Reopening an old chapter: a revised taxonomic and evolutionary concept of the *Rubus montanus* group

Znovuotevření staré kapitoly: revize taxonomického a evolučního konceptu okruhu *Rubus montanus*

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The triploid complex of *Rubus montanus* (*Rubus* ser. *Discolores*, *Rosaceae*) is a particularly intricate group due to its plasticity and variability. The representatives of this group are, notwithstanding the high number of taxon names published, often considered a single species (*Rubus montanus* s.l.). In the course of extensive field studies and herbarium revisions we revealed three widely distributed morphologically defined types that were formerly incorporated in *R. montanus* s.l. but should be treated as separate species. We analysed them using both morphological and genetic methods. We found that the species examined are identical with the formerly described *R. montanus* Lib. ex Lej., *R. bicolor* Opiz and *R. velutinus* Vest ex Tratt., and provide a taxonomic reassessment, discussion of synonymy and typification of certain taxon names, and complete their morphological description, ecological characteristics and distribution. We show that the morphological delimitation of the triploid *Discolores* microspecies is highly compatible with molecular evidence for the independent sexual origin of these units (or, rarely, extensive mutational diversification). On the contrary, the intraspecific genetic variation is rather low and only mutational, and confirms their strictly apomictic reproduction. Nevertheless, mutational load may be substantial and mutation-based and recombination-based variation overlap in some instances. Highly variable molecular genetic markers such as microsatellites are recommended as an additional line of evidence for species delimitation in any further taxonomical studies on other groups of brambles.

K e y w o r d s: apomixis, microsatellites, mutation, nomenclature, *Rubus* ser. *Discolores*, species concept, taxonomy, typification

Introduction

The *Rubus* L. sect. *Rubus* is a challenging target group for understanding species formation and rapid evolutionary processes due to its morphological and taxonomic complexity, facultative apomixis (agamospermy) and common hybridization (Holub 1992, Weber 1995, Šarhanová et al. 2012, Sochor et al. 2015). The diverse *Rubus* ser. *Discolores* (P. J. Müller) Focke (with about 80 accepted species in Europe) is, due to the whitish tomentum on the

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underside of the leaves and the lack of stalked glands, an easily separable old-established group within this section. Its taxonomic treatment was recently discussed by van de Beek (2014) who suggested further division of the series. However, to avoid any unnecessary disorder in the present work, we use the name of this series in the traditional sense (Weber 1995, Kurtto et al. 2010).

The series *Discolores* contains one diploid group (*R. ulmifolius* / *sanctus* complex) and a number of triploid and tetraploid taxa. Whereas tetraploid accessions retain a high degree of residual sexuality (and their taxonomic treatment is therefore often complicated by hybridization and recurrent formation of new apomictic genotypes), triploids do not seem to form reduced gametes and thus their reproduction is generally apomictic (Šarhanová et al. 2012). Triploid *Discolores* are rare in western Europe (Kurtto et al. 2010, D. Earl and M. Sochor, unpublished data) and the Caucasus (Sochor & Trávníček 2016); nevertheless, they form a species rich complex (with approximately 25 species) in central and south-eastern Europe (Kurtto et al. 2010, Krahulcová et al. 2013), along with numerous regionally or even widely distributed undescribed morphologically defined types ("morphotypes"; B. Trávníček and G. Király, unpublished data). This pattern is probably due to the distribution of one of the ancestors, *R. canescens* DC., which may have served as a direct pistilate parent for most of the triploids (Sochor et al. 2015). This triploid complex is particularly intricate due to morphological plasticity and variability, often making the distinction between taxa complicated. Whether the morphological variation stems from genetic diversity and what the genetic structure is within and among the morphologically defined taxa are matters that have never been studied in this group. This information is, nevertheless, critical for the sustainability of the natural species concept, not only in *Rubus* (see e.g. Nybom 1996).

In terms of taxonomic and nomenclatural difficulties, an extreme case is the triploid "*R. montanus* group". This group can be characterized morphologically by the glabrous or glabrescent first-year stem, by the ± flat leaflets with a grey to white tomentum on the underside, by the narrowly elliptical to narrowly obovate (more rarely obovate) terminal leaflet that is rounded to cordate at base, and by the usually elongated, narrowly pyramidal to almost cylindrical inflorescence; the gynoceum is glabrous or very sparsely hairy (see also the key of Trávníček & Zázvorka 2005). The name *R. montanus* Lib. ex Lej. was typified by van de Beek (1974); however, this name was not accepted until the late 1980s, when Weber (1986a) showed that the formerly (e.g. by Heslop-Harrison 1968) adopted name for this taxon (or taxon group, see below), *R. candicans* Weihe ex Rchb., is illegitimate. This standpoint (i.e. the acceptance and use of the name *R. montanus*) was later widely adopted in major Floras and bramble monographs in Europe (Weber 1987, 1995, 2016, Matzke-Hajek 1993, Maurer 1994, Holub 1995, Ranft 1995, Trávníček & Havlíček 2002, Zieliński 2004, Matzke-Hajek et al. 2005, Martinčič 2007, Ciocârlan 2009, van de Beek 2014). Mainly prior to 1950, several similar taxa were described at various taxonomic ranks; however, they were assessed by Weber (1986a, 1995) to be local biotypes or simply synonyms of *R. montanus*, a widely distributed species in Europe.

Trávníček & Zázvorka (2005) argue, however, on the basis of macromorphological characters for splitting *R. montanus*. They support the existence of *R. montanus* s. str., and, additionally, describe *R. flos-amygdalae* Trávn. et Holub as a new species, and report a third species, which they provisionally name "*R. peripragensis*". This has not been widely accepted and is assessed by the experts in different ways. Weber (in Kurtto et

al. 2010) considers *R. flos-amygdalae* to be a synonym of *R. montanus*; on the other hand, *R. flos-amygdalae* is accepted as a distinct species in several regional studies (e.g. Zieliński 2004, Kosiński 2010, Pagitz et al. 2014). *Rubus montanus* f. *macromontanus*, described by Weber (1989), gained higher taxonomic importance due to the creation of a new combination at species rank by Vannerom in Lambinon et al. (1992). This treatment is discussed by Trávníček & Zázvorka (2005) who assess this taxon as taxonomically identical with the type of *R. montanus*. Last but not least the necessity of re-assessment of certain other names probably related to *R. montanus* has also been raised (Matzke-Hajek 2001, van de Beek et al. 2017).

Because of the conflicts described above we devoted particular attention to the *R. montanus* group. In the course of extensive field studies and herbarium revisions we targeted widely distributed biotypes of this group, and analysed them using both morphological and genetic methods. In this study, we provide a nomenclatural and taxonomic reassessment (incl. identification, ecological characteristics, revised distribution data) of the taxa studied and shed light on the evolution of the triploid representatives of *R.* ser. *Discolores*.

Materials and methods

Distribution

Field studies on brambles were conducted between 2005 and 2016 at more than 2000 localities. Our data collection covers most of central Europe and the northern part of the Balkan Peninsula. For each locality, the altitude and geo-coordinates in the WGS 84 projection were determined. Distribution maps were compiled using ArcGIS software. The voucher specimens collected during recent studies (incl. specimens used for the morphological investigations) are deposited in BP and OL.

Herbarium studies

The following herbaria (acronyms according to Thiers 2017) were examined personally, using virtual herbaria accessions or on the basis of high-resolution photographs for previous records of *R. montanus* group in the area studied: B, BEOU, BP, BPU, BR, BRA, BUNS, DE, GJO, GZU, HBG, IB, JE, JPU, KOR, KRA, L, LAU, LI, LJU, NI, OL, P, PECS, PR, PRA, SAMU, SAV, SLO, SZB, W, WAG, WU, ZA, ZAHO, in addition, some specimens in private herbaria (collections of I. Bílek, P. Hrbáč, G. Király, M. Král, P. Lepší, G. Matzke-Hajek, T. Rejzek, F. Sander, V. Sedláček and V. Žíla) were also examined. We paid special attention to the reassessment of the type material of selected taxa. Earlier literature records proved to be unreliable after herbarium revisions; thus, we only included data supported by voucher specimens (see Electronic Appendix 1).

Morphological investigations

The morphological characterization of the entities belonging to the *R. montanus* group was based partly on the comprehensive study of Trávníček & Zázvorka (2005) and a recent revision of over 50 living and/or herbarium specimens for each. First-year stems

with well-developed leaves were typically examined together with the flowers and fruits on living material; abnormal and injured plants were not included in the assessments. Additional reference material for the comparative study of the species was obtained from the herbaria listed above. Altogether, we used 14 features for the characterization and differentiation of the taxa studied (Table 1).

