Palacký University Olomouc

Faculty of Science

Department of Botany

Diversity, phylogenesis and evolutionary mechanisms in the genus *Rubus*

PhD. Thesis

Mgr. Michal Sochor

Supervisor: Doc. RNDr. Bohumil Trávníček, Ph.D.

Consultant: RNDr. Radim J. Vašut, PhD.

Olomouc 2016

Bibliografická identifikace

Jméno a příjmení autora: Michal Sochor

Název práce: Diverzita, fylogeneze a evoluční mechanismy v rodu *Rubus*

Typ práce: doktorská

Pracoviště: Katedra botaniky PřF UP

Vedoucí práce: doc. RNDr. Bohumil Trávníček, PhD.

Rok obhajoby: 2016

Abstrakt: Rod ostružiník (*Rubus*), zejména jeho nejbohatší podrod (subgenus *Rubus*), představuje v Evropě a na Jižním Kavkazu jednu z taxonomicky nejkomplikovanějších skupin rostlin. Jen v Evropě je uznáváno více než 750 druhů ostružiníků, z nichž naprostá většina je polyploidních s různou mírou asexuálního rozmnožování pomocí semen (apomixe, agamospermie). Pouze málo druhů je diploidních, a tedy striktně sexuálních. Ostružiníková flóra Jižního Kavkazu je prozkoumaná jen velmi sporadicky, ačkoliv nepochybně představuje jedno z evolučních center podrodu *Rubus*. Předložená práce se zabývá třemi dílčími tématy: 1.) fylogeneze evropských ostružiníků s důrazem na nalezení vztahů a vazeb mezi diploidními a polyploidními taxony; 2.) fylogeografie diploidního okruhu *R. ulmifolius/sanctus* agg. a její vztah k evoluci polyploidního komplexu série *Discolores*; 3.) cytotypová, reprodukční a haplotypová diverzita kolchidských ostružiníků.

Práce ukazuje, že celý polyploidní komplex i přes svou bohatost a rozmanitost vznikl z genofondů pouhých 7 ancestrálních diploidních druhů nebo druhových okruhů, z nichž tři jsou pravděpodobně vyhynulé. Naopak některé diploidní druhy jižní Evropy a Makaronézie se na evoluci polyploidů zřejmě nepodílely. Jeden z předků, *R. ulmifolius/sanctus* agg., prodělal během posledního glaciálu redukci svého rozšíření a efekt hrdla lahve ve východních částech areálu. To vedlo ke snížení genetické diverzity a snad i ke snížení kompetiční schopnosti, což mohlo usnadnit expanzivní šíření nově vzniklých polyploidních apomiktů v severozápadní Evropě a na Jižním Kavkazu. Dále se ukázalo, že tito apomikti nezřídka kombinují preglaciálních genetickou divezitu diploidních předků s genofondem recentních sexuálů ze svého regionu. Ačkoliv většina recentních apomiktů vznikla až v průběhu holocénu, agamický komplex jako celek je mnohem starší, sahající *přinejmenším* do předchozího interglaciálu. Apomixe by tedy neměla být vnímána jako slepá ulička evoluce, ale jako způsob uchování a šíření genetické diverzity v prostoru a čase. Kromě výhod samotné apomixe a (allo)polyploidie tak současní apomikti mohou těžit jak z genetické diverzity svých vyhynulých (nebo geneticky značně pozměněných) předků, tak i z lokálně adaptovaných genových komplexů recentních diploidů.

Jedna z kapitol práce podává první vhled do evoluce ostružiníků západního Kavkazu – Kolchidy. Ukazuje, že v Kolchidě dominují tetraploidní linie, které jsou buď striktně sexuální (morphoser. *Glandulosi* a *Radula*), nebo apomiktické s minimální reziduální sexualitou (morphoser. *Discolores* a *Micantes*). Variabilita v plastidové DNA odhalila izolovanost kolchidských ostružiníků od recentní evropské batoflóry a omezenou účast recentních diploidů na evoluci polyploidního komplexu.

Klíčová slova: apomixe, evoluce, geografická parthenogeneze, hybridizace, ostružiník, polyploidie, *Rubus*

Počet stran: 119

Jazyk: anglický

Bibliographical identification

Author's first name and surname: Michal Sochor

Title: Diversity, phylogenesis and evolutionary mechanisms in genus *Rubus*

Type of thesis: PhD. thesis

Department: Department of Botany, Faculty of Science, Palacky University

Supervisor: doc. RNDr. Bohumil Trávníček, PhD.

Year of defence: 2016

Abstract: Genus *Rubus*, especially its richest subgenus – *Rubus*, is one of the taxonomically most complicated plant groups in Europe and the Southern Caucasus. In Europe, more than 750 species are recognised, most of which are polyploids with varied degree of asexual reproduction via seeds (apomixis, agamospermy). Only few species are diploid and thus strictly sexual. The Caucasian bramble flora is only poorly explored, although it undoubtedly represents one of the evolutionary centres of the subgenus. The thesis covers three topics: 1. Phylogenesis of European brambles with special focus on relationships among diploid and polyploid taxa; 2. Phylogeography of diploid *R. ulmifolius/sanctus* agg. and its relation to the evolution of polyploids from series *Discolores*; 3. cytotype, haplotype and reproduction diversity of Colchic brambles.

The study shows that the rich and diverse polyploid complex originated from only seven diploid species or species aggregates, of which three are probably extinct today. On contrary, some South European and Macaronesian diploids did not contribute to the evolution of polyploids. One of the diploid ancestors, *R. ulmifolius/sancus* agg., experienced a reduction in its distribution and significant bottlenecks in eastern parts of its distribution area. This led to the decrease in the genetic diversity and subsequently possibly to lower competition abilities which may have enabled an expansion of newly arisen polyploids in Northwest Europe and the Southern Caucasus. It is further shown that apomicts combine pre-glacial gene-pools of the diploid ancestors and genetic diversity of recent sexuals from their region. Although most of the recent apomicts were formed in the Holocene, the whole agamic complex is much older, its history stretching at least to the last interglacial period. Therefore, apomixis should not be seen as an evolutionary dead end, but as a way of preservation and spread of genetic diversity in space and time. In addition to the advantages of asexuality and (allo)polyploidy, apomicts can use both genetic diversity of their extinct (or markedly changed) ancestors and the locally adapted gene complexes of contemporary diploids.

One of the chapters provide the first insight into evolution of west Caucasian brambles. It shows that tetraploid accessions are prevalent and exhibit either strict sexuality (morphoser. *Glandulosi* and *Radula*), or apomixis with rare residual sexuality (morphoser. *Discolores* and *Micantes*). Plastid DNA variation revealed isolation of Colchic and European brambles and limited involvement of contemporary diploids on evolution of the polyploid complex.

Keywords: apomixis, evolution, geographic parthenogenesis, hybridization, brambles, polyploidy, *Rubus*

Number of pages: 119

Language: English

Contents

Acknowledgements

This thesis could not be finished without contribution of many people. First, I thank my supervisor, Bohumil „Bob“ Trávníček, whose laser eyes and extraordinary knowledge of plants are essential for my every work (not only) on *Rubus*. I also thank Radim J. Vašut for his invaluable help with sampling and helpful advices. Special thanks belong to Timothy F. Sharbel who accepted me in his lab in Gatersleben and supported me and my work not only financially, but also by stimulating discussions and practical advices. I also thank many other people who supported me and contributed to my work either by sampling, or advice, or both – Ľuboš Majeský, Petra Šarhanová, Martin Dančák, Martin Duchoslav, David Earl, Martin Mau, Simon Pfanzelt, Vojtěch Žíla, Michal Hroneš, Michaela Jandová, Miloslav Kitner, Zdeněk Špíšek and many others, including many members of my family. Last, but not least, I acknowledge the endless support of my girlfriend Zuzana Egertová who managed to survive all of our not really trivial holidays in the Caucasus and elsewhere and who strove to motivate me despite her natural (and understandable) dislike of those thorny beastly sweethearts.

This work was supported by the following grants: grant No. 206/08/0890 by the Grant Agency of Czech Republic, the internal grant from Palacký University (PrF-2013-003, IGA_PrF_2014001, IGA_PrF_2015_001), grant No. LO1204 (Sustainable Development of Research in the Centre of the Region Haná) from the National Program of Sustainability I, MEYS, and DFG grant BL462/11. It was also partially funded by the Apomixis Research Group (IPK). STSM grant from the COST Action FA0903 was used for financing my stay in Gatersleben.

Declaration

I hereby declare that this thesis has been worked out by myself together with listed coauthors. All literary sources cited in this thesis are listed in the References section.

Author Contributions

CHAPTER 1 Introduction and aims of the thesis

Michal Sochor (MS) wrote this text.

CHAPTER 2 How just a few makes a lot: Speciation via reticulation and apomixis on example of European brambles (*Rubus* subgen. *Rubus*, Rosaceae)

MS, B. Trávníček (BT), T. F. Sharbel and R. J. Vašut (RJV) contributed to the experimental design and/or analytical tools. MS, BT and RJV contributed to the sampling. MS performed the laboratory analyses, analyzed the data and wrote the manuscript. All of the authors contributed to and approved the final manuscript.

CHAPTER 3 Origin of apomicts as a result of the sexual ancestor's phylogeography: a model case of European and Caucasian brambles (*Rubus*, Rosaceae)

MS and BT conceived the study and prepared the plant material; MS performed plastid DNA sequencing, ecological niche modelling and data analyses and wrote the manuscript; P. Šarhanová designed the SSR sequencing and NGS data analysis; S. Pfanzelt contributed to the analytical methods of the NGS data; BT determined the plant material. All of the authors contributed to and approved the final manuscript.

CHAPTER 4 Melting pot of biodiversity: first insights into the evolutionary patterns of the Colchic bramble flora (*Rubus* subgenus *Rubus*)

MS conceived the study, performed the sampling, laboratory and data analyses and wrote the manuscript. BT and MS phenotyped the material. Both of the authors contributed to and approved the final manuscript.

CHAPTER 5 Summary and conclusions

MS wrote this text.

CHAPTER 1: Introduction and aims of the thesis

Asexuality and its role in plant evolution

Sex has been drawing attention of evolutionary biologists for centuries and it is considered a driving force of evolution of life (Flerg 2005). Numerous apparent disadvantages (e.g. disruption of advantageous genotypes, risk of Allee effect) and high costs of sexual reproduction (cost of males, cost of meiosis, production of specialized reproduction structures and mechanisms; Williams 1971) made it almost unbelievable that such system could evolve and spread via Darwinian evolution. Though, its ubiquity in nearly all lineages of Eukarya testifies the long-term importance of sex. Two main groups of hypotheses explaining the evolution of sex are often mentioned. The first group suppose that sex enhance population evolutionary potential – the sexual population is able to generate recombinant types that can make it better able to keep up with changes of the environment. The second group of hypotheses supposes benefits of sex on the level of individuals, especially via elimination or reparation of deleterious mutations (Crow 1994). Sex also maintains diploid genome constitution which brings many other advantages, e.g. enhanced evolution of new genes, and preservation of intrapopulation and intraspecific polymorphism (Flegr 2005). Reversion to asexuality thus used to be considered a blind alley of evolution (Darlington 1939).

Asexual reproduction in plants is often referred to as apomixis which may include both asexual reproduction by seeds (i.e. agamospermy) and vegetative propagation. Apomixis is nevertheless usually understood as a synonym to agamospermy in contemporary literature. In this narrow sense, it can be divided to sporophytic and gametophytic apomixis. Both types share the same basic characteristic that embryo inherits the whole genome from the mother plant without participation of male gamete, its origin is nevertheless different. In sporophytic apomicts, embryo is derived directly from somatic cells of the nucellus or the ovule integument. Such embryo is dependent on fertilization of sexually derived megagametophyte because both sexual and asexual embryos share the endosperm. Sporophytic apomixis thus leads to polyembryony and is often known as adventitious embryony (Hand and Koltunow 2014).

On the other hand, pathways of gametophytic apomixis include development of mitotically formed megagametophyte (or incorrectly, embryo sac). This can be derived either from the megaspore mother cell (MMC) or a cell with apomictic potential occupying its position (diplospory), or from a somatic cell of the ovule adjacent to the MMC (apospory; Hand and Koltunow 2014; Bicknell and Koltunow 2004). In aposporous apomicts, sexually derived and aposporous embryos can coexist within one ovule. The unreduced apomictic egg cell usually develops into embryo without fertilization; if fertilization occurs, it leads to higher ploidy level in (hybrid) offspring. The central nucleus of the unreduced megagametophyte can develop into endosperm either spontaneously in some plant groups (autonomous type), or usually requires fertilization by a sperm cell for successful endosperm development (**pseudogamy**). Thus, since successful seed development usually cannot proceed without viable endosperm, pseudogamous apomicts are dependent on pollination (Asker and Jerling 1992; Bicknell and Koltunow 2004; Hand and Koltunow 2014). A very special type of apomixis is male apomixis which involves replacement of the genetic material of the egg cell by that from the pollen. So far, it has been observed only in the Mediterranean cypress tree *Cupressus dupreziana* Camus (Pichot *et al.* 2001).

Gametophytic apomixis has many consequences on different levels. On the level of individual/genotype, it enables an effective spread in both space (effective seed dispersal, e.g. by wind or birds, and better colonization ability due to no need for sexual counterpart; Hörandl 2006) and time (recombination is suppressed and does not disrupt the genotype which can be preserved for long time periods in many geographically distant ramets). On the other hand, genetic variation within a genet can be generated mainly via mutations (which are moreover mostly deleterious), chromosome rearrangements (Richards 1996) or recombination during restitutional meiosis (van Baarlen *et al.* 2000). On the population level, apomixis *can* decrease genotypic (and possibly also allelic) diversity which leads to lower adaptability and poorer ability to respond to environmental changes. Though, empirical studies provide quite the opposite evidence. First, only few plant taxa are strict asexuals and many apomicts retain some degree of residual sexuality (Asker and Jerling 1992; Gustafsson 1947). Especially in sympatric populations of apomicts and related sexuals, many novel apomictic genotypes can be generated via hybridizations (Hörandl and Paun 2007). Second, gametophytic apomixis is almost consistently associated with polyploidy and hybridity, both of which increase heterozygosity (both observed and expected) on the individual and population levels. Consequently, populations of apomicts can exhibit as high genotypic diversity as strictly sexual populations and even higher allelic diversity (Hörandl and Paun 2007; van der Hulst *et al.* 2000). On the levels of species and genera, apomixis forms a certain reproductive isolating barrier between genotypes. Each apomictic genotype can thus be considered as a separated evolutionary unit (agamospecies) and the whole population of such genotypes as an agamic species complex.

Systematics of apomictic complexes

Due to high degree of residual sexuality and a great genotypic diversity, apomictic plants are notoriously known as a nightmare for taxonomists as well as field botanists. Since no biological species *sensu* Mayr (1942) can be delimited in asexual plants by definition, several species concepts were applied in different genera. None of them is nevertheless universally appropriate and useful in every apomictic complex. In some taxa, apomictic genotypes are grouped together with sexual progenitors into one complex which is treated as a single species. This is the case of grasses (Poaceae) where continuous morphological variation and potential interfertility among all members of the complex are observed (Kellogg 1990).

In most apomictic genera, two or more types of species are considered. Strictly sexual (diploid or polyploid) groups are treated as true biological species, whereas apomicts are classified as microspecies/agamospecies which can be grouped in macrospecies, aggregate species, circle species or other informal groups. Every formal species (i.e. microspecies) is usually formed by either a few closely related, sexually derived genotypes (e.g. some *Taraxacum* Wigg. microspecies; Majeský *et al.* 2012; 2015), or by a single genotype which accumulates only mutational intra-clonal variation without sexual recombination, as is the case of *Rubus* L. (Kraft and Nybom 1995; Nybom 1998; Šarhanová 2014), *Sorbus* L. (Lepší *et al.* 2008), according to recent taxonomical concept probably also *Boechera* Á. Löve & D. Löve (Windham and Al-Shehbaz 2007) and others. Unfortunately, this means that a species could be formed very rapidly by a single hybridization event, as well as it could go extinct easily by the death of the only ramet representing it. The resulting number of species and their instability would make the taxonomy of many genera very complicated and even unusable.

Pragmatic approaches to classification of such complexes thus add other criteria for description of a species, such as geographic distribution, ecological function and production of homogeneous offspring (e.g. Lepší *et al.* 2008). For instance, in systematics of brambles (batology), the so-called Weberian concept (Weber 1996) assumes that only widely or regionally distributed biotypes (i.e. apomictically stabilized hybrids with distribution areas larger than 50 km in diameter) should be considered a species. Local and individual biotypes should also be studied and may be given provisional names, but these should not be validated as species. This implies that not all individual shrubs can be classified in the species rank, but they usually can be assigned to higher taxa, such as series or sections. Omitting local stabilized apomicts is sometimes criticized as pseudoscientific and hampering the full view on diversity in the genus. Modifications to the Weberian concept thus emerge and advocate describing also local biotypes (Haveman and de Ronde 2013; Ryde 2011). This criticism is partly justified, of course, but considering the practical aspects and sustainability of taxonomy, such alternative tendencies are not much followed.

Geographic patterns in agamic complexes

Apparent geographic trends in distributions of asexual biotypes and their sexual relatives have been observed in many plant and animal taxa. First, apomicts are reported to exhibit larger distribution ranges than sexuals, although many exceptions exist (Bierzychudek 1985; Hörandl 2006) and, moreover, the interpretation is often dependent on the groups being compared (microspecies or whole agamic complex, all sexual progenitors or separate species) and is greatly affected by the taxonomical concept. For example, in relatively well explored European dandelions (*Taraxacum*), the complex of apomictic triploids occupies big part of Europe, whereas diploid taxa do not exceed the northern limit in Northern France, Western Germany and the Czech Republic (van Dijk 2003). Nevertheless, separate agamospecies exhibit various ranges from very small ones to those several thousand kilometers large (Kirschner *et al.* 2016; Trávníček *et al.* 2010) which may be dependent mainly on the age of the genotype and its spreading ability (B. Trávníček, pers. communication). Similarly, in the complex of *Hieracium alpinum* L., diploid cytotypes are restricted to the Eastern and Southern Carpathians, whereas triploid apomicts cover the rest of the range, including the Alps, Ural and Scandinavia (Mráz *et al.* 2009). On contrary, most agamospecies of European brambles exhibit smaller ranges than most sexual species (excluding Macaronesian endemics) and even all apomictic microspecies taken together have narrower range than the most widespread sexual diploid *R. ulmifolius–R. sanctus* agg. occurring from Macaronesia to Afghanistan and from Morocco to Northern Great Britain, or than sexual tetraploid *R. caesius* L. occupying most of Europe and big part of Asia (Kurtto *et al.* 2010).

Apomicts often tend to occupy higher altitudes and latitudes than their sexual relatives. Such a trend is well documented for many taxa (Asker and Jerling 1992), although for most of them, the patterns seem better correlated by the Pleistocene glaciations rather than altitude and latitude *per se*. This can be seen on the above-mentioned example of *H. alpinum* (Mráz *et al.* 2009), as well as in North-American *Crataegus* L. (Loo *et al.* 2012) or *Townsendia hookeri* Beaman which survived as a diploid cytotype in two distant glacial refugia and the newly arisen apomictic polyploids spread to the surrounding deglaciated areas (Thompson et Whitton 2006). Also in taxonomically rich *Taraxacum* and *Rubus* the patterns are conspicuous, as both of which are represented in

previously glaciated regions mainly by apomictic lineages, whereas sexuals (at least the diploid ones) usually do not cross the line of glaciation or are rare behind it (van Dijk 2003; Kurtto *et al.* 2010).

Different geographic distributions of sexuals and apomicts are usually termed geographic parthenogenesis and causes of this phenomenon may be manifold. Bierzychudek (1985) suggested that success of apomicts in some areas may be caused mainly by (allo)polyploidy which could be advantageous in severe and/or unstable environments. Polyploids can profit from duplicated gene copies which can gain new or slightly varied functions (neofunctionalization or subfunctionalization). This allows for ecological niche expansion and increased flexibility in responsiveness to environmental change (Maldung *et al.* 2013). Polyploidy also prevents mutational meltdown of small populations by masking deleterious recessive mutations. Polyploid populations furthermore exhibit slower genetic drift due to increased effective population size compared to diploid populations of the same absolute size (Moody *et al.* 1993, Parisod *et al.* 2010). Nevertheless, probably the greatest advantage of polyploidy is fixed heterozygosity (Brochmann *et al.* 2004). Elevated heterozygosity is typical for all polyploids by definition due to presence of multiple gene copies. It is nevertheless maintained mainly in allopolyploids which integrate two or more copies of the same gene originating from two different taxa (or distant populations) and, at the same time, often exhibit disomic pairing which disrupts free segregation (Stift *et al.* 2008). Fixation of heterozygosity is further enhanced by apomixis which also prevents segregation. This can have several ecological effects. First, polyploids may be more vigorous than either of their diploid progenitors (heterosis) and this vigour is not significantly reduced in subsequent generations which is typical for sexuals with free segregation (Johansen-Morris and Latta 2006). Second, fixed heterozygosity protects against inbreeding depression and genetic drift (Brochmann *et al.* 2004; Moody *et al.* 1993). These advantages, together with the greater potential adaptability, plasticity, and spreading abilities, likely make apomictic polyploids successful colonizers of new habitats, especially under low competition pressure, e.g. after deglaciation or human-mediated changes in the landscape.

Geographic parthenogenesis was nevertheless observed also on a single ploidy level in *Rubus* ser. *Glandulosi* (Šarhanová *et al.* 2012) indicating that the phenomenon may not be caused by differences among ploidies only. Series *Glandulosi* is a complex taxon containing around thirty-five accepted apomictic, mostly tetraploid microspecies and sexual populations with uncertain species status (usually assigned to artificially defined *R. hirtus* agg., Kurtto *et al.* 2010). Although the series has a large distribution area in Eastern, Southern and wider Central Europe and the Southern Caucasus, agamospecies could be delimited only in its northern and northwestern parts (Kurtto *et al.* 2010). The flow-cytometric seed screen performed so far confirmed this pattern as apomictically derived seeds were detected only in the Šumava Mts (South Bohemia; Šarhanová *et al.* 2012) and North Bohemia (both in the West Czech Republic; unpublished data), whereas strictly sexual populations were observed in the Western Carpathians (Šarhanová *et al.* 2012), Central Moravia (the East Czech Republic; unpublished data) and Colchis (see chapter 4). Šarhanová (2014) did not detect any genetic differentiation between the Carpathian and Bohemian populations based on nine SSR loci and suggested that differential hybrid origin and genome composition likely did not play a role in geographical parthenogenesis in this case. The Bohemian populations nevertheless exhibited elevated observed heterozygosity which was quite unexpected on

the margin of the taxon distribution. Apomixis was thus hypothesized as a factor buffering against small-population phenomena such as drift or inbreeding that decrease heterozygosity and often have deleterious effects on populations.

Alternatively, other ploidy-independent hypotheses may also apply, such as the "Red Queen" hypothesis, postulating that apomicts are not able to face a pressure of predators, pests and pathogens in lower altitudes or latitudes due to their decreased evolutionary flexibility (Verhoeven & Biere 2013). Alternatively, apomixis may be a way how to reduce gene flow from central to marginal subpopulations which prevents a fixation of local adaptations (Haag and Ebert 2004; Vrijenhoek & Parker 2009). It is also an effective solution to problems with finding a sexual counterpart under low population densities and/or in small populations (Tomlinson 1966). Subsequently, uniparental reproduction may lead to better colonization ability which form an important part of the apomicts' r-strategy (Grime 1977), being particularly advantageous for colonization of newly deglaciated areas or disturbed habitats in some, but not all agamic complexes (Hörandl 2006).

Eurasian blackberries as a model system

As noted several times above, brambles serve as one of good model systems for studies on apomixis-related phenomena, from genetics of apomixis to geographical parthenogenesis and systematics. Genus *Rubus* with its twelve (mostly polyphyletic or paraphyletic) subgenera occurs on all continents (except Antarctica) and is of great economical and ecological importance as a fruit crop, invasive weed and significant component of many plant communities (Alice and Campbell 1999). Total number of species is difficult to estimate because the latest comprehensive revision of the genus is more than a century old (Focke 1910–1914). Excluding the richest subgenus *Rubus*, there are approximately 335 species (Thompson 1997). For subgenus *Rubus* in eastern North America, Davis (1990) claimed 198 species, around 750 species have been reported for Europe (Kurtto *et al.* 2010) and many others can be found in western North America and the Caucasus (Focke 1910–1914). The total number of currently accepted species in the genus may therefore be estimated at more than 1,300. It is nevertheless highly dependent on the species concept and data availability from species rich regions, such as North America and the Caucasus (subgenus *Rubus*) and East and Southeast Asia (subgenera *Idaeobatus* and *Malachobatus*; Focke 1910–1914). In Europe, brambles have attracted a lot of attention, although they are represented by only one subgenus that contains more than three native species – subgenus *Rubus*. The long-term development of species concept resulted in acceptance of hundreds of names and refusal of other hundreds. For practical reasons, the subgenus is divided into three sections, four subsections and approximately 22 series in Europe (only native taxa; Kurtto *et al.* 2010) reflecting morphological and often also ecological similarities.

Apomixis and its evolutionary consequences have been studied predominantly in subgenus *Rubus*, although it was found also in subgenus *Malachobatus* (Amsellem *et al.* 2001), subgenus *Idaeobatus* (Pratt *et al.* 1958), and apomeiotic initials were detected in subgenera *Cylactis* and *Chamaemorus* (Czapik 1983). Reproduction in brambles is highly variable. While diploid taxa are strictly sexual, triploid accessions exhibit disrupted meiosis and seem therefore strictly apomictic (Šarhanová *et al.* 2012). The same may be expected for pentaploids. Tetraploids, which are most common in Europe (Krahulcová *et al.* 2013) and particularly in Great Britain (the author's unpublished

data), are mostly facultative apomicts with varied degree of sexuality. Both sexually and apomictically derived seeds can be formed in a single individual or even in a single flower (Šarhanová *et al.* 2012; the author's unpublished data). Apomixis in brambles is pseudogamous apospory or diplospory and its components (apomeiosis and parthenogenesis) are mutually independent. This occasionally leads to a formation of functionally haploid embryo (parthenogenetic development after meiotic reduction) or embryo with higher ploidy than that of the mother plant (fertilization of unreduced egg cell; Šarhanová *et al.* 2012). Moreover, apomixis in brambles is affected by external factors such as temperature and drought (Šarhanová *et al.* 2012; and the author's unpublished data).

Aims of the thesis

Both morphological observations and artificial crossing experiments led to conclusions that hybridization plays a major role in *Rubus* evolution and probably led to formation of many agamospecies, as well as whole series. Though, solid evidence for hybrid origin of any particular natural taxon is scarce. Neither phylogenesis nor evolutionary relationships in subgenus *Rubus* have been studied yet. Patterns of geographic parthenogenesis have been being uncovered only recently (Šarhanová *et al.* 2012; Šarhanová 2014) and there are still more questions than answers in this field. Similarly, our knowledge on the evolution and diversity of the subgenus in non-European (as well as some European) regions has not improved significantly in the last decades.

Therefore, this thesis aims to contribute to our understanding of evolution of apomictic complexes on wider geographical scale and uncover relationships and evolutionary mechanisms among different groups of European and Caucasian brambles. The thesis consists of the following parts:

CHAPTER 2: How just a few makes a lot: Speciation via reticulation and apomixis on example of European brambles (*Rubus* subgen. *Rubus*, Rosaceae)

This chapter uncovers general evolutionary patterns in the European bramble flora, evaluates the roles of hybridization and apomixis and also the roles of the sexual ancestors. Furthermore, it uncovers a spatio-temporal frame of European *Rubus* evolution for the first time.

CHAPTER 3: Origin of apomicts as a result of the sexual's phylogeography: a model case of European and Caucasian brambles (*Rubus*, Rosaceae)

This chapter concentrates on one of the diploid sexual ancestors – *R. ulmifolius* agg., and its polyploid descendants. By comparison of the diploid's phylogeography and the polyploids' distribution patterns, origin and expansion of apomicts are implied.

CHAPTER 4: Melting pot of biodiversity: first insights into the evolutionary patterns of the Colchic bramble flora (*Rubus* subgenus *Rubus*)

This part describes patterns of cytological, reproductive and molecular diversity in brambles of the Western Caucasus – one of the evolutionary hotspots of the subgenus.

CHAPTER 2:

How just a few makes a lot: Speciation via reticulation and apomixis on example of European brambles (*Rubus* subgen. *Rubus*, Rosaceae)

Michal Sochor

Radim J. Vašut

Timothy F. Sharbel

Bohumil Trávníček

2015, Molecular Phylogenetics and Evolution 89: 13–27

DOI: 10.1016/j.ympev.2015.04.007

Abstract

New species are generated by many means, among which hybridization plays an important role. Interspecific hybrids can form isolated evolutionary units, especially when mechanisms increasing viability and fertility, like polyploidy and apomixis, are involved. A good model system to study reticulate evolution in plants is *Rubus* subgen. *Rubus* (brambles, blackberries), which only in Europe includes 748 accepted species, out of which only four are sexual diploids and all others are polyploid apomicts. We employed two molecular markers (ITS and cpDNA) to shed light on the evolutionary history of European bramble flora and main processes generating such high species diversity. We distinguished just six ancestral diploids (including two extinct ones) for both markers, which gave rise to all European polyploid accessions, and revealed an extreme reticulation in bramble evolution. We furthermore detected hybridogenous origins and identified putative parents for several taxa (e.g. ser. *Nessenses*), while in other groups (e.g. ser. *Discolores*) we could also infer the direction of hybridization. By comparing different cp haplotypes having clear geographic patterns, we hypothesize that the origin of European brambles can be attributed to both Holocene species range expansion and Pleistocene climate fluctuations.

Introduction

Species-rich genera – i.e. large ones containing more than 500 species (Frodin 2004) – represent up to one quarter of all flowering plant species (Monro 2006). Besides taxonomic discussions, many factors are thought to underly such systematic patterns, among them life form, mode of dispersal, key innovations, rate of neutral evolution, co-evolution with pollinators and other various biotic and abiotic interactions leading to rapid radiation, divergence and multiple speciation events (Eriksson and Bremer 1991; Hodges and Arnold 1995; Rieseberg and Willis 2007). Hybridization has furthermore played a major role in plant speciation, often in combination with polyploidy, as it ensures rapid reproductive isolation between the hybrid and parental species, usually within one or two generations (Rieseberg and Willis 2007). On the genome level, it further enhances evolution through gene redundancy and potential subfunctionalization of duplicated genes (Comai 2005). Moreover, polyploidization, either via somatic doubling, fusion of unreduced gametes or through the so called triploid bridge, can often lead to reduction of maladaptive changes in gene expression and restoration of hybrid fertility through allopolyploidy-induced sequence elimination or changes in gene expression ameliorated by genome duplication (Rieseberg and Willis 2007). Nonetheless, meiotic aberrations and associated decreases in fertility, not to mention complete sterility, represent significant obstacles to the establishment of a hybrid lineage (Comai *et al.* 2003; Comai, 2005). One potential evolutionary solution to this problem is asexual reproduction, either by vegetative means or through seeds (apomixis or agamospermy; Asker and Jerling 1992).

Apomixis (and parthenogenesis in animals) is typically associated with both polyploidy and hybridity, since many apomictic taxa are of allopolyploid origin (Bicknell and Koltunow 2004), although it is unclear whether these traits represent cause or effect of asexual reproduction. For example, the widespread occurrence of diploid apomixis in *Boechera* Á. Löve & D. Löve, by definition implicates hybridization rather than

polyploidy as the inducer of apomixis from sexual ancestors (Beck *et al.* 2012), although analyses of genetic variation also demonstrate that diploid apomicts are not always interspecific hybrids (Lovell *et al.* 2013). Apomixis has also been reported in several autopolyploid taxa, e.g. *Townsendia hookeri* Beaman (Thompson and Whitton 2006), *Paspalum* L. (Hojsgaard *et al.* 2008) or *Ranunculus kuepferi* Greut. et Burd. (Cosendai *et al.* 2011). Hence, depending on the species context, hybridization and polyploidy may only indirectly be correlated with apomixis, for example by masking deleterious mutations accumulated during the asexual life cycle.

The adaptive and evolutionary potential of asexuality has historically been underestimated, resulting from the assumption that obligate apomicts are characterized by low genetic variability (Hörandl and Paun 2007). In contrast, accumulating evidence based upon genetic markers and population genetic methods support the opposite view. As many apomicts are facultative, whereby low levels of sexuality are maintained, backcrossing with sexual relatives is hypothesized to lead to multiple evolutionary origins of apomictic lineages, with the concomitant generation of considerable clonal diversity (van der Hulst *et al.* 2000; Paun *et al.* 2006). On the population level, apomictic lineages often show higher levels of genetic variability (e.g. observed heterozygosity) compared to their sexual relatives (Hörandl and Paun 2007), a reflection of their allopolyploid origin in addition to mutation accumulation (i.e. Muller's ratchet; Muller 1964), the latter of which generates new alleles which are subsequently redistributed into new genotypes via occasional sexual outcrossing (Asker and Jerling 1992; Majeský *et al.* 2012, 2015). Beside fixed heterozygosity, potential hybrid vigor and buffering of inbreeding depression, apomicts can take advantages from uniparental reproduction, lowered cost of sex, maintaining adapted genotypes and at the same time also from reproduction by seeds including dormancy, diaspore protection and better dispersal ability compared to vegetative reproduction (Hörandl 2006). All these factors can lead to great ecological and evolutionary success of many apomictic plant genera.

The genus *Rubus* L. is a good example of such a successful taxon, being characterized by twelve subgenera and a worldwide distribution (excluding Antarctica). It is widespread across Europe (Kurtto *et al.* 2010), with some European species having been introduced for fruit production into different parts of the world where they have repeatedly become aggressive invaders (Caplan and Yeakley 2010; Clark *et al.* 2013). The taxonomic classification of the enormous number of described species has been a challenging task for generations of researchers. A recent taxonomic approach – referred to as the *Weberian concept* (Weber 1996) – consider a species only if it is morphologically stable over wider distribution area, and ignores local morphotypes/biotypes of putatively hybrid origin. Although this approach reduced the number of accepted species considerably, 763 *Rubus* species are still recognized in Europe, of which 748 belong to the subgenus *Rubus* (brambles, blackberies; Kurtto *et al.* 2010). Morphologically similar species are clustered into *series*, which are rather artificial units with overlapping morphology. Further, out of the high number of known species in Europe, only four sexual diploids (i.e. *R. ulmifolius*, *R. canescens*, *R. incanescens* and *R. sanctus*) are known, and additionally four confirmed diploid sexuals occur in neighbouring regions of the Transcaucasia (*R. moschus*) and Macaronesia (*R. bollei*, *R. palmensis* and *R. serrae*; Gustafsson 1942; Matzke-Hajek 2001; Kurtto *et al.* 2010). It is likely that this extensive taxonomic complexity is the reason for the absence of any reliable subgenus-wide phylogenetic analysis to date.

The three above mentioned factors – apomixis, polyploidy and hybridization – are the main contributors to such complexity, as the majority of European brambles are tetraploid (with some triploid, pentaploid and hexaploid) pseudogamous apomictic lineages (Krahulcová *et al.* 2013). Reproduction is highly variable, ranging from obligate sexuality to obligate apomixis on the inter- and intraspecific levels, to the floral level within a single individual or even ovules within a single flower (Pratt and Einset 1955; Gerlach 1965; Šarhanová *et al.* 2012). Apomixis itself can furthermore combine both apospory and diplospory (Christen 1950; Pratt and Einset 1955), not to mention that the reproductive mode can be influenced by external environmental factors (Šarhanová *et al.* 2012). Especially in tetraploid taxa, the degree of residual sexuality can be considerable, as seen on both seed and seedling levels (Jennings *et al.* 1967; Nybom 1995; Kollmann *et al.* 2000). On the other hand, triploid and pentaploid accessions show almost obligate apomixis (Šarhanová *et al.* 2012). Additionally, fertilization of unreduced embryo sacs, leading to increased ploidy levels, or spontaneous development of reduced embryo sacs giving rise to polyhaploid offspring, are sometimes observed in flow-cytometric seed screen analyses of various taxa (Šarhanová *et al.* 2012) as well as in offspring from artificial crossings (Crane and Thomas 1949). The fusion of two reduced egg nuclei (i.e. automixis) has also been found (Gerlach 1965; Antonius and Nybom 1995).

Batologists (specialists on brambles) have long been aware of hybridization as the main driving force of *Rubus* evolution, as reflected in the overlapping morphology of the various *Rubus* series. Many experimental crosses have shown frequent hybridization even between distant taxa, as the fitness of F1 and subsequent hybrids are very high, sometimes higher than that of their respective parents (Lidforss 1914; Jennings *et al.* 1967; Nybom 1988). These studies have also shown improved meiosis in artificial hybrids (see also Bammi and Olmo 1966) resulting in higher pollen viability and seed set, as well as in higher degree of sexuality. Early experiments with artificial hybrids additionally revealed enormous morphological variability among hybrid progeny, much of which often resembles distantly related natural taxa (Lidforss 1914; Rozanova 1934, 1938). Importantly, these works point out that a given cytologically and morphologically defined polyploid accession can be formed not only multiple times independently, but also in several ways from the same basal ancestors (Mavrodiev and Soltis 2001). These experimental data have been confirmed for natural populations using molecular markers in several lower-level taxa (Kraft *et al.* 1995; Alice *et al.* 2001; Šarhanová 2014), although only morphological and cytological evidence has been employed to study wide-scale evolutionary patterns of European brambles.

While much is understood regarding microevolutionary processes and morphological differentiation in *Rubus*, the mechanisms of diversification, especially with regards to which species were (or still are) involved in polyploid evolution in European *Rubus*, remain unclear. In this study, we analyse chloroplast and nuclear DNA markers (Internal transcribed spacer; ITS) in a broad sample covering all major *series* within the *Rubus* subgen. *Rubus* to understand the evolutionary patterns and processes influencing their evolution, and furthermore we attempt to identify parental species associated with hybridization. While commonly used in phylogenetic studies, ITS is part of highly repetitive tandem rDNA array whose evolution is complicated by processes such as sequence homogenization, intergenic recombination and pseudogenization (Álvarez and Wendel 2003). These may lead to distortion or even loss of phylogenetic signal in hybrid complexes. On the contrary, it was documented that concerted evolution of ITS

is suppressed in polyploid apomicts (genus *Taraxacum*, Asteraceae) preserving high intraindividual variability (Záveská Drábková *et al.* 2009). Thus we aimed to quantify the intragenomic processes potentially affecting the usefulness of ITS for reconstruction of evolutionary pathways in apomictic genera.

Materials and methods

Plant material

A total of 287 individuals from 145 species were sampled throughout Europe and adjacent regions in order to cover the complete taxonomic complexity of the subgenus *Rubus*, including all four known sexual diploids from Europe, two from Macaronesia and one from Transcaucasia (Supplementary table 1). Moreover, 15 local hybrids of at least partly known origin (17 samples), 14 undetermined putatively apomictic taxa (14 samples), one series of unclassifiable facultative apomicts (ser. *Glandulosi*; 17 samples), *R. idaeus* (subgen. *Idaeobatus*) and two outgroup species (*R. odoratus* and *R.* cf. *biflorus*) were included. Taxonomy and nomenclature follow recent literature based on the so-called *Weberian* taxonomic concept (Kurtto *et al.* 2010).

Molecular methods

DNA was extracted from silica gel-dried leaves, or in few cases from herbarium specimens, following the CTAB (Cetyl Trimethyl Ammonium Bromide) protocol of Doyle and Doyle (1987) with minor modifications. For chloroplast DNA variation, four regions were tested; the *trnH–psbA* intergenic spacer (Newmaster and Ragupathy 2009) and the *psaA* coding region (primers F: GGATGCCTGTGCCCATAAGAAATCGC, R: GGATTTCTCATAGTTGGTGCTGCTGCG) revealed only low variability and were not used. The other two non-coding regions were selected for the analysis: the *matK* intron amplified with XFA and AST_R primers (Dunning and Savolainen 2010), and the *trnL-trnF* intergenic spacer with *e* and *f* primers (Taberlet *et al.* 1991). One universal primer pair, ITS1–ITS4, was used for amplification of the ITS1-5.8S rDNA-ITS2 (internal transcribed spacer) nuclear locus (White *et al.* 1990). All PCR reactions were performed in 15 μL reaction volume using *Pfu* DNA polymerase (Thermo Scientific) according to manufacturer's recomendations with 0.8 μM final concentration of each primer, 20 ng of template DNA and 0.25 u DNA polymerase. Cycling conditions were as follows: initial dentautation at 95 °C for 5 min., 37 cycles including one-minute denaturation at 95 °C, 40sec. annealing step at 48 °C, 52 °C or 59.6 °C for the *matK*, *trnL-trnF* and ITS, respectivelly, and extension step at 72 °C for 160 sec., followed by 10 min. final extension step at 72 °C. ITS PCR products were cloned into a bacterial vector prior to sequencing using the CloneJet™ PCR Cloning Kit (Thermo Scientific) with One Shot® TOP10 chemically competent cells (Invitrogen™) following manufacturer's instructions. Subsequent Templi-Phi™ reactions and Sanger sequencing of Templi-Phi products (ITS) or polyethylene glycol-purified PCR products (chloroplast markers; 10% PEG 6000 and 1.25M NaCl in the precipitation mixture) were performed on a 96-capillary ABI 3730 instrument in the IPK central sequencing facility or by Macrogen Europe. For all three markers sequencing of both DNA strands was performed to avoid any ambiguities. All sequences were deposited in NCBI Genbank and accession numbers can be obtained from Supplementary table 1.

In addition to sequencing, additional sets of ITS-transformed bacterial colonies were analyzed by PCR-RFLP (restriction fragment length polymorphism) to test for deviation from expected proportion of parental ribotypes, and thus for concerted evolution acting on these sequences. Plasmid-specific pJET1.2 primers were used for colony-specific PCR, followed by digestion with the BspLI (NlaIV) restriction enzyme, which differentiated three parental taxa with two restriction sites (*R. idaus*, *R. caesius*, *R.* sect. *Rubus*; Alice *et al.* 2001). Visualization was performed on 1.5% agarose gels stained with ethidium bromide.

Data analysis

The raw forward and reverse chloroplast sequences were aligned in the SEQMAN PRO™ module of the LASERGENE® software (ver. 11; DNASTAR Inc., WI, USA) and manually trimmed, checked and edited. Alignments and haplotype identification were performed with GENEIOUS (ver. 3.6.1.; created by Biomatters, available from www.geneious.com). A median joining algorithm was used for creating phylogenetic haplotype networks in NETWORK (ver. 4.6.1.2; Bandelt *et al.* 1999; epsilon=10) and maximum parsimony (MP) calculation was performed in the same software to reduce unnecessary median vectors and links in the final network (Polzin and Daneshmand 2003). Microspecies sharing the same haplotype were grouped together and their geographic distributions, extracted from the Atlas Florae Europaeae (Kurtto *et al.* 2010), were overlaid with sexual taxa of the same haplotype onto a single map in QGIS (ver. 2.0.1; Open Source Geospatial Foundation Project, www.qgis.org) to produce haplotype density distribution maps.

Alignment of ITS sequences was performed in MAFFT (ver. 7; Katoh and Standley, 2013). For each sequence the free energy of RNA structure and CG content were computed using the NUPACK web server (Zadeh *et al.* 2011). Single nucleotide polymorphisms (SNPs) having a frequency < 1.2 % (as calculated based on number of observations of all frequency classes in the dataset) were excluded from the analysis, as they could represent technical errors and furthermore did not contribute any phylogenetic information. Products of intraindividual recombination were distinguished from the alignment manually based on observations of stable motifs characterizing the parental species. The resulting set of non-recombinant and non-pseudogenous sequences was analyzed in SPLITSTREE using NeighborNet method (Huson and Bryant, 2006) and NETWORK (ver. 4.6.1.2; Bandelt *et al.* 1999) using the Star contraction algorithm (Forster *et al.* 2001) with default maximal radius of 5 mutations. Subsequently a median joining algorithm and MP calculation (Polzin and Daneshmand, 2003) were used for creating phylogenetic networks following recommended parameters (epsilon=10, several mutations downweighted according to their mutation frequency within the network up to a half value). The same ITS dataset was analyzed using Bayesian inference (BI) and the K80+I+G mutational model of MRBAYES (ver. 3.2; Huelsenbeck and Ronquist, 2001) in four replicate runs, with four chains each and 3 milion generations (first 40 % of them discarded as burn-in), sampling every 500th tree. Phylogenetic trees for selected non-hybridogenous taxa were calculated using maximum likelihood (ML) methods in MEGA (ver. 5.2.2; Tamura *et al.* 2011) and BI in MRBAYES with the same parameter as above, except for 600 000 generations of MCMC and HKY and HKY+I models for the cpDNA and combined cpDNA+ITS datasets, respectively. Likelihood ratio tests were performed in JMODELTEST (ver. 2.1.4;

Darriba *et al.* 2012) to choose the most appropriate mutation models for all ML and BI analysis.

For quantification of concerted evolution in ITS sequences, the expected ratio of parental ribotypes (i.e. specific types of ITS sequences) was assessed according to the ploidy of the studied individual and its parental taxa. Observed ratios of parental ribotypes were then tested against the expectations by a single-proportion exact test in NCSS software (ver. 2007; Hintze, J., www.ncss.com.).

Results

Both species specific and shared cp haplotypes in Rubus

The total length of the intergenic spacer *trnL-trnF* consensus alignment was 475 bp, including two 6 bp insertions and two deletions (6 and 8 bp) in subgen. *Idaeobatus*, and one 6–8 bp insertion in *R. caesius*. Thirteen (2.7 %) SNPs were identified within subgen. *Rubus* and 28 SNPs (5.9 %) in the total sample-set. The *matK* intron sequencing revealed no length polymorphism over a 993 bp alignment, and was characterized by 22 SNPs (2.2 %) within the subgenus *Rubus* and 47 SNPs (4.7 %) in the whole sample-set .

The combination of both markers distinguished 24 haplotypes (Supplementary table 1), including two haplotypes of *R. idaeus* (*Ida1, Ida2*) and two outgroup haplotypes (*Odo, Bfl*), which were named after the main taxa or geographic regions they represented. Only four haplotypes were found strictly in diploid species (excluding *R. idaeus*) – haplotypes *San1*, *San2* and *San3* in *R. sanctus* and haplotype *Inc* in *R. incanescens*, whereas 4 haplotypes were detected in both diploid and polyploid species (*Can1*, *Gla1*, *Ulm1* and *Ulm2*). Haplotype *Cae1* was present in tetraploid sexual *R. caesius* and in most of the apomictic hybridogenous taxa classified in sect. *Corylifolii*. Four haplotypes were detected only in polyploids from Europe (*Suber*), western Transcaucasia (*Cau*) or Madeira (*Mad1* and *Mad2*). The remaining haplotypes characterized a few polyploid taxa, and were clearly derived from *Cae1*, *Can1* and *Gla1* (see Figure 1).

In diploids, intraspecific haplotypic variability was detected only in the *R. ulmifolius/sanctus* complex. Two haplotypes (*Ulm1* and *Ulm2*) were spread across the whole western part of its distribution area with an eastern limit in the west Balkans, whereas haplotype San1 occured only in the east and south of the peninsula. In the Transcaucasian region, none of these haplotypes was detected in diploid *R. sanctus*, where it was instead characterized by the *San2* or *San3* haplotypes. On the other hand, both *Ulm1* and *Ulm2* haplotypes but none of *San* haplotypes were found in both Europaean and Caucasian polyploids. An almost opposite situation was observed in haplotype *Gla1*, which was detected in all three samples of the Caucasian endemic diploid *R. moschus* and many Europaean polyploids, whereas no polyploid with this haplotype from the Caucasus was observed. Within each polyploid microspecies, only one haplotype was found. Potential inconsistencies could be explained by inaccurate taxonomic determination (marked as cf. or aff. in Supplementary table 1), as many species were sampled in large geographic area and precise determination is often difficult due to the high morphological plasticity of brambles.

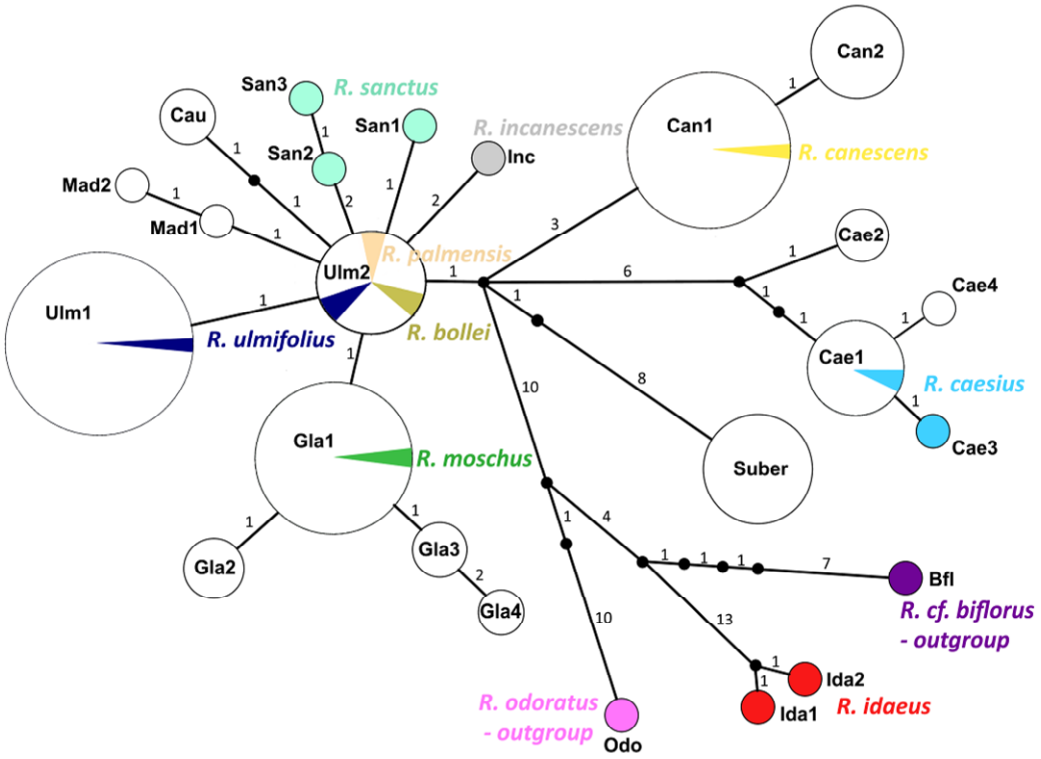


Figure 1: Median joining cp haplotype network: each detected haplotype is denoted by one circle sized according to the number of species bearing it (see a list of accessions with their haplotypes in Supplementary table 1); diploid species and *R. caesius* are plotted in color whereas apomictic microspecies are white. The number of mutational changes between haplotypes and median vectors (black dots) are shown above branches.

