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RESEARCH PAPER

Population genetic diversity and species relationships in the genus *Rhinanthus* L. based on microsatellite markers

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ABSTRACT

The genus *Rhinanthus* L. is complex, containing many taxonomically unresolved taxa. In this paper we studied genetic variation and species relationships in 15 populations of six *Rhinanthus* species from three sections. For this purpose, we developed new microsatellite primers for *R. osiliensis* and used them to investigate genetic variation in two narrow endemics (*R. osiliensis*, *R. javorkae*) and in four widespread species (*R. rumelicus* *R. wagneri*, *R. angustifolius* and *R. minor*). Species-specific private alleles were found in all species except *R. osiliensis* and *R. angustifolius*. The Bulgarian endemic *R. javorkae* showed the lowest genetic variation, followed by widespread *R. minor* and Estonian endemic *R. osiliensis*. *Rhinanthus javorkae* and *R. minor* were genetically most differentiated. Section *Cleistolemus* is weakly structured genetically, indicating close affinity between *R. osiliensis*, *R. rumelicus*, *R. wagneri* and *R. angustifolius*.

INTRODUCTION

The genus *Rhinanthus* is divided into five sections based on flower characteristics (Soó & Webb 1972) and contains about 30–40 annual species. Most species are widespread in Europe, and about ten species are endemics. The large morphological polymorphism of the species led to the description of many subspecies and ecotype variants (Soó & Webb 1972). The authors of *Flora Europaea* emphasised that in addition to ecotypic variation, the pattern of variation in the genus *Rhinanthus* is 'so reticulate and complex that there is great lack of agreement on the limits of species' (Soó & Webb 1972). Out of 36 species of *Rhinanthus* described in *Flora Europaea*, 11 taxa have an unclear status. Most taxa of provisional status have a narrow distribution and are handled as endemics within their range. Moreover, natural interspecific hybrids have been found in *Rhinanthus* (Kwak 1980; Ducarme & Wesselingh 2005, 2013; Ducarme *et al.* 2010), which makes taxonomic and phylogenetic relationships in the genus even more vague. Hence, there are many reasons why genus *Rhinanthus* has become so attractive to study in the last decade.

Taxonomic and phylogenetic relationships within the genus *Rhinanthus* L. (Orobanchaceae) have been studied several times in the past 10 years. Böhme (2001) found low genetic differentiation between the species and sections of *Rhinanthus* when using ITS sequences. Next, RAPD and ISSR markers successfully identified and discriminated hybrids between *R. minor* and *R. angustifolius* (Ducarme & Wesselingh 2005). Vrancken *et al.* (2009) described a lack of a phylogeographic pattern in the chloroplast DNA of *R. angustifolius* and suggested the presence of putative ancestral polymorphisms or hybridisations to explain this finding. AFLP polymorphism data revealed that the geographic structure of genetic variation in *R. angustifolius* is similar to other perennial species in Europe (Vrancken *et al.* 2009). In a study based on microsatellites, sequencing of cpDNA and rDNA ITS distinguished *R. minor* populations in the UK from other European *R. minor* and *R. angustifolius* populations, but did not separate subspecies of *R. minor* in the UK (Houston & Wolff 2012). Our isozyme studies demonstrated that endemic *R. osiliensis* and widespread *R. rumelicus* are closely related (Talve *et al.* 2012), section *Cleistolemus* is not monophyletic, and the taxonomic position of *R. alectorolophus* needs to be reconsidered (Oja & Talve 2012). A recent paper from Natalis & Wesselingh (2012) showed that hybridising species *R. minor* and *R. angustifolius* share pollinators, and found very weak differences in pollen transfer dynamics and respective gene flow between species. A new paper (Ducarme & Wesselingh 2013) specifies large differences in outcrossing rate between these two species, leading to introgression towards *R. angustifolius* by affecting pollination. Another paper (Vrancken *et al.* 2012) described a strong geographic genetic structure in *R. minor* and found that populations from Italy, Greece and southeast Russia were totally different from other *R. minor* populations, and may belong to an unknown taxon of *Rhinanthus*. All of these studies have significantly improved our understanding of the relationships between sections and species within the genus, however there are still many intrinsic problems to elucidate.

An assessment of genetic differentiation could help to understand the complex pattern of morphological variation within and between species. Studying genetic diversity provides information not only about present-day species diversity but also about evolutionary history and the processes that have shaped this diversity (Charlesworth & Wright 2001). Population structure also provides insight into species-wide levels of genetic diversity. Comparing rare species to common congeners, which are expected to have similar phylogenetic histories, can provide critical information about the level and distribution of genetic variation in this particular genus (Karron 1991; Gitzendanner & Soltis 2000; Cole 2003).