Table 1. – Distinctive features of the species of the *Rubus montanus* group. * the feature refers to both of the compound leaves on first-year stems and the inflorescence.

Character	*R. bicolor*	*R. montanus*	*R. velutinus*
Colour of the first-year stem (exposed to the sun)	red-violet, not spotted to slightly so	conspicuously dark violet, distinctly darkly spotted	red-violet, not spotted to distinctly so
No. of prickles on the first-year stem (per 5 cm of stem length)	(0–) 2–5	(0–) 2–5	3–7
Length of the prickles on the first-year stem (mm)	5–7 (–9)	5–7 (–9)	6–9 (–10)
Shape of the terminal leaflet*	narrowly (rarely broadly) elliptical to almost rectangular	narrowly (angular-) obovate, less often obovate	ovate to obovate, rarely narrower
Lateral margins of the terminal leaflet*	usually ± parallel	not parallel	not parallel
Ratio (%) of the length of the petiolule and the blade of the terminal leaflet of first stem leaves	33–50	25–40	25–40
Apex of the terminal leaflet*	(5–) 10–15 mm long, rather abrupt, acute	up to 5 (–10) mm long, broad, ± abrupt	(5–) 10–15 mm long, ± abrupt and acute
Serration of the terminal leaflet*	teeth rather short, not sharp	teeth rather short, not sharp	teeth often coarse and very sharp
Branchlets of the inflorescence	± thick, often straight or somewhat curved	± thin, often ascending	± thick, often straight or somewhat curved
Length of the prickles on the axis of the inflorescence (mm)	3–5 (–7)	3–5 (–7)	4–9
Colour of petals	pale (whitish) pink	pale (whitish) to bright pink	white to (rarely) pale (whitish) pink
Shape of petals	obovate to suborbicular	narrowly obovate to obovate	obovate to suborbicular
Colour of filaments	white	pale (whitish) to bright pink	white
Carpels	glabrous (rarely with few hairs)	glabrous	sparsely hairy with long hairs

Living plants

Sample set for the SSR analysis was selected in order to cover most of the triploid representatives of *R.* ser. *Discolores*, a tetraploid that shares the plastid haplotype with the triploid accessions (*R. bohemiicola*; Sochor et al. 2015) and two morphologically similar species of unknown ploidy (*R. albiflorus*, *R. arduennensis*). The ploidy levels were obtained mainly from Krahulcová et al. (2013), for a few species of unknown ploidy it

was measured using flow cytometry according to the protocol described in Šarhanová et al. (2012). Except for the *R. montanus* group there were two individuals from distant localities of most species. The three entities of the *R. montanus* group were represented by 17, 19 and 13 individual shrubs, respectively, including the holotype of *R. flos-amygdalae* and samples from the type localities of *R. macromontanus*, *R. montanus*, *R. roseolus*, *R. velutinus* and *R. rotundipetalus* (Electronic Appendix 2). The sampling was designed to include a representative part of the genetic variability of triploids in ser. *Discolores* (with special focus on the *R. montanus* group) in order to reveal the clonal structure within and among morphologically defined species, estimate intra-clonal genetic variation and evaluate the species concept in this group based on neutral molecular markers. In total, 25 entities represented by 93 individuals were analysed. Voucher specimens of the material used in the analysis were deposited in OL.

Microsatellite analyses

Genomic DNA was extracted from silica-gel-dried leaves following the CTAB (Cetyl Trimethyl Ammonium Bromide) protocol of Doyle & Doyle (1987). Ten microsatellite loci (Graham et al. 2004, 2006, Woodhead et al. 2008) were selected based on amplification efficiency and variability, and amplified using the Kapa Taq PCR Kit (Kapa Biosystems) in 10μL reactions with 7.5 ng of template DNA following the standard manufacturer's protocol (see Electronic Appendix 3 for further details). PCR products of six loci (Rubus166b, Rubus275a, ERubLR_SQ01_G16, Rub265a, Rub236b and Rubleaf86) were separated by denaturing polyacrylamide gel electrophoresis in a T-REX electrophoresis device (Thermo Scientific Owl Separation Systems, Rochester, NY, USA) on 6 %, 0.4 mm-thick polyacrylamide gels. The fragments were visualized by silver staining and their length was determined using the 30–330 bp AFLP® DNA ladder (Invitrogen, Carlsbad, CA). Four loci (RubPara_SQ005_K23, Rub238h, Rubusr47a and Rubus105b) were analysed as fluorescently labelled PCR products together with the GeneScan 600LIZ® size standard on an ABI 3730XL capillary sequencer at Macrogen Europe. In these cases, fluorescent labelling was performed in a nested PCR reaction containing three primers: a sequence-specific forward primer with M13 tail at its 5' end (used at a concentration 0.1 μM), a sequence-specific reverse primer (c = 0.4 μM), and a fluorescently 5'-modified M13 primer (NED™, PET®, VIC™ or FAM™ modification; c = 0.4 μM). To facilitate annealing of the universal M13 primer the annealing temperature was lowered to 53 °C in the last nine PCR cycles.

Basic descriptive statistics (number of alleles, expected heterozygosity) were computed in SPAGEDI (ver. 1.3; Hardy & Vekemans 2002) using a codominant data matrix. For other analyses, data were transformed to a binary matrix. The analysis of molecular variance (AMOVA) and principal coordinate analysis (PCoA) using the covariance method with standardization were performed in GENALEX (ver. 6.5; Peakall & Smouse 2012). Analyses of genotypic diversity, including histograms of genetic distances, were done using the Genotype/Genodive software package (ver. 2.0b23; Meirmans & van Tienderen 2004). Mutation and recombination as sources of intraspecific variation were also distinguished using the character incompatibility approach in module JACTAX of package PICA 4.0 (Wilkinson 2001). It is based on the simple rationale that binary unordered characters (i.e. presence or absence of alleles) cannot be present in all combinations

of character states in a population in the absence of recombination. If the characters are compatible (i.e. two binary characters form only one to three combinations), mutations are very probably the source of the observed variation (Mes 1998). Split network based on NeighbourNet method and uncorrected P-distances was computed in SPLITSTREE 4 (Huson & Bryant 2006).

Results

Nomenclature and morphological characterization

In the course of the morphological investigations of the *R. montanus* group we distinguished three widespread morphotypes, which are identical to the entities included in the identification key of Trávníček & Zázvorka (2005). Although typical specimens can be separated merely on the basis of the leafy first-year stem, the safe identification often requires information on the features of the inflorescence (shape, prickles) and flowers (petal colour, hairiness of carpels). Specimens without an inflorescence may therefore be difficult to identify, particularly in herbaria (for the distinguishing characters see Table 1 and Figs 1–3, and photographs of living plants in Electronic Appendix 7).

We compared the three morphotypes distinguished with the protologue (Lejeune 1813), and original herbarium material, and concluded that *R. montanus* in the sense of Lib. ex Lej. is present in our set, however, it is not identical to *R. montanus* sensu Trávníček & Zázvorka (2005) but to *R. flos-amygdalae* Trávn. et Holub. The lectotype specimen of *R. montanus* (deposited in BR) includes a young inflorescence, three leaves from a first-year stem and the apical leafy part of a primocane. Our identification was confirmed mainly by the form of the inflorescence and the leaves on the first-year stem. Moreover, the holotype of *R. flos-amygdalae* and the recently examined specimens from the locus classicus of *R. montanus* shared an almost identical SSR genotype. Thus, *R. flos-amygdalae* is considered to be the later heterotypic synonym of *R. montanus*. As another later synonym of *R. montanus* we found *R. roseolus* P. J. Müll. ex Boulay (Boulay 1866), whose name we also typified based on a specimen deposited in P.

