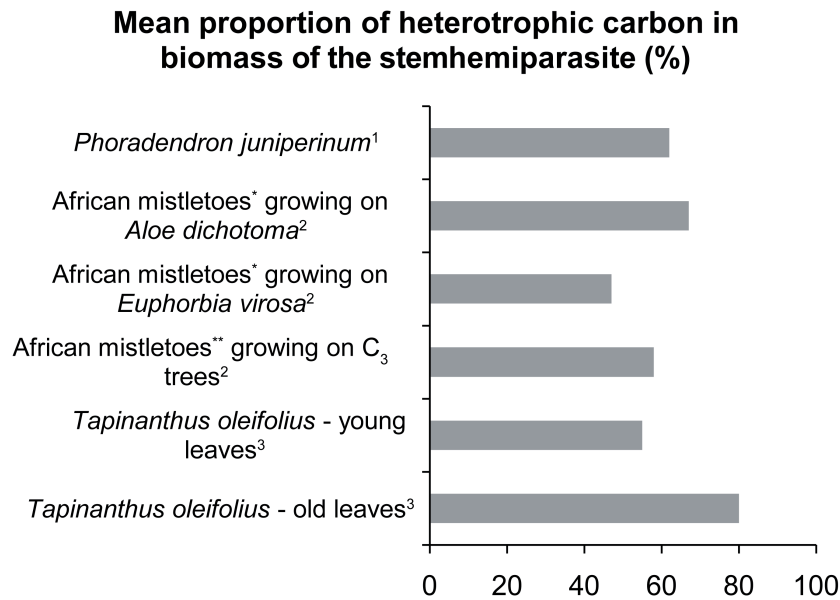

Fig 2. Mean proportion of heterotrophic carbon in different species of hemiparasitic mistletoes. In some cases, only isotopic composition was reported in the original studies. Proportion of heterotrophic carbon was then calculated using an isotope mixing model as in Těšitel et al. (2010). African mistletoes[*] = *Septulina glauca*, *Tapinanthus oleifolius*, African mistletoes[**] = *Odonthella welwitchii*, *Plicosepalus undulatus*, *Septulina glauca*, *Tapinanthus oleifolius*, *Viscum rotundifolium*; no separate data for individual species were given in the original study (Schulze et al. 1991). [1]Marshall & Ehleringer (1990), [2]Schulze et al. (1991), [3]Schulze et al. (1991).

Competitive interactions in the host-hemiparasite systems in relation to carbon budget

Despite the fact that root hemiparasites are capable of abstracting substantial amounts of organic carbon from their host, their performance is limited by competition for light with the host or surrounding plants (Matthies 1995, Keith et al. 2004, Cameron et al. 2005). In the field, this translates into the fact that root hemiparasites grow predominantly in low productive communities (Hejcman et al. 2007) in which the intensity of competition is rather restricted. A detailed mechanism of the effect of competition on hemiparasites is however not known. It is not likely that a moderate level of light deficiency would have detrimental effects on obligate hemiparasites or adult root hemiparasites given the high efficiency of heterotrophic carbon gain (Press et al. 1987, Graves et al. 1990, Těšitel et al. 2010). In contrast, seedlings of facultative hemiparasites germinate without a host induction, start growing unattached and hence, they must be extremely sensitive to decrease of irradiance at this stage of development especially considering their low rates of

photosynthesis (Seel et al. 1993, Lechowski 1996). Experiments evaluating effect of shading on different life stages of hemiparasites are missing from the literature although e.g. *Striga* spp. are known to be quite independent on its own production of photoassimilates which is indicated by the existence of mature albino plants and growth of *Striga* in complete darkness (Dörr 1997).

The benefits of the hemiparasitic lifestyle connected with efficient, yet low-cost nutrient acquisition, are highest in an environment where plant performance is limited by available nutrients and the primary production is relatively low (Phoenix & Press 2005). Under such conditions, root hemiparasites can effectively decrease growth rate of their host and hence decrease its competitive ability. In particular, annual hemiparasites tend to have highly negative effect on their host performance (Matthies 1995, Cameron et al. 2008, Shen et al. 2010). The decrease of the host biomass production is usually not compensated by the biomass production of the hemiparasite resulting in a general decrease of productivity of the whole community (Matthies 1995, Phoenix & Press 2005). The hemiparasitic systems hence do not display source-sink relationship as has been shown for holoparasitic relationships (Jeschke et al. 1997, Hibberd et al. 1998, 1999). In addition, hemiparasites accumulate nutrients and transpire huge amounts of water preventing their effective use for biomass production by surrounding autotrophic plants. Such 'luxury use' of resources (water and mineral nutrients) further decreases site productivity. Populations of root hemiparasites are therefore not passively dependent on the occurrence of low productive stands in nature but can actively reduce productivity of communities in which they grow (Cameron et al. 2005, Phoenix & Press 2005, Press & Phoenix 2005). However, nutrient-rich litter of hemiparasitic plants decomposes quickly, which may in some cases act in an opposite way enhancing nutrient cycling in communities (Quested et al. 2003).

Little is known on the effect of light competition on stem parasites due to methodical difficulties of studying ecological interactions of epiphytes. It is however likely that negative effects of light competition are less pronounced given the position that mistletoes occupy in the host canopy and rather high heterotrophic carbon acquisition (Fig. 2). Despite this, a case of a healthy host tree shading out a mistletoe has been reported and some mistletoes tend to perform better if their host grows under environmental stress such as water deficiency (Glatzel & Geils 2009). Dwarf-mistletoes (genus *Arceuthobium*) that are considered to be highly dependent on host-derived organic carbon (based on gasometric measurements (Logan et al. 2002, Bickford et al. 2005); no detailed carbon budget studies are available) tend to perform better on vigorous host that are in a good physiological state (Bickford et al. 2005, Shaw et al. 2005). Nonetheless, even these highly heterotrophic species were demonstrated to produce more aerial shoots under high irradiance which is however not likely to be underpinned by a causal dependence of parasite performance on the light level. It is more probable that the dwarf-mistletoe is indirectly positively affected by increased photosynthetic capacity of highly irradiated branch or by elevated temperature (Shaw & Weiss 2000). Drawing a general conclusion on the effect of light competition on stem hemiparasites is a difficult task due to their highly variable physiology (compare e. g. rather photosynthetic mistletoe such as *Loranthus europaeus* vs. almost non-photosynthetic *Arceuthobium* spp.) and a general deficiency of studies dealing with this topic.

Conclusions and Perspectives

Carbon budget is the key parameter of biology of individual hemiparasitic species. Hitherto published studies demonstrated that many hemiparasitic species are capable of efficient organic carbon abstraction from their hosts. It is however necessary to validate how the outcomes of the growth-chamber experiments translate into the natural conditions. More field studies are therefore needed focusing especially on the role of the heterotrophic carbon acquisition in relationships between root hemiparasites and their hosts, on the effect of infection by hemiparasitic mistletoes on the host species and on the influence of shading on mistletoes. Patterns of host-derived carbon assimilation within hemiparasitic plants and partitioning of the effects of flows of individual resources (water, mineral nutrients and organic carbon) on performance of both members of the

host-hemiparasite association present other appealing topics investigation of which would substantially push forward present knowledge of biology of hemiparasitic plants.

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4 The role of heterotrophic carbon acquisition by the hemiparasitic plant *Rhinanthus alectorolophus* in seedling establishment in natural communities: a physiological perspective.

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Introduction

Most plants are photoautotrophic organisms fixing carbon through photosynthesis. Plants are however dependent also on water and mineral nutrients which are acquired from the environment (either directly through plant roots or rhizoids or through symbiotic relations with other constituents of ecosystems, principally mycorrhizal fungi). Despite the prevailing autotrophic strategy, several plant lineages have evolved heterotrophic means of resource acquisition parasitising fungi (mycoheterotrophy; Leake 1994, Selosse & Cameron 2010) or other plants (plant-parasitism; e.g. Irving & Cameron 2009) for either all or a subset of resources required to support their vital processes. Among these strategies, hemiparasitism is a plant-specific mechanism of resource acquisition based on both autotrophy and heterotrophy. Hemiparasitic plants are green performing photosynthesis but at the same time attack vascular system of other species exploiting the host xylem sap which contains both mineral nutrients and organic carbon (Irving & Cameron 2009). Hemiparasitic species occur in the majority of terrestrial ecosystems however in temperate Eurasia, grassland communities represent the principal habitats containing the highest diversity of the hemiparasitic flora. Moreover, parasitic plants are more than botanical curiosities, their ability to suppress host species growth and affect nutrient flows in the ecosystems (Quested et al. 2003, Cameron et al. 2005, Phoenix & Press 2005) results in the hemiparasites often being considered keystone species or ecosystem engineers regulating the structure and function of host communities (Cameron et al. 2005, Press & Phoenix 2005).

The genus *Rhinanthus* (Orobanchaceae) is a common and widely distributed example of a northern-temperate root-hemiparasitic group. All of its 25-35 species are annual hemiparasites occurring in grasslands of low and medium productivity in Europe and North America (Meusel et al. 1978). As root-hemiparasites, *Rhinanthus* spp. attach to roots of their hosts by a specialized organ called haustorium providing direct luminal continuity between host and parasite xylem vessels via open conduits called oscula (Cameron et al. 2006). Host-to-parasite solute transfer therefore occurs through mass-flow, driven by a water potential gradient between the host and the parasite induced by high transpiration rate of the parasite and accumulation of osmotically-active compounds, particularly mannitol, in parasite tissues (Cameron et al. 2006). While direct luminal continuity as a mechanism for solute extraction from a host is not common to all parasitic plants, it is shared by many hemiparasitic species within the family Orobanchaceae, including the noxious hemiparasitic tropic weed *Striga* spp. (Dörr 1997). The connection to the host's xylem and the absence of a phloem connection implies that water and mineral nutrients are the most important resources obtained from the host. Their flows have indeed been quantified in numerous

hemiparasitic systems accounting for a substantial proportion of total host resources (Jiang et al. 2003, 2004, 2010) although the potential for acquisition of organic carbon is often ignored (but see Těšitel et al. 2010a). Despite low organic carbon concentration in xylem, the extensive amount of solutes acquired from the host can result also in significant heterotrophic carbon gain (Těšitel et al. 2010a) observed not only in *Rhinanthus* but also in numerous representatives of other root-hemiparasitic species including *Euphrasia* (Těšitel et al. 2010a), *Striga* (Press et al. 1987) and *Olax* (Tennakoon & Pate 1996) species. The host-derived carbon can account for 20-80% of hemiparasite dry-mass (see Těšitel et al. 2010b for a review).

The hemiparasitic lifestyle provides notable advantages to *Rhinanthus* spp. supplying relatively rich and stable resource of inorganic and organic nutrients without need of carbon investment in extensive root network or mycorrhizal associations. The efficient exploitation of host resources is probably closely related to the annual life history (Press et al. 1988, *Ehleringer* & Marshall 1995), which is shared with most of the temperate hemiparasitic Rhinanthoid Orobanchaceae and evolved independently in individual genera from perennial ancestors (Těšitel et al. 2010c). The annual life history combined with the absence of a long-living seed bank (ter Borg 1985, van Hulst et al. 1987, Kelly 1989) however requires successful seed production and seedling establishment in every season imposing an apparent constraint on persistence of *Rhinanthus* spp. populations growing in dense-sward communities dominated by perennial species. *Rhinanthus* spp. and some other hemiparasites (e.g. *Melampyrum* species; Těšitel et al. 2010c) compensate for this constraint by production of large, resource-rich seeds enabling fast growth of seedlings at the start of the growing season and hence allowing them to inhabit sites of medium productivity where no other annuals persist (Kelly 1989, Strykstra et al. 2002). Highly productive, nutrient-rich sites are however, generally unfavourable for persistence of hemiparasite populations including *Rhinanthus* spp. (van Hulst et al. 1987, Cameron et al. 2009, Fibich et al. 2010, Hejcman et al. 2011) underpinned by high intensity of competition for light at such sites (Hautier et al. 2009). In addition, the sensitivity of hemiparasites to light competition was confirmed experimentally (Matthies 1995, Keith et al. 2004, Hejcman et al. 2011) and is further supported by theoretical mathematical models suggesting an unstable population dynamics with high extinction risk (Cameron et al. 2009) or competitive exclusion (Fibich et al. 2010) at highly productive sites.

Limitation of *Rhinanthus* spp. to low and intermediate productivity grasslands by interspecific competition might appear in conflict with the reported ability of *Rhinanthus* to withdraw substantial (up to *c*. 50% of dry biomass for *Rhinanthus minor*; Těšitel et al. 2010a) amounts of organic carbon from its host, especially given that Hwangbo & Seel (2002) reported no effect of shading on biomass production of *R. minor*. However, this latter study is based on analyses of adult plants that were shaded immediately prior to anthesis, limiting their predictive power over the competitive exclusion hypothesis detailed above.

As a facultative hemiparasite, the seeds of *Rhinanthus* spp. germinate without host induction, produce green leaves and then attach to their host's roots several days after emergence. They are thus entirely reliant upon their seed reserves and own photosynthetic activity for carbon and nutrients during the short period before the attachment (Irving & Cameron 2009). This is in direct contrast to the obligate hemiparasites which usually require host-borne signalling compounds, such as strigolactones, to induce germination and that attach to their host prior the formation and emergence of green shoots (Irving & Cameron 2009). In *Rhinanthus*, the seedling stage can be therefore hypothesized as the bottle-neck stage of the populations when most mortality occurs due to competition for light and nutrients. Competitive exclusion of established *Rhinanthus* spp. is, on the other hand, hard to imagine since the mature plants can grow relatively tall and their growth can be further supported in moderately productive ecosystems (Fibich et al. 2010, Mudrák & Lepš 2010)

In the present study, we investigate the effect of light competition on performance of *Rhinanthus alectorolophus* in the context of its heterotrophic carbon acquisition. Experimental *R. alectorolophus* plants were cultivated in pots with wheat (*Triticum aestivum*; C_3 photosynthesis) and maize (*Zea mays*; C_4 photosynthesis) as host species. This allowed estimation of the amount of host-

derived carbon in hemiparasite biomass using natural abundance of carbon stable isotopes (method introduced by Press et al. 1987 for *Striga* spp. and adapted by Těšitel et al. 2010a). The shading treatments were imposed on plants at different stages of development simulating competitive pressure from co-occurring species. The main aim of this experiment hence was to test the hypothesis that the highest susceptibility of *Rhinanthus* to competition for light is at the seedling stage and to uncover a possible role of heterotrophic carbon acquisition regulating the population dynamics of *Rhinanthus* spp.

Materials and Methods
Experimental species
Rhinanthus alectorolophus (Scop.) Pollich is an annual hemiparasitic species occurring mostly in calcareous grasslands (Karlík & Poschlod 2009), however, *R. alectorolophus* was a weed in agro-ecosystems prior to the industrialisation of agriculture, infesting cereal crops most likely including wheat and maize, the species used as hosts in the present study (Skála & Štech 2000). Together with two closely related species in the genus, *R. minor* and *R. angustifolius*, *R. alectorolophus* is one of frequently used model species in ecological and ecophysiological studies of root-hemiparasites (e.g. Joshi et al. 2000, Matthies 2003, Hautier et al. 2010). Seeds of *R. alectorolophus* used in this study were collected from fruiting plants of a natural population close to Zechovice near Volyně, Czech Republic (N 49°09'28", E 13°52'13", 510 m a.s.l.).

Resolving the photosynthetic performance of R. alectorolophus *in the field*
A light-response curve of net photosynthesis over a range of actinic light intensities (0 – 1500 μmol photons m^{-2} s^{-1}) was resolved for *R. alectorolophus* plants ($n = 5$) at the site from which the seeds originated in order to capture characteristics of species photosynthesis under natural conditions. Each measured leaf was first allowed to accommodate to the maximal light intensity (1500 μmol photons m^{-2} s^{-1}) until steady-state assimilation rate developed, which was consequently recorded followed by decrease of the irradiation level and steady-state assimilation rate recording in a series of 1500, 1000, 800, 500, 300, 150, 100, 50 and 0 μmol photons m^{-2} s^{-1}. Gas-exchange measurements were conducted on leaves of *R. alectorolophus* plants using an infra red gas analyser coupled to a Li-6400 portable photosynthesis system (Li-Cor Biosciences, Lincoln, USA). Individual curves were fitted by a non-linear regression models using an equation in Lambers et al. (2008):

$$A_n = \frac{\phi I + A_{max} - \{(\phi I + A_{max})^2 - 4\theta\phi I A_{max}\}^{0.5}}{2\theta} - R_d \qquad \text{Eqn 1}$$

where A_n = rate of assimilation, A_{max} = light-saturated rate of CO_2 assimilation, ϕ = apparent quantum yield, θ = curvature factor, R_d = dark respiration during photosynthesis. A mean light-response curve (Fig. 1) was obtained by averaging the five individual light-response curves, which also allowed calculating its confidence limits.

Assessment of host-derived carbon in hemiparasite biomass
Experimental *R. alectorolophus* plants were cultivated in pots containing wheat or maize host plants in order to assess the proportion of host-derived carbon in hemiparasite biomass. *R. alectorolophus* and wheat display C_3 photosynthetic pathway while maize performs C_4 photosynthesis. As a result of their photochemical processes, C_4 plants are usually significantly more enriched in ^{13}C (expected $\delta^{13}C$ value ranges between -12 and -18‰; Smith & Epstein 1971) than C_3 plants (expected $\delta^{13}C$ value ranges between -23 and -35‰, Smith & Epstein 1971; expected $\delta^{13}C$ of *Rhinanthus* assimilates is close to the lower limit of the C_3 range due to its high stomatal conductance and low water use efficiency; Press et al. 1988, Lambers et al. 2008). It is hence possible to infer the proportion of host-derived carbon in biomass of a hemiparasitic plant by measuring the relative change in the $\delta^{13}C$ value of the C_3 parasite attached to a C_4 host compared to when it is attached to a C_3 host using a linear two-source isotope mixing model (an adjusted form of models of Marshall & *Ehleringer* 1990 and Gebauer & Meyer 2003, adopted by Těšitel et al. 2010a), relating the excess of ^{13}C in hemiparasites attached to the C_4 host compared to those attached to the C_3 host to the

difference in isotope composition between the C_3 and C_4 hosts themselves (Eq. 2).

$$\%H = \left(\frac{\delta_{P(C_4)} - \delta_{P(C_3)}}{\delta_{H(C_4)} - \delta_{H(C_3)}} \right) \cdot 100 \, [\%] \qquad\qquad \text{Eqn 2}$$

Where $\%H$ = the percentage of carbon in parasite biomass that is derived from the host, $\delta_{P(C3)} = \delta^{13}C$ of the parasite growing on the C_3 wheat host, $\delta_{P(C4)} = \delta^{13}C$ of the parasite growing on the C_4 maize host, $\delta_{H(C3)} = \delta^{13}C$ of the infected wheat host and $\delta_{H(C4)} = \delta^{13}C$ of the infected maize host. $\delta^{13}C$
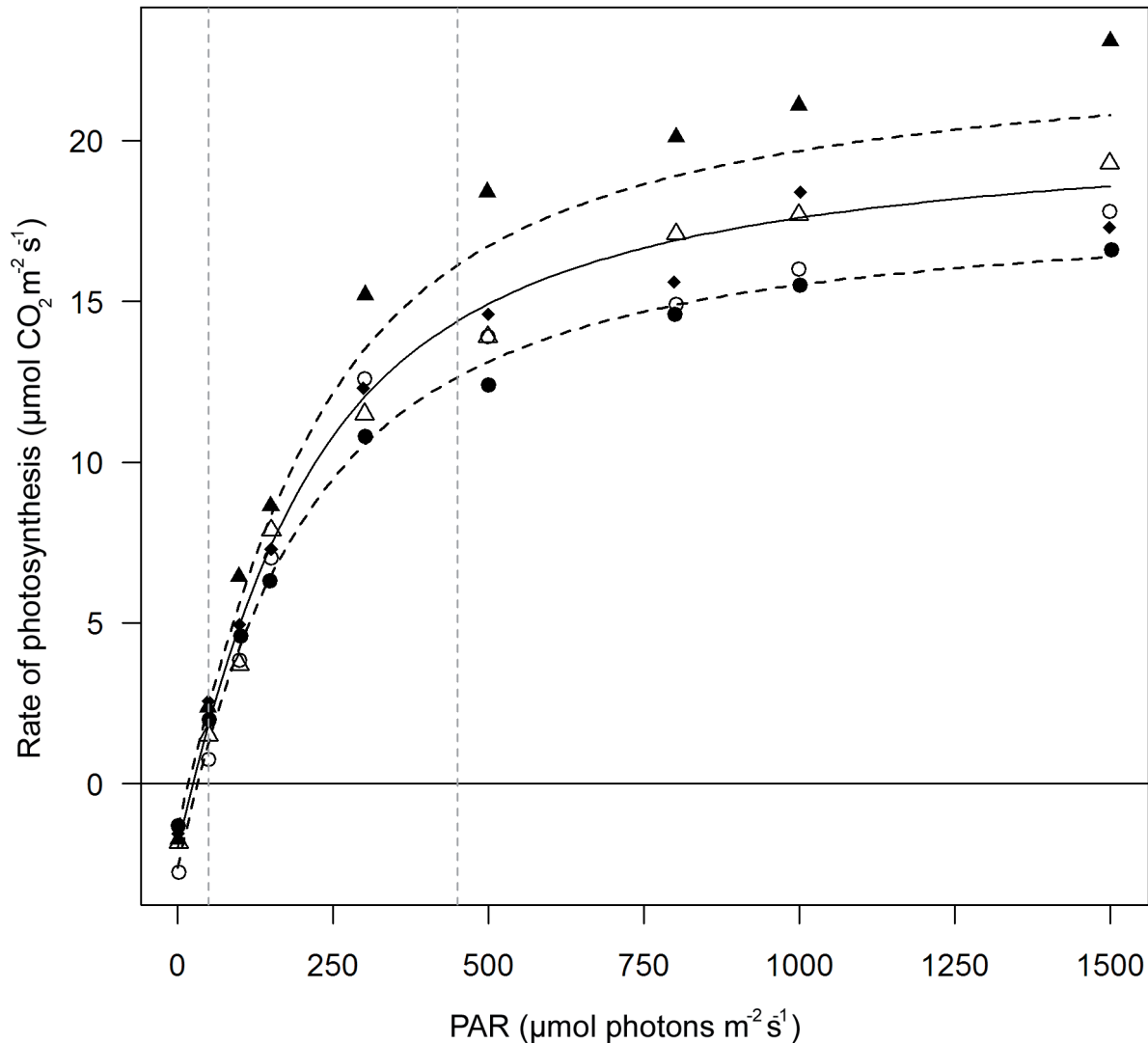
Fig. 1 Light-response curve displaying dependence of the rate of photosynthesis on the intensity of photosynthetically active radiation (PAR) in leaves of five *Rhinanthus alectorolophus* plants growing under natural conditions. The summary light-response curve (full line) and the confidence intervals (dashed lines) were calculated as means and 2 × standard errors of values predicted by light-response model of individual plants. Symbols represent raw data and are classified by plant sample identity. Mean (± standard error) parameters of the light-response curve models: A_{max} = 22.69 (±1.26) µmol CO_2 m^{-2} s^{-1}, R_d = 2.00 (±0.31) µmol CO_2 m^{-2} s^{-1}, ϕ = 0.085 (±0.004), θ = 0.467 (±0.094), compensation point: 24.81 (±3.7) µmol photons m^{-2} s^{-1}. Grey dashed lines represent PAR intensities resulting from the shading treatments: 50 µmol photons m^{-2} s^{-1} under shaded conditions, 450 µmol photons m^{-2} s^{-1} under full irradiation.

samples were taken of leaves of hosts and a leaf and stem of the upper and lower part of each parasite since we expected variation in isotopic values between these separate parts of individual hemiparasitic plants. Subsequent calculations of the proportion of host-derived carbon in the separate parts of the hemiparasite plants was based on our experimentally derived values for $\delta_{P(C4)}$ of the separate parts of the *R. alectorolophus* plant and the $\delta_{H(C4)}$ of leaf of to which the parasite was attached. The average values of $\delta_{H(C3)}$ of corresponding treatment and $\delta_{P(C3)}$ of corresponding separate part of *R. alectorolophus* plants cultivated under corresponding treatment were entered into the model as the baseline C_3 reference values. Total amount of host-derived organic carbon in above-ground hemiparasite biomass was estimated as a product of biomass dry weight and mean proportion of host-derived carbon concentration in the samples of the four separate plant parts.