For evaluation of genetic diversity among and within species of *Rhinanthus* we chose simple sequence repeat (SSR) markers. SSR, or microsatellites, are very popular markers for genetic analysis in plants because of their distribution throughout the genome, polymorphism, co-dominance, bi-parental inheritance, rapid mutation rate and high discriminating ability (Morgante & Olivieri 1993). These qualities make SSRs good tools for investigating the degree and pattern of genetic variability within and between populations (*e.g.*, Ellis *et al.* 2006; Furches *et al.* 2009; Riley *et al.* 2010) and for resolving complex relationships within closely related species groups (*e.g.,* Dunbar-Co & Wieczorek 2011; Kim *et al.* 2012; Rahemi *et al.* 2012). Variable microsatellites are also frequently used in phylogenetic analyses to study recently derived and problematic species lineages (*e.g.*, Dunbar-Co & Wieczorek 2011; Mitsui & Setoguchi 2012).

The aims of this study are to (i) develop and describe nuclear microsatellite markers for the endemic *R. osiliensis*; (ii) test these new markers for cross-amplification in five *Rhinanthus* species from different sections; (iii) characterise the genetic variation of species; and (iv) estimate genetic relationships between six *Rhinanthus* species to assess species boundaries.

MATERIAL AND METHODS

Studied species

We analysed 15 populations of six *Rhinanthus* species from three sections (Table 1). *Rhinanthus rumelicus* Velen., *R. wagneri* Degen, *R. angustifolius* C. C. Gmelin and *R osiliensis* (Ronniger & Saarsoo) Vassilcz. (=*Rhinanthus rumelicus* Velen. subsp. *osiliensis* Ronniger & Saarsoo) belong to section *Cleistolemus* Chab. The basic distributions of *R. rumelicus* and *R. wagneri* are southward (in Eastern and Central Europe and the Balkan Peninsula; Soó & Webb 1972), whereas *R. angustifolius* is frequent throughout Europe. *Rhinanthus osiliensis* is a narrow endemic from calcareous spring fens on the island of Saaremaa, Estonia. Another narrow endemic, *R. javorkae* Soó, is distributed in southwest Bulgaria and belongs to the small section *Anoectolemi* Chab; this species is found only in one location in Pirin Plane, Bulgaria (Asenov 1995). Widespread *R. minor* L. from section *Rhinanthus* was included as outgroup, to obtain an appropriate comparison among species. In this study, we follow the taxonomic classification of species according to *Flora Europaea* (Soó & Webb 1972), where all named taxa are treated as a species.

Healthy leaf samples were collected from randomly selected plants at least 3-m apart and stored in silica gel until DNA extraction. We attempted to analyse at least 15 individuals per population. In total, 296 individuals from 15 populations of six species were sampled. Populations Rosi3 and Rosi9 of *R. osiliensis* consist of approximately 2000 individuals. Population Rosi8 is small with less than 500 plants and is showing signs of decline over recent years. The population of the Bulgarian endemic *R. javorkae* (Rjav49) is small and mosaic, with approximately 1000 plants. All other studied populations are large and vigorous. Population codes, locations and coordinates are presented in Table 1. The codes of the populations are given with one-letter abbreviation of the genus and three-letter abbreviation of the specific epithet. Vouchers are kept in the herbarium of the Natural History Museum of the University of Tartu.

Primer development

In this paper we developed new microsatellite primers for *R. osiliensis* and used them to investigate genetic variation in two narrow endemics (*R. osiliensis*, *R. javorkae*) and in four widespread species within the same genus (*R. rumelicus R. wagneri*, *R. angustifolius*, *R. minor*). Total genomic DNA was isolated from silica-dried leaves using the CTAB method

Table 1. Populations and geographic locations of six *Rhinanthus* species studied.

section	species	population code	N	location	latitude N	longitude E
Cleistolemus	*R. osiliensis*	Rosi3	25	Paatsasoo, Saaremaa, Estonia	58°30′	22°18′
		Rosi8	23	Haavassoo, Saaremaa, Estonia	58°14′	22°10′
		Rosi9	25	Sutru, Saaremaa, Estonia	58°16′	22°06′
	R. rumelicus	Rrum25	19	Marchaevo, Stolichna, Sofia grad, Bulgaria	42°35′	23°09′
		Rrum63	25	Pirdop, Pirdop, Sofija, Bulgaria	42°42′	24°12′
		Rrum72	16	Zhedna, Radomir, Pernik, Bulgaria	42°25′	22°57′
		Rrum108	18	Brakyovtsi, Godech, Sofia, Bulgaaria	43°02′	23°08′
	R. wagneri	Rwag48	16	Karlova, Karlova, Plovdiv, Bulgaria	42°41′	24°54′
		Rwag67	19	Karlova, Karlova, Plovdiv, Bulgaria	42°41′	24°56′
		Rwag76	18	Razlog, Bansko, Blagoevgrad, Bulgaria	41°46′	23°25′
	R. angustifolius	Rang12	18	Tartu, Tartumaa, Estonia	58°23′	26°42′
		Rang30	14	Metsküla, Läänemaa, Estonia	58°43′	23°37′
		Rang45	19	Kirna, Jõgevamaa, Estonia	58°32′	26°14′
Rhinanthus	*R. minor*	Rmin31	18	Metsküla, Läänemaa, Estonia	58°43′	23°37′
Anoectolemi	*R. javorkae*	Rjav49	23	Razlog, Bansko, Blagoevgrad, Bulgaria	41°46′	23°25′

