

Reticulate evolution and taxonomic concepts in the *Ranunculus auricomus* complex (Ranunculaceae): insights from analysis of morphological, karyological and molecular data

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The *Ranunculus auricomus* complex is an interesting model system for studying the evolution and diversity of apomictic polyploid complexes. It comprises hundreds of agamospecies, usually referred to two distinct morphotypes (traditionally named “*R. auricomus*” and “*R. cassubicus*”) which are connected by several intermediate forms. Here we try to elucidate the evolution of apomictic “*cassubicus*” morphotypes and we test criteria for different classification concepts by combining the information of molecular phylogenetic, morphological, karyological and population genetic data (AFLPs, amplified fragment length polymorphism). Phylogenetic analysis based on sequences of the nrDNA ITS and plastid data (*matK*, *trnK*, *psbJ-psbA*) suggest a deep split between the diploid sexual species *R. notabilis* (“*auricomus*” morphotype) from the closely related allopatric taxa *R. cassubicifolius* and *R. carpaticola* (“*cassubicus*”). The apomictic “*cassubicus*” morphotypes are not monophyletic, as one, *R. hungaricus*, groups with *R. notabilis*, which may be due to hybrid origin. Morphometric studies and ploidy level determinations via Feulgen densitometry show a transition from 4x *R. hungaricus* to the 6x apomictic hybrid derivatives of *R. cassubicifolius* and *R. carpaticola*. In two accessions, AFLPs and flow cytometric data suggest local gene flow among different apomictic polyploid morphotypes. Frequent facultative sexuality of apomicts may increase genetic diversity by continuous formation of new cytotypes, local hybridization and introgression, which obstructs the fixation of distinct agamospecies. We conclude that “*R. cassubicus*” and “*R. auricomus*” cannot be regarded as species but should be treated as either informal groups, or as (notho)taxa at the sectional level. To reflect the different evolutionary processes involved, we propose a separate classification of the sexual species, *R. notabilis* and the closely related species pair *R. cassubicifolius* and *R. carpaticola*. Based on these well-defined biological species, the apomictic biotypes can be classified as nothotaxa.

KEYWORDS: apomixis, Europe, molecular systematics, morphometry, *Ranunculus auricomus* agg., taxonomy

INTRODUCTION

Apomictic polyploid complexes offer challenging questions for evolutionary research and classification. Apomixis, a mode of reproduction via asexually formed seed (Asker & Jerling, 1992; Richards, 2003), leads to the stabilization and dissemination of single genotypes. Apomictic lineages were traditionally thought to be genetically invariant lineages that originated from hybridization and polyploidization of sexual species that later became fixed by apomixis (e.g., Stebbins, 1950). Hybrid origin and highly complex and reticulate relationships within apomictic groups have been confirmed by molecular studies, e.g., in *Hieracium* s.l. (Fehrer & al., 2007a, b),

in *Antennaria* (Bayer & Chandler, 2007) and in *Boechera* (Koch & al., 2003). Studies on population genetics have shown that apomictic complexes harbour considerable genetic diversity within and among populations (review in Hörandl & Paun, 2007). Pollen usually remains functional in these lineages and functionality is reduced by meiosis and the occurrence of facultative sexually reproducing individuals has been demonstrated in several apomictic complexes (Asker & Jerling, 1992). In fact, backcrossing of apomictic individuals with sexual individuals, hybridization between apomictic lineages and facultative sexuality within populations are, along with mutations, the main factors promoting the recurrent formation of new genotypes (Hörandl & Paun, 2007). Non-maternal offspring

may also arise from a partial expression of the apomictic developmental pathway. Apomixis usually requires the coordination of embryo sac development without meiosis (apomeiosis) and the development of the egg cell without fertilization (parthenogenesis). Uncoupling of these processes leads to shifts in the ploidy levels of the offspring (Nogler, 1984a). Thus, meiosis plus parthenogenesis results in a dihaploid offspring ($n+0$); meiosis plus fertilization results in a homoploid recombined offspring ($n+n$, B_{II} hybrids) and fertilization of an apomeiotic egg cell results in an increase in ploidy level ($2n+n$, B_{III} hybrids).

From these processes, apomictic lineages with varying levels of stability and possessing distinct morphological, ecological and geographical features have originated in many genera. If such lineages are recognised as (agamo)species, apomicts would constitute half of the species diversity of the flora of the British Isles (Richards, 2003). The abundance and wide distribution of agamic complexes, as observed in, e.g., dandelions (*Taraxacum* spp.), blackberries (*Rubus fruticosus* agg.) and hawkweeds (*Hieracium* s.l.; see Hörandl, 2006a; Van Dijk, 2003), raises the need for practicable taxonomic concepts for biodiversity research and flora writing. Not only do apomictic complexes have a reticulated evolutionary structure, meaning that it is difficult to assign their position in hierarchical classifications, such complexes are also not equivalent to biological species because of the lack of interbreeding between populations. Furthermore, they are often too diverse to be regarded as ecological units. Consequently, apomictic complexes are often treated as informal groups such as aggregates (agg.) (sensu Ehrendorfer, 1973; Stace, 1998) or often simply called “complexes” (e.g., Grant, 1981). Within complexes, the formal classification of “microspecies” or “agamospecies” has been applied to apomictic lineages with a morphological and ecological distinctiveness comparable to that exhibited by sexual species (e.g., Stace, 1998). Application of agamospecies concepts requires internal constancy and stability of the apomictic lineage(s) both spatially and temporally (Weber, 1995, 1996; Hörandl, 1998). Understanding the evolutionary history, the extent of residual sexuality and the extent of internal stability of apomictic lineages is therefore essential for a practicable taxonomic concept.

The *Ranunculus auricomus* complex (*R. auricomus* agg.) is an interesting model system for studying the dynamics of evolution connected to apomixis. This complex belongs to the otherwise sexual section *R. sect. Auricomus*, which represents one of the larger clades within *Ranunculus* (Hörandl & al., 2005; Hörandl, in press) and is distributed from Europe to western Siberia, Greenland and Alaska, and from the arctic zone to the Mediterranean region (Jalas & Suominen, 1989). In Central Europe, populations colonize a broad spectrum of habitats, ranging from natural forests, wetlands and meadows to disturbed

areas (Hörandl & Paun, 2007). Within the genus *Ranunculus*, this complex has sometimes been treated as a separate section because of the great diversity of morphotypes, cytotypes and ecotypes that it contains (Borchers-Kolb, 1983; Loos, 1997) and the ca. 800 microspecies that have been described (Hörandl & Gutermann, 1998a).

Due to its high levels of diversity, the *R. auricomus* complex has often been subdivided into four morphological groups (*auricomus*, *cassubicus*, *fallax* and *monophyllus* sensu Hörandl & Gutermann, 1998a) or corresponding morphological grades, i.e., “morphological expressions” (Ericsson, 1992, 2001). Two of the most common morphotypes have frequently been classified as *R. cassubicus* L. s.l. and *R. auricomus* L. s.l. Following the suggestion of Ericsson (2001), these names are used in quotation marks throughout this paper to designate the traditionally recognized morphotypes. These morphotypes show striking differences in leaf shape. The “*R. cassubicus*” morphotypes possess basal sheaths without leaf blades (cataphylla) and large undivided basal leaves, while stem leaves may be broad or divided into segments with a smooth or toothed margin. The “*R. auricomus*” morphotypes lack basal sheaths and are characterized by the presence of heterophyllous leaves. Basal leaves can be deeply divided while stem leaves are linear with predominantly entire segments. The basal leaves of the “*auricomus*” and the “*cassubicus*” morphotypes are not homologous because they arise from different shoot systems (Hörandl & Gutermann 1998a; Lohwasser, 2001) and morphological diversity is further increased by specific features of the torus (Borchers-Kolb, 1983, 1985; Hörandl & Gutermann, 1998a). The broad series of intermediate morphotypes between the “*cassubicus*” and “*auricomus*” morphotypes have often been summarized under the names *R. fallax* (Wimm. & Grab.) Slob. and *R. monophyllus* Ovcz., though some authors have treated these four main morphotypes as formal species and have assigned distinct populations to subspecies (e.g., Marklund, 1961, 1965). The impossibility of demarcating clear boundaries between the four main morphotypes has encouraged most authors to abandon Marklund’s concept and adopt a microspecies concept for those groups of populations that are morphologically distinct (Ericsson, 1992; Hörandl, 1998).

Most of the polyploid cytotypes of the *R. auricomus* complex are apomictic, aposporous and pseudogamous (Häfliger, 1943; Rutishauser, 1954a, b; Rousi, 1956; Izmailow, 1967, 1973, 1996; Nogler, 1984b). Facultative apomixis, i.e., the occasional development of megaspore tetrads and meiotically derived embryo-sacs, has been demonstrated in “*cassubicus*” morphotypes by Izmailow (1967). In apomictic plants reduction by meiosis results in partly aborted, yet partly functional pollen (Häfliger, 1943; Izmailow, 1996; Hörandl & al., 1997). Apospory

is usually connected with parthenogenetic development of the egg cell, but uncoupling of these two processes may result in non-maternal offspring, as experimentally proven by Nogler (1984b, 1995). Nevertheless, apomixis is predominant enough to form clonal lineages, as seen in isoenzyme and DNA fingerprinting population studies (Hörandl & al., 2000, 2001; Paun & al., 2006b).

The detection of diploid species (*R. cassubicifolius* W. Koch, *R. carpaticola* Soó [“*cassubicus*” morphotype] and *R. notabilis* Hörandl & Guterm. [“*auricomus*” morphotype]; Hörandl & Guterm., 1998b) has given insights into the evolutionary history of the group. Studies on developmental biology, analysis of population genetic data, pollen studies, flow cytometry data and pollinator exclusion tests have confirmed that all three diploid taxa are sexual and outcrossing (Nogler, 1984b; Hörandl & al., 1997, 2000, 2001, 2008; Paun & al., 2006a; Hörandl, 2008). Moreover, phylogenetic analyses of the genus based on DNA sequence data and isoenzyme analysis reveal that *R. cassubicifolius* and *R. carpaticola* are closely related sister taxa and are genetically distant from *R. notabilis* (Hörandl, 2004; Hörandl & al., 2005; Paun & al., 2005). This confirms that the *R. auricomus* complex as a whole is not monophyletic; both sexual and apomictic species are distributed among clades containing other arctic and Central Asian species of *Ranunculus* in molecular phylogenetic reconstructions. The highly reticulate structure of the ITS sequence data suggests a complex hybrid origin of agamospecies (Hörandl & al., 2005). Only the “*cassubicus*” group is currently designated as monophyletic (Hörandl & al., 2005; Paun & al., 2005) and is also the morphologically most homogeneous group (Loos, 1997; Ericsson, 2001).

In recent years, intensive molecular and morphological work has elucidated the evolutionary relationships of the “*cassubicus*” morphotypes in Central and eastern Europe (Hörandl, 2002; Hörandl & Greilhuber, 2002; Paun & al., 2006a, b). The diploid and tetraploid species *R. cassubicifolius*, distributed from the Swiss Jura to the northern Prealps, is exclusively sexual whereas the closely related *R. carpaticola*, distributed in the Carpathians, has diploid sexual and apomictic hexaploid cytodesmes (Hörandl & Greilhuber, 2002; Paun & al., 2006a, b). Range fluctuations and hybridization of diploid sexual *R. carpaticola* and tetraploid *R. cassubicifolius* during or after the last glaciations have probably caused the origin of the widespread hexaploid apomictic cytotype of *R. carpaticola*, which will hereafter be referred to as *R. carpaticola* × *cassubicifolius* (Paun & al., 2006b). Reports of tetraploid *R. carpaticola* in Slovakia by Májovský & Múrin (1987) and findings of another slightly different morphotype matching *R. hungaricus* Soó by Mládenkova (2004) raise the question of whether the “*cassubicus*”-like morphotypes share a common ancestry.

In this case study we try to elucidate the evolutionary history of the Slovakian “*cassubicus*”-like populations in a larger geographical context and with a broad array of methods. The main goal of the study is to test various criteria for classification of the “*cassubicus*” morphotypes. Monophyly of the “*cassubicus*” morphotypes as a potential criterion for a supraspecific taxon is tested using DNA sequence analyses and phylogenetic reconstruction. To provide insights into the internal constancy of apomictic lineages, we also examine chromosome number distribution and ploidy levels within morphotypes via chromosome counts and DNA content measurements at the population level. Morphological distinctiveness of sexual species and apomictic morphotypes is studied on population samples using morphometric methods. To obtain further insights into dynamics of hybridization, we analyze the local genetic patterns at two sites, where different apomictic morphotypes are sympatric. This case study utilizes amplified fragment length polymorphism (AFLPs) to gain insights into the stability of sympatric apomictic lineages vs. occurrence of facultative hybridization. Based on the information gathered from all these datasets, we present a hypothesis for the evolution of these taxa and attempt to establish useful criteria for classifying this apomictic complex.