This approach assumes that ^{13}C flows from the host only in a form of xylem-mobile organic compounds and no significant refixation of root respired CO_2 by the hemiparasite occurs, effect of which was experimentally excluded by Těšitel et al. (2010a). In addition, the model assumes that no carbon isotope fractionation occurs during the transfer of solutes through haustoria and that potential difference in $\delta^{13}C$ between host bulk-leaf and xylem mobile organic compounds is either negligible or identical in both species.

Cultivation and experimental design

Seeds of the hosts were germinated on Petri dishes with moist filter paper. The seedlings were moved to 10 cm x 10 cm square pots containing substrate of peat and washed quartz sand (1:1,v/v ratio) after successful germination. Each pot contained one host plant. The plants were cultivated in a growth cabinet of Faculty of Science University of South Bohemia under light intensity of 450 (400-500) µmol m^{-2} s^{-1} (photosynthetic active radiation - PAR) with a cycle of 14h day and 10h dark at 18°C. *R. alectorolophus* seedlings (germinated on moist filter paper at 4°C) were sown at a density of three seedlings per pot *c*. 3 to 4 cm from the host plant after 7 days of host development. After having produced green cotyledon, the parasite seedlings were thinned to one per pot by removing the most and least advanced seedlings to obtain a homogeneous cohort of seedlings.

Pots were divided into four experimental groups arranged in a completely randomised design. Shading was imposed on *Rhinanthus* plantlets 8 days after sowing in the first group (referred to as shaded seedlings, *n* = 18). In the second group (*n* = 18), the same shading was imposed to young plants on day 24 after sowing (referred to as shaded young plants). The third group (*n* = 17) consisted of plants that were kept under completely dark conditions imposed at the same time as the young plant shading. The fourth group (*n* = 19) was the control growing under full light. The host plants received full light in all treatments. Shading was adjusted and checked every day to prevent direct light from coming on any part of the shaded plants. Moreover, the position of each of the pots was exchanged randomly every *c*. 7 days to diminish possible effects of irradiation heterogeneity (although this was rather minimal as indicated by the ranges of irradiation values).

The shading imposed on the experimental plants decreased the PAR intensity from 450 (400-500) µmol photons m^{-2} s^{-1} to 50 (40-60) µmol photons m^{-2} s^{-1} corresponding to rates of photosynthesis 14.25 µmol CO_2 m^{-2} s^{-1} (62.8% of A_{max} in plants growing under natural conditions) and 1.82 µmol CO_2 m^{-2} s^{-1} (8.0% of A_{max}, Fig. 1) respectively. This PAR intensity ratio of *c*. 0.1 corresponds well to values typical of light conditions above the grassland canopy and in the understory (Hautier et al. 2009). The shading was imposed by a square shield made of thick green paper (8 x 8 cm, 218 g m^{-2}) blocking the direct light. The shaded plants therefore received just indirect irradiation similar to a plant growing under natural conditions shaded by nearby competitive species. The shading shields were fixed 20 cm above the pots by a wooden support inserted into the pot substrate. This simple shield design allowed selective shading of *Rhinanthus* plants only and did not block air circulation preventing modification of air water potential or vapour pressure by shading. Several *Rhinanthus* plants grew sufficiently tall to almost interfere with the shading shield at the end of the experiment. The shield was moved upwards by several centimetres in such cases; however an additional shield was placed to the pot to keep the irradiation level within the desired range of 40-60 µmol photons m^{-2} s^{-1} (confirmed by PAR measurements). The complete

darkness treatment was imposed by covering the *Rhinanthus* plants by a 1 cm thick isolation polypropylene tube (inner diameter of 5 cm, length 20 cm) which was covered by a small tray and perforated by holes (*c*. 1 cm in diameter, 1 hole on *c*. 25 cm^2 of the tube) pointing downwards to allow as much ventilation as possible while preventing light from coming in.

This experimental design was designed to provide sufficient contrast between the light treatments rather than analyse performance or physiology of *R. alectorolophus* under exact levels of irradiation used. The shaded plants also performed better than might be expected from the light-response curves of the *R. alectorolophus* plants (Fig 1.) due to higher the effectiveness of diffuse light photosynthetic utilization (e.g. Sinclair & Shiraiwa 1992, Gu et al. 2002); nonetheless the shading apparently created a contrast of light-deficiency stressed *vs*. non-stressed plants.

Biomass harvesting and stable isotope analysis

Plants were harvested 46 days after sowing of *R. alectorolophus*. Height of each of the *R. alectorolophus* plants was measured prior to the harvest. Above ground biomass of both host plants and parasites was sampled in addition to the sampling for stable isotope analyses. All samples were dried at 80°C for 48 hours and weighed immediately. Dry weight of samples used for stable isotope analyses was included in the weight the total above-ground dry biomass produced.

The samples for carbon stable isotope analysis were homogenized separately and a 0.4 mg subset of each constituent part was analysed for ^{13}C content by a Vario MicroCube elemental analyzer (Elementar Analysensysteme, Hanau, Germany) connected to an isotope ratio mass spectrometer (IRMS Delta XL Plus, Finnigan, Germany) at the Faculty of Science, University of South Bohemia. Data were collected as atom % ^{13}C and re-expressed as delta values relative to the Pee Dee Belemnite standard (d) using Equation 3:

$$\delta^{13}C = (R_{Sample} / R_{Standard} - 1) \cdot 1000 [‰] \qquad \text{Eqn 3}$$

where R_{Sample} = ^{13}C:^{12}C ratio in the sample and $R_{Standard}$ = ^{13}C:^{12}C ratio in the Pee Dee Belemnite standard. In addition to the δ^{13}C data, the atomic N/C ratio was determined in the samples during the mass spectrometry analysis in order to resolve the nitrogen status of the experimental plants. The delta values were related to the internal working standard (cellulose, δ^{13}C = -24.389‰) which is related to the international standard saccharose (δ^{13}C = -10.40‰) obtained from the International Atomic Agency, Vienna. Quantity calibration for N/C ratio measurements was conducted using an atropine standard.

Data analysis

Factorial analyses of variance (ANOVA) were used to analyse the effects of treatments and host species identity on height and dry weight of biomass of *R. alectorolophus* and dry weight of biomass of the hosts and the estimated amount of carbon present in the hemiparasite biomass. The response variables were log-transformed to improve normality and homoscedasticity of residuals. Tukey honest significant difference post-hoc tests were calculated to test differences among individual levels of statistically significant multilevel terms.

Linear mixed-effect models were used to analyse the carbon isotope composition and N/C ratio of *R. alectorolophus* plants and the proportion of host-derived carbon in individual parts of its biomass. The analysis of "split-plot" design was used: the host species, shading and their interaction represented the "main plot" fixed factor tested against the variability among individual plants (plant identity was a random factor nested in the interaction of host species × shading). The plant parts represented the split plot factor tested against the residual variability, similarly to its interactions. *A priori* defined contrasts were employed for further detailed analyses of model results and their interpretation. The contrasts for the light treatments were defined to compare both shading treatment with the control (treatment contrasts; Crawley 2007). Custom contrasts were defined for separate *R. alectorolophus* plant parts from which isotope samples were taken, testing i) upper *vs*. lower part of the plant ii) leaf *vs*. stem in the upper part of the plant and iii) leaf *vs*. stem in the lower part of the plant. All statistical analyses were conducted in R version 2.12 (R Development

Core Team 2010). Linear mixed-effect models were calculated in package *nlme* version 3.1-97 (Pinheiro et al. 2010).

Results
Survival and growth of the experimental plants

All plant on which the total darkness treatment was imposed died within five days. In the other treatments, *Rhinanthus* plants survived until the harvest except for three shaded seedlings and one control plant. These plants were excluded from further analyses. Shading had a significant effect on biomass production in *R. alectorolophus* (Table 1, Fig. 2a). Plants of both shading treatments produced significantly less biomass compared to unshaded control plants and shaded seedlings produced significantly less biomass than shaded young plants (Fig. 2a). A different effect of shading treatments on hemiparasite height was observed (Table 1, Fig. 2b) as shaded seedlings grew significantly smaller compared to control plants while shading imposed later had no statistically significant effect on plant height (Fig. 2b). Host species identity had no significant effect on either biomass production or height of the parasites (Table 1, Fig. 2a, b).

Host biomass production significantly differed between the host species (wheat produced less biomass than maize) and across the treatments (Table 1, Fig 2c). Hosts of shaded young plants of *R. alectorolophus* produced a similar amount of biomass as hosts parasitized by control plants while the amount of biomass produced by those parasitized by shaded seedlings was significantly higher (Table 1, Fig. 2c)

Table 1 Summary of factorial analyses of variance testing the effects of shading treatments and host species identity on above-ground biomass production and height of *R. alectorolophus* and biomass of the hosts. Data were transformed by natural logarithm prior to the analysis. Statistically significant test results ($P < 0.05$) are indicated in bold.

Effect	df	*Rhinathus* biomass dry weight			*Rhinanthus* height			host biomass dry weight		
		Sum Sq.	F	P	Sum Sq.	F	P	Sum Sq.	F	P
1. Host species	1,41	0.03	0.12	0.73	0.01	0.08	0.77	9.58	97.49	**<10⁻¹⁰**
2. Shading treatment	2,41	30.37	58.04	**<10⁻⁹**	2.08	10.9	**< 0.001**	3.59	18.24	**<10⁻⁵**
1. × 2.	2,41	0.32	0.62	0.55	0.05	0.24	0.79	0.078	0.4	0.68
Residuals	41	10.73			3.91			4.03		

Carbon stable isotope composition and N/C ratio in biomass of the experimental plants

Carbon stable isotope composition of host species corresponded to values expected for C_3 and C_4 plants (Fig 3a). $\delta^{13}C$ values detected in the individual parts of *R. alectorolophus* biomass displayed substantial variability which was significantly influenced by host species identity, shading treatment, plant part (from which the sample originated) and the interactions between these factors (Table 2). *R. alectorolophus* attached to wheat was in general slightly depleted compared to the host as expected due the low water use efficiency of the parasite (Fig. 3a). *R alectorolophus* plants attached to maize were significantly enriched in ^{13}C compared to those attached to wheat ($t_{41} = 2.45$, $P = 0.0001$). Leaves were significantly depleted in ^{13}C compared to stems ($t_{130} = -5.04$, $P < 0.0001$ in the upper part of the plants; $t_{130} = -4.76$, $P < 0.0001$ in the lower part of the plants) while the difference in $\delta^{13}C$ between the upper and the lower part was non-significant ($t_{130} = 1.64$, $P = 0.10$). The main effects of the shading treatment did not yield statistically significant contrasts ($t_{41} = 0.57$, $P = 0.57$ for shaded seedlings; $t_{41} = 0.84$, $P = 0.40$ for shaded young plants) but there was a significant interaction indicating ^{13}C enrichment in both shaded treatments of *R. alectorolophus* attached to maize ($t_{41} = 3.40$, $P = 0.0015$ for shaded seedlings; $t_{41} = 2.18$, $P = 0.035$ for shaded young plants). The upper parts of shaded young plants were significantly less enriched in ^{13}C than the lower parts ($t_{121} = -3.601$, $P = 0.0005$) and their upper leaf was significantly less depleted compared the upper part of stem ($t_{121} = 2.95$, $P = 0.0038$) if compared to the situation in control plants. In addition, lower leaves of the parasites attached to maize were significantly depleted in ^{13}C

compared to the lower part of the stem (t_{121} = -2.54, P = 0.012).

The N/C atomic ratio in biomass of *Rhinanthus* was clearly affected by the shading treatments and plant part from which the sample originated (Fig 3b, Table 2) although there was also a marginally significant effect of host species identity (parasites attached to maize had slightly lower N/C ratio, t_{43} = -2.08, P = 0.043). The N/C ratio in unshaded control plants was close that of wheat host plants (incl. parasites attached to maize) while shading caused significant increase of its value in both shaded seedlings (t_{43} = 8.32, P < 0.0001) and young plants (t_{43} = 4.39, P = 0.0001). The upper parts of *Rhinanthus* displayed generally higher N/C ratio (t_{124} = 2.90, P = 0.0044), which also applied to leaves compared to stems in both upper (t_{124} = 2.02, P = 0.045) and lower (t_{124} = 4.93, P < 0.0001) parts of the plants. In addition, there was a pronounced effect of interaction between the shading treatments and plant parts. The upper parts of both shaded seedlings and shaded young plants had higher than additive N/C proportion in their upper part compared to unshaded control (t_{124} = 2.28, P = 0.024, t_{124} = 3.78, P = 0.0002, respectively). In addition, shaded seedlings had higher than additive N/C proportion in their upper leaves compared to stems (t_{124} = 2.85, P = 0.0052) and a similar pattern was present in the lower part of the shaded young plants (t_{124} = 2.04, P = 0.043).

Proportion and total amount estimation of host-derived carbon in hemiparasite biomass

Proportion of host-derived carbon in *Rhinanthus* biomass was significantly affected by the shading treatments and differed across sampled parts of the plants (Fig. 4a, Table 3). Shaded seedlings had significantly higher proportion of host-derived carbon in their biomass compared to control plants (t_{23} = 4.30, P = 0.0003). A similar, albeit smaller difference was detected between shaded young plants and control plants (t_{23} = 2.62, P = 0.015). In addition, host-derived carbon proportion was significantly lower in the upper parts of the
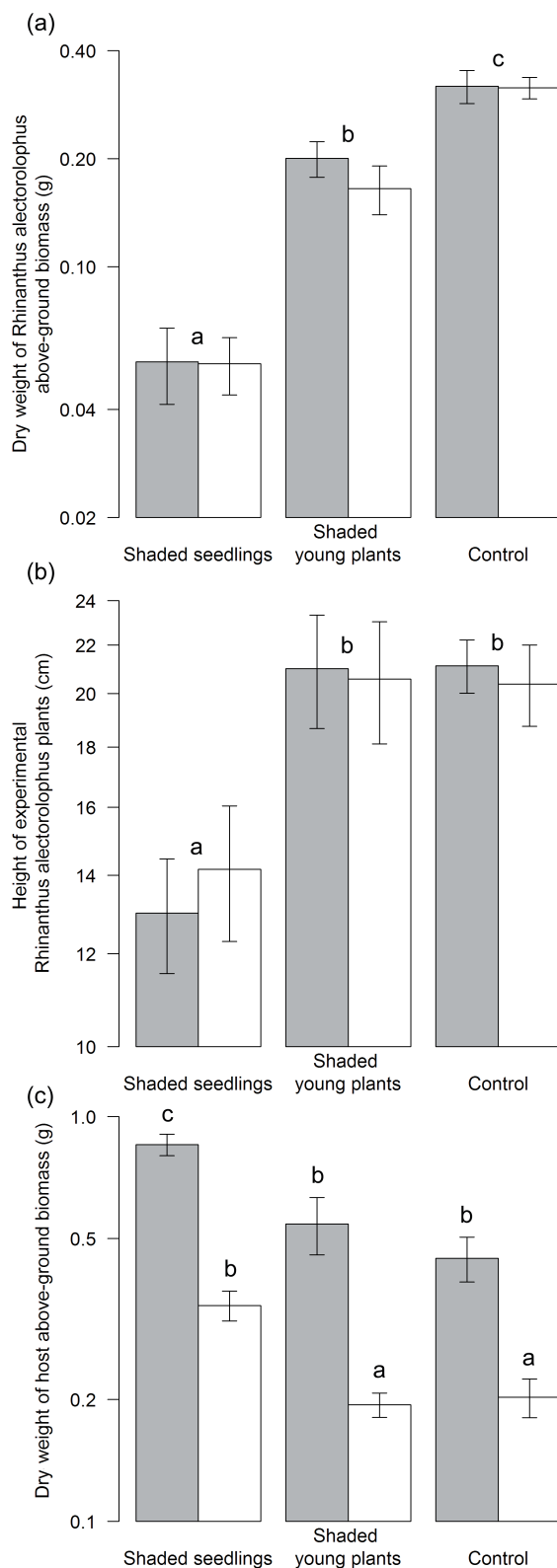
Fig. 2 Above-ground biomass production and height of *R. alectorolophus* and above-ground biomass production of its hosts under different shading treatments imposed on the hemiparasite. (a) Dry weight of above-ground biomass produced by *R. alectorolophus* (b) Height of *R. alectorolophus* (c) Dry weight of above-ground biomass produced by the hosts to which *R. alectorolophus* was attached. White bars indicate wheat or *R. alectorolophus* attached to wheat, grey bars represent maize or *R. alectorolophus* attached to maize. Note the logarithmic scale of the y-axes. Error bars represent ±1 standard error. Different letters symbolize statistically significant difference inferred from post-hoc pairwise comparisons (P < 0.05).

plants compared to the lower parts (t_{64} = -3.40, P = 0.0012). There were also significant interactions caused by higher than additive proportions of host-derived carbon in the upper parts of shaded seedlings (t_{64} = 3.31, P = 0.0015) and lower than additive proportions in the lower leaves compared to the corresponding parts of the stem (t_{64} = -4.64, P < 0.0001) in plants of the same treatment.

Not only had the shading substantial effect on the proportion of host-derived carbon in *Rhinanthus* biomass but also on the estimated total amount on carbon incorporated in the above-ground biomass (Fig. 4b, one-way ANOVA summary: log-transformed data, R^2 = 0.376, $F_{(2,23)}$ = 6.93, P = 0.0044). In contrast to the observed positive effect of shading on the proportion of host-derived carbon, its total estimated amount was significantly lower in shaded seedlings compared to the other two treatments (Fig. 4b). There was no statistically significant difference between shaded young plants and control plants.
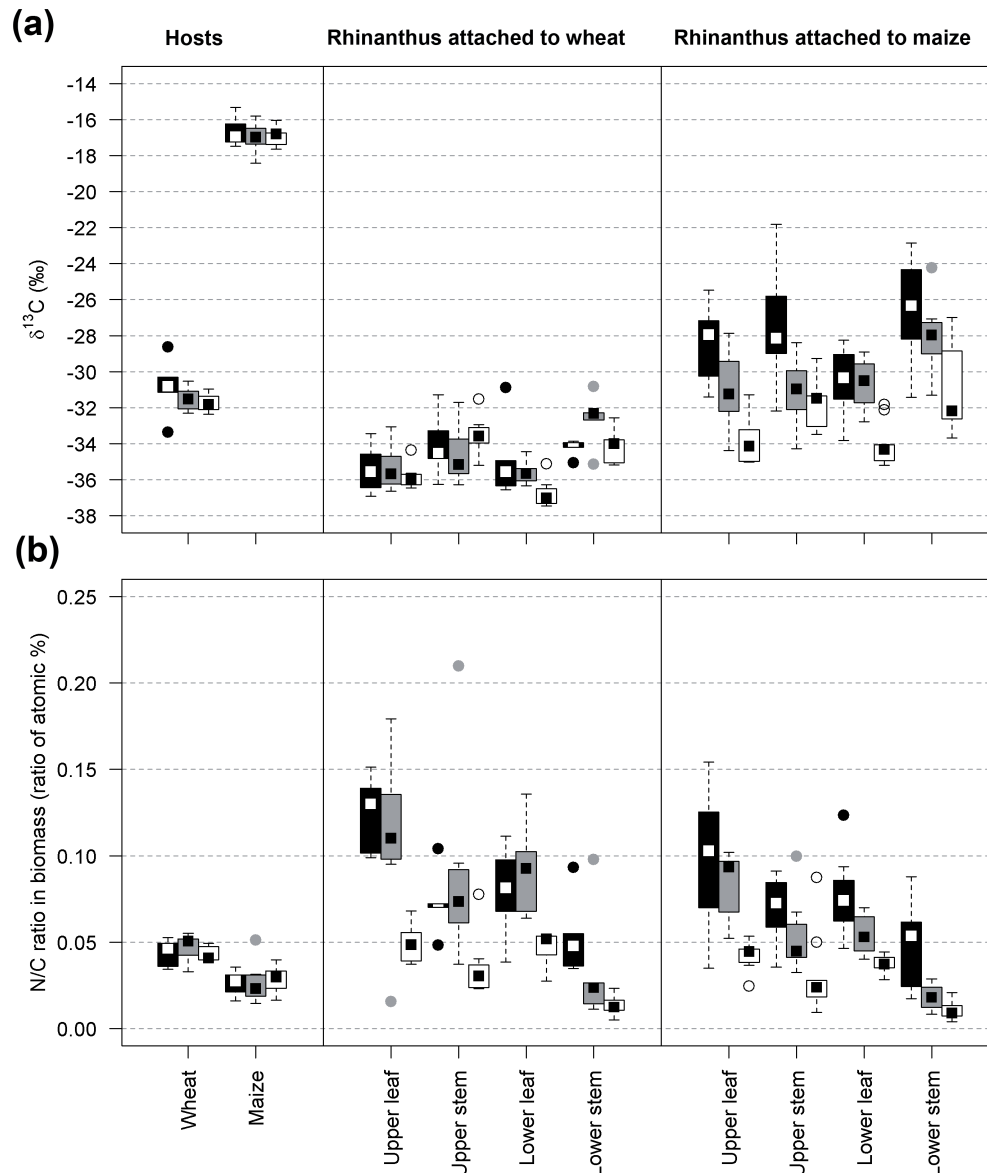
Fig. 3 (a) $\delta^{13}C$ values and (b) N/C atomic ratio in biomass of wheat (C_3 photosynthesis) and maize (C_4 photosynthesis) hosts and individual samples of *Rhinanthus alectorolophus* plants (C_3 photosynthesis) under individual shading treatments. Black, grey and white boxes indicate shaded seedlings, shaded young plants and control treatments respectively. The points in boxes represent medians, boxes represent quartiles and the lines extending from the box boundaries (whiskers) represent the range or non-outlier range of values, whichever is smaller. The non-outlier range is defined as the interval between 25% quantile – 1.5 times interquartile range and 75% quantile + 1.5 interquantile range. Any point outside this interval is considered outlier and depicted separately.

Table 2 Summary of the general linear mixed-effect models testing the effects of host species identity, shading treatment and part of the plant from which the sample was taken on the $\delta^{13}C$ value and N/C ratio in biomass of *R. alectorolophus*. Non-significant terms were omitted from the final model of N/C ratio distribution. Overall tests of the final models: $\delta^{13}C$: Likelihood ratio = 205.23, df = 17, $P < 0.0001$, N/C ratio: Likelihood ratio = 194.42, df = 12, $P < 0.0001$ (tested against the null models containing no fixed effect terms).