N = sample size.

(Doyle & Doyle 1990). The extracted DNA was dissolved in 100 µl of TE buffer and diluted to 1:10 for further polymerase chain reaction (PCR) analyses. DNA quality and quantity was determined with 1% agarose gel electrophoresis. Extracted DNA was hybridised to eight oligonucleotide repeats, $(AC)_{15}$, $(AG)_{15}$, $(AT)_{15}$, $(CG)_{15}$, $(CCG)_{10}$, $(AAC)_{10}$, $(AGG)_{10}$ and $(CAC)_{10}$, following the method of Hamilton *et al.* (1999) with some modifications. Enriched DNA fragments were cloned using pBluescript II SK-Phagemid vector and the XL1-Blue MRF' bacterial host strain (*Escherichia coli*; Stratagene, La Jolla, CA, USA). Colour-positive clones were screened for inserts using a membrane-based 'dot blot' method (Glenn & Schable 2005) and the Phototope chemiluminescent detection system (New England Biolabs, Ipswich, MA, USA). Positive clones were amplified by PCR using an MJ Research PTC-200. PCR amplifications were conducted in 25-µl volumes containing 1 µl template DNA, 0.8 µM each of primers T3 and T7 (Integrated DNA Technologies, Coralville, IA, USA), 1× Thermopol Reaction Buffer (New England Biolabs), 200 µM of each dNTP, and 0.2 U Vent (exo-) DNA polymerase (New England Biolabs). The clones were purified using a PEG precipitation procedure (Johnson & Soltis 1995). The purified PCR products were sequenced using the T3 primer, BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and Better Buffer (The Gel Company, Tielt, Belgium) on an Avant 3100 Genetic Analyser (Applied Biosystems). The T7 primer was used to generate a complementary reverse sequence for inserts containing a microsatellite motif. All sequences contigs were put together using SEQUENCHER 4.1 (GeneCodes, Ann Arbor, MI, USA).

Primers were designed using the program PRIMER 3 (Rozen & Skaletsky 2000). To use universal fluorescent M13R (AGGAAACAGCTATGACCAT) and CAGT (ACAGTCGGGC GTCATCA) primers, one primer of each pair was synthesised with a M13R or CAGT tag at the 5′ end following the procedure of Boutin-Ganache *et al.* (2001) (Table 2).

Microsatellite analyses

Six new microsatellite markers for endemic *R. osiliensis* were used to test cross-species amplification. The primers were optimised for a range of temperatures and $MgCl_2$ or $MgSO_4$ concentrations (Table 2). PCR reactions were carried out in 20-µl volume containing 1–3 µl of template DNA (*ca.* 20–40 ng), 1× Go*Taq* Flexi buffer (Promega, Fitchburg, WI, USA), 0.2 mM of each dNTP, 0.25 µM of untagged primer, 0.25 µM of fluorescent tag, 0.03 µM of the tagged primer, varying concentrations of $MgCl_2$ or $MgSO_4$ and 0.2 U Go*Taq* Flexi DNA polymerase (Promega). DNA from each sample was amplified with the common tag containing one of three fluorescent dyes, 6-FAM, PET, or VIC (Applied Biosystems). PCR was performed under the following conditions: preliminary denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 1 min, annealing temperature 52.5–64.0 °C (Table 2) for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 30 min, using a Techne TC-5000. Microsatellite alleles were detected on an Avant 3100 Genetic Analyser (Applied Biosystems) and were sized against LIZ 500 size standard (Applied Biosystems) using Peak Scanner Software version 1.0 (Applied Biosystems).

Data analysis

The program Genalex version 6 (Peakall & Smouse 2006) was used to determine the total number of alleles, species-specific unique alleles, allele frequencies, the average number of alleles per locus (A) and the observed (H_o) and expected (H_e) heterozygosities (or gene diversity; Nei 1987). Values were averaged for each species to express mean genetic variation at the population level.