MATERIALS AND METHODS

Materials. — Between 10 and 25 individuals were collected from 31 populations and pressed as herbarium specimens; fresh leaf material was collected for molecular analysis and preserved in silica gel (see Table 1). The populations designated by acronyms have previously been studied with respect to morphology by Hörandl (2002), to ploidy levels by Hörandl & Greilhuber (2002), and with respect to analysis of AFLP profiles by Paun & al. (2006a, b). All studies were conducted on the same individuals. The populations designated by numbers ranging from 5 to 43 have not been previously examined. For morphometrics, 28 populations of the “*cassubicus*” morphotypes were used; a subset of this material, comprising 19 populations with the addition of one “*auricomus*” population (43) was used for AFLP analysis. For determination of the ploidy level of the new populations, between one and three individual plants were transferred to pots and grown on until root tips were actively growing. At this point meristem tissue was removed, fixed and DNA content was studied; from this material, a single individual from each of the 14 populations was selected randomly to assess DNA sequence variation. This material also included samples from the locus classicus of *R. hungaricus* which, while not complete enough for morphometrics (flowering stages only), was useful for ploidy and DNA sequence

analysis. The distribution of the studied populations is mapped in Fig. 1. Plant material has been either stored as herbarium specimens at WU and SAV or cultivated in the experimental fields of the Botanical Garden of the University of Vienna.

DNA sequence analysis. — Sequence variation of the nrITS and three plastid regions (*matK*, flanking *trnK*

intron and the *psbJ-petA* region) was assessed in 14 individuals from different populations, representing the two main morphotypes and the main geographical regions of the sexual taxa. Protocols for DNA extraction, amplification, sequencing, and primers for ITS and *matK-trnK* were those used by Paun & al. (2005) and the *psbJ-petA* region was amplified using the *psbJ* and *petA* primers of Shaw &

Table 1. List of materials and ploidy levels of the studied populations.

Final accepted name	Population acronym or number	Chromosome no. (ploidy level), mode of reproduction	Data-sets ^a	Location	Voucher: collector(s) + collection number, date (herbarium); GenBank accession numbers (in the order ITS, <i>matK-trnK</i> , <i>psbJ-petA</i>)
<i>R. notabilis</i>	NOT1	16 (2x) ^b sexual	FDM, m ^c , s	Austria, Burgenland, Strem valley, on 1,5 km ENE Strem, 220 m, wet meadow and forest margin	Hörandl 5612+7220, 20.04.1996 (WU); AY680033; AY954115 ^d ; FJ619873
<i>R. cassubicifolius</i>	BAV1	16 (2x) ^c sexual	FDM, m ^c , A ^e	Germany, Bavaria, N. Foreland of Alps, Dobelbachgraben, W Waginger See (forest)	Hörandl 8476, 18.04.1998 (WU);
<i>R. cassubicifolius</i>	BAV2	16 (2x) ^c sexual	FDM, m ^c , s, A ^e	Germany, Bavaria, N. Foreland of Alps, Osterbuchberg, SW Grabenstätt (forest)	Hörandl 8477, 18.04.1998 (WU); AY680040; AY954112 ^d , FJ619867
<i>R. cassubicifolius</i>	SALZ	16 (2x) ^c sexual	FDM, m ^c , A ^e	Austria, Salzburg, N. Foreland of Alps, between Grabensee and Mattsee (forest)	Hörandl 8479, 19.04.1998 (WU)
<i>R. cassubicifolius</i>	HAMM	16 (2x) ^c sexual	FDM, m ^c , s, A ^e	Austria, Burgenland/Hungary (directly on the border), Hammer (forest)	Hörandl & Paun 9562, 01.05.2005 (WU); FJ619887, FJ625800, FJ619871
<i>R. cassubicifolius</i>	YBB1	32 (4x) ^e sexual	FDM, m ^c , A ^e	Austria, Lower Austria, Wulfachgraben, SE Ybbsitz (forest)	Hörandl 8472, 12.04.1998 (WU)
<i>R. cassubicifolius</i>	YBB2	32 (4x) ^e sexual	FDM, m ^c , A ^e	Austria, Lower Austria, Schwarzois, SE Ybbsitz (forest)	Hörandl 8473, 12.04.1998 (WU)
<i>R. carpaticola</i> s.str.	BUCS	16 (2x) ^c sexual	FDM, m ^c , s, A ^e	Romania, South Carpathians, Mt. Bucegi, NW Busteni (forest)	Hörandl 9126, 19.07.1998 (WU); FJ619885, FJ625798, FJ619869
<i>R. carpaticola</i> s.str.	REV1	16 (2x) ^c sexual	FDM, m ^c , s, A ^e	Slovenské rudohorie, Revúca, hill Skalka (forest)	Hörandl 8483, 01.05.1998 (WU); AY680041; AY954111 ^d , FJ619866
<i>R. carpaticola</i> s.str.	REV2	2x ^e sexual	FDM, m ^c , A ^e	Slovenské rudohorie, Revúca, hill Pavlusove bane (forest)	Hörandl 8486, 01.05.1998 (WU)
<i>R. carpaticola</i> s.str.	5	16 (2x) sexual	FDM, m	C. Slovakia, Slovenské Rudohorie, Revúca, 13.5.2003	Mládenkova C5, 13.5.2003 (SAV)
<i>R. carpaticola</i> × <i>cassubicifolius</i>	13	4x apomictic	FDM, m, s	C. Slovakia, Veľká Fatra, beginning of the valley Gaderská dolina	Mládenkova C13, 26.5.2003 (SAV); FJ619884, FJ625797, FJ619868
<i>R. carpaticola</i> × <i>cassubicifolius</i>	14	40 (5x) apomictic	FDM, m	C. Slovakia, Veľká Fatra, Gaderská dolina, near ruins of Gaderský hrad	Mládenkova C14, 26.5.2003 (SAV)
<i>R. carpaticola</i> × <i>cassubicifolius</i>	15	40 (5x, 4x–5x) apomictic	FDM, m, s	C. Slovakia, Veľká Fatra, near Čremošné, direction to the ski lift	Mládenkova C15, 27.5.2003 (SAV); FJ619886, FJ625799, FJ619870;
<i>R. carpaticola</i> × <i>cassubicifolius</i>	19	48 (6x) apomictic	FDM, m	C. Slovakia, Turčianska kotlina, Martin – Záturčie, slope near the castle	Mládenkova C19, 29.5.2003 (SAV)
<i>R. carpaticola</i> × <i>cassubicifolius</i>	TRE	48 (6x) ^e apomictic	FCM, m, A	C. Slovakia, Strážovské vrchy (near Trenčín), between Kubra and Kubrica, close to the bus-stop Kyselka (margin of <i>Carpinus</i> forest and meadow);	Mládenkova, Paun & Hörandl C29, 30.4.2004 (SAV)

Table 1. Continued.

Final accepted name	Population acronym or number	Chromosome no. (ploidy level), mode of reproduction	Data-sets	Location	Voucher: collector(s) + collection number, date (herbarium); GenBank accession numbers (in the order ITS, <i>matK-trnK</i> , <i>psbJ-petA</i>)
<i>R. carpaticola</i> × <i>cassubicifolius</i>	VRU1	48 (6x) ^e apomictic	FCM, m, A	C. Slovakia, Turčianska kotlina, Vrútky-Piatrová, behind the cottage (forest);	<i>Mládenkova, Paun & Hörandl C34</i> , 1.5.2004 (SAV)
<i>R. carpaticola</i> × <i>cassubicifolius</i>	VRU2	48 (6x) ^e apomictic	FCM, m, A	C. Slovakia, Turčianska kotlina, Vrútky-Piatrová, behind the cottage (meadow)	<i>Mládenkova, Paun & Hörandl C35</i> , 1.5.2004 (SAV)
<i>R. carpaticola</i> × <i>cassubicifolius</i>	TUR	48 (6x) ^e apomictic	FCM, m, A	C. Slovakia, Turčianska kotlina (near Martin), Turčianska Štiavnička, Révayovský park (forest);	<i>Mládenkova, Paun & Hörandl C36</i> , 1.5.2004 (SAV)
<i>R. cassubicifolius</i> × <i>carpaticola</i>	RUZ	48 (6x) ^e apomictic	FCM, m, A	C. Slovakia, Liptovská kotlina, Ružomberok, right side of the road, direction from Žilina (shrub)	<i>Mládenkova, Paun & Hörandl C40</i> , 1.5.2004 (SAV)
<i>R. carpaticola</i> × <i>cassubicifolius</i>	HRA	48 (6x) ^e apomictic	FCM, m, s, A	C. Slovakia, Liptovská kotlina, Liptovský hrádok, close to the river Váh (riverine forest)	<i>Mládenkova, Paun & Hörandl C44</i> , 2.5.2004 (SAV); FJ619888, FJ625801, FJ619872
<i>R. carpaticola</i> × <i>cassubicifolius</i>	IVAC	48 (6x, 5x) ^e apomictic	FDM, f, m, A	C. Slovakia, Liptovská kotlina, Ivachnová (forest and meadow); together with population nos. 42, 43	<i>Hörandl 8492</i> , 1.5.1998 (WU); <i>Mládenkova, Paun & Hörandl C41</i> , 2.5.2004 (SAV)
<i>R. × hungaricus</i>	8	32 (4x, 4x–5x) apomictic	FDM, m	E. Slovakia, Nízke Beskydy, Humenský Sokol, near the hill Veľká skala	<i>Mládenkova C8</i> , 23.5.2003 (SAV)
<i>R. × hungaricus</i>	10	32 (4x)	FDM, m, s	E. Slovakia, Nízke Beskydy, near NPR Humenská, by sanatorium Podskalka	<i>Mládenkova C10</i> , 23.5.2003 (SAV); FJ619890, FJ625803, FJ619875
<i>R. × hungaricus</i>	11	32 (4x) apomictic	FDM, m, s	E. Slovakia, Nízke Beskydy, near the brook Hubková close to Humenné	<i>Mládenkova C11</i> , 23.5.2003 (SAV); FJ619891, FJ625804, FJ619876
<i>R. × hungaricus</i>	18	30–32 (4x) apomictic	FDM, m, s	C. Slovakia, Veľká Fatra, Frčkov, slope called Folkušovský úšust	<i>Mládenkova C18</i> , 28.5.2003 (SAV); FJ619894, FJ625807, FJ619878
<i>R. × hungaricus</i>	37	4x and 5x, apomictic	FDM, m, s, A ^e	C. Slovakia, Turčianska kotlina (near Martin), Turčianska Štiavnička, Révayovský park (meadow); together with TUR	<i>Mládenkova, Paun & Hörandl C37</i> , 1.5.2004 (SAV); FJ619889, FJ625802, FJ619874
<i>R. × hungaricus</i>	42	4x apomictic	FCM, m, A	C. Slovakia, Liptovská kotlina, Ivachnová (meadow); together with IVAC, 43	<i>Mládenkova, Paun & Hörandl C42</i> , 2.5.2004 (SAV)
<i>R. × hungaricus</i>	20735	4x apomictic	FCM, s	Hungary, Matricum, Miskolc, Mt. Bükk, Gyertán-völgy, 200 m SW Adriantelep (locus typi)	<i>F.G. Dunkel 20735</i> , 19.4.2008 (WU); FJ619892, FJ625805, FJ619881
<i>R. carpaticola</i> × <i>cassubicifolius</i> (introgressants)	9	4x apomictic	FDM, m, s	E. Slovakia, Nízke Beskydy, Humenský Sokol, near to old water reservoir	<i>Mládenkova C9</i> , 23.5.2003 (SAV); FJ619893, FJ625806, FJ619877
“ <i>R. auricomus</i> ” morphotype (unnamed)	43	4x apomictic	FCM, A	C. Slovakia, Liptovská kotlina, Ivachnová (meadow); together with IVAC, 42	<i>Mládenkova, Paun & Hörandl C43</i> , 2.5.2004 (SAV)

^a FDM, Feulgen densitometry and chromosome counts; FCM, flow cytometry; m, morphometry; s, DNA sequences; A, AFLPs.

^b Karyological data from Hörandl & al., 2000.