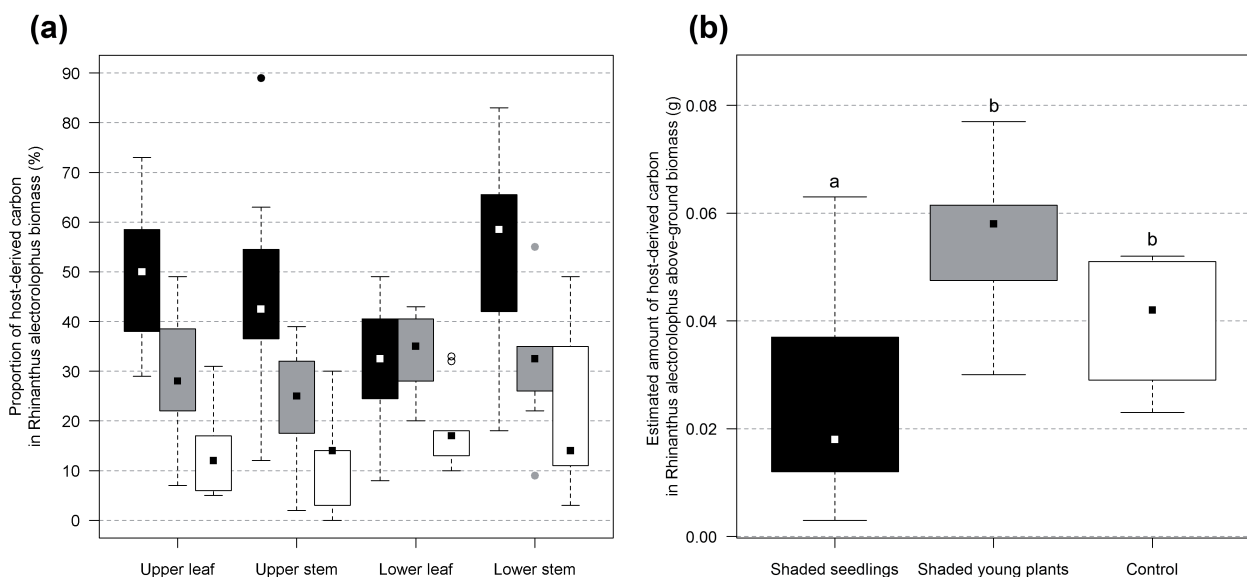|	$\delta^{13}C$ value			N/C ratio		
Effect	*df*	*F*	*P*	*df*	*F*	*P*
1. Host species	1,41	89.93	**< 0.0001**	1,41	4.07	**0.050**
2. Shading treatment	2,41	14.15	**< 0.0001**	2,41	40.89	**< 0.0001**
3. Plant part	3,121	62.74	**< 0.0001**	3,121	73.38	**< 0.0001**
1. × 2.	2,41	6.86	**0.0027**	2,41	1.33	0.28
1. × 3.	3,121	3.08	**0.0301**	3,121	1.23	0.30
2. × 3.	6, 121	4.16	**0.0008**	6, 121	4.75	**0.0002**

Fig. 4 (a) Proportion of host-derived carbon in different samples of biomass of *R. alectorolophus* attached to maize and cultivated under different shading treatments. The values were calculated from raw $\delta^{13}C$ data (Fig. 3a) using an isotope mixing model (Eqn. 2). (b) Total estimated amount of host-derived carbon in biomass of *R. alectorolophus* attached to maize and cultivated under different shading treatments. The values were calculated as a product of mean proportion of host-derived carbon in hemiparasite biomass across individual analyzed parts of a plant and the dry weight of its above-ground biomass. Black, grey and white boxes indicate shaded seedlings, shaded young plants and control treatments respectively. The points in boxes represent medians, boxes represent quartiles and the lines extending from the box boundaries (whiskers) represent the range or non-outlier range of values, whichever is smaller. The non-outlier range is defined as the interval between 25% quantile – 1.5 times interquartile range and 75% quantile + 1.5 interquantile range. Any point outside this interval is considered outlier and depicted separately. Different letters in (b) symbolize statistically significant difference inferred from post-hoc pairwise comparisons (*P* < 0.05).

Discussion

Our study unequivocally demonstrates that the growth of *Rhinanthus alectorolophus* is supported by two sources of organic carbon originating from its own photosynthetic activity and host-derived assimilates. Assimilates of autotrophic origin apparently represent the dominant component of carbon balance in adult *R. alectorolophus* plants in agreement with the high rates of photosynthesis we detected in *R. alectorolophus* plants under natural field conditions (Fig. 1). The fatal consequences of the total darkness treatment for *R. alectorolophus* identifies photosynthesis as an absolutely essential physiological process for *R. alectorolophus* in contrast to the cases of the obligate hemiparasites such as *Striga asiatica* which was shown to grow and reproduce even in absolute darkness (Rogers & Nelson 1962) or *S. hermonthica* in which albino mutants occasionally occur (Press et al. 1991).

Table 3 Summary of a linear mixed-effect model testing differences in proportion of host-derived carbon in biomass of individual parts of *R. alectorolophus* plants cultivated under the shading treatments. Plant identity was used as a random factor nested within the shading treatment. Overall test of the final model: Likelihood ratio = 71.86, *df* = 11, *P* < 0.0001 (tested against null model containing no fixed effect terms).

Effect	*df*	*F*	*P*
1. Shading treatment	2,23	9.91	**0.0008**
2. Plant part	3,64	8.12	**0.0001**
1 × 2	6,64	8.22	**< 0.0001**

Based on the key role of photosynthesis in the carbon budget of *R. alectorolophus*, the lower hemiparasite biomass production observed under both shading treatments can be attributed to a reduction of the rate of photosynthesis caused by the shading. This contrasts with the conclusions of Hwangbo & Seel (2002) who recorded little or no effects of shading on hemiparasite growth. This study however only imposed limited shading (decreasing PAR intensity to *c*. 50%) on adult *Rhinanthus minor* plants after 4.5 weeks post attachment. Not only did the shading treatments of our experiment affect the rate of assimilation, but also the quantity of host-derived carbon acquired by the hemiparasites. Shaded seedlings had the highest proportion of host-derived carbon in their biomass but we estimated that the total amount was lower compared to shaded young plants and unshaded control, which furthermore decreased their growth. In contrast, shaded young plants abstracted a similar amount of organic carbon from the host as the control plants. In addition, these plants had a larger photosynthetically active leaf area at the onset of shading, resulting in higher rate of assimilation per whole plant compared to shaded seedlings. This in turn allowed production of more leaves leading to a positive feedback underpinning differential performance of shaded seedlings and young plants.

A comparison between the proportion of host-derived carbon, its total amount and N/C ratio in the biomass of the hemiparasites (Fig 4a,b) clearly suggests that differences in assimilation rates of plants under individual shading treatment caused the principal pattern in proportion of host-derived carbon in hemiparasite biomass. The low proportion of host-derived carbon and low N/C ratio in unshaded *R. alectorolophus* were apparently caused by their high assimilation rates resulting in "dilution" of host-derived carbon and nitrogen in the hemiparasite biomass dominated by assimilates of autotrophic origin while in the shaded treatments, this effect was much lower. The detected trend of higher concentration of host-derived carbon in the lower plant parts indicate host-derived carbon being mostly directed to structural components of the lower stem parts while the autotrophic carbon is preferentially used for development of new leaves and vertical growth of the stem, i.e. producing additional photosynthetically active area. On the other hand, nitrogen is mostly directed to leaves and the upper parts of the plants (particularly in shaded treatments). These contrasting patterns suggest an intensive retranslocation and metabolic processing of the host-borne resources in the hemiparasite. A strikingly similar pattern was also detected in *Striga hermonthica* however the differential allocation of carbon originating from different sources was much more pronounced (Santos-Izquierdo et al. 2008). The elevated proportion of host-derived carbon in the upper parts of shaded seedlings indicates that under extreme deficiency of autotrophic assimilates,

host-derived carbon can be also directed to vertical growth.

The host-derived carbon acquired by *Rhinanthus* is derived from xylem-mobile organic elements (mostly organic acids and amino-acids, e.g. Alvarez et al. 2008) and the inflow of these compounds therefore occurs through mass flow driven by water potential difference maintained by elevated transpiration rate of the hemiparasite and accumulation of osmotically active sugar alcohols in hemiparasite tissues (Jiang et al. 2003, 2008). Playing the key role in determining sink strength of the hemiparasite, the transpiration rate per whole plant is dependent on its total leaf area, stomatal conductance and water vapour concentration difference between the leaves and ambient air. Of these, the effect of stomatal conductance can be assumed closely similar across *Rhinanthus* plants which keep their stomata permanently open (Jiang et al. 2003). We did not perform any detailed quantitative analysis of leaf area, but the difference in biomass production between control and shaded young plants appeared to be caused by increased leaf thickness of directly illuminated leaves and their palisade parenchyma (Fig. 5) rather than by lower leaf area of shaded young plants. The pattern whereby the total amount of host-derived carbon in biomass in depends on shading treatment (Fig. 4b) therefore suggests that the total leaf area could be the key factor underpinning the parasitic resource uptake. The last variable potentially affecting the transpiration rate, differences in interior water vapour concentration, might have developed between irradiated and shaded leaves due to their different temperature increasing the transpiration of leaves under full irradiation. Leaf temperature data acquired during the field gas-exchange measurement of light-response curves (Fig. 1) however indicated rather low temperature difference (*c*. 1°C) between leaves irradiated by 500 and 50 µmol photons m^{-2} s^{-1} resulting in relatively small decrease of transpiration rate inflicted by shading if compared to the effect of leaf area. In addition, substantial amount of host-borne resources can be acquired during the dark period of the day when host plants close their stomata while those of *Rhinanthus* remain open resulting in rather intense night-time transpiration (Press et al. 1988, Jiang et al. 2003). Due to resultant difference in sink strength between the host and hemiparasite shoots in the night, this mechanism is hypothesized to play an important role in parasitic resource acquisition by the hemiparasites (Press et al. 1988, Erhelinger & Marshall 1995) independently of light conditions during the day.

The lack of a significant difference in height between shaded young plants and control and a rather moderate decrease of vertical growth in shaded seedlings achieving more than 50% height of control plants (Fig. 2b) indicate that shaded plants mobilise their resources (acquired both autotrophically and heterotrophically) to support their vertical growth aiming to avoid shading, a fundamental process also observed in non-parasitic plants (Lambers et al. 2008). Shaded young plants were therefore able to grow as tall and suppress host growth to the same degree as the control hemiparasites despite the severe shading. This response would potentially allow the parasites to escape from shading under natural conditions in grasslands when shading is imposed by competing neighbouring plants. However, if shading is imposed in the seedling stage, parasites remain small and are unable suppress the host growth to a sufficient extent or to compete for light with the surrounding vegetation.

The sensitivity of *R. alectorolophus* to shading when imposed in a very early stage of development clearly supports the hypothesis of Těšitel et al. (2010a) explaining the conflict between heterotrophic acquisition of substantial amount of carbon and reported sensitivity to light competition in *Rhinanthus* (Matthies 1995, Hejcman et al. 2011) by inefficiency of resource uptake by unattached hemiparasite seedlings. In addition, a moderate increase of grassland productivity and hence increase of competition pressure (Hautier et al. 2009) has been reported to increase seedling mortality in hemiparasites but also fecundity of the survivors (van Hulst et al. 1987, Mudrák & Lepš 2010). Similarly, Keith et al. (2004) and Hautier et al. (2010) demonstrated that once survived the seedling stage, hemiparasites growing close to the host or attached to a fast-growing host species achieve comparatively high fecundity. All these results suggest that productivity connected with elevated mineral nutrient uptake support the growth of the hemiparasites after overcoming the seedling stage by increasing effectiveness of their photosynthetic machinery.
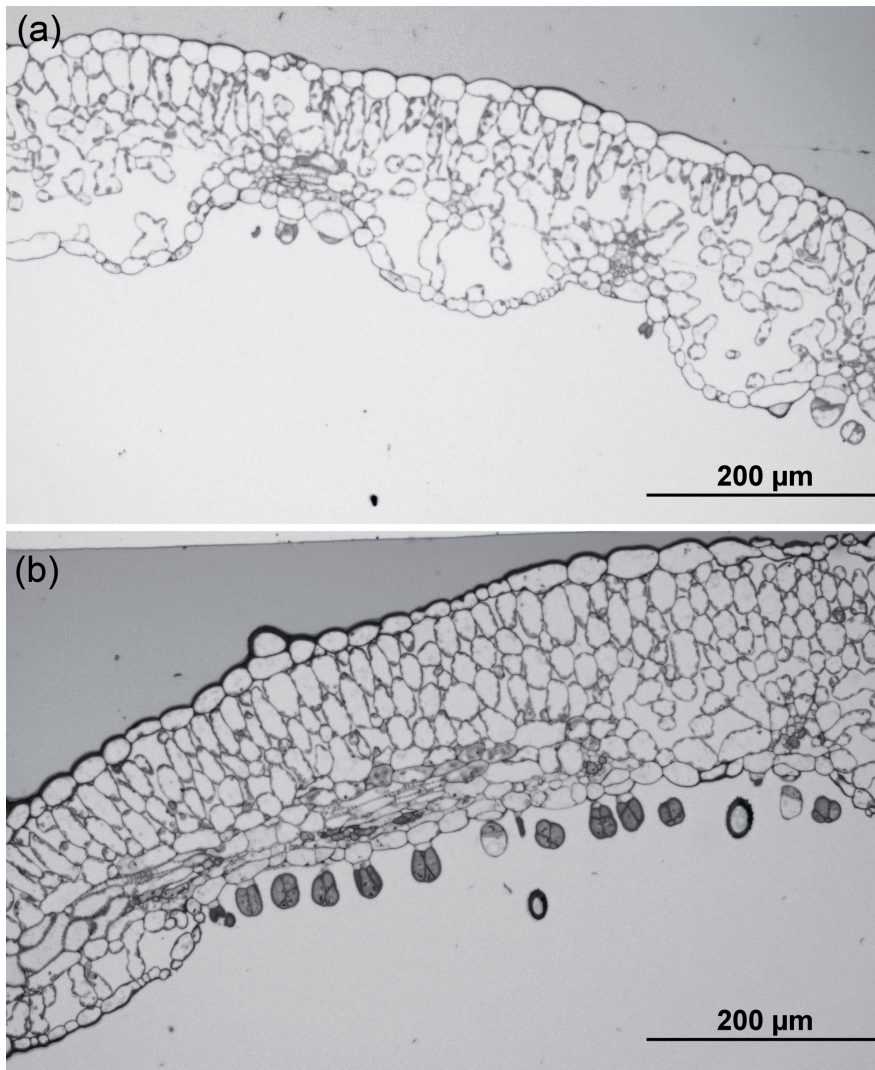
Fig. 5 Light micrographs of transverse semi-thin sections of shaded leaves (a) leaves expose to direct light (b) of the experimental *Rhinanthus alectorolophus* plants. The shade leaf is represented by a sample of upper leaf of a plant of the shaded young plant treatment and the upper leaf an unshaded plant was taken as sample of a sun leaf. Leaves were fixed in 2.5% glutaraldehyde, dehydrated with an acetone series and embedded in Spurr resin. 400 nm thick sections were cut using a Leica Ultracut UCT ultramicrotome at the Laboratory of Electron Microscopy, Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice. Sections were stained by 1% toluidine blue prior to microscopy.

The mortality of hemiparasite seedlings caused by light competition pressure from the surrounding vegetation is also determined by the quality of attachment to the host; that is, seedlings attached by multiple haustoria and to lower-order host roots take up host resources more effectively and their mortality rate is consequently lower (Keith et al. 2004). This can, at least in part, compensate for the effect of shading on the carbon balance of the hemiparasite by providing substantial amounts of host-derived carbon. Indeed, some of the shaded seedlings in our experiment managed to acquire as much host-derived carbon as plants in the other two treatments (Fig. 4b) and these individuals also performed best among the shaded seedlings in terms of biomass production. Supporting this conclusion, Hejcman et al. (2011) have demonstrated that *R. minor* is able to persist even in a highly productive grassland plots requiring however intensive seed rain from surrounding plots of low productivity. These *R. minor* plants were substantially more vigorous than plants growing in surrounding less productive plots (Hejcman pers. comm.) and probably represented a population subset that was able to acquire substantial amounts of carbon heterotrophically. Nonetheless they were still unable to establish a stable population due to the high mortality rate and subsequent low population density.

Wider perspectives

Persistence of *Rhinanthus* spp. populations in grassland communities depends on its ability to complete the life cycle. As annual hemiparasites without substantial long-living seed bank, the species must germinate, grow, flower and produce seeds in every season. This study identified the seedling stage as the bottleneck of the life cycle limiting the occurrence of *Rhinanthus* spp. to grasslands with low to moderate productivity. Such sensitivity of seedlings is in part addressed by a comparatively large seed size and early germination controlled by environmental factors allowing

rapid development and overcoming limitation in the critical recruitment stage. The ability to acquire substantial amounts of host-derived organic carbon supporting the growth of young plants however represents a further mechanism enabling *Rhinanthus* seedlings to escape from competition for light. In this way, the heterotrophic carbon acquisition can broaden the ecological range of the hemiparasites allowing their occurrence in moderately productive environment.

Acknowledgements

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Leaf excretory glands play a key role in physiology and evolution of parasitism of the Rhinanthoid Orobanchaceae

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Introduction

Hemiparasitism is a specialized strategy of resource acquisition based on a combination of autotrophy a heterotrophy, that is unique for several lineages of dicotyledonous plants (Barkman et al. 2007). Hemiparasites are green plants undertaking their own photosynthetic activity. At the same time they however attach to host root or stem by specialized transfer organs called haustoria in order to withdraw resources from host xylem (Irving & Cameron 2009). The location of the attachment to the host define two basic functional types of hemiparasites – root hemiparasites and stem hemiparasites (mistletoes). Of these, the root hemiparasites constitute an omnipresent constituent of various terrestrial plant communities in which they can play an important functional role connected mainly to their ability to suppress host growth and influence nutrient cycling (e.g. Bardget et al. 2006).

Hemiparasitic Rhinanthoid Orobanchaceae present one of the most intensively studied group of parasitic plants due to their omnipresence in plant communities of Western Eurasia making them easily accessible to numerous local researchers. The typical representatives of the group include annual hemiparasites, e.g. of genera *Melampyrum, Rhinanthus* and *Euphrasia* displaying so-called facultative hemiparasitic strategy characterized by a rather low dependency on the host species compared to other parasitic plants (Irving & Cameron 2009, Westwood et al. 2010). Facultative hemiparasites tend to produce rather large seeds which germinate without host induction. Their seedlings produce their own root system foraging for host roots to which they attach in a period ranging from several days to few weeks after germination. In addition, they are usually able to survive and some of them even reproduce generatively when grown without host under artificial conditions. Attachment to suitable host however always strongly increases their performance in terms of both growth and offspring productions. In general however, hemiparasites that remain unattached after the short post-germination period are virtually absent from natural populations.

Not only contains the Rhinanthoid clade these well-known and widespread facultative hemiparasites, but also several species displaying obligate-hemiparasitic strategy and one completely holoparasitic genus. The obligate hemipatrasites of genera *Tozzia* and *Rhynchocorys* feature an underground non-green early development stage that persists below-ground for several years after germination (Weber 1973, Kubat & Weber 1987). These species only produce green above-ground shoots prior to flowering (Weber 1973, Kubat & Weber 1987). The lifestyle of holoparasitic *Lathraea* species is closely similar except for lacking photosynthetic activity in the flowering shoots. All of these obligate hemiparasites and holoparasites produce underground rhizomes covered by fleshy scales. This structure evolved from leaves as an underground organ accumulating resources acquired from the host, which are consequently used to produce flowering shoots.

The close relationship between the leaves of facultative hemiparasites and below-ground scales of obligate hemiparasites and holoparasites is furthermore reflected by sharing closely similar glandular hairs on their surface (first described by Fedorowicz 1915) . These glands are generally

assumed to operate as hydathodes actively excreting water via guttation (Ziegler 1955, Govier et al. 1968, Weber 1975a, Renaudin & Capdepon 1977). Morphology and ultrastructure of the glands was intensively studied in *Lathraea* and *Tozzia* in the past (Ziegler 1955, Renaudin & Garrigues 1967, Weber 1975a, Renaudin & Capdepon 1977). In hemiparasites however, the function of the leaf glands was investigated only by Govier et al. (1968) who suggested them to play a key role in the acquisition of resources from host xylem by increasing the transpiration flow of the hemiparasite. The glands were also hypothesized to affect directing of resources to tissues of young developing leaves prior completion of stomatal development. This study has however been completely neglected by recent literature dealing with water regime and other aspects of physiology of the Rhinanthoid hemiparasites (e.g. Press et al. 1988, Seel & Press 1994, Lechowski 1996, Jiang et al. 2003, Jiang et al. 2004, Jiang et al. 2010).

In this paper, we present morphology and ultrastructure of excretory glands on leaves of the facultative Rhinanthoid hemiparasites *Rhinanthus alectorolophus*, *Odontites vernus* using scanning and transmission electron microscopy. In addition, presence and structure of the glands on leaves of *Melampyrum pratense* is demonstrated by light microscopy. The key point of the present study however lies in summarizing the information available from previous German and French studies (Ziegler 1955, Renaudin & Garrigues 1967, Weber 1975a, Renaudin & Capdepon 1977) which are rather difficult to access and consequent integration of the function of the glands with the current concepts of physiology of hemiparasites. In addition, recently resolved phylogenetic relationships within the Rhinanthoid clade (Benett and Mathews 2006, Těšitel et al. 2010) allow also discussing the role of the excretory glands in the evolution of parasitism in the Rhinanthoid clade of Orobanchaceae

Materials and Methods
Plant material
Leaf material was collected from *Rhinanthus alectorolophus* (Scop.) Pollich , *Odontites vernus* Dumort. and *Melampyrum pratense* L. Well-developed mature leaves of the first two species were obtained from plants cultivated in a growth chamber at the Department of Botany, Faculty of Science, University of South Bohemia. The cultivation conditions followed those described in Těšitel et al. (2011). Wheat (*Triticum aestivum* L.) was used as host. Leaves of *M. pratense* were collected in a wild population occurring in an acidophilous oak forest near Mokré in Budějovice Basin, Czech Republic (N48°57'41", E14°24'29", 420 m a.s.l.).

Sample preparation for and microscopic observation
Leaf samples of *Odontites vernus* and *Rhinanthus alectorolophus* was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH = 7.2) for 2 days at 4°C. Samples for conventional SEM were dehydrated in a graded concentration of acetone and dried using a Pelco critical point dryer CPD 2 (Ted Pella Inc., Redding, CA, USA). The samples were subsequently attached to an aluminium disc by a carbon tape and coated with gold using a sputter-coater E5100 (Polaron Equipment Ltd. – now Quorum Technologies Ltd., Ringmer, UK). The microscopic samples were finally examined with a field-emission SEM JSM 7401-F (JEOL Ltd., Tokyo, Japan) operated at 3.0 kV in GB-low mode.

A subset of glutaraldehyde-fixed samples was post-fixed in 4% osmium tetroxide for 4 h, at 4°C, washed, dehydrated in an acetone series and embedded in Spurr resin. A series of ultrathin sections was cut of these samples using a Leica UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany), counterstained with uranyl acetate and lead citrate and then examined in a TEM JEOL 1010 (JEOL Ltd., Tokyo, Japan) operated at 80 kV equipped with a MegaView III digital camera (Olympus SIS GmbH, Münster, Germany).

Additional fresh leaves of *R. alectorolophus* and *O. vernus* were attached to an aluminium disc and frozen by plunging in liquid nitrogen in order to prepare samples for cryo field emission scanning electron microscopy (cryo-SEM). These samples were subsequently transferred under vacuum into the chamber of the cryo-attachment (CryoALTO 2500, Gatan Inc., Pleasanton, CA, USA). Vacuum sublimation was performed for 10 minutes at −90 °C followed by coating of the

specimens with platinum/palladium for 2min at −140 °C. Finally, the specimens were examined in field-emission SEM JEOL 7401-F (JEOL Ltd., Tokyo, Japan) operated at 3.0 kV in GB-low mode.

Transverse sections of living *Melampyrum pratense* leaves were prepared using a razor blade. Standard light-microscopic samples was consequently prepared of this tissue. These samples were immediately examined using an Olympus BX51 microscope.