The presence of null alleles that could affect the estimators of relatedness and relationships (Wagner *et al.* 2006; Chapuis & Estoup 2007) was examined using MICROCHEKER version 2.2.3 (Van Oosterhout *et al.* 2004). In cases where null alleles were detected, the Brookfield 1 algorithm (Brookfield 1996) was used to calculate new adjusted genotypes and compile the new data set. Then, the inbreeding coefficient (F_{is}) was estimated using GENEPOP version 4.0 (Rousset 2008). To compare the effect of null alleles on the genetic distances, Chord distances (Cavalli-Sforza & Edwards 1967) were calculated using the program FreeNA (Chapuis & Estoup 2007).

Nei's genetic distances between population pairs were calculated using MSA version 4.05 (Dieringer & Schlötterer 2003), and cluster analyses on the basis of these distances were performed with PHYLIP 3.69 (Felsenstein 2004), using the

Table 2. Characteristics of six microsatellite loci in *Rhinanthus osiliensis* with primer sequences, repeat motif and reaction conditions.

locus	GenBank accession no.	primer sequence (5′–3′)	5′-tag	repeat motif	T_a (°C)	Magnesium	dye
RHOS8	GQ872188	F-CGCGTTATTGTCATCTATAGCC R-CATGAAAAAGAGCCTGTTCG	M13R	$(CT)_9(CA)_2CT(CA)_4AA(CA)_3$	57.5	3 mM $MgSO_4$	VIC
RHOS50	GQ872189	F-ACACGAGACACCGGGTAATC R-TCGGCTCTCGATTATGTTTG	CAGT	$(AC)_6GCAC$	54.8	3 mM $MgSO_4$	VIC
RHOS149	GQ872191	F-TATACCTTGGCGCTTTGTGG R-AGAAAGCTTTGGTCCTATG	CAGT	$(TG)_9$	60	4 mM $MgCl_2$	6-FAM
RHOS156	GQ872192	F-AAATGGCAAGAGTAGTTC R-AGTCGAGGCGACGAAGTG	CAGT	$CAC(CACAA)_5CAC$	53.6	2 mM $MgSO_4$	PET
RHOS203	GQ872193	F-TACGGGAGAAGGGAGAG R-CGACTCGGACTTTGGAG	M13R	$(AC)_4TA(AC)_3C(AC)_3$	52.5	4 mM $MgCl_2$	VIC
RHOS261	GQ872195	F-GCAAGCCCTAGATTGAGATT R-CCAGGTCTCAGGAGTAGTT	M13R	$(CA)_2CT(CA)_6$	60	4 mM $MgCl_2$	PET

The 5′ tag M13R (AGGAAACAGCTATGACCAT) and CAGT (ACAGTCGGGCGTCATCA) used for incorporation of the fluorescent tag.
T_a = annealing temperature.

unweighted pair group (UPGMA) method. The reliability of each node was tested using 1000 bootstrap resamplings. The final trees were viewed and edited in FigTree version 1.3.1 (Rambaut 2008).

The partitioning of genetic diversity among species, populations within species and within populations was estimated with an analysis of molecular variance (AMOVA) using ARLEQUIN version 3.1 (Excoffier *et al.* 2005). The analyses were conducted with two different data sets: (i) all populations grouped by species (six species, 15 populations); and (ii) populations of section *Cleistolemus* grouped by species (four species, 13 populations). In addition, a principal coordinates analysis (PCA) using the covariance standardised method of pair-wise Nei's genetic distances among the populations was implemented with Genalex version 6 (Peakall & Smouse 2006) to illustrate the genetic distances among populations. Two distinct species (*R. minor* and *R. javorkae*) were excluded from the PCA analysis to obtain a better picture of species boundaries in section *Cleistolemus*. Finally, a Bayesian model-based clustering approach implemented in STRUCTURE (Pritchard *et al.* 2000) was used to estimate the number of genetic clusters (K) without *a priori* knowledge of taxonomy or population location. The clustering was conducted with the admixture model and correlated allele frequencies using the 1×10^4 burn-in time periods and 1×10^4 Markov Chain Monte Carlo (MCMC) iterations. The different number of clusters (K) from 1 to 10 was tested with 10 independent runs. The optimal number of clusters (K) was found with the ΔK method (Evanno *et al.* 2005) visualised with Structure Harvester version 0.6.92 (Earl & von Holdt 2012).

RESULTS

Cross-species amplification

All six microsatellite primer pairs developed for *R. osiliensis* were successfully amplified across all six *Rhinanthus* species. In total, 296 individuals were analysed and 27 alleles detected (Table 3). Six loci yielded only eight alleles in endemic *R. javorkae,* followed by endemic *R. osiliensis* with ten alleles. Common *R. rumelicus, R. wagneri, R. angustifolius* and *R. minor* had 16, 19, 14 and 11 alleles, respectively (Table 3). The number of alleles per locus ranged from three (RHOS50 and RHOS149) to eight (RHOS156). Species-specific private alleles were found in all species except *R. osiliensis* and *R. angustifolius*. In total there were 11 private alleles, but the number dropped to six after excluding rare alleles with a frequency below 0.05 (Table 3).