It is worth noting that the name *R. fruticosus* L. was treated contrary to the original sense by Weihe & Nees (1822–1827). Neither the description, nor the illustration of *R. fruticosus* in Weihe & Nees (l.c.) provide sufficient information for an identification at a microspecies rank, nonetheless, their plant falls clearly into the *R. montanus* group. Per se it is a simple misidentification (the Linnaean *R. fruticosus* is identical with *R. plicatus* Weihe & Nees, see Weber 1986b), but *R. fruticosus* sensu Weihe & Nees (and its illustration in Weihe & Nees l.c.) is cited in several subsequent protologues, consequently, it can have a significant role in the typification of selected names. In creating the new name *R. cerasifolius*, Lefèvre (1851) refers to the illustration of "*R. fruticosus*" in Weihe & Nees (l.c.), and because there is no extant original herbarium material for *R. cerasifolius* Lefèvre, an assessment and typification of this name is needed. We therefore select plate no. 7 in Weihe & Nees (l.c.) as the lectotype, and, in order to avoid later misapplications, and underpin that choice by the designation of an epitype (as provided for in Art. 9.8 of the Melbourne Code, McNeill et al. 2012) belonging to *R. montanus* s. str. Our decision is supported by the fact that the specimen served as basis for plate no. 7, which presumably

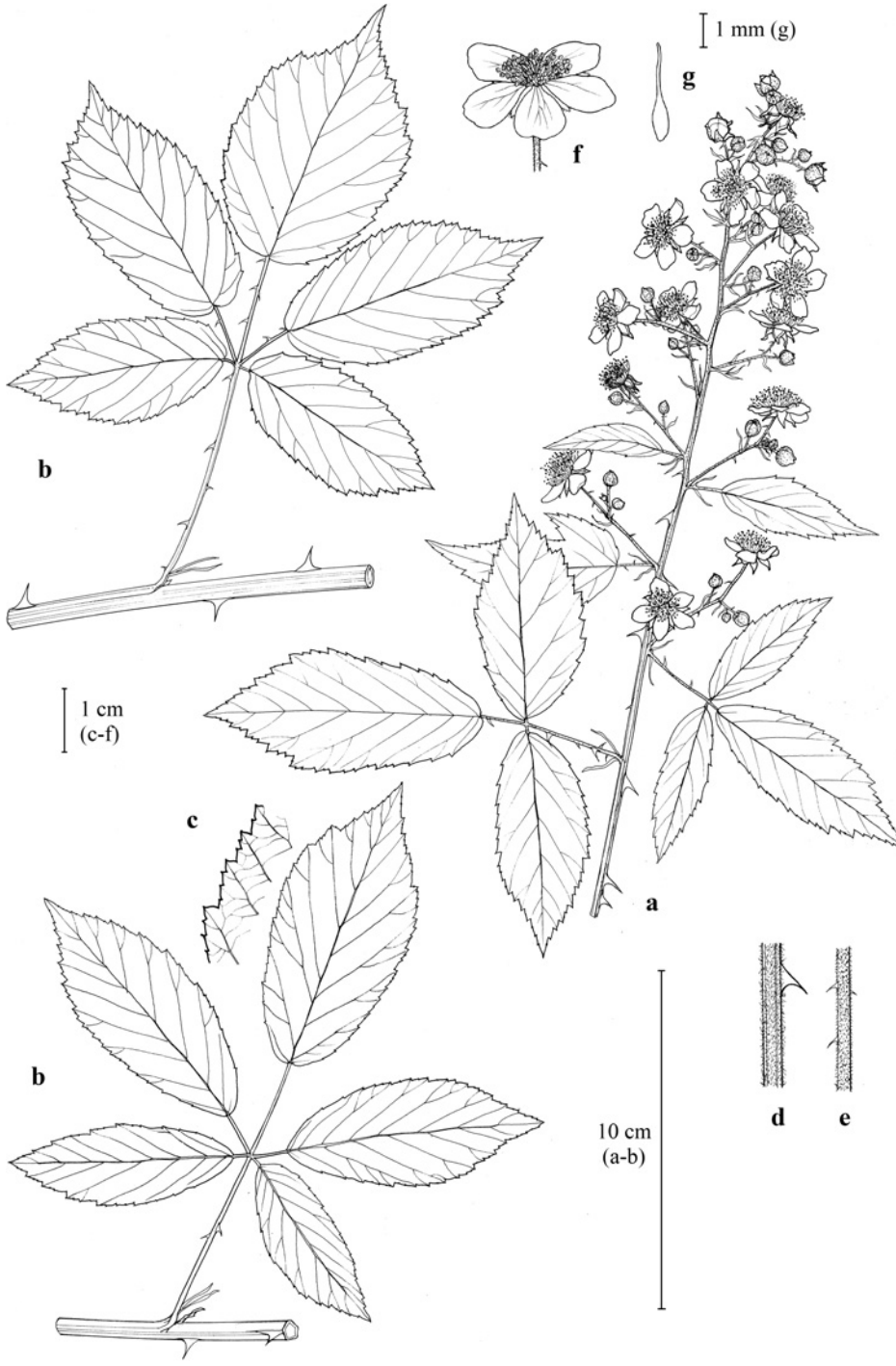


Fig. 1. – *Rubus bicolor* Opiz: a – inflorescence, b – first-year stem with leaves, c – margin of the terminal leaflet, d – inflorescence axis, e – peduncle, f – flower, g – carpel. Del. A. Skoumalová

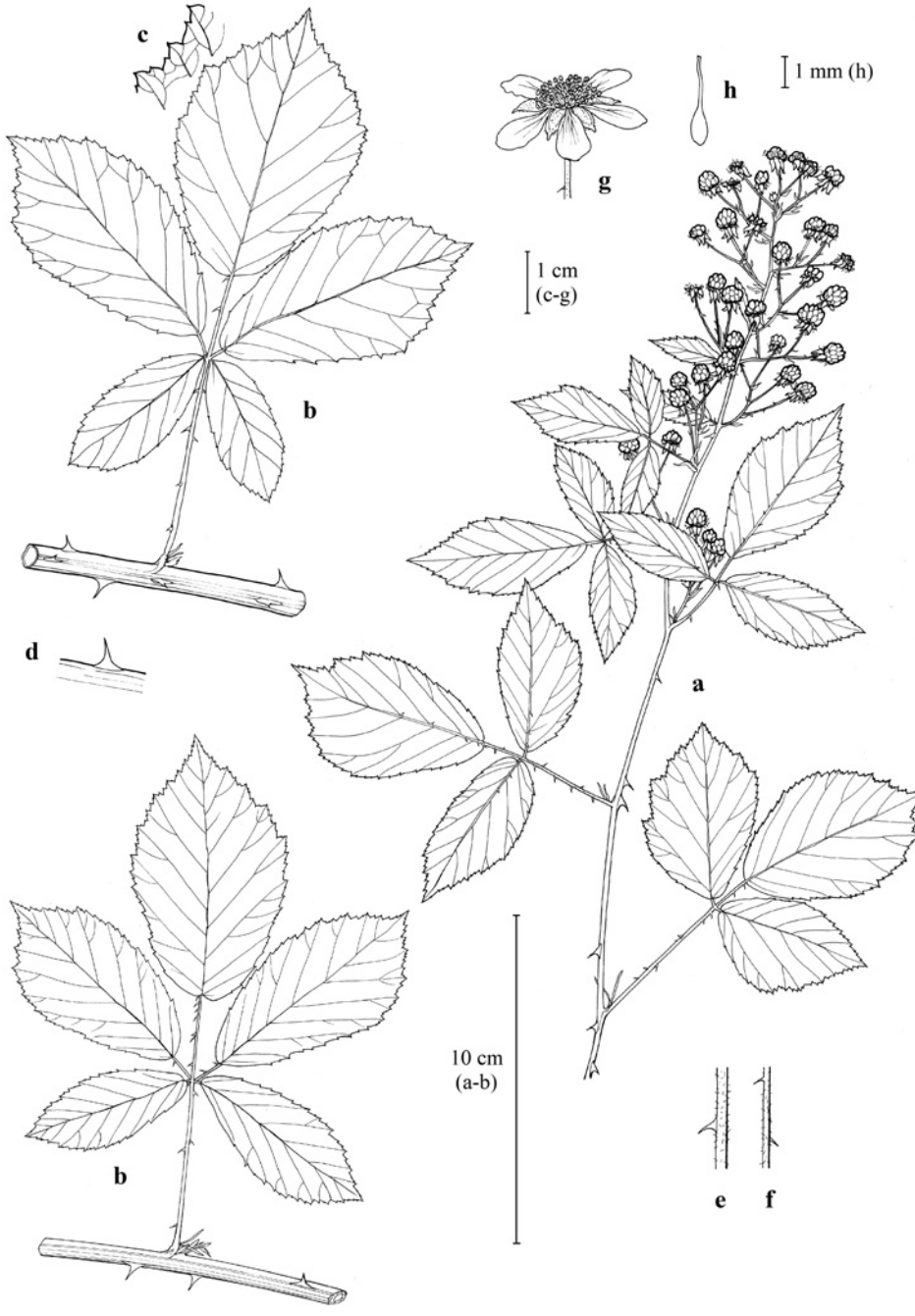


Fig. 2. – *Rubus montanus* Lib. ex Lej.: a – infructescence, b – first-year stem with leaves, c – margin of the terminal leaflet, d – detail of the first-year stem, e – inflorescence axis, f – peduncle, g – flower, h – carpel. Del. A. Skoumalová