Results

The microscopic inspections of all leaves of hemiparasites revealed presence of sessile and stalked glands similar to those previously described from *Odontites vernus* and scale leaves of *Lathraea* and *Tozzia*. These glands occupied a substantial proportion of leaf surface area in *Rhinanthus* and *Odontites* (Fig. 1a, b). Interestingly, the abaxial leaf surface consists of regions with both types of glands and gland-free regions with stomata. Presence of the glands appeared associated with vascular bundles and stomata were absent from the regions covered by the glands.
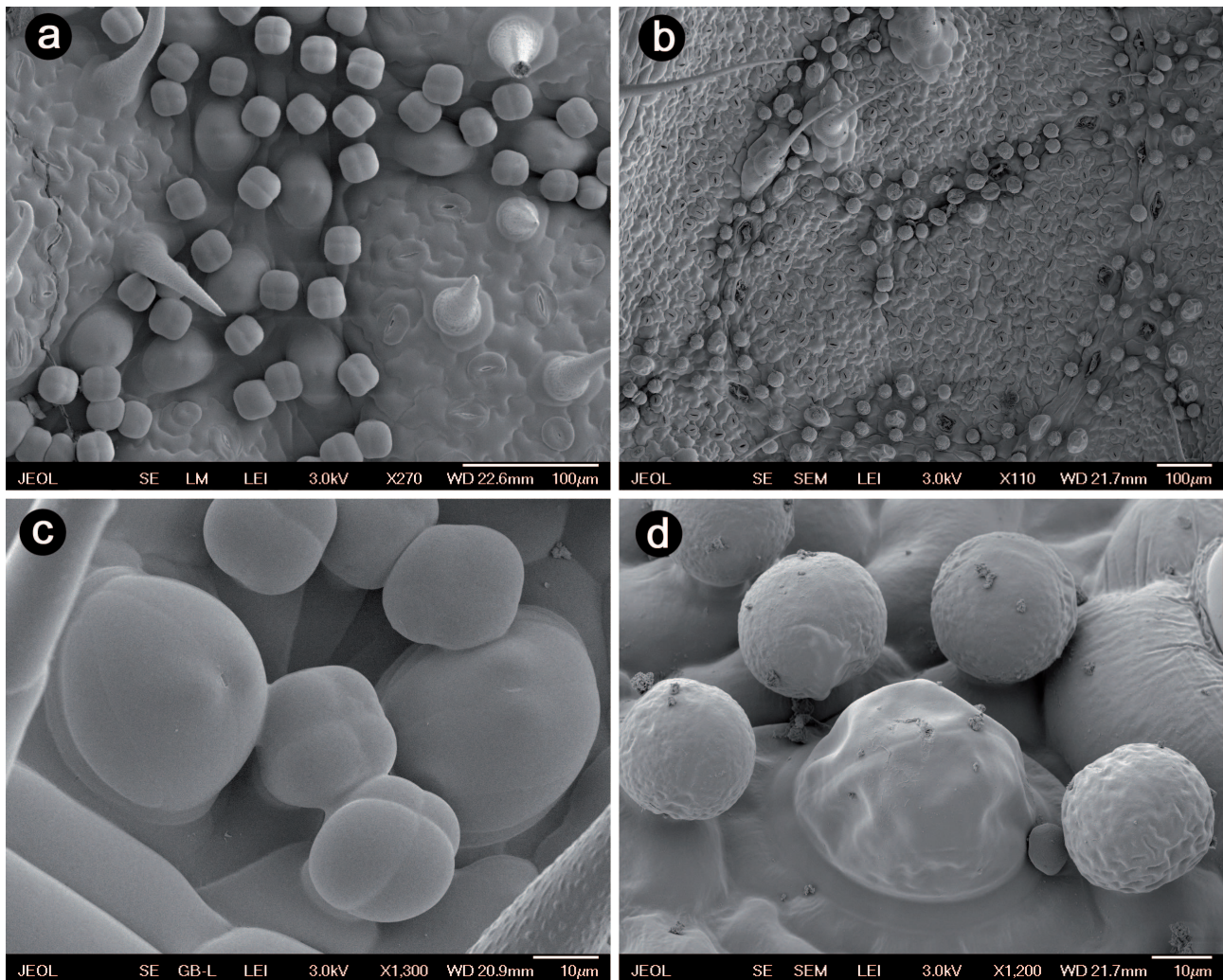
Fig. 1 Scanning electron micrographs of abaxial leaf surface of *Rhinanthus alectorolophus* and *Odontites vernus*. (a) Overview of *R. alectorolophus* leaf surface featuring stomata, non-glandular hairs, sessile and stalked glands (cryo-SEM). (b) overview of *O. vernus* leaf surface featuring stomata, non-glandular hairs, sessile and stalked glands (CPD-SEM). (c) detailed outer morphology of sessile and stalked glands of *R. alectorolophus* (cryo-SEM), (d) detailed outer morphology of sessile and stalked glands of *O. vernus* (CPD-SEM).

The outer morphology of stalked glands is defined by a head consisting typically of 4 cells in *Rhinanthus* (Fig. 1c) and 2 cells in *Odontites* (Fig. 1d). In both species, the head is supported by a single stalk cell that connects it with the leaf. The sessile glands (Fig. 1c,d), appear to have *c.* 2x larger volume compared the head of the stalked glands and occur in slightly lower frequency on leaf

surface of both *Rhinanthus* and *Odontites*. Apparently, they also consist of several (probably up to 4) cells that are arranged laterally in the gland which is apparent from shallow ridges on their surface clearly visible on Fig. 1c (and less so on Fig. 1d). Cryo-SEM images nonetheless revealed smooth surface of all of the gland cells; therefore apart from the cell connections, any structures visible on the surface or surface shrinkage visible on conventional SEM pictures (Fig. 1b,d) can be interpreted as CPD-artefacts.

The TEM inspection of the head cell of the stalked glands revealed the whole volume of the cell filled with cytoplasm, no vacuole was observed (Fig. 2a,b). The outer and to a lesser extent also inner surface of the cells displayed thick labyrinth cell wall penetrated by a high number of cytoplasmatic strains (Fig. 2c,d). Mitochondria dominated the interior part of the cytoplasm almost completely filling the cells. In addition, prominent Golgi apparatus was observed in the vicinity of the cell wall in head cells of the stalked gland of *Odontites*. The stalk cell also contained high volume of cytoplasm compared e. g. to parenchyma cells (Fig. 2a,b). Its cell wall did not however display the labyrinth structure observed in the head cells and in *Rhinanthus*, it also featured a prominent vacuole (Fig. 2a).

Fig. 2 Transmission electron micrographs of stalked glands on the abaxial leaf surface of *Rhinanthus alectorolophus* and *Odontites vernus*. (a) A stalked gland of *R. alectorolophus* consisting of a stalk cell and four head cells, (b) stalked gland of *O. vernus* consisting of a stalk cell and two head cells, (c) ultrastructure of a head cell of a stalked gland of *R. alectorolophus*; note the labyrinth cell wall and numerous mitochondria, (d) labyrinth cell wall, mitochondria and Golgi apparatus in a surface region of an *O. vernus* head cell.

The inner ultrastructure of sessile glands (Fig. 3a,b) displayed features comparable to those of stalk gland heads consisting of several (mostly four) cells extruding of the leaf surface supported by a single large basal cell nested among ordinary epidermal cells of the leaf. The immense size of the basal cells prevented its imaging of the whole cell by TEM, therefore, only its fraction

neighbouring with the extruding cells is displayed. Labyrinth cell wall was present in the extruding cells on the connection with the basal cell (Fig. 3c,d). Despite prominent vacuoles, the cells also contained rather extensive proportion of cytoplasm with numerous mitochondria. Their number was however generally lower than in the stalked gland cells. Plasmodesmata were observed connecting the extruding cells and the basal cell (Fig. 3c,d). In addition, notable apoplastic space was observed on the contact between neighbouring sessile gland cells. This was clearly visible on Fig. 3b and slightly less so on Fig. 3a (this was however caused by the fact that we did not manage to capture an ideal transversal section of a sessile gland of *Rhinanthus*).

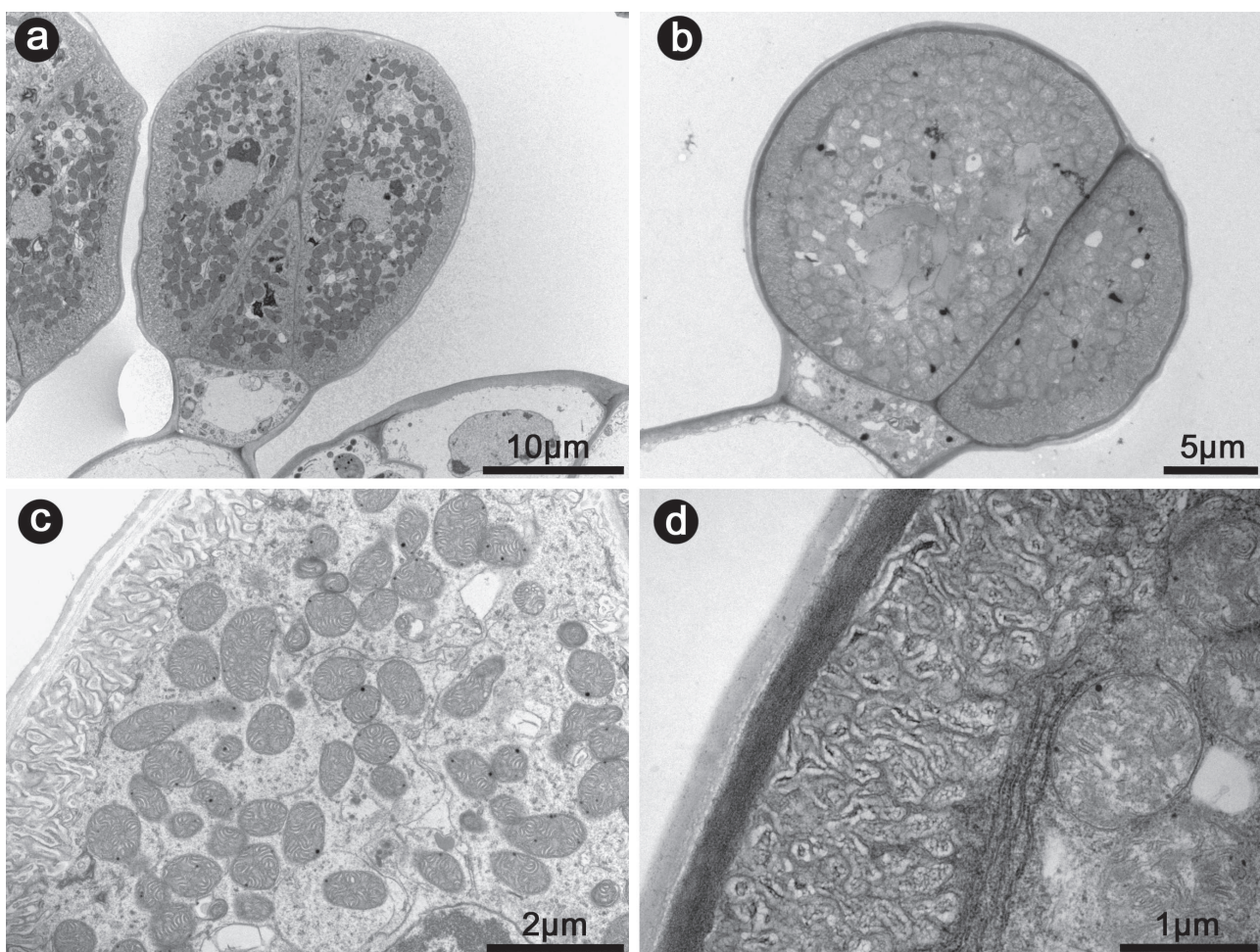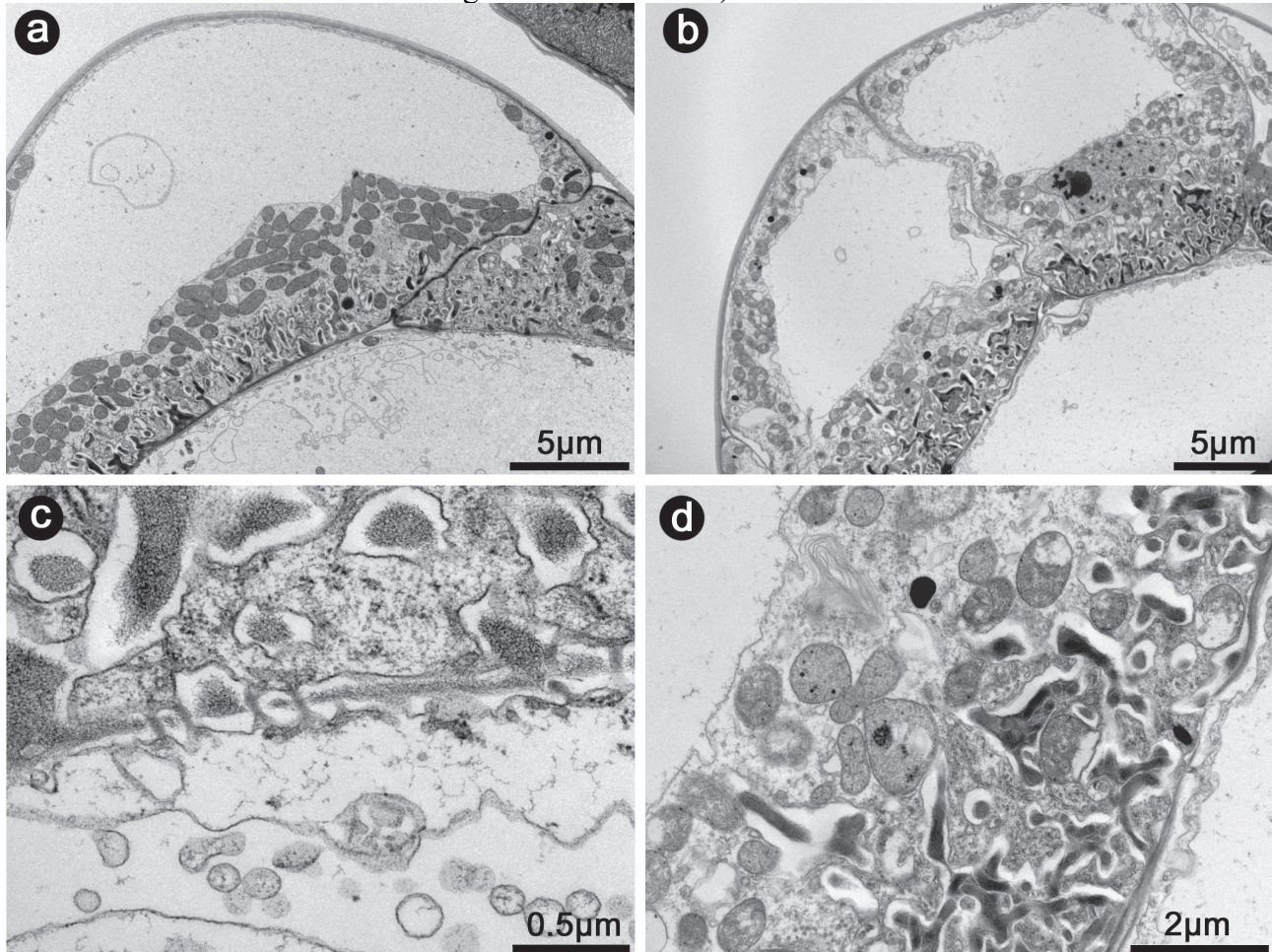**Fig. 3** Transmission electron micrographs of sessile glands on the abaxial leaf surface of *Rhinanthus alectorolophus*, *Odontites vernus* and *Melampyrum pratense*. (a) a sessile gland cell of *R. alectorolophus* with prominent vacuole, numerous mitochondria and a labyrinth cell wall in the extruding cells on the contact with the basal cell; note numerous vesicles present in the basal cell close to the contact with the extruding gland cells, (b) a sessile gland of *O. vernus* featuring ultrastructure closely similar to that of *R. alectorolophus*, (c) plasmodesmata connecting the extruding cell and the basal gland cell of *R. alectorolophus*, (d) ultrastructure of the labyrinth cell wall of the sessile gland cell on the contact with the basal gland cell in *O. vernus*; note a plasmodesma close to the right edge of the image.

Although *Melampyrum pratense* leaves were not inspected by electron microscopy, presence of both types of glands was clearly demonstrated by light-microscopic observations. The stalked glands exhibited a structure closely similar to that of *Rhinanthus* and *Odontites* (Fig. 4a) and their head consisted of four cells (Fig. 4b). The sessile glands also consisted of several cells (up to four) and appeared closely similar to those of the species inspected by electron microscopy (Fig. 4c).

Fig. 4 Light micrographs of glands present on leaves of *Melampyrum pratense*. (a) a side view of a stalked gland (b) a top view of a stalked gland demonstrating the head consisting of four cells (c) a side view of a sessile gland as seen under light microscope.
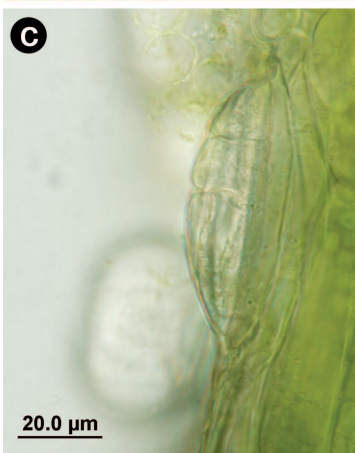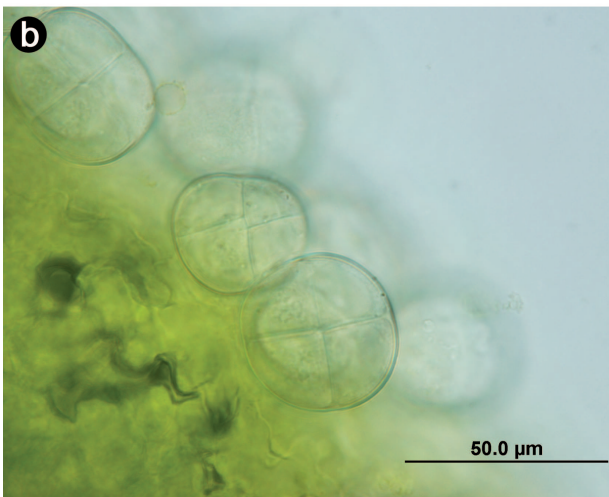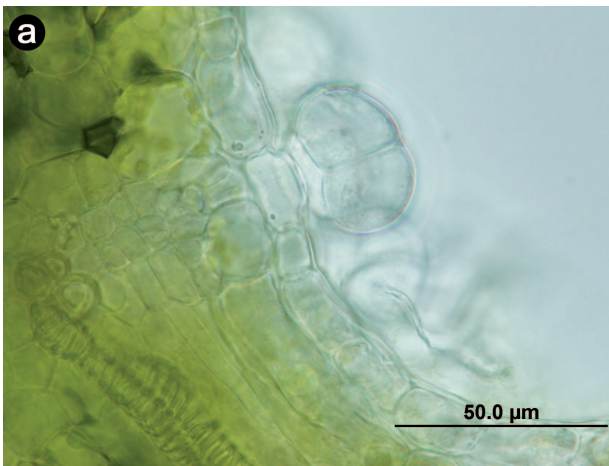
Discussion

Morphology and anatomy of the excretory glands

Both types of excretory glands observed on the leaves of all of the hemiparasites in this study clearly correspond to those reported on leaf surface of *Odontites* by Govier et al. (1968) and are apparently homologous to those present on scales of *Lathraea* and *Tozzia* (Renaudin & Garrigues 1967, Weber 1975a, Renaudin & Capdepon 1977). In particular, the anatomy of the sessile glands corresponds almost exactly to those described from *Lathraea* including also the typical apoplastic space as described by Renaudin & Garrigues (1967) who termed this structure as "sillon". Appart from few details (e.g. 2 vs. 4 celled heads of stalked glands of *Odontites* and the other two species examined here respectively), the morphology and anatomy of the glands appears rather similar across the examined species. In general however, both types of the glands can be found not only across the whole all Rhinanthoid clade but also in other related species of Orobanchaceae and the variability of their appearance and structure is likely to be considerably higher than the differences among the species presented here and the well-described cases of *Lathraea* and *Tozzia* (Fedorowicz 1915, Kaplan & İnceoğlu 2003).

The glands apparently present physiologically highly active structures based on a high content of cytoplasm in their cells, numerous mitochondria and plasmodesmata. In addition, the labyrinth cell wall present in the gland cells, closely resembling to that of transfer cells (Taiz & Zeiger 2006), substantially facilitates exchange of solutes between different cells or between the protoplasts and the exterior environment of the leaf. Despite missing direct experimental evidence on the mechanism of physiological processes in which the glands are involved, it is generally agreed that they operate as water excreting organs (Ziegler 1955, Renaudin & Garrigues 1967, Govier et al. 1968, Weber 1975a, Renaudin & Capdepon 1977).

Physiological role of excretory glands

The water regime of the Rhinanthoid hemiparasites is characterized by elevated transpiration rate (Press et al. 1988, Jiang et al. 2003). Moreover, stomata of some of the species are kept open permanently even during night-time and drought stress and are non-responsive to abscisic acid concentration effectively resulting in lack of control over transpirational water loss in these plants (Jiang et al. 2003). This unusual stomatal behaviour induces highly negative water potential in the hemiparasites directing the solute flow from host xylem to the parasite via xylem continuity present

in the haustoria (Cameron et al. 2006). The water potential difference between the host and the hemiparasite is further enhanced by content of osmotically active compounds such as sugar alcohols (Hodgson 1973).

Based on the presence of the excretory glands, the hemiparasitic Rhinanthoid Orobanchaceae posses however also another mechanism enhancing the transpiration flow. In contrast to water vapour movement through stomata, the water excretion from leaves is driven actively in this case as indicated by numerous mitochondria present in the glands. Such process has even been captured by gas-exchange measurement performed by Press et al. (1988) who detected elevated rates of night respiration and a ctight linear relationship between night respiration and transpiration rates in *Rhinanthus minor*, *Bartsia alpina* and across all analyzed hemiparasitic species (all of which were of Orobanchaceae family). Such pattern in gas-exchange corresponds exactly with what one would expect if water is excreted actively by the glands. The elevated transpiration rate in hemiparasites is generally assumed to play an important role in acquisition of resources in terms of both mineral and organic nutrients (Ehrelinger & Marshall 1995, Press 1995). Active excretion of water can be hence assumed to play an important role in parasitic uptake of the resources from the host as suggested by Govier et al. (1968). Energetic expenses of this process indicated by the elevated respiration rate can be hypothesized to be at least in part compensated by heterotrophic organic carbon acquisition (Těšitel et al. 2010b, Těšitel et al. 2010c).

Given the evidence presented here and in the earlier studies (Ziegler 1955, Renaudin & Garrigues 1967, Govier et al. 1968, Weber 1977, Renaudin & Capdepon 1977), the physiological role of the excretory glands as hydathodes appears clear. Nonetheless, any information on the exact mechanism how water is excreted is largely missing. In addition, all data on the function of the excretory glands are of rather qualitative character. Based on the gas-exchange measurements of Press et al. (1988), it appears likely that they can substantially affect the water flows in the hemiparasitic plant and present a significant carbon sink within the hemiparasitic plant. New investigations are however needed to reveal the extent to which the glands affect the water regime and nutrient acquisition of the Rhinanthoid Orobanchaceae.

Evolution of parasitism in the Rhinanthoid group

Most of the Rhinanthoid species are facultative hemiparasites nonetheless, two obligate-hemiparasitic (genus *Tozzia* and most of *Rhynchocorys* spp.) and one holoparasitic (genus *Lathraea*) lineages evolved independently in the group (Těšitel et al. 2010a). None of these plants however display primary (terminal) haustoria (Weber 1975b, Kubat & Weber 1987) or connection to phloem (Ziegler 1955, Weber 1974, Kubat & Weber 1987). This is in a clear contrast to other parasitic plant lineages in which the evolution of these more advanced types of parasitism are coupled with the evolution of a primary (terminal) haustorium and phloem connection (Irving & Cameron 2009, Westwood et al. 2010).

Such dependence of a holoparasite and long-living holoparasitic stages of obligate hemiparasites just on xylem uptake from the host is unique among parasitic plants. This is however not completely surprising since even facultative hemiparasites can acquire substantial amounts of organic carbon from the host (e.g. Tennakoon & Pate 1996, Těšitel et al 2010b, Těšitel et al. 2011). In addition, *c.* 10% of homopteran herbivores feed also exclusively on xylem (Press & Whittaker 1993) indicating a possibility of complete heterotrophy based on extraction of xylem-mobile organic elements.

Such nutritional strategy must be however based on an extensive solute flow through the parasitic organism combined with filtering of organic elements diluted in host xylem sap due to their low concentration (Press & Whittaker 1993). Consequently, large volumes of water must be excreted from the body of a xylem-feeder, which is indeed observed in the case of the xylem-feeding insects (Press & Whittaker 1993). Most of the hemiparasitic xylem-feeding plants display elevated transpiration rate allowing them to enhance the transpiration stream in a passive way resulting in effective and intensive uptake of host xylem-borne inorganic (Jiang et al. 2004, Jiang et al. 2010) and organic resources (Press et al. 1987, Těšitel et al. 2010b, Těšitel et al. 2011). The

Rhinanthoid hemiparasites have however evolved an additional mechanism of water excretion based on active energy-consuming exudation of water. Apparently, this mechanism was the key pre-adaptation which allowed evolution of the xylem tapping holoparasites and obligate hemiparasites with long-living holoparasitic stages.