Microsatellite polymorphism and genetic diversity

Locus RHOS149 showed null alleles only in one population (Rwag76). The presence of null alleles in locus RHOS261 was identified in nine studied populations (Rrum25, Rrum63,

Table 3. Allele frequencies at six microsatellite loci in six *Rhinanthus* species.

locus	allele	*R. osiliensis* N = 73	*R. rumelicus* N = 78	*R. wagneri* N = 53	*R. angustifolius* N = 51	*R. minor* N = 18	*R. javorkae* N = 23
RHOS8	174						**1.000**
	180	0.932	0.923	0.821	0.686	0.056	
	182		0.071	0.179	0.182	0.944	
	188	0.068	0.006		0.137		
RHOS50	216					**0.944**	
	218	1.000	1.000	1.000	1.000		1.000
	224					**0.056**	
RHOS149	180			**0.163**			
	188	1.000	1.000	0.798	1.000	1.000	
	192			0.038			1.000
RHOS156	158		**0.019**				
	166			0.067			1.000
	168	0.007	0.038	0.029	0.069	0.861	
	172	0.514	0.821	0.385	0.578	0.056	
	178	0.479	0.058	0.308	0.314	0.083	
	182			**0.019**			
	184		0.064	0.173	0.039		
	188			**0.019**			
RHOS203	251						**0.048**
	253						**0.952**
	261			**0.157**			
	263	0.801	0.384	0.402	0.696	0.029	
	265	0.199	0.616	0.441	0.304	0.971	
RHOS261	220		**0.013**				
	222	1.000	0.413	0.784	0.333	1.000	0.136
	224		0.533	0.176	0.324		0.864
	226		0.040	0.039	0.343		

Species-specific alleles are in bold. Frequencies were calculated for the total sample within species.
N = sample size.

Rrum72, Rrum108, Rwag67, Rwag76, Rang12, Rang30, Rang45), which indicates the 'problematic' status of this locus; RHOS261, therefore, should be used with caution. However, the tree based on Chord distances (not presented) did not show significant differences from the tree calculated using Nei's genetic distances.

Genetic diversity parameters are shown in Table 4. The highest mean percentage of polymorphic loci was found in *R. wagneri* (77.78%), followed by *R. angustifolius* and *R. minor* (66.67%). Endemics *R. osiliensis* (P = 38.89%) and *R. javorkae* (P = 33.33%) showed the lowest mean percentage of polymorphic loci.

Among studied populations, the lowest average allele number per locus was found in the Bulgarian endemic *R. javorkae* (mean A = 1.33), followed by the Estonian endemic *R. osiliensis* (mean A = 1.44). The highest allelic diversity was found in populations of *R. wagneri* (mean A = 2.33). Observed heterozygosity (H_o) was lower than expected (H_e) in all populations except Rosi3, ranging from 0.030 (Rjav49) to 0.325 (Rang45). The highest genetic diversities were found in Rwag76 (H_e = 0.448), Rang45 (H_e = 0.364) and Rrum108 (H_e = 0.314). The endemic *R. javorkae* had the lowest genetic variation (H_e = 0.056). The average population genetic diversity values per species ranged from 0.145 in endemic *R. osiliensis* to 0.391 in *R. wagneri*.

The highest inbreeding coefficient at the species level was found in endemic *R. javorkae* (F_{is} = 0.455), followed by the widespread *R. minor* (F_{is} = 0.374; Table 4). Endemic *R. osiliensis*, common *R. wagneri* and *R. angustifolius* showed similar value of the coefficient (F_{is} = 0.127, 0.126, 0.163, respectively). The inbreeding coefficient of *R. rumelicus* was around zero (F_{is} = 0.099).

Genetic differentiation and species relationships

A UPGMA dendrogram based on Nei's genetic distances illustrates the genetic relationships between populations of the studied species (Fig. 1). The genetic differentiation of endemic *R. javorkae* from section *Anoectolemi* and the widespread *R. minor* from section *Rhinanthus* are strongly supported (95%). The species from section *Cleistolemus* comprised one major cluster, where the most distant basal branch was occupied by Rwag76. Two populations of *R. osiliensis* (Rosi3, Rosi9)

Table 4. Genetic diversity values and inbreeding coefficient F_{is} at the population level in 15 populations of six *Rhinanthus* species.