Highly polymorphic Internal Transcribed Spacer sequences

In total, 503 transformed bacterial colonies were sequenced (8 colonies per taxon on average). The total length of the ITS alignment was 717 bp, including one single-nucleotide insertion in the outgroup (*R. odoratus*), one in *R. idaeus*, and a two-nucleotide length polymorphism in a poly-C region within the ingroup. The alignment included 30 bp of 18S rDNA, 255 bp of ITS1, 164 bp of 5.8S rDNA, 211 bp of ITS2 and 57 bp of 26S rDNA. CG content varied between 52.0 and 57.1 %, and the free energy of RNA structure between -252.0 and -202.5 kcal.mol^{-1} at 37 °C (see online version of the article).

As all sequences formed two distinct clusters according to the analysis of CG content and RNA structure stability, sequences exhibiting CG content lower than 55.0 % and free energy higher than approximately -226 kcal.mol^{-1} were considered as pseudogenes (n = 23). All pseudogenes exhibited 5 to 15 mutated positions within the 5.8S rDNA region, whereas only 38 out of 480 non-pseudogenous sequences were mutated in one or exceptionally in two or three positions within this region. Moreover, all pseudogene sequences formed significantly distinct clusters in MP analysis (Supplementary fig 1). Excluding pseudogenes, 186 positions (25.8 %) were polymorphic in the alignment, but pairwise sequence similarity was 98.7 %, since 108 single-nucleotide polymorphisms were colony-specific, i.e. present in only one colony in the dataset, and other 49 polymorphisms were present only in two to six colonies in the total dataset. Neglecting

these polymorphisms as random uninformative mutations (except for several species-characteristic SNPs), the number of variable sites was lowered to 91 (12.7 %) including the outgroup *R. odoratus*, or 60 (8.4 %) considering ingroups only (see the genbank sequences or online version of the article).

BspLI (NlaIV) was used for digestion of cloned PCR fragments, as it cleaves the PCR product of *R. caesius* in two sites, that of *R.* sect. *Rubus* in a single position, while not cleaving ribotypes of *R. idaeus*. Sixteen individuals of fifteen species, which are known to be hybrids between two of the mentioned taxa, were selected and 538 colonies were analyzed (34 colonies per individual on average). Eight individuals showed significant reduction of one of the parental ribotypes, in seven cases the proportion of parental sequences did not differ significantly from expectation (Table 1). In *R. dollnensis* the proportion was strongly biased (8:36), but deviation from expectation could not be tested since the origin of this species turned out to be unclear. In all cases both expected parental ribotypes and no unexpected restriction products were observed.

Table 1: Proportion of parental ITS ribotypes in hybridogenous accessions based on PCR-RFLP. Asterisks indicate significance of deviation from expected proportion (*** P<0.001; ** P<0.01; * P<0.05; ns P>0.05; *P<P0 indicates significant deviation only in one-side test).

	Number of colonies						
Accession	**Sexual parent ribotype**	**Apo-parent ribotype**	**Sum**	**Proportion of sexual's ribotype**	**Proportion of apo's ribotype**	**Expected proportion (sexual's ribotype)**	**Significant deviation**
R. scissoides[a]	2	26	28	0.071	0.929	0.5	***
R. dollnensis	8	36	44	0.182	0.818	NA	NA
R. kuleszae (N Moravia)	7	35	42	0.167	0.833	0.4	***
R. albifrons ined.	7	24	31	0.226	0.774	0.5	***
R. wahlbergii	8	35	43	0.186	0.814	0.4	***
R. nessensis	11	35	46	0.239	0.761	0.5	***
R. grossus agg.	6	24	30	0.200	0.800	0.4	*
R. fasciculatus	10	28	38	0.263	0.737	0.5	**
R. albocarpaticus ined.	12	32	44	0.273	0.727	0.4	ns (*P<P0)
R. grossus agg.	5	11	16	0.313	0.688	0.4	ns
R. subditivus ined.	12	13	25	0.480	0.520	0.5	ns
R. aff. *wahlbergii*	3	4	7	0.429	0.571	0.4	ns
R. kuleszae (c Moravia)	22	26	48	0.458	0.542	0.4	ns
R. grossus	19	21	40	0.475	0.525	0.4	ns
R. orthostachys	19	9	28	0.679	0.321	0.5	ns (*P>P0)
R. franconicus	24	4	28	0.857	0.143	0.5	***

[a] In Kurtto *et al.* (2010) incorectly named as R. scissus W.C.R.Watson (see Weber 2013).

The star contraction algorithm yielded 59 groups of similar ribotypes, which formed 7 separated clusters when analyzed by the maximum parsimony approach in NETWORK (Figure 2). Ribotype position within each cluster was dependent on algorithm parameters, but in all cases the clustering into 7 groups and constant assignment of every sequence to its respective cluster was observed when using both the MP (Figure 2; several approaches in various software used) and BI methods (Supplementary figure

2). Ribotypes of each diploid taxon and tetraploid *R. caesius* were confined to a single cluster, except for *R. bollei*, which contained two different types of ITS sequences – one close to *R. ulmifolius* (i.e. *ulmifolius* ITS ribotype group) and the second forming a distinct branch within the *glandulosi* cluster together with the geographically nearby *R. palmensis* (Figure 2). The s*uberecti* ITS cluster was formed mainly by ribotypes of *R.* subsect. *Rubus* (former *Suberecti*), but contained no diploid species. All ribotypes found in the cultivated tetraploid hybrid 'Thornfree' – probably a descendant of North American diploid representative(s) of this subsection – also clustered with this group. Ribotypes clustering with two or three groups were found in almost all natural polyploid taxa (Table 2). Tetraploid representatives of ser. *Glandulosi*, both from Europe and Transcaucasia, bore only *glandulosi* ribotypes, but two sequences *(Gla_c04*, *Gla_c05*) detected in *R.* ser. *Glandulosi* and one in *R. schleicheri* (*Schl_c05*) were placed at the base of the *caesius* branch (Figure 2). Few ribotypes could not be assigned unambiguously to any of the clusters, but were constantly placed close to them. These were composed of different sequence motifs and could be considered products of multiple recombinations (see online version of the article).

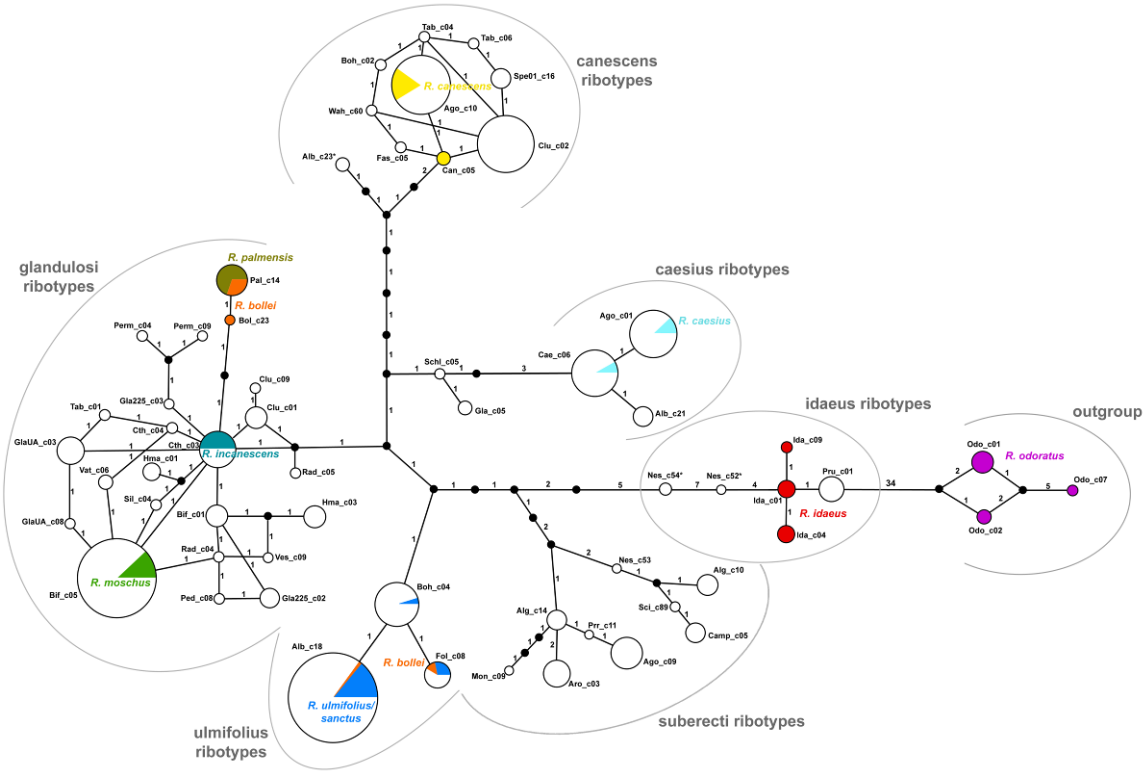


Figure 2: Median-joining network of cloned ITS sequences: each circle denote a distinct ribotype or group of similar ribotypes (circle size corresponds to the number of sequenced bacterial colonies), colonies from diploid taxa and *R. caesius* are plotted in color whereas other colonies are white. The number of mutations between ribotypes or median vectors (black dots) is shown above branches, possible recombinant ribotypes marked with an asterisk.

Table 2: Assignment of cloned ITS ribotypes of each taxon (diploid species in bold) – number of colonies clustering to respective ITS ribotype groups as defined in Figure 2 and Supplementary figure 2, number of detected pseudogenes and recombinant sequences. Intrageneric taxonomical position is indicated by section and series; note that all species are classified in subgen. *Rubus*, except for *R. idaeus* (subgen. *Idaeobatus*). For geographical origin of the individuals see Supplementary table 1.

			ITS ribotype group							
Section	**Series**	**Species (abbreviation)**	**idaeus**	**caesius**	**suberecti**	**glandulosi**	**canescens**	**ulmifolius**	**Pseudogenes**	**Recombinants**
–	–	***R. idaeus* (Ida)**	5							
Caesii	–	*R. caesius* (Cae)		6						
Rubus	*Anisacanthi*	*R. conothyrsoides* (Cth)			1	4		4		1
	Canescentes	***R. canescens* (Can)**					8			
	Discolores	*R. bifrons* (Bif)				3		3	2	
		R. bohemicola (Boh)			2		7	1		2
		R. crispomarginatus (Cri)					1	4	1	
		R. elatior (Ela)					3		4	1
		R. elegantispinosus (Ele)				8				
		R. grabowskii (Gra)			2		4		1	1
		R. montanus (Mon)			2			1	6	1
		R. praecox (Pra)					4	5		
		***R. ulmifolius/sanctus* (Ulm)**						15		
	Glandulosi	***R. moschus* (Mos)**				8				
		R. pedemontanus (Ped)				6		1		
		R. platyphyllus (Pla)				8				
		R. ser. *Glandulosi* (Gla)				13				
	Grandifolii	*R. palmensis* (Pal)				7				
	Hystrix	*R. schleicheri* (Schl)				5	4			
	Micantes	*R. clusii* (Clu)				6	4			
		R. permutabilis ined. (Perm)			1	6	4			
		R. tabanimontanus (Tab)				4	5			1
		R. vratnensis (Vra)			2	5	2			
	Mucronati	*R. hypomalacus* (Hma)				5		1		
	Nessenses	*R. nessensis* (Nes)	1		1					3
		R. scissoides (Sci)	1		7					
	Pallidi	*R. foliosus* (Fol)				6		3		
	Radula	***R. incanescens* (Inc)**				7				
		R. radula (Rad)				6		2		
		R. vatavensis ined. (Vat)				2		3	3	1
	Rhamnifolii	***R. bollei* (Bol)**				4		2		
		R. gracilis (Gra)				2		4		1
		R. rhamnifolius (Rha)						8	2	
	Silvatici	*R. silvaticus* (Sil)			1	6		3		
		R. wimmerianus (Wim)				5		3		

	Sprengeliani	*R. capricollensis* (Cap)				1		4	1	
		R. sprengelii (Spr)				1				
	Rubus	*R. constrictus* (Con)			5		2	1		
		R. divaricatus (Div)			1			4	2	
		R. perrobustus (Prr)			5		1			
		R. plicatus (Pli)			1					
		R. sulcatus (Sul)			7			1		
	Vestiti	*R. pyramidalis* (Pyr)			1	6		2		
		R. vestitus (Ves)				1		9		
Corylifolii	– (subsect. *Subidaeus*)	*R. pruinosus* (Pru)	5	1			2			
	Hystricopses	*R. dollnensis* (Dol)		1			7		1	1
	Sepincola	*R. franconicus* (Fra)		9			5			
	Subcanescentes	*R. fasciculatus* (Fas)					4	4		
	Suberectigeni	*R. orthostachys* (Orth)		7			1		1	1
	Subsilvatici	*R. camptostachys* (Camp)		6	1					1
	Subthyrsoidei	*R. albocarpaticus* ined. (Alb)		1			1	2		2
		R. albifrons ined. (Aro)			1			7		
		R. subditivus ined. (Ago)		5	1		1			3
		R. grossus (Gro)		5				3		2
		R. grossus agg. sp. 1 (Spe01)					4			
		R. grossus agg. sp. 2 (Spe02)		2				2		
		R. kuleszae (Kul)		3			2	3		3
		R. aff. *wahlbergii* (Pwa)		1				2		3
		R. wahlbergii (Wah)		1			3			1
– (hybrid)	– (hybrid)	*R. canescens* × *crispomarginatus* (CxC)					2	2		
		R. cv. Thornfree (Alg)			5					
		R. ulmifolius × *caesius* (UxC)		1				5		2
		R. caesius × *?* (Hyb)		5			3			
sum			12	54	47	135	84	114	24	31

Discussion

Hybridization is believed to play an important role in plant evolution and speciation (Rieseberg, 1995; Nolte and Tautz, 2010). Especially in many apomictic complexes it is responsible for generating very high morphological and allelic diversity, which can be fixed and preserved due to combination of asexuality and polyploidy (Asker and Jerling 1992; Hörandl and Paun 2007). European brambles seem to represent an extreme case of such a highly diverse hybridogenous complex, since very few diploid taxa are known while at the same time many hundreds of apomictic species are recognized (Kurtto *et al.* 2010). Moreover, *Rubus* is characterized by a highly variable reproductive mode, varying from obligate sexuality to obligate apomixis, and represents a good model for studying the importance of asexuality in plant evolution. In this study we provide the first molecular data covering the whole taxonomic complexity of *Rubus* subgen. *Rubus* in Europe on a large geographic scale. The two molecular markers (chloroplast DNA and ITS of nuclear rDNA) reveal very different levels of polymorphism, which might

be explained by different processes of *Rubus* evolution, but which together point to a restricted species-pool which led to the origin of this apomictic complex.

Table 3: Presence of cp haplotypes and ribotypes in higher taxonomical units (sections and series; *R. palmensis, R. bollei, R. incanescens* and unclear primary hybrids excluded). For more details see Supplementary table 1.

Section/subsection	Series	No. of species /samples (cpDNA)	Detected haplotypes (no. of species)	No. of species/colonies (ITS)	Detected ribotypes (no. of colonies)
Rubus/Rubus	*Rubus*	14/16	Suber (8) Ulm1 (4) Can1(1) Can2 (1)	5/28	Suber (19) Ulm (6) Can (3)
Rubus/Rubus	*Nessenses*	4/11	Suber (3) Ulm1 (1)	2/10	Suber (8) Ida (2)
Rubus/Hiemales	*Discolores*	35/82	Can1 (19) Ulm1 (8) Ulm2 (4) Can2 (4)	9/65	Ulm (29) Can (19) Gla (11) Suber (6)
Rubus/Hiemales	*Rhamnifolii*	8/9	Ulm1 (6) Can1 (1) Can2 (1)	2/14	Ulm (12) Gla (2)
Rubus/Hiemales	*Silvatici*	14/16	Gla1 (3) Suber (1) Ulm1 (9) Ulm2 (1)	2/18	Gla (11) Ulm (3) Suber (1)
Rubus/Hiemales	*Sprengeliani*	2/3	Suber (1) Ulm1 (1)	2/6	Ulm (4) Gla (2)
Rubus/Hiemales	*Vestiti*	6/6	Gla1 (3) Gla2 (1) Ulm1 (1) Ulm2 (1)	2/19	Ulm (11) Gla (7) Suber (1)
Rubus/Hiemales	*Micantes*	11/12	Can1 (1) Can2 (1) Gla1 (8) Gla2 (1) Ulm2 (1)	4/39	Gla (21) Can (15) Suber (3)
Rubus/Hiemales	*Mucronati*	1/1	Ulm1 (1)	1/6	Gla (5) Ulm (1)
Rubus/Hiemales	*Anisacanthi*	2/2	Gla1 (1) Ulm1 (1)	1/9	Gla (4) Ulm (4) Suber (1)
Rubus/Hiemales	*Radula*	6/9	Gla1 (2) Gla2 (1) Ulm1 (1) Ulm2 (2)	2/13	Gla (8) Ulm (5)

Rubus/Hiemales	*Pallidi*	3/3	Gla1 (1) Gla2 (1) Ulm2 (1)	1/9	Gla (6) Ulm (3)
Rubus/Hiemales	*Hystrix*	4/4	Gla1 (3) Gla3 (1)	1/9	Gla (5) Can (4)
Rubus/Hiemales	*Glandulosi*	12/35	Gla1 (7) Gla3 (2) Cau (2) Gla2 (1)	4/28	Gla (27) Ulm (1)
Corylifolii/Subidaeus	–	1/1	Cae1 (1)	1/8	Ida (5) Can (2) Cae (1)
Corylifolii/Sepincola	*Suberectigeni*	1/1	Cae1 (1)	1/8	Cae (7) Can (1)
Corylifolii/Sepincola	*Sepincola*	2/2	Cae1 (2)		
Corylifolii/Sepincola	*Subthyrsoidei*	10/19	Can1 (6) Can2 (2) Cae1 (1) Cae2 (1)	8/48	Cae (19) Ulm (17) Can (11) Suber (1)
Corylifolii/Sepincola	*Subsilvatici*	2/2	Cae1 (2)	1/7	Cae (6) Suber (1)
Corylifolii/Sepincola	*Subcanescentes*	4/5	Cae1 (3) Cae2 (1)	2/16	Ulm (11) Can (4) Suber (1)
Corylifolii/Sepincola	*Subradulae*	2/2	Cae1 (1) Cae2 (1)	-	-
Corylifolii/Sepincola	*Hystricopses*	1/2	Cae1 (1)	1/8	Can (7) Cae (1)
Caesii	–	1/7	Cae1 (1) Cae3 (1)	1/6	Cae (6)

Limited homogenization preserves high variability of ITS sequences

The internal transcribed spacer of ribosomal DNA (ITS) is a widely used marker for phylogenetic studies at the generic or infrageneric levels (reviewed by Álvarez and Wendel 2003). Nevertheless, employment of this marker can lead to serious errors if used in highly reticulate systems because of various processes of intergenic sequence homogenization in a genome, so called concerted evolution (Wendel *et al.* 1995). Considering the influences of hybridization and apomixis on *Rubus* evolution, it was necessary to evaluate the extent of this process. We thus chose hybridogenous accessions between distantly related taxa, such as *R.* ser. *Subthyrsoidei* (hybridogenous taxa from *R.* ser. *Discolores* and *R. caesius*) and ser. *Nessenses* (hybridogenous taxa probably from *R. idaeus* and *R.* ser. *Rubus*), which were fortunately distinguishable by a single restriction enzyme (BspLI). In seven individuals no significant reduction in either of the ribotypes occurred, nine accessions were partially homogenized as the observed ratio of parental ribotypes differed significantly from expected ratios (0.5 for tetraploids, 0.4 for pentaploids; Table 1). Nevertheless, in all 16 individuals both

parental ribotypes were detected, indicating only incomplete homogenization and slow concerted evolution. Although it was not the main aim of this study, it seems probable that different rates of homogenization can be observed even within a single apomictic line as suggested by two individuals of pentaploid *R. kuleszae* (Table 1) which showed different proportions of parental ribotypes. But confirmation of this hypothesis requires more cloning and genotyping data.

Homogenization, as inferred from unequal proportion of parental ribotypes, was detected in several other hybrid taxa. In recent hybrids of *Tragopogon* L. species, Kovarik *et al.* (2005) detected concerted evolution of ITS sequences which is reflected in reduction of one of the parental ribotypes during the 50 years since initial hybridization. A similar pattern was observed in allopolyploid *Gossypium* L. species (Wendel *et al.* 1995), some tetraploid *Paeonia* L. species (Sang *et al.* 1995), or in the parthenogenetic hybrid lizard *Heteronotia binoei* Gray (Hillis *et al.* 1991). Consequently, an absence of intragenomic nrDNA sequence variability was considered to be a general rule.

In contrast, more recent reports have revealed additive intra-individual variation in nrDNA, indicating preserved parental sequences in many hybrids or hybridogenous taxa. This information proved to be valuable for evolutionary inferences in *Paeonia* L. (Sang *et al.* 1995), *Rosa* L. (Ritz *et al.* 2005), *Sidalcea* A. Grey (Andreasen and Baldwin 2003), *Armeria* Willd. (Fuertes Aguilar and Nieto Feliner 2002), *Bursera* Jacq. ex L. (Weeks and Simpson 2004), *Helianthus* L. (Timme *et al.* 2007) or in the apomictic genera *Hieracium* L. (Fehrer *et al.* 2009), *Taraxacum* Weber ex F.H. Wigg. (Záveská Drábková *et al.* 2009), *Amelanchier* Medik. (Campbell *et al.* 1997) and *Boechera* Á. Löve & D. Löve (Koch *et al.* 2003), among others.

Beside orthologous ribotypes originating in sexual ancestors, part of the intra-individual ITS variability observed in this study could be explained by ITS paralogs, which represent the result of independent evolution of two or more ribotypes in a single phylogenetic lineage. As has been shown in several plant taxa, more functional sequences can coexist in a genome when concerted evolution is slower than speciation (Suh *et al.* 1993; Dubcovsky and Dvořák 1995; Buckler *et al.* 1997). If some of them escape functional constraints, they may become non-functional, i.e. pseudogenes (Buckler and Holtsford 1996; Buckler *et al.* 1997; Harpke and Peterson 2006). Here we have provided evidence for both functional ITS paralogs and pseudogenes, the latter being clearly distinguished by typical characteristics (summarized by Bailey *et al.* 2003) including low stability of secondary RNA structure, lower CG content, high mutation rate in an otherwise conservative 5.8S region as well as characteristic genetic distance variation between pseudogenes and functional ribotypes (Supplementary fig 1). Considering that pseudogenization via mutation accumulation should be a random process (i.e. Muller's ratchet; Muller 1964), the identification of three pseudogene clusters (Supplementary figure 1) implies that there have been 3 pseudogenization events in the taxa studied here. Pseudogenes were relatively rare considering all data together (4.6 %), although one accession of *R. montanus* Lej. was characterized by high levels (60%) of non-functional ribotypes. All pseudogenes were detected in polyploid accessions, especially in apomictic species of ser. *Discolores* and its descendants. Only functional ITS ribotypes were found in sexual diploids indicating that (allo)polyploidy and possibly also apomixis may be the factors triggering and preserving pseudogenes in an evolutionary lineage. Even after exclusion of pseudogenes, very high intra-individual

variability remained; the majority of which was caused by random single-nucleotide mutations.

Besides hybridization and mutation, the third factor underlying ITS sequence variability could be intergenic recombination. The majority of detected recombinant sequences contained apparent parental motifs in two blocks, indicating a single recombination event. Furthermore, several sequences were difficult to distinguish unambiguously as recombinants because the parental motives were mixed along the sequence in several blocks and could have therefore arisen via multiple recombinations. These ribotypes clustered more or less separately in phylogenetic analyses (Figure 2). Four sequences were obvious pseudorecombinants, i.e. chimeric sequence between functional ribotype and pseudogene. Whether the recombinats are of natural origin or rather represent PCR artifacts cannot be determined from our data, but chimeric ITS or ETS sequences have been detected several times before (Campbell *et al.* 1997; Barkman and Simpson 2002; Koch *et al.* 2003; Timme *et al.* 2007) and in some cases they may come to dominate the genome via concerted evolution (Álvarez and Wendel 2003).

Rubus polyploids have arisen multiple times mainly from six ancestors

European brambles (*Rubus* subgen. *Rubus*; blackberries in American English) represent a large agamic complex, i.e. most species are polyploids and reproduce asexualy through seeds (Weber 1996). Years of intensive batological research have revealed only eight diploid (and obligately sexual) species in Europe and adjacent regions, including *R. bollei* Focke (Gustafsson 1942), *R. palmensis* A.Hansen and *R. serrae* Soldano from Macaronesia (all measured by flow-cytometry; unpublished data), and *R. moschus* Juz. endemic to Transcaucasia (possibly synonymous to *R. caucasicus* Focke, the nomenclature is unresolved; Krahulcová and Holub 1997). Despite the dominance of asexuality, brambles show a very high variability in morphology (Nybom 1998), genetic markers (Clark *et al.* 2013; Šarhanová 2014; present study and unpublished SSR data) and ploidy (Krahulcová *et al.* 2013; Krahulcová and Holub 1997). The main evolutionary force generating such high variability seems to be hybridization, as has been shown in *Rubus* based upon numerous experimental crossing experiments and a few studies on natural populations (Lidforss 1914; Nybom 1995; Clark and Jasieniuk 2012; Šarhanová 2014).

Focke (1910) and later Gustafsson (1942) postulated the occurrence of so called primary species, which could represent the sexual ancestors of all polyploid taxa, and which furthermore encompass the morphological variation seen within European brambles. Although they were unsure about several taxa, their observations are in remarkable agreement with the data shown here. According to our phylogenetic analysis, all detected ITS ribotypes and chloroplast haplotypes distinguish six main clusters within the subgenus *Rubus* (Figures 1 and 2). The suggested extant ancestral diploids *R. ulmifolius*, *R. moschus* and *R. canescens* clearly define three of them; the fourth cluster contains only sequences of the other putative ancestor – tetraploid *R. caesius* – and most accessions belonging to sect. *Corylifolii*. The fifth group contains no diploid European bramble, but is defined by polyploid species of subsect. *Rubus* (former *Suberecti*), closely related to several North-American diploids. While none of these NA diploid species were analysed here, the obtained ribotypes of the *suberecti* cluster were identical to sequences of American *R. allegheniensis* (Alice and Campbell 1999) and a tetraploid putative hybridogenous descendant of this (or related) species,

frequently cultivated in Europe nowadays under name "American hybrid ('Thornfree')". Also, the *Suber* haplotype matched the sequence of *R. allegheniensis* by Fazekas *et al.* (2008). Together, these data imply that i) diploid species of subsect. *Rubus* occurred in Europe in the past and is extinct (or unknown) now, or ii) European polyploid accessions of subsect. *Rubus* arose in North America and migrated from there. The second option, however, seems much less plausible considering differences between American and European brambles (see e.g. Davis 1990) and hence natural intercontinental dispersal is likely not an evolutionary force in *Rubus*. Finally, the sixth ITS cluster is characterized by the unrelated raspberry *R. idaeus*, which is classified in the subgenus *Idaeobatus*. Cp haplotypes of this group are not shared with any stabilized apomictic accession from our sample-set, but raspberry ITS sequences were identified in *R. pruinosus* and species of ser. *Nessenses* (Table 2), indicating that *R. idaeus* shares a common ancestor with these taxa.

A brief look at phylogenetic networks from chloroplast and ITS data therefore implies that all European brambles are descendants of just six ancestral gene pools. Nevertheless, several ambiguities remain. One of them is regarding involvement of the remaining extant diploid sexuals. *Rubus incanescens*, hypothesized by Focke (1910) to be one of the ancestors, could have participated in hybridizations considering that its ribotypes cluster very closely to *R. moschus*. On the other hand, it possesses a very distinct cp haplotype not detected in any other species, and hence its role in the evolution of polyploid brambles would have been only (or predominantly) as a staminate parent. How probable this hypothesis can be is impossible to say without further studies. The species is relatively rare and has a narrow distribution area (southern France with adjacent regions, and Algiers; Focke 1910; Gustafsson 1942; Kurtto *et al.* 2010) and morphologically resembles a hybrid between ser. *Glandulosi* and ser. *Discolores* (sometimes therefore classified in ser. *Radula*; Kurtto *et al.* 2010), but it is apparently a taxonomically isolated species of probable ancient origin considering its specific cp haplotype, ploidy and morphology. The hypothesis of its involvement as staminate parent is nevertheless supported by the fact that our specimens (kindly provided by L. Belhacene) were diploid and triploid (measured by flow-cytometry, unpublished data) having similar appearance but different haplotypes, indicating at least occasional hybridization with other taxa.

Conversely, two studied diploid Macaronesian taxa, *R. bollei* and *R. palmensis*, share haplotypes with *R. ulmifolius* and many polyploid taxa, but their ribotypes form a separate branch within the *glandulosi* ITS cluster, hence it is improbable that they could be the parental species. The same seems likely for *R. serrae* from Madeira, since the only analyzed sample of this species (although of hybrid origin) bears unique cp haplotype (*Mad1* in Figure 1). Also the last Macaronesian species *R. hochstetterorum* Seub. (Matzke-Hajek 2001) is characterized by a distinct *matK* sequence (published by Schaefer *et al.* 2011). On the other hand, several morphologically diverse polyploid accessions from western Transcaucasia bear a unique cp haplotype (*Cau*) not shared with any known diploid species (Supplementary table 1). This could imply involvement of other taxa in the evolution of Caucasian polyploid complexes.

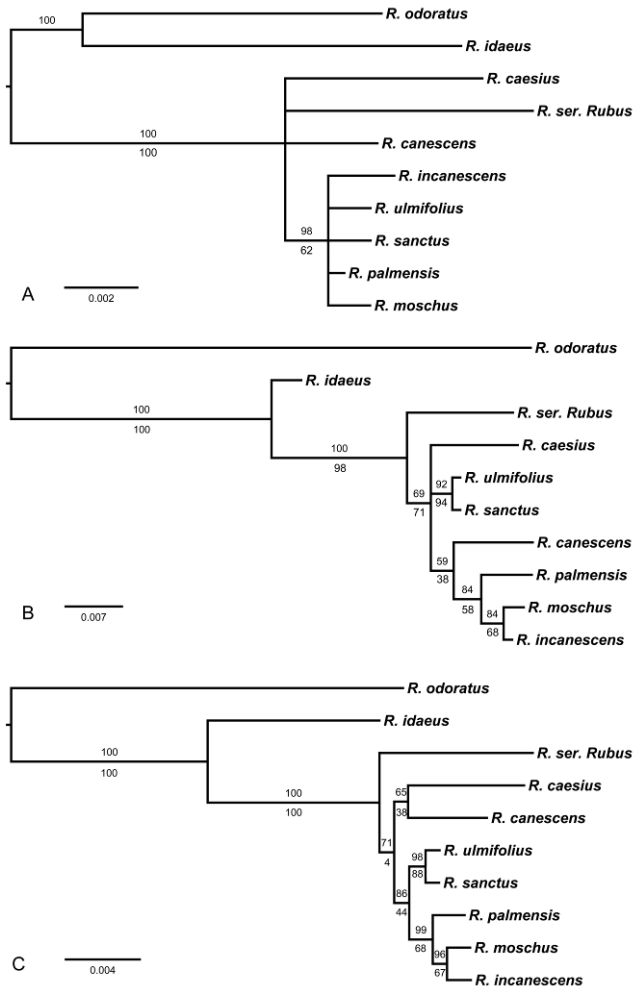


Figure 3: Phylogeny trees of basal species and other diploids based on bayesian analysis on cpDNA (A), ITS (B) and combination of both (C), rooted with outgroup *R. odoratus*; posterior probabilities shown above branches. Topology of the trees is same as for maximum likelihood analysis (bootstrap values below branches). *R.* ser. *Rubus* is represented by *R. plicatus*, *R. bollei* not included due to probable hybrid origin (excluding ulmifolius-like ribotypes same as *R. palmensis*).

A further point is the *suberecti* ITS cluster (Figure 2, Supplementary figs 1 and 2), which is clearly subdivided into two groups, both of which are found in the tetraploid American artificial hybrid 'Thornfree' as well as in several European accessions. Whether this subdivision represents two ancestral species, or rather intraspecific evolution of different paralogs, cannot be answered without extensive sampling of American brambles. Though, the *Suber* cp haplotype, which characterizes *R.* subsect. *Rubus*, does not show any variation either in our data-set (Figure 1) or when compared to the *matK* sequences of Fazekas *et al.* (2008), thus pointing to the latter hypothesis.

The last question arising from the ITS phylogenetic analysis is how and when glandulous brambles evolved. Although all accessions from *R.* ser. *Glandulosi* and related taxa were represented in the *glandulosi* ITS cluster, and most of them also share the same cp haplotype with the morphologically close diploid *R. moschus*, two ITS ribotypes of *R.* ser. *Glandulosi (Gla_c04*, *Gla_c05*) and one of *R. schleicheri* (*Schl_c05*) were placed close to the *caesius* ribotype cluster in all statistical analyses (Figure 2 and Supplementary figs 1 and 2). Both morphologically and ecologically, ser. *Glandulosi* and *R. caesius* form well distinguishable taxa with no signs of recent common origin (Tomaszewski *et al.* 2013). The exclusively tetraploid *R. caesius* is a widely distributed (Kurtto *et al.* 2010; Krahulcová *et al.* 2013) predominantly sexual species with regular meiosis (Gustafsson 1942; Christen 1950; Dowrick 1961) whose diploid ancestor is unknown and probably extinct. Our ITS data suggest (1) that this diploid probably existed shortly after diversification from other primary species, (2) that it took part in

the formation of polyploid series *Glandulosi* and (3), via autopolyploidization, the tetraploid *R. caesius* which independently evolved on the molecular (Figures 1 and 2), morphological and ecological levels.

Several apomictic lineages may have a Pleistocene origin

Based upon the relationships between some European and North American brambles, the apomictic complex of *R.* subgen. *Rubus* was suggested to be of Tertiary origin (Asker and Jerling 1992). This hypothesis cannot be entirely rejected based on our data, although considering the star-like topology of the phylograms containing primary bramble species (Figure 3), obvious differences in mutational rates between lineages and critical lack of suitable paleobotanical data, all methods of molecular dating are hence impractical (Bromham and Penny 2003). Nonetheless, clear geographic patterns were detected in two parental taxa and their descendants, which may help with approximating relative dates of hybridizations and polyploidizations. First, the aggregate species *R. ulmifolius–R. sanctus* (species concept adopted from Monasterio-Huelin and Weber 1996) bears five different cp haplotypes across the studied area. Two of them (*Ulm1*, *Ulm2*) were detected in the Macaronesian and European *R. ulmifolius* with the eastern limit in the Western Balkans, and in polyploid accessions throughout the studied area. The eastern diploid type (*R. sanctus*) has one haplotype in the Balkan Peninsula (*San1;* the *Ulm1* haplotype occurs only within hybrid zone in the Western Balkan) and two haplotypes in Transcaucasia (*San2* only in Armenia, *San3* in both Armenia and Georgia). None of these *San* haplotypes was detected in any polyploid brambles, suggesting that only the western type of *R. ulmifolius* participated in polyploid formation. Furthermore this did not necessarily occur only in Western Europe, since both the *Ulm1* and *Ulm2* haplotypes were also detected in Caucasian polyploids. These haplotypes display only one or no autapomophic polymorphisms, an observation implying an ancestral state (Figure 1), and thus at least some of the polyploids may be older than eastern *R. sanctus*. This hypothesis is further supported by the presence of *suberecti* ITS ribotypes in series *Discolores* (Table 2), since these ribotypes were not found in any diploid species. The second taxon, *R. moschus*, today endemic to Transcaucasia, shares a close morphological and molecular relationship with European polyploid ser. *Glandulosi*. On the other hand, the related polyploids from Caucasus bear a completely different haplotype (*Cau*), indicating that diploid *R. moschus* (or its close ancestor) occurred in Europe in the past and gave rise to glandulous polyploids there, together with the diploid ancestor of current *R. caesius*.

Since the haplotype distribution of the *R. ulmifolius–R. sanctus* group, as well as the occurence of *R. moschus*, correspond to known glacial refugia of thermophilous plants on the Iberian, Apennine and Balkan Peninsulas and regions of Colchis and Hyrcania (Nieto Feliner 2011; Akhani and Djamali 2010), it seems probable that distribution areas of these species were severely restricted and fragmented by Pleistocene climate fluctuations, leading to haplotype differentiation and star-like cp haplotype networks (Worth *et al.* 2011). The great climate changes characterizing the Quaternary could also be the main reason for extinction of diploid ancestors of *R.* subsect. *Rubus* and *R. caesius*, especially during the last deglaciation, as shown e.g. for *Picea critchfieldii* Jackson & Weng (Jackson and Weng 1999). If these assumptions are true, the first significant polyploidization events which generated the recent apomictic species of ser. *Glandulosi* and ser. *Discolores* must have occurred already before the last glacial maximum (LGM). Also in other genera, glacial cycles are believed to play an important role in formation and spread of apomictic polyploids due to changes in distribution

areas of sexual diploids, which increases chances for hybridization and selection for advantageous polyploid asexual genotypes for colonizing deglaciated regions (Hörandl 2009). Nevertheless, whether the phylogeographic patterns observed in European brambles originated in the last or earlier glacial periods can be only specified by additional research on fossil material, which is relatively accessible, but difficult and laborious to analyse in such a complex group (e.g. Tomlik-Wyremblewska 1995; DeVore and Pigg 2007; Bhandari *et al.* 2011).

Although at least some apo-species of subsect. *Rubus*, ser. *Glandulosi* and ser. *Discolores* seem to have a Pleistocene origin, cp haplotype ditribution maps (Figure 4) indicate that most apomictic lineages were formed only recently. The highest species diversity has accumulated in regions from the British Isles up to the central Europe (Kurtto *et al.* 2010), an area which was glaciated or significantly influenced by glaciation during the LGM and thus likely unsuitable for bramble survival (Ray and Adams 2001). Also, the diversity of polyploid species sharing cp haplotypes with *R. ulmifolius* or *R. canescens* corresponds significantly to the northern limit of these respective diploids (Figure 4a, b). A very similar pattern can be observed when comparing apomictic accessions sharing the *Gla1* haplotype to the distribution of the predominantly sexual tetraploid taxon of the ser. *Glandulosi* (since this is not a formally delimited taxon, its distribution was approximated by that of *R. hirtus agg*. sensu Kurtto *et al.* 2010; Figure 4c). Species diversity of *Cae* haplotypes is centered around the same area, obviously reflecting species density of the former haplotypes (Figure 4d). This implies that tetraploid sexuals of *R.* ser. *Glandulosi* and modern *R. caesius*, rather than their diploid ancestors, participated in formation of most allopolyploid lineages. On the other hand, the *Suber* haplotype is spread over a great part of the continent with no evident center of diversity (Figure 4e), which further supports the hypothesis of a pre-glacial origin of *R.* subsect. *Rubus*. Although slight bias due to uneven sampling and insufficient batological research in many areas of Eastern and Southern Europe cannot be ruled out as factors potentially contributing to the above mentioned geographical pattern, it can be hypothesized that *R. ulmifolius* spread from the western part of the Mediterranean along the Atlantic coast, whereas *R. canescens* and sexual *R.* ser. *Glandulosi* emerged from the Balkans, followed by post-glacial contact and hybridization in north-western continental Europe. The open landscape of the early Holocene in this area (Klerk 2002; Bos and Urz 2003) may have then become optimal for the establishment and spreading of apomictic lineages, a phenomenon which could be explained by a number of hypotheses (Rosenberg 1946; Asker and Jerling 1992; Hörandl 2009, to name but a few).

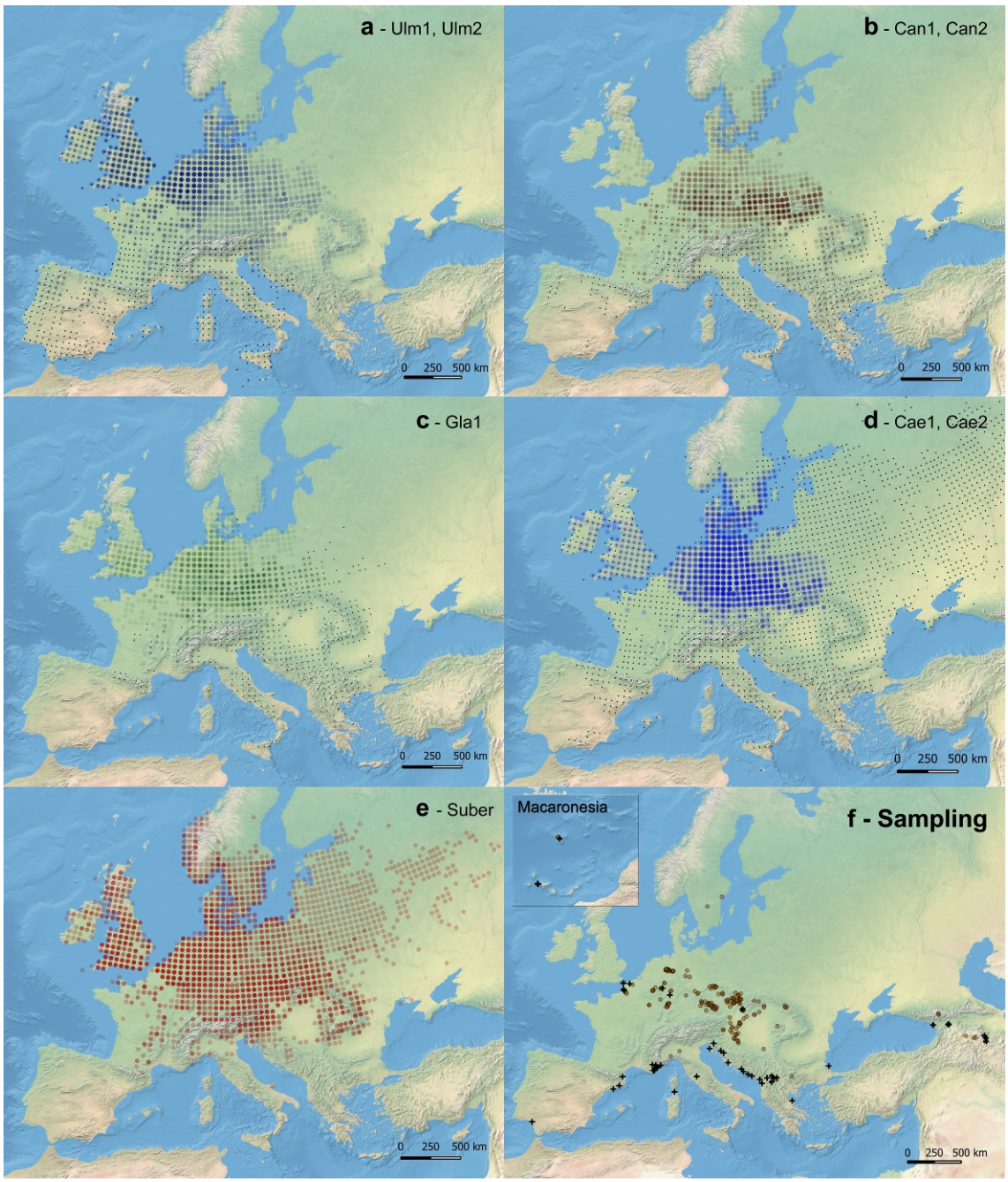


Figure 4: Distribution maps of haplotypes *Ulm1* and *Ulm2* (*a*; 34 species), *Can1* and *Can2* (*b*; 24 spp.), *Gla1* (*c*; 25 spp.), *Cae1* and *Cae2* (*d*; 13 spp.) and *Suber* (*e*; 12 spp.) compiled from data by Kurtto *et al.* (2010). Hybridogenous taxa shown as overlaying circles, i.e. darkness corresponds to the number of species bearing the haplotype and occuring in the mapping plot. Black dots shows current distribution of sexual taxa of the respective haplotype – *R. ulmifolius* (*a*), *R. canescens* (*b*), *R. hirtus* agg. (*c*) and *R. caesius* (*d*). Map *f* shows origin of the accessions used in this study – diploids as cross, polyploids as circles.

Unidirectional hybridization characterizes Rubus *hybridization*

Although our molecular data cannot be universally used for inferring origin of every hybrid taxon due to partial concerted evolution of ITS sequences and uniparental heritability of chloroplast markers, they shed light on the origin of several taxa (Tables 2 and 3). For example, while they confirm evolutionary relationships between polyploid

accessions of *R.* ser. *Discolores* and *R. ulmifolius* (cf. e.g. Tomaszewski *et al.* 2013), they also surprisingly implicate other basal species in the formation of this series, namely *R. moschus* (or its ancestor), extinct diploid(s) of *R.* ser. *Rubus*, and *R. canescens*, which even shares its haplotype with most triploid *Discolores* (Supplementary table 1). This finding is in accordance with former morphological observations (cf. Gustafsson 1939). On the other hand, most tetraploids of this series bear *Ulm* haplotypes, further supporting morphological observation of two evolutionary groups in the series (Trávníček and Zázvorka 2005). Both *R. ulmifolius* and *R. canescens* were frequently also involved in the formation of other taxa in addition to *R.* ser. *Discolores*, which is supported by shared branched trichomes on leaves of many taxa from *R.* sect. *Rubus* (Tomaszewski *et al.* 2013).

Our data further confirm ser. *Nessenses* as a hybridogenous group formed from members of *R.* ser. *Rubus* and *R. idaeus* (the hypothesis is further evidenced by morphological characters such as incompletely pinnate leaves, dark red drupelets and small prickles), the latter always being a staminate parent (Supplementary table 1). The whole sect. *Corylifolii* was confirmed to be hybridogenous from tetraploid *R. caesius* and members of *R.* sect. *Rubus* (Gustafsson 1942), since the vast majority of studied accessions contains ITS ribotypes of the *caesius* cluster (Table 2). In the case of pentaploid ser. *Subthyrsoidei*, all accessions bear *Can1* or *Can2* haplotypes and share several common traits with the triploid *Discolores* (Tomaszewski *et al.* 2013), pointing to the latter as being the pistillate parent. In contrast, all tetraploid accessions of sect. *Corylifolii* share haplotypes with *R. caesius*, as do both triploid and tetraploid primary hybrids between this species and *R. ulmifolius*. A different pattern was found in tetraploid ser. *Radula*, being hybridogens from tetraploid *Discolores* and ser. *Glandulosi*, and although most of the species share haplotype *Gla1* with the second parent (see also Šarhanová 2014), at least one (*R. radula*) bears the *Ulm1* haplotype, pointing to ser. *Discolores* being the pistillate parent (Supplementary table 1). A similar situation can be observed in ser. *Micantes* – *R. tabanimontanus*, which has the *Gla1* haplotype, whereas it is morphologically very similar to *R. gliviciensis* with the *Can1* haplotype.

Except for the last two series, all studied hybrid systems show an apparent non-randomness in parental roles. Triploid *Discolores* may be handicapped as a staminate parent due to very low pollen viability (Gustafsson 1942; Nybom 1988). In *R. caesius* the pollen viability is very high (Nybom 1985), but the pollen performance may favor it rather as a pistillate parent of most tetraploid *Corylifolii*. Slow growth of pollen tubes, incapability of fertilizing the egg cell or pollen competition have been suggested as explanations for the failure of some hybrid combinations in controlled crosses (Asker and Jerling 1992; Werlemark and Nybom 2003). These data also show that a generally assumed advantage of apomicts, whereby they benefit both from uniparental reproduction and preserved male function which enable pollination of sexual relatives (Hörandl 2006), is not a universally valid model for *Rubus*, because the more sexual taxon often serves as pollen donor, not to mention that physiological constrains seem to play the pivotal role in determining paternity in each hybridization.

Conclusions

Our data reveal two different levels of genetic diversity in European brambles. First, intra-individual polymorphism, represented by ITS data in our study, is very high mainly as a result of polyploidy, hybridization, asexuality and (in case of ITS) slow concerted evolution. Second, overall allelic diversity is extremelly low, despite the high number of studied species, as seen in both total cp haplotype diversity and differentiation of ITS ribotypes. While this low overall allelic variation may lead to an impression that such an evolutionary complex undergoes loss in adaptive potential at some point of its evolution, the ability to accumulate, preserve and eventually to also recombine high diversity in a single genome is a good argument for the opposite view on apomictic complexes. This view is further supported by the obvious evolutionary success of *Rubus* around the world, as it occupies very diverse habitats and many species have become widespread or even expansive/invasive. The same is also true for other apomictic genera, such as *Taraxacum*, *Hieracium* or *Ranunculus* L. (Asker and Jerling 1992; Hörandl and Paun 2007), making apomixis an intriguing phenomenon of plant evolution.