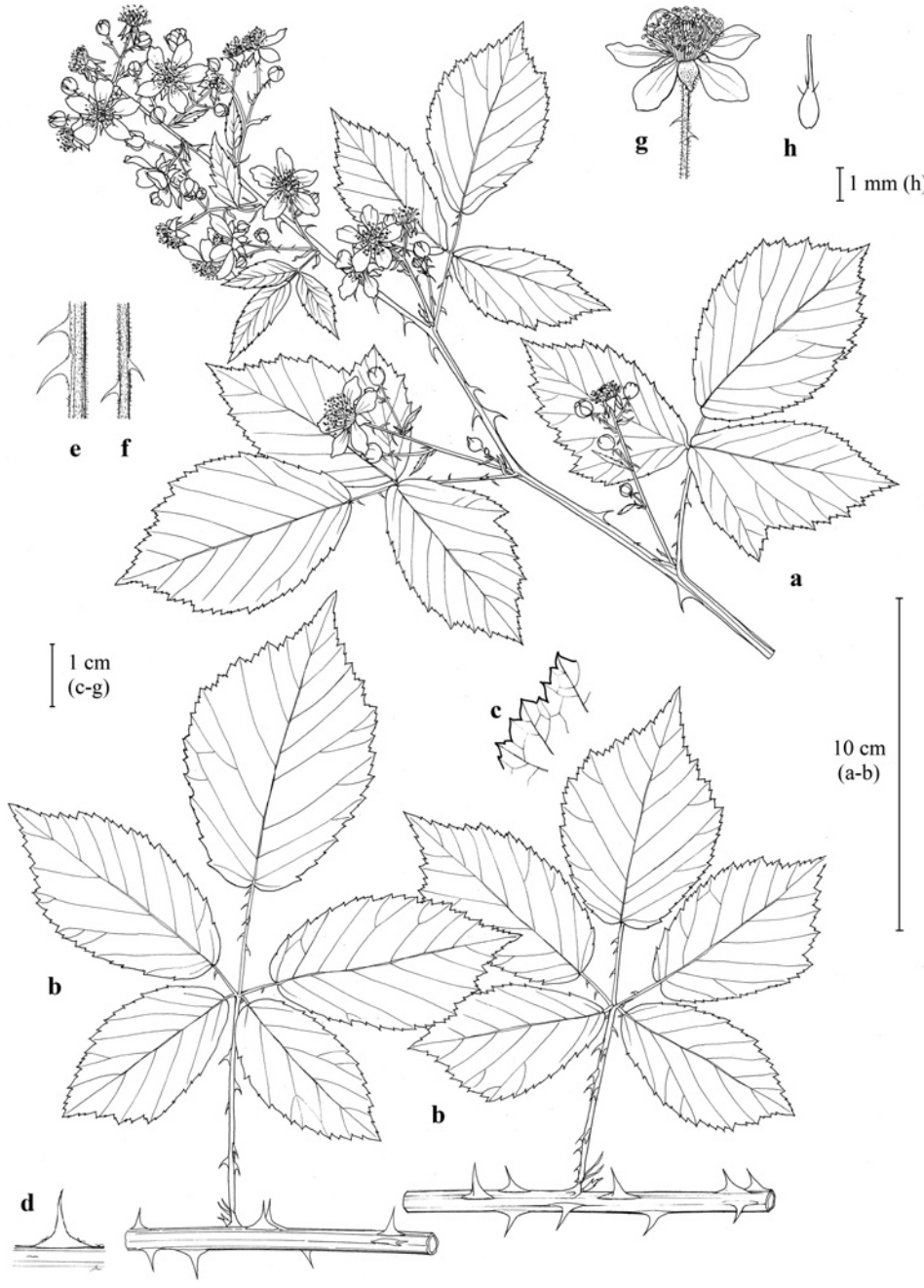


Fig. 3. – *Rubus velutinus* Vest ex Tratt.: a – inflorescence, b – first-year stem with leaves, c – margin of the terminal leaflet, d – detail of the first-year stem, e – inflorescence axis, f – peduncle, g – flower, h – carpel. Del. A. Skoumalová.

came from Westphalia where *R. montanus* is quite common and the only representative of the group; furthermore, the only specimen of "*R. fruticosus*" collected by Weihe seen by us (BR529513) is also *R. montanus* s. str. The epitype selected here is a modern, well-prepared specimen, for which SSR data are also presented.

Based on the above and because the name *R. montanus* has to be applied to the taxon described later as *R. flos-amygdalae*, we had to find the correct species name of the plant identified erroneously as *R. montanus* by Trávníček & Zázvorka (2005). After studying the protologue (Opiz 1854) and original material (single specimen in PR, comprising a well-developed inflorescence and part of a first-year stem with leaves) of the name *R. bicolor* Opiz, it appears to be the oldest name associated with this taxon. The types of *R. macromontanus* (H. E. Weber) Vannerom and *R. thyrsoideus* subsp. *candicans* var. *excelsior* P. J. Müll. ex Sudre are identical with that of *R. bicolor*, hence, they are later synonyms for that name. On the basis of the protologue (Müller 1858), *R. coarctatus* P. J. Müll. presumably also belongs to the *R. montanus* group. This name was typified by van de Beek et al. (2017) in the sense of *R. bicolor* (LAU, Müller 1515!); however, we believe that this proposal is not correct because the lectotype was not designated from the original material.

The third taxon (formerly named *R. peripragensis* ined. in Trávníček & Zázvorka 2005) is identical with *R. velutinus* Vest ex Tratt., described from Styria (south-eastern Austria; Trattinnick 1823). We designate as a lectotype a specimen from WU, which consists of part of a stem with a typical infructescence. However, to avoid any confusion over the lectotype lacking a first-year stem with leaves, we also designate an epitype consisting of a modern, well-prepared specimen of *R. velutinus* collected in Styria, for which SSR data are also presented, and which has the same SSR genotype as *R. peripragensis* ined. Furthermore, based on the protologue (Müller 1859) and original material of *R. rotundipetalus* P. J. Müll., we consider the latter taxon to also be conspecific with *R. velutinus*. These identifications are supported also by microsatellite analysis of individuals from the type localities of *R. rotundipetalus* (Wissembourg region) and *R. velutinus* (Styria), which share the same SSR genotype as *R. peripragensis* ined.

Connected with the conclusions provided above, the nomenclatural concept of the species distinguished in the *R. montanus* group reads as follows:

Rubus bicolor Opiz, Lotos 4: 70, 1854.
Loc. typ. cit.: "Hinter Selč" [now Sedlec, suburb of Prague, Czech Republic]. **Lectotype** (**designated here**, or perhaps holotype): "Hinter Selč" (Ph. M. Opiz, 11 VII 1852, PR!, see Electronic Appendix 6).
= *R. thyrsoideus* subsp. *candicans* var. *excelsior* P. J. Müll. ex Sudre, Bat. Eur. 44, 1905.
Loc. typ. cit.: "Alsace". Type (designated by Weber 1986a: 303): "zwischen Saarbrücken und Dudweiler" (F. Winter, 13 VII 1869, JE!, lectotype).
= *R. montanus* f. *macromontanus* H. E. Weber, Osnabrück. Naturwiss. Mitt. 15: 106, 1989.
Loc. typ. cit.: "Bohemia orientalis, Vamberg" [= Vamberk]. Type: "Vamberg, Ostböhmen" (J. Holub, 12 VIII 1985, HBG, holotype; PRA!, isotype, see Electronic Appendix 6).
≡ *R. macromontanus* (H. E. Weber) Vannerom in Lambinon et al., Nouv. Fl. Belg., ed. 4, 988, 1992.
≡ *R. montanus* var. *macromontanus* (H. E. Weber) Holub, Preslia 64: 129, 1993.
– *R. excelsior* P. J. Müll. ex Focke, Syn. Rub. Germ. 167, 1877, nom. nudum.
– *R. montanus* auct. mult. non Lib. ex Lej.
Illustrations: Nyárády 1956: 357, 361 (as *R. candicans* var. *typicus* and f. *excelsior*); Leute & Maurer 1977: 286 (as *R. candicans*); Weber 1989: 107 (as *R. montanus* f. *macromontanus*); Holub 1995: 111; Trávníček & Havlíček 2002: 338; Zieliński 2004: 71–72; Henker & Kieswetter 2009: 103 (all as *R. montanus*).
Chromosome number: 2n = 21 (Krahulcová et al. 2013, as *R. montanus*).