species	population code	N	*P* (%)	A	H_o	H_e	F_{is}
R. osiliensis	Rosi3	25	33.33	1.33	0.213	0.169	−0.272
	Rosi8	23	33.33	1.50	0.080	0.139	0.433
	Rosi9	25	50.00	1.50	0.100	0.128	0.221
Mean			38.89	1.44	0.131	0.145	0.127
R. rumelicus	Rrum25	19	66.67	2.17	0.144	0.221	0.147
	Rrum63	25	66.67	2.17	0.143	0.217	0.045
	Rrum72	16	50.00	1.67	0.110	0.197	0.067
	Rrum108	18	66.67	2.17	0.210	0.314	0.137
Mean			62.50	2.04	0.152	0.237	0.099
R. wagneri	Rwag48	16	66.67	2.00	0.229	0.250	0.087
	Rwag67	19	83.33	2.17	0.241	0.298	0.088
	Rwag76	18	83.33	2.83	0.258	0.448	0.203
Mean			77.78	2.33	0.243	0.332	0.126
R. angustifolius	Rang12	18	66.67	2.00	0.130	0.288	0.308
	Rang30	14	66.67	2.00	0.131	0.302	0.301
	Rang45	19	66.67	2.33	0.325	0.364	−0.119
Mean			66.67	2.11	0.195	0.318	0.163
R. minor	Rmin31	18	66.67	1.83	0.056	0.088	0.374
R. javorkae	Rjav49	23	33.33	1.33	0.030	0.056	0.455

Population codes as in Table 1.
N = sample sizes, *P* = percentage of polymorphic loci, A = observed number of alleles per locus, H_o = observed heterozygosity, H_e = expected heterozygosity, F_{is} = inbreeding coefficient.

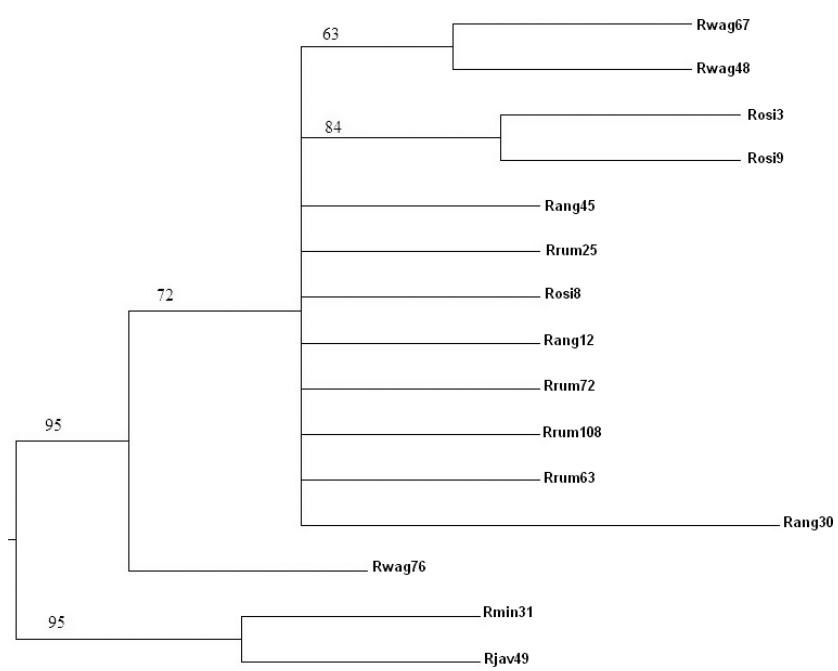

Fig. 1. Unweighted pair group dendrogram based on Nei's distances among 15 populations from six *Rhinanthus* species. Bootstrap values higher than 50% from 1000 replicates are shown above the branches. Population codes as in Table 1.

and two populations of *R. wagneri* (Rwag48, Rwag67) each formed separately supported subclusters.

The analysis of molecular variance (AMOVA) indicated that 41% of the variation was found among species, while 8% of the genetic diversity was explained by differentiation among populations within species and 51% within populations. When excluding *R. javorkae* end *R. minor* from AMOVA analyses, the differentiation among species decreased to 8%, and genetic variation among populations within species and within populations increased to 11% and 81%, respectively. In the PCA analysis, 73.29% of the total genetic variation of the populations of *R. osiliensis*, *R. rumelicus*, *R. wagneri* and *R. angustifolius* was explained with the first two axes (45.04% and 28.25%, respectively; Fig. 2). The Bayesian Structure analysis identified six genetic clusters according to ΔK (K = 6; Fig. 3). Our results showed that *R. minor* and *R. javorkae* are clearly distinct species, while *R. osiliensis*, *R. rumelicus*, *R. wagneri* and *R. angustifolius* formed four heterogeneous groups with no pure species.