Specifically in brambles, apomixis has been found in several subgenera (subgen. *Malachobatus* (Focke) Focke – Amsellem *et al.* 2001; subgen. *Idaeobatus* – Pratt *et al.* 1958; apomeiotic initials detected in subgg. *Cylactis* (Raf.) Focke and *Chamaemorus* (Focke) Focke – Czapik 1983), and hence a genetic predisposition for apomixis may be much older than *R.* subgen. *Rubus*. Additionally, this reproduction mode is not restricted to European brambles, as shown by our preliminary flow-cytometric data indicating that apomixis and polyploidy are common among Caucasian accessions. Several works have also revealed widespread apomixis in eastern North American brambles (Einset 1951; Pratt and Einset 1955; Asker and Jerling 1992). These regions may therefore represent other hotspots of parallel reticulate evolution of the subgenus *Rubus*, re-connected only recently by gene-flow between indigenous and invasive or cultivated species (Clark and Jasienuik 2012). Past and recent evolutionary processes in non-European brambles still remain to be revealed and could add valuable information to our understanding of (not only) this evolutionary and ecologically successful group, which is necessary for further studies on genetics of apomixis in higher plants.

CHAPTER 3:

Origin of apomicts as a result of the sexual ancestor's phylogeography: a model case of European and Caucasian brambles (*Rubus*, Rosaceae)

Michal Sochor
Petra Šarhanová
Simon Pfanzelt
Bohumil Trávníček

Submitted to Molecular Ecology

Abstract

Apomixis (agamospermy) occurs in a variety of plant taxa of diverse phylogenetic origins and resulted in a high abundance and wide distribution of some groups, demonstrating its important role in the evolution of plants. When and where apomicts arose, why they are so evolutionary successful and whether their success is temporally persistent are long-standing questions in evolutionary biology. We used next-generation sequencing on ten nuclear microsatellite loci, Sanger sequencing on two plastid loci and ecological niche modelling to unravel phylogeographic patterns in the widely distributed diploid sexual bramble *Rubus ulmifolius* agg. and its allopolyploid apomictic descendants of series *Discolores* and related taxa. The sampling covered a significant part of their distribution ranges in Europe and adjacent regions. The data reveal strong, continental-scale genetic structuring within this bird-dispersed diploid. Geographical patterns of genetic diversity and ecological niche models indicate its survival mainly on the Iberian Peninsula and in Morocco during the last glacial maximum, as well as severe population bottlenecks in the Eastern Mediterranean and the Caucasus, whereas low allelic diversity in Northwestern Europe stems from post-glacial re-colonization from southern refugia. The distribution of alleles among ploidy levels indicates that the first allopolyploidization events occurred prior to the last glaciation and also reflects the recent gene flow from diploids to polyploids. Because areas with a high genotype diversity of polyploid apomicts mostly exhibit a low genetic diversity of the diploid, we hypothesize that genetic deterioration resulting from genetic bottlenecks and post-glacial recolonization in the sexual ancestor may affect its competitive abilities and enable the diversification and spread of apomicts.

Introduction

Apomictic/agamospermic plants (i.e., those reproducing asexually via seeds) represent an interesting model system for research on the evolutionary importance of sex (e.g., Hojsgaard and Hörandl 2015). They also have great agricultural potential (Kandemir and Saygili 2015). Nevertheless, the high complexity of apomixis-associated phenomena renders their study challenging. For instance, gametophytic apomixis (the most frequently studied type of apomixis; sporophytic apomixis will not be considered further) is almost consistently associated with, but probably not caused by, polyploidy and hybridity (Lovell *et al.* 2013). Apomictic developmental pathways are induced and regulated by multiple loci and possibly also by epigenetic mechanisms (Hand and Koltunow 2014) and may even be modulated by environmental factors (Evans and Knox 1969; Gounaris *et al.* 1991; Šarhanová *et al.* 2012). Additionally, most apomictic taxa maintain a normal sexual pathway. Such facultative apomicts are then able to generate high genotypic diversity in sympatric populations with other apomicts or related sexuals, thus having advantages of both asexuality (stabilization of advantageous genotypes) and sexuality (generation of new genotypes; Houliston and Chapman 2004; Majeský *et al.* 2015; van der Hulst *et al.* 2003). Furthermore, many taxa exhibit geographic parthenogenesis, i.e., they show different degrees of asexuality across their distribution ranges. Polyploid apomicts usually occur in higher latitudes/altitudes than their diploid sexual relatives and tend to occupy previously glaciated areas (Hörandl 2006). Geographic parthenogenesis may be caused mainly by advantages of polyploidy and by the more effective reproduction and dispersal of apomicts in harsh environments.

Nonetheless, it was observed also on a single ploidy level within a climatically relatively homogeneous region (Šarhanová *et al.* 2012) and is likely shaped by multiple co-acting factors, including niche diversification and competition between sexuals and apomicts (Hörandl 2006).

Brambles (*Rubus* subgenus *Rubus*) may serve as typical examples of plants exhibiting geographic parthenogenesis. In Europe and adjacent regions, the taxon consists of eight extant sexual diploids, few sexual tetraploid species and more than 750 recognized polyploid apomictic microspecies with varying degrees of residual sexuality (Kurtto *et al.* 2010; Sochor and Trávníček 2016; Šarhanová *et al.* 2012). Diploids are confined to the Mediterranean, Macaronesia or warm regions of Western Asia, and only two of them can be found occasionally in temperate parts of Central (*R. canescens* DC.) or Western Europe (*R. ulmifolius* Schott; Kurtto *et al.* 2010). In contrast, polyploid species diversity is concentrated in Western and Central Europe and the Southern Caucasus. In warmer regions (e.g., the Northern Mediterranean), polyploid apomicts occur rather rarely or are locally absent and usually do not form widespread stabilized lineages (i.e., agamospecies; Kurtto *et al.* 2010; the authors' pers. obs.).

The evolution of European and Caucasian brambles is highly reticulate, as it is driven by hybridization and polyploidization. The whole polyploid complex originated from only seven diploid ancestors, of which three are now extinct or unknown (Sochor *et al.* 2015; Sochor and Trávníček 2016). Extant polyploid apomicts are thus the result of multiple hybridization events between taxa of varying ploidy levels (Sochor *et al.* 2015; Nybom 1988; and pers. obs.). Taxonomically, the subgenus *Rubu*s is divided into several infrageneric ranks, such as sections and series, although most of the taxa seem artificial. One of the most widespread and morphologically rather distinct series is *Discolores*, which includes the diploid species aggregate *R. ulmifolius–R. sanctus* (for simplicity, hereafter referred to as *R. ulmifolius* agg.) and a number of triploid and tetraploid apomicts (Kurtto *et al.* 2010; Sochor and Trávníček 2016). *R. ulmifolius* agg. proved to be one of the diploid parents of these polyploids, and it was suggested that multiple polyploidization events occurred in different time periods, at least in Western Europe. Some polyploids originated likely before the last glacial maximum, as implied from the specific distribution patterns of plastid haplotypes, but the Holocene evolution of the group could not be easily ascertained (Sochor *et al.* 2015). Especially in the Balkan and Caucasian polyploid populations, the modern involvement of the diploid is unclear, as polyploids mostly exhibit different haplotypes in the respective area (Sochor *et al.* 2015, Sochor and Trávníček 2016).

In this study, we therefore focus on the widespread and morphologically well-characterized diploid *R. ulmifolius* agg. and its polyploid descendants. Employing the next-generation sequencing of ten microsatellite loci, the Sanger sequencing of two plastid loci and ecological niche modelling, we aim 1) to disentangle the phylogeography of *R. ulmifolius* agg. (i.e., to identify glacial refugia and post-glacial recolonization routes) and 2) to shed light on the origin and expansion of polyploid apomicts from the series *Discolores*. Such information is critical for our understanding of the evolution of apomictic complexes and may help explain the evolutionary importance of apomixis as well as probable reasons for geographical parthenogenesis.

Materials and methods

Plant material

Diploid specimens of *R. ulmifolius* agg. and polyploid accessions of ser. *Discolores* and *Discolores*-like taxa were sampled across Europe, Transcaucasia and Morocco. With minor exceptions (e.g., Ireland, Transcaspian Region), the entire distribution range of *R. ulmifolius* agg. was covered. The sampling area for the diploid species aggregate was divided into 11 regions (hereafter termed "populations"), each containing 9–10 individuals (Supplementary figure 1A; for a list of populations, see Table 1). Several samples from Israel, Turkey and Macaronesia were also added. Polyploids were divided into five regions according to general distributional patterns observed in *Rubus* (Kurtto *et al.* 2010, Supplementary figure 1B). Overall, 116 individuals of *R. ulmifolius* agg. were sampled for SSR sequencing and 82 for chloroplast (cp) DNA sequencing (70 samples for SSR and cp; 46 only for SSR; 12 only for cp). Sixty-one polyploid individuals were used for SSR sequencing (of which 53 were also used for cpDNA sequencing), covering 33 microspecies and 18 undescribed morphotypes (pers. obs.) from ser. *Discolores* or *Discolores*-like taxa. Another 51 polyploid individuals of diverse taxonomic affinity but that shared chloroplast haplotypes with *R. ulmifolius* agg. were added to complete the haplotype distribution patterns. *Rubus canescens*, *R. caesius* L., *R. moschus* Juz., *R. plicatus* Weihe et Nees and tetraploid members of series *Glandulosi* (12 individuals in total) were included as an "outgroup" for SSR analysis, representing other parental species of European brambles (Supplementary table 1). In taxa with unknown ploidy levels, ploidy was determined by the flow cytometry of fresh leaves with *Solanum lycopersicum* L. or *Zea mays* L. as internal standards, stained with propidium iodide (for details, see Šarhanová *et al.* 2012). Collected herbarium vouchers are deposited at the herbarium of Palacky University in Olomouc (OL).

Molecular methods

Genomic DNA was extracted from silica-gel-dried leaves following the CTAB (Cetyl Trimethyl Ammonium Bromide) protocol of Doyle and Doyle (1987). For cp haplotype determination, 993 bp of the *matK* intron and 461 bp of the *trn*L–*trn*F spacer were amplified with XFA and AST_R primers (Dunning and Savolainen 2010) and *e* and *f* primers (Taberlet *et al.* 1991), respectively, as described by Sochor *et al.* (2015). The PCR products were purified by precipitation with polyethylene glycol (10 % PEG 6000 and 1.25 M NaCl in the precipitation mixture) and sequenced by Macrogen Europe.

For SSR analysis, we used a multiplex and barcoding approach to amplify and sequence ten SSR loci from at least four linkage groups (Supplementary table 2; Castillo *et al.* 2010; Graham *et al.* 2004; Woodhead *et al.* 2008). Only SSR loci with a repeat unit length of at least 3 bp were selected. The 192 individual DNA samples that were used for SSR sequencing were divided into two sample sets, each containing 96 individuals. By appending 8-nucleotide barcodes to the 5' tail of both the forward and reverse primer sequences, we created tagged primers that were specific for each sample set and locus (40 tagged primers in total for 10 loci and two sample sets; the barcode being identical for F and R primers of one specific set and locus). Primers were ordered from Metabion International AG (Planegg/Steinkirchen, Germany). The web tool MULTIPLX 2.1 (Kaplinski *et al.* 2005) was used to define primer groupings within each of the two

sample sets in order to identify the optimal primer compatibility and to avoid undesired primer pairings. The tool was run with the default settings, and 'Calculating scores' was set to 'primer-primer any'. Two multiplex groups were suggested for each sample set. Multiplex PCR reactions were performed in 96-well microtiter plates for each of the multiplex groups using a Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany). The PCR conditions and primer list are given in Supplementary table 2. The PCR products were then pooled, and a total of 96 libraries were prepared for the paired-end sequencing of the SSR amplicons on an Illumina MiSeq at TraitGenetics GmbH (Gatersleben, Germany).

Molecular data analysis

cpDNA sequence editing, alignments and haplotype identification were performed in GENEIOUS (ver. 7.1.7., Biomatters, Auckland, New Zealand). Haplotype assignment and naming followed that of Sochor *et al.* (2015). A median-joining algorithm was used to create phylogenetic haplotype networks in NETWORK (ver. 4.6.1.2; Bandelt *et al.* 1999). GENEIOUS was also used to process the Illumina SSR sequence data. The reads were demultiplexed based on the barcodes that were specific for each sample set and locus (single mismatch allowed). All of the reads were mapped to a reference sequence specific for the respective locus, manually trimmed after the tenth nucleotide (from the 3' end) of each primer, and *de novo* assembled with strict algorithm parameters that were optimized for each locus separately (maximum mismatches per read 1 %, maximum gap size >150 bp, minimum overlap >40 bp). Contigs with the highest coverage (usually 10^2–10^3 reads per locus and individual sample) were then considered alleles. Contigs with coverage lower than 10 % of that of the contig with the highest coverage were always excluded as non-specific PCR products or polymerase/sequencing errors. In cases where a higher number of different "allelic" contigs than expected (based on ploidy level) were observed, all of these sequences were examined in an alignment with other sequences from the sample set, and apparently erroneous sequences were discarded. All of the resulting allele sequences were visually checked for possible errors, manually edited and named in order to enable further binary/codominant data analyses. Both plastid and nuclear SSR sequences were deposited in NCBI GenBank (accession numbers KU895556–KU895795).

A Mantel test for the isolation by distance hypothesis, Principal coordinates analysis (PCoA) based on a genetic distance matrix and population genetic summary statistics (allele numbers, heterozygosity, and F_{IS} according to Wright 1951) were performed in GENALEX (ver. 6.501; Peakall and Smouse 2012). F-statistics according to Weir and Cockerham (1984) and Fisher's exact probability test for genetic population differentiation were calculated in GENEPOP (ver. 4.0.11; Markov chain burn-in 10,000; 100 batches; 5000 iterations per batch; Raymond and Rousset 1995; Rousset 2008). For the detection of population structure in the diploid specimens, a Bayesian clustering algorithm was used as implemented in STRUCTURE (ver. 2.2; Pritchard *et al.* 2000) using a codominant data matrix. The algorithm parameters were as follows: burn-in 50,000; number of iterations 400,000; number of runs 10 for each K from 1 to 12; admixture model; no prior information was used. The most appropriate K was selected by computing Pr(X|K) and ΔK in STRUCTURE-SUM (ver. 2009; Ehrich 2006). The individuals' Q-values were averaged in CLUMPP (ver. 1.1.2; Jakobsson and Rosenberg 2007). The STRUCTURE results were displayed graphically by the software DISTRUCT (ver. 1.1; Rosenberg 2003). An analysis of allelic (*Hd*) and nucleotide diversity (*Pi*) was

performed with allelic sequences in DNASP (ver. 5.0; Librado and Rozas 2009). All of the maps were produced using QGIS (ver. 2.0.1; Open Source Geospatial Foundation Project, www.qgis.org).

Ecological niche modelling

In order to complement our molecular data, ecological niche modelling was performed using the maximum entropy approach as implemented in MAXENT (ver. 3.3.3k; Phillips *et al.* 2006). Current and past maps of habitat suitability of *R. ulmifolius* agg. were constructed based on 19 biologically relevant climatic variables compiled in the WorldClim database (Hijmans *et al.* 2005; http://www.worldclim.org). Out of these, six variables (bioclim 6, 7, 10, 11, 16 and 17) were excluded from the models due to high correlation with other variables (Pearson correlation coefficient >0.9; calculated in SDMTOOLBOX, ver. 1.1c; Brown 2014). Bioclimatic layers from the present and coordinates of 178 known localities (based on our observations or extracted from herbarium databases) served as training data. Background points (10,000) were selected only from areas of a maximum distance of 200 km from the presence points as extracted using the convex-hull algorithm in SDMTOOLBOX. Presence data were chosen and filtered through the same software in order to cover evenly the whole species distribution area with a minimum distance of 20 km between species records to avoid potential sampling bias (Merow *et al.* 2013). Various values of MAXENT model parameters were explored, and the resulting models were compared to the known distribution area (Kurtto *et al.* 2010, Monasterio-Huelin and Weber 1996, pers. obs.). In the final models, the regularization multiplier was set to 2.5, clamping and fading by clamping was allowed, and other model parameters were left as defaults. As projection input data, bioclimatic layers from three paleoclimate models (CCSM, MIROC-ESM and MPI-ESM-P) of the Last Glacial Maximum (LGM; 22 ky BP) and the mid-Holocene (6 ky BP) and one model of the last interglacial period (LIG; 120–140 ky BP) were used (Brady *et al.* 2012; Sueyoshi *et al.* 2013; Otto-Bliesner *et al.* 2006; Giorgetta *et al.* 2013). All of the climatic variables had a spatial resolution of 2.5 arc-minutes. Each ecological niche model was averaged based on 15 independent MAXENT runs.

Results

Chloroplast variation patterns differ in diploids and polyploids

Nine haplotypes were identified within *R. ulmifolius* agg. based on 9 single nucleotide polymorphisms (SNPs, 0.9 %) in the 993 bp *matK* alignment and 3 SNPs (0.7 %) in the 455 bp *trnL-trnF* alignment (Supplementary table 3). The highest cp-haplotype diversity in diploids was observed in Morocco (4 haplotypes) and Western Transcaucasia (3 haplotypes, but only one common; Fig. 1). In Western and Central Europe, including the northern parts of the Balkans, only two haplotypes were found (*Ulm1* and *Ulm2*) across all ploidy levels. Only four haplotypes were shared with polyploid accessions – two commonly (*Ulm1*, *Ulm2*) and two only rarely (*San1*, *San3*). Whereas the *Ulm1* and *Ulm2* haplotypes were widespread from Macaronesia to the Western Balkans in diploids, most haplotypes were only regionally distributed. Haplotype distribution was almost incongruent between diploids and polyploids in Transcaucasia; although neither *Ulm1* nor *Ulm2* was detected in Caucasian *R. ulmifolius* agg., these were the most

common haplotypes in polyploids in that region (Fig. 1). Transcaucasia was also characterized by the highest haplotype diversity in polyploids (4 haplotypes in total; Fig. 1B).

Table 1: Within-population characteristics: number of individuals (N); number of alleles (N_a); effective number of alleles (Ne); Shannon information index (I); observed, expected and unbiased expected heterozygosity (H_o, H_e, and uH_e, respectively); F_{IS} according to Weir & Cockerham (1984; WC84) and Wright (1951; Wr); number of private alleles (A_p); and locally common alleles found in ≤ 25 % of populations (A_{lc}).

Pop.	Abbrev.	N	N_a	N_e	I	H_o	H_e	uH_e	F_{IS} (WC84)	F_{IS} (Wr)	A_p	A_{lc}
Armenia	Am	9	2.300	1.805	0.643	0.400	0.419	0.444	0.1042	0.041	0.500	0.300
Georgia	Ge	10	2.600	1.958	0.676	0.387	0.400	0.421	0.0907	0.022	0.000	0.700
Balkans – Central	BC	10	3.800	2.419	0.887	0.390	0.459	0.483	0.2014	0.129	0.100	0.700
Balkans – West	BW	10	3.900	2.387	0.998	0.460	0.539	0.568	0.2036	0.223	0.100	0.700
Balkans – North	BN	10	3.500	2.210	0.905	0.478	0.502	0.529	0.1056	0.057	0.000	0.100
France – South	FS	10	3.800	2.185	0.861	0.390	0.450	0.474	0.1847	0.104	0.600	0.400
France – North	FN	9	2.600	1.819	0.640	0.225	0.381	0.403	0.4542	0.348	0.000	0.100
Great Britain	GB	9	2.400	1.550	0.527	0.251	0.310	0.329	0.2537	0.150	0.000	0.000
Spain – North	SN	10	3.500	2.271	0.875	0.430	0.471	0.496	0.139	0.069	0.500	0.300
Morocco – North	MN	10	3.500	2.127	0.850	0.478	0.454	0.478	0.0047	-0.077	0.100	0.400
Morocco – South	MS	10	2.900	2.173	0.764	0.360	0.432	0.454	0.2164	0.153	0.000	0.300

Nuclear SSR allelic variation reveals complex geographic patterns

In total, 148 alleles were distinguished for ten SSR loci based predominantly on SNPs. Because SSR length polymorphism was randomly distributed and seemed therefore mostly homoplasic, it was considered only exceptionally to further distinguish common alleles. Sixteen alleles were unique for *R. ulmifolius* agg., 55 alleles were shared between this diploid taxon and polyploid accessions, and 54 alleles were polyploid-specific or shared with other diploid taxa and could therefore be considered non-*ulmifolius* in origin. Twenty alleles were identified only in "outgroup" taxa (Table 2). Alleles could be further distinguished according to their geographic distributions. The "eastern" populations were considered Armenia (Am), Georgia (Ge) and Balkan–Central (BC); all others were considered "western". Balkan populations (BC, BW, and BN) were considered to represent a transition zone based on allele distribution maps (Fig. 2; Supplementary fig. 2) and population genetic clustering (see below). Conspicuously numerous alleles were shared between eastern diploid *R. ulmifolius* agg. and eastern polyploids (hereafter as E/E alleles; 10), between western diploid and western polyploids (W/W; 14) and between western diploid and *all* (both western and eastern) polyploids (W/a; 13; Table 2). Several alleles were population-specific, especially in the East – two E/0 alleles (not present in polyploids) and three E/E alleles specific for Am, three E/0 specific for the Balkan populations and one E/E allele for Ge (Supplementary fig. 2).

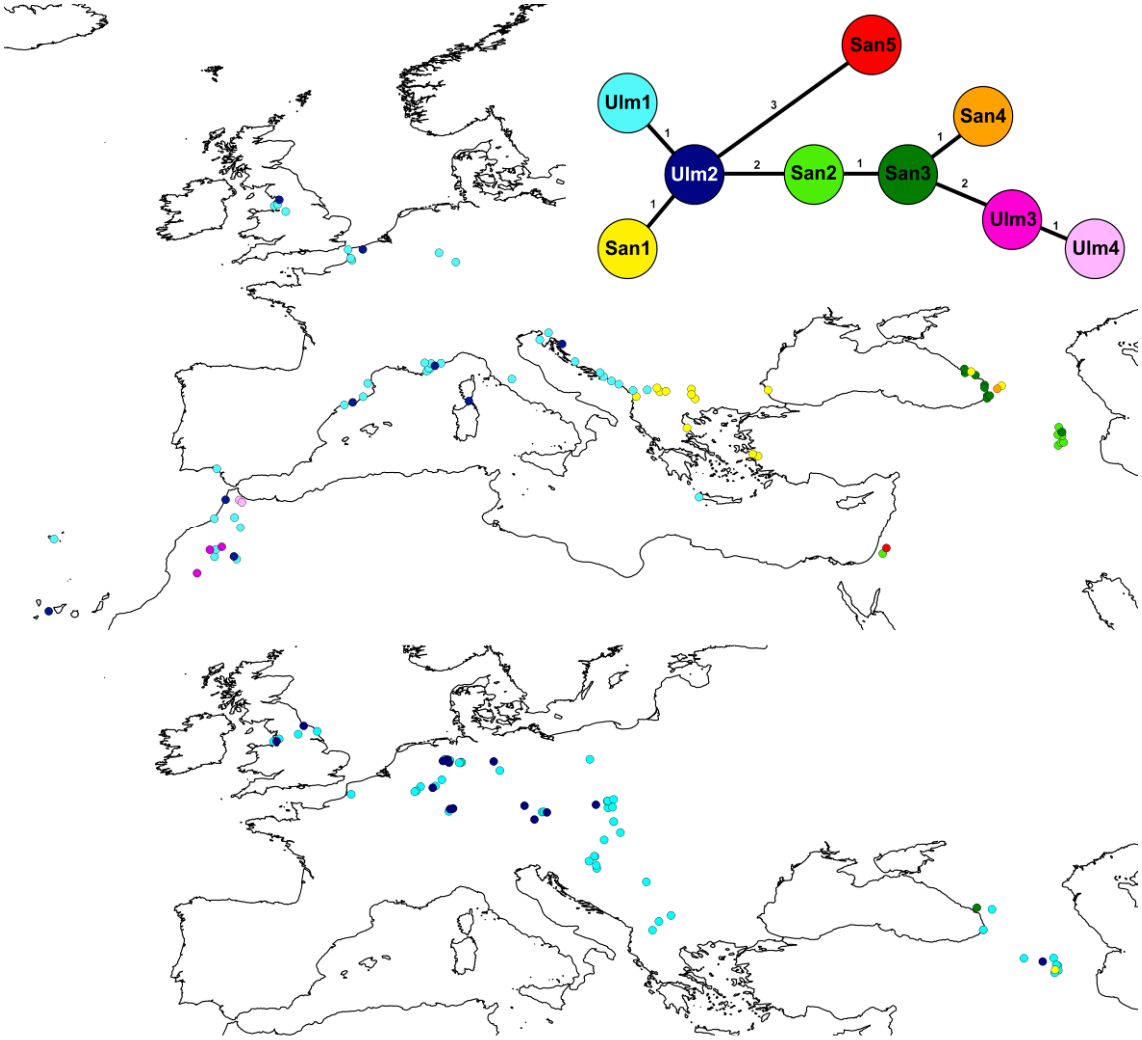


Figure 1: Detected cp-haplotypes in diploid accessions (above; N = 82) and polyploid accessions (below; N = 82).

		diploid			
		E	**W**	**a**	**0**
polyploid	**E**	10	0	3	6
	W	0	14	3	23
	a	1	13	11	25
	0	8	7	1	19

Table 2: Distribution of SSR alleles in eastern (E), western (W), both (a = all) or none (0) diploid *R. ulmifolius* agg. and polyploids; the Balkans were considered a transition zone

Genetic diversity indices (number of alleles, heterozygosity, Shannon information index) were lowest for populations Am, Ge, Great Britain (GB) and France–North (FN). Mediterranean populations exhibited a high and mutually comparable diversity (Table 1). The inbreeding coefficient F_{IS} always exhibited positive values [except for the value for Morocco–North (MN) when calculated using the method of Wright (1951); Table 1], being highest in FN [0.454, Wright's method; 0.348, the method of Weir and Cockerham (1984)] and only slightly affected by the Wahlund effect of population subdivision [F_{IS} for French and German subpopulations of 0.397 and 0.368 according to Weir and Cockerham's method, or 0.306 and 0.044 according to Wright's method, respectively]. Among-population comparisons of allelic (*Hd*) and nucleotide (*Pi*)

diversity resulted in three groups. First, Am, Ge and BC exhibited low values of both *Hd* (<0.444) and *Pi* (<0.0055). Second, FN and GB exhibited high *Pi* and low *Hd*. Third, all of the other populations were characterized by both high *Pi* (>0.473) and high *Hd* (>0.0061; Fig. 3).

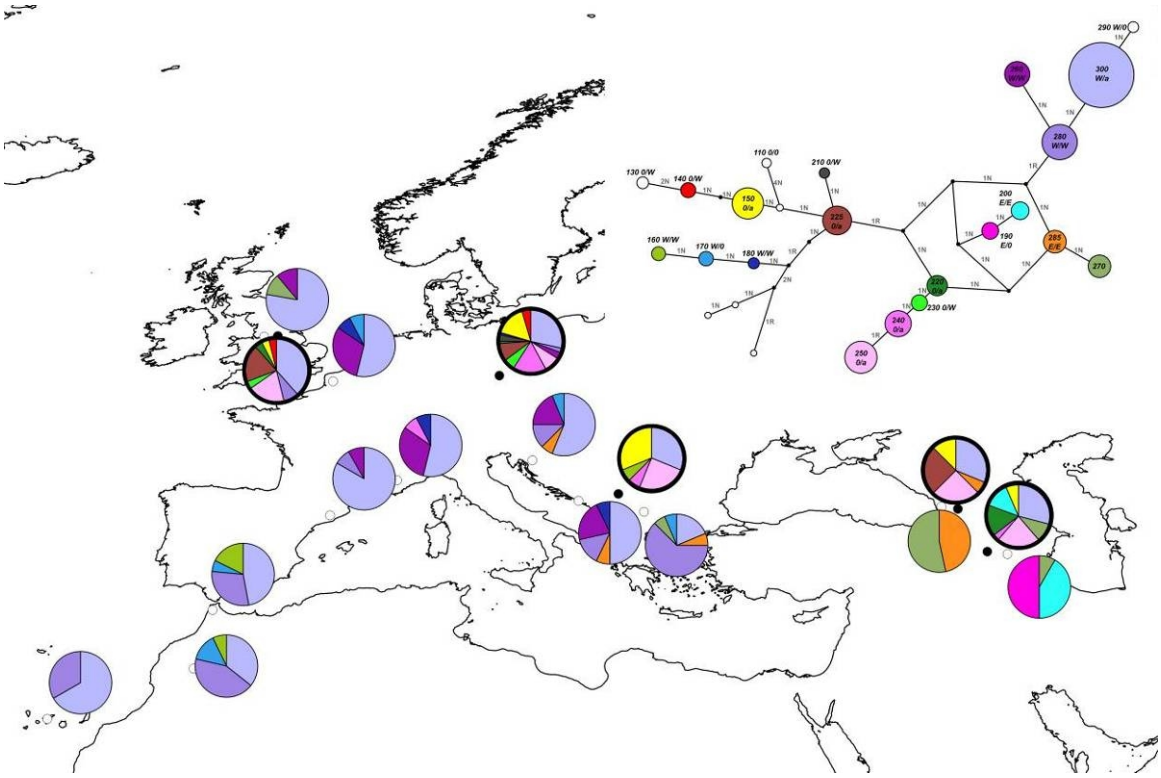


Figure 2: SSR allele distribution patterns in R. ulmifolius agg. (thin pie charts) and polyploid accessions (in bold) using an example of locus 01B06. Each colour represents one allele and its relative frequency in a population (pie charts). The circle size in the phylogenetic network corresponds to the allele occurrence in the total dataset; N and R indicate the numbers of single nucleotide mutations and changes in microsatellite repeat number, respectively. Distribution maps for other loci can be found in Supplementary fig. 2.

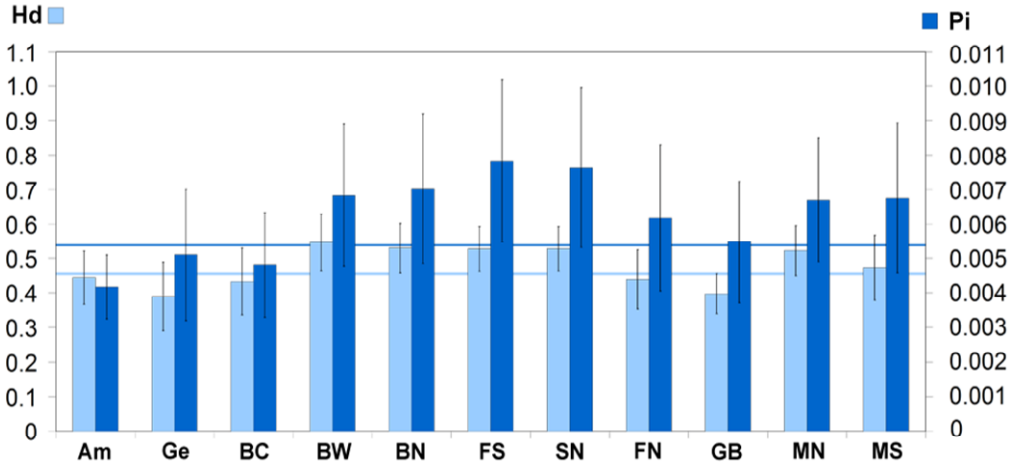


Figure 3: Allelic (Hd) and nucleotide diversity (Pi) within populations of R. ulmifolius agg.; error bars show standard errors of a mean among loci.

A Bayesian population clustering analysis of *R. ulmifolius* agg. resulted in the highest statistical support for K=4 and 8 [based on mean DeltaK and L(K); data not shown]. For both K values, Am clustered separately, and Ge clustered together with a part of BC, the latter of which showed a gradual transgression towards BW. All of the western populations were highly variable for K=8 (with the exception of GB), showing high levels of admixture. For K=4, the western population GB was at the one end, and MS and MN were at the other end, forming two homogeneous clusters and reflecting a North-South gradient in genetic structuring along the Atlantic coast (Fig. 4). Clear East-West and South-North gradients were also observed in the PCoA plot (Fig. 5). Pair-wise F_{ST} indices confirmed the high degree and significance of genetic differentiation among most of the non-neighbouring populations, with western populations being less strongly differentiated than the eastern ones (Supplementary table 4). These geographic patterns resulted in a significant positive correlation of geographic and genetic distances between individuals when the whole sampling area was considered (R^2=0.316, P<0.0001; Supplementary fig. 3). When each region was analysed separately, significant correlation was detected only in FN and BC, which were subdivided into two geographic clusters (not shown in the thesis).

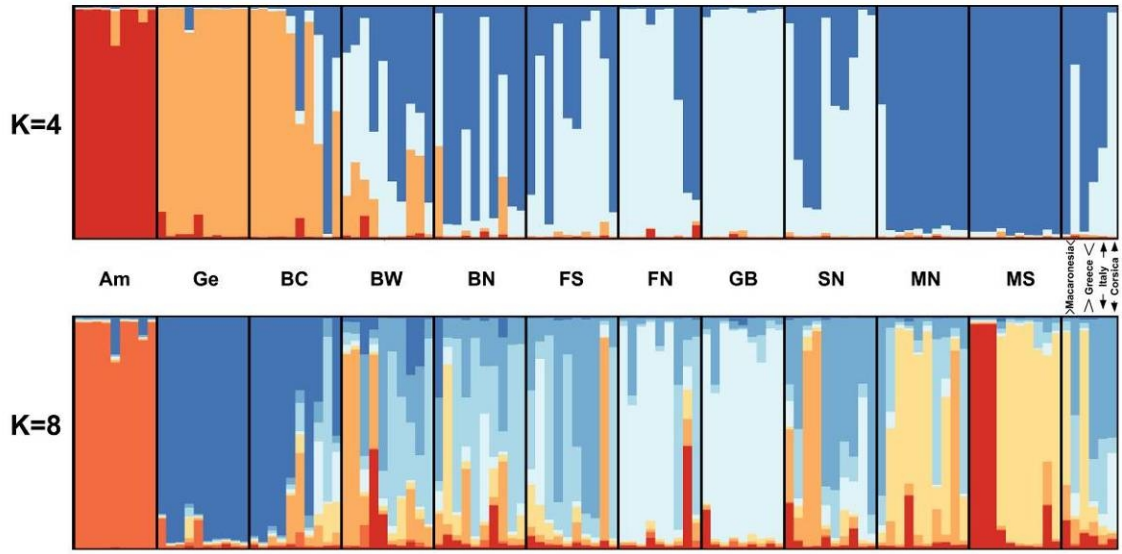


Figure 4: STRUCTURE diagram of *R. ulmifolius* agg. for two different values of K.

Ecological niche modelling suggests uneven habitat loss during LGM

Ecological niche modelling was able to reveal all of the areas with the extant occurrence of *R. ulmifolius* agg. (Fig. 6A). Projections on climatic layers of the last glacial maximum (LGM) provided slightly different results depending on the models used for paleoclimatic reconstruction (Fig. 6B, C). The main differences were observed in the Adriatic shelf, the Apennine Peninsula, the Atlantic shelf and the Southern coasts of the East Mediterranean basin. All of the models indicated the existence of suitable climatic conditions on both sides of the Strait of Gibraltar, along the Atlantic coast and in the Levant. On the other hand, the Black Sea coast and Transcaucasia exhibited a reduction of climatically suitable habitats during the LGM (Fig. 6B, C). Changes in MAXENT model parameters, input data or background selection had only very minor effects on the final results and were generally less influential than were the projection layers (not

shown). Models of the mid-Holocene and the last inter-glacial period climate did not reveal any significant differences in suitable habitats compared to the extant distribution (not shown in the thesis).

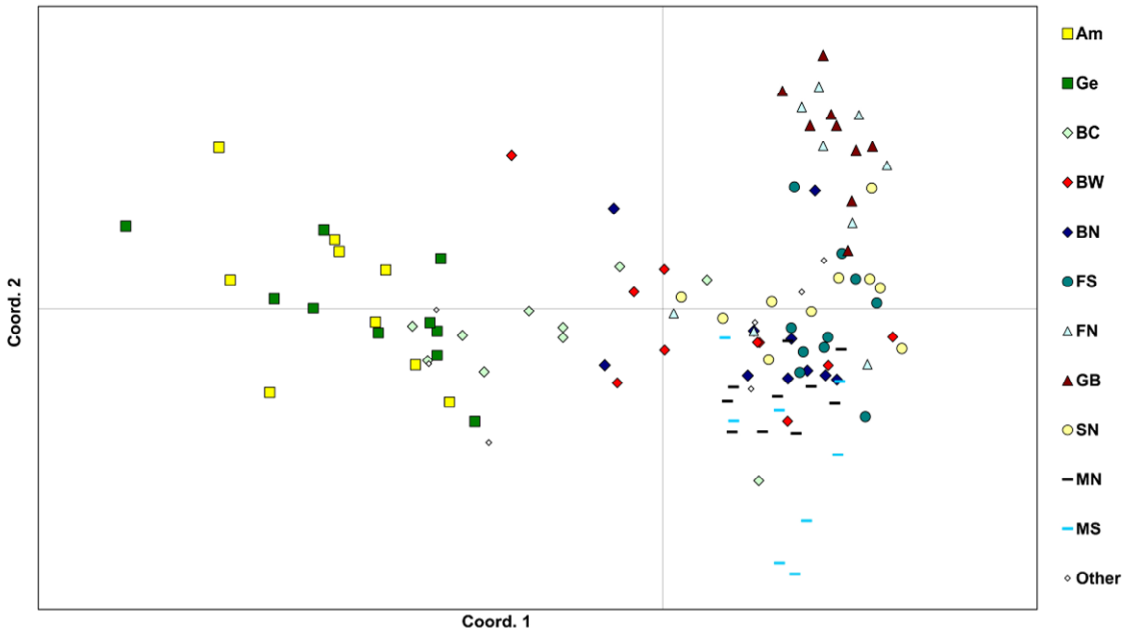


Figure 5: PCoA plot of *R. ulmifolius* agg. The axes explain 27.0 % and 11.5 % of the total variation, respectively.

Discussion

In this study, we used two chloroplastic and ten nuclear loci to study phylogeographic and evolutionary patterns in a taxonomically extremely complex group of European brambles. Our NGS approach provided sequence information of SSR alleles. SSRs are known for their high degree of homoplasy when only length polymorphism is taken into account (Selkoe and Toonen 2006). This can be confirmed by our data because SSR length was mostly not correlated with single nucleotide polymorphisms in flanking regions. The use of the SSR markers that were used here thus cannot be recommended for fragment-analysis-based studies with a divergent sample set. Although our primary aim was to reveal geographic patterns among diploid and (allo)polyploid accessions, the combination of the vegetation dynamics caused by the Quaternary climatic oscillations, hybridizations and polyploidization events with limited methodological possibilities of allopolyploid data analysis made this task very complicated. Despite the fact that these complex patterns cannot be visualized by standard analytical methods (such as multivariate or clustering analysis), we managed to disentangle the phylogeographic processes by combining different approaches.

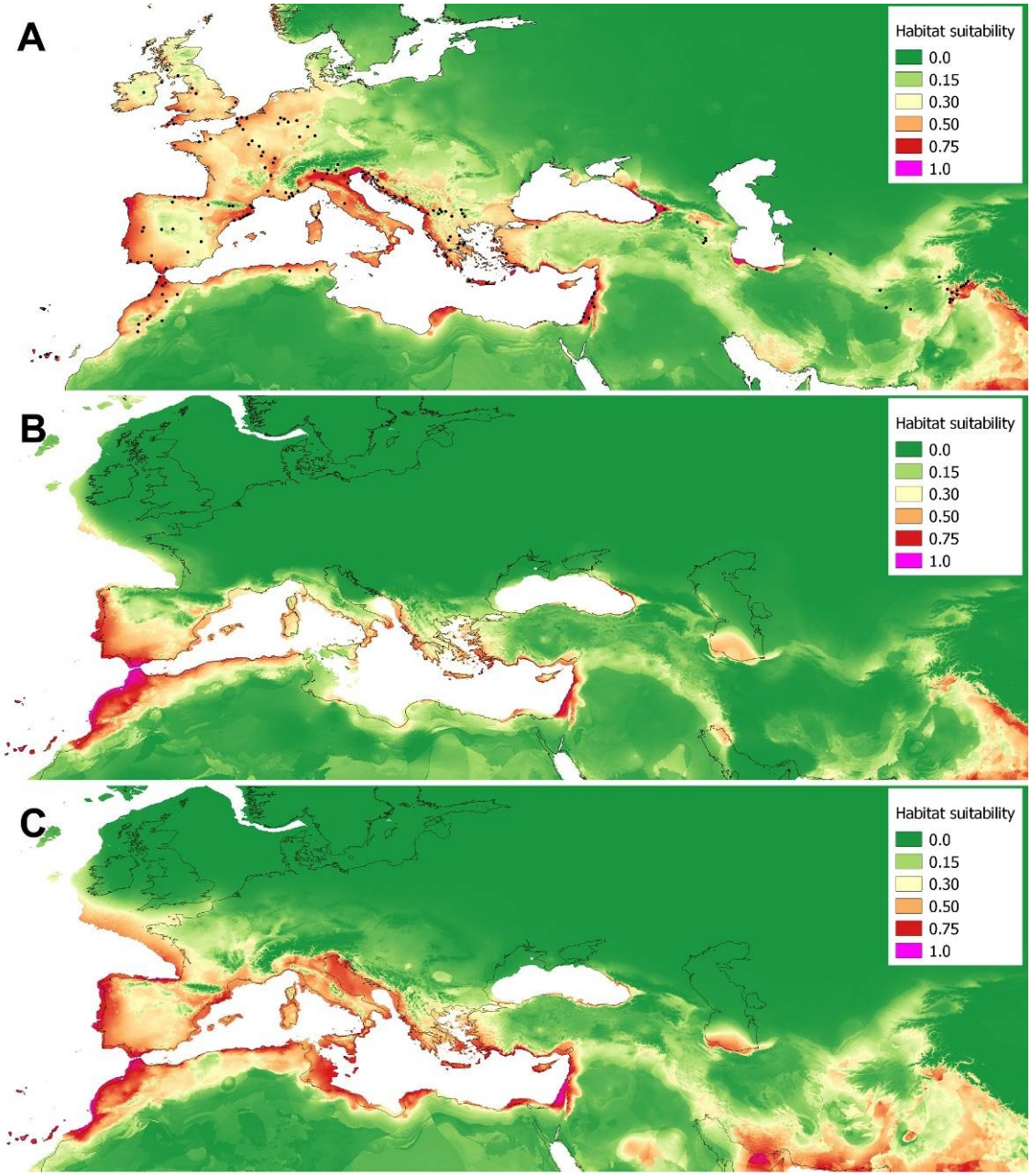


Figure 6: MaxEnt model of habitat suitability for *R. ulmifolius* agg. (A) present situation (known localities used as training data are shown as dots), (B) the LGM based on the CCSM model, and (C) the LGM based on the MIROC model.

Strong genetic structure indicates weak long-distance gene flow

Metapopulation genetic structure is shaped by many processes, among which gene flow (in plants mainly via seed or pollen dispersal) plays a major role (Bohonak 1999). Effective long-distance seed dispersal by large birds is well documented for many wetland plants (van Leeuwen *et al.* 2012) but remains enigmatic for other plant groups, as its effectiveness can be influenced by many often poorly understood factors (Nathan *et al.* 2008; Schupp *et al.* 2010; Sosa *et al.* 2013). Brambles are typical bird-dispersed plants with a wide spectrum of dispersers of diverse migratory and feeding behaviours (Jordano 1982; Rejmánek 2015). Spanish populations of *R. ulmifolius* were found to be dispersed mainly by various migrant birds (Jordano 1982), which may lead to the

assumption of intensive gene flow and weak geographical genetic structure. Such a pattern was actually observed in several fleshy-fruit shrubs and trees, such as *Sorbus aucuparia* L. (Raspé *et al.* 2000) or *Prunus spinosa* L. (Mohanty *et al.* 2002). In contrast, both our nuclear and chloroplast data show clear inter-regional differences in *R. ulmifolius* agg., especially in the Mediterranean and Transcaucasia (Figs 1, 2 and 4), where F_{ST} indices indicate very strong differentiation even between neighbouring regions (Supplementary table 4), such as Am and Ge separated by the Lesser Caucasus range, which is unsuitable for the species (Juzepczuk 1952), or Ge and BC connected by only a narrow strip of ecologically suitable Black Sea coast (Fig. 6A; Davis and Meikle 1972). The structuring of genetic variation (Figs 4 and 5) also indicates the strong influence of only short- or medium-distance gene flow in the diploid accessions, leading to significant isolation by distance on the (supra)continental scale, but not within narrowly defined regions. These findings agree with our previous studies on other bramble species using SSR and cpDNA markers, which revealed some degree of genetic differentiation within (ecologically rather heterogeneous) regions of Colchis and Central Europe (Sochor *et al.* 2015; Sochor and Trávníček 2016; Šarhanová *et al.* in prep.). Floristic and reproduction mode analyses further suggested the absence of gene flow on the scale of hundreds of kilometres in Central and West European brambles (Haveman *et al.* 2016; Šarhanová *et al.* 2012). A similarly strong genetic structure was observed in another bird-dispersed taxon, *Frangula alnus* Mill., mainly in populations from Mediterranean mountain ranges. Although low differentiation was detected among temperate populations of that species, this did not reflect the recent gene flow, which could not be estimated precisely due to low observed genetic variation (Hampe *et al.* 2003).

High genetic variability and its geographic structuring in *R. ulmifolius* agg. are reflected in its morphological diversity, which has caused much confusion in taxonomy. The latest taxonomic revision by Monasterio-Huelin and Weber (1996) resulted in the acceptance of just two names – *R. ulmifolius* Schott for western populations up to the Western Balkans and *R. sanctus* Schreber for eastern populations from the Balkans eastwards (see also Kurtto *et al.* 2010). Our data confirm this concept, as the Balkans indeed form a transition zone between the eastern and western gene pools, as seen in both studied markers. Because we did not examine morphological variation, the further taxonomical distinction (e.g., between Am and Ge) or re-classification of the species is beyond our scope. However, because the two taxa are closely related and *R. ulmifolius* is by far the most widely known name within the complex, we use the informal name *R. ulmifolius* agg. for both.

In addition to the above-discussed isolation by distance, some degree of connectedness between neighbouring regions was observed by both nuclear and plastid markers in *R. ulmifolius* agg. (Figs 1 and 2). Cp-haplotype *San1* (characteristic for the Southern Balkans) was detected in population Ge, and *San3* (common in Ge) was found in Am. Distinction between occasional long-distance colonization events and consecutive "stepping stone" expansion is impossible in these cases due to the disjunctive sampling design. A specific situation was nevertheless observed for the Moroccan haplotypes *Ulm3* and *Ulm4*, which derived from Caucasian haplotype *San3* (Fig. 1). Homoplasic independent origins of these haplotypes are improbable because *San3* differs from the most basal haplotype *Ulm2* in three mutations and likely originated in the Caucasus (see Sochor *et al.* 2015). The Caucasus and Morocco have probably never been connected by suitable habitats, as seen from ecological niche modelling (Fig. 6), and an ancient long-

distance migration followed by further mutation accumulation thus seems a likely explanation and is further supported by two SSR alleles that are shared between the two regions (160 of locus 01M20 and 230 of 53E02; Supplementary fig. 2).

Population bottlenecks shaped the genetic diversity of R. ulmifolius agg.

Extensive meta-analyses of various plant and animal species showed a significant decrease in within-population genetic diversity from the Eastern towards the Western Mediterranean on both the European and African sides of the basin (Fady and Conord 2010; Conord *et al.* 2012). The authors explained this common (but not universal) pattern particularly by different climates during the Last Glacial Maximum (LGM). Although their assumptions on paleoclimate are somewhat contradictory to modern models (Brady *et al.* 2012; Sueyoshi *et al.* 2013; Giorgetta *et al.* 2013), climatic variables can indeed explain the observed patterns. Our ecological niche reconstructions for *R. ulmifolius* agg. confirm an exceptional reverse trend typical for chamaephytes (Conord *et al.* 2012). All three LGM climatic models assumed a widely suitable environment in Morocco and the Southern Iberian Peninsula, whereas its extent was significantly reduced in the Eastern Mediterranean (except for Levant) and Transcaucasia (Fig. 6, Supplementary fig. 2), the extent of reduction being dependent on the model used. At the same time, bioclimatic layers 1 (mean annual temperature) and 13 (precipitation of the wettest month) gave the highest contributions to the model (29.3 and 20.8 %, respectively), with layer 13 being positively correlated with predicted habitat suitability (data not shown). This supports the hypothesis that the higher genetic diversity of chamaephytes in the Western Mediterranean stems from the availability of snow protecting over-wintering buds (Conord *et al.* 2012), whereas it may have a negative effect on other life forms. Molecular data confirmed the supposed habitat reduction in the east because populations Am, Ge and BC exhibited low allelic (*Hd*) and nucleotide (*Pi*) diversity – indicators of long-lasting or repeated population bottlenecks (Kiefer *et al.* 2009; Avise 2000). On the other hand, high values of *Hd* and *Pi* in West-Mediterranean populations imply long-term demographic stability (or multiple colonizations and admixed populations). Furthermore, severe bottlenecks are likely responsible for the loss of nuclear alleles and cp-haplotypes in eastern *R. ulmifolius* agg. but not in eastern polyploids, as illustrated by the high number of W/a alleles (Table 2) and the absence of *Ulm1* and *Ulm2* haplotypes in the eastern diploids (Fig. 1).

The last glacial cycle likely produced the South-North gradient in the diploids' genetic structure. Populations GB and FN exhibited low *Hd* but high *Pi*, which is characteristic for populations founded from several diverged sources (Avise 2000). Our data are thus consistent with a leading-edge expansion model, which supposes the gradual colonization of free (northern) areas by only a fraction of genotypes from the closest populations (Nieto Feliner 2014; Hewitt 2004), in case of *R. ulmifolius* mainly from different parts of the Iberian Peninsula (Fig. 6; Supplementary table 4). This inevitably leads to lower allelic/haplotypic diversity with increasing latitude. Moreover, so-called surfing on the wave of advance often causes significant changes in allelic frequencies as a result of repeated founder effects and strong genetic drift (Excoffier and Ray 2008; Edmonds *et al.* 2004). Although gene surfing is a more or less theoretical concept with rather scarce empirical evidence (Graciá *et al.* 2013, Hallatschek *et al.* 2007), this phenomenon can be observed for a few alleles *in R. ulmifolius* as well (Fig. 2 and Supplementary fig. 2). With denser sampling along the Atlantic coast, the species may be a good model for population genetic studies of range expansion.