^c Morphological data from Hörandl & al., 2001.

^d Sequences from Paun & al., 2005; all others new here. Outgroup, GenBank accession numbers: *R. fluitans*: AY680069; AY954129^d, FJ619880; *R. gmelinii*: AY68003; AY954128^d, FJ619879.

^e Chromosome and AFLP data after Hörandl & Greilhuber, 2002, Paun & al., 2006a, b.

al. (2007). The new sequences were combined with five sequences available from Paun & al. (2005; *R. notabilis*, diploid *R. cassubicifolius*, diploid *R. carpaticola* and two outgroup species, *R. fluitans* and *R. gmelinii*.) The outgroup species *R. gmelinii* is sister to the *R. auricomus* clade on a basal branch, *R. fluitans* is a member of the next sister clade in Paun & al. (2005). Origin of materials and GenBank accession numbers are listed in Table 1.

Sequences of the ITS, *matK* and the adjacent *trnK* regions were aligned visually, based on the alignment available from Paun & al. (2005) and sequences of the *psbJ-psbA* region were aligned using ClustalX (Thompson & al., 1997). Subsequent manual corrections to both datasets were carried out with BioEdit (version 7.0.9.0; Hall, 1999). Insertion/deletion events (indels) were coded as binary characters for their presence or absence following the “simple indel coding method” (Simmons & Ochoterena, 2000) using the program SeqState (version 1.36; Müller, 2005).

A maximum parsimony analysis on the combined matrix was conducted using a heuristic search strategy with 1,000 replicates, random sequence addition, TBR branch swapping, Multrees on and all characters unordered and equally weighted. Multistate taxa were interpreted as polymorphisms. Gaps occurred in the ingroup

only as single bases and were therefore treated as a fifth base for coding purposes (two larger indels, 9 bp and 3 bp, were detected in the plastid sequences but were only present in outgroup taxa and were not regarded as relevant here). A strict consensus tree was computed from the 96 most parsimonious trees. Bootstrap support was assessed using non-parametric bootstrapping (Felsenstein, 1985) using 1,000 replicates, random sequence addition and NNI (nearest neighbour interchange) branch swapping and these values are shown on the corresponding branches of the cladogram. Separate analyses of the ITS region alone revealed a tree topology similar to the combined matrix, whereas analysis of the plastid region resulted in a largely unresolved tree (not shown). To further examine the basic grouping of diploid sexual taxa, a separate analysis was conducted on the combined matrix including the diploid sexual accessions only (BAV2, REV1, BUCS, HAMM, NOT1) using the same settings and outgroup as above, but just performing 100 bootstrap replicates. All analyses were conducted using PAUP vers. 4.0b (Swofford, 2003).

Karyological analysis. — Root tips of potted plants were pre-treated with colchicine, fixed in a mixture of ethanol-acetic acid, and stored for several weeks in the freezer. Feulgen staining was conducted synchronously with fixed root tips of *Pisum sativum* ‘Kleine Rheinländerin’

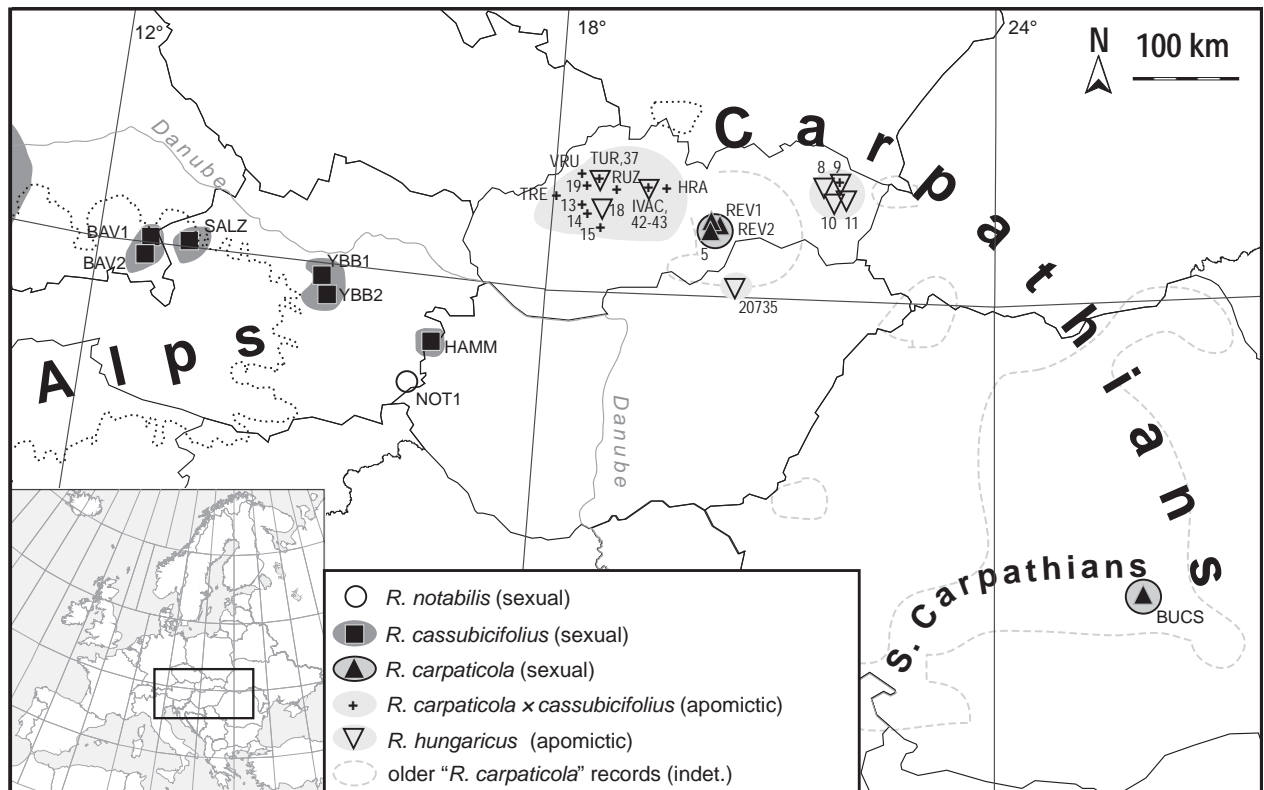


Fig. 1. Geographical distribution of studied populations (see Table 1). The dotted line indicates the extent of the ice shield of the last glacial maximum.

seedlings as semi-internal standard (semi-internal because the specimen and standard were fixed not synchronously but at a time interval of several weeks). The staining methods followed closely Greilhuber & Tensch (2001). Whenever it was possible, chromosomes were counted from the same slides used for DNA content measurements, aided by a drawing device (Zeiss) if required. Nuclear DNA content was measured by Feulgen DNA–image densitometry (Vilhar & al., 2001) using CIREs (Cell Image Retrieval and Evaluation System, Kontron, Munich), with a 63× immersion objective, ‘local’ background determination (around each nucleus separately), a green interference filter and the green channel of the CCD camera. One root tip was measured each from the standard and the *Ranunculus* specimens. A variable number of *Ranunculus* sp. nuclei of known replication status (20 to 220 early prophase and late telophases, exceptionally interphase nuclei) and a similar number of *P. sativum* late telophase nuclei were measured from each plant. The average coefficient of variation (CV) within slide and nucleus class was 2.4% in *Ranunculus* species and 2.0% in *P. sativum*. The 2C-value in pg of the *Ranunculus* specimens was calculated from the ratio of their Feulgen stain amount, corrected for G1/G0 nuclear cycle state, and that of the standard (in arbitrary units), multiplied by the 2C genome size of *P. sativum*, i.e., 8.84 pg (Greilhuber & Ebert, 1994). Genome size terminology follows Greilhuber & al. (2005), in that “holoploid genome size” (= C-value) and “monoploid genome size” (= Cx-value) are used to describe nuclear DNA content variation. The term ‘DNA ploidy’ (Suda & al., 2006) is used for indicating ploidy levels inferred via genome size measurement. Statistics analyses (*t*-test and correlation) were done using Excel and SPSS version 10. (SPSS, Chicago).

DNA ploidy determination (Suda & al., 2006) via flow cytometry was performed for two accessions where *R. hungaricus* is sympatric with other apomictic morphotypes: Turčianska Štiavnička: *R. hungaricus* (population 37, 2 individuals) and 6x *R. carpaticola* × *R. cassubicifolius* (population TUR, 2 individuals); Ivachnová: *R. hungaricus* (population 42, 2 individuals), hexaploid *R. carpaticola* × *R. cassubicifolius* (population IVAC, 6 individuals), and an undescribed “*auricomus*” morphotype with deeply divided basal leaves (population 43, 2 individuals). Additional measurements were also performed on each two specimens from the populations HAMM and on materials from the type locality of *R. hungaricus*. Nuclear DNA contents (C-values) were evaluated together with previous data on hexaploid individuals (published in Paun & al., 2006a). Methods followed Paun & al., 2006a, using *Hordeum vulgare* as standard (5.02 pg/IC; Dolezel & al., 1998).

Morphometric studies. — From the previous study conducted by K. Klimová (see Mládenková, 2004), measurements of the most important differential characters

were selected. Because some data was missing for some individuals (e.g., data on floral characters), the final dataset was reduced to eight characters (Table 2). *Ranunculus notabilis* was not included here due to the presence of heterophyllous sequences of basal leaves that are not homologous to the basal leaves of “*cassubicus*” morphotypes (Hörandl & Gutermann, 1998a; Lohwasser, 2001). The population sample from the locus classicus of *R. hungaricus* also had to be excluded because the sample included only flowering plants. Altogether 482 individuals from 28 populations were used for analysis. From this matrix, a principal component analysis was conducted and plotted in a scatter diagram. Canonical discriminant analysis was performed to test the group membership of the two sexual species (*R. carpaticola*, *R. cassubicifolius*), according to their geographical differentiation (Alps and Prealps/Carpathians) (CDA1). The whole dataset was analysed with four defined groups, based on mode of reproduction and putative evolutionary origin as suggested by DNA sequence analysis: (1) diploid and tetraploid sexual *R. cassubicifolius*, (2) diploid *R. carpaticola*, (3) the 6x and 5x apomicts *R. carpaticola* × *R. cassubicifolius* (clade I in Fig. 2), (4) the 4x apomict *R. hungaricus* (CDA2), clade II and population 37 (Fig. 2). Individuals of population 9 were assigned to either groups (3) or (4) according to their morphotypes. Boxplots of the four most discriminant characters were plotted for these groups. All statistical analyses were conducted using the program SPSS for Windows (vers. 11.0).

AFLP analysis. — Total genomic DNA isolation and fingerprinting were done as described by Paun & al. (2006a). Three selective primer combinations were used: *EcoRI* ACC (NED)–*MseI* CATA, *EcoRI* ACA (6-FAM)–*MseI*CTGA and *EcoRI* ACG (HEX)–*MseI* CTCG. Selective PCR products, together with an internal size standard

Table 2. List of morphometric characters used for multivariate analyses (with acronyms).