Rubus montanus Lib. ex Lej., Fl. Spa 2: 317, 1813.
Loc. typ. cit.: "Malmedy". Type (designated by van de Beek 1974: 73): "Bois en Goudoufat" [Malmedy, Belgium], (M.-A. Libert, s.d., BR!, lectotype, see Electronic Appendix 6).
≡ *R. thyrsoideus* [var.] *montanus* (Lej.) Nyman, Consp. Fl. Eur. 1: 216, 1878.
≡ *R. macrophyllus* subsp. *montanus* (Lej.) Sudre, Rubi Eur. 49, 1909.
= *R. cerasifolius* Lefèvre, Journal de Senlis 21 (518): 1, 1851, non Lefèvre et P. J. Müll. 1859.
Loc. typ. cit.: "Waligny". **Lectotype** (**designated here**): Plate 7, Weihe & Nees 1822–27, Rubi Germanici.
Epitype (**designated here**): "Bramsche, bushes 1.6 km ESE of Achmer village, N52°23'24", E07°57'27"" (B. Trávníček, 17 VIII 2011, OL 34752!, see Electronic Appendix 6).
= *R. roseolus* P. J. Müll. ex Boulay, Ronces Vosg. 2: 25, 1866.
Loc. typ. cit.: [Vosges] **Type** (**designated here**): "Foręt de Saint-Gorgon, prés de Rambervillers" (N. Boulay, 27 VI 1865, P 01817212!, lectotype, see Electronic Appendix 6; P 01817214!, isolectotype; – P 01817213 with a fruiting specimen constitutes a later collection and thus it is not part of the original material).
= *R. flos-amygdalae* Trávn. et Holub, Preslia 77: 12, 2005.
Loc. typ. cit.: "Czech Republic; N Moravia, Vidnava". Type: "Czech Republic; N Moravia, Vidnava" (B. Trávníček, 26 VII 2001, OL 5071!, holotype, see Electronic Appendix 6).
– *R. fruticosus* auct. non L. 1753: Weihe & Nees, Rubi Germ. 24, 1822.
– *R. candicans* Weihe ex Reichenb., Fl. Germ. Exc. 601, 1832, nom. illeg. (superflous name for *R. silesiacus* Weihe)
Illustrations: Weber 1973: 444 (as *R. candicans*); Weber 1995: 380, figs 332 and 333 (as *R. montanus*); Trávníček & Zázvorka 2005: 13–16 (figs 7–10, as *R. flos-amygdalae*); Pagitz et al. 2014: fig. 9 (as *R. flos-amygdalae*).
Chromosome number: 2n = 21 (Krahulcová et al. 2013, as *R. flos-amygdalae*).

Rubus velutinus Vest ex Tratt., Rosac. Monogr. 3: 47, 1823.
Loc. typ. cit.: "Stiria". **Lectotype** (**designated here**): s. loc. (Ch. Vest, s. d., WU!, see Electronic Appendix 6).
Epitype (**designated here**): "Styria, Seiersberg-Pirka, 0,3 km E of Tobelbad village, coniferous stands along a forest road" (B. Trávníček, 6 VIII 2013, OL 34754!, see Electronic Appendix 6).
= *R. rotundipetalus* P. J. Müll., Jahresber. Pollichia 82, 1859.
Loc. typ. cit.: "in der Buchbach und in einem Seitentälchen der Lauterbach". Lectotype (designated by van de Beek & Matzke-Hajek in Matzke-Hajek 2001): "a la fin du Bosquet du Langenberg" (Ph. J. Müller, 28 VII 1858, Müller 6108 in LAU!, see Electronic Appendix 6).
Illustrations: not known.
Chromosome number: 2n = 21, det. A. Krahulcová, ined. (voucher in PRA: "Praha, Bohnice, eastern part of Čimický háj forest", leg. J. Holub, 29 X 1994).

Patterns in the genetic variability

At the ten loci studied, a total of 130 different SSR alleles and average expected heterozygosity of 0.733 were recorded. Intraspecific variability across the 25 morphotypes accounted for only 20 % of the overall variation, whereas 80 % of the variation was detected among species based on AMOVA. Each microspecies/morphotype was characterized by a unique dominant genotype, which exhibited small deviations in a number of individuals. Individuals from the type localities of *R. montanus*, *R. flos-amygdalae* and *R. roseolus* formed a genetic cluster together with other individuals determined as *R. montanus*. Similarly, material from the type localities of *R. velutinus* and *R. rotundipetalus* fell well within the variaton of *R. velutinus*, and material from the locus classicus of *R. macromontanus* was similar in its SSR genotype to other conspecific individuals (Fig. 4, Electronic Appendix 2). Material from the type locality of *R. bicolor* was not included in the SSR analysis, because the original locality has changed significantly since then, and all three taxa of the *R. montanus* group investigated by us occur in this area.

In most cases, intraspecific variation was due to one or two alleles per locus that differed from the dominant genotype in two or four base pairs, i.e. in one or two SSR repeats

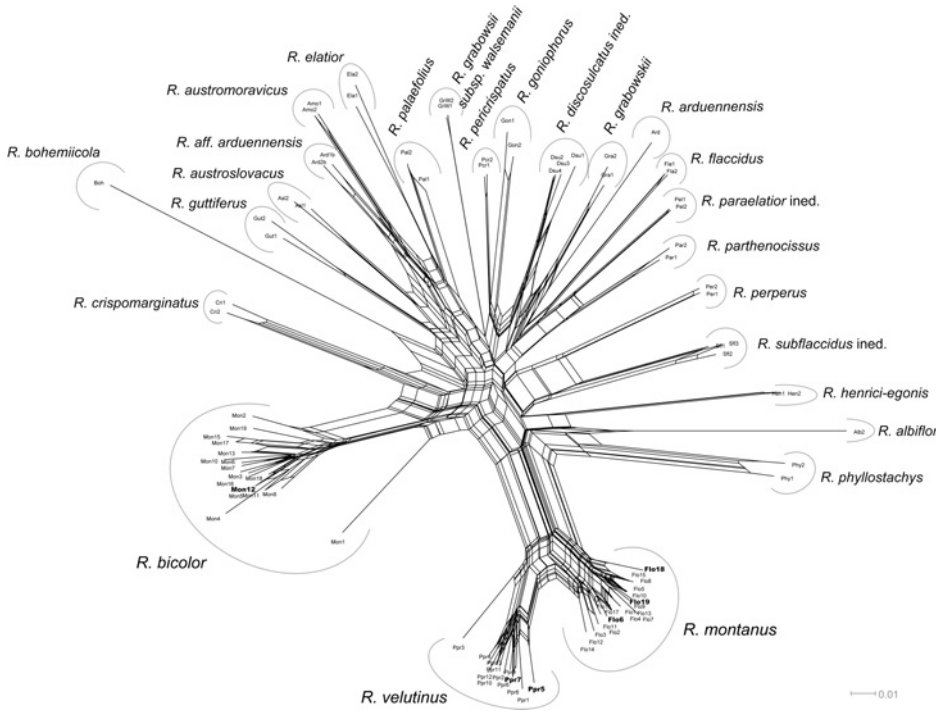


Fig. 4. – SplitsTree NeigbourNet based on SSR data of the accessions studied; individuals from type localities (incl. those of synonyms) are shown in bold (see also Electronic Appendix 2).

(Electronic Appendix 2), which implies a profound influence of mutations on intraspecific variability. This observation is in accordance with very low matrix incompatibility count (MIC) values recorded for each microspecies separately (not more than 61; Table 2), whereas for the whole species set (one individual per species) MIC = 3005 and also for the three species of the *R. montanus* group pooled the value was quite high (MIC = 349). On the other hand, when only *R. montanus* and *R. velutinus* were included, MIC = 107, whereas the pairs *R. bicolor* + *R. montanus*, and *R. bicolor* + *R. velutinus* resulted in MIC = 210 and 225, respectively.

Histograms of pairwise genetic distances among individuals did not provide a clear borderline between mutation-mediated and recombination-mediated variation. The highest peak indicated that recombination resulted in a mean pairwise distance of around 27 differences, but no single peak indicating mutations could be identified. Instead, two small peaks were recorded; one with a mean around 5 differences, which is the intraspecific distance, and a second one around 12 differences, which is the genetic distance between *R. montanus* and *R. velutinus* (Electronic Appendix 4). Setting any particular threshold between both types of variation was therefore difficult. Considering a threshold of 7 differences, each species would be formed by one or a few distinct genotypes. With a threshold of 10, each species would be formed by a single genotype (except for *R. bicolor* with one deviating individual) and every genotype would be characteristic

Table 2. – Descriptive statistics for the *Rubus montanus* group and all the samples. N – number of individuals studied, N_a – number of alleles per locus in the following order: Rubusr47a/Rubus105b/Rubus166b/Rubus275a/ERubLR_SQ01_G16/RubPara_SQ005_K23/ Rub265a/Rub236b/Rub238h/Rubleaf86, He – mean expected heterozygosity, MIC – matrix incompatibility count, NG – number of genotypes distinguished at the given distance threshold (T), NG_e – effective number of genotypes distinguished at the given threshold, DG – genotype diversity at the given threshold. * Counted for one randomly chosen sample of each microspecies due to software limitation and good comparability.