Polyploid hybrids may profit from the genetic deterioration of their parents

Hybridization and polyploidization undoubtedly play an important role in plant evolution, although it is unclear whether they enhance or hinder diversification (Madlung 2013). In European brambles, allopolyploids are stabilized by apomixis, which makes each hybrid genotype a distinct evolutionary unit by enabling spread in both time and space, as apomictic hybrids are able to quickly colonize unoccupied areas (Hörandl 2011) and thus increase the probability of survival in case of dramatic environmental change. At the same time, residual sexuality via preserved meiosis (both male and female) or via fertilization of unreduced female gametes does not exclude them from further evolution (Šarhanová *et al.* 2012; Sochor *et al.* 2015). Sexual diploids and apomictic polyploids are nevertheless often geographically separated with limited overlap – the former are distributed mainly in southern areas, whereas the latter occupy mostly colder temperate regions of Central and Western Europe and Transcaucasia (Kurtto *et al.* 2010; Sochor and Trávníček 2016). This so-called geographic parthenogenesis has been described for many plant and animal taxa and different geographical variables (altitude, latitude, glaciation, human disturbance etc.), but its reasons remain more or less hypothetical, partly because there are probably many factors acting together to shape patterns of geographic parthenogenesis (Hörandl 2006).

Asexuals usually occupy marginal habitats, very often deglaciated areas. Post-glacial migrations likely enabled the hybridization of formerly isolated taxa – a prerequisite (or at least an indirect correlate) for apomixis in most plants (Hörandl 2006, Lovell *et al.* 2013). Newly arisen apomicts could then take advantage of (1) (allo)polyploidy (e.g., elevated heterozygosity reflected by heterosis; the masking of deleterious mutations; gene redundancy; or loss of self-incompatibility; Comai 2005; Madlung 2013); (2) asexuality (better colonization ability due to no need for a reproduction mate; reproductive assurance in low population densities; resistance to negative effects of population fragmentation and inbreeding; and absence of gene flow from core habitats which, in case of sexuals, prevents adaptation to extreme conditions and may thus drive selection for reproductive isolation between core and marginal populations, e.g., via asexuality; Haag and Ebert 2004, Vrijenhoek and Parker 2009); (3) reduced pressure of predators, pests and pathogens (Verhoeven and Biere 2013); and (4) free niches after deglaciation and Neolithic revolution in Europe (Matzke-Hajek 1997). Two main hypotheses have been further proposed to explain early competitive interactions between sexuals and apomicts. The “general-purpose genotype” model suggests selective advantages for and the spread of few apomictic genotypes with high ecological tolerance (generalists) and thus with high competitive ability compared to sexuals. On the other hand, the “frozen niche-variation model” predicts the coexistence of sexuals and many apomictic genotypes specialized to narrow, non-overlapping niches (or subsequent exclusion of sexuals if the generation of these apomictic specialists is too fast; Vrijenhoek and Parker 2009). Both models may be valid in brambles, as a huge number of diverse apomictic lineages exists (often represented by just few ramets), as well as several extremely widespread genotypes exhibiting apparently broad ecological tolerance (Kurtto *et al.* 2010).

Importantly, our molecular data strongly support the so-called “metapopulation hypothesis”. Originally, this hypothesis postulated genetic bottlenecks in marginal populations and subsequent drift and inbreeding, which have very different effects on sexuals than on apomicts (Haag and Ebert 2004). Here, we evidence the genetic deterioration in *R. ulmifolius* agg. in Northwestern Europe due to post-glacial re-

colonization and in eastern populations due to severe bottlenecks at the LGM. Both Northwestern Europe and Transcaucasia are hotspots of polyploid species diversity, although polyploid apomicts (often only local hybrids) can be found elsewhere within the distribution range of *R. ulmifolius* (Kurtto *et al.* 2010; Sochor and Trávníček 2016). Diploid and polyploid brambles generally occupy slightly different ecological niches, as seen in their distribution in the Mediterranean (diploids occupy mainly hot and dry coastal habitats, whereas polyploids prefer inland and mountainous habitats) and in the pattern of geographical parthenogenesis (see above). However, in regions with low diploid allelic diversity, they are not ecologically separated (pers. obs.), indicating that their niches partly overlap. In addition to the above-mentioned hypotheses, we therefore hypothesize that the genetic deterioration of diploid *R. ulmifolius* agg. may have enabled the diversification and spread of apomicts in NW Europe and Transcaucasia. In contrast, competition among genetically diverse diploids and its polyploid descendants likely limits the spread of the latter in the Mediterranean.

Apomicts integrate gene pools of both pre- and post-glacial diploids

In our previous papers, we hypothesized that although most polyploid *Rubus* species/taxa originated in the Holocene and that their spread may have been driven by human-mediated changes in landscape beginning only a few millennia ago (Sochor *et al.* 2015; Šarhanová *et al.* in prep.), several must have been formed before the LGM (or any similarly strong environmental event), e.g., members of *R.* subsect. *Rubus*, *R.* "*hirtus*" agg., or some Caucasian polyploids (Sochor *et al.* 2015). This is strongly supported by the present data. While high numbers of W/W and E/E alleles point to a recent origin of western and eastern apomicts, respectively, multiple a/a and especially W/a alleles indicate that a large proportion of eastern polyploids' genomes derived from an ancient diploid gene pool before it was restricted by the LGM bottlenecks in the east. The same is probable also for western apomicts, although the molecular evidence is less clear due to better continuity of populations (and thus smaller genetic changes) in the Western Mediterranean (Fig. 6). Moreover, several region-specific alleles shared between di- and polyploids imply the origin of Caucasian polyploids in a small area of their current occurrence and only limited spread outside of it. A noticeable exception is *R. armeniacus* Focke, which is a European cultivated and invasive crop, but there have never been doubts about its Caucasian origin (Focke 1910). Here, we confirm its formation in Armenia because it shares several private alleles with Armenian *R. ulmifolius* agg. (it was hence treated as an Armenian polyploid in Fig. 2 and Supplementary figs 1B and 2).

At the same time, we found only two eastern polyploid accessions sharing the cp-haplotype with the eastern diploid, while all others bore the "western" haplotypes *Ulm1* and *Ulm2* or haplotypes derived from other diploid ancestors (see also Sochor *et al.* 2015; and Sochor and Trávníček 2016). Previously, the absence of shared haplotypes led us to assume that eastern *R. ulmifolius* agg. either did not participate in recent hybridizations or that it served predominantly as a pollen parent. In the light of the nuclear SSR data, the latter hypothesis is more likely. The situation is somewhat paradoxical because Caucasian polyploids (at least in Colchis and with exception of the ecologically different series *Glandulosi*) exhibit only limited sexuality (Sochor and Trávníček 2016) and are thus expected to serve mainly as pollen donors in hybridizations (Šarhanová *et al.* in prep.). Unidirectional hybridization is nevertheless a ubiquitous phenomenon not only in plants but also in animals and fungi and may be

hypothetically caused by many pre- or post-zygotic mechanisms, including genetic imprinting, epigenetic effects or cytoplasmic–nuclear interactions (Turelli and Moyle 2007), or exogenous factors (Muir *et al.* 2015). It was also assumed to play a role in the origin of several other *Rubus* taxa, such as pentaploid *Corylifolii* accessions. In that case, triploids always serve as acceptors of diploid pollen from *R. caesius* probably due to their low pollen viability and frequent production of unreduced megaspores (Sochor *et al.* 2015). For Caucasian polyploids, this explanation would be only speculative, as the pollen viability is unknown (however, low viability would not be surprising considering the high proportion of unreduced megaspores; Sochor and Trávníček 2016). Among many other hypotheses, the "SI × SC rule" is noteworthy, which states that pollen from self-incompatible species is able to fertilize ovules from self-compatible species but not *vice versa* (Zhou *et al.* 2008). The physiological mechanism of this rule is only partly characterized and shares at least three common factors with the self-incompatibility system in tomato (Baek *et al.* 2015). Because self-incompatibility was observed in *R. ulmifolius* and is often broken by polyploidy (Comai 2005; Tammisola and Ryynänen 1970), this rule may be a possible explanation for the observed pattern.

Conclusion

Using the next-generation sequencing of nuclear microsatellites, the Sanger sequencing of non-coding plastid regions and ecological niche modelling, we shed light on the phylogeography of *R. ulmifolius* agg. – one of the only three extant diploid species (excluding *R. idaeus*) that gave origin to the extremely rich European and Caucasian bramble flora. The complex patterns of allelic and nucleotide diversity resulted from Pleistocene and Holocene climate changes, which also drove the evolution of apomictic brambles (Haveman *et al.* 2016; Sochor *et al.* 2015). However, associations between the evolution of apomicts and the phylogeography of a sexuals have, to our knowledge, never been explicitly studied. The present data show that apomictic taxa not only preserve alleles lost in the diploid ancestors due to past genetic bottlenecks but also integrate these old alleles with younger ones from the same or different diploid taxa via recent gene flow. Then, facultative apomicts can profit both from the advantageous genetic constitution of their asexual ancestors (which preserved the old alleles via apomixis and polyploidy) and from the local adaptation of recent sexual populations. This makes them a very flexible evolutionary system and a highly successful plant group. Additionally, in regions where diploids' genetic diversity was deteriorated by environmental conditions in the past, apomicts seem to have a competitive advantage that could promote their further spread and diversification. Genetic bottlenecks in sexuals may thus serve as another explanation for geographic parthenogenesis in plants. Overall, our data demonstrate a tight interplay between the evolutionary histories of apomicts and their sexual ancestors.

CHAPTER 4:

Melting pot of biodiversity: first insights into the evolutionary patterns of the Colchic bramble flora (*Rubus* subgenus *Rubus*)

Michal Sochor

Bohumil Trávníček

2016, Botanical Journal of the Linnean Society

DOI: 10.1111/boj.12436

Abstract

The Caucasus is a biodiversity hotspot of global significance, containing a number of highly diverse and species rich plant taxa. The region is also thought to be an important evolutionary hotspot for *Rubus* subgenus *Rubus*. However, Caucasian brambles have only been poorly studied to date and our knowledge of their evolutionary mechanisms, systematics, and taxonomic variability remains rudimentary. The objectives of this study therefore were to shed light on the evolution, diversity and reproduction modes of *Rubus* in one of the two Caucasian glacial refugia – Colchis. Flow cytometry measurements were used to estimate DNA ploidy level, a flow cytometric seed screen was conducted to determine reproduction mode, and Sanger sequencing of two non-coding plastid regions was used to reveal phylogenetic patterns. The most common ploidy level was tetraploid, followed by diploid and rarely triploid. Intra-individual variation in reproduction mode was rather low, as the "morphoseries *Glandulosi* and *Radula*" exhibited strict sexuality and other taxa were mostly apomictic. A few exceptions were observed that deserve further attention, for example, sexuality induced hypothetically by haploid pollen or by environmental conditions, a high proportion of triploid embryos, or polyspermy. Plastid haplotype variability revealed specific, ancient evolutionary patterns with limited involvement of extant diploid taxa, and recent isolation from European brambles. We provide the very first insight into the variability and evolution of Colchic brambles, which can serve as a starting point for further systematic and evolutionary studies.

Introduction

The Caucasus is among the 25 most threatened biodiversity hotspots in the world with a high level of endemism (Myers *et al.* 2000) and one of the richest floras of its latitude (Zimina 1978). One reason for this is the presence of two glacial refugia within this relatively small area, which enabled survival of the temperate flora, including tertiary relics. These refugia are Hyrcania, located along the southern Caspian coast, and Colchis in the West of the Southern Caucasus (Tarkhnishvili *et al.* 2012). The so-called "Colchic Triangle", delimited by the ranges of the Greater and Lesser Caucasus in the North, East, and South and the Black Sea in the West, is characterized by particularly high plant diversity and endemism due to the Pleistocene development and geographical isolation of the region, which together resulted in new free niches and the subsequent adaptive radiation and speciation of some families (Kikvidze and Ohsawa 2001). Currently, more than 4,100 species of vascular plants are recognized in Georgia (Gagnidze 2005), of which around 3,000 are reported in Colchis (Kikvidze and Ohsawa 2001). However, the taxonomic and floristic literature is mostly several decades old, or it is based on former treatments. Further detailed investigations will presumably result in the discovery of many species that are new either for science or for the region, and especially so for complicated and complex taxa, e.g. *Orobanche* L. (Piwowarczyk 2015) or *Astragalus* L. (Ganbarov and Ibrahimov 2015).

The genus *Rubus* L., specifically its subgenus *Rubus* (blackberries, brambles), unambiguously belongs among the most diverse and yet least explored Colchic taxa (Juzepczuk 1925; Gagnidze 2005). Generally, the evolution of brambles is driven by extensive hybridization, polyploidy, and asexual reproduction by seeds (apomixis), the last of which enables the stabilization of the hybrid state (Gustafsson 1942; Pratt *et al.* 1958; Sochor *et al.* 2015). *Rubus* subgenus *Rubus* in Europe and adjacent regions thus

constitutes an assemblage of 1) few "true biological" (sensu Mayr 1942) sexual species (both diploid and tetraploid); 2) stabilized polyploid, mostly facultative asexual agamospecies derived from both extant and extinct sexual ancestors; and 3) various products of ancient and recent hybridizations (Sochor *et al.* 2015). Moreover, the presence of geographical parthenogenesis (Šarhanová *et al.* 2012) or autosegregation (Gustafsson 1942), among others, further increases the complexity of evolutionary patterns in some taxa. Manifold taxonomic concepts have been applied to deal with such a difficult subject, among which only one, termed "*Weberian batology*" (Holub 1997; Weber 1996), is widely accepted in Europe where *Rubus* systematics has advanced the most. It represents a pragmatic, morphology-based agamospecies concept (Haveman 2013) and postulates that only widely or regionally distributed biotypes should be considered species. Local and individual biotypes should also be studied and may be given provisional names, but these should not be validated as species (Weber 1996). Unfortunately, this implies that not all individual shrubs can be classified at the species rank, but they can usually be assigned to higher taxa, such as series or sections, although these often reflect only morphological similarities, not phylogenetic relationships (Sochor *et al.* 2015), and their function is only rather pragmatic.

Although still vastly enigmatic, the Colchic diploid taxa are amongst the relatively best explored. *Rubus sanctus* Schreber is closely related to the Western European *R. ulmifolius* Schott. Thorough taxonomic revision by Monasterio-Huelin and Weber (1996) led to synonymization of many names and resulted in the acceptance of just two names in the complex: *R. ulmifolius* (syn. *R. discolor* Weihe et Nees, *R. rusticanus* Mercier etc.) for western populations and *R. sanctus* (syn. *R. sanguineus* Friv., *R. anatolicus* (Focke) Hausskn. etc.) for Balkan, Caucasian, and other eastern plants. The second diploid species, *R. moschus* Juz., the only known extant diploid member of series *Glandulosi*, is endemic of the Lesser Caucasus and is often erroneously treated under the name *R. caucasicus* Focke of unclear status (Juzepczuk, 1925). The third diploid, *R. canescens* DC., is often treated under such names as *R. tomentosus* Borkh. and *R. lloydianus* Genev., but otherwise it does not seem to cause any taxonomic difficulties. The systematics of polyploid groups is not so clear-cut, however. In the three main floras covering the Southern Caucasian region (Juzepczuk 1941; Juzepczuk 1952; Kutateladze 1980), 44 species are mentioned in total and only 24 of them have been recorded in Colchis (including the adjacent area of Borjomi and its vicinity). That is a very low number compared to the extremely high morphological diversity observed in the field (see also Juzepczuk 1925). Moreover, at least five of these species (*R. caucasicus* Focke, *R. candicans* Weihe, *R. hirtus* Waldst. et Kit., *R. serpens* Wiehe, and *R. ponticus* Juz.) can be considered doubtful or aggregate taxa (Kurtto *et al.* 2010 and pers. obs.) and seven others (*R. abchaziensis* (Sudre) Sudre, *R. adzharicus* Sanadze, *R. carthalinicus* Juz., *R. miszczenkoi* Juz., *R. nakeralicus* Sanadze, *R. ochtodes* Juz. and *R. platyphylloides* Sanadze) are probably known only from the type locality or a very restricted area (Juzepczuk 1925; Juzepczuk 1952; Kutateladze 1980). Moreover, some of these names are based on insufficient herbarium material and/or a single individual specimen of somewhat extreme appearance (Juzepczuk 1925). All of these names must be used very carefully and call for the critical systematic revision of Colchic brambles.

Colchis appears to represent a very specific and isolated centre of *Rubus* evolution, which shares several common ancestors with the European bramble flora (Sochor *et al.* 2015). Considering the very poor knowledge of the genus in the Caucasus, we aim to unravel general patterns in 1) cytological variability, 2) reproduction mode, and 3)

plastid DNA variation in Colchic brambles, which together reflect fundamental evolutionary mechanisms and processes. By this, we intend to provide new insights into the evolutionary mosaic of apomictic complexes, and establish a foundation for future biosystematic research on the Caucasian brambles. This will enable further progress in other fields of science, such as biogeography, evolutionary or reproductive biology (e.g. Sochor *et al.* 2015; Šarhanová *et al.* 2012).

Materials and methods

Plant material

Plant material was collected during three field trips in 2013 to 2015 at approximately 17 wider localities in Racha, Lechkhumi, Zemo Svaneti, Adjara, Samegrelo, Imereti, and Abkhazia (Fig. 1). As type locality of *R. moschus*, the surroundings of Borjomi were also explored, although this area lies beyond the Meskheti/Likhi Range and does not formally belong to Colchis. Altogether, 70 individuals were studied, but not all analyses could be performed for every individual due to the limited accessibility of appropriate material. In total, 52 individual plants were used for flow cytometry measurement (FCM), 22 plants were sampled for flow cytometric seed screen (FCSS), and 65 for plastid (cp) DNA sequencing, including one sample of *R. idaeus* L. as an outgroup. The sample-set was divided into 36 morphological groups (hereafter termed morphotaxa) based on herbarium material and preliminary field observations and assigned to putative higher-level taxa (see e.g. Weber 2005). Since their identity with European taxa is often debatable, we use the designation "morphoseries", implying that while these taxa *may* be of different origin they share the basic morphological traits with the respective European series. All herbarium vouchers are deposited in the herbarium of Palacký University in Olomouc (OL) and scans can be obtained from the corresponding author upon request.

Flow cytometric analysis

Ploidy levels of studied accessions were assessed based on the relative fluorescence of stained nuclei, as determined by FCM of fresh leaves or, in a few cases, by FCSS of dried fruits, using a BD Accuri C6 (BD Biosciences, Franklin Lakes, NJ, USA) or a Partec CyFlow ML (Sysmex Partec, Görlitz, Germany) flow cytometer. Reproduction mode was assessed from the relative position of the peaks for embryo, endosperm, and an internal standard by FCSS (for details see Matzk *et al.* 2000; Šarhanová *et al.* 2012). As internal standards, *Solanum lycopersicum* 'Stupické polní rané' (2C = 1.96 pg; Doležel *et al.* 1989) or *Glycine max* 'Polanka' (2C = 2.5 pg; Doležel *et al.* 1994) were used for FCM and *Zea mays* 'CE-777' (2C=5.43 pg; Lysák and Doležel 1998) for FCSS. Leaf tissues (or seed) of the sample and standard were chopped together with a razor blade in 0.5 mL LB01 buffer (Doležel *et al.* 1989; 15 mM Tris, 2mM EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.1 % Tritone X-100, 30 g/L PVP40, and 550 µL/L 2-mercaptoethanol [pH = 8.0]). The suspension was filtered through a 42 µm nylon mesh and stained with 20 µL of propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI). At least 3,000 particles were measured within the size limits of the sample and the standard only. BD Accuri C6 (BD Biosciences) or FlowMax (Sysmex Partec) software was used to calculate peak positions and

coefficients of variation (CV). The highest CV value for accepted peaks was approx. 5.0 %. For ploidy level calibration, genotypes of *R. moschus* (2n = 14; chromosomes counted by Krahulcová and Holub 1997; documented by herbarium specimen collected by Trávníček under no. R266/11 deposited in OL herbarium) and *R. bifrons* Vest (2n = 28; counted by Tesařová (2012); documented by herbarium specimen *Dus2* in OL herbarium) were also measured.

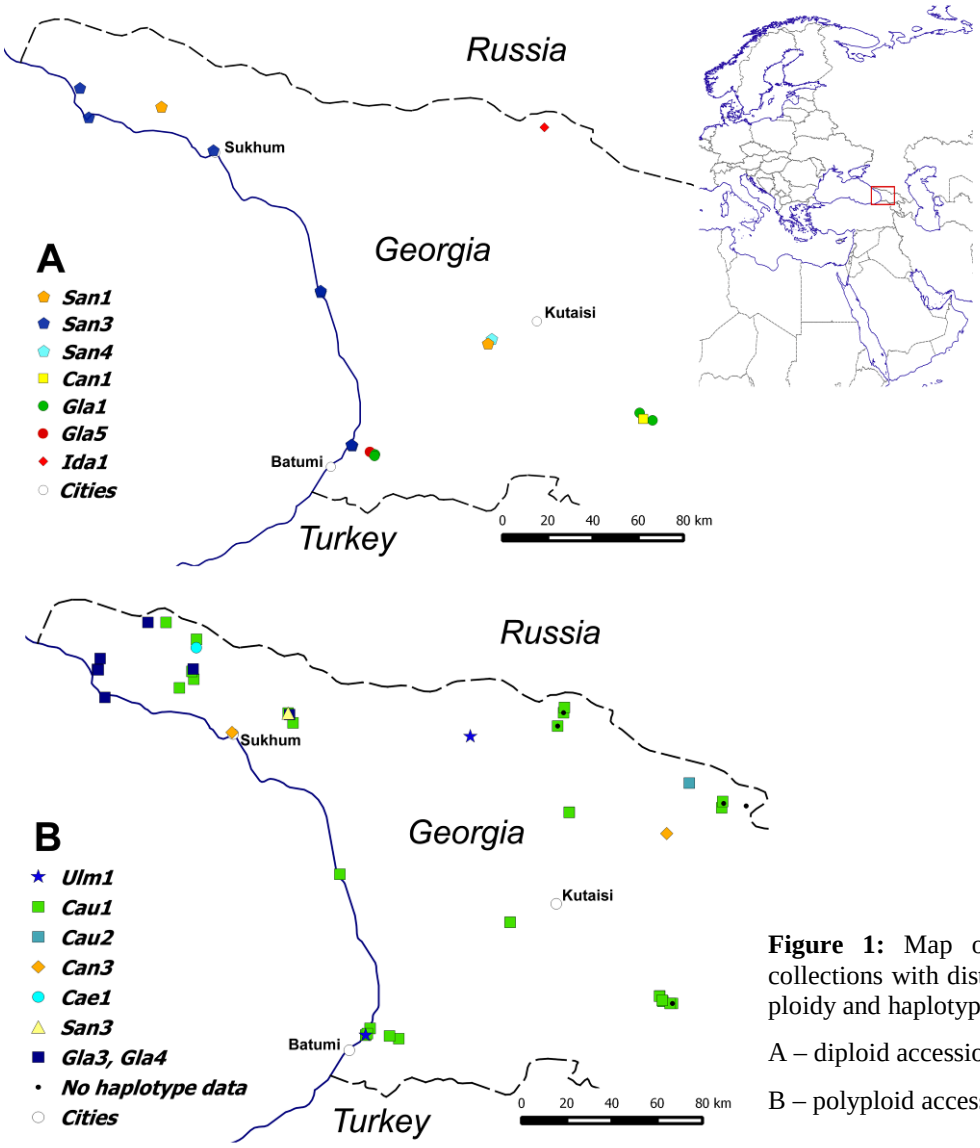


Figure 1: Map of studied collections with distinguished ploidy and haplotypes:

A – diploid accessions,

B – polyploid accessions.

Plastid DNA analysis

DNA was extracted from silica gel-dried leaves following the CTAB (cetyltrimethylammonium bromide) protocol of Doyle and Doyle (1987). Two plastid regions were analysed: the *matK* intron amplified with XFA and AST_R primers (Dunning and Savolainen 2010) and the *trnL-trnF* intergenic spacer with *e* and *f* primers (Taberlet *et al.* 1991). PCR reactions were performed in a 15 µL reaction volume using *Pfu* DNA polymerase (Thermo Scientific) according to manufacturer's recommendations with 0.8 µM final concentration of each primer, 20 ng of template DNA and 0.25 u DNA polymerase. Cycling conditions were as follows: initial

denaturation at 95 °C for 5 min, 37 cycles including one-minute denaturation at 95 °C, 40s annealing at 48 °C or 52 °C for *matK* and *trnL-trnF*, respectively, and extension step at 72 °C for 160 s, followed by 10min final extension at 72 °C. Polyethylene glycol-purified PCR products (10 % PEG 6000 and 1.25M NaCl in the precipitation mixture) were sequenced using the Sanger method at Macrogen Europe in both DNA-strand directions to avoid any ambiguities. Sequence editing, alignments, and haplotype identification were performed in GENEIOUS (ver. 7.1.7.; created by Biomatters, available from www.geneious.com). All haplotypes were compared to the sequences by Sochor *et al.* (2015) and named accordingly. A median-joining algorithm was used to create a phylogenetic haplotype network in NETWORK (ver. 4.6.1.2; Bandelt *et al.* 1999). All sequences were deposited in NCBI Genbank (accession numbers KT581122 – KT581217).

Results

Tetraploid is the most common ploidy level in Colchic brambles

The relative fluorescence (calculated as the ratio of sample/*Solanum*) for diploid taxa was observed within the interval 0.324 to 0.420 for PI staining (*R. moschus* control 0.339 for PI and 0.305 for DAPI). Tetraploids fell within the intervals 0.675 to 0.822 and 0.602 to 0.725 for PI and DAPI, respectively (Table 1; *R. bifrons* control 0.791 and 0.725). Within-ploidy variation in DNA content was quite high and corresponded to morphological affinity, since the diploid species of series *Glandulosi*, *R. moschus*, exhibited relative fluorescence ratios of 0.324 with PI (~2C=0.634 pg), whereas a fluorescence ratio of 0.420 (~2C=0.822 pg) was observed in *R. sanctus* (series *Discolores*). Tetraploids exhibited similar variation among the series (see Table 1).

Out of the 34 studied morphotaxa of blackberries (*R.* subgenus *Rubus*), four were observed to be diploid – *R. sanctus*, *R. moschus* (with two slightly different morphotypes), and one putative hybrid *R. sanctus* × *canescens* (Table 1). Since *R. canescens* is a strictly diploid species (Krahulcová *et al.* 2013), it was not included in the flow cytometry analysis, although the species was observed also in the vicinity of Borjomi. Only one sampled individual was triploid. It belongs to "morphoseries *Radula*" and can only be a local hybrid of tetraploid *R.* "morphoseries *Discolores*" (reduced female gamete; see Supplementary table 1) and diploid *R. moschus* (reduced male gamete), both of which commonly occur at the site. All other studied morphotaxa (85 %) were tetraploid and no higher ploidy levels were found (Table 1).

Glandulosi and Radula are obligate sexuals, other taxa mostly apomictic

The flow cytometric seed screen was able to reveal meiotic reduction and subsequent fertilization of the female gamete as well as the pollen contribution in most cases. Most embryos were developed either via a normal sexual pathway (i.e. the reduced megagametophyte giving rise to 4x embryo and 6x endosperm by double fertilization in 4x mother plants) or via standard pseudogamous apomixis (i.e. the unreduced megagametophyte, unfertilized egg cell, and fertilized central cell; 4x embryo and usually 10x endosperm in 4x mother plants; Table 2), both preserving the mother plant ploidy level in offspring. Deviations leading to lower or higher DNA content in

embryos were detected in 30 seeds (15 %; Table 2). Obligate sexuality was observed in all accessions of "morphoseries *Glandulosi*" and "*Radula*", while other taxa exhibited prevalent apomixis with a low proportion of residual sexuality (Table 2).

Table 1. Mean relative fluorescence (MRF) of 2C nuclei stained with propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) as a ratio of sample to standard (all values are calculated relative to the internal standard *Solanum lycopersicum*, 2C = 1.96 pg) and estimated DNA ploidy level of studied morphotaxa. Number of sampled individuals and total number of measurements per morphotaxon are provided, together with the method used (flow cytometry measurement [FCM] or flow cytometric seed screen [FCSS]).

Morphotaxon	Morphoseries	#Ind./#Meas. (PI + DAPI)	MRF (PI)	MRF (DAPI)	Est. ploidy level
R. cf. *ibericus* Juz.	*Discolores*	1/1 (FCM)	0.801	-	4x
R. morphotaxon *dis1*	*Discolores*	1/1 (FCM)	0.717	-	4x
R. morphotaxon *dis2*	*Discolores*	1/9 (FCSS)[1]	0.784 ± 0.065	-	4x
R. morphotaxon *dis3*	*Discolores*	1/6 (FCSS)[2]	0.822 ± 0.052	-	4x
R. morphotaxon *dis4*	*Discolores*	1/1 (FCM)	0.689	-	4x
R. morphotaxon *dis5*	*Discolores*	1/1 (FCM)	0.767	-	4x
R. morphotaxon *dis6*	*Discolores*	1/2 (FCM)	0.729 ± 0.092	-	4x
R. morphotaxon *dis8*	*Discolores*	1/1 (FCM)	-	0.695	4x
R. morphotaxon *dis9*	*Discolores*	1/1 (FCM)	-	0.692	4x
R. morphotaxon *dis10*	*Discolores*	1/1 (FCM)	-	0.687	4x
R. morphotaxon *dis11*	*Discolores*	1/1 (FCM)	-	0.686	4x
R. morphotaxon *dis12*	*Discolores*	1/1 (FCM)	-	0.674	4x
R. morphotaxon *dis13*	*Discolores*	1/1 (FCM)	-	0.702	4x
R. morphotaxon *dis14*	*Discolores*	1/1 (FCM)	-	0.713	4x
R. morphotaxon *dis15*	*Discolores*	1/1 (FCM)	-	0.710	4x
R. morphotaxon *dis16*	*Discolores*	1/1 (FCM)	-	0.721	4x
R. morphotaxon *dis17*	*Discolores*	1/1 (FCM)	-	0.703	4x
R. sanctus Schreb.	*Discolores*	2/2 (FCM)	0.420 ± 0.001	-	2x
R. "*hirtus*" agg.	*Glandulosi*	3/4 + 2/2 (FCM)	0.667 ± 0.042	0.609 ± 0.001	4x
R. cf. *platyphyllus* C. Koch	*Glandulosi*	11/15 + 2/2 (FCM)	0.675 ± 0.114	0.602 ± 0.002	4x
R. moschus Juz. (morphotype 2)	*Glandulosi*	2/2 (FCM)	0.324 ± 0.039	-	2x
R. moschus Juz. (morphotype 1)	*Glandulosi*	4/9 (FCM)	0.337 ± 0.041	0.305	2x
R. cf. *peruncinatus* (Sudre) Juz.	*Micantes*	1/6 (FCSS)[2]	0.771 ± 0.075	-	4x
R. morphotaxon *mic1*	*Micantes*	1/1 (FCM)	0.709	-	4x
R. morphotaxon *mic2*	*Micantes*	2/2 (FCM)	0.732 ± 0.037	-	4x
R. morphotaxon *mic3*	*Micantes*	1/1 (FCM)	-	0.614	4x
R. morphotaxon *mic4*	*Micantes*	1/1 (FCM)	-	0.602	4x
R. morphotaxon *mic5*	*Micantes*	1/1 (FCM)	-	0.657	4x
R. morphotaxon *rad1*	*Radula*	1/3 (FCM)	0.699 ± 0.037	-	4x
R. morphotaxon *rad2*	*Radula*	1/1 (FCM)	0.568	-	3x
R. morphotaxon *rad3*	*Radula*	1/1 (FCM)	-	0.696	4x
R. canescens x *sanctus*	- (hybrid)	1/1 (FCM)	0.365		2x
R. morphotaxon *cor1*	- (section *Corylifolii)*	1/1 (FCM)	-	0.691	4x
R. idaeus L.	- (subgenus *Idaeobatus*)	1/2 (FCSS)[1]	0.346 ± 0.073	-	2x

[1] Based on standard sexually developed embryos only.
[2] Based on standard apomictically developed embryos only.

The male contribution to the embryo and/or endosperm generally corresponded to the ploidy level of the respective taxon (i.e. haploid sperm in diploid taxa and diploid sperm in tetraploid taxa). Nevertheless, two tetraploid individuals from "morphoseries *Radula* and *Glandulosi*" produced predominantly triploid embryos with pentaploid endosperm, indicating pollination by haploid pollen. Similarly, tetraploid *R.* cf. *peruncinatus* (Sudre) Juz. produced 38 % pentaploid embryos, presumably derived from fertilization of an unreduced egg cell by haploid pollen. One seed with heptaploid embryo and several seeds with exceptionally high ploidy levels of the endosperm (up to 13x) were also observed in this accession.

Table 2: Assessment of reproduction mode using flow cytometric seed screen (FCSS).

				Origin of embryo (%)				Pollen ploidy[1] (%)		
Sample code	Morphotaxon (ploidy)	Morphoseries	#seeds	Red. Fert.	Red. Unfert.	Unred. Fert.	Unred. Unfert.	n=1x	n=2x	n>2x
MS57/13	*R.* morphotaxon *dis2* (4x)	*Discolores*	12	92	0	8	0	0	92	8
MS58/13	*R.* morphotaxon *dis3* (4x)	*Discolores*	10	10	10	20	60	0	70	30
MS30/14	*R.* morphotaxon *dis4* (4x)	*Discolores*	10	10	0	0	90	0	80	20
MS41/14	*R.* morphotaxon *dis5* (4x)	*Discolores*	10	0	0	0	100	0	50	50
MS42/14	*R.* morphotaxon *dis6* (4x)	*Discolores*	10	0	0	10	90	0	90	10
MS33/14B	*R.* "*hirtus*" agg. (4x)	*Glandulosi*	10	100	0	0	0	0	100	0
MS42/13A	*R.* cf. *platyphyllus* (4x)	*Glandulosi*	7	86	0	14	0	0	100	0
MS42/13D	*R.* cf. *platyphyllus* (4x)	*Glandulosi*	9	100	0	0	0	0	100	0
MS44/13	*R.* cf. *platyphyllus* (4x)	*Glandulosi*	10	100	0	0	0	0	100	0
MS46/13C	*R.* cf. *platyphyllus* (4x)	*Glandulosi*	10	100	0	0	0	0	100	0
MS46/13D	*R.* cf. *platyphyllus* (4x)	*Glandulosi*	4	100	0	0	0	0	100	0
MS53/13[2]	*R.* cf. *platyphyllus* (4x)	*Glandulosi*	10	100	0	0	0	100	0	0
MS56/13[3]	*R.* cf. *platyphyllus* (4x)	*Glandulosi*	10	100	0	0	0	0	100	0
MS52/13	*R.* cf. *platyphyllus* (4x)	*Glandulosi*	6	100	0	0	0	0	100	0
MS51/13O	*R. moschus* morphotype 1 (2x)	*Glandulosi*	10	100	0	0	0	100	0	0
MS36/14B	*R. moschus* morphotype 2 (2x)	*Glandulosi*	10	100	0	0	0	100	0	0
MS59/13	*R.* cf. *peruncinatus* (4x)	*Micantes*	11[4]	0	0	45	55	27[5]	18[5]	55[5]
MS44/14	*R.* morphotaxon *mic1* (4x)	*Micantes*	10	10	0	0	90	0	70	30
MS45/14	*R.* morphotaxon *mic2* (4x)	*Micantes*	10	0	0	0	100	0	70	30
MS37/14	*R.* morphotaxon *rad1* (4x)	*Radula*	8	100	0	0	0	87	13	0
MS15/15	*R.* morphotaxon *rad3* (4x)	*Radula*	3	100	0	0	0	0	100	
MS54/13	*R. idaeus*(2x)	- (subgenus *Idaeobatus*)	2	100	0	0	0	100	0	0

Red./Unred. = reduced/unreduced megagametophyte, Fert./Unfert. = fertilized/unfertilized.

[1] Note that in a few cases more than one sperm cell may be involved and/or fusion of more than two maternal nuclei may occur.

[2] Maternal plant could not be measured by flow cytometry measurement (fresh material was not preserved) but probably tetraploid based on morphology and cpDNA haplotype. Fruits collected on natural locality with excess of diploid *Rubus moschus*.

[3] Fruits obtained by greenhouse cultivation.

[4] Two seeds were excluded as no endosperm peak was observed.

[5] Male contribution was impossible to assess due to extraordinarily high ploidy of endosperm indicating involvement of more than three nuclei in endosperm formation and possibly also unusual embryo origin in this individual.

Haplotypic variation does not correspond to morphology

Within 63 samples from 35 species and morphotaxa of "true" brambles (*R.* subgenus *Rubus*), 13 plastid DNA haplotypes were distinguished (Fig. 2) by 13 single nucleotide polymorphisms (1.3 %) in 993 bp *matK* alignment and 18 polymorphic sites (3.9 %) in the 461 bp *trnL-trnF* alignment, including one 6 bp insertion in the *Cae1* haplotype (see the online version of the article). Most haplotypes were restricted to either diploid or polyploid accessions; the only exception was haplotype *San3* which was shared between *R. sanctus* and tetraploid "morphotaxon *dis8*". The most common haplotypes were *Cau1* and locally also *Gla3*, both of which were found in morphologically very distinct polyploid accessions. Three haplotypes were observed in diploid *R. sanctus*, but only one was common and widespread (*San3*). The other two were detected in one (*San4*) or two individuals (*San1*). Both morphotypes of *R. moschus* bore *Gla1* and *Gla5* haplotypes. No morphoseries with more than one accession were defined by a single haplotype. The highest haplotype diversity was observed in "morphoseries *Discolores*", which had seven haplotypes in total (Supplementary table 1).

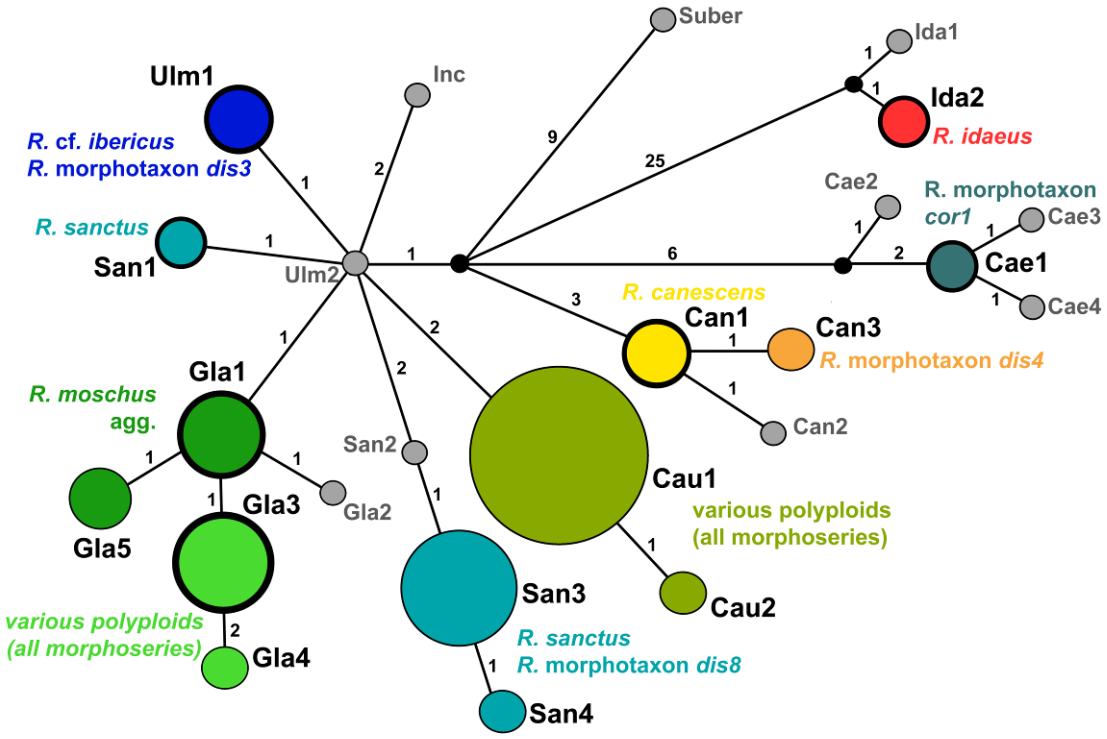


Figure 2: Median-joining plastid haplotype network. Each detected haplotype is denoted by one circle sized according to the number of individuals bearing it. Haplotypes detected by Sochor *et al.* (2015) but not in the present study are shown as small grey circles. Haplotypes detected in both Europe and Colchis are in bold. The number of mutational changes between haplotypes and median vectors (black dots) are shown above branches.

Discussion

Specific ploidy-level pattern in Colchic brambles

Since our sampling covers a great part of Colchis as well as an extensive range of morphological variability, we provide a very first insight into overall cytological diversity in Colchic *Rubus*. The dominance of tetraploid accessions seems slightly deviant relative to both European and North American true brambles. Among North American bramble taxa, ploidy levels are more diverse, including chromosome counts up to nonaploidy in Eastern North America and up to dodecaploidy in Western North America along with high proportions of triploid and diploid accessions (Thompson 1997). Similarly, European brambles exhibit a higher proportion and also more common occurrence of triploid microspecies, especially in Central Europe (Krahulcová *et al.* 2013; and pers. obs.), with most triploids belonging to series *Discolores* (predominantly originating from *R. canescens* as a pistillate ancestor; Sochor *et al.* 2015), or to subsection *Rubus* (Krahulcová *et al.* 2013). In Colchis, however, the haplotype is rarely shared between *R. canescens* and the polyploids (see below), thus indicating only limited involvement of this diploid in Colchic bramble evolution (*cf.* Sochor *et al.* 2015). Subsection *Rubus* (former *Suberecti*) is probably completely absent in the Colchic flora (Juzepczuk 1941). Also, pentaploid and hexaploid accessions, which comprise 7 % of the European bramble flora and a significant part of bramble vegetation in Europe, belong mainly to section *Corylifolii*, a taxon containing (geno)types derived from hybridizations between *R. caesius* L. and members of section *Rubus* (Sochor *et al.* 2015). During three field trips (totalling together around 7 weeks) only one member of section *Corylifolii* was found in Colchis, and its mention in the literature is also rare (Juzepczuk 1941). Thus, *R. caesius* does not seem to hybridize as often in the Western Caucasus as in Europe, although it was found at two sites in Racha and occurs elsewhere in Colchis (Juzepczuk 1952; Kutateladze 1980). Nevertheless, some *Corylifolii* accessions have been observed in Armenia (R. J. Vašut, pers. comm.). The lack of pentaploid lineages may also be related to the scarcity of triploids, which often serve as pistillate parents in pentaploid formation (Sochor *et al.* 2015). The distinctive cytological patterns observed in Colchis, therefore, seem to be shaped mainly by the lack of a few particular taxa, although detailed mechanisms cannot be hypothesized yet.

Peculiar reproduction modes contradict the cytological variability

Despite many cytological and embryological studies in the past (e.g. Longley 1924; Gustafsson 1942; Czapik 1983), we still have only limited knowledge of the natural reproduction processes in brambles, even without mentioning the role of natural selection acting on seeds and seedlings. In addition to artificial crossings (Lidforss 1914; Nybom 1995), it is mainly the development of the FCSS method that has revealed a surprisingly high proportion of sexually derived embryos even in putatively strict apomicts (Šarhanová *et al.* 2012). Although these recombinant seeds do not necessarily have to increase the genetic variability of a population, FCSS has proved to be a suitable method for assessing the degree of residual sexuality of each species and its potential role in the evolution of apomictic genera (Dobeš *et al.* 2014).

Colchic brambles seem to exhibit lower intra-individual variability than European tetraploids (*cf.* Šarhanová *et al.* 2012), as most accessions studied are either strict sexuals or almost strict apomicts (Table 2). The former group includes "morphoseries *Glandulosi*", where only 1 out of 96 embryos originated from an unreduced (though fertilized) megagametophyte. This agrees with the finding of Šarhanová *et al.* (2012) in series *Glandulosi* from the Carpathians. In contrast, most accessions from "morphoseries *Discolores*" and "*Micantes*" exhibited prevalent apomixis with only occasional sex. This is a surprising finding considering the lack of widely distributed morphotypes in our sample set. Although our sampling was not focused on the identification of widely distributed morphotaxa, just one morphotype was sampled more than once. Repeated observations of a single morphotype at different localities were quite rare, as well. This contrasts with the opposite pattern observed in Europe, where several (genetically uniform) accessions exhibit very wide distribution areas (Kurtto *et al.* 2010), and, at the same time, they produce a quite high proportion of sexually derived seeds (Šarhanová *et al.* 2012; and unpublished data). The only exception within the apomictic morphoseries was "morphotaxon *dis2*", with all its egg cells fertilized and mostly reduced (Table 2). This shrub was sampled on a coastal swamp, not more than 500 m from the coast at sea level. It may therefore be hypothesized that environmental factors (such as salt stress), rather than genotype, may have increased the frequency of meiotic megagametophytes in this case, as had previously been observed in the aposporous grass *Cenchrus ciliaris* L. (Gounaris *et al.* 1991) and suggested in *Rubus* (Šarhanová *et al.* 2012).

Two tetraploid (sexual) individuals produced mainly triploid embryos (*R.* cf. *platyphyllus* C. Koch and *R.* morphotaxon *rad1*; Table 2 and online version of the article). Both co-occurred with diploid *R. moschus* agg. which probably served as a pollen donor. Therefore, fertilization of a diploid egg cell by haploid sperm does not seem to be rare in Colchis, even though this is not consistent with the scarcity of triploid accessions detected. Together with approximately 75% of aborted seeds in these individuals (data not shown), this observation implies strong postzygotic selection against triploid embryos following their early development. Further strong selection may also be expected at the seedling and later stages of development, as demonstrated recently, e.g. in poplar hybrids (Lindtke *et al.* 2014), or during the reproductive phase when triploid hybrids fail to reproduce apomictically. Alternatively, high abortion and low germination rates could be caused by an imbalance in the maternal:paternal contribution to the endosperm (Haig 2013). Nevertheless, this seems unlikely for *Rubus* as it is characterized by relaxed requirements for specific ratios of parental genomes in the endosperm (Šarhanová *et al.* 2012). Interestingly, a high proportion of seeds with exceptionally high ploidy levels of the embryo (5x or 7x) and endosperm (up to 13x; see the online version of the article) was detected in one individual of *R.* cf. *peruncinatus* ("morphoseries *Micantes*"). Based on embryological observations, Gerlach (1965) described the fusion of an egg nucleus with two sperm nuclei (originating from the same pollen tube or two different ones) in *R. caesius* and did not exclude the possibility of additional fertilization by a synergid or other nuclei in the megagametophyte. Hypothetically, these exceptional seeds could therefore be formed either via endoreduplication, or due to the involvement of more sperm cells or megagametophyte cells in the embryo and endosperm formation. Although these mechanisms have provoked some controversy in the past, with potentially important evolutionary implications (Dowrick 1961), they have never been extensively studied and their significance in natural populations remains enigmatic.

Haplotype diversity points to isolated evolution of Colchic brambles and regional differentiation

Colchic and European brambles share several ancestral taxa that have participated in the origin of both apomictic complexes via hybridization and polyploidization (Sochor *et al.* 2015). This is supported by our data since six out of thirteen detected haplotypes (excluding *R. idaeus*) are shared with European *Rubus*. Nevertheless, their distributions differ significantly between the regions both in frequency and across ploidy levels. Although many haplotypes are shared between diploids and polyploids in Europe, just one such haplotype (*San3*, originating in *R. sanctus*) was observed in the Colchic accessions (Fig. 2). Moreover, this case may be rather exceptional given that *R. sanctus* apparently rarely serves as a female parent in hybridizations not only in Colchis but also in the Balkans or Armenia (Sochor *et al.* 2015; and the author's unpublished data). The absence of *R. moschus* haplotypes *Gla1*/*Gla5* in Colchic polyploids implies, too, that recent gene flow from this diploid to polyploid accessions is limited (if not absent). However, data from Europe show that this (or closely related) diploid gave rise to the European tetraploid accessions probably in the previous interglacial period (Sochor *et al.* 2015).

Another example of a historical connection between Europe and Colchis is the *Ulm1* haplotype, which is the most common haplotype in Western European diploid *R. ulmifolius*, but was found only in two tetraploid accessions in Colchis. Three haplotypes (*Cae1*, *Gla3* and *San1*) were detected in both regions at the same ploidy level, indicating either shared ancestry, or recent gene-flow (in the case of *San1*, which is characteristic for Balkan *R. sanctus*). On the contrary, seven haplotypes are, as far as we know, specific to the Southern Caucasus (*Cau1*, *Cau2*, *San3*, *San4*, *Gla5*, *Gla4* and *Can3*; see also Sochor *et al.* 2015) and point to the long-term isolation of Caucasian and European brambles.

The haplotype distribution patterns can be explained by preglacial connections of the Caucasian and European bramble floras and their subsequent isolation leading to parallel evolutionary pathways in the two regions. Molecular data from Armenian brambles furthermore points to differentiation even between the East and West Caucasus (Sochor *et al.* 2015; and the author's unpublished data), and some geographic differentiation was detected even within Colchis. The most striking regional differences were observed in "morphoseries *Glandulosi*"; *R. moschus* is apparently restricted to the Western Lesser Caucasus (see also Juzepczuk 1925), where it often dominates the bramble shrub forest vegetation. Meanwhile, *R.* "*hirtus*" agg. (i.e. all undeterminable members of series *Glandulosi* without leaf tomentum, irrespective of colour of stem glands) occurs commonly in the westernmost part of the Lesser Caucasus and also dominates some of the westernmost parts of the Greater Caucasus, but is considerably rarer elsewhere (Juzepczuk 1952). On the other hand, *R.* cf. *platyphyllus* is the most common bramble species in the Central Greater Caucasus and in a somewhat sparsely tomentose form also in a small region in Abkhazia, incidentally coinciding with the distribution of the *Gla3* haplotype (Fig. 1, Supplementary table 1).