1. Diameter of the stem (between rosette and the lowest stem leaf (ST_D) [mm]
Lowest stem leaf, middle segment
2. Number of teeth on the margin (NR_TEETH)
3. Ratio of length/maximum width (L_LW); width measured excluding teeth
4. Position of the maximum width in relation to length of the middle segment (POS_L)
5. Width at the base of the middle segment (BASE_W) [mm]
Torus (measured at fruiting stage)
6. Height of androclinium (ANDRO) [mm]
7. Height/width ratio of gynoclinium (FR_LW); height and width measured excluding carpellophores
8. Length of the longest carpellophore (CARP) [mm]

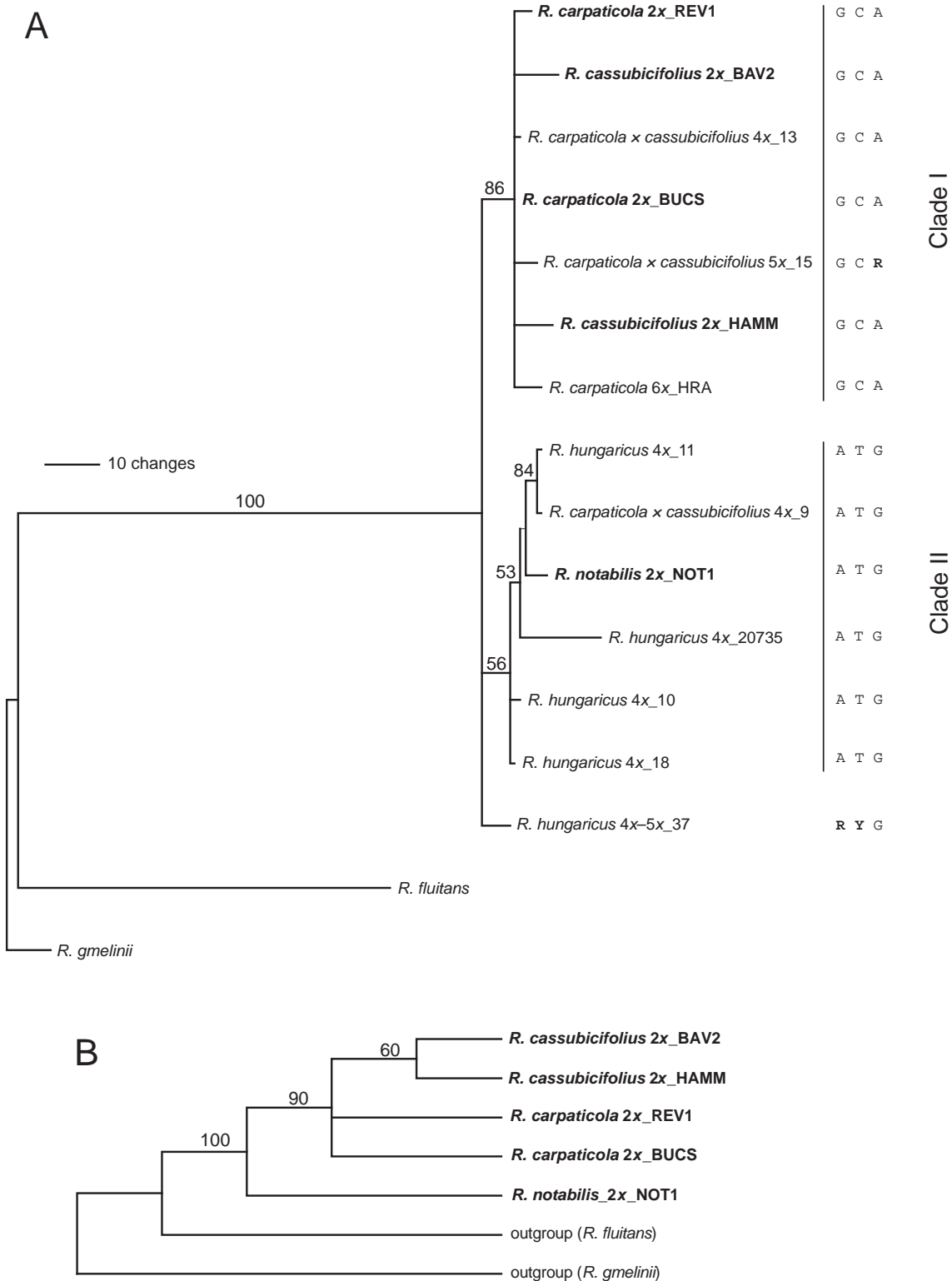


Fig. 2. Phylogenetic analyses based on the combined DNA sequence data matrix. Accessions given with ploidy levels and population acronyms: sexual taxa are shown in bold. Bootstrap support is shown above branches. A, strict consensus tree (phylogram) of eight most parsimonious trees based on diploid and polyploid accessions. Accessions are designated with the species name, ploidy level and population acronym or number (see Table 1). Additive bases at three parsimony-informative sites of the ITS region (43, 74, and 488) are given in bold. B, single most parsimonious tree of diploid sexual accessions only.

(GeneScan ROX 500, PE Applied Biosystems) were run on a 5% denaturing polyacrylamide gel on an automated sequencer (ABI 377, Perkin Elmer). Fragments in the range 50–500 bp were aligned with ABI Prism GeneScan 2.1 Analysis Software (PE Applied Biosystems) and visualized, scored and exported as binary presence/absence matrix using Genographer (version 1.6, available from <http://hordeum.msu.montana.edu/genographer/>). All 505 individuals included in the present analyses (see Table 1) have been genotyped in the same batch. Replicates and blind samples were routinely used to test the reproducibility of AFLP fingerprinting and contamination (Bonin & al., 2004); non-reproducible fragments were removed from further analyses. An unrooted neighbor joining dendrogram on the genetic distance of Nei & Li (1979) was generated and bootstrapped (Felsenstein, 1985) using 10,000 replicates with PAUP* 4.0b10 (Swofford, 2003). The Split-Decomposition network method using the program Splits Tree 4.0 beta (Huson, 1998) and uncorrected *P*-distances (Hamming distances) were used to investigate and visualize local inter-“specific” reticulations at two sites where different morphotypes grow sympatrically (TUR+37 and IVAC+42+43, Table 1). The robustness of the network was tested by 1,000 bootstrap replicates. A goodness of fit value indicates the degree of correlation between the graph and the information contained in the dataset.

RESULTS

Phylogenetic analysis of DNA sequence data. —

When aligned, the combined data matrix had a length of 2,982 characters with 2,791 constant and 100 parsimony-informative characters. The heuristic search of the combined matrix revealed 96 most parsimonious trees with a length of 229 steps, a consistency index of 0.836 and a retention index of 0.880. The strict consensus tree reveals two clades plus one accession (37) on an unresolved branch (Fig. 2A): clade I is supported by a bootstrap value of 86% and comprises all sexual cytotypes of *R. carpaticola* and *R. cassubicifolius*, plus most of the polyploid accessions *R. carpaticola* × *cassubicifolius*. Clade II has a bootstrap support of 56% and comprises the sexual diploid species *R. notabilis*, all tetraploid accessions of *R. hungaricus* and one accession from a mixed population of *R. carpaticola* × *cassubicifolius* and *R. hungaricus* (population 9). In the ITS region, additive bases occurred in the polyploids on parsimony-informative sites in three positions (Fig. 2A). The tetraploid accession 37 from a mixed population of *R. hungaricus* and *R. carpaticola* × *cassubicifolius* had two additive sites in the ITS region, combining the different phylogenetic signals of clades I and II.

A single, most parsimonious tree was obtained in the analysis of the diploid sexual accessions (Fig. 2B)

separating the *R. cassubicifolius*–*carpaticola* clade from *R. notabilis* with moderate bootstrap support (90%) and grouping the two accessions of *R. cassubicifolius* with a support of 60%.

Karyological data. — Feulgen densitometry, conducted on 34 individuals, revealed 5 diploids ($2n = 16$; pop. 5), 24 tetraploids ($2n = 32$; pops. 8, 9, 10, 11, 13, 15, 18), 3 pentaploids ($2n = 40$; pops. 14, 15) and two hexaploids ($2n = 48$; pop. 19). Chromosome counts or approximate counts were possible in all diploids, in ten of the tetraploids, in two of the pentaploids and in both hexaploids (Table 1). A summary list of the DNA content measurements assessed using Feulgen DNA image densitometry (FDM) and flow cytometry (FCM) is presented in Table 3. Based on the Cx-value of diploids, the tetraploids, pentaploids and hexaploids have 1.06-fold, 0.95-fold, and 0.88-fold the DNA amount in the monoploid genome. The pentaploids closely match the expected mean between tetraploids and hexaploids (Table 3; Fig. 3). With FCM, performed on different plants, a lower Cx-value for diploids, compared

Table 3. Previously unpublished DNA content measurements using Feulgen densitometry (FDM) and flow cytometry (FCM).

Final accepted name	Population designation	Method	DNA content in pg (2C), mean (standard deviation), no. of individuals
<i>R. cassubicifolius</i>	HAMM	FCM	6.42 (0.278), 3
<i>R. carpaticola</i>	5	FDM	5.97 (0.037), 5
<i>R. carpaticola</i> × <i>cassubicifolius</i>	13	FDM	12.74 (0.254), 3
<i>R. carpaticola</i> × <i>cassubicifolius</i>	14	FDM	14.10 (0.036), 2
<i>R. carpaticola</i> × <i>cassubicifolius</i>	15	FDM	13.27, 1 14.24, 1
<i>R. carpaticola</i> × <i>cassubicifolius</i>	19	FDM	15.73 (0.187), 2
<i>R. × hungaricus</i>	8	FDM	13.24 (0.211), 2
<i>R. × hungaricus</i>	10	FDM	12.35 (0.111), 8
<i>R. × hungaricus</i>	11	FDM	12.54 (0.154), 5
<i>R. × hungaricus</i>	18	FDM	12.65 (0.204), 2
<i>R. × hungaricus</i>	37	FCM	12.36, 1
<i>R. × hungaricus</i>	42	FCM	11.85, 1
<i>R. × hungaricus</i>	20735	FCM	12–13, 4 ^b
<i>R. carpaticola</i> × <i>cassubicifolius</i>	9	FDM	12.66 (0.143), 3
“ <i>R. auricomus</i> ”	43	FCM	12.124 (0.006), 2

^aSee Table 1.

^bNo exact values (herbarium material).

to that of tetraploids, is not evident, but the samples differ taxonomically (Tables 1, 3). Cx-values decrease with increasing ploidy level, so that tetraploids, pentaploids and hexaploids have on average 0.93-fold, 0.83-fold, and 0.81-fold the DNA content of the diploids in the monoploid genome. While neither methodological nor biological reasons for these moderate incongruences can be identified or excluded, the ploidy levels can be determined.

At taxon level, a comparison can be made between the present data and previously published C-values (Hörandl & Greilhuber, 2002) in *R. carpaticola* (formerly *R. carpaticola*, 2x) and in *R. carpaticola* × *cassubicifolius* (formerly *R. carpaticola*, 6x). The present C-values are marginally lower than previously established values by 0.975-fold and 0.977-fold, respectively, and are thus in reasonable agreement. The ratio of the Cx-values of the diploid versus the hexaploid taxon was 1.140 in Hörandl & Greilhuber (2002) and is now 1.139. Thus, both studies confirm a lower monoploid genome size in the hexaploid taxon, as is frequently observed in polyploids. The present tetraploids, most of them belonging to *R. hungaricus* (see Table 1), cannot be directly compared at taxon level with previous data. The previous study indicated a somewhat lower than expected Cx-value in tetraploid *R. cassubicifolius* by 0.923-fold, when compared with the diploid *R. cassubicifolius* (Hörandl & Greilhuber, 2002). In contrast, the present tetraploids, measured with FDM (*R. hungaricus* and *R. cassubicifolius* s.l.), have a slightly higher Cx-value than expected from the present diploids (see above).

DNA content measurements using flow cytometry revealed 2C values similar to densitometry-derived values and can be partitioned into four categories corresponding to ploidy levels: diploids, tetraploids, pentaploids and hexaploids (Table 3). The specimens from the locus classicus of *R. hungaricus* were confirmed as tetraploid. One individual of *R. hungaricus* (37) collected

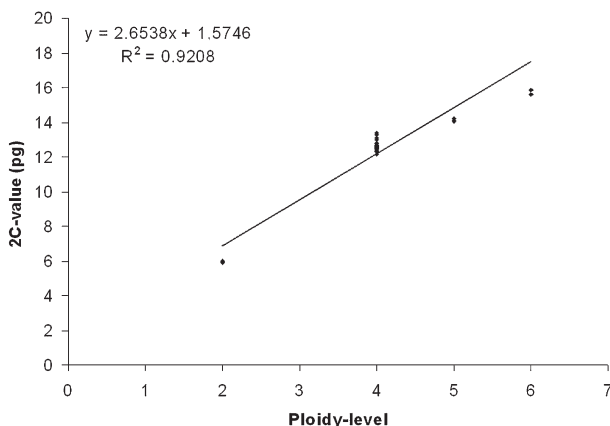


Fig. 3. Correlation test of DNA content and ploidy levels based on measurements with Feulgen densitometry; $P < 0.001$ (t -test).

at Turčianska Štiavnička was tetraploid, one was pentaploid, whereas two individuals of *R. carpaticola* × *cassubicifolius* (TUR) were confirmed as hexaploid. At the location Ivachnová, eight individuals of *R. carpaticola* × *cassubicifolius* (IVAC) were hexaploid, confirming earlier results (Hörandl & Greilhuber, 2002), but two pentaploid individuals were also detected. *Ranunculus hungaricus* (population 42) was confirmed as tetraploid by analysis of two individuals. The “auricomus”-like morphotype in the meadow habitat (population 43) was determined as tetraploid based on examination of two individuals. The 2C-values for the DNA ploidy levels obtained with flow cytometry closely resembled the densitometry-derived 2C-values. For diploid *R. cassubicifolius*, the flow cytometric measurements on population HAMM were slightly higher (6.22–6.74 pg, $N = 3$) than densitometry-derived values on other populations of this taxon (6.20–6.30 pg, $N = 48$; see Hörandl & Greilhuber, 2002). Diploid *R. carpaticola* has slightly lower values than diploid *R. cassubicifolius* with both methods (flow cytometry: 5.94–6.10 pg, $N = 2$, and Feulgen densitometry: 6.12 pg, $N = 54$; Hörandl & Greilhuber, 2002). The reason for the difference may be either technical or biological, but is yet to be clarified.