Xylem-tapping holoparasitic strategy comes with certain constraints connected with the need of acquisition of large amount of xylem sap. Therefore, all Rhinanthoid Orobanchaceae displaying this strategy are long-living species with rather slow ontogeny in contrast to predominantly annual facultative hemipasites (Těšitel et al. 2010a). Long-term storage of resources in rhizomes also limits their occurrence to rather stable environments (mostly forests) with porous soil types. On the contrary, there is one crucial advantage of xylem-tapping holoparasites in comparison to phloem-feeders. In contrast to parasitic solute uptake from phloem, effective xylem sap translocation from host to parasite does not require establishing biochemical compatibility allowing unloading host phloem sap to parasite transfer cells (Hibberd & Seel 2001, Irving & Cameron 2009) and in general the number of developmental checkpoints, where host-resistance reaction can occur, appears lower in xylem-feeders than in phloem-feeders (Cameron & Seel 2007, Thorogood & Hiscock 2010). Therefore, the host range of the Rhinanthoid xylem-tapping holoparasites is generally broader than that of most phloem-tapping species, some of which are even strict single host species specialists (Irving & Cameron 2009). For example, *Lathraea squamaria* is known to parasitize mostly deciduous trees or shrubs of genera *Alnus*, *Corylus, Fagus* and *Salix* but also accepts conifer hosts, e.g. *Picea* or even introduced *Metasequia* trees (Heide-Jørgensen 2008).

Acknowledgements

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6

Variation in the *Melampyrum sylvaticum* group in the Carpathian and Hercynian region: two lineages with different evolutionary histories

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Introduction

The hemiparasitic genus *Melampyrum* (*Orobanchaceae*) is an important part of the European flora and is most diverse in the Balkan Peninsula followed by Caucasus and temperate Europe (Meusel et al. 1978). *Melampyrum* originated probably in the Mid Tertiary (Wolfe et al. 2005), evolved a number of species a few of which migrated outside Europe and the Caucasus and constituted taxa that do not occur in Europe (e.g. *Melampyrum roseum* and a few related species in E Asia, *M. lineare* in E North America; Soó 1926–1927, Štech 1998). Having survived the Quaternary climatic cycles, temperate species of *Melampyrum* maintained a high diversity, unlike most of the European Tertiary flora (Ložek 1973, Lang 1994). Their diversity might have even increased as a result of isolated evolution in glacial refuges.

The genus *Melampyrum* is still actively speciating, which has resulted in the evolution of several complexes of closely related microspecies that are hardly distinguishable from each other, of which the *M. nemorosum* and *M. sylvaticum* groups are good examples. The origin and distribution pattern of individual microspecies are supposed to have been predominantly affected by the migration of populations, their isolation and subsequent coming into contact in the late Pleistocene and Holocene (Wesselingh & van Groenendael 2005).

The *M. sylvaticum* group is a widespread element of the European montane and subalpine flora. Its geographical range covers mountain ranges from W Europe (the Pyrenees, Scottish Highlands; Dalrymple 2007) to the Urals and lowlands in the boreal zone (Meusel et al. 1978). Forming large populations, it is relatively common in most of its range. Nonetheless, it is sometimes considered rare or even endangered in countries near its geographical boundary, e.g. in Britain (Dalrymple 2007).

Three taxa are usually distinguished at the species level in the *M. sylvaticum* group, based on anther length, corolla size and colour. *Melampyrum sylvaticum* L. s. str. defined by short anthers and a small yellow corolla (see Soó & Webb 1972 or Těšitel & Štech 2007 for the exact range of values) is the most widespread type, believed to grow across the entire range of the group (Meusel et al. 1978). Although certain levels of variability in *M. sylvaticum* s. str. are reported from the Alps (e.g. Ronniger 1911, Soó 1926–1927), there are currently no other species level taxa in this group (Soó & Webb 1972). Long anthers and long (large) corolla characterize *M. herbichii* Woł. and *M. saxosum* Baumg., which differ from each other only in their corolla colour, being yellow and white, respectively. Beside being similar in terms of morphology, the centre of the geographical distribution of the latter two species is in the Eastern Carpathians (Soó 1926–1927, Jasiewicz 1958, Paucă & Nyárady 1960, Soó & Webb 1972) and they are also often reported from the Southern Carpathians (Paucă & Nyárady 1960, Soó & Webb 1972). The taxonomic concept of the group is complicated by populations exhibiting diagnostic traits that are intermediate between those of *M. sylvaticum* s. str. and the Eastern Carpathian species. These are frequent in the Western Carpathians and eastern part of the Hercynian Massif, where there is a large zone of morphologically transitional types (Jasiewicz 1958, Štech & Drábková 2005, Těšitel & Štech 2007). Nonetheless, it is not clear whether this morphological similarity reflects genetic similarity with the Eastern Carpathian populations, which has resulted from gene flow across the boundary

between the Eastern and Western Carpathians. An alternative hypothesis is that the similarity in morphological features resulted from convergent evolution under similar ecological conditions in both mountain ranges.

The main objective of this study was to evaluate the pattern of variation in the M. sylvaticum group across the Hercynian Massif and the Eastern and Western Carpathians using molecular markers and modern morphological methods. We asked the following questions: (i) What is the relationship between populations occurring on the opposite sides of the biogeographical boundary between the Eastern and Western Carpathians? Are they closely related or do they present two distinct lineages within the complex? (ii) Can we detect gene flow between the two Carpathian massifs? (iii) Do the Eastern Carpathian populations display any morphological or genetic variation (in non-coding loci) associated with the two corolla colour forms?

Material and methods

Material

The present study is based on plant material from 31 populations (596 individual specimens) of the *M. sylvaticum* group collected from the Eastern and Western Carpathian region and Hercynian Massif (Table 1, Fig. 1). Up to 31 plants were sampled from each population and used in the morphometric analysis of all the specimens collected (596 plants). The corolla of one flower per plant was put into an Eppendorf-tube filled with concentrated (96%) ethanol (denatured) and stored for digitalization. Several leaves or bracts from up to three plants per population were desiccated using silica-gel and kept at –20°C for DNA extraction (in total, 72 specimens were analyzed using molecular tools). The other parts of each plant were processed as a standard herbarium voucher and are in the herbarium of the Faculty of Science, University of South Bohemia (CBFS).

Some of the samples used in the present investigation were originally collected for a previous study of variation in the *M. sylvaticum* group (Těšitel & Štech 2007). Unfortunately, this material was destroyed during processing for the previous analysis and, therefore, not available for the current morphometric analyses. Nonetheless, those samples are a valuable source of material for the molecular part of this study (Table 1). These populations were chosen so as to represent all the mountain ranges from where the samples for our previous study originated (Těšitel & Štech 2007).

Digitalization and morphometric analysis

Thin plate spline method with sliding semilandmarks (Bookstein 1997, Zelditsch et al. 2004) was employed to analyze corolla shape. This is an efficient way of describing the outline of an object, in particular the edges (presence of which causes difficulties when outline-based methods are used) and has been used in a number of studies on the variation in shape of biological objects (e.g. Neustupa & Hodač 2005, Macholán 2006). Preliminary trials clearly showed that this method is superior to the traditional distance-based morphometrics used previously (Štech & Drábková 2005, Těšitel & Štech 2007). The semilandmarks managed to capture e.g. variation in the corolla curvature, an important diagnostic character completely overlooked by the conventional approach based on a series of linear measurements of corolla shape (see Těšitel & Štech 2007).

The corollas kept in ethanol were flattened and scanned at 1200 dpi using CanoScan 4200 (Canon Inc., Tokyo, Japan). The images were saved as RGB colour images in JPG format (low compression). 27 landmarks were digitized on the outline of each corolla (Fig. 2a), using version 2.05 of tpsDig software (Rohlf 2006). The images were ordered randomly before performing the landmark digitization, which should minimize subjective bias caused by potential similar misplacement of some landmarks in successive images. 25 landmarks were defined as semilandmarks allowing them to slide along the abscissa between their neighbours during the superimposition. Although landmarks 11, 12 and 21 seem to be well defined in two dimensional space (Fig. 2a), we decided to use them as semilandmarks. True landmarks have a slightly higher influence on the analysis than semilandmarks (Zelditsch et al. 2004), which is undesirable in the case of these points. Their position is strongly affected by bending of the lower corolla lip, which

occurs when the three dimensional corolla is flattened, and the curvature of the corolla base, which is more or less stochastic and potentially connected to the phenological stage of individual flowers. Nonetheless, there was no apparent difference in the results when we performed a reference analysis in which these points were true landmarks.

Individual landmark constellations were aligned using the Procrustes super-imposition (Zelditsch et al. 2004) in tpsRelw, version 1.42 (Rohlf 2005). A maximum of 10 iterations was allowed in the superimposition procedure aiming to minimize the bending energy among the shapes. Resulting scatter of superimposed landmarks can be seen in Fig. 2b. Relative warp analysis (RWA, Rohlf 1993) was subsequently performed with the parameter α set to 0 (resulting in shape principal component analysis) using tpsRelw, version 1.42 software (Rohlf 2005). Centroid size (i.e. sum of distances between individual landmarks and the central point defined as the hypothetical centre of gravity) was extracted during the superimposition procedure and employed in subsequent analyses as a measure of size independent of shape.

Anther length was measured in individual flowers in addition to the acquisition of corolla shape and size data. The measurements were done under a dissection microscope. The metering accuracy was 0.05 mm.

Table 1 List of the details of the samples of the *Melampyrum sylvaticum* group used in this study. Localization, nuclear DNA (ITS) haplotypes and chloroplast DNA (*trn*L–*trn*T) haplotypes are indicated. Samples marked by anasterisk are from populations included also in our previous study (Těšitel & Štech 2007). Numbers of specimens analyzed by morphometric and molecular methods are indicated before and after slash, respectively.

No.	Country	Locality	Latitude	Longitude	Altitude (m)	Date of sampling	ITS haplotypes	cpDNA haplotypes	Corolla color	Number of specimens
1*	Ukraine	Rakhiv: spruce forest on slope ca 2 km ESE of the town	48°02'36"N	24°15'13"E	950	30.6.2005	B	a	yellow	27/3
2*	Ukraine	Yasinya: forest edge abbuting the ski slopes at the tourist resort ca 8 km W of the town	48°14'25"N	24°14'24"E	1410	12.7.2005	B, B1	a4	yellow	30/3
3	Romania	Staţiunea Durău resort: side of path between Finţinele and Dochia chalets on N slope of Mt Ceahlau, ca 3 km ESE of the mountain resort	46°59'03"N	25°57'25"E	1610	2.7.2006	A6, A7	a6	white	30/3
4	Romania	Vatra Dornei: rocky massif Piatrele Doamnei ca 1 km SW of Mt Rărau, ca 20 km NE of the town	47°26'51"N	25°33'53"E	1590	3.7.2006	A, A5	a	yellow	30/3
5	Romania	Vatra Dornei: side of path in a spruce forest on the ridge between Mt Rărau and Mt Giumalau, ca 13 km NE of the town	47°27'01"N	25°29'60"E	1416	4.7.2006	A	a2	yellow	20/2
6	Romania	Vatra Dornei, Mt Giumalau: *Pinus mugo* vegetation on the E slope of the mountain ca 250 m E of the summit, ca 11 km NE of the town	47°26'13"N	25°29'04"E	1788	4.7.2006	---	---	yellow	27/---
7	Romania	Vatra Dornei: mountain meadows of Poiană Obcina Mică ca 5 km NE of the town	47°22'46"N	25°22'39"E	1250	5.7.2006	A, A2	a3	mixed	29/3
8	Romania	Vatra Dornei: side of path in meadows on the NE bundary of the town	47°21'38"N	25°22'14"E	946	5.7.2006	A	a3	yellow	22/3
9	Romania	Gura Haitii: *Pinus mugo* vegetation along a path ca 1 km S of the rocky massif Stîncile doispresece apostolii	47°13'04"N	25°13'28"E	1589	5.7.2006	A	a	white	30/3
10	Romania	Iacobeni: edge of spruce forest on the S slope of Mt Tarniţa ca 5 km W of the village	47°24'49"N	25°14'13"E	1421	6.7.2006	A	a	yellow	27/4
11	Romania	Rotunda settlement: *Pinus mugo* vegetation around a path ca 1.5 km SE of the summit of Mt Omu, ca 8 km SE of the settlement	47°29'18"N	25°06'23"E	1737	7.7.2006	A	a	white	30/3
12	Romania	Rotunda settlement: side of the road between the Rotunda settlement and the Pasul Rotunda saddle, ca 2 km SW of the settlement	47°33'27"N	25°01'14"E	1128	8.7.2006	A	a	mixed	29/3
13	Romania	Rotunda settlement: side of the road between the Rotunda settlement and the Pasul Rotunda saddle, ca 1.5 km SW of the settlement	47°33'33"N	25°01'52"E	1080	8.7.2006	A	a3	white	27/2
14	Romania	Daneşti (Izvoru Oltului): spruce forest and spring area on N slope of a hill ca 1 km NW of the village	46°34'46"N	25°46'47"E	908	1.7.2006	A4, A5, A7	a1	yellow	29/3
15	Romania	Baile Tuşnad: edge of a spruce forest at the western boundary of the town ca 1 km SW of the railway station	46°08'42"N	25°51'09"E	683	30.6.2006	A, A1	a, a5	yellow	31/2

No.	Country	Locality	Latitude	Longitude	Alt.	Date			Colour	
16	Romania	Timişu de Jos: side of a path in the valley of the Şipaia creek ca 2 km SE of the railway station	45°3441"N	25°3813"E	838	28.8.2006	A, A2, A3	a	yellow	24/3
17*	Slovakia	Runina: alpine pastures at Sedlo pod Ďurkovcom Saddle, 3.2 km NNE of the village	49°05'08"N	22°2524"E	1128	8.7.2005	B	a	yellow	29/3
18*	Czech Republic	Zalány: *Picea abies* forest at the N border of the village	49°38'35"N	13°5125"E	645	12.7.2005	B	b1	yellow	26/3
19*	Czech Republic	Ovesná: *Picea abies* forest next to the railway station	48°48'26"N	13°5621"E	740	15.6.2006	B	b	yellow	30/2
20	Czech Republic	Volary: edge of forest ca 1 km W of Mt Doupná hora, ca 3.5 km ESE of the town	48°53'45"N	13°5528"E	790	21.6.2006	---	---	yellow	30/---
21	Czech Republic	Javorník: edge of meadow ca 700 m S of the village	49°07'58"N	13°3937"E	900	21.6.2006	---	---	yellow	15/---
22	Czech Republic	Pec pod Sněžkou: edge of forest near the lower station of the cableway to Mt Sněžka.	50°42'28"N	15°4402"E	870	17.6.2006	B	b	yellow	24/2
23*	Czech Republic	Kvilda: group of spruce trees on a knoll in valley of Kvildský potok stream 0.5 km E of the village	49°01'04"N	13°3502"E	1045	11.7.2004	B	b1	yellow	--/2
24*	Czech Republic	Karlov, Velká kotlina Valley: montane spruce forest ca 1.5 km SE of peak of Mt. Vysoká Hole	50°03'10"N	17°14'52"E	1110	7.7.2004	B	b	yellow	--/2
25*	Slovakia	Mt. Veľký Rozsutec: spruce forest on the N slope of the mountain	49°14'20"N	19°0620"E	1385	22.6.2004	B	b	yellow	--/2
26*	Slovakia	Trangoška: montane meadows on the S slope of Mt. Chopok, ca 100 meters N of the Kosodrevina Hotel	48°55'57"N	19°3528"E	1525	26.6.2004	B	b	yellow	--/2
27*	Slovakia	Huty: montane forest near the starting point of the pathway leading to Mt. Biela skala, ca 2 km E of the village	49°13'24"N	19°3559"E	930	1.7.2004	B	b	yellow	--/2
28*	Slovakia	Lysá Poľana: meadow beside the road between the village and a gamekeeper's lodge, ca 3 km S of the village	49°14'27"N	20°0605"E	1005	4.7.2004	B	b	yellow	--/2
29	Slovakia	Oravská Polhora: alpine meadows with *Pinus mugo* shrubs on Mt. Babia hora, ca 0.5 km SSW of the summit, ca 8 km NE of the village	49°34'09"N	19°31'34"E	1580	6.7.2008	B	b	mixed	--/3
30*	Ukraine	Burkut: alpine meadows on Mt. Chivchin, ca. 0.5 km N of the summit, ca 8.5 km S of the village	47°52'09"N	24°4238"E	1640	9.7.2003	A	b	white	--/2
31*	Ukraine	Lazeshchina: alpine pastures between Mt. Hoverla and Mt. Pietrosh ca 2.75 km W of the Hoverla summit, ca 12 km S of the village	48°09'37"N	24°2750"E	1570	11.7.2003	A	c	yellow	--/2
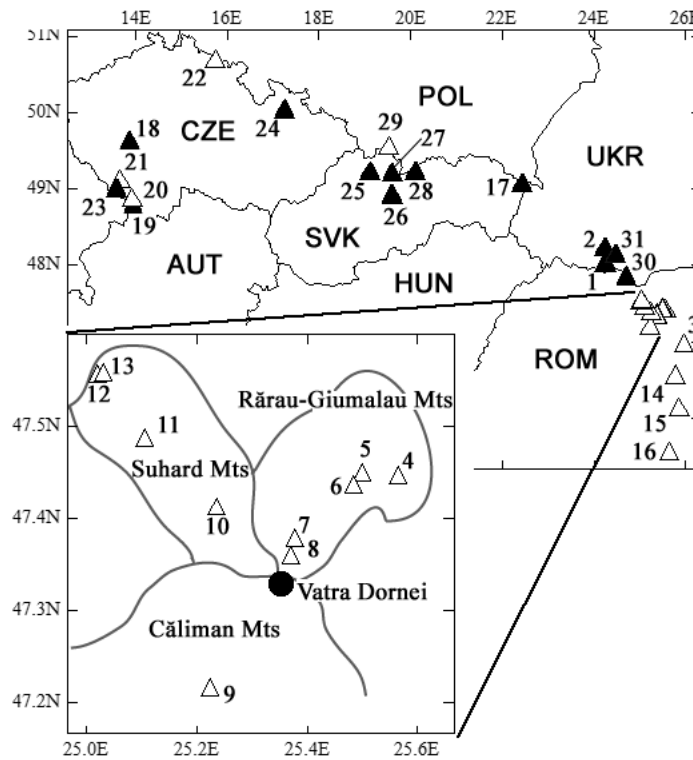
Fig. 1 Map of the *Melampyrum sylvaticum* group localities included in this study. A magnified view of the surroundings of the town of Vatra Dornei is provided as many samples were collected in this area. Populations displayed by ▲ were sampled in our previous study (Těšitel & Štech 2007) and those depicted by △ were sampled in this study. Borders of the following Central and Eastern European countries are shown: CZE – Czech Republic, AUT – Austria, SVK – Slovakia, POL – Poland, UKR – The Ukraine, ROM – Romania, HUN – Hungary.

Fig. 2 (a) Position of landmarks on flattened corolla. Landmarks are marked by boxes, semilandmarks by circles. (b) Scatter plot of superimposed specimens.

DNA sequencing

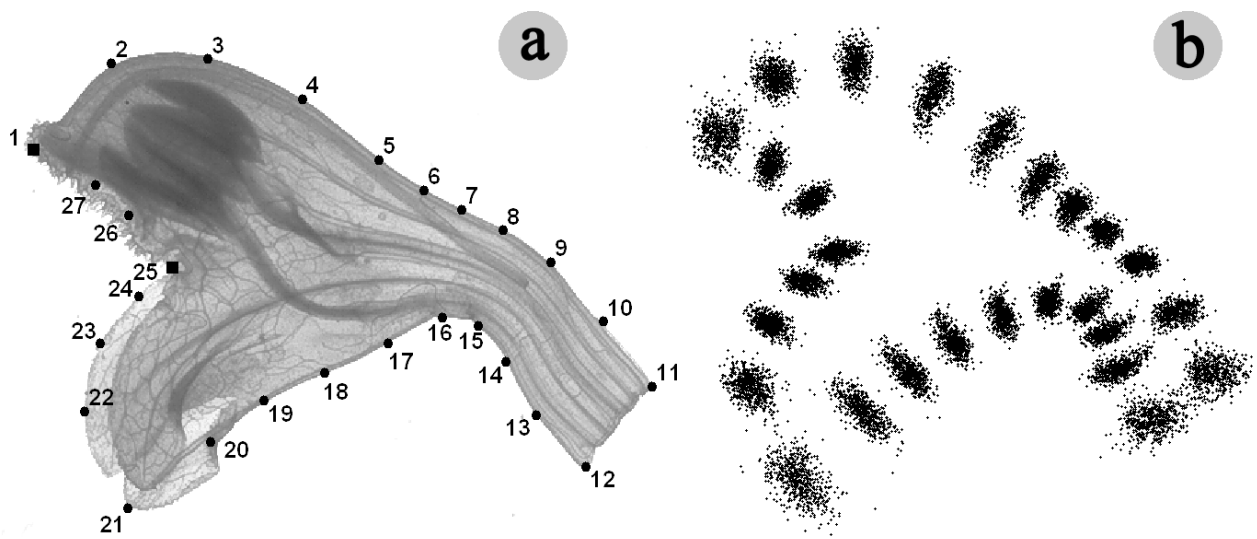DNA was extracted from dried leaf tissue using a commercial Invitek Plant Extraction Kit (Invitrogen) and following the standard protocol provided by the manufacturer. Polymerase chain reaction (PCR) performed on a Biometra T3000 thermal cycler was employed to amplify the *trn*L-*trn*T region of chloroplast DNA and the ITS1, 5.8S and ITS2 region of ribosomal DNA under the following conditions. PCR was performed in a total volume of 25 μl consisting of 1X PCR Buffer, 200 μM each of dNTPs, 1.25U Taq DNA polymerase (TopBio), 1 μl DNA template solution and 7.5 pmol of each of the primers *trn*L (5'-GAGATTTTGAGTCTCGCGTGTC-3'; primer d in Taberlet et al. 1991), *trn*T2F (5'-CAAATGCGATGCTCTAACCT-3'; Cronn et al. 2002) for cpDNA amplification, or plant-specific ITS1P (5'-CTTTATCATTTAGAGGAAGGAAG-3'; Selosse et al. 2002) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al. 1990) for ITS. The amplification profile for cpDNA consisted of denaturation at 95°C (300 s), 30 cycles of denaturation at 95°C (60 s), annealing at 62°C (90 s), extension at 72°C (90 s) and final extension at 72°C (600 s). The amplification profile for ITS consisted of denaturation at 95°C (300 s), 32 cycles of denaturation at 95°C (60 s), annealing at 52°C (90 s), extension at 72°C (90 s) and final extension at 72°C (600 s). The PCR products were subsequently purified using JetQuick PCR Purification Kit (Genomed). Sequencing reaction was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in the Sequencing Centre of the University of South Bohemia.

The sequencing procedure resulted in unambiguous data for both loci. The nucleotide 583 in the ITS alignment was the only exception, oscillating erratically between C and T. It was treated as an ambiguous base Y in all specimens, which set its influence in any analysis to zero. The ITS data otherwise displayed complete concerted evolution (see e.g. Álvarez & Wendel 2003 for explanation) and were directly used in sequence grouping for haplotype definition.

Data analysis

We employed standard statistical techniques for detecting variability in morphological characters. The axes constructed by the relative warp analysis (RWA) are suitable for direct visualization by ordination plots as this method is identical with a principal component analysis (PCA), if an appropriate parameter setting is applied. Proportions of within-population variation were calculated using an expected mean square procedure (EMS; Quinn & Keough 2002). Indicators of populations were regarded as random-effect predictors in the calculation. Differences in the quantitative morphometric traits among populations displaying different corolla colours were analyzed only within the North-Eastern Carpathian region due to lack of morphological data for the Western Carpathian populations and the uniformity of the South-Eastern Carpathian populations. An analysis of variance (ANOVA) and a redundancy analysis (RDA) based on the relative warp scores were used to test the relationship between corolla colour and univariate morphometric characters, and corolla shape. Based on the population means or mean relative warp scores of populations (corolla shape), these tests treated populations as independent observations. Corolla colour entered this analysis as a predictor defined as a binary-coded two variable matrix (describing presence of yellow/white colour in the population); hence populations of mixed or transitional colours received 1 for both predictor variables.

We used Statistica for Windows, version 6.0 (StatSoft 2001) for basic statistical procedures, graphical visualization of data and calculation of EMS for univariate variables. Package R, version 2.3.1 (R Development Core Team 2006) was employed for ANOVA calculations. Canoco for Windows, version 4.53 (ter Braak & Šmilauer 2002) was used for the multivariate statistics and for an extraction of sum of squares from the relative warps, which served as a basis for subsequent manual calculation of EMS using a formula in Quinn & Keough (2002). A PCA based on consensual landmark configurations was computed in PAST package, version 1.67 (Hammer et al. 2001) using a singular value decomposition algorithm (which improved the PCA stability when more variables than samples were present in the analysis).