Within-region geographic structuring of bramble flora is further supported by the lack of widely distributed apomictic morphotypes (see above). Such strong differentiation

most likely reflects the high diversity of topographic, soil, climatic, and other environmental conditions in the region, forming diverse niches and also ecological or physical barriers to gene-flow (Kikvidze and Ohsawa 2001). Survival of multiple species in different parts of Colchis during the last glacial maximum, post-glacial recolonization routes delimited by their ecological requirements (Álvarez *et al.* 2009) and subsequent hybridizations in contact zones could further shape patterns of species and genetic diversity. At the same time, the long history of human impact on the landscape may have speeded up bramble evolution in the region as it did in Central Europe, although there it led to range expansion of apomicts rather than their geographic differentiation (Matzke-Hajek 1997).

Conclusion and future directions

In this paper we provide the first insights into evolutionary mechanisms and patterns of Colchic brambles. We believe that these data will trigger further evolutionary and taxonomic studies, which will profit not only from traditional morphological methods, but also from cytological and molecular analyses. FCM proved to be an easy-to-use method for detecting diploid accessions, which constitute very important units in *Rubus* evolution and may still be overlooked in the Caucasus. Moreover, knowledge of ploidy level can help to distinguish different taxa and contribute to revealing their evolutionary origins (cf. Šarhanová *et al.* 2012). FCSS, as a method of estimating reproduction mode, provides useful information as to the degree of apomixis and can detect strictly (or predominantly) sexual taxa, which cannot be treated as agamospecies. Colchic brambles can furthermore serve as a good model system for studies of reproduction-related phenomena, as indicated by several of the above-mentioned findings. Molecular methods can provide additional information for phylogenetic systematics. Highly variable sequences (e.g. microsatellites) reveal allelic and clonal diversity and help us to distinguish clonal genotypes – a prerequisite for agamospecies delimitation. They can also confirm recent hybridization events (e.g. Clark and Jasieniuk 2012; and the author's unpublished data). Phylogenetically more conserved molecular markers (e.g. *cp*DNA) can detect different origins of morphologically similar morphotypes while pointing to ancient ancestry, as shown in this and previous studies (Sochor *et al.* 2015). Morphology-based Weberian concept may furthermore help to establish a pragmatic classification system, although it should not form an obstacle for sampling and studying locally distributed biotypes. By utilizing multiple methods and approaches, further studies on Colchic brambles will undoubtedly bring very interesting results with important implications for biogeography, evolution of polyploids and apomixis, plant breeding, etc.

CHAPTER 5: Summary and conclusions

Summary and conclusions

Title: Diversity, phylogenesis and evolutionary mechanisms in the genus *Rubus*

European and Caucasian brambles appear to be among the top taxa in category "taxanomist's nightmare, evolutionist's delight" (MacIntyre 1967). This thesis shows that it is a highly dynamic group with history of extensive hybridization. The apomictic European and Caucasian blackberries mostly originate from only four extant diploid species or species aggregates (*R. ulmifolius–sanctus* agg., *R. canescens*, *R. moschus*, *R. idaeus*) and three extinct species (diploid members of *R.* subsect. *Rubus*, an ancestor of *R. caesius*, an unknown Caucasian diploid), and many species are derived from two tetraploid sexual taxa (*R. caesius*, *R.* ser. *Glandulosi*). Strict or prevalent sexuality of *R. caesius* and *R.* ser. *Glandulosi* and residual sexuality of other biotypes have led to extreme morphological and genetic diversity, especially in free deglaciated regions and in areas with low competition of the sexual ancestors, i.e. in Northwest Europe and the Southern Caucasus.

The Quaternary climate fluctuations and human impact on the landscape probably were the most influential evolutionary drivers as most apomictic lineages were formed only in the Holocene as a result of post-glacial migrations. From this point of view, indeed, apomixis may be seen as a temporary phenomenon or blind alley of evolution (Darlington 1939). On the other hand, there is evidence that the apomicts existed already before the last glacial period. It is highly probable that many of the pre-glacial apomictic lineages went extinct, but some of them survived the last glacial maximum and thus preserved parts of their sexual ancestors' gene pools that would otherwise disappear. Moreover, apomictic lineages represent novel, independent evolutionary units which only occasionally accept genes from contemporary diploids, utilizing both ancient adaptive variation and new locally advantageous alleles. Apomixis thus represents a way of spread of genetic variation in space and time and its preservation for further "evolutionary use".

Although this thesis brings many new insights into the evolutionary mechanisms and trends in apomictic brambles, there are still many questions to be answered. Besides specific taxonomical questions regarding relationships among particular taxa, it is especially genetics and regulation of apomixis that is very poorly known in *Rubus*. Batology and other studies on brambles are therefore far from exhausted and will definitely bring very interesting results in the future.

Shrnutí a závěr

Název práce: Diverzita, fylogeneze a evoluční mechanismy v rodu *Rubus*

Evropské a kavkazské ostružiníky patří mezi přední zástupce kategorie „noční můra taxonoma, rozkoš evolucionisty“ (MacIntyre 1967). Práce ukazuje, že je to velmi dynamická skupina poznamenaná častou hybridizací. Všechny původní ostružiníky Evropy a Kavkazu jsou totiž potomky pouhých čtyř recentních diploidních druhů nebo druhových komplexů (*R. ulmifolius–sanctus* agg., *R. canescens*, *R. moschus*, *R. idaeus*) a tří vyhynulých (diploidi z *R.* subsect. *Rubus*, předchůdce *R. caesius* a neznámý diploid ze západu Jižního Kavkazu) a mnoho apomiktických mikrospecií je odvozeno ze dvou tetraploidních sexuálních taxonů (*R. caesius* a *R. ser. Glandulosi*). Úplná nebo převládající sexualita *R. caesius* a *R. ser. Glandulosi* a reziduální sexualita jiných biotypů tak vedla k extrémní morfologické a genetické diverzitě, zejména v územích postižených zaledněním a v oblastech se sníženými kompetičními tlaky ze strany sexuálních předků, tedy v severozápadní Evropě a na Jižním Kavkazu.

Čtvrtohorní klimatické změny a anthropogenní změny v krajině byly pravděpodobně nejsilnějšími evolučními tlaky, jelikož většina apomiktických linií vznikla až v holocénu jako důsledek postglaciálních migrací. Z tohoto pohledu se apomixe opravdu může jevit jen jako dočasný fenomén – slepá ulička evoluce (Darlington 1939). Na druhou stranu, v práci je předložena řada nepřímých důkazů, že apomiktické ostružiníky existovaly již před posledním glaciálním maximem. Z nich velmi pravděpodobně mnoho vyhynulo, ale alespoň některé přežily až do holocénu a zachovaly taky část ancestrálních genofondů, které by jinak vymizely. Navíc, apomiktické linie reprezentují nové, nezávislé evoluční jednotky jen příležitostně přijímající genetický materiál recentních diploidů. Jsou tak schopny využívat jak adaptivní variabilitu svých dávných předků, tak i nové, lokálně výhodné alely recentních diploidních populací. Apomixe tak spíše než slepou uličku evoluce představuje způsob šíření genetické variability v prostoru a čase a její uchování pro následné „evoluční využití“.

Ačkoliv tato dizertační práce přináší řadu nových poznatků o evolučních mechanismech a trendech apomiktických ostružiníků, zůstává v této oblasti stále mnoho nezodpovězených otázek. Kromě taxonomických problémů kolem příbuznosti konkrétních taxonů je to zejména genetika a regulace apomixe, která je u ostružiníků jen velmi málo prozkoumaná. Batologie a další vědní obory zabývající se ostružiníky tak zdaleka nejsou vyčerpané a v budoucnu jistě přinesou řadu zajímavých poznatků.

References

Akhani H, Djamali M. **2010**. Plant biodiversity of Hyrcanian relict forests, N Iran: an overview of the flora, vegetation, palaeoecology and conservation. *Paistan. Journal of Boany* **42**: 231–258.
Alice L, Campbell C. **1999**. Phylogeny of *Rubus* (Rosaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. *American Journal of Botany* **86**: 81–97.
Alice L, Eriksson T, Eriksen B, Campbell C. **2001**. Hybridization and gene flow between distantly related species of *Rubus* (Rosaceae): evidence from nuclear ribosomal DNA internal transcribed spacer region. *Systematic botany* **26**: 769–778.
Álvarez I, Wendel J. **2003**. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular phylogenetics and evolution* **29**: 417–434.
Álvarez N, Thiel-Egenter C, Tribsch A, Holderegger R, Manel S, Schönswetter P, Taberlet P, Brodbeck S, Gaudeul M, Gielly L, *et al.* 2009. History or ecology? Substrate type as a major driver of patial genetic structure in Alpine plants. *Ecology Letters* **12**: 632–640.
Amsellem L, Noyer J-L, Hossaert-McKey M. **2001**. Evidence for a switch in the reproductive biology of *Rubus alceifolius* (Rosaceae) towards apomixis, between its native range and its area of introduction. *American Journal of Botany* **88**: 2243–2251.
Andreasen K, Baldwin BG. **2003**. Nuclear ribosomal DNA sequence polymorphism and hybridization in checker mallows (*Sidalcea*, Malvaceae). *Molecular Phylogenetics and Evolution* **29**: 563–581.
Antonius K, Nybom H. **1995**. Discrimination between sexual recombination and apomixis/automixis in a *Rubus* plant breeding programme. *Hereditas* **213**: 205–213.
Asker SE, Jerling L. **1992**. *Apomixis in plants*. CRC Press.
Avise J. **2000**. *Phylogeography: the history and formation of species*. Harvard University Press, Cambridge.
Baek YS, Covey PA, Petersen JJ, Chetelat RT, McClure B, Bedinger P A. 2015. Testing the SI x SC rule: Pollen-pistil interactions in interspecific crosses between members of the tomato clade (*Solanum* section *Lycopersicon*, Solanaceae). *American Journal of Botany* **102**: 302–311.
Bailey C, Carr T, Harris S, Hughes C. **2003**. Characterization of angiosperm nrDNA polymorphism, paralogy, and pseudogenes. *Molecular Phylogenetics and Evolution* **29**: 435–455.
Bammi R, Olmo H. **1966**. Cytogenetics of *Rubus*. V. Natural Hybridization Between i PJ Muell. and i Willd. *Evolution* **20**: 617–633.
Bandelt HJ, Forster P, Rohl A. **1999**. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37–48.
Barkman T, Simpson B. **2002**. Hybrid origin and parentage of *Dendrochilum acuiferum* (Orchidaceae) inferred in a phylogenetic context using nuclear and plastid DNA sequence data. *Systematic Botany* **27**: 209–220.
Beck JB, Alexander PJ, Allphin L, Al-Shehbaz IA, Rushworth C, Bailey CD, Windham MD. **2012**. Does hybridization drive the transition to asexuality in diploid *Boechera*? *Evolution* **66**: 985–95.
Bhandari S, Paudayal KN, Momohara A. **2011**. Climate change on the basis of plant macrofossil assemblages from the Late Quaternary sediments from the Mulpani section of the Gokarna Formation, Kathmandu Valley, Nepal. *Journal of Stratigraphic Association of Nepal* **7**: 47–58.
Bicknell R, Koltunow A. **2004**. Understanding apomixis: recent advances and remaining conundrums. *The Plant Cell Online* **16**: 228–246.
Bierzychudek P. **1985**. Patterns in plant parthenogenesis. *Experientia* **41**: 1255–1264.
Bohonak AJ. **1999**. Dispersal, gene flow, and population structure. *The Quarterly Review of Biology* **74**: 21–45.
Bos JAA, Urz R. **2003**. Late Glacial and early Holocene environment in the middle Lahn river valley (Hessen, central-west Germany) and the local impact of early Mesolithic people – pollen and macrofossil evidence. *Vegetation History and Archaeobotany* **12**: 19–36.
Brady EC, Otto-Bliesner BL, Kay JE, Rosenbloom N. **2012**. Sensitivity to glacial forcing in the CCSM4. *Journal of Climate* **26**: 1901–1925.
Brochmann C, Brysting AK, Alsos IG, Borgen L, Grundt HH, Scheen A-C, Elven R.. **2004**. Biological relevance of polyploidy : ecology to genomics. *Biological Journal of the Linnean Society* **82**: 521–536.
Bromham L, Penny D. **2003**. The modern molecular clock. *Nature reviews. Genetics* **4**: 216–24.
Brown JL. **2014**. SDMtoolbox: a python-based GIS toolkit for landscape genetic, biogeographic and species distribution model analyses. *Methods in Ecology and Evolution* **5**: 694–700.
Buckler ES, Holtsford TP. **1996**. *Zea* ribosomal repeat evolution and substitution patterns. *Molecular biology and evolution* **13**: 623–32.
Buckler E, Ippolito A, Holtsford T. **1997**. The evolution of ribosomal DNA divergent paralogues and phylogenetic implications. *Genetics* **145**: 821–832.
Campbell CS, Wojciechowski MF, Baldwin BG, Alice L a, Donoghue MJ. **1997**. Persistent nuclear ribosomal DNA sequence polymorphism in the *Amelanchier* agamic complex (Rosaceae). *Molecular biology and evolution* **14**: 81–90.
Caplan JS, Yeakley JA. **2010**. Water relations advantages for invasive *Rubus armeniacus* over two native ruderal congeners. *Plant Ecology* **210**: 169–179.
Castillo NRF, Reed BM, Graham J, Fernandez-Fernandez F, Bassil N V. **2010**. Microsatellite markers for raspberry and blackberry. *Journal of the American Society for Horticultural Science* **135**: 271–278.
Clark L V, Jasieniuk M. **2012**. Spontaneous hybrids between native and exotic *Rubus* in the Western United States produce offspring both by apomixis and by sexual recombination. *Heredity* **109**: 320–328.
Clark L V., Evans KJ, Jasieniuk M. **2013**. Origins and distribution of invasive *Rubus fruticosus* L. agg. (Rosaceae) clones in the Western United States. *Biological Invasions* **15**: 1331–1342.
Comai L. **2005**. The advantages and disadvantages of being polyploid. *Nature reviews. Genetics* **6**: 836–846.

Comai L, Tyagi AP, Lysak MA. **2003**. FISH analysis of meiosis in *Arabidopsis* allopolyploids. *Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology* **11**: 217–26.

Conord C, Gurevitch J, Fady B. **2012**. Large-scale longitudinal gradients of genetic diversity: a meta-analysis across six phyla in the Mediterranean basin. *Ecology and evolution* **2**: 2600–14.

Cosendai A, Rodewald J, Horandl E. **2011**. Origin and distribution of autopolyploids via apomixis in the alpine species *Ranunculus kuepferi* (Ranunculaceae). *Taxon* **60**: 355–364.

Crane M, Thomas P. **1949**. Reproductive versatility in Rubus; raspberry-blackberry hybrids. *Heredity* **3**: 99–107.

Crow JF. **1994**. Advantages of sexual reproduction. *Developmental Genetics* **15**: 205–213.

Czapik R. **1983**. Embryological problems in *Rubus* L. In: Erdelská O, ed. Fertilization and embryogenesis in ovulated plants. Bratislava: Veda, 375–379.

Darlington CD. **1939**. *The evolution of genetic systems*. Cambridge: Cambridge University Press.

Darriba D, Taboada GL, Doallo RR, Posada D. **2012**. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**: 772.

Davis H. **1990**. Studies in *Rubus*. *Castanea* **55**: 22–30.

Davis P, Meikle R. **1972**. 11. Rubus L. In: Davis P, ed. Flora of Turkey and the East Aegean Islands, vol. 4. Edinburgh University Press, 30–40.

DeVore ML, Pigg KB. **2007**. A brief review of the fossil history of the family Rosaceae with a focus on the Eocene Okanogan Highlands of eastern Washington State, USA, and British Columbia, Canada. *Plant Systematics and Evolution* **266**: 45–57.

Dobeš C, Lückl A, Hülber K, Paule J. **2013**. Prospects and limits of the flow cytometric seed screen – insights from *Potentilla* sensu lato (Potentilleae, Rosaceae). *The New Phytologist* **198**: 605–616.

Doležel J, Doleželová M, Novák FJ. **1994**. Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*). *Biologia Plantarum* **36**: 351–357.

Doležel J, Binarová P, Lucretti S. **1989**. Analysis of nuclear DNA content in plant cells by flow cytometry. *Biologia Plantarum* **31**: 113–120.

Dowrick G. **1961**. Biology of reproduction in *Rubus*. *Nature* **191**: 680–682.

Doyle JJ, Doyle JL. **1987**. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Dubcovsky J, Dvořák J. **1995**. Ribosomal RNA multigene loci: nomads of the Triticeae genomes. *Genetics* **140**: 1367–77.

Dunning LT, Savolainen V. **2010**. Broad-scale amplification of *matK* for DNA barcoding plants, a technical note. *Botanical Journal of the Linnean Society* **164**: 1–9.

Edmonds CA, Lillie AS, Cavalli-Sforza LL. **2004**. Mutations arising in the wave front of an expanding population. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 975–979.

Ehrich D. **2009**. Structure-sum-2009. A series of R functions for summarizing the outputs of the program Structure ver. 2.2.

Einset J. **1951**. Apomixis in American polyploid blackberries. *American Journal of Botany* **38**: 768–772.

Eriksson O, Bremer B. **1991**. Fruit characteristics, life forms, and species richness in the plant family Rubiaceae. *American Naturalist* **138**: 751–761.

Evans LT, Knox RB. **1969**. Environmental control of reproduction in *Themeda australis*. *Australian Journal of Botany* **17**: 375–389.

Excoffier L, Ray N. **2008**. Surfing during population expansions promotes genetic revolutions and structuration. *Trends in Ecology and Evolution* **23**: 347–351.

Fady B, Conord C. **2010**. Macroecological patterns of species and genetic diversity in vascular plants of the Mediterranean basin. *Diversity and Distributions* **16**: 53–64.

Fazekas A, Burgess K, Kesanakurti P. **2008**. Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. *PLoS One* **3**.

Fehrer J, Krak K, Chrtek J. **2009**. Intra-individual polymorphism in diploid and apomictic polyploid hawkweeds (*Hieracium*, Lactuceae, Asteraceae): disentangling phylogenetic signal, reticulation, and noise. *BMC evolutionary biology* **9**: 239.

Flegr J. **2005**. *Evoluční biologie*. Praha: Academia.

Focke W. **1910–1914**. *Species ruborum*. Stuttgart: Schweizerbartsche Verlagsbuchhandlung.

Forster P, Torroni A, Renfrew C, Rohl A. **2001**. Phylogenetic star contraction applied to Asian and Papuan mtDNA evolution. *Molecular Biology and Evolution* **18**: 1864–1881.

Frodin D. **2004**. History and concepts of big plant genera. *Taxon* **53**: 753–776.

Fuertes Aguilar J, Nieto Feliner G. **2003**. Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae). *Molecular Phylogenetics and Evolution* **28**: 430–447.

Gagnidze R. **2005**. *Vascular plants of Georgia. A nomenclatural checklist*. Georgian Academy of Sciences: Tbilisi.

Ganbarov DS, Ibrahimov AS. **2015**. *Astragalus dasyanthus* L . (Fabaceae) a new species to the flora of Azerbaijan. *International Journal of Multidisciplinary Research and Development* **2**: 426–427.

Gerlach D. **1965**. Befruchtung und Autogamie bei *Rubus caesius*. *Biologisches Zentralblatt* **84**: 611–633.

Giorgetta MA, Jungclaus J, Reick CH, Legutke S, Bader J, Böttinger M, Brovkin V, Crueger T, Esch M, Fieg K, *et al.* 2013. Climate and carbon cycle changes from 1850 to 2100 in MPI-ESM simulations for the Coupled Model Intercomparison Project phase 5. *Journal of Advances in Modeling Earth Systems* **5**: 572–597.

Gounaris EK, Sherwood RT, Gounaris I, Hamilton RH, Gustine DL. **1991**. Inorganic salts modify embryo sac development in sexual and aposporous *Cenchrus ciliaris*. *Sexual Plant Reproduction* **4**: 188–192.

Graciá E, Botella F, Anadón JD, Edelaar P, Harris DJ, Giménez Andrés. **2013**. Surfing in tortoises? Empirical signs of genetic structuring owing to range expansion. *Biology Letters* **9**: 20121091.
Graham J, Smith K, MacKenzie K, Jorgenson L, Hackett C, Powell W. **2004**. The construction of a genetic linkage map of red raspberry (*Rubus idaeus* subsp. *idaeus*) based on AFLPs, genomic-SSR and EST-SSR markers. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik* **109**: 740–9.
Grime JP. **1977**. Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. *The American Naturalist* **111**: 1169–1194.
Gustafsson Å. **1947**. Apomixis in higher plants III. Biotype and species formation. *Lunds universitets årsskrift* **43**: 181–370.
Gustafsson Å. **1939**. Differential polyploidy within the blackberries. *Hereditas* **25**: 33–47.
Gustafsson Å. **1942**. The origin and properties of the European blackberry flora. *Hereditas* **28**: 249–277.
Haag CR, Ebert D. **2004**. A new hypothesis to explain geographic parthenogenesis. *Annales Zoologici Fennici* **41**: 539–544.
Haig D. **2013**. Kin conflict in seed development: an interdependent but fractious collective. *Annual review of cell and developmental biology* **29**: 189–211.
Hallatschek O, Hersen P, Ramanathan S, Nelson DR. **2007**. Genetic drift at expanding frontiers promotes gene segregation. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 19926–30.
Hampe A, Arroyo J, Jordano P, Petit RJ. **2003**. Rangewide phylogeography of a bird-dispersed Eurasian shrub: contrasting Mediterranean and temperate glacial refugia. *Molecular Ecology* **12**: 3415–3426.
Hand ML, Koltunow AMG. **2014**. The genetic control of apomixis: Asexual seed formation. *Genetics* **197**: 441–450.
Harpke D, Peterson A. **2006**. Non-concerted ITS evolution in *Mammillaria* (Cactaceae). *Molecular phylogenetics and evolution* **41**: 579–593.
Haveman R. **2013**. Freakish patterns - species and species concepts in apomicts. *Nordic Journal of Botany* **31**: 257–269.
Haveman R, Bijlsma R-J, de Ronde I, Schaminée JHJ. **2016**. Capricious, or tied to history's apron strings? Floristic regions in north-west European brambles (*Rubus* subgenus *Rubus*, Rosaceae). *Journal of Biogeography*: n/a–n/a.
Haveman R, de Ronde I. **2013**. The role of the Weberian Reform in European Rubus research and the taxonomy of locally distributed species – which species should we describe? *Nordic Journal of Botany* **31**: 145–150.
Hewitt GM. **2004**. Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society B: Biological Sciences* **359**: 183–195.
Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. **2005**. Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* **25**: 1965–1978.
Hillis DM, Moritz C, Porter CA, Baker RJ. **1991**. Evidence for biased gene conversion in concerted evolution of ribosomal DNA. *Science* **251**: 308–310.
Hodges S, Arnold M. **1995**. Spurring plant diversification: are floral nectar spurs a key innovation? *Proceedings of the Royal Society of London.* **262**: 343–348.
Hojsgaard D, Hörandl E. **2015**. A little bit of sex matters for genome evolution in asexual plants. *Frontiers in Plant Science* **6**: 1–6.
Hojsgaard D, Schegg E, Valls JFM, Martínez EJ, Quarin CL. **2008**. Sexuality, apomixis, ploidy levels, and genomic relationships among four *Paspalum* species of the subgenus *Anachyris* (Poaceae). *Flora* **203**: 535–547.
Holub J. **1997**. Some considerations and thoughts on the pragmatic classification of apomictic *Rubus* taxa. *Osnabrücker Naturwissenschaftliche Mitteilungen* **23**: 147–155.
Hörandl E. **2009**. Geographical parthenogenesis: opportunities for asexuality. In: Schön I, Martens K, van Dijk P, eds. Lost sex: The evolutionary biology of parthenogenesis. Springer, 161–186.
Hörandl E. **2006**. The complex causality of geographical parthenogenesis. *New Phytologist* **171**: 525–538.
Hörandl E. **2011**. Evolution and biogeography of alpine apomictic plants. *Taxon* **60**: 390–402.
Hörandl E, Paun O. **2007**. Patterns and sources of genetic diversity in apomictic plants: implications for evolutionary potentials. In: Hörandl E, Grossniklaus U, van Dijk PJ, Sharbel TF, eds. Apomixis: evolution, mechanisms and perspectives. Rugell: A.R.G.Gantner Verlag, 169–174.
Houliston GJ, Chapman HM. **2004**. Reproductive strategy and population variability in the facultative apomict Hieracium pilosella (Asteraceae). *American Journal of Botany* **91**: 37–44.
Huelsenbeck J, Ronquist F. **2001**. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.
Hulst RG van der, Mes TH, Den Nijs JC, Bachmann K. **2000**. Amplified fragment length polymorphism (AFLP) markers reveal that population structure of triploid dandelions (*Taraxacum officinale*) exhibits both clonality and recombination. *Molecular ecology* **9**: 1–8.
Huson DH, Bryant D. **2006**. Application of phylogenetic networks in evolutionary studies. *Molecular biology and evolution* **23**: 254–67.
Christen HR. **1950**. Untersuchungen über die Embryologie pseudogamer und sexueller *Rubus*-Arten. *Ber. Schweiz. Bot. Ges.* **60**: 153–198.
Jackson ST, Weng C. **1999**. Late quaternary extinction of a tree species in eastern North America. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 13847–52.
Jakobsson M, Rosenberg NA. **2007**. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**: 1801–1806.

Jennings D, Craig D, Topham P. **1967**. The role of the male parent in the reproduction of Rubus. *Heredity* **22**: 43–55.
Johansen-Morris AD, Latta RG. **2006**. Fitness consequences of hybridization between ecotypes of *Avena barbata*: hybrid breakdown, hybrid vigor, and transgressive segregation. *Evolution* **60**: 1585–1595.
Jordano P. **1982**. Migrant birds are the main seed dispersers of blackberries in southern Spain. *Oikos* **38**: 183–193.
Juzepczuk S V. 1952. Genus 514. *Rubus* L. In: Grossheim AA, ed. Flora of the Caucasus, vol. 5. Academy of Sciences of USSR, Moscow, 44–58.
Juzepczuk S V. 1941. Genus 734. *Rubus*. In: Komarov VL, ed. Flora of U.S.S.R, vol. 10.5–58.
Juzepczuk S V. 1925. Material dlja izuchenia jezhevik kavkaza. *Trudy po Prikladnoj Botanike i Selekktsii* **14**: 139–169.
Kandemir N, Saygili I. **2015**. Apomixis: new horizons in plant breeding. *Turkish Journal of Agriculture and forestry* **39**: 1–8.
Kaplinski L, Andreson R, Puurand T, Remm M. **2005**. MultiPLX: automatic grouping and evaluation of PCR primers. *Bioinformatics* **21**: 1701–1702.
Katoh K, Standley DM. **2013**. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.
Kellogg EA. **1990**. Variation and species limits in agamospermous grasses. *Systematic Botany* **15**: 112–123.
Kiefer C, Dobes C, Sharbel TF, Koch MA. **2009**. Phylogeographic structure of the chloroplast DNA gene pool in North American *Boechera*–a genus and continental-wide perspective. *Molecular phylogenetics and evolution* **52**: 303–311.
Kikvidze Z, Ohsawa M. **2001**. Richness of Colchic vegetation: comparison between refugia of south-western and East Asia. *BMC Ecology* **1**: 6.
Kirschner J, Oplaat C, Verhoeven KJF, Zeisek V, Uhlemann I, Trávníček B, Räsänen J, Wilschut RA, Štěpánek J. **2016**. Identification of oligoclonal agamospermous microspecies: taxonomic specialists versus microsatellites. *Preslia* **88**: 1–17.
Klerk P de. **2002**. Changing vegetation patterns in the Endinger Bruch area (Vorpommern, NE Germany) during the Weichselian Lateglacial and early Holocene. *Review of Palaeobotany and Palynology* **119**: 275–309.
Koch MA, Dobeš C, Mitchell-Olds T. **2003**. Multiple hybrid formation in natural populations: Concerted evolution of the internal transcribed spacer of nuclear ribosomal DNA (ITS) in North American *Arabis divaricarpa* (Brassicaceae). *Molecular Biology and Evolution* **20**: 338–350.
Kollmann J, Steinger T, Roy B. **2000**. Evidence of sexuality in European *Rubus* (Rosaceae) species based on AFLP and allozyme analysis. *American Journal of Botany* **87**: 1592–1598.
Kovarik A, Pires JC, Leitch AR, Lim KY, Sherwood AM, Matyasek R, Rocca J, Soltis DE, Soltis PS. **2005**. Rapid concerted evolution of nuclear ribosomal DNA in two *Tragopogon* allopolyploids of recent and recurrent origin. *Genetics* **169**: 931–44.
Kraft T, Nybom H. **1995**. DNA fingerprinting and biometry can solve some taxonomic problems in apomictic blackberries (Rubus subgen. Rubus). *Watsonia* **343**: 329–343.
Kraft T, Nybom H, Werlemark G. **1995**. *Rubus vestervicensis* (Rosaceae) — its hybrid origin revealed by DNA fingerprinting. *Nordic Journal of Botany* **15**: 237–242.
Krahulcová A, Trávníček B, Šarhanová P. **2013**. Karyological variation in the genus Rubus, subgenus *Rubus* (brambles, Rosaceae): new data from the Czech Republic and synthesis of the current knowledge of European species. *Preslia* **85**: 19–39.
Krahulcová A, Holub J. **1997**. Chromosome number variation in the genus *Rubus* in the Czech Republic. I. *Preslia* **68**: 241–255.
Kurtto A, Weber HE, Lampinen R, Sennikov AN (Eds.). **2010**. *Atlas florae Europaeae. Distribution of vascular plants in Europe, vol. 15: Rosaceae (Rubus)*. Helsinki: Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo.
Kutateladze SI. **1980**. Genus 12. Rubus L. In: Ketskhoveli NN, ed. Flora of Georgia, vol. 6. 95–135.
Lepší M, Vít P, Lepší P, Boublík K, Suda J. **2008**. *Sorbus milensis*, a new hybridogenous species from northwestern Bohemia. *Preslia* **80**: 229–244.
Librado P, Rozas J. **2009**. DNASP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451–1452.
Lidforss B. **1914**. Resume seiner Arbeiten tiber *Rubus*. *Zeitschrift für Induktive Abstammungs- und Vererbungslehre* **12**: 1–13.
Lindtke D, Gompert Z, Lexer C, Buerkle CA. **2014**. Unexpected ancestry of *Populus* seedlings from a hybrid zone implies a large role for postzygotic selection in the maintenance of species. *Molecular Ecology* **23**: 4316–4330.
Lo EYY, Stefanović S, Dickinson TA. 2013. Geographical parthenogenesis in Pacific Northwest hawthorns (*Crataegus*: Rosaceae). *Botany* **91**: 107–116.
Longley AE. **1924**. Cytological studies in the genus *Rubus*. *American Journal of Botany* **11**: 249–282.
Lovell JT, Aliyu OM, Mau M, Schranz ME, Koch M, Kiefer C, Song B-H, Mitchell-Olds T, Sharbel TF. **2013**. On the origin and evolution of apomixis in *Boechera*. *Plant reproduction* **26**: 309–15.
Lysák MA, Doležel J. **1998**. Estimation of nuclear DNA content in *Sesleria* (Poaceae). *Caryologia* **51**: 123–132.
MacIntyre GT. **1967**. Foramen pseudovale and quasi-mammals. *Evolution* **21**: 834–841.
Madlung A. **2013**. Polyploidy and its effect on evolutionary success: old questions revisited with new tools. *Heredity* **110**: 99–104.
Majeský Ľ, Vašut RJ, Kitner M. **2015**. Genotypic diversity of apomictic microspecies of the *Taraxacum scanicum* group (*Taraxacum* sect. *Erythrosperma*). *Plant Systematics and Evolution* **301**: 2105–2124.

Majeský L, Vašut RJ, Kitner M, Trávníček B. **2012**. The pattern of genetic variability in apomictic clones of *Taraxacum officinale* indicates the alternation of asexual and sexual histories of apomicts. *PloS one* **7**: e41868.
Matzk F, Meister A, Schubert I. **2000**. An efficient screen for reproductive pathways using mature seeds of monocots and dicots. *The Plant Journal* **21**: 97–108.
Matzke-Hajek G. **2001**. A revision of Macaronesian *Rubus* taxa (Rosaceae). *Edinburgh Journal of Botany* **58**: 371–382.
Matzke-Hajek G. **1997**. Zur Evolution und Ausbreitung apomiktischer *Rubus*-Arten (Rosaceae) in Offenland-Ökosystemen. *Bulletin of the Geobotanical Institute ETH, Zürich* **63**: 33–44.
Mavrodiev E, Soltis D. **2001**. Recurring polyploid formation: an early account from the Russian literature. *Taxon* **50**: 469–474.
Mayr E. **1942**. *Systematics and origin of species*. Columbia University Press, New York.
Merow C, Smith MJ, Silander JA. **2013**. A practical guide to MAXENT for modeling species' distributions: what it does, and why inputs and settings matter. *Ecography* **36**: 1058–1069.
Mohanty A, Martín JP, Aguinagalde I. **2002**. Population genetic analysis of European *Prunus spinosa* (Rosaceae) using chloroplast DNA markers. *American Journal of Botany* **89**: 1223–1228.
Monasterio-Huelin E, Webert HE. **1996**. Taxonomy and nomenclature of *Rubus ulmifolius* and *Rubus sanctus* (Rosaceae). *Edinburgh Journal of Botany* **53**: 311–322.
Monro A. **2006**. The revision of species-rich genera: a phylogenetic framework for the strategic revision of *Pilea* (Urticaceae) based on cpDNA, nrDNA, and morphology. *American journal of botany* **93**: 426–441.
Moody ME, Muellert LD, Douglas E. **1993**. Genetic variation and random drift n autotetraploid populations.
Mráz P, Chrtek J, Šingliarová B. **2009**. Geographical parthenogenesis, genome size variation and pollen production in the arctic-alpine species *Hieracium alpinum*. *Botanica Helvetica* **119**: 41–51.
Muir G, Fleming C, Schlötterer C. **2001**. Three divergent rDNA clusters predate the species divergence in *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. *Molecular Biology and Evolution* **18**: 112–119.
Muir G, Ruiz-Duarte P, Hohmann N, Mable BK, Novikova P, Schmickl R, Guggisberg A, Koch MA. 2015. Exogenous selection rather than cytonuclear incompatibilities shapes asymmetrical fitness of reciprocal *Arabidopsis* hybrids. *Ecology and Evolution* **5**: 1734–1745.
Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA, Kent J. **2000**. Biodiversity hotspots for conservation priorities. *Nature* **403**: 853–858.
Nathan R, Schurr FM, Spiegel O, Steinitz O, Trakhtenbrot A, Tsoar A. **2008**. Mechanisms of long-distance seed dispersal. *Trends in Ecology and Evolution* **23**: 638–647.
Newmaster SG, Ragupathy S. **2009**. Testing plant barcoding in a sister species complex of pantropical *Acacia* (Mimosoideae, Fabaceae). *Molecular ecology resources* **9**: 172–80.
Nieto Feliner G. **2011**. Southern European glacial refugia: a tale of tales. *Taxon* **60**: 365–372.
Nieto Feliner G. **2014**. Patterns and processes in plant phylogeography in the Mediterranean Basin. A review. *Perspectives in Plant Ecology, Evolution and Systematics* **16**: 265–278.
Nolte AW, Tautz D. **2010**. Understanding the onset of hybrid speciation. *Trends in genetics* **26**: 54–58.
Nybom H. **1995**. Evaluation of interspecific crossing experiments in facultatively apomictic blackberries (*Rubus* subgen. *Rubus*) using DNA fingerprinting. *Hereditas* **122**: 57–65.
Nybom H. **1988**. Apomixis versus sexuality in blackberries (*Rubus* subgen. *Rubus*, Rosaceae). *Plant systematics and evolution* **160**: 207–218.
Nybom H. **1985**. Pollen viability assessments in blackberries (*Rubus* subgen. *Rubus*). *Plant Systematics and Evolution* **150**: 281–290.
Nybom H. **1998**. Biometry and DNA fingerprinting detect limited genetic differentiation among populations of the apomictic blackberry *Rubus nessensis* (Rosaceae). *Nordic Journal of Botany* **18**: 323–333.
Otto-Bliesner BL, Marshall SJ, Overpeck JT, Miller GH, Hu A, members CLIP. **2006**. Simulating arctic climate warmth and icefield retreat in the Last Interglaciation. *Science* **311**: 1751–1753.
Parisod C, Holderegger R, Brochmann C. **2010**. Evolutionary consequences of autopolyploidy. *New Phytologist* **186**: 5–17.
Paun O, Stuessy TF, Hörandl E. **2006**. The role of hybridization, polyploidization and glaciation in the origin and evolution of the apomictic *Ranunculus cassubicus* complex. *The New phytologist* **171**: 223–36.
Peakall R, Smouse PE. **2012**. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research - an update. *Bioinformatics* **28**: 2537–2539.
Phillips S, Anderson R, Schapire R. **2006**. Maximum entropy modeling of species geographic distributions. *Ecological Modelling* **190**: 231–259.
Pichot C, El Maataoui M, Raddi S, Raddi P. **2001**. Surrogate mother for endangered Cupressus. *Nature* **412**: 39.
Piwowarczyk R. **2015**. *Orobanche zajaciorum* (Orobanchaceae): a new species from the Caucasus. *Phytotaxa* **201**: 214–220.
Polzin T, Daneshmand S V. **2003**. On Steiner trees and minimum spanning trees in hypergraphs. *Operations Research Letters* **31**: 12–20.
Pratt C, Einset J. **1955**. Development of the embryo sac in some American blackberries. *American Journal of Botany* **42**: 637–645.
Pratt C, Einset J, Clausen RT. **1958**. Embryology, breeding behavior and morphological characteristics of apomictic, triploid *Rubus idaeus* L. *Bulletin of the Torrey Botanical Club* **85**: 242–254.
Pritchard JK, Stephens M, Donnelly P. **2000**. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Raspé O, Saumitou-Laprade P, Cuguen J, Jacquemart a. L. **2000**. Chloroplast DNA haplotype variation and population differentiation in *Sorbus aucuparia* L. (Rosaceae: Maloideae). *Molecular Ecology* **9**: 1113–1122.
Ray N, Adams JM. **2001**. A GIS-based vegetation map of the world at the last glacial maximum (25,000–15,000 BP). *Internet Archeology* **11**: 0–15.
Raymond M, Rousset F. **1995**. GENEPOP (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. *Journal of Heredity* **86**: 248–249.
Rejmánek M. **2015**. Invasion of *Rubus praecox* (Rosaceae) is promoted by the native tree *Aristotelia chilensis* (Elaeocarpaceae) due to seed dispersal facilitation. *Gayana. Botánica* **72**: 27–33.
Rieseberg L. **1995**. The role of hybridization in evolution: old wine in new skins. *American Journal of Botany* **82**: 944–953.
Rieseberg LH, Willis JH. **2007**. Plant speciation. *Science* **317**: 910–914.
Richards AJ. **1996**. Genetic variability in obligate apomicts of the genus *Taraxacum*. *Folia Geobotanica & Phytotaxonomica* **31**: 405–414.
Ritz CM, Schmuths H, Wissemann V. **2005**. Evolution by reticulation: European dogroses originated by multiple hybridization across the genus *Rosa*. *The Journal of heredity* **96**: 4–14.
Rosenberg NA. **2003**. DISTRUCT: A program for the graphical display of population structure. *Molecular Ecology Notes* **4**: 137–138.
Rosenberg O. **1946**. The influence of low temperatures on the development of the embryo-sac mother cell in *Lilium longiflorum* Thunb. *Hereditas* **31**: 65–92.
Rousset F. **2008**. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* **8**: 103–106.
Rozanova MA. **1934**. Modes of form genesis in the genus *Rubus*. *Journal of Botany of URRS*. **19**: 376–384.
Rozanova MA. **1938**. About the polymorphic origin of species. *Comptes Rendus de l'Academie des Sciences de l'URRS*. **18**: 681–684.
Rozas J, Rozas R. **1997**. DNASP version 2.0: A novel software package for extensive molecular population genetics analysis. *Computer applications in the biosciences : CABIOS* **13**: 307–311.
Ryde U. **2011**. Arguments for a narrow species concept in *Rubus* sect. *Corylifolii*. *Nordic Journal of Botany* **29**: 708–721.
Sang T, Crawford DJ, Stuessy TF. **1995**. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: Implications for biogeography and concerted evolution. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 6813–6817.
Selkoe KA, Toonen RJ. **2006**. Microsatellites for ecologists: A practical guide to using and evaluating microsatellite markers. *Ecology Letters* **9**: 615–629.
Schaefer H, Hardy OJ, Silva L, Barraclough TG, Savolainen V. **2011**. Testing Darwin's naturalization hypothesis in the Azores. *Ecology Letters* **14**: 389–396.
Schupp EW, Jordano P, Gómez JM. **2010**. Seed dispersal effectiveness revisited: A conceptual review. *The New phytologist*: 333–353.
Sochor M, Vašut RJ, Sharbel TF, Trávníček B. **2015**. How just a few makes a lot: Speciation via reticulation and apomixis on example of European brambles (*Rubus* subgen. *Rubus*, Rosaceae). *Molecular Phylogenetics and Evolution* **89**: 13–27.
Sosa PA, González-González EA, González-Pérez MA, Pérez de Paz PL. **2013**. Contrasting patterns of genetic differentiation in macaronesian lineages of *Ilex* (Aquifoliaceae). *Botanical Journal of the Linnean Society* **173**: 258–268.
Stift M, Berenos C, Kuperus P, van Tienderen PH. **2008**. Segregation models for disomic, tetrasomic and intermediate inheritance in tetraploids: a general procedure applied to *Rorippa* (yellow cress) microsatellite data. *Genetics* **179**: 2113–2123.
Sueyoshi T, Ohgaito R, Yamamoto A, Chikamoto MO, Hajima T, Okajima H, Yoshimori M, Abe M, O'ishi R, Saito F, *et al.* 2013. Set-up of the PMIP3 paleoclimate experiments conducted using an Earth system model, MIROC-ESM. *Geoscientific Model Development* **6**: 819–836.
Suh Y, Thien L, Reeve H, Zimmer E. **1993**. Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA in Winteraceae. *American Journal of Botany* **80**: 1042–1055.
Šarhanová P. **2014**. Microevolutionary processes of apomictic *Rubus* subgenus *Rubus*. PhD. Thesis, depon. in library of Palacky University in Olomouc.
Šarhanová P, Vašut RJ, Dančák M, Bureš P, Trávníček B. **2012**. New insights into the variability of reproduction modes in European populations of *Rubus* subgen. *Rubus*: How sexual are polyploid brambles? *Sexual Plant Reproduction* **25**: 319–335.
Taberlet P, Gielly L, Pautou G, Bouvet J. **1991**. Universal primers for amplification of 3 noncoding regions of chloroplast DNA. *Plant Molecular Biology* **17**: 1105–1109.
Tammisola J, Ryynänen A. **1970**. Incompatibility in *Rubus arcticus* L. *Hereditas* **66**: 269–277.
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. **2011**. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution* **28**: 2731–2739.
Tarkhnishvili D, Gavashelishvili A, Mumladze L. **2012**. Palaeoclimatic models help to understand current distribution of Caucasian forest species. *Biological Journal of the Linnean Society* **105**: 231–248.
Tesařová M. **2012**. Stanovení velikosti genomu u vybraných druhů rodu *Rubus* metodou průtokové cytometrie. Master thesis, depon. in library of Palacky University in Olomouc.

Thompson MM. **1997**. Survey of chromosome numbers in *Rubus* (Rosaceae: Rosoideae). *Annals of the Missouri Botanical Garden* **84**: 128–164.
Thompson SL, Whitton J. **2006**. Patterns of recurrent evolution and geographic parthenogenesis within apomictic polyploid Easter daises (*Townsendia hookeri*). *Molecular ecology* **15**: 3389–3400.
Timme RE, Simpson BB, Linder CR. **2007**. High-resolution phylogeny for *Helianthus* (Asteraceae) using the 18S-26S ribosomal DNA external transcribed spacer. *American Journal of Botany* **94**: 1837–1852.
Tomaszewski D, Zieliński J, Gawlak M. **2013**. Foliar indumentum in central-European *Rubus* species (Rosaceae) and its contribution to the systematics of the group. *Nordic Journal of Botany* **31**: 1–10.
Tomlik-Wyremblewska A. **1995**. Pollen morphology of the genus *Rubus*: I. Introductory studies on the European representatives of the subgenus *Rubus* L. *Acta Societatis Botanicorum Poloniae* **64**: 187–203.
Tomlinson J. **1966**. The advantages of hermaphroditism and parthenogenesis. *Journal of Theoretical Biology* **11**: 54–58.
Trávníček B, Zázvorka J. **2005**. Taxonomy of *Rubus* ser. *Discolores* in the Czech Republic and adjacent regions. *Preslia* **77**: 1–88.
Trávníček B, Kirschner J, Štěpánek J, Vašut RJ. **2010**. *Taraxacum* Wiggers - pampeliška (smetánka). Květena České republiky 8. Praha: Academia, 23–269.
Turelli M, Moyle LC. **2007**. Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. *Genetics* **176**: 1059–1088.
van Baarlen P, van Dijk PJ, Hoekstra RF, de Jong JH. **2000**. Meiotic recombination in sexual diploid and apomictic triploid dandelions (*Taraxacum officinale* L.). *Genome* **43**: 827–835.
van Der Hulst RGM, Mes THM, Falque M, Stam P, Den Nijs JCM, Bachmann K. **2003**. Genetic structure of a population sample of apomictic dandelions. *Heredity* **90**: 326–335.
van Dijk PJ. **2003**. Ecological and evolutionary opportunities of apomixis: insights from *Taraxacum* and *Chondrilla*. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **358**: 1113–1121.
van Leeuwen CHA, van der Velde G, van Groenendael JM, Klaassen M. **2012**. Gut travellers: Internal dispersal of aquatic organisms by waterfowl. *Journal of Biogeography* **39**: 2031–2040.
Verhoeven KJ, Biere A. **2013**. Geographic parthenogenesis and plant-enemy interactions in the common dandelion. *BMC Evolutionary Biology* **13**: 23.
Vrijenhoek RC, Parker DE. **2009**. Geographical parthenogenesis: General purpose genotypes and frozen niche variation. In: Schön I, Martens K, van Dijk P, eds. Lost sex: The evolutionary biology of parthenogenesis. Springer, 99–131.
Weber HE. **1996**. Former and modern taxonomic treatment of the apomictic *Rubus* complex. *Folia Geobotanica et Phytotaxonomica* **31**: 373–380.
Weber HE. **2013**. Nomenklatur und Taxonomie zweier Brombeerarten aus der Verwandtschaft der Fuchsbeere (*Rubus nessensis* Hall). *Drosera* **2011**: 107–111.
Weeks A, Simpson B. **2004**. Molecular genetic evidence for interspecific hybridization among endemic Hispaniolan *Bursera* (Burseraceae). *American Journal of Botany* **91**: 976–984.
Weir BS, Cockerham CC. **1984**. Estimating F-Statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.
Wendel JF, Schnabel A, Seelanan T. **1995**. Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). *Proceedings of the National Academy of Sciences of the United States of America* **92**: 280–4.
Werlemark G, Nybom H. **2003**. Pollen donor impact on progenies of pseudogamous blackberries (*Rubus* subgen. *Rubus*). *Euphytica* **133**: 71–80.
White TJ, Bruns T, Lee S, Taylor JW. **1990**. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR Protocols: A guide to methods and applications. New York: Academic Press, Inc., 315–322.
Whitfield J, Lockhart P. **2007**. Deciphering ancient rapid radiations. *Trends in ecology & evolution* **22**: 258–65.
Williams GC. **1971**. *Group selection: A controversy in biology*. Chicago: Aldine Atherton.
Windham MD, Al-Shehbaz IA. 2007. New and noteworthy species of *Boechera* (Brassicaceae) III: Additional sexual diploids and apomictic hybrids. *Harvard Papers in Botany* **12**: 235–257.
Woodhead M, McCallum S, Smith K, Cardle L, Mazzitelli L, Graham J. **2008**. Identification, characterisation and mapping of simple sequence repeat (SSR) markers from raspberry root and bud ESTs. *Molecular Breeding* **22**: 555–563.
Worth JRP, Marthick JR, Jordan GJ, Vaillancourt RE. **2011**. Low but structured chloroplast diversity in *Atherosperma moschatum* (Atherospermataceae) suggests bottlenecks in response to the Pleistocene glacials. *Annals of botany* **108**: 1247–56.
Wright S. **1951**. The genetical structure of populations. *Annals of eugenics* **15**: 323–354.
Zadeh JN, Steenberg CD, Bois JS, Wolfe BR, Pierce MB, Khan AR, Dirks RM, Pierce NA. **2011**. NUPACK: Analysis and design of nucleic acid systems. *Journal of Computational Chemistry* **32**: 170–173.
Záveská Drábková L, Kirschner J, Štěpánek J, Záveský L, Vlček Č. **2009**. Analysis of nrDNA polymorphism in closely related diploid sexual, tetraploid sexual and polyploid agamospermous species. *Plant Systematics and Evolution* **278**: 67–85.
Zhou R, Gong X, Boufford D, Wu C-I, Shi S. **2008**. Testing a hypothesis of unidirectional hybridization in plants: Observations on *Sonneratia*, *Bruguiera* and *Ligularia*. *BMC Evolutionary Biology* **8**: 149.
Zimina RP. **1978**. The main features of the Caucasian Natural landscapes and their conservation, USSR. *Arctic and Alpine Research* **10**: 479–488.