Morphological data. — Principal component analysis of populations extracted eight components; the first two components had Eigenvalues of > 1 (Appendix 1 in the online version of this article) and in combination possess the highest cumulative variance of nearly 54%. Component 1 has the highest correlation with the length/width ratio of stem leaf segment and component 2 with the length of carpellophores (Appendix 2 in the online version of this article). The scatter plot (Fig. 4) shows two weakly separated clouds connected by a transitional zone, comprising tetraploid and pentaploid populations (represented by dots). The left cloud comprises tetraploid and pentaploid cytodesmes of *R. hungaricus*, whereas the right one is internally more heterogeneous and comprises 2x, 4x, 5x and 6x apomictic and sexual cytodesmes. The sexual cytodesmes (2x and 4x *R. cassubicifolius* and 2x *R. carpaticola*; filled symbols) show differentiation along the axis corresponding to factor 2 as previously demonstrated in Hörandl (2002), but are here connected by a broad zone of intermediate forms representing the hexaploid apomictic hybrid derivatives of the two sexual taxa (represented by the cross symbol); for molecular evidence of hybrid origin see Paun & al. (2006b).

A canonical discriminant analysis grouping the sexual species *R. cassubicifolius* and *R. carpaticola* (CDA1) reveals one canonical discriminant function with an Eigenvalue of 2.607 and a canonical correlation of 0.850 (Wilks’ lambda = 0.277, Chi-square = 300.163, $df = 8$, $P < 0.001$). The canonical discriminant function has the highest within-group correlation to the variable number of teeth, followed by length of carpellophores (see

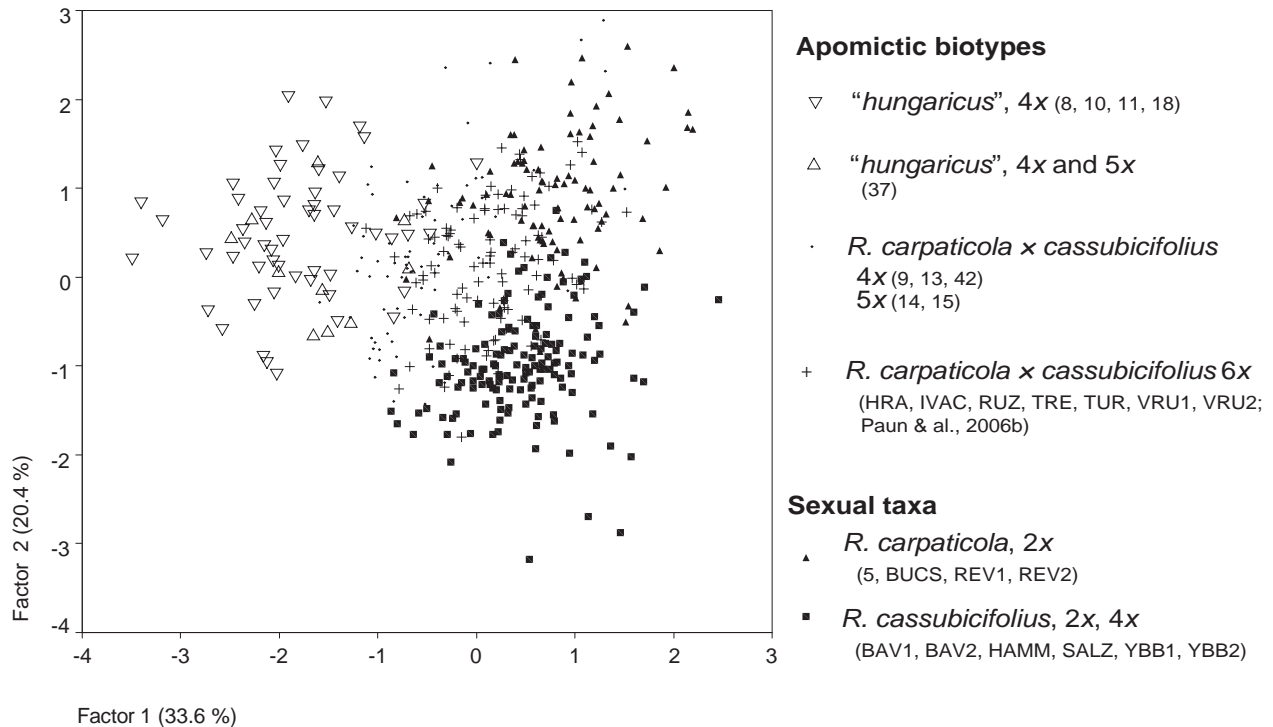


Fig. 4. Principal component analysis of morphometric data of all “*cassubicus*” morphotypes. Percentages of total variance explained by the first two factors are given in parentheses.

Appendix 3 in the online version of this article for correlations of variables and the canonical function). Altogether 93.8% of the original cases (n = 240) were correctly classified (see results of classification in Table 4).

The canonical discriminant analysis of the final four taxa (*R. cassubicifolius*, *R. carpaticola*, *R. carpaticola* × *cassubicifolius*, *R. hungaricus*) is represented in Appendices 4–6 in the online version of this article. The variables with the greatest contribution to the discriminant functions were length/width ratio of stem leaf segment in the first function, number of teeth of the stem leaf segment in the second and the position of the maximum width of the stem leaf segment in the third one (Appendix 6 in the online version of this article). A 3D scatter plot of the three functions shows a weak separation of *R. hungaricus*, whereas the three other groups were strongly

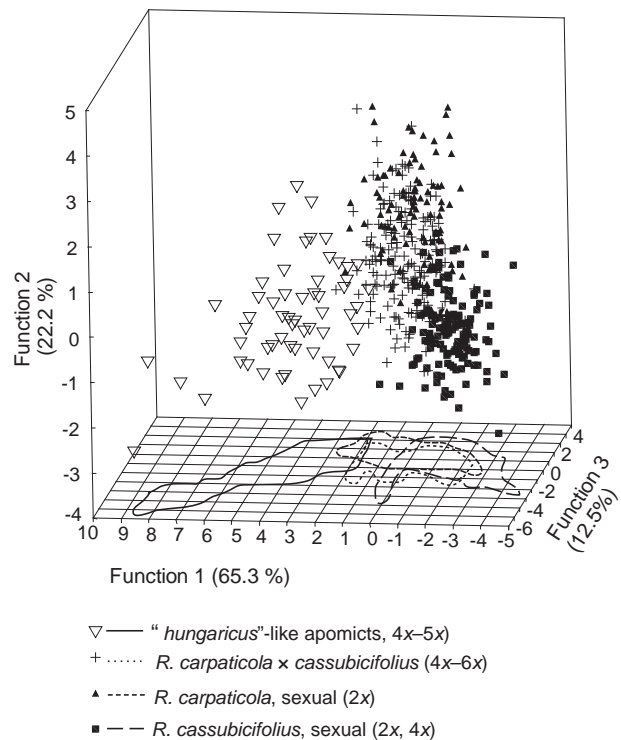


Fig. 5. Discriminant function analysis of morphometric data with four predefined groups which cluster corresponding to taxa. Percentages of total variance explained by the functions are given in parentheses.

Table 4. Classification results of discriminant analysis 1 of eight morphological characters with two predefined groups corresponding to the geographical groups of sexual species: number and percentages (rounded) of correctly classified cases.

		Predicted group membership		Total
		1	2	
1	<i>R. cassubicifolius</i>	139 (95.2%)	7 (4.8%)	146
2	<i>R. carpaticola</i>	8 (8.5%)	86 (91.5%)	94

overlapping (Fig. 5). The discriminant analysis showed that only 84.0% of original cases were correctly classified (Table 5), indicating that the inclusion of apomictic hybrid derivatives creates a morphological transition zone. The highest frequencies of incorrect classifications occurred between *R. carpaticola* and *R. carpaticola* × *cassubicifolius*, and between *R. hungaricus* and *R. carpaticola* × *cassubicifolius* in population 9. Boxplots of these four groups confirm a strong overlap of variation in all discriminating characters except for the length/maximum width ratio, which separates *R. hungaricus* from the other groups (Fig. 6).

AFLP data. — The three AFLP primer combinations yielded 359 reproducible fragments, of which 7.5% were monomorphic. In the 505 individuals analysed, AFLPs identified 291 unique multilocus genotypes. In the apomictic populations, the presence of 18 clones formed by between two and 33 individuals was indicated. All identified clonal lineages were restricted to individual populations. In the neighbor joining analysis, seven major clusters were observed corresponding to different ploidy levels and/or geographical areas, but just four of them were strongly supported (BS > 80%). The three populations (pops. 37, 42, 43) that were not included in previous

analyses (i.e., Paun & al., 2006a, b) form a single cluster (BS 91%) and within each of these three populations, the majority of individuals have identical genotypes and form local clones (Fig. 7).

Split decomposition analyses evidenced a reticulate relationship within the 21 individuals from Turčianska Štiavnička (populations TUR and 37) and also for the 93 individuals from Ivachnová (populations IVAC, 42 and 43). The network-like connection between different morphotypes in populations TUR and 37 suggests the presence of local gene flow among co-occurring microspecies, together with frequent recombination events within population 37 (Fig. 8A). At Ivachnová, reticulations are indicated within IVAC and respectively between the populations 42 and 43, while the simple connection between morphotypes suggests infrequent local gene flow between them (Fig. 8B). Another indication of putative gene-flow between morphotypes was given by private markers for different groups. From the 21 novel fragments generated in the allopolyploid *R. carpaticola* × *cassubicifolius* as compared with its parents (Paun & al., 2006b), 6 were found to be shared with *R. hungaricus*. None of them was fixed in any of the morphotypes, supporting the occurrence of localized gene flow.

Table 5. Classification results of discriminant analysis 2 of eight morphological characters with four predefined groups corresponding to taxa: number and percentages (rounded) of correctly classified cases (see also Fig. 5).

Predefined groups	Predicted group membership				Total
	1	2	3	4	
1 <i>R. cassubicifolius</i>	130 (89.0%)	6 (4.1%)	10 (6.8%)	0 (0.0%)	146
2 <i>R. carpaticola</i>	8 (8.5%)	76 (80.9%)	10 (10.6%)	0 (0.0%)	94
3 <i>R. carpaticola</i> × <i>cassubicifolius</i>	20 (11.0%)	16 (8.8%)	144 (79.6%)	1 (0.6%)	181
4 <i>R. hungaricus</i>	0 (0.0%)	0 (0.0%)	6 (10.0%)	54 (90.0%)	60

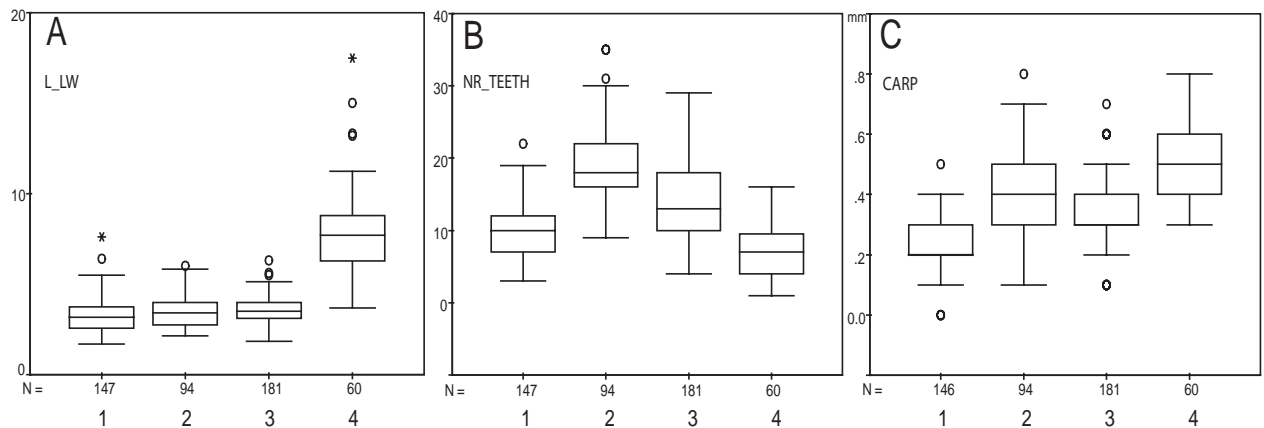


Fig. 6. Boxplots of three discriminating characters, groups as in Fig. 4. 1, *R. cassubicifolius*, 2x, 4x; 2, *R. carpaticola*, 2x; 3, *R. carpaticola* × *cassubicifolius*, 4x–6x apomictic cytotypes; 4, apomictic *R. hungaricus*. A, length/width ratio of middle segment of lowest stem leaf; B, no. of teeth of middle segment of lowest stem leaf; C, length of carpellophores.

homozygosity, e.g., that observed in the inbreeding coefficients of BUCS inferred from analysis of allozyme data (Hörandl & Greilhuber, 2002), may also influence sequence polymorphisms (Glémin & al., 2006).