Sequences of each of the analyzed loci were aligned using Clustal W (Thomson et al.1994)

and the alignment was subsequently improved manually. Identical sequences were grouped to define haplotypes. Phylogenetic network of nuclear and chloroplast haplotypes was constructed by means of statistical parsimony (Posada & Crandall 2001) using software package TCS, version 1.21 (Clement et al. 2000). Indels were treated as independent binary characters (coded as A for absence and C for presence as TCS does not support 0/1 coding). Individual gap positions were treated as missing data.

Results

Continuous morphometric characters

Within-population variation accounted for 50.3% of the variation in the shape of the corolla, 32.9% of that of the corolla centroid size and 23.8% of anther length (inferred from EMS analyses). Variation in all analyzed traits displayed continual patterns, which were more or less congruent with each other.
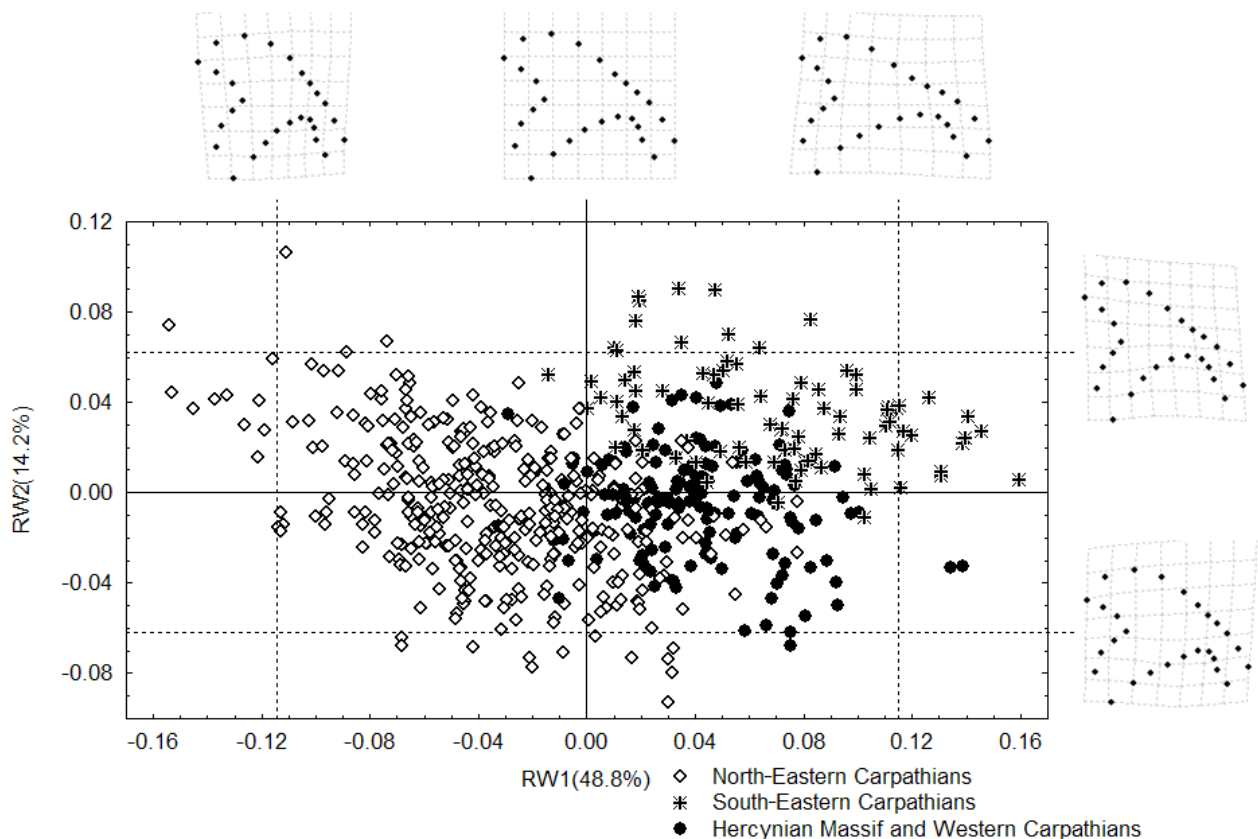

Fig. 3 RWA ordination plot based on variation in corolla shape of individual plants. Points representing the specimens are classified according to the geographical distribution of the populations. Mean corolla shape and the shape changes associated with the first two principal warps are depicted (shapes corresponding to ± 2 SD positions are displayed on each axis).

Classification of individual specimens (Fig. 3) or populations (Fig. 4) in the relative warp ordination plots revealed that plants growing in different geographical regions tend to concentrate in certain parts of the ordination space. Most of the specimens (and all when the consensual corolla shapes within populations are considered) from the North-Eastern Carpathians (north of the southern slopes of the Câliman Mts and Ceahlau Massif) occupy the left side of the first ordination axis, and tend to have a concave shaped shorter corolla with a slightly more prominent lower lip. Differing mainly in the convex shape of their corolla, the Western Carpathian and Hercynian plants (populations) are generally located on the opposite side of this gradient. Three populations in the South-Eastern Carpathians (south of the northern limit of the Harghita Mts) differ from both of

these groups, especially those in the geographically proximate North-Eastern Carpathians. Featuring very long and strongly convexly curved corollas, these plants appear similar to some extreme specimens from the Western Carpathian – Hercynian region.
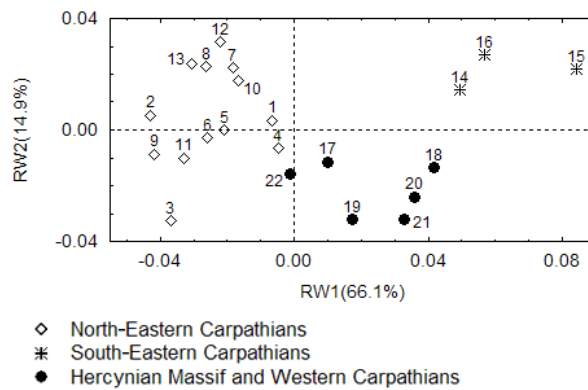
Fig. 4 RWA ordination plot based on consensual corolla shapes in each population. Percentages of variance explained by the axes correspond only to the variation among populations. The populations are labelled with the numbers of the localities (Table 1).
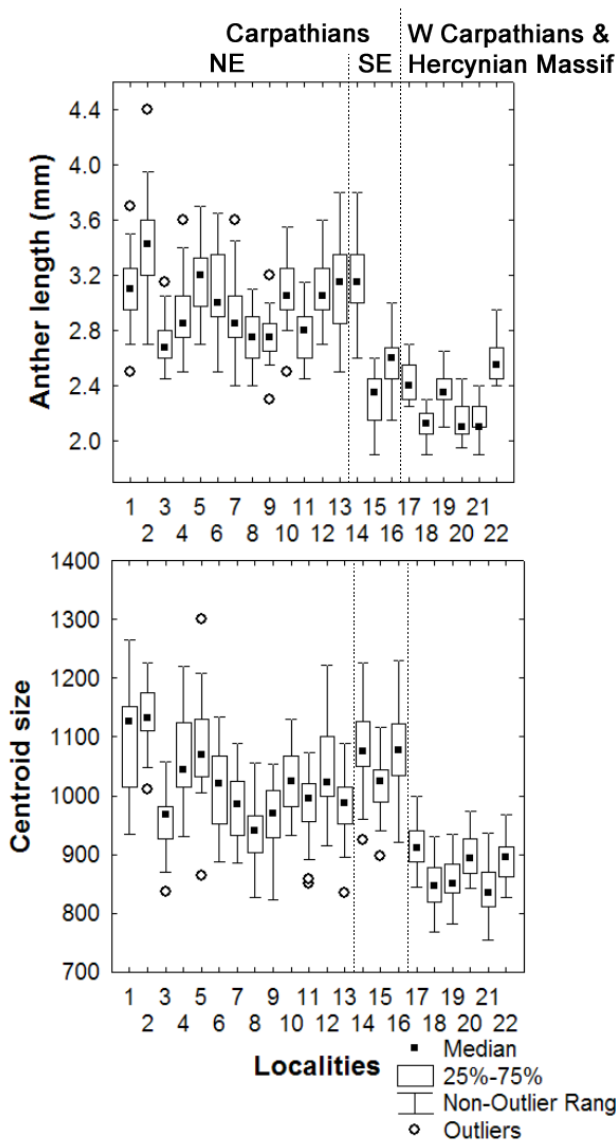
Fig. 5 Box-and-whisker plots displaying the values of anther length (AL) and centroid size at individual localities. Population numbers correspond to those in Table 1. Their geographical location is indicated by dotted lines separating populations in the North-Eastern Carpathians (1–13), South-Eastern Carpathians (14–16) and Western Carpathians and Hercynian Massif (17–22).

The plot of variation in univariate morphometric characters showed similar opposite tendencies in samples from North-Eastern Carpathians versus Hercynian and Western Carpathian populations (Fig. 5). The former group has longer anthers and larger corollas than the latter group. The three populations in the southern part of the Eastern Carpathians differ in that their corollas are very large but anther length is variable, as one population has long and the other two rather short anthers, similar to the Hercynian specimens.

Variation revealed by molecular markers
Both loci analyzed were variable enough to provide valuable information on the relationships among the populations. Ten haplotypes were detected in the *trn*L-*trn*T region of cpDNA (Table 2). Most of them in two haplotype lineages (**a, b**), which differed in two relatively large indel mutations (Table 2, Fig. 6a). Within each of these lineages there is a basic and widespread haplotype (haplotypes a and b) from which other generally much less frequent haplotypes were derived (these are marked by numbers) by both indel and point substitutions. Haplotype c could not be assigned to either of the large lineages and formed an independent group characterized by a unique indel combination, which positioned it between haplotype groups **a** and **b**. Lineage **a** was found in the whole Eastern Carpathian region including the Bukovské vrchy Mts (Fig. 6b), with its basic haplotype present in most of the specimens and populations (Table 1, Fig 6a). The derived haplotypes were either characteristic of small populations or only found in one population, resulting in comparatively high genetic differentiation among populations. In contrast, within-population variation was rather low as multiple (two) haplotypes were found only in one population (Table l). The Hercynian and Western Carpathian populations are similar in only containing haplotype b and its variant b1, distinguished by a point substitution present in two populations in the southern half of Bohemia (Table 1). Haplotype c was only found in a single population on Mt Hoverla (Table 1, Fig. 6b).

The ITS haplotypes could be assigned to the two major lineages **A** and **B**, which differ in the number of single base substitutions and two three-base indels (Table 3). The most parsimonous network describing relationships among individual haplotypes revealed a very pronounced genetic differentiaton (corresponding to a high number of missing haplotypes) between these haplotypic groups (Fig. 7a). Occurring at more than one site, the derived haplotypes were in general not characteristic of individual populations. There was more than one haplotype in many Eastern-Carpathian populations despite the small number of specimens analyzed per population (Tables 1, 3). This was particularly pronounced in the three populations in the southern part of the mountain range where almost no two plants share the same haplotype. Therefore, the genetic pattern is characterized by low differentiation between populations and high within-population variation, at least in the Eastern Carpathians where the genetic variability is high enough for such an estimation. The geographical range of lineage **A** includes most of the Eastern Carpathian sites except for those occurring near the Eastern-Western Carpathian boundary (Fig. 7b). In contrast to the cpDNA lineages, lineage **B** is not restricted to the Hercynian and Western Carpathian populations but also occurs in the Eastern Carpathians. The valley of the Tisa River, which crosses the Eastern Carpathians in the Ukraine, appears to be its eastern limit (Fig. 7b).

Variation in both molecular markers appears substantially higher in the Eastern Carpathians than in the western populations, which were found to be almost uniform. In spite of clear differentiation between the Eastern Carpathian and the western populations revealed by both molecular markers, the geographical borders of the distributions of the haplotype lineages do not coincide, resulting in discordance of the phylogeographic patterns. That is, there is a transitional zone on the boundary between the Eastern and Western Carpathians.

Table 2 Chloroplast DNA haplotypes defined by variable positions in the *trn*L-*trn*T cpDNA region. Substitutions within indels are illustrated by white font on black background. Number of plants and populations (sites) in which individual haplotypes were found are indicated.

| Haplo-type	GenBank accession number	Number of plants (sites)	131	306	307-315	347-355	484	559	615-622	637	678-723	798-799
a	EU653274	27 (10)	A	C	-----	--------	C	G	--------	A	TTATATTTCTAGAGACACTATAT---------------	--
a1	EU653275	3 (1)	A	C	-----	--------	C	G	--------	A	TTATATTTCTAGAGA**T**ACTATAT---------------	--
a2	EU653276	2 (1)	A	C	-----	--------	C	A	--------	A	TTATATTTCTAGAGACACTATAT---------------	--
a3	EU653277	8 (3)	A	C	-----	--------	C	G	--------	A	TTATATTTCTAGAGACACTATATTTATATTTCTAGAGACACTATAT	--
a4	EU653278	3 (1)	A	C	TATAA	--------	C	G	--------	C	---	--
a5	EU653279	1 (1)	A	C	-----	--------	C	G	--------	A	TTATATTTCTAGAGACACTATAT---------------	GC
a6	EU653280	3 (1)	A	G	-----	--------	T	G	--------	A	TTATATT**A**TAGAGACACTATAT---------------	--
b	EU653282	17 (8)	A	C	-----	--------	C	G	AATATAGA	A	---	--
b1	EU653283	5 (2)	C	C	-----	--------	C	G	AATATAGA	A	---	--
c	EU653281	2 (1)	A	C	-----	AGTAATTAA	C	G	--------	A	---	--

Table 3 Nuclear DNA haplotypes defined by variable positions in the internal transcribed spacer (ITS1, 5.8S, ITS2) sequences. Number of plants and populations (sites) in which individual haplotypes were found are indicated.

Haplo-type	GenBank accession number	Number of plants (sites)	50	59	86-87	91	187	406	408-409	414	442	509	558-560	567	571-572	577-579	597
A	EU624125	27 (13)	C	G	TC	A	C	T	GT	C	A	A	---	C	CG	---	C
A1	EU624126	1 (1)	C	G	TC	C	C	T	GT	C	A	A	---	C	CG	---	C
A2	EU624127	2 (2)	C	G	TC	A	C	T	GT	C	G	A	---	C	CG	---	C
A3	EU624128	1 (1)	C	G	CC	C	C	T	GT	C	A	A	---	C	CG	---	C
A4	EU624129	1 (1)	C	G	TC	C	C	T	GT	C	G	A	---	C	CG	---	C
A5	EU624130	3 (2)	C	A	TC	C	C	T	GT	C	A	A	---	C	CG	---	C
A6	EU624131	1 (1)	C	G	CT	C	C	T	GT	C	G	A	---	C	CG	---	C
A7	EU624132	3 (2)	T	G	CT	C	C	T	GT	C	G	A	---	C	CG	---	C
B	EU624133	28 (13)	C	A	TC	A	A	C	GT	T	A	C	TTG	T	TC	GTA	A
B1	EU624134	2 (1)	C	A	TC	A	A	C	AC	T	A	C	TTG	T	TC	GTA	A
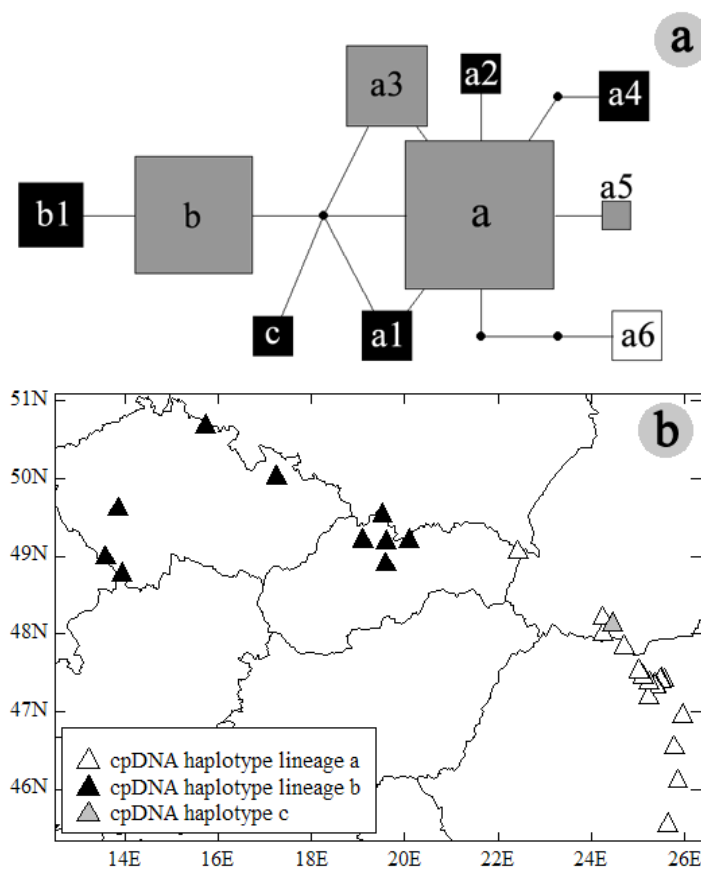
Fig. 6 (a) The most parsimonous phylogenetic network of the cpDNA haplotypes of the *Melampyrum sylvaticum* group detected in the populations studied. Size of boxes is proportional to the number of plant specimens in which individual haplotypes were detected. Small circles symbolize missing haplotypes. Corolla colour trait is mapped onto the network: haplotypes of populations with only yellow-coloured flowers ■ , haplotypes of populations with only white-coloured flowers □, haplotypes of populations with flowers of both colour types or mixed corolla colour ■. (b) Map displaying the distribution of the cpDNA haplotype lineages of the *Melampyrum sylvaticum* group in the populations studied.
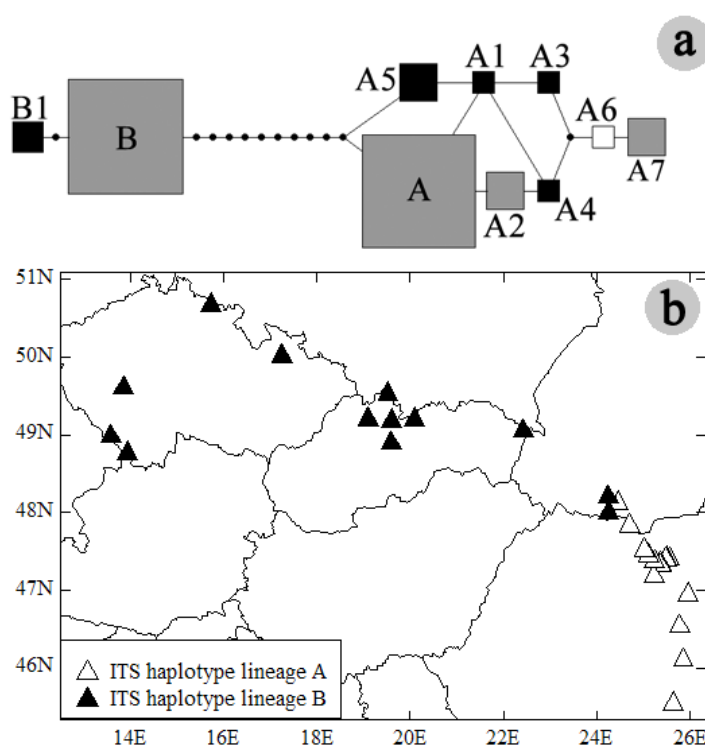
Fig. 7 (a) The most parsimonous phylogenetic network of the ITS haplotypes of the *Melampyrum sylvaticum* group detected in the populations studied. Size of boxes is proportional to the number of plant specimens in which individual haplotypes were detected. Small circles symbolize missing haplotypes. Corolla colour trait is mapped onto the network haplotypes of populations with only yellow-coloured flowers ■, haplotypes of populations with only white-coloured flowers □, haplotypes of populations with flowers of both colour types or mixed corolla colour ■. (b) Map displaying distribution of the ITS haplotype lineages of the *Melampyrum sylvaticum* group in the populations studied.

Variation in corolla colour

There were both yellow- and white-flowered plants in the North-Eastern and Western Carpathian populations of the *Melampyrum sylvaticum* group (Figs 8a, 8b) but only yellow-flowered plants in the populations sampled in the South-Eastern Carpathians and Hercynian Massif. White-flowered specimens were frequent in the North-Eastern Carpathian region, where they formed entire populations, but very rare in the Western Carpathians where only one population was found with yellow, intermediate pale yellow and almost white-flowered specimens on Mt Babia hora (population no. 29, Fig. 8e). The same within-population pattern in corolla colour was recorded at one North-Eastern Carpathian site near Vatra Dornei (population no. 7). In the population below the Rotunda saddle in the Suhard Mts (no. 12) there were plants with intermediate pale-yellow flowers and both extreme corolla colours (Fig. 8d). There was a continuum in corolla colour from white (or almost white in the first two cases) to yellow (Fig. 8a) in all these populations. Slight differences in colour were rarely observed even among flowers on an individual plant (Fig. 8c).
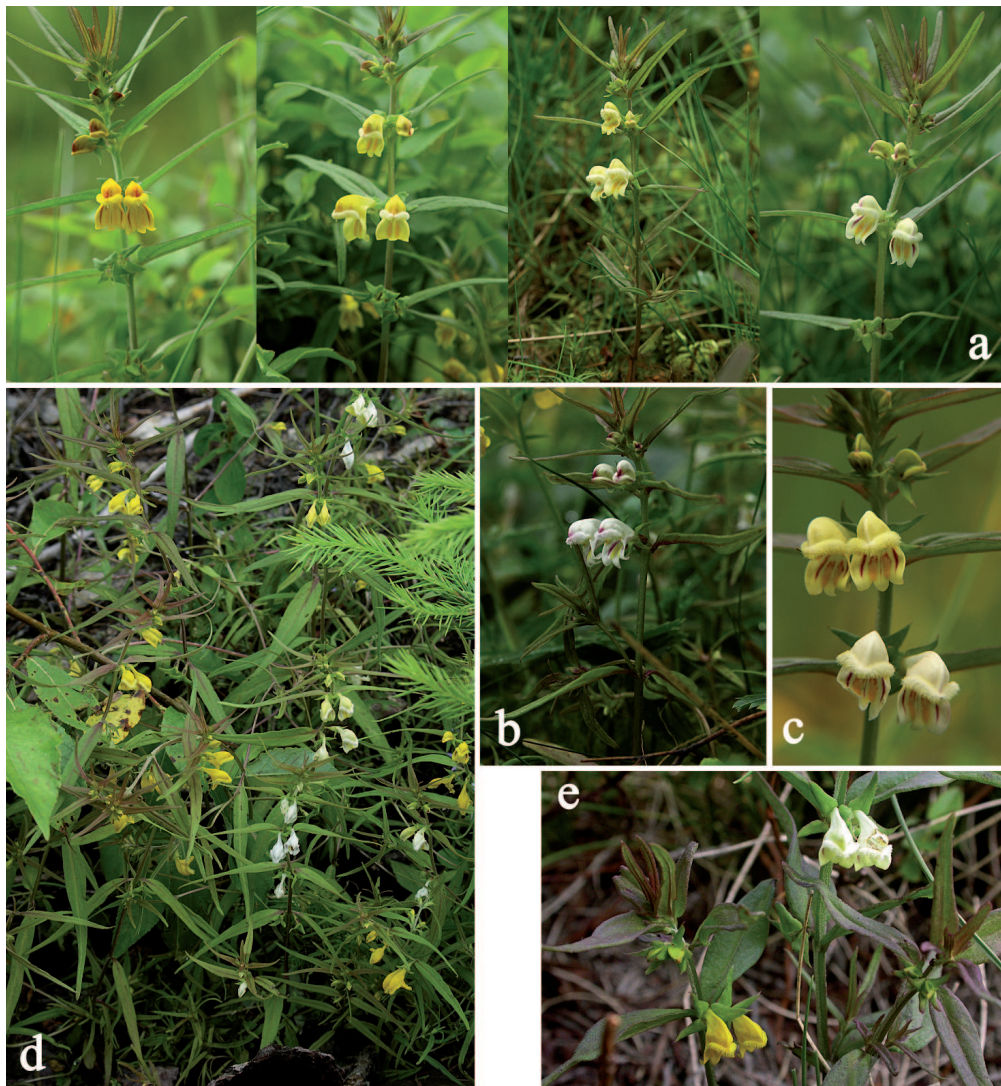

Fig. 8 Variation in the corolla colour of populations of the *Melampyrum sylvaticum* group. (a) Variation in corolla colour of the population growing in the Poiană Obcina Mică meadows near Vatra Dornei, Romania. The plants are ordered from the yellow on the left to the lightest (almost white) specimen on the right. 5 July 2006. (b) A typical example of a white-flowered plant, Ceahlau Massif, 2 July 2006. (c) An unusual plant with flowers of different colours at different nodal positions, Poiană Obcina Mică meadows near Vatra Dornei, 5 July 2006. (d) A mixture of yellow-, white- and pale-yellow-flowered plants growing along the side of the road near Pasul Rotunda saddle, 8 July 2006. (e) Yellow- and whitish-flowered specimens from Mt Babia hora, Slovakia, 6 July 2008.