	R. bicolor	*R. montanus*	*R. velutinus*	Total sample (25 spp.)
N	17	19	13	92
N_a	3/2/5/12/9/2/1/5/2/3	3/5/3/9/3/2/1/4/2/1	3/5/4/7/3/3/1/4/2/2	14/15/17/29/20/6/2/1 3/4/9
H_e	0.489	0.511	0.541	0.733
MIC	61	23	10	3005*
NG/ NG_e/DG (T=0)	17 / 17.0 / 1.000	13 / 9.0 / 0.941	12 / 11.3 / 0.987	82 / 70.5 / 1.000
NG/ NG_e/DG (T=7)	2 / 2.0 / 1.125	1 / 1.0 / 0.000	2 / 1.2 / 0.154	31 / 10.5 / 0.914
NG/NG_e/DG (T=15)	1 / 1.0 / 0.000	1 / 1.0 / 0.000	1 / 1.0 / 0.000	18 / 5.9 / 0.839

for a single species (except for *R. velutinus* and *R. montanus*, which share the same genotype; see Electronic Appendix 2).

These patterns were supported by the PCoA (Electronic Appendix 5) and Neighbour-Net (Fig. 4) algorithms, which clustered individuals of the same microspecies together and separately from other microspecies. Only *R. montanus* and *R. velutinus* formed two very close clusters in both analyses (with a small overlap in the PCoA).

Distribution and ecology

The distribution of the *R. montanus* group was, due to the special nomenclatural and taxonomic problems, not sufficiently defined until the 1970s. This applies both for local/regional or national floras, and even the assessment in Flora Europaea (Heslop-Harrison 1968) for the group under the name *R. candicans* is false, which report it occurring at some of the peripheries of Europe (e.g. Ireland, Portugal, Turkey) without reliable records. Later on, however, considerable progress was made in the clarification of its range. It was shown that *R. montanus* (s.l.) is not present on the Iberian Peninsula (Monasterio-Huelin 1993) and its distribution in central Europe was also precisely characterized (Weber 1973, 1986a, 1987, 1995, Holub 1995, Zieliński 2004). On the contrary, the situation remained rather unclear in south-eastern Europe. This duality (well-defined in the west and unclear in the south-east) is precisely illustrated by the map in Kurtto et al. (2010, map. 4003) that assigned *R. montanus* (s.l.) as occurring in the entire Balkans with only question marks (despite the fact that scattered records are mentioned even by Weber 1973 and 1986a for that area). These doubts are, nevertheless, substantiated, because the Balkan Peninsula was practically unexplored from the point of view of modern batology (Kurtto et al. 2010, Király et al. 2013), and, although there are records of the *R. montanus* group from more countries in the region (Albania: Vangjeli 2015; Bosnia and Herzegovina: Weber 1973; Croatia: Purgar et al. 2007, Nikolić 2015; Romania: Nyárády

1956; Serbia: Josifović 1972), these are often unreliable due to the lack of herbarium material. In the well-explored western part of the range *R. montanus* s.l. is present in northern France, the Benelux Union, Switzerland, northern Italy and commonly in Germany, Austria, Czech Republic and Slovakia. In the eastern part, it is missing in the forest steppe belt in the lowlands of the Pannonian Basin, but occurs in mountains in Hungary, Slovenia, Slovakia and southern Poland.

There is little published information on the distribution of the species included in the *R. montanus* group. The chorology of *R. bicolor* has never been previously discussed, which is because a new taxonomic treatment of the group was only recently established. Trávníček & Zázvorka (2005) provide a preliminary distribution map of *R. montanus* s. str. (under the name *R. flos-amygdalae*) that shows the species as widespread in Czech Republic and with some localities also in Austria, Germany, Poland and Slovakia. Kosiński (2010) summarizes its known localities in Poland and Royer et al. (2016) report it from a few sites in northern France. *R. velutinus* is published under this name solely from the type locality (Styria, Austria; Trattinnick 1823). Furthermore, it is reported from central Bohemia (as *R. peripragensis* ined.) by Trávníček & Zázvorka (2005), and, lastly, from northern France (as *R. rotundipetalus*) by Royer et al. (2016).

In the course of our recent field studies, we found representatives of the *R. montanus* group at approximately 1200 localities in 14 European countries. The associated herbarium studies served mainly taxonomic purposes; however, we also found some older collections in diverse herbaria in other countries that complemented the distribution mainly at the edges of the species ranges.

Rubus bicolor is undoubtedly the most widespread species within this group (Fig. 5, Electronic Appendix 1). It is distributed nearly throughout the entire area mapped by Kurtto et al. (2010, map. 4003) for *R. montanus* s.l. (we do not know of any voucher specimens for Switzerland and the Netherlands). Its fine scale regional distribution requires revision in the western part of its range, e.g. in the German federal states. As a chorological novelty, we recorded the presence of *R. bicolor* in northern Italy and extensively in the northern Balkans. We confirmed its occurrence in western Romania, Bosnia and Herzegovina, Croatia (both in the Adriatic and Slavonian regions) and northern Serbia (isolated in the Fruška Gora Mts). In term of ecology, *R. bicolor* is relatively highly ecologically tolerant, which might account for its wide distribution. We recorded it at various altitudes from 130 to 870 m above sea level, and, among the lowermost localities there are sites both in the sub-Mediterranean (Croatia) and subcontinental (Hungary and Romania) parts of its range. It grows on soils developed both on acidic (e.g. mica) and alkaline (e.g. limestone) bedrocks, and on a wide range of soils from coarse to very fine texture. It occurs in several forest habitats, such as beech and oak-hornbeam dominated communities and their derivatives, and often in coniferous plantations. Compared to both the other groups of species, it is also found in semi-dry oak forest (e.g. Turkey oak communities in the Pannonian Basin).

Rubus montanus seems to be a sub-Atlantic central-European species whose range considerably overlaps that of *R. bicolor* in the west; however, it is much rarer in the eastern regions (Fig. 6, Electronic Appendix 1). As additions to the distribution map drawn by Trávníček & Zázvorka (2005, under the name *R. flos-amygdalae*), we also recorded it in other federal states in Germany (e.g. Saxony, Hesse) and Austria (Burgenland, Salzburg, and Vorarlberg). It is certainly present in Belgium (but hitherto confirmed only

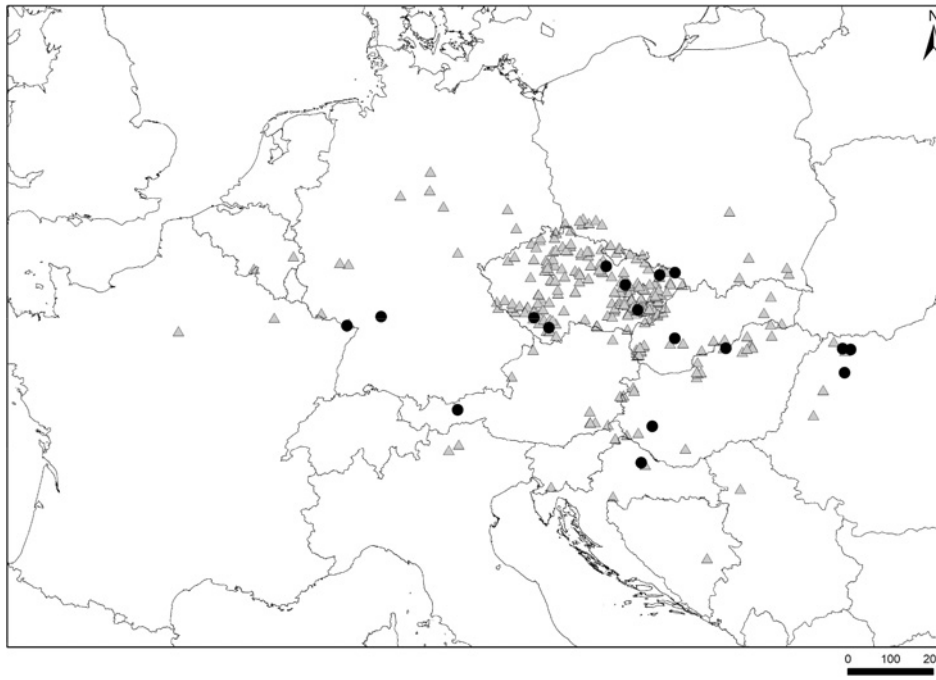


Fig. 5. – Distribution of *Rubus bicolor* Opiz based on localities supported by herbarium vouchers. Grey triangles are vouchers without an SSR analysis and black dots indicate those with an SSR analysis, respectively.

