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ORIGINAL ARTICLE

Ecotypes and genetic structure of *Rhinanthus alectorolophus* (Orobanchaceae) in southwestern Germany

Thekla Pleines · Korinna Esfeld · Frank R. Blattner · Mike Thiv

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Abstract European annual species of the genus *Rhinanthus* often exhibit seasonal ecotypic variation, a phenomenon also known from related genera of hemiparasitic Orobanchaceae. Populations with different flowering times exist, correlated with differences in a number of morphological characters. The present study evaluates the correlation of morphological characters and genetic differentiation of populations of *Rhinanthus alectorolophus*. Thirty-nine populations of three different subspecies from southwestern Germany were sampled. A total of 798 individuals were used for morphological analyses and 187 of these for AFLP analyses. Principal component analysis showed that morphological variation is mostly continuous. In a discriminant analysis based on morphological characters, only 89.7 % of all individuals were correctly assigned to their previously determined subspecies, indicating that subspecies identification is ambiguous for some populations. Using AFLP data and Bayesian assignment analysis, the sampled individuals could be grouped in three genetic clusters which do not correspond to the three subspecies. Instead, the clustering shows a clear geographic pattern and a Mantel test likewise revealed a significant correlation between genetic and geographic distances. Correlations of genetic distances with differences in morphological characters were weak and mostly insignificant. The results indicate that the subspecies of *R. alectorolophus* do not form discrete entities and that the character combinations distinguishing them are homoplastic.

Keywords AFLP · Isolation by distance · Morphology · Orobanchaceae · *Rhinanthus alectorolophus* · Ecotypes · Subspecies

Introduction

Many European annual species of hemiparasitic Orobanchaceae, especially in the genera *Rhinanthus*, *Euphrasia*, and *Melampyrum*, exhibit high ecotypic variability (Karlsson 1974). Populations with different flowering times and differences in associated morphological characters can be found. Early-flowering populations have fewer and longer internodes, as well as fewer branches and intercalary leaves compared to late-flowering populations. This phenomenon was described by Wettstein (1895) as "seasonal dimorphism". Wettstein interpreted it as the result of adaptation to different kinds of meadows, which led to very recent speciation events. However, in most species more than two seasonal forms can be recognized. In addition to morphological characters correlated with flowering time, there are differences between populations from different elevations. For example, forms from higher elevations (montane or alpine forms) often consist of smaller plants with fewer branches than lowland forms (e.g., Sterneck 1901). Because of the complicated morphological patterns not

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T. Pleines (✉) · K. Esfeld · M. Thiv
Department of Botany, Stuttgart State Museum of Natural History, Stuttgart, Germany
e-mail: thpleines@yahoo.de

K. Esfeld
Institute of Plant Sciences, University of Bern, Bern, Switzerland

F. R. Blattner
Departmentof Genebank, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany

only related to flowering time, Soó (1929) preferred to use the term "pseudoseasonal polymorphism".

Some authors doubt if the seasonal forms are distinguishable from each other at all. For example, Soó (1929) mentioned that the pseudoseasonal forms in *Rhinanthus* are connected with each other by a nearly continuous spectrum of intermediate forms. This view was similarly expressed by other authors (e.g., Karlsson 1974; Campion-Bourget 1982). Karlsson (1974) argued that morphological variation is more or less continuous, and that the described ecotypes are forms which are adapted to more widespread habitats.

There are two main hypotheses on the time of origin of ecotypes in Orobanchaceae. Most authors regarded the seasonal ecotypes in hemiparasitic Orobanchaceae as recently evolving forms, linked to the origin of different meadow types within historical times (e.g., Wettstein 1895, 1900; Karlsson 1974). Alternatively, the forms could be much older, e.g., dating back at least to the last ice-age, so that several pre-adapted ecotypes existed before the species migrated into anthropogenic habitats [Soó 1929; Schwarz 1935; Krause 1944; Bolliger 1989 for *Odontites lanceolata* (Gaudin) Reichenbach].

Several experiments have been conducted in *Rhinanthus* to investigate the genetic fixation of characters linked to ecotypes and the impact of environmental factors on these morphological traits. Campion-Bourget (1982) studied plants of *Rhinanthus alectorolophus* (Scop.) Pollich from four different ecotypes (lowland aestival, autumnal, and intermediate ecotypes, and a montane autumnal ecotype) cultivated on different hosts and found significant differences in plant vigor depending on the host species. Most of the studied morphological characters important for the classification were altered ex situ compared to natural conditions, although some differences between ecotypes could still be detected in culture on the same hosts. Campion-Bourget (1982) concluded that the morphological variability of *R. alectorolophus* is mainly caused by the host plants. However, four characters, e.g., the numbers of internodes and of intercalary leaves, retained stable differences between ecotypes in culture. In contrast, flowering times were similar for all ecotypic variants under identical growing conditions. In addition, crossing experiments showed that flowering time and the number of internodes are genetically heritable and intermediate individuals result from crosses between individuals from early- and late-flowering populations (Campion-Bourget 1982). Zopfi (1993b) conducted cultivation experiments using Swiss *R. alectorolophus* populations. Important morphological features, in particular the number of internodes, were conserved under different meadow management regimes and on different hosts. Thus, these adaptations can be assumed to have a genetic background and to be, at least to a certain extent, stable. However, Zopfi (1993b) reported an obvious influence of the host plants on the rate of development as well. Similar conclusions were also drawn from studies on other species, such as *Rhinanthus glacialis* Personnat (Zopfi 1995) and *Rhinanthus serotinus* (Schönh.) Oborny (Mizianty 1978).

So far, the relationships between morphological and seasonal variation and environmental factors are not well understood. Accordingly, the taxonomic treatment of such ecotypic forms has long been a topic of debate. Among Rhinantheae genera, different taxonomic treatments have been proposed by several authors. In *Rhinanthus*, many ecotypes have originally been described as species, however mostly as part of species groups or species in the broader sense (e.g., Wettstein 1895, 1900; Sterneck 1901). Soó (1929) treated them as subspecies, whereas Mizianty (1978) preferred the varietal rank.

Not much is known about the genetic structure of ecotypes in hemiparasitic Orobanchaceae. To our knowledge, the only study is by Kolseth and Lönn (2005), who conducted a population genetic study of *Euphrasia stricta* J.P.Wolff ex J.F.Lehm. on Gotland (Sweden) and found three genetically separated groups. Two of these groups corresponded to two early-flowering varieties, whereas three later-flowering varieties were combined in the third group. This indicates that seasonal forms might indeed be genetically separated.