Supplementary information

Chapter 2, Supplementary table 1: List of studied accessions, their herbarium voucher numbers, taxonomic position, geographic origin, obtained cp haplotypes, genbank accession numbers and ploidy level measured by flow cytometry (FC), flow cytometry seed screen (FCSS) or published earlier for the respective microspecies (Krahulcová *et al.* 2013, Kurtto *et al.* 2010).

Herbarium voucher	Taxon	Section	Series	Latitude	Longitude	Locality	Haplotype	Genebank accession no.	Ploidy level
NA[ITS]	*R. odoratus*	-	-	49°35'09"N	17°14'59"E	Czechia, Olomouc - cultivation	Odo	KM036728, 37172, 37678-37685	2x
NA	R. cf. *biflorus*	-	-	27°46'32"N	86°43'19"E	NE Nepal, near Khumjung	OutBfl	KM036730, 37179	NA
OL-Ida02	*R. idaeus*	-	-	49°35'59"N	16°54'59"E	cE Czechia, c Moravia, distr. Prostějov	Ida1	KM036844, 37173	2x
OL-Ida03	*R. idaeus*	-	-	50°02'41"N	15°44'06"E	cN Czechia, E Bohemia, Pardubice	Ida1	KM036845, 37174	2x
NA	*R. idaeus*	-	-	50°46'15"N	14°46'32"E	N Czechia, N Bohemia, Jablonné v Podještědí	Ida2	KM036625, 37176	2x
NA[ITS]	*R. idaeus*	-	-	48°05'50"N	24°24'24"E	SW Ukraine, Zakarpatska oblast, near Rachiv	Ida2	KM036626, 37175, 37405-37411	2x
NA	*R. idaeus*	-	-	43°06'29"N	42°44'40"E	NW Georgia, Svaneti, near Mestia	Ida2	KM036673, 37177	2x (FCSS)
OL-AM32/2	*R. caesius*	*Caesii*	-	38°56'56"N	46°11'45"E	S Armenia, Syunik, near Meghri	Cae1	KM036650, 37159	4x
OL-AM8	*R. caesius*	*Caesii*	-	39°42'38"N	45°12'28" E	c Armenia, near Areni	Cae3	KM036651, 37169	4x
OL-25456	*R. caesius*	*Caesii*	-	48°51'18"N	16°43'29"E	SE Czechia, S Moravia, Nové Mlýny	Cae1	KM036838, 37156	4x
NA	*R. caesius*	*Caesii*	-	49°00'20"N	20°43'17"E	cE Slovakia, near Spišské Podhradie	Cae1	KM036545, 37157	4x
OL-25622	*R. caesius*	*Caesii*	-	49°48'40"N	18°01'28"E	NE Czechia, N Moravia, near Bílovec	Cae1	KM036546, 37152	4x
NA[ITS]	*R. caesius*	*Caesii*	-	49°57'24"N	02°13'47"E	SE France, Picardie, near Amiens	Cae1	KM036547, 37142, 37232-37236	4x
NA	*R. caesius*	*Caesii*	-	42°12'08"N	20°59'21"E	S Kosovo, near Jazhincë	Cae1	KM036548, 37143	4x
OL-R143/11[ITS]	*R. conothyrsoides*	*Rubus*	*Anisacanthi*	52°19'38"N	07°38'35"E	NW Germany, Nordrhein-Westfalen, Ibbenbüren	Gla1	KM036561, 36856, 37281-37288	4x
OL-R189/11	*R. infestus*	*Rubus*	*Anisacanthi*	52°15'41"N	08°47'05"E	NW Germany, Nordrhein-Westfalen, near Bad Oeynhausen	Ulm1	KM036628, 36927	4x
OL-24830[ITS]	*R. canescens*	*Rubus*	*Canescentes*	48°30'39"N	18°24'34"E	cW Slovakia, district Zlaté Moravce	Can1	KM036550, 36889, 37245-37252	2x
NA	*R. canescens*	*Rubus*	*Canescentes*	43°28'30"N	06°55'39"E	SE France, Provence-Alpes-Côte d'Azur, near Fréjus	Can1	KM036652, 36978	2x
NA	*R. canescens*	*Rubus*	*Canescentes*	41°50'08"N	43°15'53"E	c Georgia, near Borjomi	Can1	KM036653, 36979	2x
OL-24885	*R. canescens*	*Rubus*	*Canescentes*	41°40'17"N	20°51'12"E	NW Macedonia, dist. Mavrovo and Rostusha	Can1	KM036551, 36890	2x
OL-24917	*R. canescens*	*Rubus*	*Canescentes*	44°45'09"N	15°22'29"E	NW Croatia, Licko-senjska županija, near Ličko Lešće	Can1	KM036888	2x
OL-R169/13	*R.* aff. *arduennensis*	*Rubus*	*Discolores*	49°09'57"N	08°00'55"E	W Germany, Rheinland-Pfalz, near Landau	Ulm1	KM036643, 37023	NA
OL-R256/13	*R. arduennensis*	*Rubus*	*Discolores*	50°04'20"N	10°39'53"E	c Germany, Bayern, near Königsberg	Can1	KM036644, 36976	NA
NA	*R. armeniacus*	*Rubus*	*Discolores*	49°18'05"N	08°05'32"E	W Germany, Rheinland-Pfalz, near Neustadt	Ulm2	KM036529, 37131	4x
OL-24949	*R. armeniacus*	*Rubus*	*Discolores*	49°34'39"N	17°17'13"E	cE Czechia, c Moravia, Olomouc	Ulm2	KM036528, 36850	4x

OL-R192/10	*R. austromoravicus*	*Rubus*	*Discolores*	49°10'52"N	17°20'45"E	cE Czechia, S Moravia, near Otrokovice	Can1	KM036524, 37061	3x
OL-R19/12	*R. austromoravicus*	*Rubus*	*Discolores*	47°21'55''N	17°49'01''E	NW Hungary, Györ region, near Bakonyszentászló	Can1	KM036525, 37171	3x
OL-25605	*R. austromoravicus*	*Rubus*	*Discolores*	49°27'15"N	18°15'40"E	NE Czechia, NE Moravia, near Rožnov pod Radhoštěm	Can1	KM036526, 36885	3x
OL-R197/10	*R. austroslovacus*	*Rubus*	*Discolores*	49°08'50"N	17°23'06"E	cE Czechia, S Moravia, near Otrokovice	Can1	KM036531, 37059	3x
OL-24836	*R. austroslovacus*	*Rubus*	*Discolores*	48°21'47"N	18°28'02"E	cW Slovakia, district Zlaté Moravce	Can1	KM036532, 36886	3x
OL-R154/12	*R. austroslovacus*	*Rubus*	*Discolores*	49°19'41"N	08°14'53"E	W Germany, Rheinland-Pfalz, near Haßloch	Can1	KM036533, 36887	3x
OL-25591	*R. bifrons*	*Rubus*	*Discolores*	49°08'31"N	13°52'22"E	SW Czechia, S Bohemia, near Volyně	Ulm1	KM036536, 37050	4x
OL-Dus[ITS]	*R. bifrons*	*Rubus*	*Discolores*	49°22'46"N	18°01'14"E	E Czechia, E Moravia, dist. Vsetín	Ulm1	KM036539, 37210-37215, 37668-37669	4x (FC)
OL-R144/09	*R. bifrons*	*Rubus*	*Discolores*	49°08'30"N	13°52'20"E	SW Czechia, S Bohemia, near Volyně	Ulm1	KM037138	4x
OL-24833	*R. bifrons*	*Rubus*	*Discolores*	48°30'40"N	18°24'34"E	cW Slovakia, district Zlaté Moravce	Ulm1	KM036538, 36921	4x
OL-R100/12	*R. bifrons*	*Rubus*	*Discolores*	45°34'00''N	17°20'32''E	N Croatia, Bjelovarsko-bilogorska županija, near Sirač	Ulm1	KM036537, 36920	4x
OL-R145/12	*R. bifrons*	*Rubus*	*Discolores*	49°18'05"N	08°05'28"E	W Germany, Rheinland-Pfalz, near Neustadt	Ulm1	KM036540, 36922	4x
OL-R149/09[ITS]	*R. bohemiicola*	*Rubus*	*Discolores*	49°18'22"N	13°41'35"E	SW Czechia, S Bohemia, near Horažďovice	Can1	KM036541, 37065, 37216-37225	4x
OL-25638	*R. bohemiicola*	*Rubus*	*Discolores*	49°16'45"N	13°53'22"E	S Czechia, S Bohemia, near Strakonice	Can1	KM036542, 36974	4x
OL-R140/10	*R. crispomarginatus*	*Rubus*	*Discolores*	49°16'39"N	17°54'46"E	E Czechia, E Moravia, dist. Vsetín	Can1	KM036558, 37063	3x
OL-R18/09[ITS]	*R. crispomarginatus*	*Rubus*	*Discolores*	48°32'39''N	21°23'15''E	NE Hungary, Borsod-Abaúj-Zemplén, near Gönc	Can1	KM036559, 36891, 37276-37280	3x
OL-R81/09	*R. crispomarginatus*	*Rubus*	*Discolores*	48°03'56''N	20°35'28''E	NE Hungary, Borsod-Abaúj-Zemplén, near Bükkszentkereszt	Can1	KM036560, 36892	3x
OL-R21/12	*R. discosulcatus* ined.	*Rubus*	*Discolores*	47°14'06''N	17°51'29''E	W Hungary, Veszprém region, near Zirc	Can1	KM036564, 37107	3x
OL-R123/09[ITS]	*R. elatior*	*Rubus*	*Discolores*	48°55'33"N	14°24'45"E	SW Czechia, S Bohemia, near České Budějovice	Can1	KM036566, 37120, 37307-37677	3x
OL-R147/09	*R. elatior*	*Rubus*	*Discolores*	49°17'29"N	13°52'37"E	SW Czechia, S Bohemia, near Strakonice	Can1	KM036567, 37108	3x
OL-R173/11[ITS]	*R. elegantispinosus*	*Rubus*	*Discolores*	52°16'45"N	08°09'32"E	NW Germany, Niedersachsen, near Osnabrück	Can1	KM036568, 37081, 37312-37319	4x
OL-R204/11	*R. elegantispinosus*	*Rubus*	*Discolores*	52°18'23"N	08°19'40"E	NW Germany, Niedersachsen, near Osnabrück	Can1	KM036569, 37088	4x
OL-R148/12	*R. flaccidus*	*Rubus*	*Discolores*	49°18'48"N	08°06'17"E	W Germany, Rheinland-Pfalz, near Neustadt	Ulm1	KM036573, 37127	3x
OL-R160/12	*R. flaccidus*	*Rubus*	*Discolores*	49°20'29"N	08°16'44"E	W Germany, Rheinland-Pfalz, near Haßloch	Ulm1	KM036574, 37085	3x
OL-R196/10	*R. flos-amygdalae*	*Rubus*	*Discolores*	49°08'50"N	17°23'05"E	cE Czechia, S Moravia, near Otrokovice	Can1	KM036575, 37111	3x
OL-R185/10	*R. flos-amygdalae*	*Rubus*	*Discolores*	49°12'04"N	17°24'05"E	cE Czechia, S Moravia, near Otrokovice	Can1	KM036576	3x
OL-24959	*R. flos-amygdalae*	*Rubus*	*Discolores*	49°49'23"N	18°01'13"E	NE Czechia, N Moravia, near Bílovec	Can1	KM036577, 36975	3x
OL-R184/11[ITS]	*R. flos-amygdalae*	*Rubus*	*Discolores*	52°23'14"N	07°57'27"E	NW Germany, Niedersachsen, near Osnabrück	Can1	KM036578, 36894	3x
OL-R174/11	*R. geniculatus*	*Rubus*	*Discolores*	52°22'08"N	08°05'02"E	NW Germany, Niedersachsen, near Wallenhorst	Ulm1	KM036582, 37019	4x

OL-R218/12	*R. geniculatus*	*Rubus*	*Discolores*	50°24'07"N	05°51'53"E	E Belgium, Walloon Region, near Stavelot	Ulm1	KM036583, 37128	4x
V. Žíla 5517/12	*R. goniophorus*	*Rubus*	*Discolores*	50°29'33"N	08°33'30"E	cW Germany, Hessen, near Wetzlar	Can1	KM036601, 37089	3x
V. Žíla 5516/12	*R. goniophorus*	*Rubus*	*Discolores*	50°25'38"N	08°25'42"E	cW Germany, Hessen, near Weilmünster	Can1	KM036602, 37075	3x
OL-25457[ITS]	*R. grabowskii*	*Rubus*	*Discolores*	50°09'03"N	15°50'43"E	cN Czechia, E Bohemia, near Hradec Králové	Can1	KM036603, 37060, 37367-37372, 37686	3x
OL-R163/12	*R. grabowskii*	*Rubus*	*Discolores*	49°07'11"N	05°30'24"E	NE France, Lorraine, near Verdun	Can1	KM036607, 37090	3x
OL-R118/09	*R. guttiferus*	*Rubus*	*Discolores*	48°52'03"N	14°21'03"E	SW Czechia, S Bohemia, near Český Krumlov	Can2	KM036611, 37057	3x
OL-R44/09	*R. guttiferus*	*Rubus*	*Discolores*	48°26'04"N	21°18'28"E	NE Hungary, Borsod-Abaúj-Zemplén, near Gönc	Can2	KM036612, 36899	3x
OL-R95/10	*R. guttiferus*	*Rubus*	*Discolores*	47°53'20"N	19°56'37"E	N Hungary, Heves region, near Mátraszentimre	Can2	KM036613, 36900	3x
OL-24840	*R. guttiferus*	*Rubus*	*Discolores*	48°27'43"N	18°18'21"E	cW Slovakia, district Zlaté Moravce	Can2	KM036738, 37110	3x
OL-R116/09	*R. henrici-egonis*	*Rubus*	*Discolores*	48°51'38"N	14°20'53"E	SW Czechia, S Bohemia, near Český Krumlov	Can2	KM036615, 37052	3x
OL-R9/12	*R. henrici-egonis*	*Rubus*	*Discolores*	48°31'16"N	18°25'28"E	cW Slovakia, district Zlaté Moravce	Can2	KM036616, 36901	3x
OL-R90/10	*R. henrici-egonis*	*Rubus*	*Discolores*	47°53'42"N	19°51'42"E	N Hungary, Heves region, near Mátraszentimre	Can2	KM036617, 36902	3x
OL-R178/11	*R. lindebergii*	*Rubus*	*Discolores*	52°22'08"N	08°05'02"E	NW Germany, Niedersachsen, near Osnabrück	Ulm1	KM036637, 37094	4x
OL-MD	*R.* cf. *moestus*	*Rubus*	*Discolores*	49°18'27"N	17°56'56"E	E Czechia, E Moravia, near Vsetín	Can2	KM036678, 36981	4x (FC)
OL-R209/10[ITS]	*R. montanus*	*Rubus*	*Discolores*	49°08'31"N	17°13'40"E	SE Czechia, S Moravia, distr. Kroměříž	Can1	KM036840, 37106, 37656-37661, 37430-37433	3x
OL-24837	*R. montanus*	*Rubus*	*Discolores*	48°27'49"N	18°18'36"E	cW Slovakia, district Zlaté Moravce	Can1	KM036718	3x
OL-24932	*R. montanus*	*Rubus*	*Discolores*	46°49'50"N	15°33'11"E	SE Austria, Steiermark, near Leibnitz	Can1	KM036717, 37099	3x
OL-R164/12	*R. montanus*	*Rubus*	*Discolores*	49°07'11"N	05°30'24"E	NE France, Lorraine, near Verdun	Can1	KM036720, 37082	3x
OL-R94/12	*R. montanus*	*Rubus*	*Discolores*	45°56'32"N	16°53'45"E	N Croatia, Bjelovarsko-bilogorska županija, near Bjelovar	Can1	KM036719, 36907	3x
OL-R227/12	*R. palaefolius*	*Rubus*	*Discolores*	50°38'52"N	07°00'07"E	W Germany, Nordrhein-Westfalen, near Bonn	Ulm1	KM036731, 37125	3x (FC)
OL-R231/12	*R. palaefolius*	*Rubus*	*Discolores*	50°45'28"N	07°10'14"E	W Germany, Nordrhein-Westfalen, near Bonn	Ulm1	KM036732, 37126	3x
NA	*R. parthenocissus*	*Rubus*	*Discolores*	49°02'54"N	14°25'55"E	SW Czechia, S Bohemia, near České Budějovice	Can1	KM036733, 37109	3x
R194/10	*R. parthenocissus*	*Rubus*	*Discolores*	49°10'52"N	17°20'45"E	SE Czechia, distr. Kroměříž	Can1	KM037053	3x
OL-25619	*R. parthenocissus*	*Rubus*	*Discolores*	49°49'23"N	18°01'13"E	NE Czechia, N Moravia, near Bílovec	Can1	KM036734, 37083	3x
OL-R119/09	*R. pericrispatus*	*Rubus*	*Discolores*	48°54'52"N	14°19'57"E	SW Czechia, S Bohemia, near České Budějovice	Can1	KM036736, 37064	3x
OL-R18/12	*R. pericrispatus*	*Rubus*	*Discolores*	47°21'34"N	17°49'01"E	NW Hungary, Györ region, near Bakonyszentászló	Can1	KM036737, 37073	3x
OL-24945	*R. peripragensis* ined.	*Rubus*	*Discolores*	50°41'32"N	14°51'27"E	N Czechia, N Bohemia, near Stráž pod Ralskem	Can1	KM036756, 37115	3x[1]
OL-25589	*R. perperus*	*Rubus*	*Discolores*	49°02'50"N	14°25'55"E	SW Czechia, S Bohemia, Hluboká nad Vltavou	Can1	KM036742, 37054	3x
OL-R12/12	*R. perperus*	*Rubus*	*Discolores*	48°32'01"N	18°24'48"E	cW Slovakia, distr. Partizánske	Can1	KM036743, 36908	3x
OL-R33/10	*R. perperus*	*Rubus*	*Discolores*	47°39'46"N	18°24'07"E	N Hungary, Komárom-Esztergom region, Tata	Can1	KM036744, 37112	3x

OL-R130/10	*R. phyllostachys*	*Rubus*	*Discolores*	49°18’47"N	17°37’59"E	E Czechia, E Moravia, distr. Kroměříž	Can1	KM036748, 37077	3x
OL-R108/12	*R. phyllostachys*	*Rubus*	*Discolores*	45°45’20’’N	17°18’47’’E	N Croatia, Bjelovarsko-bilogorska žup., near Grubišno Polje	Can1	KM036749, 37070	3x
OL-R158/12[ITS]	*R. phyllostachys*	*Rubus*	*Discolores*	49°20’29"N	08°16’44"E	W Germany, Rheinland-Pfalz, near Haßloch	Can1	KM036750, 37071	3x
OL-R102/12	*R.* aff. *phyllostachys*	*Rubus*	*Discolores*	45°34’00’’N	17°20’32’’E	N Croatia, Bjelovarsko-bilogorska županija, near Sirač	Can1	KM036563, 36893	NA
OL-R30/12	*R. pilosipraecox*	*Rubus*	*Discolores*	47°10’49’’N	17°48’24’’E	NW Hungary, Veszprém region, near Hárskút	Can2	KM036752, 37084	4x (FC)
OL-R159/11	*R. polyanthemus*	*Rubus*	*Discolores*	52°24’47"N	07°55’44"E	NW Germany, Niedersachsen, near Bramsche	Ulm2	KM036755, 36986	4x
OL-25624	*R. portae-moravicae*	*Rubus*	*Discolores*	49°48’09"N	18°00’39"E	NE Czechia, N Moravia, near Bílovec	Ulm1	KM036754, 37132	4x
OL-AM36	*R.* aff. *praecox* agg.	*Rubus*	*Discolores*	39°08’43"N	46°26’03"E	SE Armenia, Syunik, near Kapan	Ulm1	KM036679, 37028	NA
OL-AM20	*R.* aff. *praecox* agg.	*Rubus*	*Discolores*	39°29’30"N	46°18’45"E	SE Armenia, Syunik, near Goris	Ulm1	KM036659, 37024	NA
OL-24842	*R.* aff. *praecox* agg.	*Rubus*	*Discolores*	48°27’39"N	18°18’11"E	cW Slovakia, district Zlaté Moravce	Can1	KM036762, 36911	4x
OL-24863	*R. praecox*	*Rubus*	*Discolores*	44°43’00"N	20°28’04"E	cN Serbia, near Beograd	Ulm1	KM036763, 37114	4x
OL-R223/11[ITS]	*R. praecox*	*Rubus*	*Discolores*	49°07’49"N	14°00’33"E	SW Czechia, S Bohemia, near Volyně	Ulm1	KM036757, 37139, 37481-37489	4x
OL-25592	*R. praecox*	*Rubus*	*Discolores*	47°49’25"N	18°49’49"E	S Slovakia, near Štúrovo	Ulm1	KM036758, 37021	4x
OL-R105/12	*R. praecox*	*Rubus*	*Discolores*	45°45’20’’N	17°18’47’’E	N Croatia, Bjelovarsko-bilogorska žup., near Grubišno Polje	Ulm1	KM036759, 37100	4x
OL-R16/12	*R. praecox*	*Rubus*	*Discolores*	47°21’33’’N	17°48’21’’E	NW Hungary, Györ region, near Bakonyszentászló	Ulm1	KM036760, 37101	4x
OL-R187/11	*R. praecox*	*Rubus*	*Discolores*	52°25’10"N	07°56’38"E	NW Germany, Niedersachsen, near Bramsche	Ulm1	KM036761, 37096	4x
OL-R230/12	*R. pseudargenteus*	*Rubus*	*Discolores*	50°38’52"N	07°00’07"E	W Germany, Nordrhein-Westfalen, near Bonn	Ulm2	KM036766, 36987	NA
OL-AM25/30	*R. sanctus* [2]	*Rubus*	*Discolores*	39°26’32"N	46°26’08"E	SE Armenia, near Goris	San2	KM036691, 37016	2x
OL-AM25/6	*R. sanctus* [2]	*Rubus*	*Discolores*	39°26’32"N	46°26’08"E	SE Armenia, near Goris	San3	KM036692	2x
OL-AM34	*R. sanctus* [2]	*Rubus*	*Discolores*	38°54’12"N	46°17’16"E	S Armenia, Syunik, near Meghri	San2	KM036693, 37017	2x
OL-AM38	*R. sanctus* [2]	*Rubus*	*Discolores*	39°12’01"N	46°25’27"E	SE Armenia, Syunik, Kapan	San2	KM036694, 37018	2x
NA	*R. sanctus* [2]	*Rubus*	*Discolores*	42°23’18"N	41°33’60"E	W Georgia, Samegrelo-Zemo Svaneti, Anaklia	San3	KM036689, 37014	2x
NA	*R. sanctus* [2]	*Rubus*	*Discolores*	42°23’41’’N	41°33’57"E	W Georgia, Samegrelo-Zemo Svaneti, Anaklia	San3	KM036690, 37015	2x
OL-24874	*R. sanctus* [2]	*Rubus*	*Discolores*	41°59’46"N	21°33’06"E	N Macedonia, near Skopje	San1	KM036801, 37129	2x
OL-24876	*R. sanctus* [2]	*Rubus*	*Discolores*	41°58’22"N	21°09’35"E	NW Macedonia, distr. Zhelino	San1	KM036802, 37072	2x
OL-24891	*R. sanctus* [2]	*Rubus*	*Discolores*	42°12’08"N	20°59’21"E	S Kosovo, near Jazhincë	San1	KM036803, 36916	2x
OL-24896	*R. sanctus* [2]	*Rubus*	*Discolores*	41°39’54"N	19°40’57"E	N Albania, distr. Lezhë	San1	KM036805, 37013	2x
OL-24903	*R. sanctus* [2]	*Rubus*	*Discolores*	42°26’22"N	18°35’23"E	W Montenegro, near Herceg Novi	Ulm1	KM036807, 36944	2x
NA	*R. sanctus* [2]	*Rubus*	*Discolores*	42°38’23"N	18°07’49"E	S Croatia, Dubrovnik	Ulm1	KM036808, 36945	2x
OL-24906	*R. sanctus* [2]	*Rubus*	*Discolores*	42°55’07"N	17°37’37"E	S Bosnia and Herzegovina, Neum	Ulm1	KM036809, 36946	2x
NA	*R. sanctus* [2]	*Rubus*	*Discolores*	39°42’02"N	22°52’30"E	cE Greece, Thessalia Sterea Ellada, Agiokampos	San1	KM036688, 37012	2x

OL-25620[ITS]	*R. sanctus* [2]	*Rubus*	*Discolores*	42°04'08"N	27°58'01"E	SE Bulgaria, distr. Burgas, Sinemorec	San1	KM036795, 37133, 37596-37600	2x
OL-R35/12	*R. subflaccidus* ined.	*Rubus*	*Discolores*	47°04'01''N	17°35'25''E	NW Hungary, distr. Veszprém, near Ajka	Can1	KM036783, 37102	NA
OL-25547	*R. ulmifolius*	*Rubus*	*Discolores*	32°40'09"N	16°53'12"W	Madeira, near Funchal	Ulm1	KM036671, 37026	2x (FC)
GAT-FB12-15	*R. ulmifolius*	*Rubus*	*Discolores*	37°08'06"N	06°39'49"W	SW Spain, Andalusia, near Punta Umbría	Ulm1	KM036814, 36947	2x
OL-24804	*R. ulmifolius*	*Rubus*	*Discolores*	43°43'58''N	06°48'07"E	SE France, Alpes-Maritimes, near Cannes	Ulm1	KM036816, 37103	2x
OL-24806	*R. ulmifolius*	*Rubus*	*Discolores*	43°16'22''N	06°38'46''E	SE France, Provence-Alpes-Côte d'Azur, Cogolin	Ulm1	KM036817, 37116	2x
OL-24807	*R. ulmifolius*	*Rubus*	*Discolores*	43°46'19''N	06°22'40''E	SE France, Provence-Alpes-Côte d'Azur, near Trigance	Ulm1	KM036818, 37034	2x
OL-24809	*R. ulmifolius*	*Rubus*	*Discolores*	43°15'15''N	06°31'37''E	SE France, Provence-Alpes-Côte d'Azur, Cogolin	Ulm1	KM036819, 37097	2x
OL-24815	*R. ulmifolius*	*Rubus*	*Discolores*	41°17'53"N	01°52'24"E	NE Spain, Catalunya, near Barcelona	Ulm2	KM036820, 37010	2x
OL-24816	*R. ulmifolius*	*Rubus*	*Discolores*	41°39'19"N	02°31'50"E	NE Spain, Catalunya, near Sant Celoni	Ulm1	KM036821, 37035	2x
OL-24818	*R. ulmifolius*	*Rubus*	*Discolores*	42°29'43"N	02°49'16"E	S France, Languedoc-Roussillon, near Le Boulou	Ulm1	KM036822, 37087	2x
OL-MD-K4	*R. ulmifolius*	*Rubus*	*Discolores*	28°07'57"N	17°13'15"W	Canary Islands	Ulm2	KM036703, 37005	2x
MS14/13	*R. ulmifolius*	*Rubus*	*Discolores*	43°35'33''N	07°02'08''E	SE France, Alpes-Maritimes, near Cannes	Ulm2	KM036705, 37007	2x (FC)
NA	*R. ulmifolius*	*Rubus*	*Discolores*	43°23'43''N	06°43'43''E	SE France, Provence-Alpes-Côte d'Azur, Fréjus	Ulm1	KM036706, 37029	2x
NA	*R. ulmifolius*	*Rubus*	*Discolores*	43°45'14''N	07°25'16''E	SE France, Alpes-Maritimes, near Monaco	Ulm1	KM036707, 37030	2x
OL-24895	*R. ulmifolius*	*Rubus*	*Discolores*	42°05'09"N	20°22'34"E	NE Albania, near Kukës	Ulm1	KM036804, 36942	2x
OL-24898	*R. ulmifolius*	*Rubus*	*Discolores*	42°02'41"N	19°29'05"E	N Albania, distr. Shkodër	Ulm1	KM036806, 36943	2x
OL-24909	*R. ulmifolius*	*Rubus*	*Discolores*	43°09'48"N	17°24'56"E	c Croatia, Splitsko-dalmatinska županija, near Vrgorac	Ulm1	KM036797, 36939	2x
OL-24913	*R. ulmifolius*	*Rubus*	*Discolores*	43°51'25"N	15°50'45"E	c Croatia, Šibensko-kninska županija, near Vodice	Ulm1	KM036798, 36940	2x
OL-24918	*R. ulmifolius*	*Rubus*	*Discolores*	44°57'39"N	15°01'50"E	NW Croatia, Ličko-senjska županija, near Senj	Ulm2	KM036799, 37008	2x
OL-24928	*R. ulmifolius*	*Rubus*	*Discolores*	45°39'49"N	14°10'41"E	SW Slovenia, Postojna, near Pivka	Ulm1	KM036800, 36941	2x
OL-R178/12	*R. ulmifolius*	*Rubus*	*Discolores*	50°12'29"N	01°49'38"E	NE France, Picardie, near Abbeville	Ulm1	KM036810, 37086	2x (FC)
OL-R183/12	*R. ulmifolius*	*Rubus*	*Discolores*	50°20'10"N	01°44'56"E	NE France, Picardie, near Abbeville	Ulm1	KM036811, 37032	2x (FC)
OL-R187/12	*R. ulmifolius*	*Rubus*	*Discolores*	50°52'15"N	01°34'59"E	NE France, Nord-Pas-de-Calais, near Calais	Ulm1	KM036812, 37033	2x
OL-R192/12[ITS]	*R. ulmifolius*	*Rubus*	*Discolores*	50°53'08"N	02°30'42"E	NE France, Nord-Pas-de-Calais, near Dunkerk	Ulm2	KM036813, 37009, 37601-37610	2x
OL-Ulm02	*R. ulmifolius*	*Rubus*	*Discolores*	45°13'29"N	13°37'27"E	NW Croatia, Istria, near Poreč	Ulm1	KM036796, 37056	2x
NA	*R. ulmifolius*	*Rubus*	*Discolores*	42°45'43"N	11°52'27"E	c Italy, N Lazio, near Acquapendente	Ulm1	KM036815, 36948	2x
V. Žíla	*R. ulmifolius*	*Rubus*	*Discolores*	50°05'43''N	08°21'08''E	W Germany, Hessen, near Wiesbaden	Ulm1	KM036823, 37140	2x
V. Žíla	*R. ulmifolius*	*Rubus*	*Discolores*	50°40'31''N	07°17'49''E	W Germany, Nordrhein-Westfalen, near Bonn	Ulm1	KM036824, 37130	2x
25574	*R. ulmifolius*	*Rubus*	*Discolores*	41°23'08''N	09°10'22''E	S Corsica, Bonifacio	Ulm2	KM036704, 37006	2x
OL-R140/11	*R. winteri*	*Rubus*	*Discolores*	52°20'12"N	07°38'44"E	NW Germany, N Nordrhein-Westfalen	Ulm2	KM036837, 37062	4x

OL-AM10	*R.* sp.	*Rubus*	*Discolores*[3]	39°41'43"N	45°27'05"E	c Armenia, Vajoc Dzor, near Vayk	Ulm2	KM036658, 36992	NA
OL-AM39-WF	*R.* sp.	*Rubus*	*Discolores*[3]	39°19'42"N	46°22'27"E	S Armenia, Syunik, near Kapan	Gla4	KM036712, 37047	NA
OL-25003	*R.* sp.	*Rubus*	*Discolores*[3]	42°23'29"N	41°34'11"E	W Georgia, Samegrelo-Zemo Svaneti, Anaklia	Cau	KM036656, 37038	NA
OL-AM2	R. sp.	*Rubus*	*Discolores*	40°11'34"N	44°30'54"E	c Armenia, Jerevan	Can1	KM036685, 36982	NA
OL-R184/13	*R. elegans*	*Rubus*	*Glandulosi*	49°02'40"N	08°06'52"E	W Germany, Rheinland-Pfalz, near Landau	Gla1	KM036661, 36993	NA
Ol-R167/11	*R. hilsianus*	*Rubus*	*Glandulosi*	52°15'46"N	07°59'01"E	NW Germany, Niedersachsen, near Osnabrück	Gla1	KM036618, 36868	NA
OL-R211/11	*R. iuvenis*	*Rubus*	*Glandulosi*	51°11'07"N	07°31'10"E	W Germany, Nordrhein-Westfalen, near Halver	Gla3	KM036627, 36870	NA
OL-R311/13	*R. lividus*	*Rubus*	*Glandulosi*	50°11'10"N	12°17'20"E	W Czechia, W Bohemia, near Aš	Gla1	KM036677, 36999	4x
OL-R266/11[ITS]	*R. moschus*	*Rubus*	*Glandulosi*	49°59'33"N	14°33'39"E	Czechia, Praha-Průhonice - cultivation	Gla1	KM036721, 37124, 37434-37441	2x (FC)
OL-25000	*R. moschus*	*Rubus*	*Glandulosi*	41°49'46"N	43°18'50"E	c Georgia, near Borjomi	Gla1	KM036680, 37000	2x (FC)
NA	*R. moschus*	*Rubus*	*Glandulosi*	41°51'43"N	43°14'50"E	c Georgia, near Borjomi	Gla1	KM036681, 37001	2x
OL-24940[ITS]	*R. pedemontanus*	*Rubus*	*Glandulosi*	50°47'36"N	14°52'34"E	NW Czechia, N Bohemia, near Liberec	Gla1	KM036740, 36876, 37462-37468	5x
OL-24963	*R. pedemontanus*	*Rubus*	*Glandulosi*	50°47'14"N	14°02'12"E	NW Czechia, N Bohemia, near Děčín	Gla1	KM036741, 36985	5x
OL-R159/10	*R. pedemontanus*	*Rubus*	*Glandulosi*	49°11'10"N	13°29'47"E	SW Czechia, SW Bohemia, near Sušice	Gla2	KM036739, 37036	5x
OL-R202a/13	*R. pedemontanus*	*Rubus*	*Glandulosi*	49°01'27"N	07°53'10"E	E France, Alsace, near Haguenau	Gla1	KM036684, 37003	5x
OL-R300/13	*R. perlongus*	*Rubus*	*Glandulosi*	50°15'44"N	12°23'49"E	W Czechia, W Bohemia, near Sokolov	Gla1	KM036686, 37004	4x (FC)
NA	*R.* cf. *platyphyllus*	*Rubus*	*Glandulosi*	41°51'41"N	43°14'45"E	c Georgia, near Borjomi	Cau	KM036682, 37041	NA
OL-25002	*R.* cf. *platyphyllus*	*Rubus*	*Glandulosi*	41°50'35"N	43°15'37"E	c Georgia, near Borjomi	Cau	KM036701, 37046	4x (FC)
OL-24996	*R.* cf. *platyphyllus*	*Rubus*	*Glandulosi*	43°05'22"N	42°44'34"E	NW Georgia, Svaneti, near Mestia	Cau	KM036698, 37043	4x (FC)
OL-24997[ITS]	*R.* cf. *platyphyllus*	*Rubus*	*Glandulosi*	43°06'44"N	42°44'51"E	NW Georgia, Svaneti, near Mestia	Cau	KM036699, 37044, 37578-37585	4x (FC)
OL-24998	*R.* cf. *platyphyllus*	*Rubus*	*Glandulosi*	43°01'56"N	42°42'43"E	NW Georgia, Svaneti, near Mestia	Cau	KM036700, 37045	4x (FC)
OL-R183/10	R. sp.[4]	*Rubus*	*Glandulosi*	48°57'55"N	14°32'13"E	SW Czechia, S Bohemia, near České Budějovice	Gla1	KM036847, 37104	4x (FCSS)
OL-R225/11[ITS]	R. sp.[4]	*Rubus*	*Glandulosi*	49°01'49"N	14°19'04"E	SW Czechia, S Bohemia, near České Budějovice	Gla1	KM036586, 37079, 37351-37357	NA
OL-24964	R. sp.[4]	*Rubus*	*Glandulosi*	49°38'34"N	17°21'40"E	cE Czechia, c Moravia, near Olomouc	Gla1	KM036595, 36866	4x (FCSS)
OL-Tes2/10	R. sp.[4]	*Rubus*	*Glandulosi*	49°48'52"N	18°00'09"E	NE Czechia, N Moravia, near Bílovec	Gla1	KM036597, 37051	4x (FC)
NA	R. sp.[4]	*Rubus*	*Glandulosi*	49°8'N	20°8'E	cN Slovakia, Vysoké Tatry	Gla1	KM036598	NA
OL-Zdi	R. sp.[4]	*Rubus*	*Glandulosi*	50°46'08"N	14°52'40"E	NW Czechia, N Bohemia, near Liberec	Gla1	KM036599, 37122	4x (FC)
OL-25616	R. sp.[4]	*Rubus*	*Glandulosi*	41°52'00"N	23°20'18"E	SW Bulgaria, distr. Blagojevgrad, near Bansko	Gla1	KM036592, 36864	NA
OL-24826	R. sp.[4]	*Rubus*	*Glandulosi*	51°49'41"N	10°36'12"E	cN Germany, W Sachsen-Anhalt, near Wernigerode	Gla1	KM036590, 36862	NA
OL-24827	R. sp.[4]	*Rubus*	*Glandulosi*	49°06'16"N	18°06'43"E	E Czechia, E Moravia, near Valašské Klobouky	Gla1	KM036591, 36863	NA

OL-24855	R. sp.[4]	*Rubus*	*Glandulosi*	48°14’07"N	24°11’20"E	SW Ukraine, Zakarpatska oblast, near Rachiv	Gla1	KM036587, 37091	NA
OL-24857[ITS]	R. sp.[4]	*Rubus*	*Glandulosi*	48°05’57"N	24°23’55"E	SW Ukraine, Zakarpatska oblast, near Rachiv	Gla1	KM036588, 36861, 37358-37365	NA
OL-25006	R. sp.[4]	*Rubus*	*Glandulosi*	41°51’43"N	43°14’50"E	c Georgia, near Borjomi	Cau	KM036667, 37040	4x (FC)
OL-24881	R. sp.[4]	*Rubus*	*Glandulosi*	41°40’17"N	20°51’12"E	NW Macedonia, dist. Mavrovo and Rostusha	Gla1	KM036589, 37092	NA
OL-R104/12	R. sp.[4]	*Rubus*	*Glandulosi*	45°45’20’’N	17°18’47’’E	N Croatia, Bjelovarsko-bilogorska žup., near Grubišno Polje	Gla3	KM036846, 36859	NA
OL-R133/12	R. sp.[4]	*Rubus*	*Glandulosi*	46°30’18’’N	16°40’05’’E	W Hungary, Zala, district Letenyei	Gla1	KM036585, 36860	NA
OL-R161/12	R. sp.[4]	*Rubus*	*Glandulosi*	49°07’11"N	05°30’24"E	NE France, Lorraine, near Verdun	Gla1	KM036594, 36865	NA
OL-R197/12	R. sp.[4]	*Rubus*	*Glandulosi*	50°49’50"N	02°57’09"E	NW Belgium, W Vlaanderen, near Ypry	Gla1	KM036593, 36984	NA
OL-25611	R. sp.[4]	*Rubus*	*Glandulosi*	46°34’02’’N	22°42’09"E	NW Romania, Bihor, near Stei	Gla1	KM036596, 36867	NA
OL-MD-K6[ITS]	*R. palmensis*	*Rubus*	*Grandifolii*	28°07’27"N	17°13’27"W	Canary Islands, c part of La Gomera	Ulm2	KM036683, 37002, 37455-37460	2x (FC)
OL-25641	*R. apricus*	*Rubus*	*Hystrix*	49°16’45"N	13°53’22"E	SW Czechia, S Bohemia, near Strakonice	Gla3	KM036527, 36849	4x
OL-25648	*R. bavaricus*	*Rubus*	*Hystrix*	49°22’04"N	12°53’07"E	W Czechia, W Bohemia, near Domažlice	Gla1	KM036534, 36851	4x
OL-24941	*R. koehleri*	*Rubus*	*Hystrix*	50°47’36"N	14°52’34"E	NW Czechia, N Bohemia, near Liberec	Gla1	KM036630, 36871	4x
OL-25617[ITS]	*R. schleicheri*	*Rubus*	*Hystrix*	50°44’00"N	15°00’51"E	NW Czechia, N Bohemia, near Liberec	Gla1	KM036777, 37068, 37533-37542	4x
OL-24943	*R. acanthodes*	*Rubus*	*Micantes*	50°40’48’’N	14°51’00’’E	NW Czechia, N Bohemia, near Stráž pod Ralskem	Gla1	KM036515, 36848	4x
OL-R95/12[ITS]	R. aff. *clusii*	*Rubus*	*Micantes*	45°56’32’’N	16°53’45’’E	N Croatia, Bjelovarsko-bilogorska županija, near Bjelovar	Gla1	KM036555, 36855, 37258-37267	NA
OL-25633	*R. clusii*	*Rubus*	*Micantes*	48°42’11"N	14°41’09"E	SW Czechia, S Bohemia, Novohradské hory	Gla2	KM036556, 36951	4x
OL-VD	*R. glivicensis*	*Rubus*	*Micantes*	49°32’10"N	17°22’12"E	cE Czechia, c Moravia, near Olomouc	Can1	KM036600, 36896	4x
OL-25645	*R. chaerophyllus*	*Rubus*	*Micantes*	50°51’N	14°28’E	NW Czechia, N Bohemia, near Děčín	Gla1	KM036554, 36854	4x
OL-R179/12	*R. micans*	*Rubus*	*Micantes*	50°14’15"N	01°51’25"E	NE France, Picardie, near Abbeville	Gla1	KM036714, 36875	4x
OL-R234/12[ITS]	*R. permutabilis* ined.	*Rubus*	*Micantes*	49°16’38"N	16°25’14"E	cE Czechia, distr. Brno-venkov, Veverská Bitýška	Can2	KM036745, 36909, 37469-37479	5x (FC)
OL-R240/12	*R. permutabilis* ined.	*Rubus*	*Micantes*	49°27’05"N	16°16’12"E	cE Czechia, Vysočina, near Bystřice pod Pernštejnem	Can2	KM036746, 36910	5x (FC)
OL-25005	*R.* cf. *peruncinatus*	*Rubus*	*Micantes* [3]	41°49’46’’N	43°18’50’’E	c Georgia, near Borjomi	Cau	KM036657, 37039	4x (FCSS)
OL-R171/11	*R. raduloides*	*Rubus*	*Micantes*	52°16’45"N	08°09’32"E	NW Germany, Niedersachsen, near Osnabrück	Gla1	KM036772, 36878	NA
OL-25649	*R. silvae-bohemicae*	*Rubus*	*Micantes*	49°30’57"N	12°47’12"E	W Czechia, W Bohemia, distr. Domažlice	Ulm2	KM036784, 36880	4x
OL-25634	*R. silvae-norticae*	*Rubus*	*Micantes*	48°50’49"N	14°27’18"E	SW Czechia, S Bohemia, near Český Krumlov	Gla1	KM036786, 36882	4x
OL-24946[ITS]	*R. tabanimontanus*	*Rubus*	*Micantes*	50°41’32’’N	14°51’27’’E	NW Czechia, N Bohemia, near Stráž pod Ralskem	Gla1	KM036794, 37105, 37586-37595	4x
OL-R158/99[ITS]	*R. vratnensis*	*Rubus*	*Micantes*	49°28’39"N	13°20’40"E	W Czechia, W Bohemia, distr. Klatovy	Gla1	KM036829, 36883, 37629-37637	4x
OL-R201/11[ITS]	*R. hypomalacus*	*Rubus*	*Mucronati*	52°13’18"N	08°40’12"E	NW Germany, Nordrhein-Westfalen, near Bünde	Ulm1	KM036624, 36926, 37390-37396	4x

OL-R156/11	*R. ammobius*	*Rubus*	*Nessenses*	52°23'01"N	07°55'04"E	NW Germany, SW Niedersachsen, near Bramsche	Ulm1	KM036523, 36919	4x
OL-24860	*R. nessensis*	*Rubus*	*Nessenses*	48°04'23"N	24°12'53"E	SW Ukraine, Zakarpatska oblast, near Rachiv	Suber	KM036726, 36956	4x
OL-24951	*R. nessensis*	*Rubus*	*Nessenses*	49°22'42''N	17°43'13''E	cE Czechia, E Moravia, near Bystřice pod Hostýnem	Suber	KM036725, 36967	4x
OL-25590[ITS]	*R. nessensis*	*Rubus*	*Nessenses*	49°48'20"N	18°01'19"E	NE Czechia, N Moravia, near Bílovec	Suber	KM036724, 37134, 37442-37445	4x
OL-R181/12	*R. nessensis*	*Rubus*	*Nessenses*	50°14'15"N	01°51'24"E	NE France, Picardie, near Abbeville	Suber	KM036727, 36957	4x
OL-R208/12	*R. scissoides*[5]	*Rubus*	*Nessenses*	50°26'57"N	05°57'55"E	E Belgium, Walloon Region, near Spa	Suber	KM036781, 36961	4x
OL-R285/13	*R. scissoides*[5]	*Rubus*	*Nessenses*	48°53'50''N	14°53'59''E	SW Czechia, S Bohemia, Novohradské hory	Suber	KM036696, 36972	4x
OL-25607	*R. scissoides*[5]	*Rubus*	*Nessenses*	49°25'19"N	18°20'33"E	E Czechia, Beskydy Mts., near Rožnov pod Radhoštěm	Suber	KM036780, 36960	4x
OL-R243/11[ITS]	*R. scissoides*[5]	*Rubus*	*Nessenses*	48°44'00"N	14°45'56"E	SW Czechia, S Bohemia, Novohradské hory	Suber	KM036778, 37135, 37543-37550	4x
OL-25597	*R. scissoides*[5]	*Rubus*	*Nessenses*	48°54'59"N	14°31'50"E	SW Czechia, S Bohemia, near České Budějovice	Suber	KM036779, 37136	4x
OL-R217/13	*R. scissus*[6]	*Rubus*	*Nessenses*	49 40 45N	07°02'56''E	W Germany, Rheinland-Pfalz, near Birkenfeld	Suber	KM036695, 36971	4x
OL-R181/11[ITS]	*R. foliosus*	*Rubus*	*Pallidi*	52°23'14"N	07°57'27"E	NW Germany, Niedersachsen, near Bramsche	Gla1	KM036579, 36858, 37328-37336	4x
OL-R169/11	*R. loehrii*	*Rubus*	*Pallidi*	52°13'55"N	08°01'26"E	NW Germany, Niedersachsen, near Osnabrück	Ulm2	KM036638, 36872	NA
OL-R178/13	*R. tereticaulis*	*Rubus*	*Pallidi*	49°01'25''N	08°02'38''E	W Germany, Rheinland-Pfalz, near Karlsruhe	Gla2	KM036702, 37037	NA
NA	*R. epipsilos*	*Rubus*	*Radula*	49°05'37"N	14°11'47"E	SW Czechia, S Bohemia, near Vodňany	Ulm2	KM036570, 37121	4x (FC)
NA	*R. indusiatus*	*Rubus*	*Radula*	48°38'47"N	13°24'40"E	SE Germany, E Bayern, near Passau	Ulm2	KM036843, 37123	4x (FC)
OL-25635	*R. muhelicus*	*Rubus*	*Radula*	49°03'52"N	14°25'27"E	SW Czechia, S Bohemia, Hluboká nad Vltavou	Gla2	KM036722, 36952	4x
OL-25630	*R. perpedatus*	*Rubus*	*Radula*	49°22'04"N	12°53'07"E	W Czechia, W Bohemia, near Domažlice	Gla1	KM036747, 36877	4x
OL-24821	*R. radula*	*Rubus*	*Radula*	51°44'02"N	11°13'49"E	cN Germany, W Sachsen-Anhalt, near Quedlinburg	Ulm1	KM036770, 36933	4x
OL-24832[ITS]	*R. radula*	*Rubus*	*Radula*	48°30'42"N	18°24'31"E	cW Slovakia, district Zlaté Moravce	Ulm1	KM036769, 36932, 37517-37524	4x
OL-R84/12	*R. radula*	*Rubus*	*Radula*	46°01'19''N	16°50'58''E	N Croatia, Bjelovarsko-bilogorska županija, near Kapela	Ulm1	KM036771, 36934	4x
OL-R127/09	*R. vatavensis* ined.	*Rubus*	*Radula*	49°00'30"N	14°08'29"E	SW Czechia, S Bohemia, near Prachatice	Gla1	KM036826, 37067	4x (FC)
OL-VZ13[ITS]	*R. vatavensis* ined.	*Rubus*	*Radula*	49°15'01"N	13°46'06"E	SW Czechia, S Bohemia, near Strakonice	Gla1	KM036830, 37078, 37638-37643, 37664-37667	4x (FC)
OL-25575[ITS]	*R. incanescens*	*Rubus*	*Radula*[7]	43°25'06''N	02°00'24"E	S France, Midi-Pyrénées, near Revel	Inc	KM036674, 36997, 37412-37418	2x (FC)
OL-25576	*R.* cf. *incanescens*	*Rubus*	*Radula*[7]	43°26'25''N	02°05'15''E	S France, Midi-Pyrénées, near Revel	Gla1	KM036675, 36998	3x (FC)
OL-R154/11	*R. gelertii*	*Rubus*	*Rhamnifolii*	52°24'03"N	07°53'41"E	NW Germany, Niedersachsen, near Bramsche	Can1	KM036581, 36895	4x
OL-R153/12	*R. gracilis*	*Rubus*	*Rhamnifolii*	49°19'41"N	08°14'53"E	W Germany, Rheinland-Pfalz, near Haßloch	Ulm1	KM036605, 36925	4x
OL-25610[ITS]	*R. gracilis*	*Rubus*	*Rhamnifolii*	49°25'12"N	18°20'06"E	E Czechia, Beskydy Mts., near Rožnov pod Radhoštěm	Ulm1	KM036604, 37093, 37373-37379	4x