There were no significant relationships between the variation in corolla colour and anther length (logarithmic transformation; ANOVA, $F_{2,10} = 2.59$, $P = 0.226$), centroid size (square-rooted; ANOVA, $F_{2,10} = 1.73$, $P = 0.124$) or corolla shape (RDA, Monte-Carlo permutation test with 999 permutations $F = 2.47$, $P = 0.146$). There was also no apparent agreement between corolla colour and genetic variation in either of the analyzed loci (Fig. 6a, 7a). Moreover, there were no conspicuous patterns in the geographical distributions of populations featuring different corolla colours (Fig. 9).
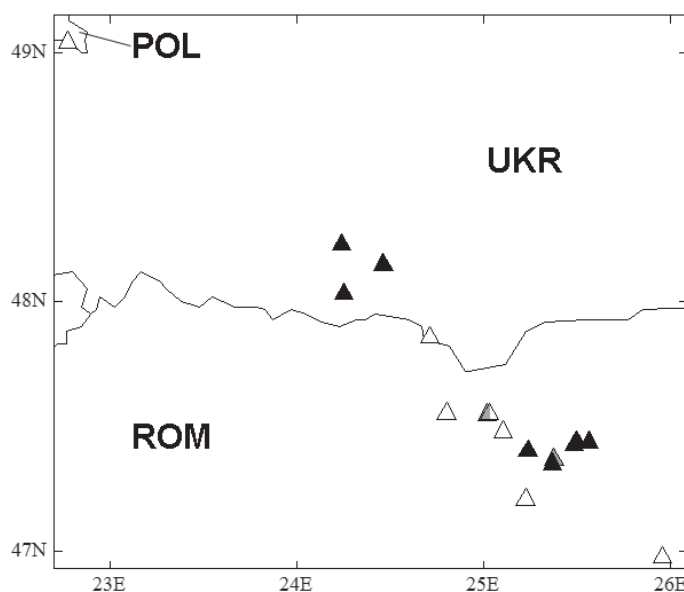

Fig. 9 Map of the localities for the *Melampyrum sylvaticum* group in the northern part of the Eastern Carpathians for which reliable data are available. Position of an additional Polish white-flowered population is estimated on the basis of data in Zając & Zając (200l) and on the web site of the Bieszczady National Park (Anonymus 2008). The populations are classified according to corolla colour (▲ yellow corolla, △ white corolla, ▲ populations with mixed- and pale-yellow-flowered plants). Borders of countries are shown: UKR – The Ukraine, ROM – Romania, POL – Poland.

Discussion

Differentiation and gene flow on the border between the Eastern and Western Carpathians

Our analyses revealed solid and in general concordant phylogeographic patterns in variability in all continuous morphometric characters and both molecular markers. The differentiation between the Eastern Carpathian and western populations suggested by previous studies (e.g. Jasiewicz 1958, Těšitel & Štech 2007) is clearly supported by two distinct lineages within the *Melampyrum sylvaticum* group in our data. The differences delimiting the Eastern and Western Carpathian populations were especially pronounced in the ITS sequences and in congruent patterns in variation of several morphological characters (despite continual nature of their variation and the overlaps). The marked differences in the western (**B**) and eastern (**A**) ITS haplotype lineages is good evidence that the Eastern Carpathian and western types were isolated from one another for a long time in their evolutionary history.

The origin of the transitional zone observed in the border region between the Eastern and Western Carpathians can be attributed to the meeting and subsequent hybridization of the two lineages. Although there are few samples from this area, it is likely that this zone extends from the Tisa valley (and neighbouring Pass of Yablunjitsa) to the Bukovské vrchy Mts (probably its western margin). Reaching only around 850 and 700 m a.s.l., respectively, these sites are very low and narrow parts of the Carpathian ridge, and are likely to impede the migration of alpine and upper-montane floristic elements. The pattern in variation apparently reflects these gene-flow barriers, although the *M. sylvaticum* group is generally regarded as a montane to subalpine taxon (Soó & Webb 1972, Šípošová 1997, Štech 2000) growing in mountain spruce (less frequently beech) forests

and ascending to the tree-line. This description of its ecology is however based predominantly on observations in the Alps or Western Carpathians, where many populations indeed grow under spruce or beech forest canopy albeit not in heavily shaded areas (e.g. populations 19, 23 and 27 in the present dataset; Table 1). By contrast, all the populations in the North-Eastern Carpathians apparently prefer open habitats, either natural subalpine grasslands or dwarf-pine vegetation near the tree-line or man-made meadows, clearings and road sides at lower altitudes. This may account for the limited gene flow from the Eastern Carpathians westwards but not in the reverse direction. The low altitude of the Eastern Slovakian part of the main Carpathian ridge combined with the comparatively ineffective myrmecochorous seed dispersal strategy of *Melampyrum* (Winkler & Heinken 2007) may have prevented a mass migration of the central Western Carpathian populations in an easterly direction. The region between the Vysoké Tatry and Bukovské vrchy Mts comprises ca l00 km wide zone within which the altitude only fluctuates between 500 and 800 m a.s.l. The natural vegetation of this region is a continuous closed-canopy beech forest unfavourable for *M. sylvaticum*. On the other hand, *M. sylvaticum* might have migrated at certain periods in the Holocene when *Picea abies* forests formed a more substantial part of the local vegetation, probably between ca 8000 and 4000 BP (Latałova & van der Knaap 2006). The presence of western-type ITS haplotype in the Eastern Carpathian populations west of the Tisa valley may, therefore, be attributed to gene flow from the central part of the Western Carpathians in the past and subsequent introgression.

The pronounced genetic and morphological differences in the *M. sylvaticum* group on the East-West Carpathian boundary are similar to the recently reported patterns in genetic variation in *Hypochaeris uniflora* (Mráz et al. 2007) and *Campanula alpina* (Ronikier et al. 2008). The relatively wide transitional zone is in agreement with the continuous nature of the biogeographical boundary characterized by a gradual decrease in the diversity of Eastern Carpathian alpine floristic elements (such as R*hododendron kotschyi*, *Alnus viridis*, *Laserpitium krapfii* subsp. *krapfii* and several diploid species of *Hieracium*; Polívka et al. 1928, Mráz & Szeląg 2004) in a westerly direction (Zemanek 1991). Many lower-montane species (i.e. those occurring mainly in beech forests), however, crossed this border and reached the Western Carpathians (e.g. *Veronica urticifolia*, *Aconitum moldavicum*, *Aposeris foetida*) and even the Hercynian Massif (e.g. *Anthriscus nitida* and *Doronicum austriacum*; Slavík 1997, Štech 2004). The latter case was recently well documented for *Rosa pendulina* using a phylogeographic study based on chloroplast DNA sequence variation (Fér et al. 2007).

Evolution of the extant variation pattern and its palaeoecological background

The high level of molecular variation in both chloroplast and nuclear DNA sequences in populations in the Eastern Carpathians indicates that large populations sufficient to maintain such variability have been present there for a long time. These loci are almost uniform in the Hercynian and Western Carpathian populations, which indicates markedly different evolutionary histories. Populations in the North-Eastern Carpathians probably survived the last glacial period (Weichsel, Würm) in one, or more likely, several refuges located probably either in the Eastern Carpathians or their vicinity. Locations and size of these favourable sites might have been relatively dynamic and dependent on climatic oscillations. Evolution in refuges that were isolated but connected periodically can result in the observed pattern in the genetic variation. Molecular uniformity of the populations in the Western Carpathian and Hercynian Massif indicate a recent (Holocene) migration from refuges located probably in perialpine areas.

Several recent studies have demonstrated that it is highly likely that a glacial refuge existed in the Eastern Carpathians, which supports our hypothesis of the long-term persistence of the *M. sylvaticum* group in this region. Robust evidence comes from a review of palaeobotanical finds of charcoal in Central Europe (Willis & van Andel 2004), which indicates the presence of *Picea* (one of the main *M. sylvaticum* group host species; e.g. Štech 2000) and *Alnus* in the eastern surroundings of the North-Eastern Carpathians between 35 000 and 20 000 years BP (calibrated ^{14}C chronology), i.e. during a significant part of the Last Glacial Maximum (LGM). Genetic and pollen

data indicate that *P. abies* survived in the North-Eastern Carpathian region (Tollefsrud et al. 2008). In addition, the North-Eastern Carpathian populations of *Pinus mugo*, another important species associated with the *M. sylvaticum* group, differ morphologically from other Central European and Balkan populations, which indicates their genetic isolation and that they have probably been present in the region for a long time (Boratyńska et al. 2004). This is also supported by the results of several palynological sequences that indicate the presence of *Pinus* (probably *P. mugo*) in the late ice age (Farcas et al. 1999, Feurdean 2004), and that a refuge or several isolated refuges suitable for *Melampyrum* might have existed at favourable sites at the base of the mountains in the North-Eastern Carpathian region.

It is suggested that Siberian taiga-type boreal forest existed in the Western Carpathians during the LGM (Jankovská & Pokorný 2008). This does not accord with our hypothesis that the Hercynian Massif and Western Carpathians were recolonized by *M. sylvaticum* during the Holocene, as it suggests the species might have survived in the area during the full-glacial period. On the other hand, Tollefsrud et al. (2008) have demonstrated not only the survival of *Picea abies*, a characteristic and often dominant tree species in European boreo-montane forests, in the Western Carpathians during the Weichselian Ice Age but also very low genetic diversity in populations of this species in this region. These authors suggest a bottleneck resulting from a substantial decrease in population size during either the LGM or Younger Dryas (Tollefsrud et al. 2008), associated with a decrease in the area covered by vegetation favourable for *M. sylvaticum* and its putative local extinction. Moreover, the present distribution of *M. sylvaticum* is limited by the Uralian mountain range and does not extend substantially into Siberia (Meusel et al. 1978), where the vegetation is nowadays analogous with that reconstructed by Jankovská & Pokorný (2008). It is possible that the distribution of *Melampyrum* is limited by permafrost as it germinates in autumn and has an active overwintering stage with roots. This would account for its present distribution limit and extinction in the Western Carpathians if the reconstructed LGM forest grew on permafrost (Jankovská & Pokorný 2008). The conditions in the Eastern Carpathians were certainly more favourable as at least the southern part of the mountain range was in the permafrost free zone even during the LGM (Taberlet et al. 1998) and permafrost free sites could have occurred at more northerly situated sites (e.g. on southern slopes).

The substantial divergence between *Picea abies* genetic lineages in the northern and southern parts of the Eastern Carpathians (Tollefsrud et al. 2008) suggests a possible explanation for the morphological divergence and differences in the pattern of genetic variation found between the *M. sylvaticum* group populations occurring in these regions. Both species have similar ecological preferences and, therefore, might share the same evolutionary history characterized by the isolation in the past of the populations inhabiting the South- and North-Eastern Carpathians. The little data on *Melampyrum*, however, make this hypothesis very speculative. Nonetheless, it is an interesting idea worthy of further study.

Taxonomic conclusions

Our results demonstrate that the current taxonomic concept of the *Melampyrum sylvaticum* group (Jasiewicz 1958, Soó & Webb 1972) needs to be reviewed. The nonsignificant relationships between corolla colour and other traits, lack of a pattern in the geographical distributions of populations with different corolla colours and the presence of whitish-flowered specimens in the Western Carpathians decrease the taxonomic value of this character. Therefore, we propose that *M. saxosum* and *M. herbichii* are conspecific as their delimitation is based entirely on corolla colour. Under the terms of the priority rule, the correct name for most Eastern Carpathian plants is *Melampyrum saxosum* Baumg., as this name was published earlier (Baumgarten 1816) than *Melampyrum herbichii* Woł. (Wołoszczak 1887). Nonetheless, this nomenclatorical solution must be regarded as preliminary. The final designation of plant names must be based on type herbarium vouchers, which have not yet been studied.

We suggest the Central European populations of the *M. sylvaticum* group be classified into two species *M. sylvaticum* s. str. and *M. saxosum* differing in the shape and size of the corolla and

anther length. These species have different evolutionary histories and geographic distributions, with the approximate border zone between them on the Eastern-Western Carpathian boundary. Nevertheless, this morphological delimitation between these species applies only to the populations in the northern part of the Carpathian mountain range. The presence of morphologically specific populations in the South-Eastern Carpathians that are genetically closer to the North-Eastern Carpathian samples prevents the generalization of this delimitation between *M. sylvaticum* s. str. and *M. saxosum*, which requires further study (especially the collection of more samples from the Southern Carpathian region).

Acknowledgements

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7 Cytotype distribution and seasonal variation in the *Odontites vernus* group in Central Europe

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Introduction

The genus *Odontites* Ludw. (Rhinanthoid clade of Orobanchaceae; Benett & Mathews 2006, Těšitel et al. 2010) comprises annual or perennial hemiparasitic herbs of dwarf-shrubs (Bolliger 1993, 1996). It displays the highest species and life-form diversity in the Western-Mediterranean area. In Central Europe however, it is represented only by two taxa of annual herbs, *Odontites vernus* group and *O. luteus*.

The *O. vernus* group is one of the most common hemiparasitic plants of the Central European flora occurring across the whole region (Kubát 2000). It is also by far the most widespread group within the genus *Odontites* as its geographical range extends from the western coast of Europe to the Himalayas and Siberia (Bolliger 1996). As suggested by its indication as "group", it is assumed to represent a taxonomically complicated aggregate of several variable microspecies which are difficult to identify. Three taxa on species level are usually recognized in the *O. vernus* group comprising *O. littoralis* (Fr.) Fr., *O. vernus* (Bellardi) Dumort. and *O. vulgaris* Moench of which the first grows on the maritime coast of Western Europe and does not occur in Central Europe.

Odontites vernus and *O. vulgaris* are defined on the basis ploidy level and seasonal variation. *O. vernus* is an early-flowering (late May-June) tetraploid species (2n = 4x = 40) while *O. vulgaris* is diploid (2n = 2x = 18 or 20) and flowers later with the onset of flowering after mid-July (Snogerup 1983, Michalková 1998). Some authors nonetheless consider these species conspecific and delimit them only as subspecies (*O. vernus* subsp. *vernus* and *O. vernus* subsp. *serotinus* e.g. Kubát 2000) despite their breeding incompatibility (Snogerup 1983). Such treatment of the group is based on lack of any reliable morphological characters separating the diploids and tetraploids except for those based on plant architecture.

The *O. vernus* group displays a substantial variation in traits related to plant architecture (i.e. number of internodes, number of branches etc.) which is a common feature of most annual hemiparasites of the Rhinanthoid clade (e.g. Ronniger 1911, Zopfi 1993a, 1995, 1998a, 1998b). Due to its close relation to phenology of the plants, it is usually termed as seasonal variation (or dimorphism). This phenomenon typical of annual hemiparasitic Orobanchaceae was subject of numerous studies revealing the evolutionary source and genetic basis of the seasonal variation and revealing its close relationships with the environmental gradients on which the species occur and differential grassland management (Zopfi 1993b, 1998a, Štech 1998, Těšitel 2005). Therefore, the seasonal types are usually called ecotypes as they comply with the definition of this term as distinct genetic lineages within a species, adapted to particular environmental conditions (Begon et al. 1990). In the *O. vernus* group, however, this type of ecotypic variation is reported to coincide with the cytotypic variation. According to the current taxonomic treatment (Schneider 1964, Bolliger 1996, Kubát 2000), the tetraploid *O. vernus* (s. str.) corresponds to an early-flowering ecotype while the diploid *O. vulgaris* is considered a late-flowering ecotype. These types also differ in their

ecological ranges as *O. vernus* grows predominantly as an agricultural weed while *O. vulgaris* is considered ruderal species occurring also on pastures and mesotrophic and flood-plain meadows in Central Europe (Kubát 2000).

The current concept of the *O. vernus* group appears well developed given the literature resources that mostly agree with each other. Nonetheless, there are still several issues that call for investigation. Despite the reproductive barrier between *O. vernus* and *O. vulgaris* (Snogerup 1983), transitional types between these two have been constantly reported (Schneider 1964, Bolliger 1996, Kubát 2000). Therefore, the delimitation between these two types can be assumed rather difficult if relying just on morphology. This is a crucial problem since the descriptions of ecology and geographical distribution of cytotypes and seasonal ecotypes, i.e. the concept of the group, are mostly based on field sampling and herbarium vouchers explicitly assuming the close relationship between morphology, phenology and ploidy level. The ploidy level itself was however rarely identified by means of karyology. Representing the key basis for any hypothesis on evolution of polyploid taxa (e.g. Kolář et al. 2009, Trávníček et al. 2010), the reported distribution of cytotypes in the *O. vernus* group hence lacks support of direct evidence. In addition, 18 and 20 chromosomes are frequently reported as 2n for *O. vernus* without any further details on the importance of this cytotypic variation.

The aim of this study therefore was to revisit the geographical distribution of cytotypes and in the context of the seasonal variation in *O. vernus* group in Central Europe. An extensive screening of ploidy level in the populations was conducted by flow-cytometry (FCM) allowing rapid and accurate identification of ploidy level of analyzed specimens (Suda & Pyšek 2010). The flow-cytomteric approach was nonetheless supported with classical karyology that provided exact chromosome numbers in selected populations.

Material and methods
Field sampling and morphological analysis
In total, 109 population samples (2–15 randomly selected individuals per population) of the *O. vernus* were collected in Czech Republic, Slovakia and Austria (Fig. 1). Fresh specimens were transported to the laboratory to perform the flow-cytometric analysis (see next paragraph for details). In addition, the morphological characters related to seasonal variation were recorded, i.e. number of vegetative internodes (count of internodes between cotyledons and the uppermost pair of branches) and number of intercalary internodes (count of internodes between the uppermost pair of branches and lowermost flowers). The sum of these two values was also analyzed as the total internode number. The analysis of morphological characters was not possible for all populations analyzed by flow-cytometry since some of the populations were sampled prior to flowering or during late fruiting season, which prevented reliable counting of internodes. All plant samples were processed as standard herbarium vouchers and are kept in the herbarium of the Faculty of Science, University of South Bohemia (CBFS).

Flow cytometry
DNA ploidy levels were determined using Partec PA II flow cytometer (Partec GmbH., Münster, Germany) equipped with a mercury arc lamp. The samples were prepared following the simplified two-step protocol using Otto buffers (Doležel et al. 2007). About $0.25cm^2$ of intact leaf tissue (field-collected leaves, stored in plastic bags for max. 1 week at 4°C) was chopped with a sharp razor blade together with an appropriate volume of the internal standard (*Glycine max* 'Polanka', 2C = 2.50pg; Doležel et al. 1994) in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween-20). The suspension was filtered through a 42-mm nylon mesh and incubated for *ca* 1 min. at room temperature. After incubation, 1 mL of the staining solution was added. The staining solution consisted of 1 mL of Otto II buffer (0.4M $Na_2HPO_4.12H_2O$), 2-mercaptoethanol (2 µl/ml) and the fluorochrome DAPI (4 µg/ml). Samples were run on the flow cytometer after about one minute of staining and the fluorescence intensity of 3000–5000 particles was recorded. The *Odontites* material proved to be problematic due to sensitivity to the state of the leaf material and

fast deterioration of nuclei suspension (<10 min. after isolation). Histograms with coefficients of variation for the G_0/G_1 peaks of both the analyzed *Odontites* sample and the standard below 4% were accepted. Pooled samples of up to 5 individuals could be used due to high-resolution histograms. However, in some populations there was high amount of nuclei with 4C DNA content. In such a case, each plant was separately re-analyzed. Results of the ploidy levels analysis were calibrated by populations in which direct chromosome counts were performed.
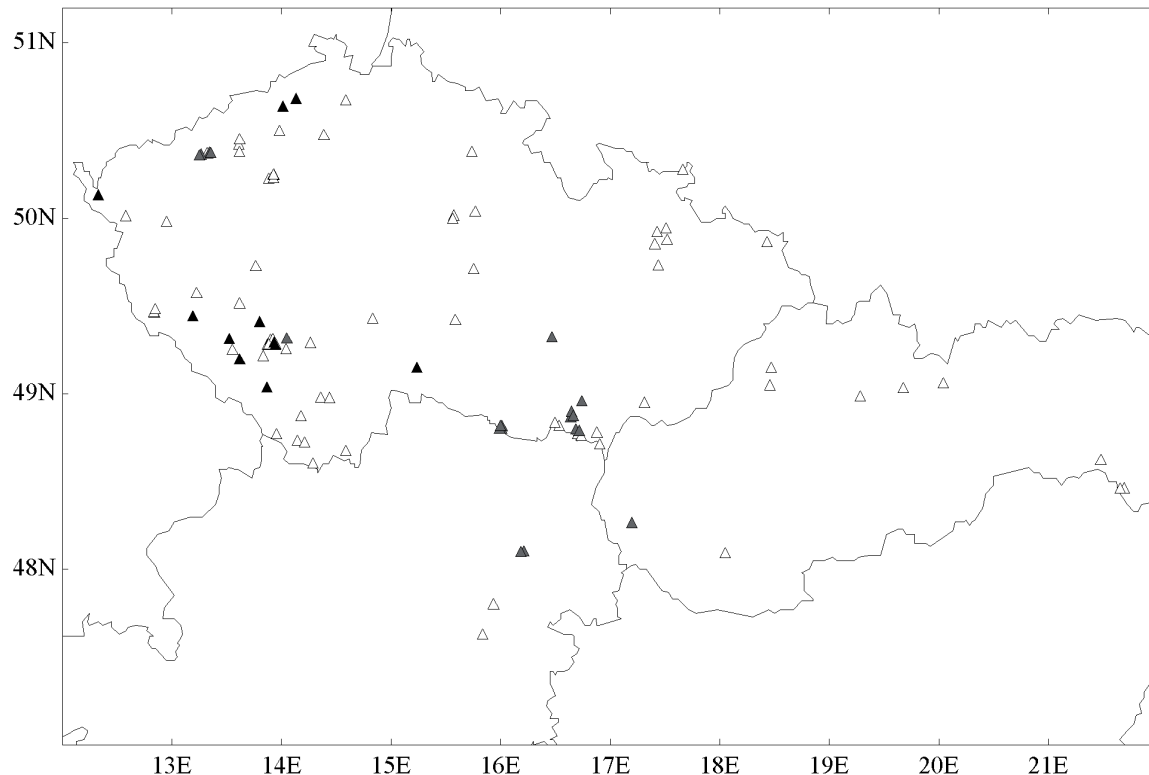

Fig. 1 Distribution map of population samples used in the present study. The populations are classifiaed according the types defined on in the *Odontites vernus* group in Central Europe on the basis of cytotypic and seasonal variation. Late-flowering diploid populations, early-flowering tetraploid populations and late-flowering tetraploid poulations are depicted as white, black and grey triangles respectively

For the genome size estimation, the same internal standard and the same method of sample preparation and staining was used, only replacing DAPI for propidium iodide (50µg/ml) and RNase IIa (50µg/ml). The genome size was determined using Partec CyFlow SL flow cytometer equipped with a 532nm (green) diode-pumped solid-state laser (100 mW output). One individual per sample was measured and the fluorescence intensity of 5000 particles was recorded. Histograms with coefficients of variation for the G_0/G_1 peaks of both the analyzed *Odontites* sample and the standard below 4.5% were considered. Each individual was analysed three times in three different days and the average value was used as the genome size to minimize random instrumental error. If the variation range within the three measurements exceeded 2% of the average value, the most outlying measurement was discarded and the sample was re-analysed.