To investigate whether this is a general phenomenon, we studied populations of *R. alectorolophus* (Scop.) Pollich in southwestern Germany. This species is annual and uses a variety of plants as hosts, especially Poaceae and Fabaceae species (Zopfi 1993a, b). The main pollinators are bumblebees (Kwak 1977), and the seeds are usually not dispersed very far (Bullock et al. 2003). Chromosome counts for *R. alectorolophus* revealed $2n = 22$, with 14 large and 8 small chromosomes (Löve 1982), with no indication of polyploidy, This species is highly polymorphic, exhibiting ecotypic variation as described above. Wettstein (1900) described an early-flowering taxon [*Alectorolophus alectorolophus* (Scop.) Stern.], a late-flowering taxon (*Alectorolophus patulus* Stern.), and an alpine taxon (*Alectorolophus kerneri* Stern.) within *R. alectorolophus* sensu lato. Sterneck (1901) additionally recognized *Alectorolophus modestus* Stern. and *Alectorolophus ellypticus* Stern. within this group, as well as *A. alectorolophus* subsp. *medius* and subsp. *buccalis*. Later authors accepted varying numbers of taxa, mostly under the genus name *Rhinanthus*, and preferred different taxonomic levels for them (for a review see Zopfi 1993a). In Germany, Zopfi (2002) recognized six subspecies—*R. alectorolophus* subsp. *alectorolophus*, subsp. *buccalis* (Wallr.) Schinz and Thell., subsp. *kerneri* (Stern.) Soó, subsp. *modestus* (Chabert) Soó, subsp. *patulus* (Stern.) Soó, and subsp. *semleri* (Stern.) Soó. This

classification by Zopfi (2002) is used in the present study of *R. alectorolophus* in the German federal state of Baden-Württemberg where the species occurs in almost all parts except for the Black Forest region. In a study of *R. alectorolophus* in Switzerland, Zopfi (1993a) found seven morphologically different ecotypes, correlated with different meadow and grassland habitats. Not all of these ecotypes corresponded to formally described subspecies, but the author refrained from giving formal names to the groups he found. In this study, we used this alternative morphological classification into seven informal ecotypes to see whether our results are robust.

The following questions are addressed in this study: (1) can morphological entities be found which correspond to the three subspecies or five informally named ecotypes found in *R. alectorolophus* in the study area? (2) Do these groups reflect genetic units indicating that the ecotypes have evolved only once? Or, (3) if the morphological entities are not genetically distinct groups, is the genetic divergence correlated with geographic distance indicating recently evolved taxa of multiple origins?

Materials and methods

Selection of taxa

Samples from 39 populations of *R. alectorolophus* were collected in spring and summer 2004 (Table 1, Fig. 1). These populations were distributed all over Baden-Württemberg to cover the geographical variation within the study area (Fig. 1). At the same time we aimed to include all a priori distinguishable subspecies and informal ecotypes. Analyses of herbarium specimens (mostly at Stuttgart State Museum of Natural History, herbarium STU) revealed that *R. alectorolophus* subsp. *alectorolophus*, subsp. *semleri*, and subsp. *patulus* can be considered native in Baden-Württemberg. These three subspecies were also sampled in our study. Subspecies *alectorolophus,* with five to nine non-flowering internodes, is described as flowering in May and June and growing in different types of meadows (Zopfi 2002). The populations of this subspecies in our study were collected in May and June from hay meadows, traditional orchards, other meadows, and in one case at the edge of a forest. Subspecies *semleri* has 10–12 internodes below the inflorescence and is said to flower in July and August and to grow in montane meadows (Zopfi 2002). The populations in our study were collected between May and the beginning of July from roadside grasslands, dry grasslands, a former heathland, a traditional orchard, and other meadow habitats. The three populations assigned to subsp. *patulus* in our study were collected between end of June and beginning of August from the edge of a forest, grassland on a levee, and a roadside grassland. According to Zopfi (2002), this subspecies flowers between July and September and occurs in montane meadows. However, because the subspecies and their distribution in Germany are not well studied, these descriptions might not be complete.

The five informal ecotypes described by Zopfi (1993a) that could be detected in our study partly correspond to formally described subspecies ("alectorolophus", "semleri", and "patulus"), whereas the ecotypes "mesobromion" and "paludosus" are partly intermediate to the subspecies or exhibit other character combinations. Ecotype "mesobromion" is described as growing in nutrient-poor colline or montane meadows and flowering in June (Zopfi 1993a). This ecotype has more and shorter internodes below the inflorescence than ecotype "alectorolophus". Ecotype "paludosus" is described from colline or montane litter meadows that are rather damp and flowers from June to early July (Zopfi 1993a). The plants are higher than those of ecotype "semleri" (Zopfi 1993a). Table 1 shows which informal ecotype and subspecies each population was assigned to.

To test whether the results are influenced by the taxonomic subspecies classification scheme, the analyses were repeated with an alternative classification using the informal ecotypes defined by Zopfi (1993a, b) in Swiss *R. alectorolophus.* Five of these ecotypes were identified in our sample—"alectorolophus", "mesobromion", "paludosus", "semleri", and "patulus".

About 20 plants per population were collected. Voucher specimens are deposited in STU.

Morphological data

Fourteen quantitative morphological characters (Table 2) were measured in a total of 798 dried individuals of all populations except population 15. That site had been mowed early, so that the main inflorescence of most individuals was lacking, and important characters could not be measured. Furthermore, six ratios were calculated from the morphological data to include some relative values (Table 2). Mostly, the same characters as analyzed by Zopfi (1993a) were used to make both studies comparable. In addition, we measured some bract characters (bract length, width, and shape, and the number of bract teeth; see Table 2). The morphological data matrix can be obtained from the corresponding author upon request.

AFLP data

Total genomic DNA was extracted from 187 individuals (four to five individuals per population except population one, which was not sampled for genetic analysis). The

Table 1 Sampling information about the 39 populations of *Rhinanthus alectorolophus* from Baden-Württemberg included in this study