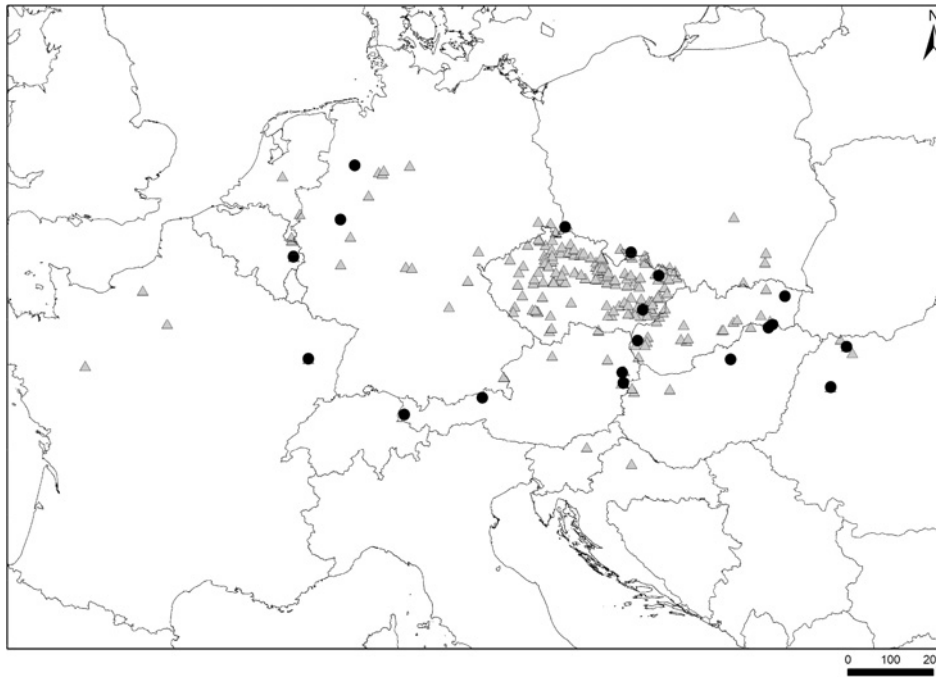


Fig. 6. – Distribution of *Rubus montanus* Lib. ex Lej. For legends see Fig. 5.

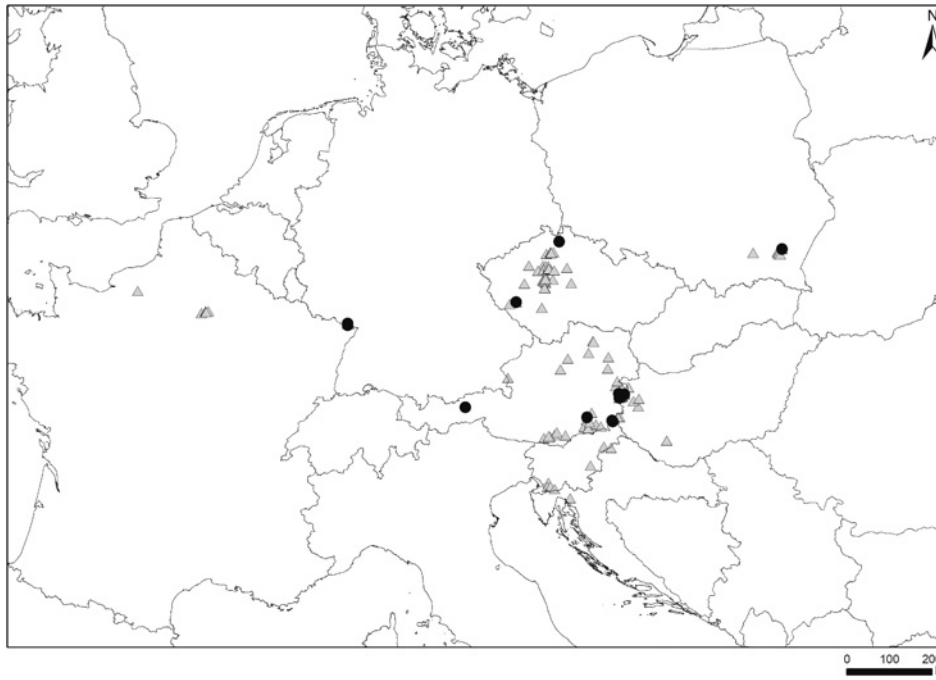


Fig. 7. – Distribution of *Rubus velutinus* Vest ex Tratt. For legends see Fig. 5.

at the type locality near Malmedy), France and the Netherlands. We considerably increased the number of sites recorded in Slovakia, confirmed its occurrence in northern and western Hungary, north-western Romania and (at two remote localities) in Croatia and Slovenia. Typical habitats of this species are fringes and young stands in beech and oak-hornbeam forests, mostly on deep clayey or loamy, slightly acidic or basic soils. Regarding altitude, its distribution is similar to that of *R. bicolor* (from 150 to 680 m above sea level). We also know of localities where it occurs together with *R. bicolor*.

Rubus velutinus is undoubtedly the species in this group with the smallest distribution (Fig. 7, Electronic Appendix 1). We circumscribed the core area of its distribution south-east of the Alps along with several smaller areas. The core area is situated in the hilly parts of the south and south-eastern foreground of the Alps (south-eastern Austria, northern Slovenia, with few localities in the area bordering Croatia and the western part of Hungary). In this region, *R. velutinus* is often the most common species of the *R. montanus* group. Somewhat isolated outposts were recorded in north-eastern parts of the Alps near Vienna, in central and south-western Bohemia, in Istria, and a very distant one in the Rhineland-Palatinate in western Germany. Interestingly, this species has not yet been found in the Carpathians, but several localities are recorded for it in an isolated area in south-eastern Poland. It was reported also from northern France (Royer et al. 2016); we know of a few vouchers from there. *R. velutinus* is a slightly acidophilous species, occurring mainly in areas covered by tertiary sediments (acidic gravel or clay), or in mountains of flysch bedrock.

Discussion

Both recombination and mutations contribute to diversification of ser. Discolores

In general, there are two main sources of variation in agamic complexes. First, residual sexuality in apomicts leads to gene-flow from sexually reproducing plants to apomicts and the formation of new apomictic lineages. Second, somaclonal variation may come from point mutations, autosegragation, somatic crossing-over etc., and may accumulate in asexual lineages (Asker & Jerling 1992). Residual sexuality via meiotically reduced megaspores or microspores is almost absent in triploid *Rubus* accessions, probably due to aberrant meiosis (Gustafsson 1943, Šarhanová et al. 2012). Our data nevertheless point to large interspecific distances that are unlikely to have resulted from the accumulation of mutations. The history of sexual recombination was also supported by the character incompatibility analysis (Table 2). It is therefore probable, that most of the species are of independent sexual origin, most likely via hybridization of a tetraploid facultative apomict (reduced gamete) and a diploid sexual (reduced gamete). Considering distribution patterns of plastid haplotypes (see Sochor et al. 2015), a cross between *R. canescens* (sexual diploid; pistillate parent) and a tetraploid accession of ser. *Discolores* (with some degree of apomixis; staminate parent) may be hypothesized as a likely scenario. A potential chance for this creation of new hybridogenous genotypes in central Europe is relatively high. This fact is becoming apparent especially in the (so far rather understudied) south-east of the region, where both potential parental taxa meet and several new morphologically distinct biotypes have been discovered (B. Trávníček and G. Király, unpublished data).