The well-supported split between the sexual species provides the basic phylogenetic framework for understanding the evolution of the polyploid taxa. The analysis of polyploid derivatives, together with the sexual progenitors, renders the latter necessarily paraphyletic, which is an artefact of tree-building methods if progenitors and derivatives are analyzed together (e.g., Hörandl, 2006b). In the interpretation of tree topologies including polyploid taxa of hybrid origin, caution is recommended with the inclusion of the ITS region in the analysis. Additive parental ITS copies may have already been homogenized towards one parental species via concerted evolution. In agamic lineages, polymorphisms in the ITS sequence may persist due to the lack of meiotic recombination (e.g., Campbell

& al., 1997). The low frequencies of additive sites in our polyploid apomicts compared to other studies (e.g., in *Amelanchier*; Campbell & al., 1997) suggests that either concerted evolution may have homogenized the ITS copies in the hybrids before they shifted to apomixis, or that rare ITS copies have remained undetected (Bayer & Chandler, 2007). Multiple hybridizations may have also resulted in complex patterns of concerted evolution, affecting parental taxa (Koch & al., 2003). Concerted evolution of multiple ITS copies in polyploids, allied to faster sequence divergence of asexuals compared to sexuals (Birky, 1996), may influence phylogenetic reconstructions.

The main phylogenetic signals separating the sexual taxa and the clades I and II (Fig. 2A) renders the accessions with a “*cassubicus*” morphotype as non-monophyletic: the tetraploid *R. hungaricus* accessions, with the exception of population 37, form a clade together with *R. notabilis*, the diploid sexual representative of the “*auricomus*”

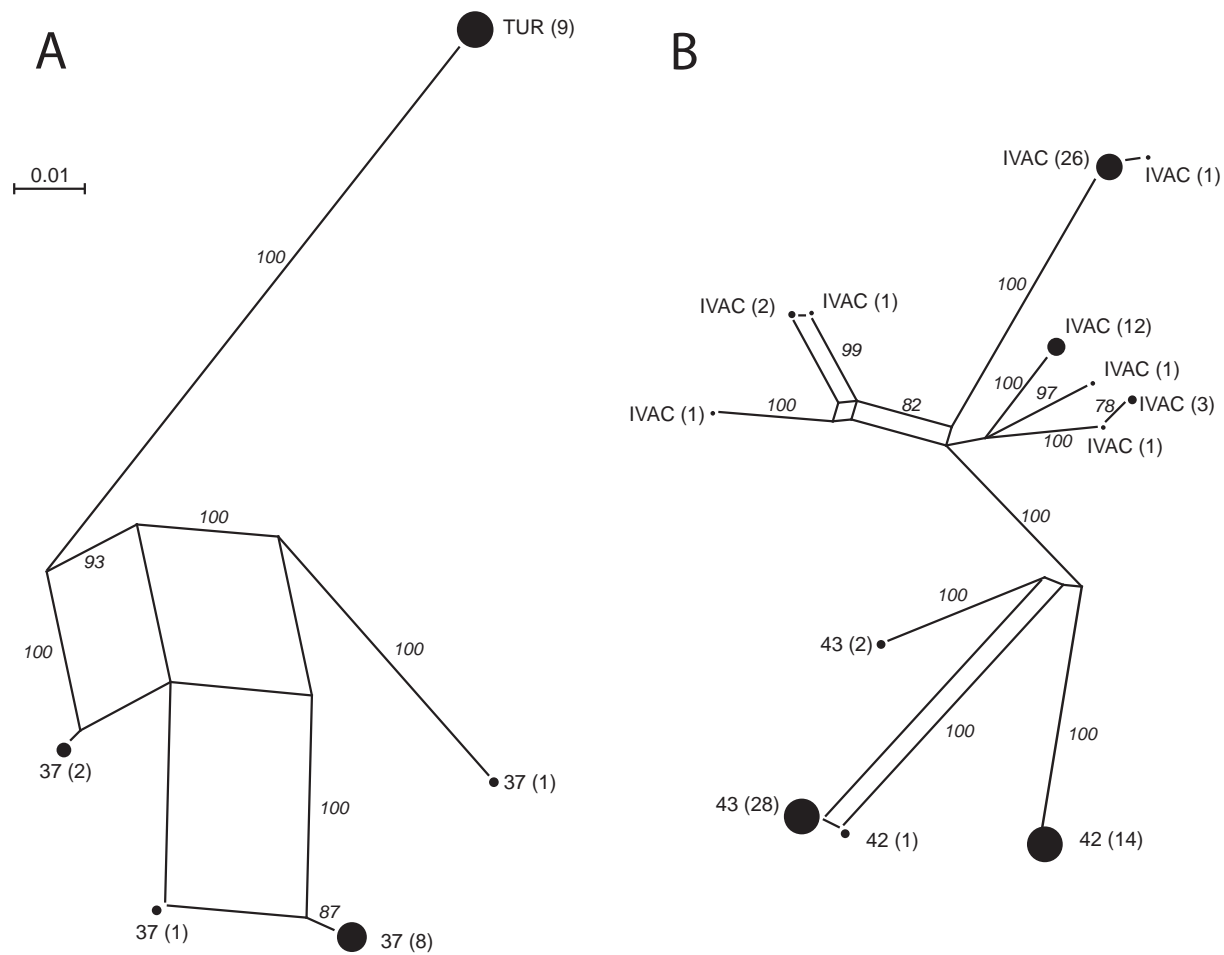


Fig. 8. Split decomposition networks for sympatric populations of *R. carpaticola* × *cassubicifolius* and *R. hungaricus*. A, Turčianska kotlina (populations TUR+37; goodness of fit 99.6%); B, Ivachnová (populations IVAC+42+43; goodness of fit 73.2%). The number of individuals sharing a particular genotype is indicated in brackets. Values on the branches indicate bootstrap percentages.

morphotype (see Hörandl & Gutermann, 1998b). The incongruence of morphology and DNA sequence data suggest a complex evolutionary origin of *R. hungaricus*, perhaps via allopolyploidy involving sexual ancestors of *R. notabilis* and diploid *R. carpaticola* (Fig. 9). In the ITS region, additive copies in the original primary hybrids may have been homogenized towards one parental species and caused the grouping with *R. notabilis* in the phylogenetic tree. Experimental crosses of *R. notabilis* with diploid *R. carpaticola* revealed highly sterile F₁ hybrids (Hörandl, 2008). Apomixis might have arisen after the hybrid origin of *R. hungaricus* and probably aided in overcoming meiotic disturbances and minority cytotype disadvantages in newly arisen allotetraploid pseudogamous hybrids (Levin, 2002). Recent hybridization between the extant sexual taxa *R. notabilis* and diploid *R. carpaticola* is unlikely because of geographical isolation (Fig. 1). The tetraploid individual from population 9 occurs in a population where both *hungaricus* and *cassubicifolius* morphotypes grow together; it groups with population 11 from the same area and is probably a local introgressed lineage. Population 37, comprising 4x and 5x individuals, can be interpreted as a recent reticulation event and a secondary local hybrid derivative of 6x *R. carpaticola* × *R. cassubicifolius* and 4x *R. hungaricus*. The phylogenetic position of this sample remains unresolved and additive bases are present

at two parsimony-informative sites in the ITS region. The hypothesis of secondary hybridization among hexaploid and tetraploid apomicts is supported by the occurrence of pentaploids and a reticulate structure of the AFLP data (see below). A more detailed analysis of ITS polymorphisms exceeds the scope of this paper.

The evolution of different cytotypes. — AFLP data suggest the fragmentation of a widespread sexual “*cassubicus*” ancestor into narrower, geographically isolated areas due to glaciation in the Pleistocene (see comprehensive discussion in Paun & al., 2006b; Figs. 7, 9). Recent dispersal or gene flow between these geographically isolated populations is unlikely. In contrast, drift may influence the genetic structure of small isolated populations, particularly in HAMM (a singular population on the Austrian/Hungarian border), but also in BUCS (South Carpathians). Moreover, a mutually exclusive clustering of individuals into their populations in neighbor joining trees is expected in AFLP datasets displaying low levels of population differentiation (e.g., F_{st} values = 0.07; see Hollingsworth & Ennos, 2004; in our study, F_{st} values for diploids were 0.06; see Paun & al., 2006a). In addition, the tree topology can be strongly influenced by different ploidy levels. The autotetraploid *R. cassubicifolius* from Lower Austria has a much higher frequency of specific fragments than the diploid populations (Paun & al., 2006b), which

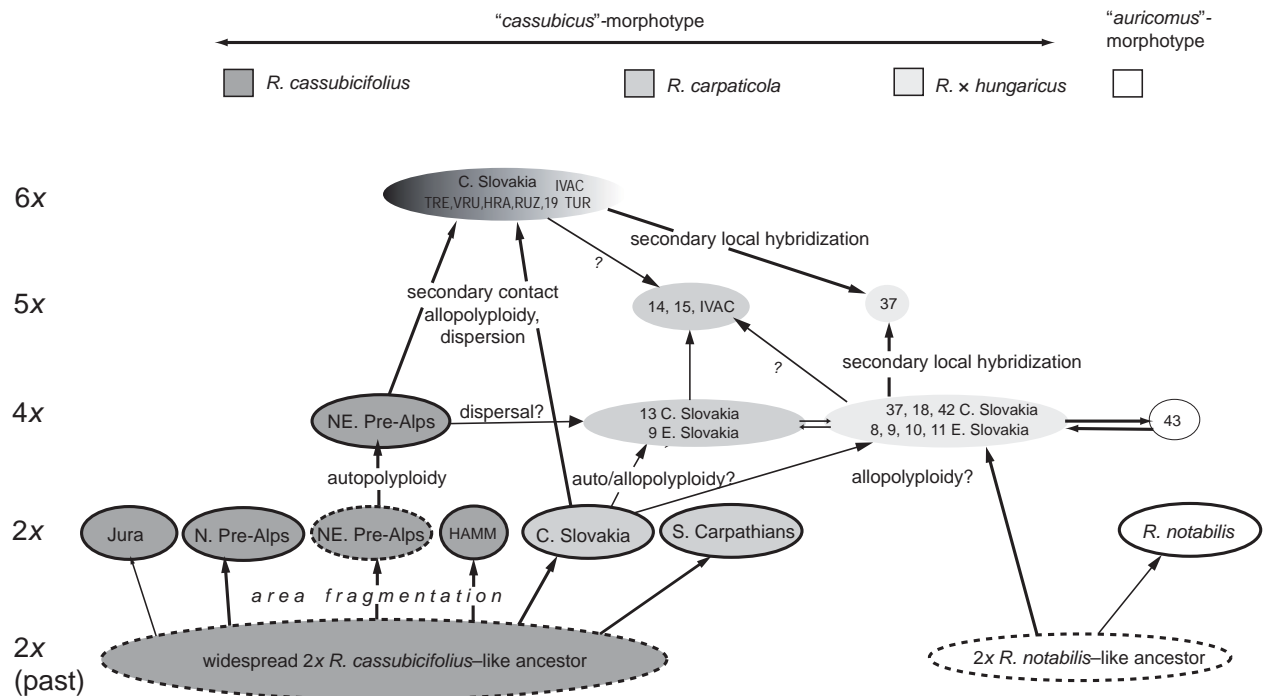


Fig. 9. Hypothetical outline of the evolution of the *Ranunculus auricomus* complex. Shading indicates morphotypes, ploidy levels are indicated on the left. Arrows indicate possible evolutionary pathways. Bold arrows are supported by molecular data. Dashed outlines of bubbles indicate hypothetical ancestors. Solid bold lines represent extant sexual populations and solid lines extant apomict populations.

may cause its separation from the diploid *R. cassubicifolius* in tree reconstructions. In the apomictic populations, the two main clusters correspond to the predominant ploidy levels, hexaploid vs. tetraploid (Fig. 7); all clusters representing different polyploid levels have bootstrap support, which contrasts with the absence of support for the geographical clusters of the diploids. These factors may also explain why the topology of the NJ tree presents a pattern strongly influenced by geography, but does not reflect the morphology-based grouping of the sexual taxa (see also Meudt & Clarke, 2007; Guo & al., 2008).