OL-25448	*R. laciniatus*	*Rubus*	*Rhamnifolii*	52°26'35''N	16°54'20''E	W Poland, Poznań	Ulm1	KM036635, 36929	4x
OL-R62/98	*R. nemoralis*	*Rubus*	*Rhamnifolii*	49°54'36''N	18°24'20''E	NE Czechia, NE Moravia, near Bohumín	Ulm1	KM036723, 37095	4x
OL-R194/11[ITS]	*R. rhamnifolius*	*Rubus*	*Rhamnifolii*	52°14'32"N	08°45'14"E	NW Germany, Nordrhein-Westfalen, near Bad Oeynhausen	Ulm1	KM036774, 37022, 37525-37532, 37663, 37665	NA
OL-R192/11	*R. rhombifolius*	*Rubus*	*Rhamnifolii*	52°15'03"N	08°49'16"E	NW Germany, Nordrhein-Westfalen, near Bad Oeynhausen	Ulm1	KM036773, 36935	4x
OL-R216/11	*R. stereacanthos*	*Rubus*	*Rhamnifolii*	51°09'09"N	07°34'27"E	NW Germany, Nordrhein-Westfalen, near Meinerzhagen	Ulm1	KM036790, 36938	NA
OL-R133/11	*R. stimulifer*	*Rubus*	*Rhamnifolii*	47°52'48"N	16°33'09"E	NE Austria, Burgenland, near Eisenstadt	Can2	KM036791, 36913	4x
OL-MD-K8	*R. bollei*	*Rubus*	*Rhamnifolii*[8]	28°09'46"N	17°17'59"W	Canary Islands, W part of La Gomera	Ulm2	KM036647, 36989	2x (FC)
OL-MD-K9[ITS]	*R. bollei*	*Rubus*	*Rhamnifolii*[8]	28°09'24"N	17°16'09"W	Canary Islands, W part of La Gomera	Ulm2	KM036648, 36990, 37226-37231	2x (FC)
OL-25637	*R. ambrosius*	*Rubus*	*Rubus*	49°07'57"N	14°00'03"E	SW Czechia, S Bohemia, near Volyně	Suber	KM036521, 36968	3x
OL-R330/13	*R.* aff. *barrandienicus*	*Rubus*	*Rubus*	50°01'36''N	15°50'32''E	cN Czechia, E Bohemia, near Pardubice	Can2	KM036646, 36977	3x (FC)
OL-R261/13	*R. barrandienicus*	*Rubus*	*Rubus*	49°19'52''N	14°11'03''E	SW Czechia, S Bohemia, near Písek	Suber	KM036645, 36970	3x
OL-R210/12	*R. bertramii*	*Rubus*	*Rubus*	50°26'57"N	05°57'55"E	E Belgium, Walloon Region, near Spa	Suber	KM036535, 36954	4x
OL-R155/12	*R. canaliculatus*	*Rubus*	*Rubus*	49°20'26"N	08°14'54"E	W Germany, Rheinland-Pfalz, near Haßloch	Suber	KM036552, 36969	3x (FC)
OL-24935[ITS]	*R. constrictus*	*Rubus*	*Rubus*	46°49'53"N	15°33'11"E	SE Austria, Steiermark, near Leibnitz	Suber	KM036557, 36955, 37268-37275	4x
OL-R152/12[ITS]	*R. divaricatus*	*Rubus*	*Rubus*	49°19'41"N	08°14'53"E	W Germany, Rheinland-Pfalz, near Haßloch	Ulm1	KM036924, 37293-37297	3x
OL-24938	*R. graecensis*	*Rubus*	*Rubus*	49°38'29"N	17°21'37"E	cE Czechia, c Moravia, near Olomouc	Suber	KM036606, 37137	3x
OL-R180/12	*R. integribasis*	*Rubus*	*Rubus*	50°14'15"N	01°51'24"E	NE France, Picardie, near Abbeville	Ulm1	KM036629, 36928	NA
OL-R244/12[ITS]	*R. perrobustus*	*Rubus*	*Rubus*	49°28'39"N	16°30'30"E	cE Czechia, S Moravia, distr. Blansko	Can1	KM036764, 36912, 37490-37495	3x (FC)
OL-24952[ITS]	*R. plicatus*	*Rubus*	*Rubus*	49°21'25''N	17°43'49''E	cE Czechia, E Moravia, near Bystřice pod Hostýnem	Suber	KM036842, 36958, 37480	4x (FCSS)
OL-R202/12	*R. plicatus*	*Rubus*	*Rubus*	50°29'08"N	05°53'48"E	E Belgium, Walloon Region, near Spa	Suber	KM036753, 36959	4x
OL-R160/11	*R. senticosus*	*Rubus*	*Rubus*	52°24'47"N	07°55'44"E	NW Germany, SW Niedersachsen, near Bramsche	Ulm1	KM036782, 36936	4x
OL-24858	*R. sulcatus*	*Rubus*	*Rubus*	48°03'43"N	24°12'30"E	SW Ukraine, Zakarpatska oblast, Rachiv	Suber	KM036793, 36964	4x
OL-24958[ITS]	*R. sulcatus*	*Rubus*	*Rubus*	49°48'20''N	18°1'19"E	NE Czechia, N Moravia, near Bílovec	Suber	KM036792, 36963, 37570-37577	4x
OL-R151/11	*R. vigorosus*	*Rubus*	*Rubus*	52°25'48"N	07°45'10"E	NW Germany, SW Niedersachsen, near Bramsche	Ulm1	KM036828, 36949	4x
OL-R165/11	*R. adspersus*	*Rubus*	*Silvatici*	52°25'33"N	07°52'52"E	NW Germany, SW Niedersachsen, near Bramsche	Ulm1	KM036516, 36917	4x
OL-R152/11	*R. amisiensis*	*Rubus*	*Silvatici*	52°25'12"N	07°45'13"E	NW Germany, SW Niedersachsen, near Bramsche	Ulm1	KM036522, 36918	4x
OL-R158/11	*R. egregius*	*Rubus*	*Silvatici*	52°23'18"N	07°55'24"E	NW Germany, SW Niedersachsen, near Bramsche	Gla1	KM036565, 36857	4x
OL-R139/11	*R. gratus*	*Rubus*	*Silvatici*	52°20'12"N	07°38'44"E	NW Germany, Nordrhein-Westfalen	Ulm1	KM036608, 37080	4x

OL-R118/12	*R. juennensis*	*Rubus*	*Silvatici*	46°19'12"N	17°13'07"E	SW Hungary, Somogy, near Somogyszob	Ulm1	KM036676, 37027	4x (FC)
OL-R206/12	*R. leucandrus* subsp. *belgicus*	*Rubus*	*Silvatici*	50°26'57"N	05°57'55"E	E Belgium, Walloon Region, near Spa	Ulm1	KM036636, 36930	NA
OL-R167/12	*R. macrophyllus*	*Rubus*	*Silvatici*	50°06'06"N	02°09'05"E	NE France, Picardie, near Abbeville	Gla1	KM036640, 36874	4x
OL-R51/12	*R. macrophyllus*	*Rubus*	*Silvatici*	46°50'24"N	17°15'59"E	W Hungary, Zala region, near Keszthely	Gla1	KM036639, 36873	4x
OL-R150/11	*R. platyacanthus*	*Rubus*	*Silvatici*	52°25'48"N	07°45'10"E	NW Germany, SW Niedersachsen, near Bramsche	Ulm1	KM036841, 37020	4x
OL-R136/11	*R. sciocharis*	*Rubus*	*Silvatici*	52°18'23"N	10°50'40"E	cN Germany, E Niedersachsen, near Wolfsburg	Ulm2	KM036776, 36879	4x
OL-R145/11	*R. schlechtendali*	*Rubus*	*Silvatici*	52°21'05"N	07°42'33"E	NW Germany, Nordrhein-Westfalen, Ibbenbüren	Ulm1	KM036787, 36937	NA
OL-R148/11[ITS]	*R. silvaticus*	*Rubus*	*Silvatici*	52°26'11"N	07°44'20"E	NW Germany, SW Niedersachsen, near Bramsche	Gla1	KM036785, 36881, 37551-37560	4x
OL-24848[ITS]	*R. wimmerianus*	*Rubus*	*Silvatici*	49°01'10"N	18°07'29"E	W Slovakia, Trenčín region, near Dubnica nad Váhom	Suber	KM036835, 36966, 37648-37655	4x
OL-R15/12	*R. wimmerianus*	*Rubus*	*Silvatici*	47°21'33"N	17°48'21"E	NW Hungary, Györ region, near Bakonyszentászló	Suber	KM036834, 36965	4x
OL-25618[ITS]	*R. capricollensis*	*Rubus*	*Sprengeliani*	49°48'54"N	18°00'35"E	NE Czechia, N Moravia, near Bílovec	Ulm1	KM036553, 36923, 37253-37257	4x
OL-R212/12[ITS]	*R. sprengelii*	*Rubus*	*Sprengeliani*	50°26'01"N	05°54'20"E	E Belgium, Walloon Region, near Spa	Suber	KM036789, 36962, 37569	4x
OL-R283/13	*R. sprengelii*	*Rubus*	*Sprengeliani*	48°57'24"N	14°44'13"E	SW Czechia, S Bohemia, near Třeboň	Suber	KM036697, 36973	4x
NA	*R. brunneri* Maurer	*Rubus*	*Vestiti* [9]	46°57'50"N	16°42'17"E	W Hungary, Vas region, near Körmendi	Gla1	KM036649, 36991	4x (FC)
OL-R190/11	*R. buhnensis*	*Rubus*	*Vestiti*	52°15'41"N	08°47'05"E	NW Germany, Nordrhein-Westfalen, near Bad Oeynhausen	Gla1	KM036543, 36853	4x
NA	*R. gizellae*	*Rubus*	*Vestiti*	47°00'36"N	16°47'12"E	W Hungary, Vas region, near Vasvár	Gla1	KM036664, 36994	4x (FC)
NA[ITS]	*R. pyramidalis*	*Rubus*	*Vestiti*	50°43'26"N	06°14'19"E	NW Germany, SW Nordrhein-Westfalen, near Aachen	Ulm1	KM036768, 36931, 37508-37516	4x
OL-R157/12[ITS]	*R. vestitus*	*Rubus*	*Vestiti*	49°20'29"N	08°16'44"E	W Germany, S Rheinland-Pfalz, near Neustadt	Ulm2	KM036827, 37011, 37619-37628	4x
OL-R120/12	*R. willibaldi-maureri* ined.	*Rubus*	*Vestiti*	46°20'11"N	17°11'05"E	SW Hungary, Somogy region, near Csurgó	Ulm1	KM036836, 36950	NA
OL-25631	*R. passaviensis*	*Rubus*	*Vestiti*[10]	48°47'54"N	14°23'37"E	SW Czechia, S Bohemia, near Český Krumlov	Gla2	KM036735, 36953	4x
OL-AM33	*R.* cf. *zangezurus*	*Rubus*	NA	38°57'03"N	46°10'33"E	S Armenia, Syunik, near Meghri	Gla4	KM036713, 37048	NA
OL-R142/12[ITS]	*R. pruinosus*	*Corylifolii*	subsect. *Subidaeus*[11]	58°00'06"N	14°26'59"E	S Sweden, near Jönköping	Cae1	KM036765, 37154, 37496-37503	5x
OL-NA	*R. dollnensis*	*Corylifolii*	*Hystricopses*	50°45'28"N	14°46'24"E	N Czechia, N Bohemia, near Jablonné v Podještědí	Cae1	KM036660, 37161	5x (FC)
OL-24961[ITS]	*R. dollnensis*	*Corylifolii*	*Hystricopses*	49°35'51"N	17°2'51"E	cE Czechia, c Moravia, near Olomouc	Cae1	KM036562, 37170, 37673-37306	5x (FC)
OL-R114/12[ITS]	*R. franconicus*	*Corylifolii*	*Sepincola*	46°15'33"N	17°10'18"E	SW Hungary, Somogy, near Csurgó	Cae1	KM036580, 37158, 37337-37350	4x
OL-R199/11	*R. hadracanthos*	*Corylifolii*	*Sepincola*	52°13'35"N	08°41'19"E	NW Germany, Nordrhein-Westfalen, near Bünde	Cae1	KM036614, 37147	4x
OL-24838[ITS]	*R. fasciculatus*	*Corylifolii*	*Subcanescentes*	48°27'49"N	18°18'36"E	cW Slovakia, district Zlaté Moravce	Cae1	KM036571, 37145, 37320-37327	4x

NA	*R. holosericeus*	*Corylifolii*	*Subcanescentes*	47°23'39"N	16°34'59"E	W Hungary, Vas region, near Koszeg	Cae1	KM036668, 37166	5x (FC)
OL-R136/12	*R. holosericeus*	*Corylifolii*	*Subcanescentes*	46°42'16"N	16°28'53"E	W Hungary, Zala, district Lenti	Cae1	KM036619, 37148	5x
OL-24835	R. mancus ined.	*Corylifolii*	*Subcanescentes*	48°21'41"N	18°28'00"E	cW Slovakia, district Zlaté Moravce	Cae2	KM036641, 37155	NA
OL-25612	*R. mollis*	*Corylifolii*	*Subcanescentes*	50°49'20"N	14°27'58"E	N Czechia, N Bohemia, near Česká Kamenice	Cae1	KM036715, 37150	4x
OL-24957[ITS]	*R. orthostachys*	*Corylifolii*	*Suberectigeni*	49°48'22"N	18°1'17"E	NE Czechia. N Moravia, near Bílovec	Cae1	KM036729, 37153, 37446-37454	4x
NA	*R.* aff. *fabrimontanus*	*Corylifolii*	*Subradulae*	46°24'13"N	17°27'49"E	SW Hungary, Somogy region, near Nagybajom	Cae1	KM036663, 37163	6x (FC)
OL-R304/13	*R. fabrimontanus*	*Corylifolii*	*Subradulae*	50°26'09"N	11°55'44"E	E Germany, Sachsen, near Burgstein	Cae2	KM036662, 37162	5x
OL-R198/11[ITS]	*R. camptostachys*	*Corylifolii*	*Subsilvatici*	52°14'32"N	08°45'14"E	NW Germany, Nordrhein-Westfalen, near Bad Oeynhausen	Cae1	KM036549, 37144, 37237-37244	4x
OL-R185/11	*R. ferocior*	*Corylifolii*	*Subsilvatici*	52°23'14"N	07°57'27"E	NW Germany, SW Niedersachsen, near Bramsche	Cae1	KM036572, 37146	4x
OL-R28/12[ITS]	*R. albifrons* ined.	*Corylifolii*	*Subthyrsoidei*	47°14'36"N	17°46'52"E	NW Hungary, distr. Veszprém, near Bakonybél	Can1	KM036530, 37113, 37202-37209	NA
OL-Alb02	*R. albocarpaticus* ined.	*Corylifolii*	*Subthyrsoidei*	49°05'29"N	17°07'46"E	SE Czechia, S Moravia, near Kyjov	Can1	KM036518, 37049	5x[1]
OL-Alb03[ITS]	*R. albocarpaticus* ined.	*Corylifolii*	*Subthyrsoidei*	49°19'17"N	17°23'27"E	cE Czechia, c Moravia, near Kroměříž	Can1	KM036519, 37074, 37190-37196	5x[1]
OL-24829	*R. albocarpaticus* ined.	*Corylifolii*	*Subthyrsoidei*	49°06'44"N	18°06'29"E	E Czechia, E Moravia, near Valašské Klobouky	Can1	KM036520, 36884	5x[1]
OL-25632[ITS]	*R. subditivus* ined.	*Corylifolii*	*Subthyrsoidei*	48°45'45"N	14°44'31"E	SW Czechia, S Bohemia, near Nové Hrady	Cae2	KM036517, 37141, 37180-37189	4x[12]
OL-R322/13	*R. gothicus*	*Corylifolii*	*Subthyrsoidei*	50°03'57"N	15°15'16"E	cNW Czechia, c Bohemia, near Kolín	Cae1	KM036665, 37164	4x (FC)
OL-R331/13	*R. gothicus*	*Corylifolii*	*Subthyrsoidei*	50°01'36"N	15°50'32"E	cN Czechia, E Bohemia, near Pardubice	Cae1	KM036666, 37165	4x (FC)
OL-25594[ITS]	*R. grossus* agg. (sp. 1)	*Corylifolii*	*Subthyrsoidei*	49°24'34"N	12°50'11"E	W Czechia, W Bohemia, near Domažlice	Can1	KM036788, 37117, 37561-37564	NA
OL-25596[ITS]	*R. grossus* agg. (sp. 2)	*Corylifolii*	*Subthyrsoidei*	49°48'31"N	18°01'19"E	NE Czechia, N Moravia, near Bílovec	Can2	KM036839, 37066, 37565-37568	NA
V. Žíla[ITS]	*R. grossus*	*Corylifolii*	*Subthyrsoidei*	49°17'26"N	13°52'41"E	SW Czechia, S Bohemia, near Strakonice	Can1	KM036609, 36897, 37380-37389	5x
V. Žíla	*R. grossus*	*Corylifolii*	*Subthyrsoidei*	49°05'37"N	12°38'48"E	SE Germany, Bayern, near Cham	Can1	KM036610, 36898	5x
OL-Kul01[ITS]	*R. kuleszae*	*Corylifolii*	*Subthyrsoidei*	49°19'43"N	16°51'06"E	cE Czechia, c Moravia, near Vyškov	Can2	KM036631, 37069, 37419-37425	5x
OL-25593[ITS]	*R. kuleszae*	*Corylifolii*	*Subthyrsoidei*	49°48'28"N	18°01'18"E	NE Czechia, N Moravia, near Bílovec	Can2	KM036632, 37058, 37426-37429	5x
OL-24845	*R. kuleszae*	*Corylifolii*	*Subthyrsoidei*	48°27'23"N	18°17'27"E	cW Slovakia, district Zlaté Moravce	Can2	KM036634, 36906	5x
OL-R243/12	*R. kuleszae*	*Corylifolii*	*Subthyrsoidei*	49°28'39"N	16°30'30"E	cE Czechia, W Moravia, near Kunštát	Can2	KM036633, 36905	5x (FC)
OL-R263/11[ITS]	*R.* aff. *wahlbergi*	*Corylifolii*	*Subthyrsoidei*	50°36'55"N	14°15'18"E	cNW Czechia, N Bohemia, distr. Litoměřice	Can1	KM036767, 37119, 37504-37507	NA
OL-R143/12	*R. wahlbergii*	*Corylifolii*	*Subthyrsoidei*	58°46'22"N	17°05'16"E	SE Sweden, Södermanland, near Nyköping	Can1	KM036832, 36914	5x
OL-R279/11[ITS]	*R. wahlbergii*	*Corylifolii*	*Subthyrsoidei*	50°03'55"N	17°40'34"E	NE Czechia, N Moravia, near Krnov	Can1	KM036831, 37055, 37644-37647	5x

A. Theofilovski	*R.* cf. *wahlbergii*	*Corylifolii*	*Subthyrsoidei*	41°49'25"N	21°28'04"E	cN Macedonia, distr. Studenichani	Can1	KM036833, 36915	5x
OL-24834	*R. bifrons* x *R.* ser. *Glandulosi*	NA (hybrid)	NA (hybrid)	48°31'08"N	18°24'39"E	cW Slovakia, district Zlaté Moravce	Gla1	KM036544, 36852	NA
OL-R35/08	*R. bifrons* x *R.* ser. *Glandulosi*	NA (hybrid)	NA (hybrid)	49°29'50"N	18°10'41"E	E Czechia, Beskydy Mts., near Rožnov pod Radhoštěm	Gla1	KM037076	4x (FC)
OL-24825	*R. caesius* x ?	NA (hybrid)	NA (hybrid)	45°03'06''N	09°45'38''E	N Italy, Emilia Romagna, near Piacenta	Cae1	KM036655, 37160	3x (FC)
OL-24843[ITS]	*R. caesius* x ?	NA (hybrid)	NA (hybrid)	48°27'39"N	18°17'21"E	cW Slovakia, district Zlaté Moravce	Cae1	KM036620, 37149, 37397-37404	NA
M. Lepší[ITS]	*R. canescens* x *R. crispomarginatus*	NA (hybrid)	NA (hybrid)			SW Czechia, S Bohemia	Can1	KM036623, 36904, 37289-37292	4x (FC)
NA	*R. canescens* x *R. sanctus*	NA (hybrid)	NA (hybrid)	41°50'08"N	43°15'53"E	c Georgia, near Borjomi	Can1	KM036654, 36980	3x (FC)
OL-24812	*R. canescens* x *R. ulmifolius*	NA (hybrid)	NA (hybrid)	43°24'05"N	06°34'48"E	SE France, Provence-Alpes-Côte d'Azur, near Fréjus	Can1	KM036622, 36903	NA
NA	R. cv. Thornfree (American hybrid)	NA (hybrid)	NA (hybrid)	49°26'25"N	18°11'50"E	Czechia, Rožnov pod Radhoštěm - cultivation	Ulm2	KM036642, 36988, 37197-37201	4x (FC)
OL-25548	R. cf. serrae x ?	NA (hybrid)	NA (hybrid)	32°40'07"N	16°52'51"W	Madeira, near Funchal	Mad2	KM036672, 36996	3x (FC)
OL-25542	*R.* cf. x *wolfredoi-wildpretii*	NA (hybrid)	NA (hybrid)	32°45'15"N	17°01'19"W	Madeira, near Vargem	Ulm1	KM036669, 37025	3x (FC)
OL-25544	*R.* cf. x *wolfredoi-wildpretii*	NA (hybrid)	NA (hybrid)	32°45'13"N	17°07'45"W	Madeira, near Calheta	Mad1	KM036670, 36995	3x (FC)
OL-R235/12	*R. idaeus* x *R. caesius*	NA (hybrid)	NA (hybrid)	49°16'38"N	16°25'14"E	cE Czechia, distr. Brno-venkov, Veverská Bitýška	Ida2	KM036751, 37178	3x (FC)
NA	*R. moschus* x *R.* cf. ser. *Discolores*	NA (hybrid)	NA (hybrid)	41°50'21"N	43°15'47"E	c Georgia, near Borjomi	Cau	KM036687, 37042	3x (FC)
OL-24846	*R.* ser. *Glandulosi* x ?	NA (hybrid)	NA (hybrid)	48°27'27"N	18°18'21"E	cW Slovakia, district Zlaté Moravce	Gla1	KM036621, 36869	NA
OL-24933	*R.* ser. *Glandulosi* x ?	NA (hybrid)	NA (hybrid)	46°49'50"N	15°33'11"E	SE Austria, Steiermark, near Leibnitz	Gla1	KM036584, 37098	NA
OL-MS15/13	*R. ulmifolius* x ?	NA (hybrid)	NA (hybrid)	43°35'33''N	07°02'08''E	SE France, Alpes-Maritimes, near Cannes	Can1	KM036708, 36983	2x (FC)
OL-MS17/13	*R. ulmifolius* x ?	NA (hybrid)	NA (hybrid)	43°28'30''N	06°55'39''E	SE France, Provence-Alpes-Côte d'Azur, near Fréjus	Ulm1	KM036709, 37031	2x (FC)
OL-MS09/13	*R. ulmifolius* x *R. caesius*	NA (hybrid)	NA (hybrid)	44°35'37''N	08°39'55''E	NW Italy, Piemont, near Ovada	Cae1	KM036711, 37168	3x (FC)
OL-R189/12[ITS]	*R. ulmifolius* x *R. caesius*	NA (hybrid)	NA (hybrid)	50°55'30"N	01°42'51"E	NE France, Nord-Pas-de-Calais, near Calais	Cae1	KM036825, 37151, 37611-37618	4x (FC)
OL-R191/12	*R. ulmifolius* x *R. caesius*	NA (hybrid)	NA (hybrid)	50°53'08"N	02°30'42"E	NE France, Nord-Pas-de-Calais, near Dunkerk	Cae4	KM036710, 37167	4x (FCSS)

Notes:

NA - Not Available

[ITS] Sample used for ITS sequencing

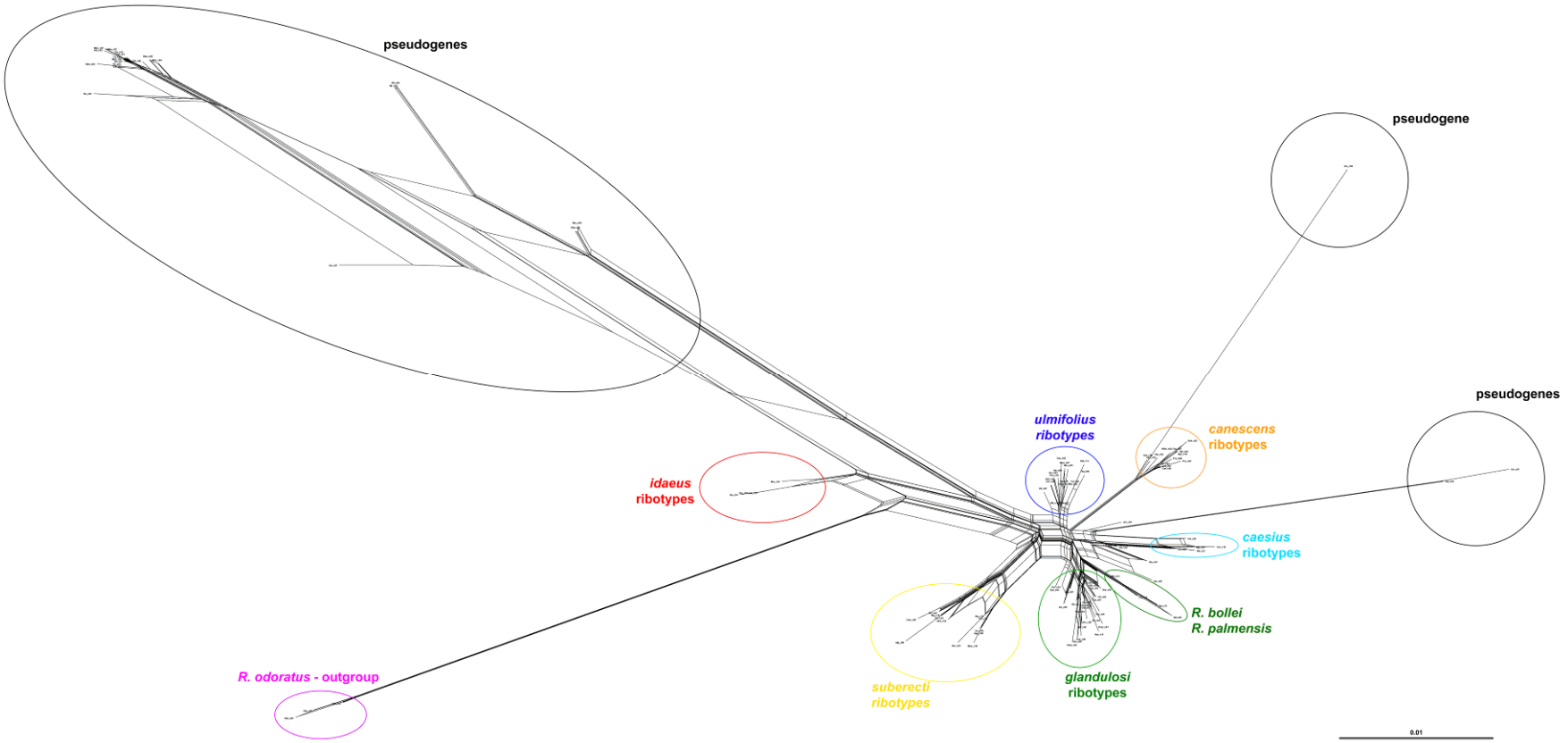
[1] Determined by A. Krahulcová.

[2] Member of *R. ulmifolius* agg.; acknowledging haplotype differentiation, we adopt two-species concept by Monasterio-Huelin et Weber (1996), although delimitation of both species is difficult in western Balkan Peninsula.

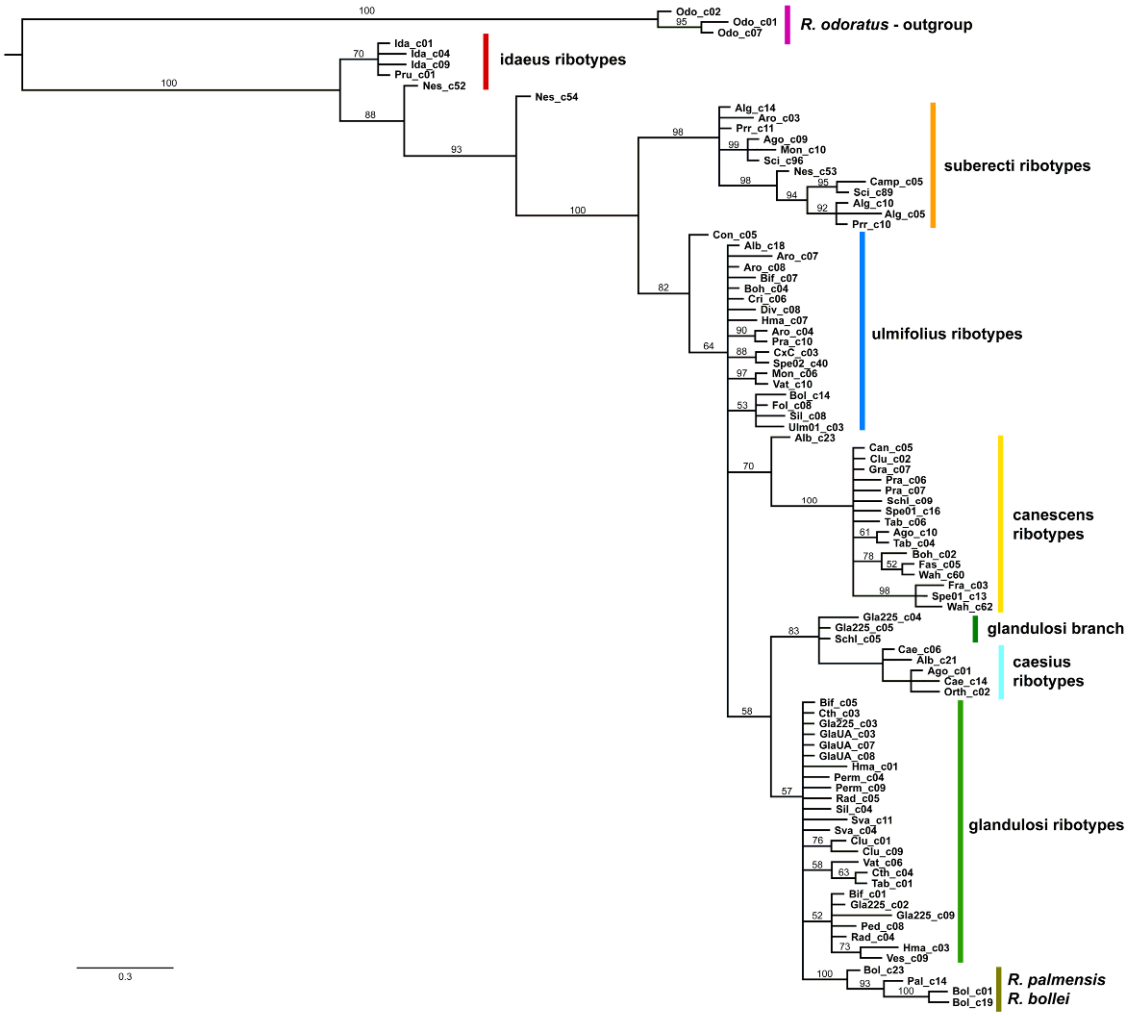
[3] Based on morphological affinity to the Europaean ser. *Discolores,* ser. *Radula or ser. Micantes.*

[4] Taxon from ser. *Glandulosi* with unclear delimitation, mostly tetraploid and highly sexual, partly similar to *R. hirtus* agg. sensu Kurtto *et al.* (2010).

[5] In Kurtto *et al.* (2010) incorectly named as *R. scissus* (see Weber 2013).
[6] In Kurtto *et al.* (2010) named as *R. ochracanthus* et Sennikov.
[7] According to Kurtto *et al.* (2010);'s original designation to ser. *Grandifolii* seems to be more appropriate.
[8] Only formal designation, as diploid taxon difficult to designate to any particular series.
[9] All traits point rather to ser. *Pallidi*.
[10] Sometimes categorized in ser. *Radula* (also in Kurtto *et al.* 2010).
[11] Subsect. *Subidaeus* is not subdivided to series; all other taxa within sect. *Corylifolii* belong to subsect. *Sepincola*.
[12] Published as *R. gothicus* in Krahulcová *et al.* (2013).



Chapter 2, Supplementary figure 1: SplitsTree maximum parsimony network of unique ITS sequences, including pseudogenes.



Chapter 2, Supplementary figure 2: BI phylogram of distinct cloned ITS sequences; posterior probabilities shown above branches.

Chapter 3, Supplementary table 1: List of studied accessions with taxonomic determination, population assignment, locality and cp-haplotype

Individual	Collection no.	SSR	Pop.	Species	Loc.	Latitude	Longitude	Haplotype
ulm-am1	AM25/30	Y	Am	*R. ulmifolius* agg.	SE Armenia, near Goris	39º26'32"N	46º26'08"E	San2
ulm-am10	AM38	Y	Am	*R. ulmifolius* agg.	SE Armenia, Syunik, Kapan	39º12'01"N	46º25'27"E	San2
ulm-am2	AM24A	Y	Am	*R. ulmifolius* agg.	SW Azerbaijan, Qubadli, near Sanasar	39º25'59"N	46º25'06"E	San3
ulm-am3	AM25/21	Y	Am	*R. ulmifolius* agg.	SW Azerbaijan, Qubadli, near Sanasar	39º26'32"N	46º26'08"E	San2
ulm-am4	AM25/6	Y	Am	*R. ulmifolius* agg.	SE Armenia, near Goris	39º26'32"N	46º26'08"E	San3
ulm-am5	AM30/2	Y	Am	*R. ulmifolius* agg.	S Armenia, Syunik, near Vardanidzor	38º59'07"N	46º12'23"E	
ulm-am6	AM34	Y	Am	*R. ulmifolius* agg.	S Armenia, Syunik, near Meghri	38º54'12"N	46º17'16"E	San2
ulm-am8	AM35/1	Y	Am	*R. ulmifolius* agg.	S Armenia, Syunik, near Shvanidzor	38º57'53"N	46º22'21"E	San2
ulm-am9	AM35/11	Y	Am	*R. ulmifolius* agg.	S Armenia, Syunik, near Shvanidzor	38º57'53"N	46º22'21"E	
ulm-ge1	MS047/13	Y	Ge	*R. ulmifolius* agg.	W Georgia, Samegrelo-Zemo Svaneti, Anaklia	42º23'18"N	41º34'00"E	San3
ulm-ge10	MS046B/14	Y	Ge	*R. ulmifolius* agg.	W Georgia, Imereti, near Kutaisi	42º11'01"N	42º28'04"E	San1
ulm-ge2	MS048A/13	Y	Ge	*R. ulmifolius* agg.	W Georgia, Samegrelo-Zemo Svaneti, Anaklia	42º23'41"N	41º33'57"E	San3
ulm-ge3	MS048B/13	Y	Ge	*R. ulmifolius* agg.	W Georgia, Samegrelo-Zemo Svaneti, Anaklia	42º23'41"N	41º33'57"E	
ulm-ge4	MS048E/13	Y	Ge	*R. ulmifolius* agg.	W Georgia, Samegrelo-Zemo Svaneti, Anaklia	42º23'41"N	41º33'57"E	
ulm-ge5	MS035/14	Y	Ge	*R. ulmifolius* agg.	W Georgia, Adjara, Chakvi	41º42'58"N	41º43'57"E	San3
ulm-ge6	MS040F/14	Y	Ge	*R. ulmifolius* agg.	W Georgia, Adjara, Chakvi	41º43'14"N	41º43'43"E	San3
ulm-ge7	MS040M/14	Y	Ge	*R. ulmifolius* agg.	W Georgia, Adjara, Chakvi	41º43'14"N	41º43'43"E	San3
ulm-ge8	MS040H/14	Y	Ge	*R. ulmifolius* agg.	W Georgia, Adjara, Chakvi	41º43'14"N	41º43'43"E	
ulm-ge9	MS046A/14	Y	Ge	*R. ulmifolius* agg.	W Georgia, Imereti, near Kutaisi	42º11'01"N	42º28'04"E	San4
ulm-bc1	BG-3A	Y	BC	*R. ulmifolius* agg.	SW Bulgaria, near Sandanski	41º30'12"N	23º22'00"E	
ulm-bc10	MS080/12	Y	BC	*R. ulmifolius* agg.	S Kosovo, near Jazhincë	42º12'08"N	20º59'21"E	San1
ulm-bc2	BG-4/1A	Y	BC	*R. ulmifolius* agg.	SW Bulgaria, near Sandanski	41º31'27"N	23º23'29"E	San1
ulm-bc3	BG-4/4A	Y	BC	*R. ulmifolius* agg.	SW Bulgaria, near Sandanski	41º31'42"N	23º24'25"E	
ulm-bc4	BG-5	Y	BC	*R. ulmifolius* agg.	SW Bulgaria, near Stara Kresna	41º47'51"N	23º09'29"E	San1
ulm-bc5	BG-8A	Y	BC	*R. ulmifolius* agg.	SW Bulgaria, near Rila village	42º07'57"N	23º09'14"E	San1
ulm-bc6	MS068/12	Y	BC	*R. ulmifolius* agg.	N Macedonia, near Skopje	41º59'46"N	21º33'06"E	San1
ulm-bc7	MS070/12	Y	BC	*R. ulmifolius* agg.	NW Macedonia, distr. Zhelino, near Rogle	41º58'22"N	21º09'35"E	San1
ulm-bc8	MS083/12	Y	BC	*R. ulmifolius* agg.	S Kosovo, near Prizren	42º10'21"N	20º49'32"E	
ulm-bc9	MS084/12	Y	BC	*R. ulmifolius* agg.	S Kosovo, near Prizren	42º11'59"N	20º40'40"E	
ulm-bw1	MS088/12	Y	BW	*R. ulmifolius* agg.	N Albania, distr. Lezhë	41º39'54"N	19º40'57"E	San1

ulm-bw10	MS103/12	Y	BW	*R. ulmifolius* agg.	central Croatia, Splitsko-dalmatinska županija, Šestanovac	43º27’13"N	16º54’44"E	
ulm-bw2	MS090/12	Y	BW	*R. ulmifolius* agg.	N Albania, distr. Shkodër	42º02’41"N	19º29’05"E	Ulm1
ulm-bw3	MS092/12	Y	BW	*R. ulmifolius* agg.	SE Montenegro, near Petrovac	42º11’58"N	18º58’02"E	
ulm-bw4	MS095/12	Y	BW	*R. ulmifolius* agg.	W Montenegro, near Herceg Novi	42º26’22"N	18º35’23"E	Ulm1
ulm-bw5	MS096/12	Y	BW	*R. ulmifolius* agg.	S Croatia, Bjelovarsko-bilogorska županija, near Bjelovar	42º37’31"N	18º10’29"E	
ulm-bw6	MS098/12	Y	BW	*R. ulmifolius* agg.	S Croatia, Bjelovarsko-bilogorska županija, near Slano	42º47’18"N	17º53’27"E	
ulm-bw7	MS099/12	Y	BW	*R. ulmifolius* agg.	S Bosnia and Herzegovina, Neum	42º55’07"N	17º37’37"E	Ulm1
ulm-bw8	MS101/12	Y	BW	*R. ulmifolius* agg.	S Croatia, Dubrovačko-neretvanska županija, near Ploče	43º04’05"N	17º26’02"E	
ulm-bw9	MS102/12	Y	BW	*R. ulmifolius* agg.	S Croatia, Splitsko-dalmatinska županija, near Gradac	43º09’48"N	17º24’56"E	Ulm1
ulm-bn1	MS104/12	Y	BN	*R. ulmifolius* agg.	central Croatia, Splitsko-dalmatinska županija, near Split	43º35’16"N	16º27’34"E	
ulm-bn10	MS005A/13	Y	BN	*R. ulmifolius* agg.	NE Italy, near Padova	45º26’58"N	11º50’45"E	
ulm-bn2	MS106/12	Y	BN	*R. ulmifolius* agg.	c Croatia, Šibensko-kninska županija, near Vodice	43º51’25"N	15º50’45"E	Ulm1
ulm-bn3	MS113/12	Y	BN	*R. ulmifolius* agg.	NW Croatia, Ličko-senjska županija, near Senj	44º57’39"N	15º01’50"E	Ulm2
ulm-bn4	MS108/12	Y	BN	*R. ulmifolius* agg.	N Croatia, Zadarska županija, near Starigrad	44º15’14"N	15º32’07"E	
ulm-bn5	MS152/12	Y	BN	*R. ulmifolius* agg.	N Croatia, Krk	45º04’34"N	14º40’36"E	
ulm-bn6	Ulm02	Y	BN	*R. ulmifolius* agg.	N Croatia, Istra, near Poreč	45º13’29"N	13º37’27"E	Ulm1
ulm-bn7	MS124/12	Y	BN	*R. ulmifolius* agg.	SW Slovenia, Postojna, near Pivka	45º39’49"N	14º10’41"E	Ulm1
ulm-bn8	MH80/13	Y	BN	*R. ulmifolius* agg.	SW Slovenia, N Istria, Strunjan	45º31’35"N	13º36’41"E	
ulm-bn9	MH-IT/13	Y	BN	*R. ulmifolius* agg.	NE Italy, near Montfalcone	45º47’17"N	13º35’20"E	
ulm-fs1	MS006/13	Y	FS	*R. ulmifolius* agg.	NW Italy, near Piacenza	45º03’06"N	09º45’38"E	
ulm-fs10	LM08/12	Y	FS	*R. ulmifolius* agg.	SE France, Provence-Alpes-Côte d’Azur, Cogolin	43º15’15"N	06º31’37"E	Ulm1
ulm-fs2	MS008/13	Y	FS	*R. ulmifolius* agg.	NW Italy, near Ovada	44º35’37"N	08º39’55"E	
ulm-fs3	MS014/13	Y	FS	*R. ulmifolius* agg.	SE France, Alpes-Maritimes, near Cannes	43º35’33"N	07º02’08"E	Ulm2
ulm-fs4	MS018/13	Y	FS	*R. ulmifolius* agg.	SE France, Provence-Alpes-Côte d’Azur, Cannes/Fréjus	43º28’30"N	06º55’39"E	
ulm-fs5	MS020/13	Y	FS	*R. ulmifolius* agg.	SE France, Provence-Alpes-Côte d’Azur, Fréjus	43º23’43"N	06º43’43"E	Ulm1
ulm-fs6	MS026/13	Y	FS	*R. ulmifolius* agg.	SE France, Provence-Alpes-Côte d’Azur, near Monaco	43º45’14"N	07º25’16"E	Ulm1
ulm-fs7	MS003A/14	Y	FS	*R. ulmifolius* agg.	SE France, Rhône-Alpes, near Vienne	45º25’34"N	04º49’28"E	
ulm-fs8	MS005A/14	Y	FS	*R. ulmifolius* agg.	SE France, Languedoc-Roussillon, near Avignon	43º56’48"N	04º32’09"E	
ulm-fs9	LM06/12	Y	FS	*R. ulmifolius* agg.	SE France, Provence-Alpes-Côte d’Azur, near Trigance	43º46’15"N	06º23’32"E	Ulm1
ulm-fn10	Ulm-VŽ2	Y	FN	*R. ulmifolius* agg.	W Germany, near Bonn	50º40’31"N	07º17’49"E	Ulm1
ulm-fn2	R178/12 (F12-Ulm)	Y	FN	*R. ulmifolius* agg.	NE France, Picardie, near Abbeville	50º12’29"N	01º49’38"E	Ulm1
ulm-fn3	R183/12 (F15-Ulm)	Y	FN	*R. ulmifolius* agg.	NE France, Picardie, near Abbeville	50º20’10"N	01º44’56"E	Ulm1
ulm-fn4	R186/12 (F17-Ulm)	Y	FN	*R. ulmifolius* agg.	NE France, Nord-Pas-de-Calais, near Calais	50º27’50"N	01º34’48"E	

ulm-fn5	R187/12 (F18-Ulm)	Y	FN	*R. ulmifolius* agg.	NE France, Nord-Pas-de-Calais, near Calais	50º52’15"N	01º34’59"E	Ulm1
ulm-fn6	R188/12 (F19-Ulm)	Y	FN	*R. ulmifolius* agg.	NE France, Nord-Pas-de-Calais, near Calais	50º52’35"N	01º39’33"E	
ulm-fn7	R190/12 (F21-Ulm)	Y	FN	*R. ulmifolius* agg.	NE France, Nord-Pas-de-Calais, near Calais	50º58’11"N	02º01’08"E	
ulm-fn8	R192/12 (F22-Ulm)	Y	FN	*R. ulmifolius* agg.	NE France, Nord-Pas-de-Calais, near Dunkerk	50º53’08"N	02º30’42"E	Ulm2
ulm-fn9	Ulm-VŽ1	Y	FN	*R. ulmifolius* agg.	W Germany, near Frankfurt am Main	50º05’43"N	08º21’08"E	Ulm1
ulm-gb1	D03	Y	GB	*R. ulmifolius* agg.	c Great Britain, England, near Preston	53º45’00"N	02º43’00"W	Ulm2
ulm-gb2	D04	Y	GB	*R. ulmifolius* agg.	c Great Britain, England, near Preston	53º45’00"N	02º43’00"W	
ulm-gb3	D05	Y	GB	*R. ulmifolius* agg.	c Great Britain, England, near Northwich	53º15’00"N	02º19’50"W	Ulm1
ulm-gb4	D06	Y	GB	*R. ulmifolius* agg.	c Great Britain, England, near Southport	53º41’20"N	02º49’42"W	
ulm-gb5	D07	Y	GB	*R. ulmifolius* agg.	c Great Britain, England, near Southport	53º42’00"N	02º51’00"W	Ulm1
ulm-gb6	D08	Y	GB	*R. ulmifolius* agg.	c Great Britain, England, near Southport	53º42’00"N	02º51’00"W	
ulm-gb7	D09	Y	GB	*R. ulmifolius* agg.	c Great Britain, England, near Southport	53º42’00"N	02º51’00"W	
ulm-gb8	D10	Y	GB	*R. ulmifolius* agg.	c Great Britain, England, near Southport	53º39’00"N	02º59’00"W	Ulm1
ulm-gb9	D11	Y	GB	*R. ulmifolius* agg.	c Great Britain, England, near Wigan	53º35’00"N	02º43’00"W	
ulm-sn1	MS006A/14	Y	SN	*R. ulmifolius* agg.	NE Spain, Catalunya, near Vidreres	41º46’24"N	02º44’20"E	
ulm-sn10	MD-Ulm4/12	Y	SN	*R. ulmifolius* agg.	S France, Languedoc-Roussillon, near Le Boulou	42º29’43"N	02º49’16"E	Ulm1
ulm-sn2	MS006B/14	Y	SN	*R. ulmifolius* agg.	NE Spain, Catalunya, near Vidreres	41º46’24"N	02º44’20"E	
ulm-sn3	MS018/14	Y	SN	*R. ulmifolius* agg.	NE Spain, Catalunya, near Tarragona	41º08’25"N	01º20’50"E	Ulm1
ulm-sn4	MS019A/14	Y	SN	*R. ulmifolius* agg.	NE Spain, Catalunya, near Barcelona	41º29’42"N	02º11’30"E	
ulm-sn5	MS019C/14	Y	SN	*R. ulmifolius* agg.	NE Spain, Catalunya, near Barcelona	41º29’42"N	02º11’30"E	
ulm-sn6	MS020A/14	Y	SN	*R. ulmifolius* agg.	S France, Languedoc-Roussillon, near Perpignan	42º34’21"N	02º50’45"E	
ulm-sn7	MD-Ulm1/12	Y	SN	*R. ulmifolius* agg.	NE Spain, Catalunya, near Barcelona	41º17’53"N	01º52’24"E	Ulm2
ulm-sn8	MD-Ulm2/12	Y	SN	*R. ulmifolius* agg.	NE Spain, Catalunya, near Sant Celoni	41º39’19"N	02º31’50"E	Ulm1
ulm-sn9	MD-Ulm3/12	Y	SN	*R. ulmifolius* agg.	NE Spain, Catalunya, near Figueres	42º21’19"N	03º02’12"E	
ulm-mn1	FB-12-18B	Y	MN	*R. ulmifolius* agg.	S Portugal, Faro region, near Monchique	37º18’57"N	08º35’47"W	
ulm-mn10	FB12-01	Y	MN	*R. ulmifolius* agg.	S Portugal, Faro region, near Faro	37º08’06"N	07º54’34"W	
ulm-mn2	FB12-9B-1	Y	MN	*R. ulmifolius* agg.	S Spain, Andalucía, near Estepona	36º33’40"N	05º11’55"W	
ulm-mn3	MS007A/14	Y	MN	*R. ulmifolius* agg.	N Morocco, Chefchaouen	35º10’16"N	05º15’23"W	Ulm4
ulm-mn4	MS009A/14	Y	MN	*R. ulmifolius* agg.	N Morocco, near Chefchaouen	34º55’32"N	05º32’41"W	Ulm4
ulm-mn5	MS010A/14	Y	MN	*R. ulmifolius* agg.	N Morocco, near Moulay Driss Zerhoun	34º04’20"N	05º33’17"W	Ulm1
ulm-mn6	MS011/14	Y	MN	*R. ulmifolius* agg.	N Morocco, near Azrou	33º25’09"N	05º10’39"W	Ulm1
ulm-mn7	MS016A/14	Y	MN	*R. ulmifolius* agg.	NW Morocco, Rabat	34º00’27"N	06º49’12"W	Ulm1
ulm-mn8	MS017A/14	Y	MN	*R. ulmifolius* agg.	NW Morocco, near Larache	35º11’49"N	06º06’41"W	Ulm2