Population	Individuals in morphological dataset	Individuals in AFLP dataset	Collection date	Voucher (STU)	Subspecies (Zopfi 2002)	Infomal ecotype (Zopfi 1993a)
P01	13	–	2004	*Sauer s.n.*	*alectorolophus*	"alectorolophus"
P02	23	5	24/5/2004	*Pleines 104*	*alectorolophus*	"alectorolophus"
P03	23	5	15/6/2004	*Pleines 114*	*semleri*	"semleri"
P04	20	5	17/5/2004	*Pleines 102*	*alectorolophus*	"alectorolophus"
P05	28	5	20/5/2004	*Pleines 103*	*alectorolophus*	"alectorolophus"
P06	19	5	17/5/2004	*Pleines 100*	*semleri*	"semleri"
P07	22	5	24/5/2004	*Pleines 105*	*alectorolophus*	"alectorolophus"
P08	30	5	18/5/2004	*Thiv 4003*	*alectorolophus*	"alectorolophus"
P09	23	5	1/6/2004	*Pleines 106*	*semleri*	"paludosus"
P10	18	5	7/7/2004	*Pleines 129*	*semleri*	"paludosus"
P11	20	5	16/6/2004	*Wörz 24.06.16.03*	*alectorolophus*	"alectorolophus"
P12	22	5	24/6/2004	*Pleines 119*	*semleri*	"paludosus"
P13	22	5	4/8/2004	*Pleines 210*	*patulus*	"patulus"
P14	20	5	11/5/2004	*Thiv 4001*	*semleri*	"mesobromion"
P15	–	5	22/6/2004	*Pleines 117*	?	?
P16	22	5	22/6/2004	*Pleines 118*	*patulus*	"paludosus"
P17	20	4	11/6/2004	*Thiv 4011*	*alectorolophus*	"alectorolophus"
P18	20	5	4/7/2004	*Pleines 128*	*patulus*	"paludosus"
P19	20	5	8/6/2004	*Joßberger s.n.*	*alectorolophus*	"mesobromion"
P20	19	4	8/6/2004	*Joßberger s.n.*	*alectorolophus*	"alectorolophus"
P21	20	4	3/6/2004	*Wörz 24.06.03.02*	*alectorolophus*	"alectorolophus"
P22	20	5	8/6/2004	*Joßberger s.n.*	*semleri*	"semleri"
P23	20	5	26/5/2004	*Engelhardt s.n.*	*alectorolophus*	"alectorolophus"
P24	25	5	1/7/2004	*Pleines 127*	*semleri*	"paludosus"
P25	19	5	1/7/2004	*Pleines 126*	*semleri*	"paludosus"
P26	22	5	29/6/2004	*Pleines 121*	*semleri*	"paludosus"
P27	22	5	2/6/2004	*Thiv 4010*	*alectorolophus*	"mesobromion"
P28	20	5	15/6/2004	*Joßberger s.n.*	*semleri*	"semleri"
P29	20	5	11/5/2004	*Thiv 4002*	*alectorolophus*	"mesobromion"
P30	20	5	9/6/2004	*Pleines 112*	*alectorolophus*	"alectorolophus"
P31	22	5	29/6/2004	*Pleines 122*	*semleri*	"paludosus"
P32	21	5	9/6/2004	*Pleines 113*	*alectorolophus*	"alectorolophus"
P33	20	5	15/6/2004	*Joßberger s.n.*	*semleri*	"paludosus"
P34	21	5	31/5/2004	*Thiv 4005*	*alectorolophus*	"alectorolophus"
P35	22	5	30/5/2004	*Thiv 4004*	*semleri*	"semleri"
P36	21	5	16/6/2004	*Herwanger s.n.*	*semleri*	"paludosus"
P37	19	5	7/6/2004	*Koch s.n.*	*semleri*	"mesobromion"
P38	20	5	17/6/2004	*Koch s.n.*	*semleri*	"semleri"
P39	20	5	21/6/2004	*Herwanger s.n.*	*alectorolophus*	"mesobromion"

Vouchers are deposited in herbarium STU

extraction was done using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

The AFLP protocol followed Vos et al. (1995) with minor modifications. After testing 12 primer combinations on eight individuals, five combinations of *Eco*RI primer E01 (5′-GACTGCGTACCAATTCA-3′) and *Mse*I primers M02 (5′-GATGAGTCCTGAGTAAC-3′) were chosen: E32(E01 + AC)/M48(M02 + AC), E32/M50(M02 + AT'), E37(E01 + CG)/M47(M02 + AA), E37/M48, and E37/M49(M02 + AG). The selective amplification products were separated on a 6 % polyacrylamide gel on an ABI Prism 377 automated sequencer, together with an internal standard (GeneScan 500 ROX, Applied Biosystems). Three

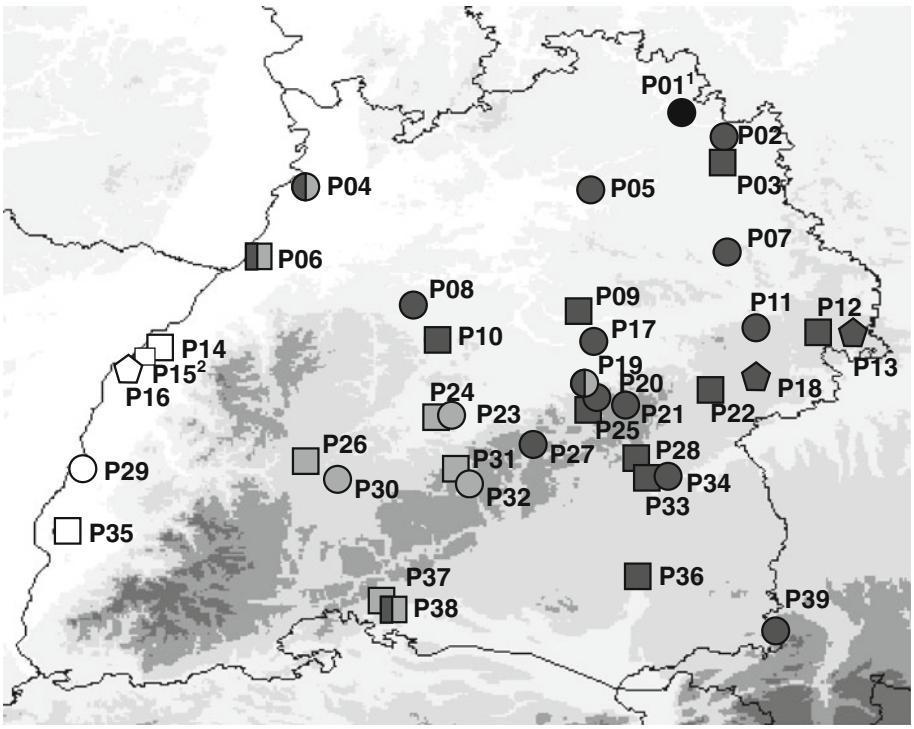

Fig. 1 Map of Baden-Württemberg with location of the 39 populations used in this study. *Circles*: *Rhinanthus alectorolophus* subsp. *alectorolophus*; *squares*: subsp. *semleri*; pentagons: subsp. *patulus*. *Colors* are according to genetic clusters obtained by Bayesian assignment analysis based on AFLP data. *White*: genetic cluster A; *gray*: genetic cluster B, *dark gray*: genetic cluster C. *1:* population without genetic data; 2: population without morphological data

Table 2 Characters used for morphometric analyses of *Rhinanthus alectorolophus* in southwestern Germany