The morphological differentiation of the closely related taxa *R. cassubicifolius* and *R. carpaticola* matches with the main geographical divide (Alps vs. Carpathians) and to allozyme population clusters (Hörandl & Greilhuber, 2002; Hörandl, 2002, 2004). The two taxa differ in their ecology, *R. cassubicifolius* growing in wet forest habitats (swamps and riverine forests dominated by alders), *R. carpaticola* occurring in mesic to dry forests (in the forest floors of oak, hornbeam, or beech forests). In cultivation characters remain constant, contradicting the hypothesis that environmental conditions influence characters of leaves (Hörandl, pers. obs.) and overall the data suggest a rather young, perhaps still incomplete allopatric speciation process from a widespread ancestor. The sexual autopolyploid cytotype of *R. cassubicifolius* is morphologically and ecologically similar to the diploid one (Hörandl, 2002) and forms a separate cluster in AFLP marker analysis, but has an ITS sequence identical to diploid *R. cassubicifolius* (population BAV1; Hörandl & al., 2005).

The hexaploid populations express a high morphological variation overlapping that of both parental taxa (*R. cassubicifolius* and *R. carpaticola*), thus blurring the morphological differences that would be otherwise more readily observed in the sexual taxa alone (see Figs. 4, 5). These hexaploids do not show any novel morphological features when compared to sexual parents. Thus, the morphological data strongly corroborate a hybrid origin of hexaploids from diploid *R. carpaticola* and tetraploid *R. cassubicifolius*, as suggested by Paun & al. (2006b). For the origin of tetraploid cytotypes in clade I, different origins can be hypothesized: (1) they may have arisen as autotetraploid derivatives of diploid *R. carpaticola* or derivatives of autopolyploid *R. cassubicifolius* after an event of long distance dispersal or major range fluctuations. (2) Hybrid origin from *R. carpaticola* × *R. notabilis*, with a later fixation of a “*cassubicus*”-like genotype. (3) They may be secondary hybrids and backcrosses between 4x *R. hungaricus* and 6x *R. carpaticola* × *cassubicifolius*. The two latter hypotheses are supported by the somewhat intermediate morphology of these populations (Fig. 4).

The non-linear increase in DNA content supports the hypothesis that the 4x *R. hungaricus* cytotypes are not derivatives of the sexual *R. cassubicifolius*-*carpaticola*

alliance alone, as the mean values for tetraploids are proportionally too high in relation to those of the diploids (Fig. 3). This is unusual considering the general tendency for reduction of DNA content in polyploids (Leitch & Bennett, 2004; but see Leitch & al., 2008), which is also observed in the autotetraploid and hexaploid hybrid derivatives of diploid *R. cassubicifolius* (Hörandl & Greilhuber, 2002; Fig. 3). Considering the results of DNA sequence analysis, genome size measurements and morphological distinctiveness, we regard a hybrid origin involving at least two different sexual species as the most plausible hypothesis (Fig. 9).

A more pronounced differentiation of stem leaf segments with long and irregular teeth plus longer carpellophores discriminate *R. hungaricus* from the other “*cassubicus*”-like taxa. It is not possible to exclude the possibility that other sexual, extinct or unknown species, or other extant agamospecies, such as those from population 43, were involved in the origin of this morphotype. In *Antennaria*, several different sexual species have been involved in the origin of the apomictic *A. rosea* complex (Bayer & Chandler, 2007). In *Hieracium* subg. *Pilosella*, a comparable group with facultative apomixis, interspecific and interploidal hybridization has led to relationships of high complexity (Fehrer & al., 2007a, b).

Stability of apomictic lineages. — The occurrence of different cytotypes within the main discernible morphotypes raises questions about the stability of apomictic polyploid lineages, because cytotype diversity probably arose from facultative or incomplete sexuality. Such events are more frequent in “*cassubicus*” morphotypes than in other taxa of the *R. auricomus* complex because of the regular formation of functional megaspore tetrads (Izmailow, 1967). Megaspores usually degenerate due to developmental problems, but occasionally form reduced embryo sacs. Aposporous initials arise in parallel and compress the megaspore tetrads aside because of intensive growth, but occur only in ca. 25% of the ovules studied (Izmailow, 1967). This strikingly low frequency of aposporous embryo sac formation may increase the chance of occasional survival of meiotically derived, functional megaspores (Izmailow, 1967). These mechanisms may explain the higher frequencies of facultative or partly expressed sexual, interspecific or interploidal hybridization and thus the higher diversity of cytotypes in the “*R. cassubicus*” group than in other taxa of the *R. auricomus* aggregate. Jankun & Izmailow (1965) reported chromosome numbers of $2n = 24, 32, 40, 44, 48$ and 64 for “*cassubicus*” morphotypes from Poland and all ploidy levels may occur within a single microspecies (e.g., *R. marginicolus* Jas.). Different ploidy levels occur regularly within populations. High clonal diversity and facultative recombination within the Slovakian populations (Paun & al., 2006a; Figs. 8, 9) suggests the occurrence of rather frequent sexual events in

this complex. Hexaploid apomicts in Poland undergo more regular pollen formation (Jankun, 1966), which may also increase the quality of seed set in pseudogamous plants (Hörandl, 2008). Because of reduction of meiotic disturbances in hexaploids (Izmailow, 1967), sexuality may also maintain the hexaploid level by forming B_{II} hybrids.

Hybridization between the different apomictic morphotypes probably occurs in at least two sites: (1) Turčianska Štiavnička, where populations 37 and TUR grow together. The occurrence of tetraploid and pentaploid individuals of *R. hungaricus* (pop. 37), additive sites in the ITS region and reticulate relationships in the AFLP data (Fig. 8A) suggest local hybridization between *R. hungaricus* and the hexaploid *R. carpaticola* × *cassubicifolius*-cytotype, whereby population 37 maintains the morphological features of *R. hungaricus*. The low percentage of specific AFLP bands generated in the hexaploid *R. hungaricus* individuals supports the hypothesis of a secondary local introgression as the origin of tetraploid *R. hungaricus* from hexaploid cytotypes. (2) In the meadows at the location Ivachnová, a morphologically quite distinct tetraploid cytotype, with heterophyllous and deeply divided basal leaves (unnamed; population 43; see Mládenkova, 2004) grows in sympatry with one pentaploid cytotype of IVAC, in a predominantly hexaploid population. Some individuals morphologically resemble *R. hungaricus* (designated as population 42) and cluster in the AFLP NJ tree together with the main clone of population 43 and some individuals of the above discussed population 37 (Fig. 7). A more detailed split decomposition analysis of individuals from this site maintains the hexaploid IVAC cytotypes on a separate, well-supported branch (Fig. 8B). *Ranunculus hungaricus* (pop. 42) forms a clone of 14 individuals, but also reticulates with some of the genotypes of population 43, which is otherwise clonal (Fig. 7). Here either local introgression or an evolutionary origin of tetraploid cytotypes matching *R. hungaricus* from the tetraploid apomictic “*auricomus*” morphotype is possible. In both locations, the occurrence of both clones and deviating genotypes in both morphotypes is probably caused by facultative sexuality. Local hybridization and introgression may explain the observed morphological “continuum” between the two main apomictic morphotypes.

To summarize, our data provide the following information for classification: (1) the “*cassubicus*” morphotypes do not represent a monophyletic group, because *R. hungaricus* is probably of hybrid origin involving a “*auricomus*”-like ancestor; (2) the sexual taxa, *R. carpaticola* and *R. cassubicifolius*, are closely related, and parents of an allohexaploid apomictic lineage; (3) the apomictic taxa *R. carpaticola* × *R. cassubicifolius* and *R. hungaricus* can be separated morphologically, but are connected by several intermediate forms; (4) internal stability of apomictic taxa is low because of hybridization among lineages and cytotypes.

CONCLUSION

Taxonomy. — The formal classification of apomictic complexes remains problematic in both theory and practice. Our study demonstrates that even fairly uniform morphotypes such as the “*cassubicus*” group do not represent monophyletic groups with a common evolutionary origin. The morphological homogeneity observed in the “*R. cassubicus*” group (Ericsson, 1992, 2001; Loos, 1997; Hörandl, 1998) is probably caused by two main factors: (1) a low morphological differentiation of sexual progenitor taxa, resulting in distinct hybrid morphotypes only if a more distantly related sexual taxon was involved in the origin of allopolyploid agamospecies; (2) an instability of cytotypes because of facultative sexual processes and hybridization, thus obstructing a fixation of distinct characters. A primary phenetic approach is problematic because morphological similarity is neither indicative of common ancestry nor common evolutionary history. Hybrids of morphologically different sexual species may have segregated in the F₂ or later generations, creating a broad variety of morphotypes. According to all present information available, apomixis does not arise in F₁ hybrids of sexual species, but requires several generations of inter-crossing and polyploidization (e.g., Van Dijk & Vijverberg, 2005). Consequently, apomixis may “freeze” the diversity of hybrid swarms. Reticulate evolution may best explain the frequently observed mosaic patterns of morphological characters in apomictic complexes (e.g., Fröhner, 1990; Kirschner & Štěpánek, 1996). Our case study demonstrates that the “*cassubicus*” and “*auricomus*” morphotypes cannot be classified as species and that infraspecific taxonomic concepts such as the “main species concept” of Marklund (1961, 1965) fail because a hierarchical system cannot reflect the interspecific reticulate relationships. Such concepts have been abandoned in the *R. auricomus* complex (Ericsson, 1992, 2001) and most of the other apomictic genera (Stace, 1998). Such groups are better treated informally as complexes, aggregates (e.g., Stace, 1998), “collective groups” (Hörandl & Gutermann, 1998a) or as (notho-)sections or subsections (e.g., Fröhner, 1990: *Alchemilla*; Weber, 1996: *Rubus* sect. *Rubus*; Kirschner & Štěpánek, 1996: *Taraxacum*), which best reflect the diversity harboured in such groups. The “*cassubicus*” morphotypes could eventually be treated as a (notho)subsection *Cassubici* Loos within the large section *Auricomus* Schur (Hörandl, in press).

On species level, an improvement in classification can be gained from the separation of exclusively sexual taxa from the polyploid apomictic derivatives, as proposed by Grant (1981), Weber (1996) and Kirschner & Štěpánek (1996). Diploid sexual species originated from different and likely earlier evolutionary processes than agamospecies. These species can be readily classified according to

broadly applicable criteria, e.g., under biological, phylogenetic or morphological species concepts. Contrary to apomictic complexes, a phenetic grouping as the primary operational approach remains valid (e.g., Stuessy, 1990). The apomictic plants can be often discriminated from the sexual species by having aborted petals and high frequencies of aborted pollen (e.g., Izmailow, 1996; Hörandl & al., 1997). Novel methodical techniques, such as flow cytometric seed screening, allow for a rapid assessment and discrimination of modes of reproduction (Matzk & al., 2000; Hörandl & al., 2008). A formal separation of the diploid sexual species improves the understanding of phylogenetic relationships, biogeographical patterns and evolutionary processes of a complex within the rest of the genus. Separating sexual species allows the exclusion of polyploid hybrid derivatives from standard tree-building phylogenetic analysis, where they potentially introduce paraphyly (Hörandl, 2006b).

Separating sexual species is reasonable if sufficient reproductive barriers exist against backcrossing of apomictic and sexual lineages. This concept will, however, be problematic if apomictic genotypes are continuously formed via fertilization of sexual individuals by pollen from apomictic plants, as has probably occurred in *Boechera* (Koch & al., 2003). It will also fail if facultative sexuality occurs too frequently to allow the fixation of morphotypes (e.g., in *Hypericum perforatum* L.; Barcaccia & al., 2007). In our case study, the sexual taxa appear to be reproductively well isolated from the apomictic cytotypes: triploids, as possible backcrosses, were not observed. Furthermore, AFLP data always separates sexual from apomictic clusters (Fig. 7). Diploid sexual *R. notabilis* is also reproductively isolated from other local sympatric apomictic derivatives, as assessed by earlier isoenzyme and karyological studies of mixed *R. notabilis*-*R. variabilis* populations (Hörandl & al., 2000, 2001).