The triploid hybrids seem to reproduce asexually (Šarhanová et al. 2012) and accumulate only somatic mutations, as indicated by the relatively small intraspecific genetic distances and low MIC within species. Nevertheless, the mutational load may be substantial and mutation-based and recombination-based variation overlap in some instances (Electronic Appendix 5). As we used only microsatellite markers, which are known for their high mutation rate, our data may overestimate the true genome-wide intraspecific variability. Intraclonal variation in apomicts was nevertheless also recorded by other markers, such as AFLP, which even have a higher variability than microsatellites among clonal mates in apomictic dandelions (Van der Hulst et al. 2003, Majeský et al. 2012). A high rate of accumulation of mutations is also recorded in a transcriptome of apomictic *Ranunculus auricomus* agg. (Pellino et al. 2013). In spite of the prediction of Muller's ratchet model (Muller 1964), these mutations need not necessarily have negative effects on fitness. On the contrary, accumulation of mutations can enhance (and be enhanced by) niche specialization (Pellino et al. 2013) and may even result in the so called Meselson effect, i.e. high divergence among alleles at the same locus with their subsequent specialization to divergent functions (Welch & Meselson 2000). Therefore, accumulation of mutations may represent an adaptive and diversifying force, rather than a simple, irreversible journey to a dead end.

Rubus ser. Discolores microspecies are distinct, rather homogenous asexual lineages

The accumulation of mutations in asexual lineages, if reflected in morphology, can result even in speciation, as has been shown for bdelloid rotifers (Fontaneto et al. 2007). This

may also be the case for *R. montanus* and *R. velutinus*. Genetic distance between these taxa is much smaller than between the other species studied (although larger than the intraspecific average; Fig. 4, Electronic Appendix 4) and the allelic differences show the typical (although extreme in extent) mutational pattern (Electronic Appendix 2). Both species are also similar in a number of morphological characters (Table 1). Therefore, they could have been derived either from the same/similar parents via two independent sexual events, or they originated from a single hybrid genotype via the accumulation of mutations. None of these scenarios can be excluded based on our data. The clustering of both morphotypes into two separate genetic groups (Fig. 4) and the significant morphological differences (Table 1) nevertheless justify their distinction as two microspecies.

As discussed above, most of the triploid *Discolores* microspecies studied nonetheless appear to be of independent sexual origin and their intraspecific variability is only mutational. Such a species concept is not universal in apomictic complexes. In some taxa, such as grasses (*Poaceae*), apomictic genotypes are grouped together with sexual progenitors into one complex, which is treated as a single species for practical reasons (Kellogg 1990). A different species concept is applied e.g. in the genus *Taraxacum* F.H. Wigg., where every formal species is formed by one or (rather rarely) a few closely related, sexually derived genotypes (Majeský et al. 2012, 2015). This approach may nevertheless result in the polyphyly of some taxa. A narrow (monoclonal) species delimitation is therefore used in many of the intensively studied agamic complexes, e.g. *Sorbus* L. (Lepší et al. 2008), and based on the most recent taxonomic concept probably also *Boechera* Á. Löve et D. Löve (Windham & Al-Shehbaz 2007). In *Rubus*, this concept was supposed to be based on morphological studies and partly also confirmed or supported by molecular methods in a few tetraploid taxa (Kraft & Nybom 1995, Nybom 1998, Šarhanová et al. 2017).

From our study, we conclude that the morphological delimitation of triploid *Discolores* microspecies is highly compatible with the molecular evidence for independent sexual origin (or extensive mutational diversification) of these evolutionary units, and that intraspecific genetic variation is rather low and only mutational. Considering the many taxonomical difficulties and errors in the past, highly variable molecular genetic markers (e.g. microsatellites, AFLP) can be recommended as an additional line of evidence that enables an independent evaluation of species delimitation in any further taxonomical studies in other groups of brambles, as well as in other apomictic genera.

Can Rubus montanus s.l. contribute to answering fundamental questions about Rubus taxonomy?

Rubus montanus s.l. was a widely accepted species occurring throughout central- and western-European countries with old batological traditions, thus its split raises further taxonomical questions. The basic idea of the division of this group sprung unequivocally from small but consistent macromorphological differences, which were subsequently supported also by genetic investigations. The apparent question is whether this train of thought may be applicable when considering other groups of European brambles. In our opinion, it is, especially in strictly or generally apomictic taxa of (usually) independent sexual origin. On the other hand, it can be misleading for sexual plants and plants for which the breeding strategies are unclear. Thus, we expect similar convincing outputs

when working on other triploids or tetraploids in *R.* sect. *Discolores*, and we presume further splits in several widespread, probably "collective" species (such as the *R. praecox* group).

The description of new species in the subgenus *Rubus* has probably culminated in western Europe, but not yet in the south-east. Nevertheless, there are reassessments of seemingly solved species throughout Europe that are mainly associated with the increase in the number of species. These processes are extraordinarily rapid in the case of brambles; monographs and maps become obsolete in 10–20 years. A plausible solution may be to retain the recently divided species as aggregates, e.g. in distribution atlases. This method is, however, unsuitable for apomictic taxa, and is only useful in special cases, i.e. in groups that consist of proven closely related species.

Another issue is the possibility of differentiation via mutations within a single clone. This is an interesting finding that has been implied by some batologists. In theory, it can result in speciation (see above); nevertheless, it can cause great difficulties for descriptive taxonomy, which refers to lineages delimited by very small differences (e.g. only by a single morphological feature and genetic marker). They can be neglected (by inclusion with the main biotype) or can be described as species. In the latter case, however, great patience is needed; we recommend the inclusion for an interim period with a preliminary name, and repeated discussions with related experts (as we did, by the way, also in the case of the well-defined species in the *R. montanus* group).

One of the disputed taxonomical questions is the significance of the size and structure of the distribution area of *Rubus* biotypes. Weber (1995) and Holub (1997) state that only stable morphotypes with a reliable distribution area can be accepted as species. However, the "necessary" extent of the distribution for a species to be accepted is still being debated and whether it has any role questioned (Ryde 2011, Haveman & de Ronde 2013). The *R. montanus* s.l. group provides no support because each of the three circumscribed species has an extensive range (the diameters of their range exceeds 800 km) and according to Weber in Kurtto et al. (2010) can be classified as "widely distributed species". Other distinct (morphologically well defined) biotypes in the extended sample set (including the other triploid biotypes examined in this study) have distribution areas with diameters of at least 50 km. On the other hand, during this study we recognized several (possibly triploid) distinct morphotypes, which were restricted almost exclusively to a single locality. For practical reasons, we did not include them in the taxonomic system presented.

One important point that needs to be raised is the usefulness of herbaria for batological purposes. Of course, they are vitally important for the typification and understanding of older nomenclatural positions and when characterizing the patterns of distribution in poorly explored regions. Curiously, compared to other previously studied taxonomic groups of brambles, we found few useful historical herbarium specimens of the *R. montanus* group in the herbaria studied.

To conclude, this work revives many unanswered general questions and provides small pieces of information that may help to provide answers in the future. To improve our knowledge and increase the number of taxonomical treatments, repeated rethinking, a patient approach and progressive collection and integration of morphological, genetic and distribution data for each species are needed.

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Souhrn

Triploidní komplex *Rubus montanus* agg. (*Rubus* ser. *Discolores*, Rosaceae) je taxonomicky obzvláště obtížná skupina kvůli své plasticitě a také pro značnou morfologickou podobnost jeho zástupců, kteří, nehledě na značný počet dosud publikovaných jmen, byli dosud často považováni za jediný druh (*Rubus montanus* s.l.). V průběhu rozsáhlých terénních studií a herbářových revizí jsme v uvedeném okruhu potvrdili existenci tří morfologicky zřetelně definovaných typů s širokým rozšířením (střední Evropa a přilehlá území), které byly analyzovány jak morfologickými, tak molekulárně genetickými metodami. Výsledky tohoto studia ukazují, že tyto typy lze hodnotit jako druhy, pro něž lze použít již dávno validně publikovaná jména *R. montanus* Lej. (s. str.), *R. bicolor* Opiz a *R. velutinus* Tratt. V práci je provedeno taxonomické přehodnocení a diskutována synonymika a typifikace souvisejících druhových jmen a zároveň jsou uvedeny morfologické charakteristiky, ekologické vazby a mapy podrobnějšího rozšíření všech těchto druhů. Ukázalo se, že morfologické vymezení triploidních zástupců (druhů) v rámci ser. *Discolores* je kompatibilní s genetickou diferenciací prokázanou pomocí použitých molekulárních metod, přičemž tyto metody podávají důkazy pro převážně nezávislý sexuální hybridogenní původ těchto jednotek. Naopak vnitrodruhová genetická variabilita je spíše malá a jen mutačního původu, a potvrzuje tak striktně apomiktický způsob rozmnožování u těchto jednotek. Akumulace mutací může být nicméně někdy natolik významná, že rozsah takto vzniklé variability se může překrývat s rozsahem genetické proměnlivosti sexuálního původu. Vysoce variabilní molekulární markery, jako např. mikrosatelity, lze na základě této práce doporučit jako další, na morfologii nezávislou, metodu vymezení druhů pro další taxonomické studie na ostružinících.

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