Character	Explanation
Plant height (cm)	
Plant height without inflorescence (cm)	
Length of lowest five internodes (cm)	
Number of sterile internodes	Number of internodes below the inflorescence
Number of fertile branches	Number of fertile primary branches
Number of sterile branches	Number of sterile primary branches
Number of intercalary leaves	Number of leaves between uppermost branches and lowest flower
Stem leaf length (cm)	Length of uppermost stem leaf
Stem leaf width (cm)	Width of uppermost stem leaf
Number of stem leaf teeth	Number of teeth of uppermost stem leaf
Bract length (cm)	Length of middle bract
Bract width (cm)	Width of middle bract
Number of bract teeth	Number of teeth of middle bract
Corolla length (cm)	
Average internode length (cm)	= Plant height without inflorescence/number of sterile internodes
Internode to leaf length ratio	= Average internode length/stem leaf length
Proportion of lowest five internodes	= Length of lowest 5 internodes/plant height without inflorescence
Inflorescence to plant height ratio	= Inflorescence length/plant height
Stem leaf shape	= Stem leaf length/stem leaf width
Bract shape	= Bract length/bract width

For ratios, the calculation from measured characters is given

samples per primer pair were replicated with a new PCR. Automatic lane tracking was performed in GeneScan 3.1 (Applied Biosystems) and refined by hand. Bands were scored manually using the software Genotyper 2.4 (Applied Biosystems) and a presence/absence matrix was created (available from the corresponding author upon request).

Data analysis

The morphometric measurements resulted in complete data with no missing values for 553 individuals. This dataset was used to conduct a principal components analysis (PCA) on scaled variables using the software R 2.9.0 (R Core Team 2009). Beside this analysis of single individuals, we conducted a PCA on population means for each character, since a PCA is likely to confound within- and between-population variation when single individuals are analyzed (James and McCulloch 1990). For both PCAs, two variables were excluded when there were strong correlations (>0.9) between variables. This was the case for plant height (strongly correlated with plant height without inflorescence) and the length of the lowest five internodes (strongly correlated with average internode length).

Discriminant analyses on the same individual data and population means were performed in SPSS v.16 (SPSS Inc.) to test a priori classifications into three subspecies or five informal ecotypes, as well as the assignment to one of the three genetic clusters obtained from the Bayesian assignment analysis. Group probabilities were calculated from the given frequency in the sample. Apart from simple reclassification, a leave-one-out cross-validation approach was used. In this approach, a discriminant analysis is performed for each individual so that this individual is not used for the calculation of discriminant function but is classified with only the information based on all other individuals.

Based on the AFLP matrix, Nei-Li distances (Nei and Li 1979) between samples were calculated in Treecon v.1.3b (Van de Peer and de Wachter 1994). Samples P8-3, P9-2, P13-3, P14-2, P16-3, and P21-4 were excluded because of missing data. Based on this distance matrix, a principal coordinates analysis (PCO) was performed in SPSS.

The AFLP data were furthermore analyzed with the software AFLP-SURV v.1.0 (Vekemans et al. 2002), calculating Nei's D distances between populations after Lynch and Milligan (1994), as well as 1,000 bootstrap replicates. A neighbor-joining tree was constructed using the NEIGHBOR program of the Phylip v.3.69 software package (Felsenstein 2005).

Using the software Structure v.2.3.2 (Pritchard et al. 2000; Falush et al. 2007), the number of genetic groups was determined through Bayesian assignment analysis. An admixture model and an initial value of the parameter for degree of admixture $\alpha = 1.0$ were used in this analysis. Allele frequencies were considered to be independent. The burn-in length was set to 100,000, followed by 200,000 iterations for $K = 1$ to $K = 12$. In Arlequin v.3.5.1.2 (Excoffier and Lischer 2010), analyses of molecular variance (AMOVA) were performed with 1,000 permutations and pairwise genetic differences.

To evaluate possible correlations between genetic distances and differences in single morphological characters, Mantel tests were performed in R using the respective function of the package ade4 v.1.4-11 (Dray and Dufour 2007). These tests were performed with 1,000 replications, including all individuals with genetic and the relevant morphological data. Further, Mantel tests were conducted to evaluate correlation between genetic and geographic distance. These analyses were done using the whole dataset of genetically fingerprinted individuals as well as within the genetic clusters defined by Structure. In the latter case, admixed populations were assigned to the genetic cluster of which they had the largest proportion.

Results

For the state of Baden-Württemberg, we identified three *Rhinanthus* subspecies, namely *R. alectorolophus* subsp. *alectorolophus*, subsp. *semleri*, and subsp. *patulus* in our field collections. Of the informal ecotypes defined by Zopfi (1993a, b), five were identified in our sample. Since our results were similar for both morphological classifications, we restrict the results to those obtained by classification into three subspecies.

Morphological variation among individuals

The PCA on morphological data on individuals without missing data resulted in five components with an eigenvalue >1, explaining 75.5 % of the variance in the dataset. The first principal component (PC 1) is mainly influenced by characters concerning the length and number of internodes up to the inflorescence (number of internodes, proportion of the lowest five internodes, length of the lowest five internodes, and average internode length). PC 2 shows a high correlation to leaf characters (stem leaf length, bract length and width, and number of stem leaf teeth). PC 3 is influenced by bract characters (number of bract teeth and bract shape), as well as the number of intercalary leaves, whereas PC 4 and PC 5 are mostly influenced by the inflorescence to plant height ratio and number of intercalary leaves, respectively. Scatterplots of the first two components, explaining 25.0 and 23.1 % of the variance, respectively, are shown in Fig. 2a and b. In the scatterplots, no well-defined clusters are visible. Although the subspecies occupy different parts of the plot, there is considerable overlap (Fig. 2a). When the same samples are coded according to genetic clusters derived from Bayesian assignment analysis, no structure is visible at all, and the clusters appear mixed (Fig. 2b).