The separation of *R. notabilis* from *R. cassubicifolius* and *R. carpaticola* as two different species is reasonable considering morphological differences (Hörandl & Gutermann 1998b, c), phylogenetic distance within the genus (Hörandl & al., 2005; Paun & al., 2005), population genetic differentiation of allozyme patterns (Hörandl, 2004) and high sterility of F_1 hybrids in experimental crosses (Hörandl, 2008). *Ranunculus cassubicifolius* and *R. carpaticola* have originated from an allopatric, but perhaps not yet completed, speciation process. They may be also treated as subspecies, but the genetic differentiation is manifested in allozyme patterns and partly by DNA sequence data. Genetic distance methods and population aggregation analysis, as inferred from allozyme analysis and morphology, are still regarded as equivalent to tree building methods for species-level taxonomy (e.g., Sites & Marshall, 2003).

Recently, Soltis & al. (2007) proposed recognizing autopolyploid cytotypes as separate species if diagnostic

morphological characters are available. The concept is theoretically founded on a distinct evolutionary origin of autopolyploids and of the expected reproductive barriers of different ploidy levels. A formal classification of autopolyploids as subspecies, as suggested by Rowley (2007), would be supported by geographical and genetic differentiation of cytodesmes as seen in allozyme patterns (Hörandl & Greilhuber, 2002) and AFLP profiles (Paun & al., 2006b), but is contradicted by the lack of diagnostic macro-morphological characters (Hörandl, 2002); potential microcharacters (e.g., size of pollen and stomata) need to be studied. Facing the diversity of cytotypes in the whole complex, we concur with Rowley (2007) that naming autopolyploids as species is not a practical approach.

As for the apomictic cytotypes, three options are available for their classification: (1) agamospecies, (2) subspecies (of a sexual taxon), or (3) as hybrid derivatives of sexual taxa (nothotaxa). A case-wise and pragmatic approach seems the best solution for classifying apomictic complexes (Stace, 1998). In our case, the application of an agamospecies concept in the sense of Grant (1981), Stace (1998) and Hörandl (1998) is problematic due to the low stability of cytotypes and the scarcity of diagnostic morphological features. Even the main discernible morphotypes, i.e., the apomictic *R. cassubicifolius* derivatives and *R. hungaricus*, are connected by a broad range of intermediate forms and seem to have at least occasional gene flow. Facultative sexuality and local hybridization may result in multiple origins of similar morphotypes, which seriously undermines the assumption that phenetically similar populations in an area also possess an historical evolutionary coherence. Consequently, the pragmatic “minimum distribution area” concept proposed by Weber (1995, 1996) is not readily applicable.

A treatment of apomictic cytotypes as subspecies or varieties might be useful for autopolyploid apomictic derivatives of sexual taxa, as proposed by Bayer (1991) for *Antennaria friesiana* and *A. monocephala*. If no reticulate evolution was involved, and morphological diversification remains low, a hierarchical infraspecific classification can well reflect the diversification of cytotypes. In our case, the evidence available does not support a hypothesis of an autopolyploid origin of apomictic cytotypes.

For the *Ranunculus* taxa studied here, broadly defined nothotaxa are probably the best solution to reflect the evolutionary origin and relationships of the “*cassubicus*” morphotypes. A concept of nothotaxa has already been proposed for the whole *R. auricomus* complex by Savulescu (1953), but unfortunately it was not based on well-defined sexual parental species. With the present information on the sexual species, the hybrid formula *R. carpaticola* × *R. cassubicifolius* can be readily applied to the polyploid cytotypes of clade I (Fig. 2). The

distribution of this apomictic hybrid derivative may be wider than currently known, since similar morphotypes have been described as species by Jasiewicz (1956) from southern Poland, as *R. niepolomicensis* Jasiewicz (pentaploid after Jankun & Izmailow, 1965), *R. czywczyński* Jasiewicz, and *R. hanna* Jasiewicz. According to figures in Jankun & Izmailow (1965), several other unnamed hexaploid morphotypes in Poland probably belong to this nothotaxon. *Ranunculus olgae* Soó and *R. cassubicus* sensu Soó (1965) are also a morphological match for this taxon. Chromosome numbers and the mode of reproduction of these populations need to be studied.

For *R. hungaricus*, a hybrid origin is strongly suggested by our data and it can be postulated from analysis of DNA sequence data that the sexual species *R. notabilis* was involved in its origin, however more detailed information on evolutionary origin would still require further study. Nevertheless, we regard it as useful to designate *R. × hungaricus* as a nothotaxon to differentiate it formally from normal sexual species of the *R. auricomus* complex. A concept of nothotaxa might, in general, be a practicable solution for many other agamospecies if hybrid origin and parentage can be assessed with some certainty.

Nomenclature. — The original circumscription of *R. carpaticola* Soó (1965: 402) includes both diploid sexual and apomictic hybrid derivatives. Here we restrict use of the name to the diploid sexual cytotype in order to separate it from its apomictic hybrid derivative *R. carpaticola* × *R. cassubicifolius* (Fig. 1).

The leaf characteristics of the holotype of *R. carpaticola* Soó “in silvis montanis ap. Várhegy pr. opp. Huszt, 14. 8. 1960, R. de Soó” (BP) fall within the range of variation exhibited by both the sexual species and the apomictic hybrid taxon (photo in Soó, 1965: Fig. 34). Unfortunately, the present methodology allows for neither an assessment of chromosome number nor of mode of reproduction from this 40-years old herbarium specimen (see, e.g., Tensch & al., 2004). It is therefore impossible to establish whether this individual belongs to the diploid sexual taxon or the apomictic polyploid hybrid. For a precise application of the name, we designate here the following epitype to fix the name to the diploid sexual species: “*Ranunculus carpaticola*. Flora Slovaciae centralis, Skalka nad Revúca, J. Májovský s.n., 1972, cyto: $2n = 16$ ” (SAV). The population on the hill at Skalka, on the border of the Revúca village in Central Slovakia, is entirely diploid and sexual based on population genetic analyses and flow cytometric studies (Hörandl, 2002; Hörandl & Greilhuber, 2002; Paun & al., 2006a, b; Hörandl & al. 2008). This species includes reports of “*R. revucensis*” Májovský & Murín (1987), as nomen nudum, with a chromosome number of $2n = 16$.

The holotype of *R. × hungaricus* Soó (1965; “*R. hungaricus* Soó, rev. Soó. 6457. *Ranunculus cassubicus* L.

Bükk hegység, Gyertánvölgy, Borsod megye, Majus 4, Budai J.”, BP!) matches well to the Slovakian material. It also shows the characteristic irregular margin (mixed long and short teeth) of the stem leaf segments, which helps to discriminate the taxon from *R. carpaticola* × *cassubicifolius*. Since new material from the type locality of *R. × hungaricus* fits phylogenetically, morphologically and cytologically to the Slovakian material, the application of the name is well supported.

Ranunculus carpaticola differs from *R. cassubicifolius* by possession of a higher number of teeth on the middle segment of the stem leaves (9)16–22(35), by longer carpelophores (0.1)0.3–0.4(0.8) mm long, and by different allozyme patterns (Hörandl, 2002; Hörandl & Greilhuber, 2002). *Ranunculus cassubicifolius* W. Koch has a lower number of teeth (3)7–12(22) on the middle segment of the stem leaves and possesses shorter carpelophores, (0)0.2–0.3(0.5) mm long; Fig. 6B, C. Detailed descriptions and figures are available in Borchers-Kolb (1985), Hörandl & Gutermann (1998b) and Hörandl (2002).

Finally, we would like to clarify some confusion about misapplications of the name *R. carpaticola* Soó. Jalas & Suominen (1989) regarded *R. carpaticola* as a synonym of *R. cassubicus-auricomus* Schiller (1917) and, using this name (changed to *R. cassubico-auricomus*), provided a distribution map in “Atlas Florae Europaeae”. *Ranunculus carpaticola* was validly described by Soó (1965) as a species, without citing any synonyms; the premature “synonymy” in Soó (1964) has no effect as *R. carpaticola* appears there as nomen nudum. Jalas (1988) argued that the older, hyphenated name used by Schiller should be regarded as a valid species name (but see McNeill & al., 2006: Art. H.10.3). Schiller (1917), without considering apomixis in this group, recognized between “main species” and “adaptive transition forms”. He believed that one species may change into another by “a long-term change of the structure of the plasma” and described *R. cassubicus-auricomus* as such an “adaptive intermediate transitional form” between two main species, *R. cassubicus* L. and *R. auricomus* L. He neither called these forms explicitly species nor hybrids. The current *IBCN* does not provide any possibility for the naming of “adaptive transitional forms” but designates hyphenated epithets as hybrid formulae (McNeill & al., 2006: Art. H.10.3). Therefore, we regard the hyphenated Schiller names as hybrid formulae and not as species names. As such, the name *R. cassubicus-auricomus* is neither applicable to the sexual species *R. carpaticola* as circumscribed here, nor to the hybrid *R. carpaticola* × *cassubicifolius*, which has no “*auricomus*”-like parent. Savulescu (1953) regarded *R. cassubicus-auricomus* Schiller as a synonym of the older name *R. × fallax* (Wimm. & Grab.) Slob., clearly designating it as a nothotaxon. *Ranunculus auricomus* L. and *R. cassubicus* L. are species of unknown mode of

reproduction and uncertain taxonomic circumscription. The Linnaean names *R. auricomus* L. s.str. and *R. cas-subicus* L. s.str. are, according to their types, not referable to any central European species (see Kvist, 1987; Ericsson, 1992; Jarvis, 2007). Thus, Schiller's hybrid formula is meaningless because it is not based on clearly defined sexual species.

ACKNOWLEDGEMENTS

The study was supported by the Austrian Research Foundation (FWF), through research grant P-19006-B03 to EH, an Erwin Schrödinger fellowship to OP (J26406-B03), and a Ph.D. research grant from the Austrian Exchange Service (ÖAD), North-South Dialogue program, to KE. We thank Franz Dunkel for a collection of *R. hungaricus* from the locus classicus and Eva Schönbeck-Temesy for translation of the original Hungarian text of Schiller (1917).

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Appendix 1. Eigenvalues and variance explained by the eight components extracted by principal component analysis of morphological characters.

Component	Eigenvalues	% of variance	Cumulative variance%
1	2.684	33.554	33.554
2	1.634	20.422	53.976
3	.906	11.323	65.299
4	.808	10.096	75.395
5	.656	8.202	83.596
6	.619	7.743	91.340
7	.358	4.476	95.816
8	.335	4.184	100.000

Appendix 2. Correlations of variables and the first two components of principal component analysis of morphological characters.

	Components	
	1	2
ANDRO	.671	.085
CARP	-.333	.725
ST_D	.725	.391
NR_TEETH	.563	.649
FR_LW	-.372	.558
POS_L	-.557	-.131
B_LW	-.764	.160
BASE_W	.500	-.414

Appendix 3. Pooled within-group correlations between discriminating variables and the standardized canonical discriminant function of CDA1 of morphological characters.

	Function 1
NR_TEETH	.658
CARP	.608
FR_LW	.433
POS_L	-.265
ST_D	.219
BASE_W	-.131
ANDRO	.108
B_LW	.082

Appendix 4. Eigenvalues and variance of the three canonical discriminant functions of CDA2 of morphological characters.

Function	Eigenvalue	% of variance	Cumulative variance%	Canonical correlation
1	2.874	65.3	65.3	.861
2	.978	22.2	87.5	.703
3	.552	12.5	100.0	.596

Appendix 5. Wilks' lambda and statistical significance of canonical functions of CDA2 of morphological characters.

Test of function(s)	Wilks' lambda	Chi-square	df	P
1 through 3	.084	1173.746	24	.000
2 through 3	.326	531.805	14	.000
3	.644	208.398	6	.000

Appendix 6. Pooled within-group correlations between discriminating variables and standardized canonical discriminant functions of CDA2 of morphological characters^a.

	Functions		
	1	2	3
B_LW	.695	-.288	-.470
CARP	.477	.442	-.052
ANDRO	-.352	.251	-.309
BASE_W	-.285	-.072	-.069
NR_TEETH	-.135	.815	.125
ST_D	-.237	.333	-.006
POS_L	.236	-.385	.643
FR_LW	.278	.310	.316

^aSee Table 2.