DISCUSSION

Successful cross-species amplification is a critical step for studying species relationships using microsatellites. In this paper, six microsatellite primer pairs were developed for the rare endemic *R. osiliensis* and used to study genetic variation and differentiation of six species of *Rhinanthus* from three different sections. All six loci amplified a high number of unique alleles, and are good tools for use in further studies. Five of the six primers amplified at least one species-specific allele. When adding the two other papers reporting nuclear microsatellite loci for two common species from genus *Rhinanthus* (Ducarme *et al.* 2008; Houston & Wolff 2009), researchers can now use three different sets of microsatellites for detailed investigation of this complicated genus. There are some important advantages when studying species using microsatellites. Nuclear microsatellites have a rapid mutation rate and are distributed throughout the genome, and are therefore better at indicating recent genetic changes in species (Yamada & Maki 2012). At the same time, different microsatellite loci may yield conflicting results; the number of null alleles can increase when different species are studied using microsatellite markers developed for other species, or the amplification of microsatellites could fail when more distant species are studied (Roa *et al.* 2000; Wu & Hu 2010). Therefore, using microsatellite markers for several different species, including rare endemics, will significantly improve our knowledge about genetic relationships in the whole genus.

Genetic diversity is an essential component for the vitality and persistence of populations and species. Therefore, evaluating genetic diversity is especially important in rare and endemic taxa. Genetic variation in the endemic *R. osiliensis* and *R. javorkae* was remarkably lower than in the common *R. rumelicus*, *R. wagneri* and *R. angustifolius*. These results confirmed our previous findings based on isoenzyme data (Oja & Talve 2012; Talve *et al.* 2012). Our data are also consistent with the common concept where narrowly distributed species have lower levels of genetic diversity compared to widespread ones (Hirai *et al.* 2012; Yamada & Maki 2012). Rare and endemic species that form small populations usually have reduced genetic diversity due to bottlenecks, genetic drift and inbreeding (Barrett & Kohn 1991; Frankham 1998; Talve *et al.* 2012). The Bulgarian endemic *R. javorkae*, which is found only in one location on Pirin Mountain, showed the lowest genetic diversity. The relatively high genetic diversity of Rosi3, which is a vital population with more than 1000 individuals, is an expected result. This finding could be due to several factors. First, it could be related to the unusual habitat type; population Rosi3 is located near the sea, whereas the typical populations, *e.g.*, Rosi8 and Rosi9, are surrounded with forests. Supposedly, the efficiency of seed dispersal can be higher in open and windy conditions in Rosi3 and might cause the population to become more heterogeneous. This, in turn, decreases crossing between closely related individuals (biparental inbreeding), leading to a decrease in the frequency of homozygotes and an increase in heterozygotes (Delph 2004). Second, hybridisation and introgression, which is common in the genus *Rhinanthus* (Kwak 1980; Ducarme & Wesselingh 2005; Ducarme *et al.* 2010;

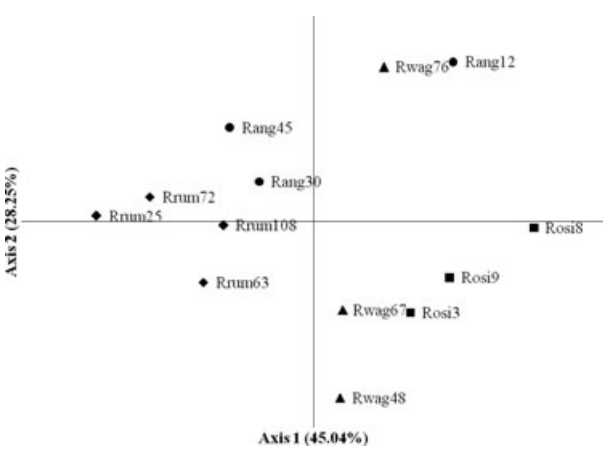

Fig. 2. Principal coordinates analysis (PCA) based on Nei's genetic distances between populations of *R. osiliensis* (■), *R. rumelicus* (♦), *R. wagneri* (▲) and *R. angustifolius* (●) from section *Cleistolemus*. The first two axes explained 73.29% of the total variation. Population codes as in Table 1.

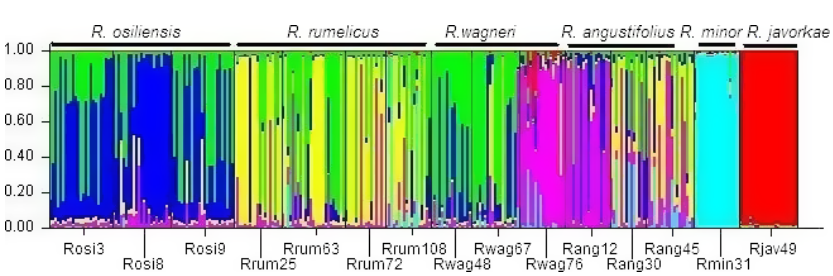

Fig. 3. Bar graph of six *Rhinanthus* species. The Bayesian analysis identified six genetic clusters according to the ΔK method (K = 6, ΔK = 6.18; Evanno *et al.* 2005). Each vertical bar is an individual, with coloured partitioning according to genetic clusters. Black vertical lines represent the populations. Below the graph are population codes and above the graph are the six species.