In the discriminant analysis based on the individual dataset without missing values, 89.7 % of all individuals

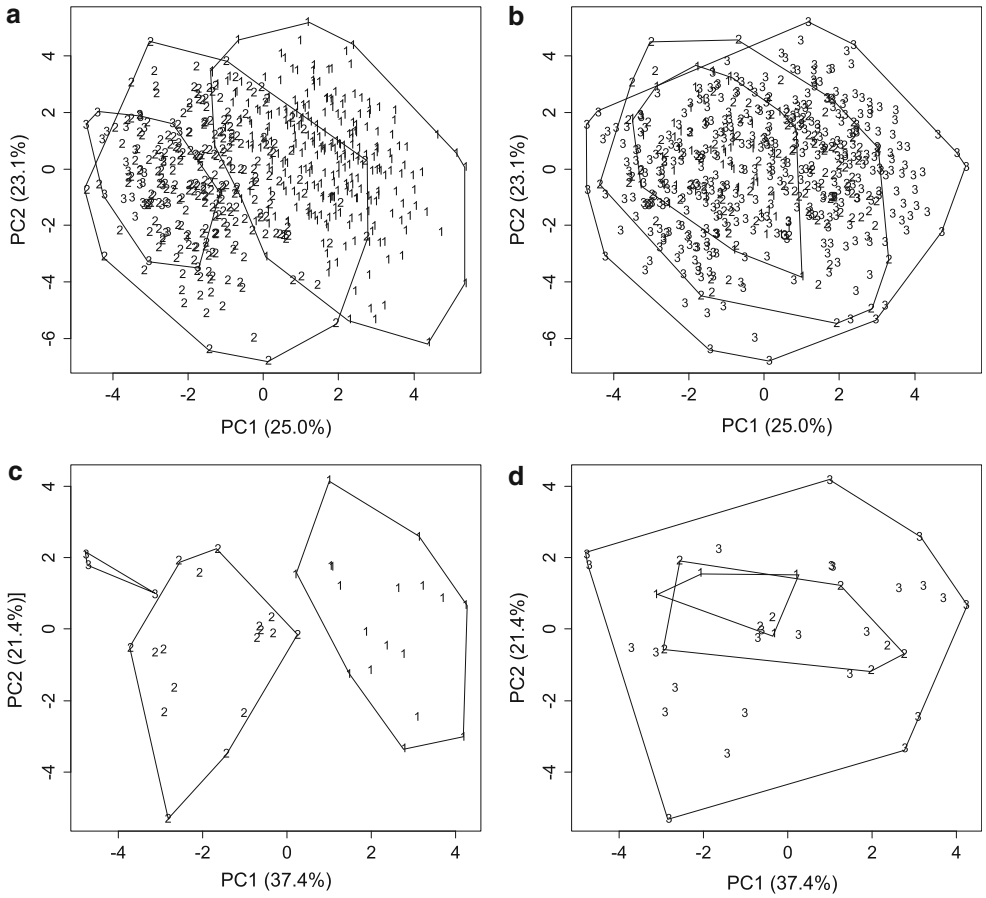

Fig. 2 First two principal components of PCAs on data from 20 morphological characters and ratios of 553 individuals (**a**, **b**) and population means (**c**, **d**) of *Rhinanthus alectorolophus* from Baden-Württemberg. Data points are labeled according to subspecies (**a**, **c**. *1*, subsp. *alectorolophus*; *2*, subsp. *semleri*; *3*, subsp. *patulus*) or genetic cluster derived from Bayesian assignment analysis (**b**, **d**. *1*, cluster A; *2*, cluster B; *3*, cluster C)

were correctly assigned to their respective subspecies (88.2 % when cross-validation was performed, see Table 3). Wilks' lambda values show how much the a priori groups differ in a certain variable, lower values indicating more differentiation. The smallest Wilks' lambda values for the discrimination between subspecies

Table 3 Results from discriminant analyses for subspecies of *Rhinanthus alectorolophus* in southwestern Germany using individual as well as population means data

Subspecies	Predicted group membership			Total	% Correctly classified
	ssp. *alect*	ssp. *semleri*	ssp. *patulus*		
Individuals					
ssp. *alect*	263/262	11/12	0/0	274	96.0/95.6
ssp. *semleri*	25/27	205/199	12/16	242	84.7/82.2
ssp. *patulus*	0/0	9/10	28/27	37	75.7/73.0
Total	288/289	225/231	40/43	553	89.7/88.2
Population means					
ssp. *alect*	18/17	0/1	0/0	18	100.0/94.4
ssp. *semleri*	0/4	17/9	0/4	17	100.0/52.9
ssp. *patulus*	0/0	0/1	3/2	3	100.0/66.7
Total	18/17	17/11	3/6	38	100.0/73.7

Predicted group memberships and percentages of correctly classified cases are given for original grouped cases (first number) as well as cross-validated grouped cases (second number)

were found for the number of sterile internodes (Wilks' lambda = 0.321, $p < 0.001$), the proportion of the lowest five internodes (Wilks' lambda = 0.513, $p < 0.001$), and the length of the lowest five internodes (Wilks' lambda = 0.699, $p < 0.001$). The discriminant analysis using the same data but grouping into three genetic clusters revealed by the Structure analysis was correct for 71.8 % of all individuals (70.1 % with cross-validation, see Table 4). This analysis showed less significant results than the ones for the discrimination between subspecies. The most important characters for the distinction between the three genetic clusters, receiving lowest Wilks' lambda values, are length of the lowest five internodes (Wilks' lambda = 0.929, $p < 0.001$), average internode length (Wilks' lambda = 0.951, $p < 0.001$), and proportion of the lowest five internodes (Wilks' lambda = 0.953, $p < 0.001$). These characters were also important for the distinction between the morphologically defined subspecies, but their higher Wilks' lambda values show less differentiation between the genetic clusters in these characters.

Morphological variation among populations

The PCA on population means of morphological data resulted in four principal components with an eigenvalue >1. These components explain 84.6 % of the variance. The most important characters for PC 1 are characters related to length and number of internodes, i.e., number of internodes, proportion of lowest five internodes, average internode length, and internode length to leaf length ratio. The main characters influencing PC 2 describe leaf characters (e.g., stem leaf length, bract length, and number of stem leaf teeth). PC 3 is mostly influenced by bract index, number of bract teeth, and the number of intercalary leaves, whereas plant height without inflorescence and the inflorescence index are important for PC 4. Scatterplots using the first two components are shown in Fig. 2c and d. The subspecies are relatively well separated, but they do not form distinct clusters (Fig. 2a). There is no visible structure if genetic clusters inferred by Bayesian assignment analysis are considered (Fig. 2b).

The discriminant analysis showed that 100 % of the populations (73.7 % using cross-validation) could be correctly assigned to their subspecies. For the discrimination of subspecies, the most important characters with the smallest Wilks' lambda values are the number of internodes (Wilks' lambda = 0.194, $p < 0.001$), the proportion of lowest five internodes (Wilks' lambda = 0.273, $p < 0.001$), and the number of intercalary leaves (Wilks' lambda = 0.402, $p < 0.001$). When the assignment to the three genetic clusters inferred by Bayesian assignment analysis was tested, 94.6 % of all populations were correctly classified using morphological characters (62.2 % using cross-validation). The three genetic clusters are best distinguished by the length of the lowest five internodes (Wilks' lambda = 0.900, $p = 0.166$), the ratio of inflorescence length to plant height (Wilks' lambda = 0.910, $p = 0.200$), and the bract length (Wilks' lambda = 0.932, $p = 0.302$). However, Wilks' lambda values are high and insignificant even for these characters (≥0.900).