Chromosome counting

Chromosome counts were determined in the diploid population Tatinná (N50°23.050', E 13°37.041') and the tetraploid population Tišnov (N49°19.623', E16°28.002') to calibrate the results of flow cytometry. Chromosomes were counted in apical root meristems of seedlings that were germinated from seeds collected at the localities. To induce germination, the seeds were placed on a moist filter paper in a Petri dish and kept at 4°C until germination occurred (ca 6 weeks). The seedlings were pre-treated with a saturated water solution of p-dichlorobenzene for 3 hours at room temperature, fixed in a mixture of ethanol and acetic acid (3:1) for 24 hours at 4°C, and stored in

70% ethanol at 4°C. Maceration lasted about 3–5 min. in a mixture of ethanol and hydrochloric acid (1:1). The apical part of the root was then cut and squashed using a cellophane square (Murín 1960) and stained for 1 h in a 10% Giemsa–Romanowski solution in 0.2M sodium phosphate buffer pH 7.2. At least three samples (achenes originating from different individuals) per population were analyzed, and at least two mitoses per plant were studied.

Data analysis

Variability in the internode characters and its relationship to the cytotypic and phenological variation was analyzed by linear mixed-effect models and analyses of variance. The counts of internodes were log-transformed prior to these statistical analyses. Distribution of variance in the numbers of internodes was analyzed by a restricted maximal likelihood linear mixed-effect model with population and type included as random factors. Internode number differences among the types defined by phenology and cytometry were analyzed by one-way analyses of variance (ANOVA) which were calculated on the level of individual plants and population means. The ANOVAs were followed by Tukey honest significance difference tests to test the differences among individual pairs of the types.

Results

The FCM screening of the *O. vernus* group across Central Europe revealed presence of two cytotypes (Tables 1, 2; Fig. 2). These were confirmed as diploids ($2n = 2x = 18$) and tetraploids ($2n = 4x = 40$) by direct microscopic inspection of mitotic metaphase chromosomes (Fig. 3). The diploids formed a single phenologically variable group of populations within which the earliest onset flowering however did not occur before early July. By contrast, two clearly defined seasonal types were found within the tetraploids. The early-flowering tetraploids flowered from late May to late June while the late-flowering population of the same cytotype usually started to flower in late July, i.e. later than most of the diploid populations which can be also considered as late-flowering. The difference between the seasonal types of the tetraploids was reflected by only a small albeit significant difference in relative fluorescence (Table 2, Welch *t* test: $t = -7.175$, $df = 85.05$, $p < 0.0001$) but not in the absolute genome size (Table 2). This small difference was nonetheless never recorded in a simultaneous FCM analysis of a mixed sample of early- and late-flowering tetraploids. The overall pattern of cytotypic and seasonal variation allowed a definition of three distinct types within the *O. vernus* group in Central Europe based on phenology and ploidy level of populations: late-flowering diploids (late 2x), early-flowering tetraploids (early 4x) and late-flowering tetraploids (late 4x).

Not only differed these types in phenology but also in numbers of internodes on their stems, i.e. characters connected with the seasonal variation in hemiparasitic Orobanchaceae. Distribution of variance in the number of vegetative internodes, number of intercalary internodes and the total number of internodes has clearly demonstrated that the highest proportion of the variation in all these characters lies on the level of the types, while variation among populations within types displayed the lowest albeit statistically significant component of variance (Table 3). Further tests of the effect of type on the seasonal characters by analyses of variance yielded statistically significant results in all of the characters on the level of both individual plants and population means (Table 4). Values of all internode parameters were lowest in the early-flowering tetraploids (Fig. 3). This applies in particular to intercalary internodes number of which mostly equalled one and reached two only in rare cases compared to 3 – 5 most frequently present in plants of the late-flowering type (Fig. 4). Nonetheless, the vegetative and total internode numbers also allowed clear identification of this type in the whole dataset. Morphological differences between the late-flowering diploid and tetraploid cytotypes were much less pronounced. The tetraploids however had significantly higher number of vegetative internodes, which was also reflected by the total internode count (Fig. 4). By contrast, these two types did not significantly differ in the number of intercalary internodes.
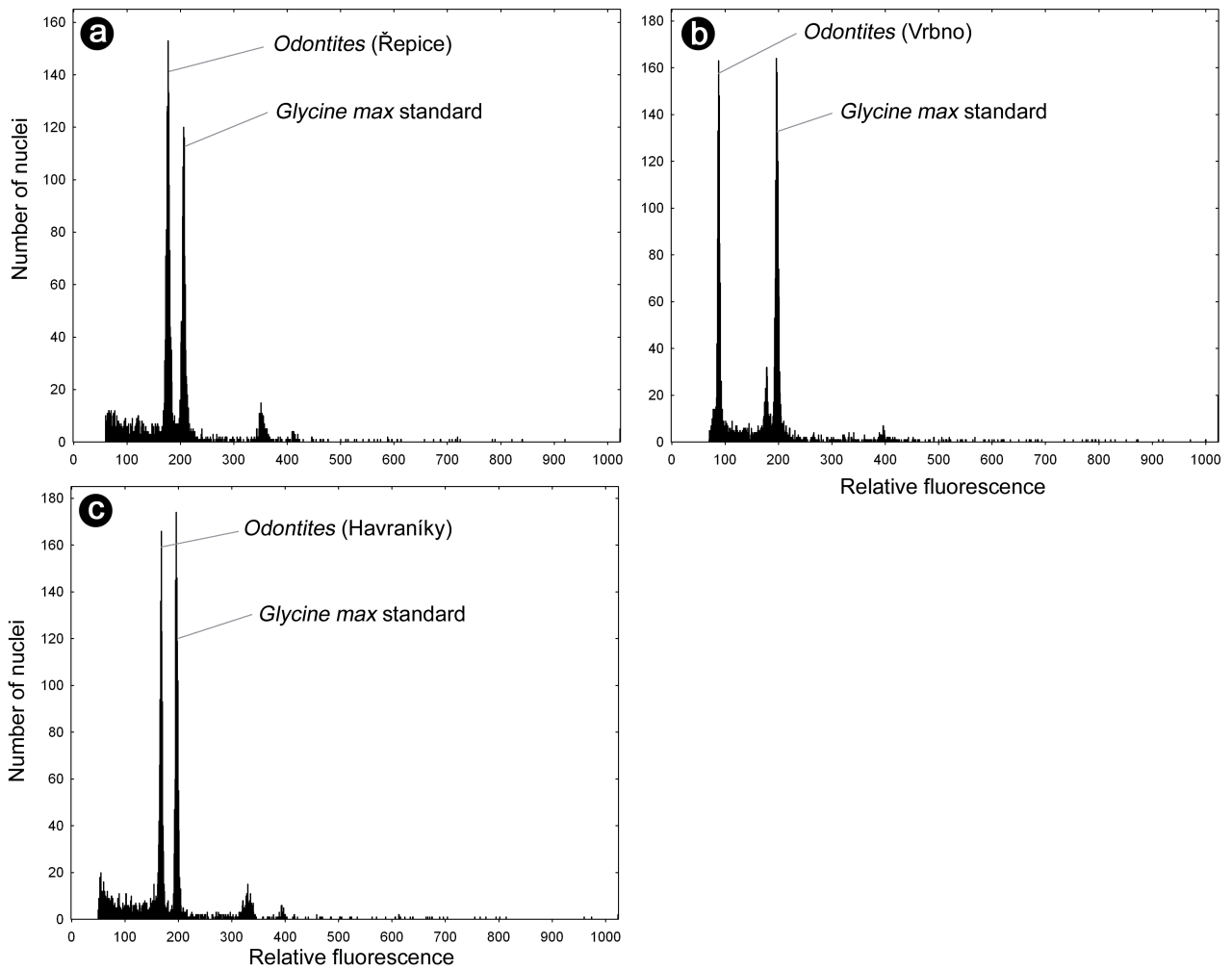
Fig. 2 Flow cytometric profiles of three phenological and cytological *types of the Odontites vernus* group occurring in Central Europe (a) early-flowering tetraploid (b) late-flowering diploid (c) late-flowering tetraploid.
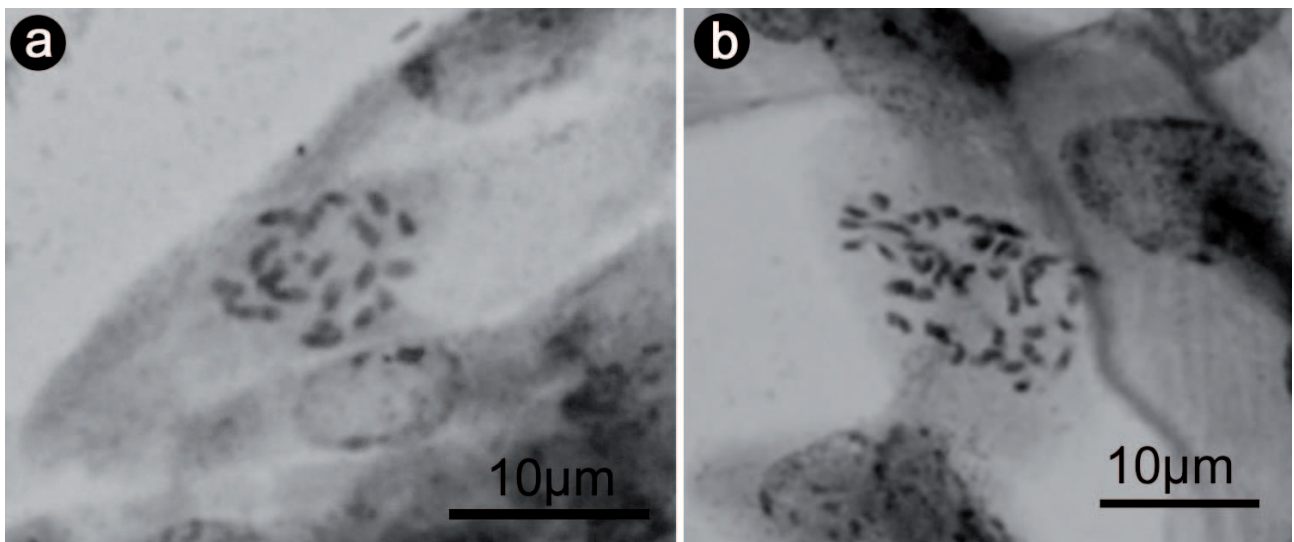

Fig. 3 Light-micrographs of mitotic metaphase chromosomes of (a) diploid (2x = 18) cytotype (population Tatinná) (b) tetraploid (2x = 40) cytotype (population Tišnov) of the *O. vernus* group.

Table 1 Genome sizes and their variability across selected population samples of the *Odontites vernus* group. C- and Cx-values are indicated in pg of DNA.

Type	Population	*n*	2C-value	Mean 2C-value	SE	Variation (%)	Mean Cx-value
Late 2x	Dětřichov	3	1.19	1.20	0.01	1.7	0.6
Late 2x	Jihlava	3	1.19				
Late 2x	Vrbno	3	1.21				
Early 4x	Cheb	3	2.31	2.35	0.02	2.4	0.59
Early 4x	Hostovice	3	2.37				
Early 4x	Řepice	3	2.36				
Late 4x	Havraníky	3	2.37	2.36	0.01	0.5	0.59
Late 4x	Tišnov	3	2.36				
Late 4x	Úhošť 1	3	2.35				

Table 2 Relative fluorescence of samples analyzed by flow-cytometry. The relative fluorescence is given as the sample:*Glycine max* standard ratio of fluorescences.

Type	*n*	Mean relative fluorescence	SE
Late 2x	227	0.442	0.001
Early 4x	63	0.848	0.001
Late 4x	40	0.860	0.001

Table 3 Distribution of variance in seasonal morphological characters in the *Odontites vernus* group inferred from variance components calculation. Tests of statistical significance of the higher hierachical levels of variation (types, populations) are displayed. Internode counts data were log-transformed prior to the statistical analysis. Statistically significant tests of variance components ($P < 0.05$) are displyed in bold.

	% Variance	Likelihood ratio	*df*	*P*
Number of vegetative internodes				
type	76.37	51.56	2	**<0.0001**
among populations	6.50	58.00	6	**<0.0001**
within populations	17.13			
Number of intrecalary internodes				
type	55.68	38.82	2	**<0.0001**
among populations	8.84	41.10	6	**<0.0001**
within populations	35.48			
Number of total internodes				
type	83.51	61.42	2	**<0.0001**
among populations	5.17	61.26	6	**<0.0001**
within populations	11.32			

Table 4 Summary of analyses of variance testing the effect of the *Odontites verrnus* types on values of seasonal characters at the levels of individual specimens and population means. The type identity is used as a single predictor in all one-way ANOVA models.Internode counts data were log-transformed prior to the statistical analysis. Statistically significant tests of variance components (P <0.05) are displyed in bold.

Morphological character	Level of testing	*df*	R^2	*F*	*P*
Number of vegetative internodes	individual plants	2, 459	0.569	303.31	**< 10⁻⁶**
	population means	2, 51	0.713	63.5	**< 10⁻⁶**
Number of intrecalary internodes	individual plants	2, 459	0.380	140.57	**< 10⁻⁶**
	population means	2, 51	0.559	32.38	**< 10⁻⁶**
Number of total internodes	individual plants	2, 459	0.693	516.96	**< 10⁻⁶**
	population means	2, 51	0.000	85.101	**< 10⁻⁶**

The geographical distribution of populations revealed contrasting patterns in ranges of individual types in the Czech Republic within the territory of which, the sampling is dense enough to be representative. The diploids are present across the whole country and were mostly found in ruderal communities or mesotrophic meadows often including those on floodplains. By contrast, the distribution of the other two types appeared restricted to particular regions of the country. The early tetraploids were found on numerous sites across South Bohemia and on two sites in North Bohemia growing always as agricultural weed or on fallows. The late tetraploids display rather clear affinity to the warm yet hilly regions of the country occurring mostly in communities that could be classified as xeric steppic grasslands. On these localities, the populations were most often found on microsites (such as pathway edges) that were at least slightly disturbed and displayed certain aspects of ruderal sites. The only exception of the general tendency of late tetraploids to grow in the warmest regions of the country was a population of late tetraploids detected on one site in South Bohemia in the region where the early tetraploids occur. Despite rather dense sampling in this area we recorded only this singular occurrence (Fig. 1).
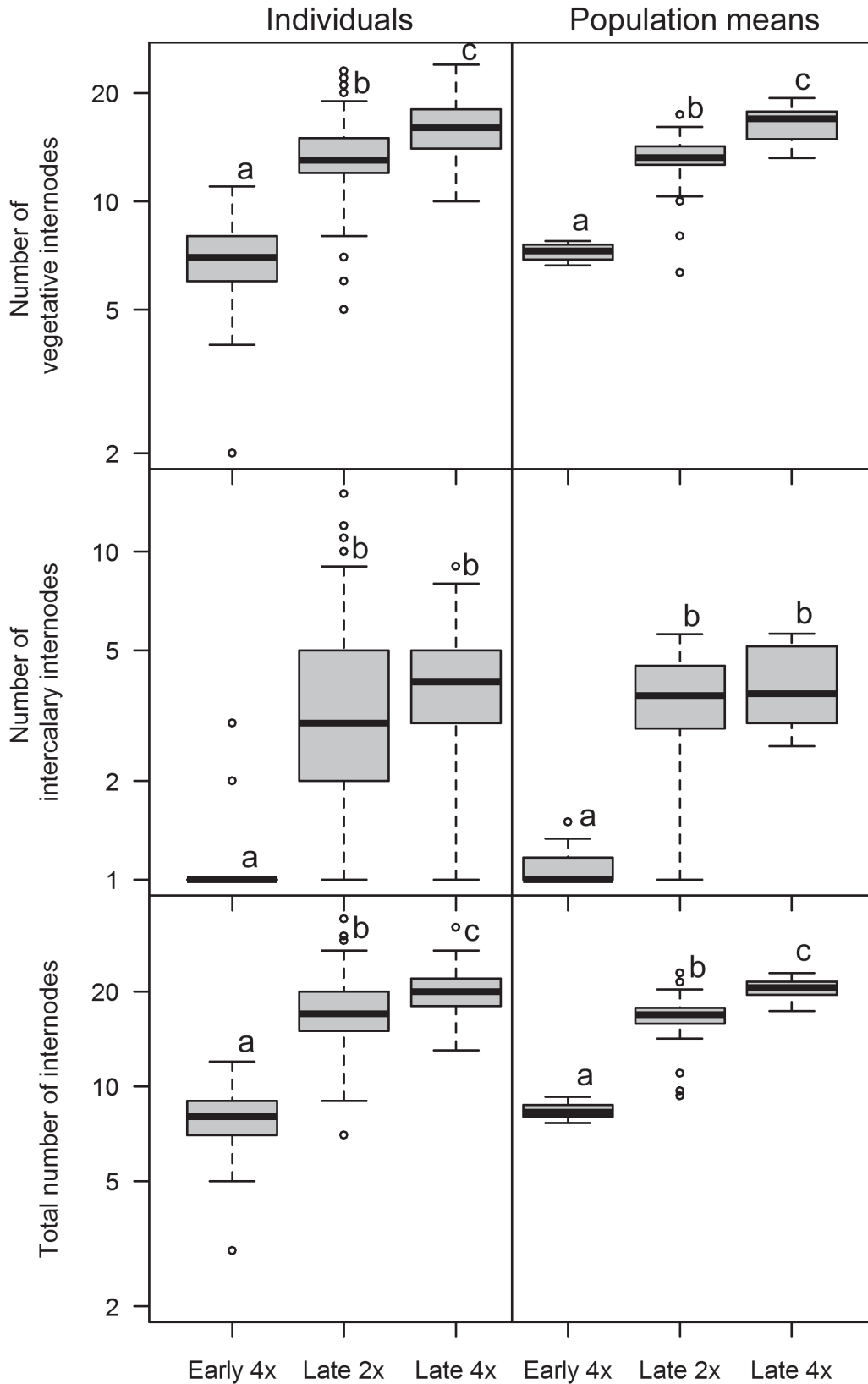
Fig. 4 Box-and-whisker plots demonstrating relationships between counts of internodes and phenological and cytological types of the *Odontites vernus* group in Central Europe displayed on the level of individual plants and mean internode counts per population. Note the logarithmic scale of the y-axes. Median, quartiles, non-outlier ranges and outliers are depicted as bold line, boxes, whiskers and points respectively. Different letters at the upper limit of the non-outlier range indicate statistically significant ($P < 0.05$) difference inferred from post-hoc Tukey HSD multiple

comparison tests applied to ANOVA models summarized in Table 4.

Discussion

Cytotypic variation

The extensive screening of ploidy level in populations of the *Odontites vernus* group in Central Europe by FCM suggests necessity of a substantial update of the concept of the *O. vernus* group. For the first time, our study has demonstrated existence of late-flowering tetraploid populations in the *O. vernus* group. This type in not only a new addition to the flora of Central Europe but also presents hitherto undescribed entity in the group at the global scale. The late-flowering tetraploids apparently present a termophilous floristic element suggesting a possibility of its range extending substantially in the southerly direction within the range of the group, which reaches as south as the Mediterranean coast (Bolliger 1996). This probably explains why it has never been recorded in any earlier study since most of these focused on the *O. vernus* group in Northern Europe (e.g. Schneider 1964, Snogerup 1983).

Apart from revealing the existence of the late-flowering tetraploids, the FCM screening has supported the other aspects of the current concept of the *O. vernus* group. All early-flowering plants were confirmed as tetraploids while all diploids were late-flowering. In addition, the sites of occurrence of both of these types comply with the description of their ecology in local floras (Kubát 2000, Michalková 1997) and monographs based mainly on northern European populations (Schneider 1964, Snogerup 1983). In the diploid type, we did not detect any variation in the relative fluorescence that would correspond to the reported existence of two chromosome numbers $2n = 18$ and $2n = 20$ (cf. Schneider 1964 and Snogerup 1983). Therefore, all of the analyzed diploid populations can be assumed to have $2n = 18$ chromosomes.

Seasonal variation

The analysis of the seasonal morphological characters has supported the clear delimitation of the early- and late-flowering types on the basis of the internode numbers. In particular, the intercalary internode number presents a clear-cut identification character in this respect as suggested by previous studies (Schneider 1964, Snogerup 1983, Bolliger 1996, Kubát 2000). In general, the overlaps in the internode numbers between the early and late types appear very restricted. This applies especially on the tetraploids within which the seasonal types are clearly separated on the basis of morphology. On the other hand, late diploids and tetraploids cannot be clearly delimited from each other on the basis of the internode numbers (Fig. 3). Despite this lack of clear separation, the tetraploids of the analyzed populations had significantly higher vegetative internode number than the diploids which was also reflected by the total internode counts.

In general, the tetraploid types appear highly specialized in the seasonal characters forming clearly distinct ecotypes comparable e.g. to some of the most extreme ecotypes present in *Euphrasia rostkoviana* in the Alps (Zopfi 1998b). The values of the seasonal morphological characters in the diploid type appear positioned in between those of the tetraploid seasonal ecotypes and also to display more variability. The diploids hence display greater plasticity in the seasonal variation coinciding with their broad ecological range but apparently lack any tendency to form distinct seasonal ecotypes.

Geographical distribution and breeding barriers

The diploid type to the *O. vernus* group is an omnipresent species in the Central European landscape (Kubát 2000); however the distribution of the tetraploid types is very different. Although we revealed a fairly restricted geographical range of the early tetraploids (Fig, 1), it is quite likely that this type was be much more abundant in the past due to numerous historical records throughout the Czech Republic (Kubát 2000). Intensification of agriculture and improved weed control has however resulted in its extinction in most of the country. In the past, however, its geographical range was probably as broad as that of the diploids. By contrast, the late-flowering tetraploids clearly tend to occur in regions with warm and rather continental climate. Such range limit is probably related with their high number of internodes resulting in a requirement of long growth

season to complete the life cycle. Even the South-Bohemian late tetraploid population occurring outside these warm regions was found in a district that displays rather mild climate. Interestingly, both habitat preferences and geographical range of the late-flowering tetraploid type correspond closely to those of *O. luteus* which is a rather distantly related species that could be also considered as a distinct autumnal type flowering in August-September (Bolliger 1996, Kubát 2000).

In general, the geographical distribution patterns of the group can be considered mostly sympatric (early tetraploids – diploids, late tetraploids – diploids) or sympatric-parapatric (early tetraploids – late tetraploids) though more data are needed to shape a more definite and accurate picture distribution patterns. Due this level of sympatry, the principal barriers preventing inter-breeding between the types therefore consist of incompatibility due to different ploidy level (Snogerup 1983) and habitat and phenological differentiation of the tetraploid types which rarely share similar habitats and their flowering seasons virtually do not overlap.

Taxonomic conclusion

In general, the pattern of variation in the *O. vernus* group appears to comply better with the two-species concept recognizing tetraploid *O. vernus* and diploid *O. vulgaris* at the species level (Bolliger 1996). The existence of the two seasonal ecotypes which are moreover likely to be reproductively isolated could be reflected by a description of the late ecotype as a new taxon at the subspecies level. Apparently, the treatment currently used e.g. in the Flora of the Czech Republic (Kubát 2000) delimiting the tetraploid and diploid plants as *O. vernus* subsp. *vernus* and subsp. *serotinus* fails to handle with the late-flowering tetraploid plants. From this perspective, this concept appears outdated.

Wider perspectives

In this study, we have clearly demonstrated the cytotypic variation, its relationship to the seasonal variation and the patterns of geographical distribution of cytotypes and seasonal types of the *Odontites vernus* group in Central Europe. New questions have however arisen on the evolutionary origin of these types variability. Given the almost constant monoploid genome size (Cx-value) across all types and lack of any co-occurring closely related *Odontites* species as a potential partner for hybridization (Bolliger 1996), it appears likely that the tetraploids originated via autopolyploidy and (Trávníček et al. 2010). On the other hand different base chromosome numbers ($x = 9$ vs. $x = 10$ in tetraploids and diploids respectively) and reported variability in this character in the diploid indicate a possibility of a more complicated process during the evolution of tetraploids. The evolutionary relationships between the tetraploids present another appealing question. Both scenarios of either independent origin of early- and late- flowering tetraploids or a single polyploidization event and subsequent differentiation of the seasonal types can be hypothesized.

Acknowledgements

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