Natalis & Wesselingh 2012), could increase the genetic diversity. We found relatively low genetic diversity and high F_{is} values in the small isolated population Rosi8, which is in agreement with conclusions that fragmentation of habitats affects population size and also genetic diversity (Kiviniemi 2008). The future of this population is unclear because it has become overgrown with bushes. Thus, our data support the general view that genetic impoverishment may indicate vanishing populations (Reed & Frankham 2003). In addition, a putative hybridisation event between *R. javorkae* and *R. wagneri* could be suggested from our results. First, *R. javorkae* revealed two presumable species-specific alleles in two different loci (allele 192 in loci RHOS149 and allele 166 in loci RHOS156), but these two alleles have also been found at low frequencies in one population of *R. wagneri* (Rwag76) in close proximity to the *R. javorkae* population. Second, Bayesian clustering showed similarity of some individuals of Rwag76 to *R. javorkae* (Rjav49). Furthermore, some intermediate individuals similar to *R. javorkae* have been found in Rwag76 (Talve, unpublished observations). Hence, our results support and expand previous studies on the hybridisation between species of *Rhinanthus* (Ducarme & Wesselingh 2005; Ducarme *et al.* 2010; Natalis & Wesselingh 2012). Surprisingly, widespread *R. minor* showed lower genetic diversity in all genetic statistics compared to the other three common species (*R. angustifolius*, *R. rumelicus*, *R. wagneri*). In addition, a high inbreeding coefficient suggests predominant autogamy, which is consistent with previous studies (Oja & Talve 2012; Ducarme & Wesselingh 2013). This could be derived directly from the flower morphology of *R. minor*, where the stigma is situated close to the anthers, and could induce autonomous self-pollination (Kwak 1979; Ducarme & Wesselingh 2013). Conversely, *R. angustifolius* needs bumblebee visitation for pollination (Kwak 1979, 1980) and its assessed outcrossing rate was remarkably high compared to *R. minor* (Ducarme & Wesselingh 2013).

The mating system and dispersal ability of a plant species directly affect its genetic variation and determine the genetic structure of populations within species (Gitzendanner & Soltis 2000). Most of the studied populations undergo non-randomly mating, as indicated by the positive inbreeding coefficient. One reason for this is the low distribution ability because the large seeds of *Rhinanthus* lack specific dispersal mechanisms. The previously mentioned lack of pollinators can also induce inbreeding in populations. Most *Rhinanthus* species are pollinated by bumblebees (Kwak 1979, 1980; Ducarme & Wesselingh 2005), and usually their flying distances are short or even within the same inflorescence, which can cause self-fertilisation. These results are also in accordance with our previous studies (Oja & Talve 2012; Talve *et al.* 2012).

The microsatellite loci clearly showed that *R. minor* from section *Rhinanthus* and *R. javorkae* from section *Anoectolemi* are genetically differentiated from section *Cleistolemus*. The distinctness of *R. minor* has previously been shown using isozymes (Oja & Talve 2012), and this result is in accordance with morphology-based taxonomy: section *Rhinanthus* is characterised by short, rounded teeth in the upper lip of the corolla, whereas the other two studied sections have long teeth (Soó & Webb 1972). The four sampled species from section *Cleistolemus* – *R. osiliensis*, *R. rumelicus*, *R. wagneri* and *R. angustifolius* – are genetically close to each other according to the microsatellite loci. The isozyme study also confirmed the close affinity of these four species (Oja & Talve 2012). Obscure species boundaries could be due to a relatively recent split of these species, or to regular hybridisation events and introgression of genetic material from one taxon to the other. For example, hybrids between *R. minor* and *R. angustifolius* have been detected in the field according to morphological characteristics (Kwak 1980) as well as using molecular markers (Ducarme & Wesselingh 2005; Ducarme *et al.* 2010). Furthermore, Ducarme *et al.* (2010) have pointed out that hybrids can always be found when these two species grow together within a population, and usually introgression towards *R. angustifolius* will occur. Ducarme & Wesselingh (2005) have shown that hybrids are very variable and many of them are so close to the parental species that only genetic markers are able to reveal hybridisation and introgression. Thus, we designed microsatellite primers for the endemic *R. osiliensis* and successfully amplified them in different species from different sections. The obtained SSR results enabled us to define genetic relationships between the species and sections of *Rhinanthus*.

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