Genetic variation and population structure

Based on three replicate samples per primer pair, reproducibility of AFLP banding patterns was good (between 89.0 and 96.2 %, depending on the primer pair). Using the software Structure, $K = 4$ received the highest likelihood

Table 4 Results from discriminant analyses for genetic clusters of *Rhinanthus alectorolophus* in southwestern Germany using individual as well as population means data

Genetic cluster	Predicted group membership			Total	% Correctly classified
	Cluster A	Cluster B	Cluster C		
Individuals					
Cluster A	7/6	1/1	101/102	109	6.4/5.5
Cluster B	3/3	25/22	29/32	57	43.9/38.6
Cluster C	11/14	9/11	360/355	380	94.7/93.4
Total	21/23	35/34	490/489	546	71.8/70.1
Population means					
Cluster A	7/7	0/0	1/1	8	87.5/87.5
Cluster B	0/0	3/0	1/4	4	75.0/0.0
Cluster C	0/4	0/5	25/16	25	100.0/64.0
Total	7/11	3/5	27/21	37	94.6/62.2

Genetic clusters were inferred by Bayesian assignment analysis and the software Structure. Predicted group memberships and percentages of correctly classified cases are given for original grouped cases (first number) as well as cross-validated grouped cases (second number)

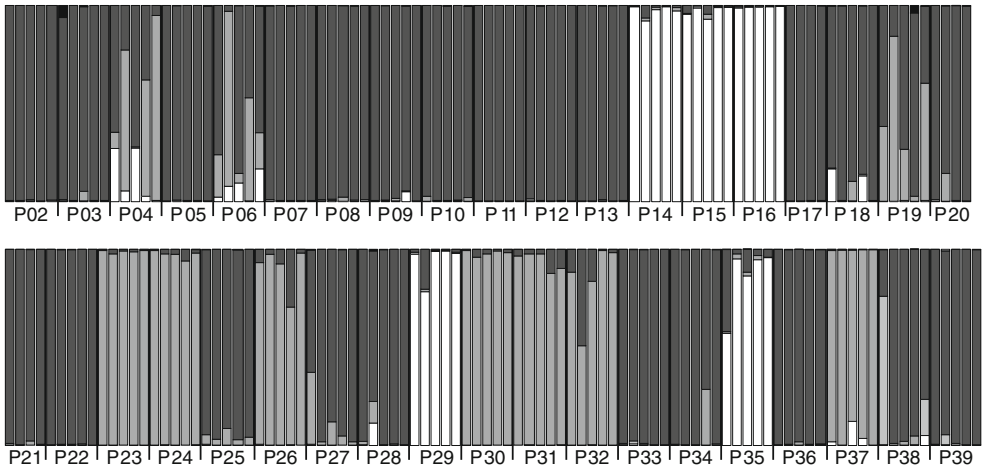

Fig. 3 Results of the Bayesian assignment analysis using Structure with $K = 4$ on AFLP data from 38 populations of *Rhinanthus alectorolophus*. *White*: genetic cluster A; *gray*: genetic cluster B, *dark gray*: genetic cluster C; *black*: genetic cluster D

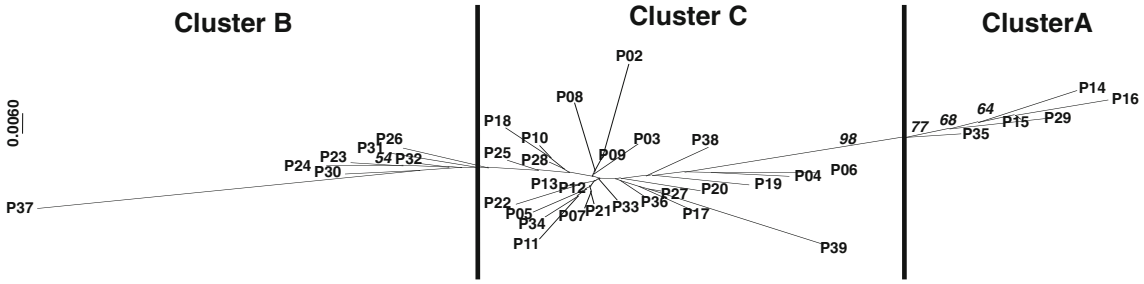

Fig. 4 Neighbor-joining tree of 38 populations of *Rhinanthus alectorolophus* from Baden-Württemberg based on Nei's D distances calculated from AFLP data. Lines and capital letters show the genetic clusters found in the structure analysis

and this is the smallest value of the plateau (Fig. S1a), which indicates that this K could be chosen (Pritchard et al. 2000). However, one of these inferred groups showed only minor contributions to a few individuals (cluster D in Fig. 3). Therefore, all individuals and populations were assigned to one of three genetic clusters (A–C). These three genetic clusters show a clear geographical structure. Cluster A consists of populations from the upper Rhine Valley. Cluster B occurs mainly in central-southern Baden-Württemberg, especially in the Swabian Alb, whereas cluster C is found in the remaining area of Baden-Württemberg (Fig. 1). Populations P04, P06, P19, and P38 are composed of individuals with major contributions from both cluster B and cluster C. Another method of choosing the best K (Evanno et al. 2005) pointed to $K = 2$ (Fig. S1b), in which case the two clusters inferred correspond to cluster A and cluster B + C. We decided to use the division into three clusters for further analysis and discussion to avoid losing information about the geographically meaningful distinction between cluster B and cluster C.

The unrooted neighbor-joining tree of populations (Fig. 4) shows a relatively clear separation between the populations belonging to cluster A and those belonging to cluster B and C. This is also the branch with the highest support (bootstrap value = 98 %). Most other relationships did not receive bootstrap support. Nevertheless, the populations belonging to cluster B form a recognizable group, with cluster C in an intermediate position between cluster A and cluster B. The subspecies are scattered over the tree (data not shown) and there are only two branches leading to groups of three or more populations belonging to the same subspecies. The first group is formed by populations P17, P27, and P39, the second group contains populations P05, P07, P11, P21, and P34. In both cases, the populations belong to subsp. *alectorolophus*.

The principal coordinates analysis based on Nei–Li distances resulted in 26 coordinates with an eigenvalue >1 and explaining a total of 89.2 % of the genetic variation. The scatterplot of the first two principal coordinates (Fig. 5a, b), explaining 38.8 and 10.8 % of the variation, respectively, does not show clear clusters. Genetic clusters inferred by Bayesian assignment analysis (Fig. 5b) can be found in different parts of the graph, but with some overlap, especially between cluster B and cluster C. The

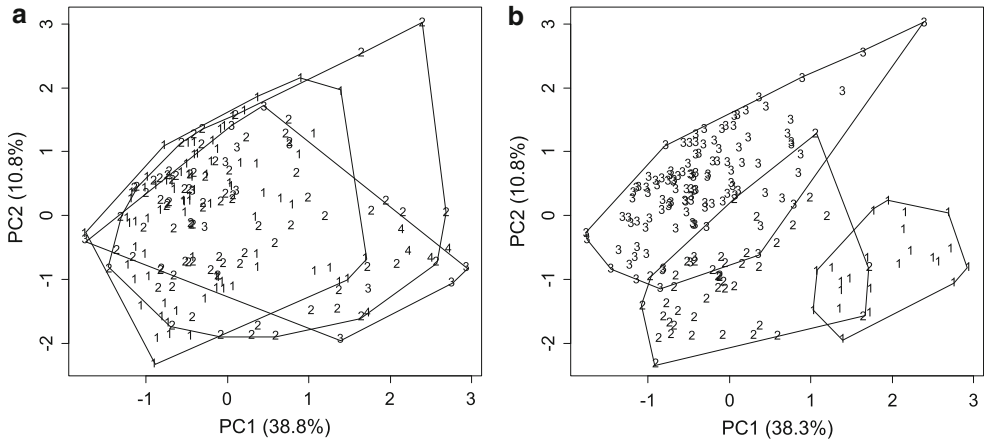

Fig. 5 Scatterplot of the first to principal coordinates obtained by a PCO on a Nei–Li distance matrix based on AFLP data from *Rhiananthus alectorolophus* from Baden-Württemberg. **a** Subspecies (*1*, subsp. *alectorolophus*; *2*, subsp. *semleri*; *3*, subsp. *patulus*). **b** Genetic cluster according to Structure analysis (*1*, cluster A; *2*, cluster B; *3*, cluster C)

morphology-based classification into subspecies does not seem to be mirrored in the AFLP-based genetic structure (Fig. 5a), since the subspecies are mostly mixed in this scatterplot.

The Mantel test indicated a significant correlation between genetic and geographic distances in the whole dataset of genotyped individuals ($r = 0.471$, $p = 0.001$). Within the genetic clusters inferred by Bayesian assignment analysis, correlations are detectable as well. For clusters A ($r = 0.440$, $p = 0.001$) and B ($r = 0.488$, $p = 0.001$), correlations were in a similar range as in the whole dataset, whereas the correlation was weaker, but still significant, in cluster C ($r = 0.231$, $p = 0.001$).

AMOVAs performed in Arlequin based on AFLP data revealed that <0.1 % of the variation was found between subspecies, 29.8 % among populations within subspecies, and 70.2 % within populations. For comparison, we also computed an AMOVA to test the three genetic clusters. In this case, 13.9 % of the variation was among genetic clusters, 20.8 % among populations within genetic clusters, and 65.3 % within populations.

Correlations of morphological characters with AFLP data

Correlations of genetic distances with single morphological characters were weak and mostly insignificant. The only significant correlation was found between genetic distance and differences in the number of internodes below the inflorescence ($r = 0.096$, $p = 0.019$). When only data from one genetic cluster were taken into account, more characters showed significant correlations with genetic distance. In cluster B, this was the case for the number of sterile internodes ($r = 0.146$, $p = 0.050$) and the number of sterile branches ($r = 0.161$, $p = 0.040$). For cluster C, significant correlations between genetic distance and number of sterile internodes ($r = 0.114$, $p = 0.018$), as well as between genetic distance and the ratio of stem leaf length to stem leaf width ($r = 0.093$, $p = 0.034$) could be found. Within cluster A, no significant correlations could be observed at all.

Discussion

In this study we aimed to elucidate whether it is possible to reliably distinguish subspecies of *R. alectorolophus* using morphological characters, and if they are genetically differentiated. Beside the subspecies classification we also tested the classification into five informally named ecotypes described by Zopfi (1993a, b). Our analyses yielded very similar results with either classification, probably because both classifications are largely relying on the same morphological characters. This shows that both classifications are comparable, and while we will mostly discuss the subspecies classification, the same conclusions can be drawn for the classification into five ecotypes.

Some of our results support the assumption that the subspecies identified a priori in our samples represent morphologically well-separated groups. For example, in an analysis of variance (ANOVA), most characters showed significant differences between subspecies (data not shown). These results are in accordance with previous studies of *Rhinanthus* species, where most morphological characters investigated showed differences between ecotypes (Zopfi 1993a, 1995). This could indicate that the ecotypes or subspecies are relatively well characterized morphologically.

However, there were a considerable number of misclassified samples between subsp. *alectorolophus* and

semleri, and between subsp. *semleri* and *patulus*. Together with the lack of clear clusters in the PCA scatterplots (Fig. 2a, c), this indicates that the subspecies in our study area are not clearly separated from each other by morphological characters, but that a continuous gradient exists for most or even all of them. Furthermore, several populations found in our study exhibited character state combinations not directly in accordance with the descriptions of subspecies or ecotypes and their habitats. This made identification of infraspecific taxa difficult in some cases, and is another indication that the delimitation of groups within the variation of morphological characters is arbitrary. The conclusion that morphological variation is continuous was also drawn by other authors (Karlsson 1974; Mizianty 1978; Zopfi 1993a).

In the past, cultivation experiments have shown that the important morphological characters correlated to flowering time are genetically fixed (Campion-Bourget 1982; Zopfi 1993b). However, many characters are to a certain extent plastic, depending on the host species and/or environmental conditions. For example, plant height is greatly influenced by nutrient supply by the hosts (Zopfi 1993b). In contrast, the number of internodes is stable under different cultivation conditions and on different hosts at least in the first cultivated generation (Zopfi 1993b, 1995), even if Campion-Bourget (1982) found that the number of internodes was reduced in cultivation but still differing between ecotypes. Because of its stability and strong correlation with flowering time in hemiparasitic Orobanchaceae (Bolliger 1989; Zopfi 1995), the number of internodes is one of the main characters for the distinction of seasonal ecotypes. In our study, the number of internodes was not only important for subspecies delimitation, but also one of the few characters that exhibit correlation to genetic distance. Mantel tests between differences in morphological characters and genetic distances showed weak but significant correlations for the number of sterile internodes in the whole dataset as well as in two of the three genetic clusters inferred by Bayesian assignment analysis. The absence of significant correlations within cluster A might partly be the result of the lower sample size. The positive correlation between differences in the number of internodes and genetic distance might give a hint that this character is determined by one or several genes under environmental selection. However, this result should be handled with care, as the significance of the correlations is relatively low. The genetic fixation was supported by crosses performed between individuals of an early-flowering and a late-flowering population, where the F_1 generation was intermediate for important morphological characters such as the number of internodes (Campion-Bourget 1982). Another morphological character that was found to be relatively stable in earlier studies is the contraction of the lowest internodes found in some ecotypes (Mizianty 1978; Zopfi 1993b, 1995). This character did not exhibit significant correlation to genetic distance in our study, but it was important for the morphological distinction of genetic clusters in the discriminant analyses and was the character with most significant differences in an ANOVA (data not shown). However, similar to the differences in morphological characters revealed in the Mantel test, it is unclear whether this character differs between the geographically separated genetic clusters because of inherent genetic differences, or because specific habitats and thus subspecies are not equally common in all geographical regions.

Our results show that subspecies identified based on morphology are not genetically differentiated. Most Mantel tests between differences in morphological characters and genetic distances did not show significant correlations either within single genetic clusters or the whole dataset. The PCO scatterplot (Fig. 5a) indicates that populations belonging to the same subspecies do not form genetic clusters on the scale of the study area. Furthermore, the high Wilks' lambda values in the discriminant analysis indicate as well that the three genetic clusters revealed by the Structure analysis do not show clear differences in the morphological characters measured.

The genetic structure inferred from our AFLP data showed a clear separation between plants from the southern Upper Rhine valley (cluster A) and plants from the rest of Baden-Württemberg (Fig. 1). This separation is also visible in the neighbor-joining tree of the populations (Fig. 4) and probably reflects limited dispersal over the Black Forest mountains, where *R. alectorolophus* is rare (Philippi 1996; Wörz et al. 2010). Outside the Rhine valley, a distinction between cluster B and C can be seen, with several populations showing admixture between these two clusters. These results are concordant with isolation by distance pattern in the AFLP data. An explanation for this pattern might be that long distance gene flow in *Rhinanthus* is probably limited. *Rhinanthus* species are mostly pollinated by bumblebees (Kwak 1977), and their seeds are probably dispersed rather locally, although restricted wind dispersal may be possible (Bullock et al. 2003). Historical patterns such as recolonization routes after the ice ages might also play a role, especially in the distinction between cluster A and clusters B/C, but the small geographic scope of our study does not allow us conclusions in this regard.

In every genetic cluster inferred by Bayesian assignment analysis, more than one ecotype or subspecies can be found. Therefore, the genetic structure found in the dataset can be assumed to be mainly a geographical one. This is supported by the Mantel tests showing significant correlations between genetic and geographic distances. Thus, the influence of the ecotypic differentiation seems to be at best of marginal importance for the genetic structure in the

dataset. This is also reflected in the AMOVA results, where <0.1 % of the variation was found among subspecies. Only the two groups of populations of subsp. *alectorolophus* visible in the neighbor-joining tree might show a certain hint to the colonization of suitable habitats by pre-adapted ecotypes. However, since the relationships in the neighbor-joining tree do not receive statistical bootstrap support, this finding should not be over-interpreted.

Despite their genetic fixation, morphological characters correlated with seasonal differentiation seem to be less useful for taxonomic delimitation in hemiparasitic Orobanchaceae than other morphological characters. For example, Bolliger (1989) found that morphological characters such as leaf form and flower size are taxonomically more important in *O. lanceolata* than characters linked to flowering time. For *Euphrasia* in Sweden, Karlsson (1976) concluded that characters such as flower size, leaf indumentum, and general leaf form are more important than characters linked to seasonal variation.

Our data indicate parallel evolution of the morphology of ecotypes in *Rhinanthus* since the genetic variation is linked to geography, and not to morphological units at subspecies level. The forms differing in flowering time were often regarded to be reproductively isolated and probably early stages on the way to the formation of new species (e.g., Wettstein 1895). In fact, their flowering time can be extended because of their lateral branches. For example, while most individuals of subsp. *alectorolophus* are unbranched, there are often several individuals in a population that do have branches. Furthermore, Zopfi (1993b) found an influence of the host on the rate of development, which could lead to variation in flowering time as well. Thus, although flowering by and large at different times, the subspecies are probably not completely genetically isolated from each other. Nevertheless, the management of *Rhinanthus* meadow habitats presents a strong selection pressure on flowering time and associated morphological characters such as the number of internodes, since plants that have not set fruit when the meadow is mowed or grazed, normally cannot reproduce anymore.

The pattern of multiple origins of subspecies found in our study suggests that morphological characters such as the number of internodes can evolve relatively fast. It is possible that the underlying genetic mechanism is relatively simple and influenced by only few genes, as also hypothesized by Zopfi (1993a) based on the presumed young age of the ecotypes which probably evolved relatively recently in man-made habitats (Campion-Bourget 1982). The characters discerning the ecotypes might be able to evolve rapidly, permitting the adaptation of populations to changing environment in only a few generations. Despite some weak correlations between morphological characters and genetic distances, it seems plausible to assume that populations adapted very quickly to the habitats to which they migrated. Moreover, they might still be able to adapt quickly if management practices in their habitat change. Rapid adaptive evolution in only a few generations was shown to exist in other plants. For example, Franks et al. (2007) studied heritable changes in flowering time of *Brassica rapa* L. (Brassicaceae) after several years of drought. They found that their study populations had acquired an earlier flowering time after only a few generations through natural selection. Similarly, Dlugosch and Parker (2008) found evolution in plant growth and flowering time in introduced populations of *Hypericum canariense* L. (Hypericaceae) in <25 generations.

While adaptations to different meadow habitats involving populations with diverging flowering times exist not only in hemiparasitic Orobanchaceae but also in other meadow species, circumstances may vary between taxa. For example, Reisch and Poschlod (2009) studied two ecotypes of *Scabiosa columbaria* L. (Caprifoliaceae) adapted to grazing or mowing, respectively, and differing in plant height and in flowering time. They found higher degrees of differentiation between mowed and grazed sites in the same region than between populations from the same ecotype but different regions (Reisch and Poschlod 2009). This is in clear contrast to our results in *R. alectorolophus*, where other genetic mechanisms might play a role in flowering time regulation.

To date, the exact genetic or regulatory basis for ecotypic differentiation in *Rhinanthus* and other hemiparasitic Orobanchaceae is unknown. Rapid parallel evolution between different populations might be a reasonable hypothesis that could be tested in future studies. For example, long-term cultivation experiments with different management treatments may be able to show changes in flowering time and associated morphological characters that the cultivation experiments conducted in the past did not show (Campion-Bourget 1982; Zopfi 1993b, 1995).

Our study suggests that, while the mechanisms behind the ecotypic variation still remains unknown, the ecotypes in *Rhinanthus* do not form genetically coherent groups, but rather morphological forms that originated multiple times. Consequently, the use of formal ranks for these groups is probably only marginally meaningful in this genus.

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