ulm-mn9	FB12-15	Y	MN	*R. ulmifolius* agg.	S Spain, Andalucía, near Huelva	37º08'06"N	06º39'49"W	Ulm1
ulm-ms1	MS012A/14	Y	MS	*R. ulmifolius* agg.	c Morocco, near Tinerhir	31º34'36"N	05º35'12"W	Ulm2
ulm-ms10	MS015L/14	Y	MS	*R. ulmifolius* agg.	c Morocco, Ouzoud	32º00'55"N	06º43'08"W	Ulm3
ulm-ms2	MS012C/14	Y	MS	*R. ulmifolius* agg.	c Morocco, near Tinerhir	31º34'36"N	05º35'12"W	
ulm-ms3	MS012E/14	Y	MS	*R. ulmifolius* agg.	c Morocco, near Tinerhir	31º34'36"N	05º35'12"W	Ulm1
ulm-ms4	MS013/14	Y	MS	*R. ulmifolius* agg.	c Morocco, Taliouine	30º31'30"N	07º54'19"W	Ulm3
ulm-ms5	MS014A/14	Y	MS	*R. ulmifolius* agg.	c Morocco, near Demnate	31º43'27"N	06º58'17"W	Ulm1
ulm-ms6	MS014B/14	Y	MS	*R. ulmifolius* agg.	c Morocco, near Demnate	31º43'27"N	06º58'17"W	
ulm-ms7	MS014C/14	Y	MS	*R. ulmifolius* agg.	c Morocco, near Demnate	31º43'27"N	06º58'17"W	Ulm3
ulm-ms8	MS015A/14	Y	MS	*R. ulmifolius* agg.	c Morocco, Ouzoud	32º00'55"N	06º43'08"W	Ulm1
ulm-ms9	MS015J/14	Y	MS	*R. ulmifolius* agg.	c Morocco, Ouzoud	32º00'55"N	06º43'08"W	
ulm-mac1	MD-Ulm-K4/13	Y	Misc.	*R. ulmifolius* agg.	Canary Islands, La Gomera	28º07'57"N	17º13'15"W	Ulm2
ulm-mac2	AK-6-Mad/13	Y	Misc.	*R. ulmifolius* agg.	Madeira	32º40'09"N	16º53'12"W	Ulm1
ulm-med1	ZŠ-Ulm-Kor/13	Y	Misc.	*R. ulmifolius* agg.	S Corsica, Bonifacio	41º23'08"N	09º10'22"E	Ulm2
ulm-med2	PM-San-Gr/13	Y	Misc.	*R. ulmifolius* agg.	c Greece, Agiocampos	39º42'02"N	22º52'30"E	San1
ulm-med4	San-Tr1/14	Y	Misc.	R. ulmifolius agg.	W Turkey, Izmir distr., Gümüldür	38º03'39"N	27º00'24"E	San1
ulm-med5	San-Tr2/14	Y	Misc.	R. ulmifolius agg.	W Turkey, Izmir distr., Ephesus	37º56'13"N	27º20'37"E	San1
ulm-med6	San-Tr3/14	Y	Misc.	R. ulmifolius agg.	W Turkey, Izmir distr., Ephesus	37º56'41"N	27º20'25"E	
ulm-med3	PS-San-Cr/14	Y	Misc.	*R. ulmifolius* agg.	Crete	35º24'00"N	24º06'00"E	Ulm1
ulm-med8	Ulm-RJV10	Y	Misc.	*R. ulmifolius* agg.	c Italy, near Acquapendente	42º45'43"N	11º52'27"E	Ulm1
am-poly6	AM36	Y	poly-Am	*R.* aff. *praecox* agg.	SE Armenia, Syunik, near Kapan	39º08'43"N	46º26'03"E	Ulm1
arm1	F1-Arm	Y	poly-Am	*R. armeniacus*	W Germany, Rheinland-Pfalz, near Neustadt	49º18'05"N	08º05'32"E	Ulm2
arm2	MS004/12	Y	poly-Am	*R. armeniacus*	Germany, Sachsen-Anhalt, near Halberstadt	51º43'45"N	11º14'17"E	
arm3	MS155/12	Y	poly-Am	*R. armeniacus*	cE Czechia, c Moravia, Olomouc	49º34'38"N	17º17'13"E	Ulm2
am-poly1	AM37/5	Y	poly-Am	*R.* ser. *Discolores*	SE Armenia, Syunik, near Kapan	39º08'09"N	46º28'02"E	Can3
am-poly2	AM39-WF	Y	poly-Am	*R.* ser. *Discolores*	SE Armenia, Syunik, near Kapan	39º19'42"N	46º22'27"E	Gla4
am-poly3	AM30/1	Y	poly-Am	*R.* ser. *Discolores*	S Armenia, Syunik, near Meghri	38º59'07"N	46º12'23"E	Ulm1
am-poly4	AM10	Y	poly-Am	*R.* ser. *Discolores*	c Armenia, Vayots Dzor, near Vayk	39º41'43"N	45º27'05"E	Ulm2
am-poly5	AM2	Y	poly-Am	*R.* ser. *Discolores*	c Armenia, Yerevan	40º11'34"N	44º30'54"E	Can1
ge-poly6	MS059/13	Y	poly-Ge	*R.* cf. *peruncinatus*	c Georgia, near Borjomi	41°49'46"N	43°18'50"E	Cau
ge-poly2	MS057/13	Y	poly-Ge	*R.* ser. *Discolores*	W Georgia, Samegrelo-Zemo Svaneti, Anaklia	42º23'29"N	41º34'11"E	Cau
ge-poly3	MS022/14	Y	poly-Ge	*R.* ser. *Discolores*	N Georgia, Racha, Ambrolauri	42º34'04"N	43º17'02"E	Can3
ge-poly4	MS030/14	Y	poly-Ge	*R.* ser. *Discolores*	N Georgia, Racha-Lechkhumi-Kvemo Svaneti, Tsageri	42º39'34"N	42º46'21"E	Cau

ge-poly5	MS042/14	Y	poly-Ge	*R.* ser. *Discolores*	SW Georgia, Adjara, near Batumi	41º43’14"N	41º43’43"E	Cau
ge-poly1	MS029/14	Y	poly-Ge	R. ser. *Micantes*	N Georgia, Racha-Lechkhumi-Kvemo Svaneti, Tsageri	42º39’34"N	42º46’21"E	Cau
bal-poly1	MS067/12	Y	poly-Bal	*R.* aff. *praecox* agg.	S Serbia, near Vranje	42º35’58"N	22º01’21"E	Ulm1
bal-poly2	MS071/12	Y	poly-Bal	*R.* aff. *praecox* agg.	NW Macedonia, near Gostivar	41º44’01"N	20º49’58"E	
bal-poly3	MS075b/12	Y	poly-Bal	*R.* aff. *praecox* agg.	S Kosovo, Kaçanik	42º13’26"N	21º15’20"E	Ulm1
bal-poly4	RO-5	Y	poly-Bal	*R.* aff. *praecox* agg.	SW Romania, Caras-Severin	44º40’52"N	21º42’39"E	
bal-poly5	MS052/12	Y	poly-Bal	*R.* ser. *Discolores*	W Ukraine, Zakarpatska oblast, Mukacheve	48º26’14"N	22º45’17"E	
ard2	R169/13	Y	poly-EU	*R.* aff. *arduennensis*	W Germany, Rheinland-Pfalz, near Landau	49º09’57"N	08º00’55"E	Ulm1
ard1	R256/13	Y	poly-EU	*R. arduennensis*	c Germany, Bayern, near Königsberg	50º04’20"N	10º39’53"E	Can1
amo	RJV005/12	Y	poly-EU	*R. austromoravicus*	E Czech Republic, NE Moravia, near Ostrava	49º27’15"N	18º15’40"E	Can1
bif1	MS018/12	Y	poly-EU	*R. bifrons*	W Slovakia, near Zlaté Moravce	48º30’40"N	18º24’34"E	Ulm1
bif2	R145/12 (F1-Bif)	Y	poly-EU	*R. bifrons*	W Germany, Rheinland-Pfalz, near Neustadt	49º18’05"N	08º05’28"E	Ulm1
boh	R221/11	Y	poly-EU	*R. bohemiicola*	S Czechia, S Bohemia, near Strakonice	49º16’45"N	13º53’22"E	Can1
moe	Moe	Y	poly-EU	*R.* cf. *moestus*	E Czechia, E Moravia, near Vsetín	49º18’27"N	17º56’56"E	Can2
ele	R173/11	Y	poly-EU	*R. elegantispinosus*	NW Germany, Niedersachsen, near Osnabrück	52º16’45"N	08º09’32"E	Can1
fla1	R148/12 (F3-Fla)	Y	poly-EU	*R. flaccidus*	W Germany, Rheinland-Pfalz, near Neustadt	49º18’48"N	08º06’17"E	Ulm1
fla2	R160/12 (F7-Fla)	Y	poly-EU	*R. flaccidus*	W Germany, Rheinland-Pfalz, near Haßloch	49º20’29"N	08º16’44"E	Ulm1
flo	Flo01 R196/10	Y	poly-EU	*R. flos-amygdalae*	cE Czechia, S Moravia, near Otrokovice	49º08’50"N	17º23’05"E	Can1
gen1	R174/11	Y	poly-EU	*R. geniculatus*	NW Germany, Niedersachsen, near Wallenhorst	52º22’08"N	08º05’02"E	Ulm1
gen2	R218/12 (F28-Gen)	Y	poly-EU	*R. geniculatus*	E Belgium, Walloon Region, near Stavelot	50º24’07"N	05º51’53"E	Ulm1
gon1	Gon-VŽ1	Y	poly-EU	*R. goniophorus*	cW Germany, Hessen, near Weilmünster	50º25’42"N	08º35’28"E	Can1
gon2	Gon-VŽ2	Y	poly-EU	*R. goniophorus*	cW Germany, Hessen, near Wetzlar	50º29’33"N	08º33’30"E	Can1
gra	Gra01	Y	poly-EU	*R. grabowskii*	cN Czechia, E Bohemia, near Hradec Králové	50º09’03"N	15º50’43"E	Can1
gut	R44/09	Y	poly-EU	*R. guttiferus*	NE Hungary, Borsod-Abaúj-Zemplén, near Gönc	48º26’04"N	21º18’28"E	Can2
hen	R9/12	Y	poly-EU	*R. henrici-egonis*	cW Slovakia, district Zlaté Moravce	48º31’16"N	18º25’28"E	Can2
mon	MS128/12	Y	poly-EU	*R. montanus*	SE Austria, Steiermark, near Leibnitz	46º49’50"N	15º33’11"E	Can1
pal1	R227/12 (F31-Pal)	Y	poly-EU	*R. palaefolius*	W Germany, Nordrhein-Westfalen, near Bonn	50º38’52"N	07º00’07"E	Ulm1
pal2	R231/12 (F32-Pal)	Y	poly-EU	*R. palaefolius*	W Germany, Nordrhein-Westfalen, near Bonn	50º45’28"N	07º10’14"E	Ulm1
par	Par01	Y	poly-EU	*R. parthenocissus*	SW Czechia, S Bohemia, near České Budějovice	49º02’54"N	14º25’55"E	Can1
pcr	R18/12	Y	poly-EU	*R. pericrispatus*	NW Hungary, Györ region, near Bakonyszentászló	47º21’34"N	17º49’01"E	Can1
per	Per01	Y	poly-EU	*R. perperus*	SW Czechia, S Bohemia, Hluboká nad Vltavou	49º02’50"N	14º25’55"E	Can1
phy	R158/12 (F7-Phy)	Y	poly-EU	*R. phyllostachys*	W Germany, Rheinland-Pfalz, near Haßloch	49º20’29"N	08º16’44"E	Can1
pol	R159/11	Y	poly-EU	*R. polyanthemus*	NW Germany, Niedersachsen, near Bramsche	52º24’47"N	07º55’44"E	Ulm2

pmo	PMo01	Y	poly-EU	*R. portae-moravicae*	NE Czechia, N Moravia, near Bílovec	49º49’14"N	18º01’39"E	Ulm1
pra1	Pra02	Y	poly-EU	*R. praecox*	S Slovakia, near Štúrovo	47º49’25"N	18º49’49"E	Ulm1
pra2	Pra01 R223/11	Y	poly-EU	*R. praecox*	SW Czechia, S Bohemia, near Volyně	49º07’49"N	14º00’33"E	Ulm1
psa	R230/12 (F31-Psa)	Y	poly-EU	*R. pseudargenteus*	W Germany, Nordrhein-Westfalen, near Bonn	50º38’52"N	07º00’07"E	Ulm2
win	R140/11	Y	poly-EU	*R. winteri*	NW Germany, N Nordrhein-Westfalen, near Ibbenbüren	52º20’12"N	07º38’44"E	Ulm2
acan	D01	Y	poly-GB	*R. anglocandicans*	E Great Britain, Filey	54°12’N	00°17’W	Ulm1
armp	D02	Y	poly-GB	*R. armipotens*	c Great Britain, England, near Kidderminster	52°24’N	02°20’W	
bou	R02	Y	poly-GB	*R. boudiccae*	c Great Britain, England, Southport	53°38’N	03°00’W	
card	R03	Y	poly-GB	*R. cardiophyllus*	c Great Britain, England, near Southport	53°33’N	03°04’W	Ulm1
ciss	R04	Y	poly-GB	*R. cissburiensis*	c Great Britain, England, near Preston	53°43’N	02°40’W	Ulm1
furn	R06	Y	poly-GB	*R. furnarius*	c Great Britain, England, near Barnard Castle	54°28’N	01°57’W	Ulm1
incu	R08	Y	poly-GB	*R. incurvatus*	c Great Britain, England, near Kirkby	53°31’N	02°50’W	
nwin1	D12	Y	poly-GB	*R.* ser. *Discolores*	c Great Britain, England, near Robin Hood’s Bay	54°26’N	00°33’W	Ulm2
nwin2	D16	Y	poly-GB	*R.* ser. *Discolores*	c Great Britain, England, near Southport	53°36’N	02°50’W	Ulm2
subin	R14	Y	poly-GB	*R. subinermoides*	c Great Britain, England, near Bebington	53°21’N	03°02’W	
cae1	Cae03	Y	outgroup	*R. caesius*	SE Czechia, S Moravia, Nové Mlýny	48º51’18"N	16º43’29"E	Cae1
cae2	MS082/12	Y	outgroup	*R. caesius*	S Kosovo, near Jazhincë	42º12’08"N	20º59’21"E	Cae1
can1	RO-4	Y	outgroup	*R. canescens*	SW Romania, Caras-Severin	44º42’11"N	21º43’44"E	
can2	MS019/13	Y	outgroup	*R. canescens*	SE France, Provence-Alpes-Côte d’Azur, near Fréjus	46º28’30"N	06º55’39"E	Can1
can3	MS055/13	Y	outgroup	*R. canescens*	c Georgia, near Borjomi	41º50’08"N	43º15’53"E	Can1
mos1	MS049/13	Y	outgroup	*R. moschus*	c Georgia, near Borjomi	41°49’46"N	43°18’50"E	Gla1
mos2	MS034/14	Y	outgroup	*R. moschus*	SW Georgia, Adjara, near Batumi	41º40’55"N	41º50’29"E	Gla1
mos3	MS036/14	Y	outgroup	*R. moschus*	SW Georgia, Adjara, near Batumi	41º40’23"N	41º50’58"E	Gla5
mos4	MS039/14	Y	outgroup	*R. moschus*	SW Georgia, Adjara, near Batumi	41º41’27"N	41º49’30"E	Gla1
pli	MS158/12	Y	outgroup	*R. plicatus*	cE Czechia, E Moravia, near Bystřice pod Hostýnem	49º21’25"N	17º43’49"E	Suber
gla1	R183/10	Y	outgroup	*R.* ser. *Glandulosi*	SW Czechia, S Bohemia, near České Budějovice	48º57’55"N	14º32’13"E	Gla1
gla2	MS046/12	Y	outgroup	*R.* ser. *Glandulosi*	W Ukraine, Zakarpatska oblast, near Rakhiv	48º14’07"N	24º11’20"E	Gla1
	AM19	N	-	*R.* ser. *Discolores*	SE Armenia, Syunik, Vorotan	39°29’05"N	46°08’25"E	Ulm1
	AM21	N	-	*R.* ser. *Discolores*	SE Armenia, Syunik, near Goris	39°29’30"N	46°18’45"E	Ulm1
	AM23	N	-	*R.* ser. *Discolores*	SW Azerbaijan, Qubadli, near Sanasar	39°26’23"N	46°23’57"E	Ulm1
	AM26dis	N	-	*R.* ser. *Discolores*	S Armenia, Syunik, near Kajaran	39°10’42"N	46°14’03"E	San1
	AM4	N	-	*R.* ser. *Discolores*	c Armenia, Ararat distr., near Artashat	39°54’00"N	44°35’56"E	Ulm1
	D15	N	-	*R.* ser. *Discolores*	c Great Britain, England, Southport	53°38’13"N	02°50’11"W	Ulm1

MS01/15	N	-	*R.* ser. *Discolores*	Abkhazia, near Sukhumi	43°05’17"N	41°17’55"E	San3
MS058/13	N	-	*R.* ser. *Discolores*	NW Georgia, Zemo Svaneti, between Mestia and Zugdidi	42°59’15"N	42°15’14"E	Ulm1
AM37/2	N	-	*R.* ser. *Discolores*	SE Armenia, Syunik, near Kapan	39º08’09"N	46º28’02"E	Can3
R165/11	N	-	*R. adspersus*	NW Germany, SW Niedersachsen, near Bramsche	52°25’33"N	07°52’52"E	Ulm1
AM20-Dis	N	-	*R.* aff. *praecox* agg.	SE Armenia, Syunik, near Goris	39°29’30"N	46°18’45"E	Ulm1
MS075a/12	N	-	*R.* aff. *praecox* agg.	NW Macedonia, near Gostivar	41°40’17"N	20°51’12"E	Ulm1
R152/11	N	-	*R. amisiensis*	NW Germany, SW Niedersachsen, near Bramsche	52°25’12"N	07°45’13"E	Ulm1
R156/11	N	-	*R. ammobius*	NW Germany, SW Niedersachsen, near Bramsche	52°23’01"N	07°55’04"E	Ulm1
Bif01	N	-	*R. bifrons*	NE Czechia, N Moravia, near Bílovec	49°48’8"N	18°0’47"E	Ulm1
Bif-Dus	N	-	*R. bifrons*	E Czechia, E Moravia, dist. Vsetín	49°23’5"N	18°2’35"E	Ulm1
R144/09	N	-	*R. bifrons*	SW Czechia, S Bohemia, near Volyně	49°07’09"N	13°52’57"E	Ulm1
MS018/12	N	-	*R. bifrons*	cW Slovakia, district Zlaté Moravce	48°30’40"N	18°24’34"E	Ulm1
R100/12	N	-	*R. bifrons*	N Croatia, Bjelovarsko-bilogorska županija, near Sirač	45°34’00”N	17°20’32”E	Ulm1
MS-cap	N	-	*R. capricollensis*	NE Czechia, N Moravia, near Bílovec	49°48’54"N	18°00’35"E	Ulm1
MS043/14	N	-	*R.* cf. *ibericus*	SW Georgia, Adjara, near Batumi	41°41’28”N	41°42’18”E	Ulm1
R152/12 (F5-Div)	N	-	*R. divaricatus*	W Germany, Rheinland-Pfalz, near Haßloch	49°19’41"N	08°14’53"E	Ulm1
R153/12 (F5-Grac)	N	-	*R. gracilis*	W Germany, Rheinland-Pfalz, near Haßloch	49°19’41"N	08°14’53"E	Ulm1
RJV001/12	N	-	*R. gracilis*	E Czechia, Beskydy Mts., near Rožnov pod Radhoštěm	49°25’12"N	18°20’6"E	Ulm1
R139/11	N	-	*R. gratus*	NW Germany, Nordrhein-Westfalen, Ibbenbüren	52°20’12"N	07°38’44"E	Ulm1
R201/11	N	-	*R. hypomalacus*	NW Germany, Nordrhein-Westfalen, near Bünde	52°13’18"N	08°40’12"E	Ulm1
R189/11	N	-	*R. infestus*	NW Germany, Nordrhein-Westfalen, near Bad Oeynhausen	52°15’41"N	08°47’05"E	Ulm1
R180/12 (F13-Int)	N	-	*R. integribasis*	NE France, Picardie, near Abbeville	50°14’15"N	01°51’24"E	Ulm1
R118/12	N	-	*R. juennensis*	SW Hungary, Somogy, near Somogyszob	46°19’12”N	17°13’07”E	Ulm1
R206/12	N	-	*R. leucandrus* subsp. *belgicus*	E Belgium, Walloon Region, near Spa	50°26’57"N	05°57’55"E	Ulm1
R178/11	N	-	*R. lindebergii*	NW Germany, Niedersachsen, near Osnabrück	52°22’08"N	08°05’02"E	Ulm1
R169/11	N	-	*R. loehrii*	NW Germany, Niedersachsen, near Osnabrück	52°13’55"N	08°01’26"E	Ulm2
R62/98	N	-	*R. nemoralis*	NE Czechia, NE Moravia, near Bohumín	49°54’38"N	18°24’23"E	Ulm1
R150/11	N	-	*R. platyacanthus*	NW Germany, SW Niedersachsen, near Bramsche	52°25’48"N	07°45’10"E	Ulm1
MS057/12	N	-	*R. praecox*	cN Serbia, near Beograd	44°43’00"N	20°28’04"E	Ulm1
R105/12	N	-	*R. praecox*	N Croatia, Bjelovarsko-bilogorska žup., near Grubišno Polje	45°45’20”N	17°18’47”E	Ulm1
R16/12	N	-	*R. praecox*	NW Hungary, Györ region, near Bakonyszentászló	47°21´33“N	17°48’21”E	Ulm1
R187/11	N	-	*R. praecox*	NW Germany, Niedersachsen, near Bramsche	52°25’10"N	07°56’38"E	Ulm1
F30-Pyr	N	-	*R. pyramidalis*	NW Germany, SW Nordrhein-Westfalen, near Aachen	50°43’26"N	06°14’19"E	Ulm1

MS003/12	N	-	*R. radula*	cN Germany, W Sachsen-Anhalt, near Quedlinburg	51°44’02"N	11°13’49"E	Ulm1
MS017/12	N	-	*R. radula*	cW Slovakia, district Zlaté Moravce	48°30’42"N	18°24’31"E	Ulm1
R84/12	N	-	*R. radula*	N Croatia, Bjelovarsko-bilogorska županija, near Kapela	46°01’19’’N	16°50’58"E	Ulm1
R194/11	N	-	*R. rhamnifolius*	NW Germany, Nordrhein-Westfalen, near Bad Oeynhausen	52°14’32"N	08°45’14"E	Ulm1
R192/11	N	-	*R. rhombifolius*	NW Germany, Nordrhein-Westfalen, near Bad Oeynhausen	52°15’03"N	08°49’16"E	Ulm1
R160/11	N	-	*R. senticosus*	NW Germany, SW Niedersachsen, near Bramsche	52°24’47"N	07°55’44"E	Ulm1
R136/11	N	-	*R. sciocharis*	cN Germany, E Niedersachsen, near Wolfsburg	52°18’23"N	10°50’40"E	Ulm2
R145/11	N	-	*R. schlechtendalii*	NW Germany, Nordrhein-Westfalen, Ibbenbüren	52°21’05"N	07°42’33"E	Ulm1
SiB01	N	-	*R. silvae-bohemicae*	W Czechia, W Bohemia, distr. Domažlice	49°30’57"N	12°47’12"E	Ulm2
R216/11	N	-	*R. stereacanthos*	NW Germany, Nordrhein-Westfalen, near Meinerzhagen	51°09’09"N	07°34’27"E	Ulm1
AM35/16	N	-	*R. ulmifolius* agg.	S Armenia, Syunik, near Shvanidzor	38°57’53"N	46°22’21"E	San2
HL1/14	N	-	*R. ulmifolius* agg.	Israel, Jerusalem	31°45’24"N	35°10’19"E	San2
HL2/14	N	-	*R. ulmifolius* agg.	Israel, Jerusalem	31°48’N	35°15’E	San5
LM03/12	N	-	*R. ulmifolius* agg.	SE France, Alpes-Maritimes, near Cannes	43°43’56"N	6°48’01"E	Ulm1
LM05/12	N	-	*R. ulmifolius* agg.	SE France, Provence-Alpes-Côte d’Azur, Cogolin	43°16’22"N	06°38’46"E	Ulm1
MS05/15	N	-	*R. ulmifolius* agg.	Abkhazia, near Gudauta	43°11’42"N	40°43’38"E	San1
MS086/12	N	-	*R. ulmifolius* agg.	NE Albania, near Kukës	42°05’09"N	20°22’34"E	Ulm1
MS097/12	N	-	*R. ulmifolius* agg.	S Croatia, Dubrovnik	42°38’23"N	18°07’49"E	Ulm1
MS21/15	N	-	*R. ulmifolius* agg.	Abkhazia, near Gagra	43°16’35"N	40°17’53"E	San3
MS23/15	N	-	*R. ulmifolius* agg.	Abkhazia, near Pitsunda	43°08’57"N	40°20’40"E	San3
MS24/15	N	-	*R. ulmifolius* agg.	Abkhazia, Sukhumi	43°00’23"N	41°00’4"E	San3
Ulm01	N	-	*R. ulmifolius* agg.	E Bulgaria, Sinemorec	42º04’08"N	27º58’01"E	San1
R157/12 (F7-Ves)	N	-	*R. vestitus* Weihe	W Germany, S Rheinland-Pfalz, near Neustadt	49°20’29"N	08°16’44"E	Ulm2
R151/11	N	-	*R. vigorosus*	NW Germany, SW Niedersachsen, near Bramsche	52°25’48"N	07°45’10"E	Ulm1

Chapter 3, Supplementary table 2: List of studied SSR loci; LG - linkage group, V - volume of the primer (20µM) used in 10µL reaction, c - final concentration of each primer in multiplex PCR.

Locus name	Locus abbrev.	LG	Primer F	Primer R	Multiplex set	V [uL]	c [µM]	Reference
ERubLR_SQ01_B06	01B06	4	CCTCTACACCACCCCATCAG	CGTCATCGTCATCTCTCTCG	1	0.15	0.3	Woodhead *et al.* 2008
Rubusr76b F	76b	2	CTCACCCGAAATGTTCAACC	GGCTAGGCCGAATGACTACA	1	0.25	0.5	Graham *et al.* 2004
ERubLR_SQ19_1_A05	191A05	5	GTTTGCTTCCTTTCGTAGTC	TATACTAATGGCCACCTTGG	1	0.25	0.5	Woodhead *et al.* 2008
RubPara_SQ007_O09	07O09	2	CATGGAAAACCATGCATCATA	GCTTTGTCCAAAAGTGCTGT	1	0.30	0.6	Woodhead *et al.* 2008
RiM015	RiM015	3	CGACACCGATCAGAGCTAATTC	ATAGTTGCATTGGCAGGCTTAT	1	0.20	0.4	Castillo *et al.* 2010
ERubLR_SQ07_2_H02	72H02	4	TGGCAATCAACCACTCTGTG	CAAACTGACAAACGCTCTTCC	2	0.15	0.3	Woodhead *et al.* 2008
RubLR_SQ05_3_E02	53E02	5	GTCACACAAGGCTACCAAG	ATTGAACTGGTCAACAATGC	2	0.15	0.3	Woodhead *et al.* 2008
ERubLR_SQ01_M20	01M20	5	TTACGAACACCCATTAATTTAAGTC	AATCCTGAGACCGACGAGTG	2	0.50	1.0	Woodhead *et al.* 2008
RhM023	RhM023	?	CGACAACGACAATTCTCACATT	GTTATCAAGCGATCCTGCAGTT	2	0.10	0.2	Castillo *et al.* 2010
RubfruitC1	RubfruitC1	2	CACGAGCTTCATCCTCTTCC	ATCCAAAGCTTTTGCGATTG	2	0.15	0.3	Graham *et al.* 2004

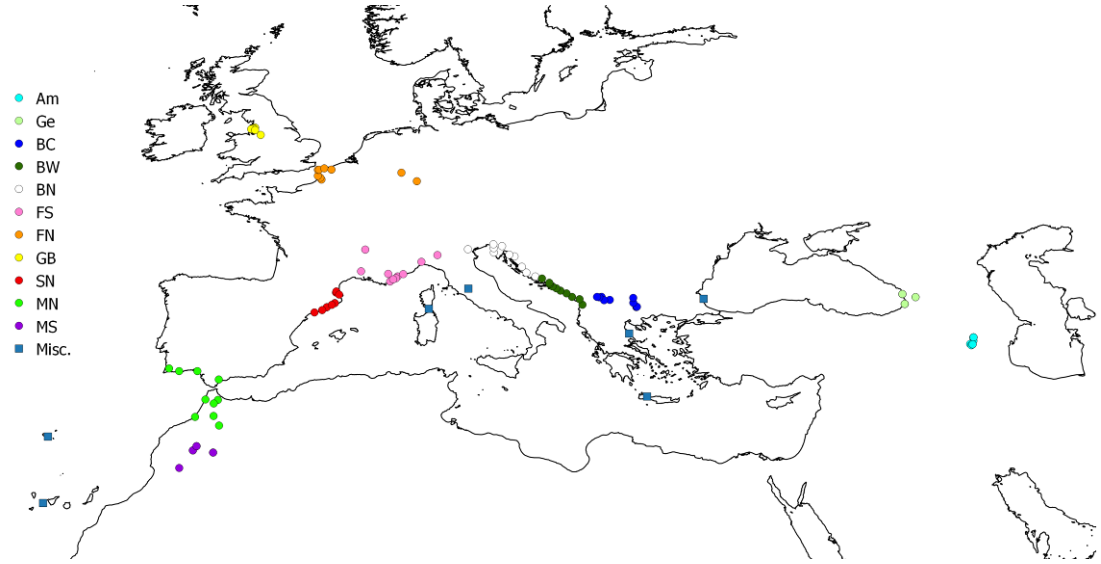
Chapter 3, Supplementary table 3: Polymorphisms in cpDNA alignment.

	position in the alignment											
	MatK									*trnL-trnF*		
Haplotype	17	146	199	306	436	457	561	837	921	54	71	76
Ulm2	A	T	C	C	A	G	T	A	C	A	A	G
San1	.	.	.	.	.	.	.	.	.	.	G	.
San2	G	.	.	.	.	.	.	.	.	.	.	A
San3	G	.	.	.	.	.	.	C	.	.	.	A
San4	G	C	.	.	.	.	.	C	.	.	.	A
San5	.	.	.	T	.	A	C	.	.	.	.	.
Ulm1	.	.	.	.	.	.	.	.	.	T	.	.
Ulm3	G	.	T	.	.	.	.	C	T	.	.	A
Ulm4	G	.	T	.	C	.	.	C	T	.	.	A

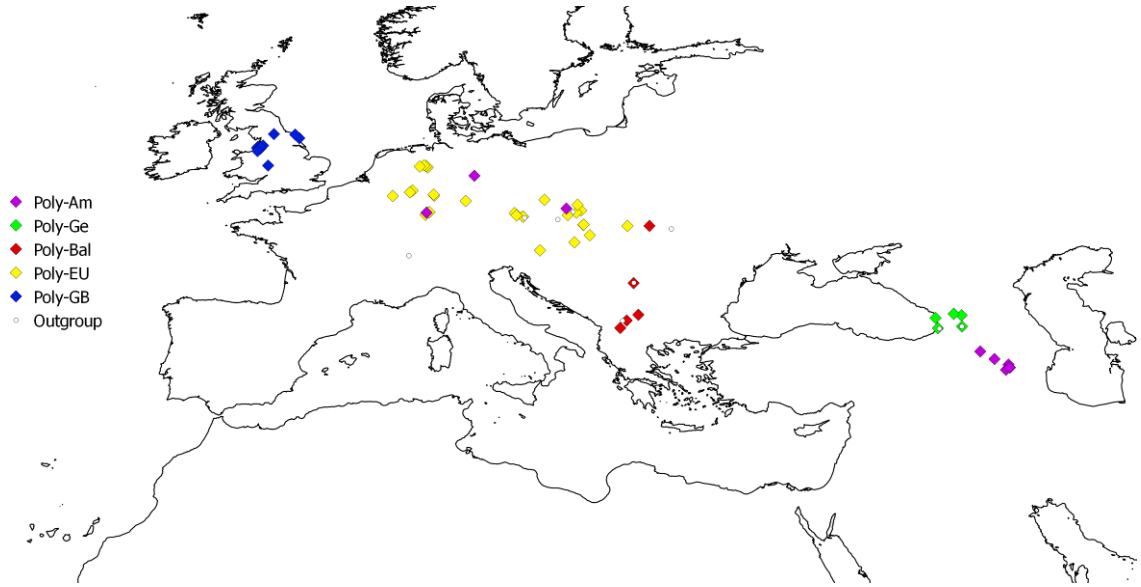
Chapter 4, Supplementary table 4: Pairwise FST indices (below diagonal) and P-values of test for population differentiation (above diagonal, non-significant values in bold).

Population	**Am**	**Ge**	**BC**	**BW**	**BN**	**FS**	**FN**	**GB**	**SN**	**MN**	**MS**
Am	-	HS*	HS*	HS*	HS*	HS*	HS*	HS*	HS*	HS*	HS*
Ge	0.231	-	HS*	HS*	HS*	HS*	HS*	HS*	HS*	HS*	HS*
BC	0.267	0.219	-	0.000	0.000	HS*	HS*	HS*	HS*	0.000	HS*
BW	0.300	0.265	0.116	-	**0.672**	0.006	0.000	0.000	0.001	0.000	0.000
BN	0.334	0.301	0.163	-0.025	-	0.033	0.001	0.000	0.030	0.011	0.000
FS	0.397	0.388	0.235	0.040	0.031	-	**0.065**	0.000	**0.062**	0.000	0.000
FN	0.428	0.419	0.263	0.076	0.070	0.047	-	**0.398**	0.009	0.000	HS*
GB	0.491	0.483	0.350	0.186	0.160	0.160	0.000	-	0.000	0.000	HS*
SN	0.383	0.367	0.230	0.046	0.025	0.015	0.040	0.121	-	0.000	0.000
MN	0.377	0.359	0.198	0.037	0.014	0.074	0.148	0.261	0.071	-	0.028
MS	0.409	0.397	0.266	0.105	0.087	0.108	0.217	0.334	0.111	0.026	-

*HS (Highly Significant) - at least one of the individual tests being combined yielded a zero P-value estimate

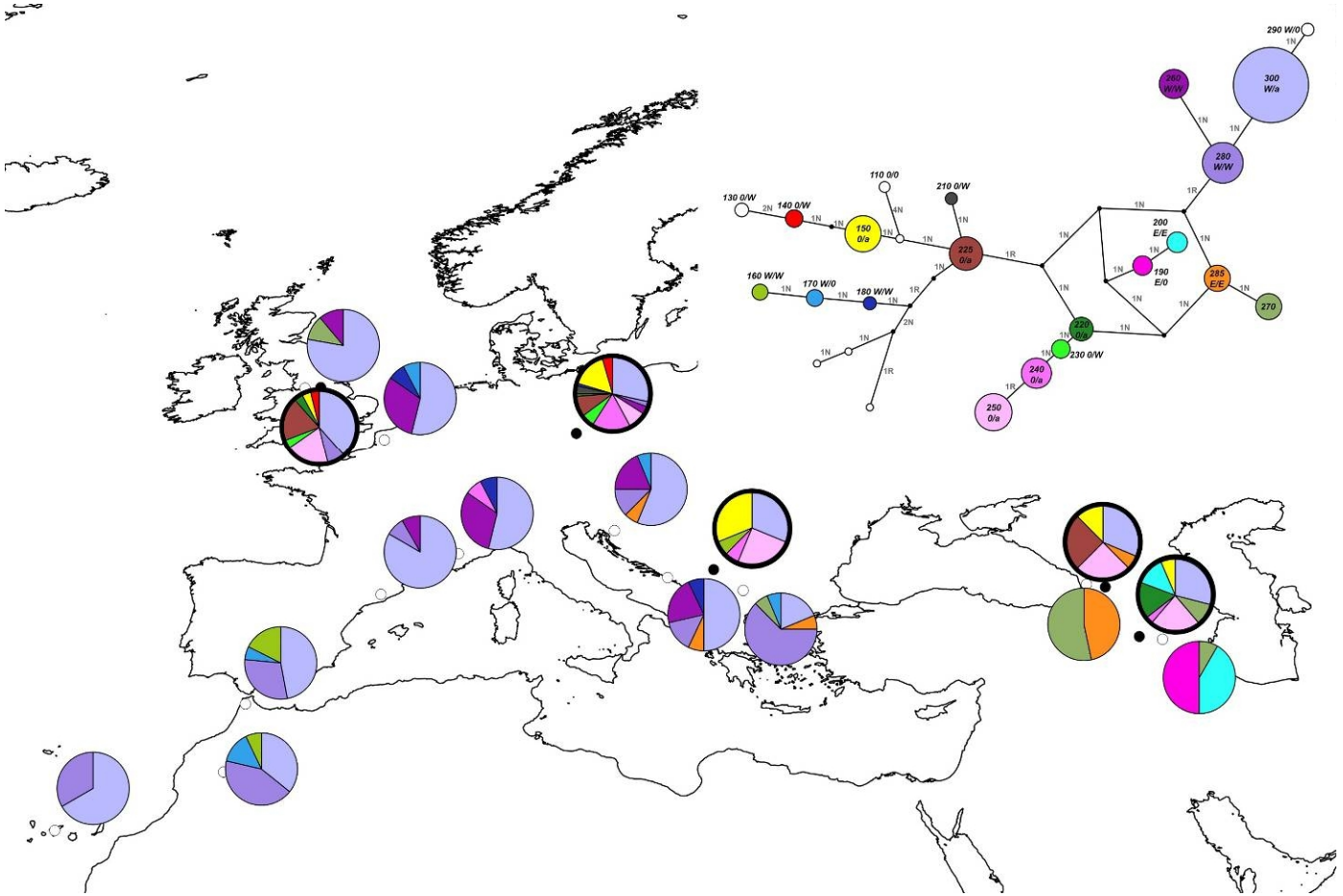


Chapter 3; Supplementary figure 1A: Sampling design and population delimitation of *R. ulmifolius* agg.

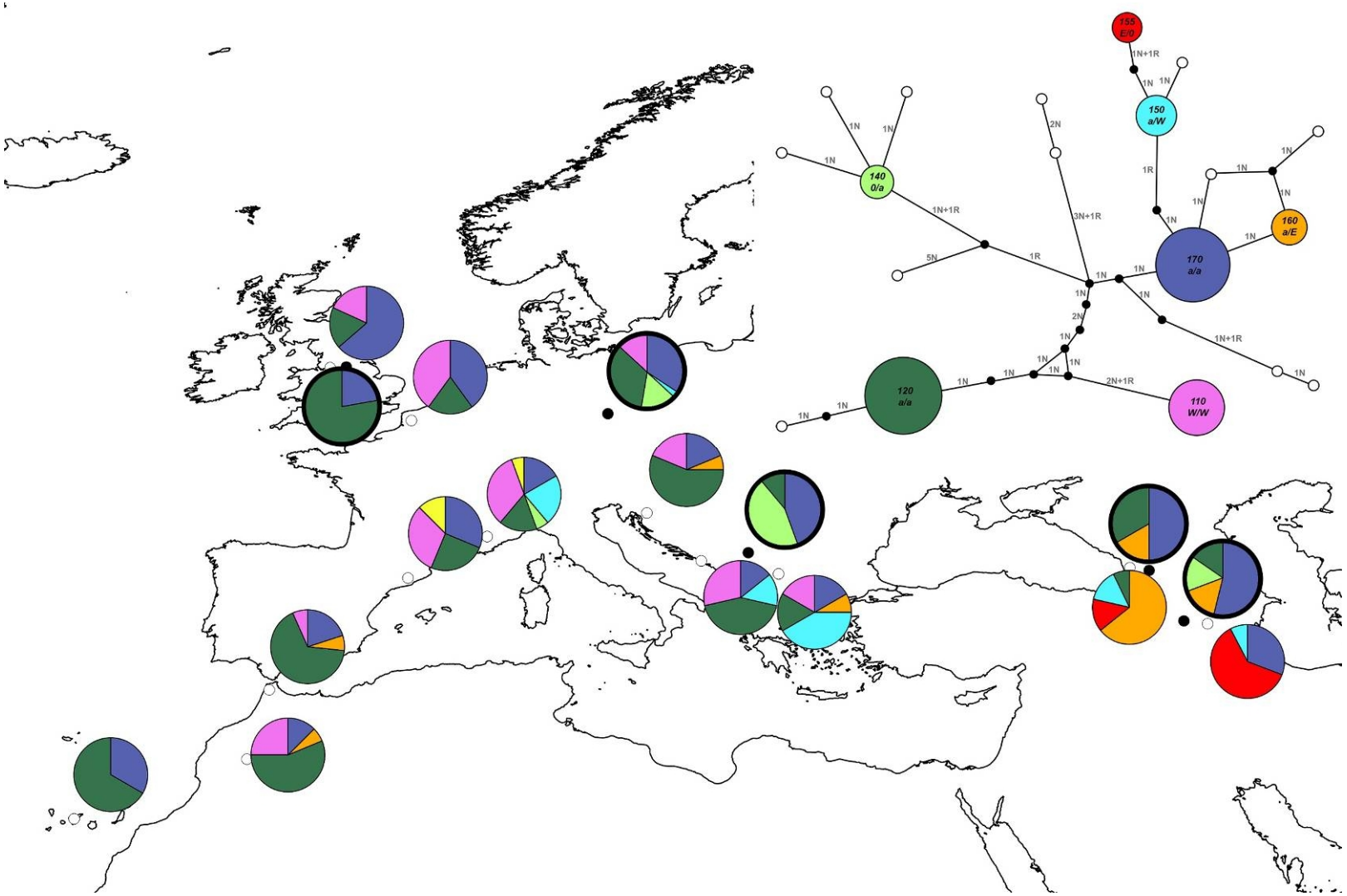


Chapter 3; Supplementary figure 1B: Sampling design and population delimitation of polyploid and outgroup *Rubus* accessions, the three Poly-Am samples in cetral Europe are *R. armeniacus*.

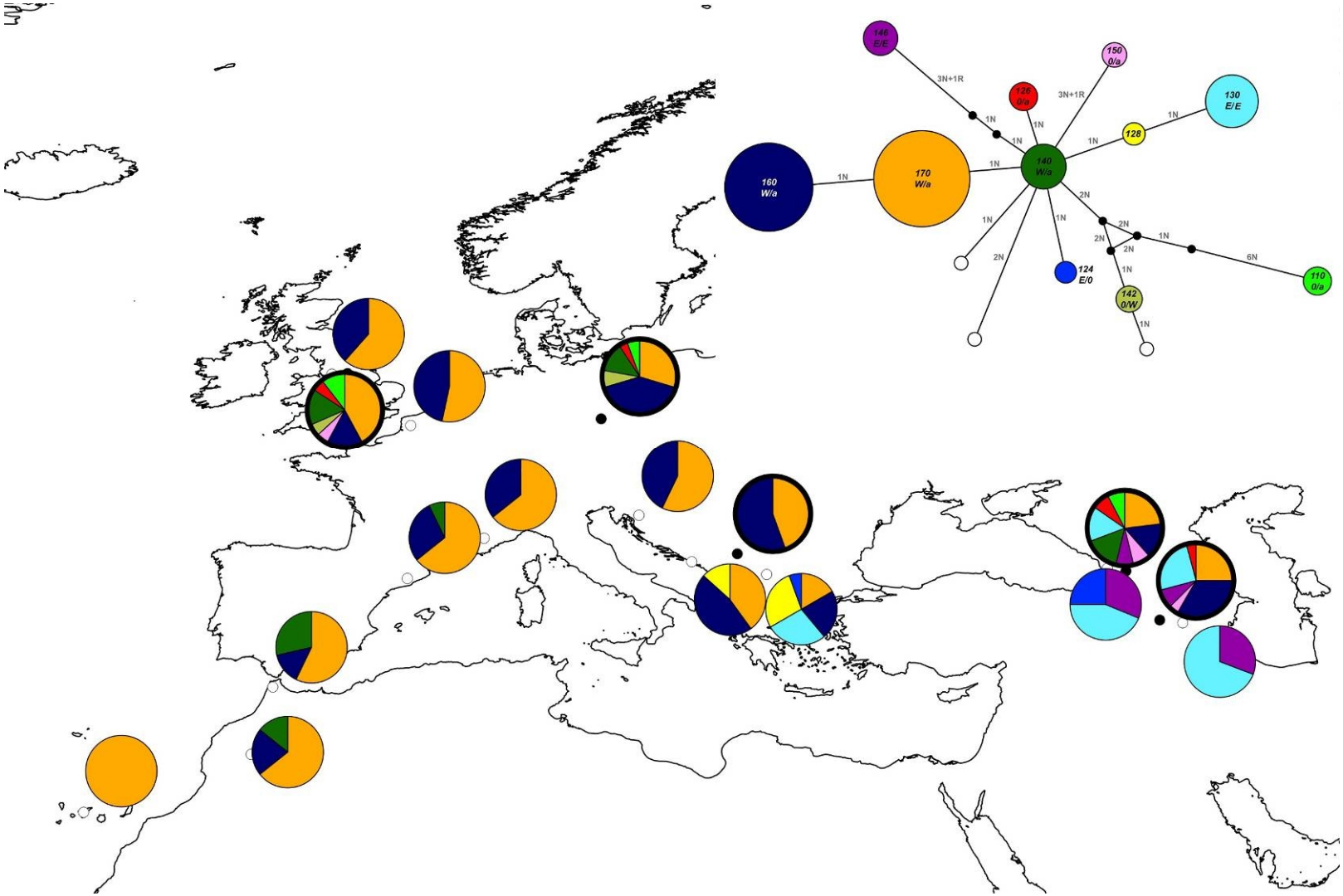
Chapter 3, Supplementary figure 2: SSR allele distribution patterns in *R. ulmifolius* agg. (thin pie-charts) and polyploid accessions (in bold) for each locus. Each color represents one allele and its relative frequency in a population (pie-charts). Circle size in phylogenetic network corresponds to allele occurrence in total data-set; N and R indicate numbers of single nucleotide mutations and changes in microsatellite repeat number, respectively.



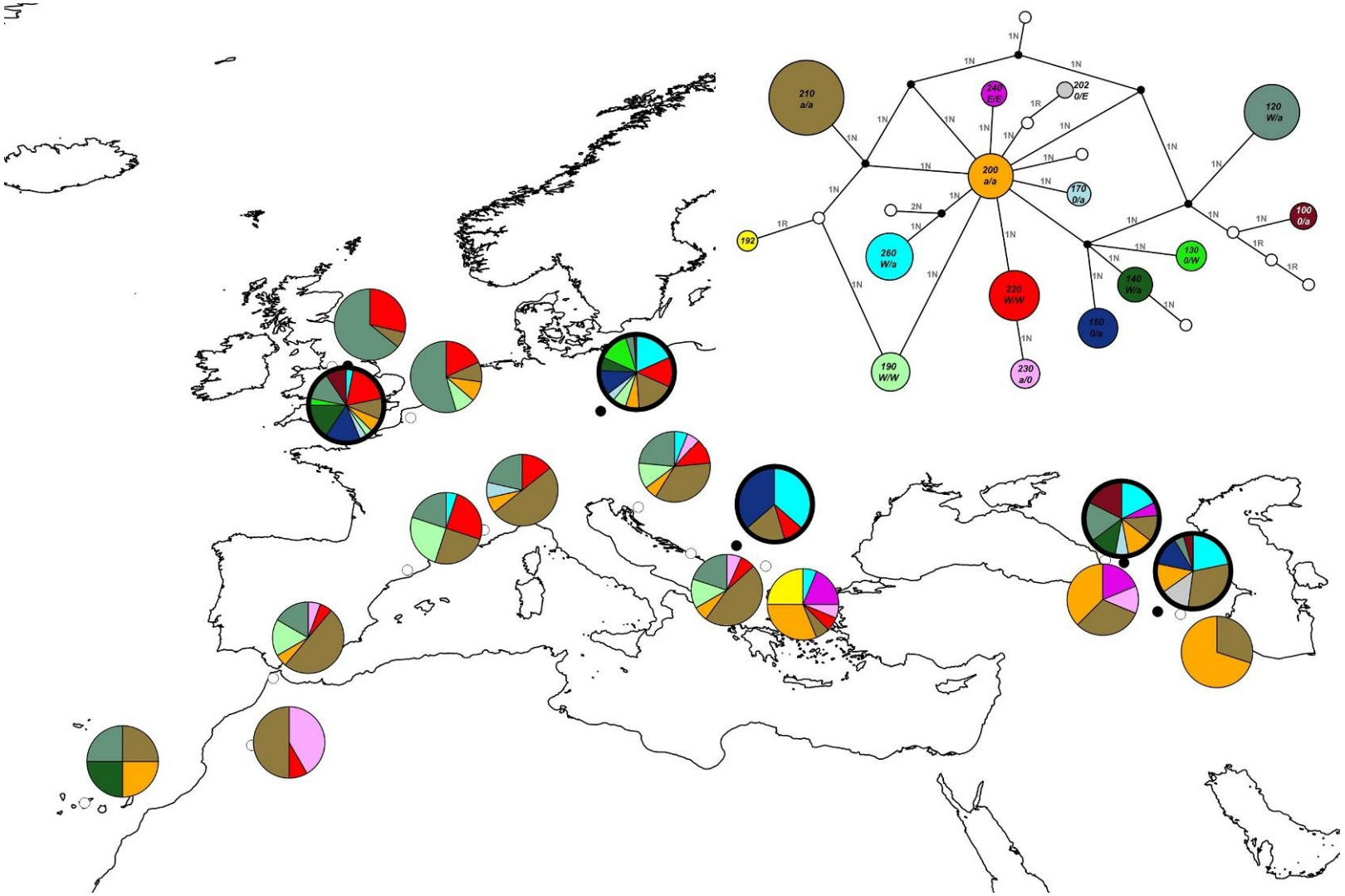
A: Locus 01B06



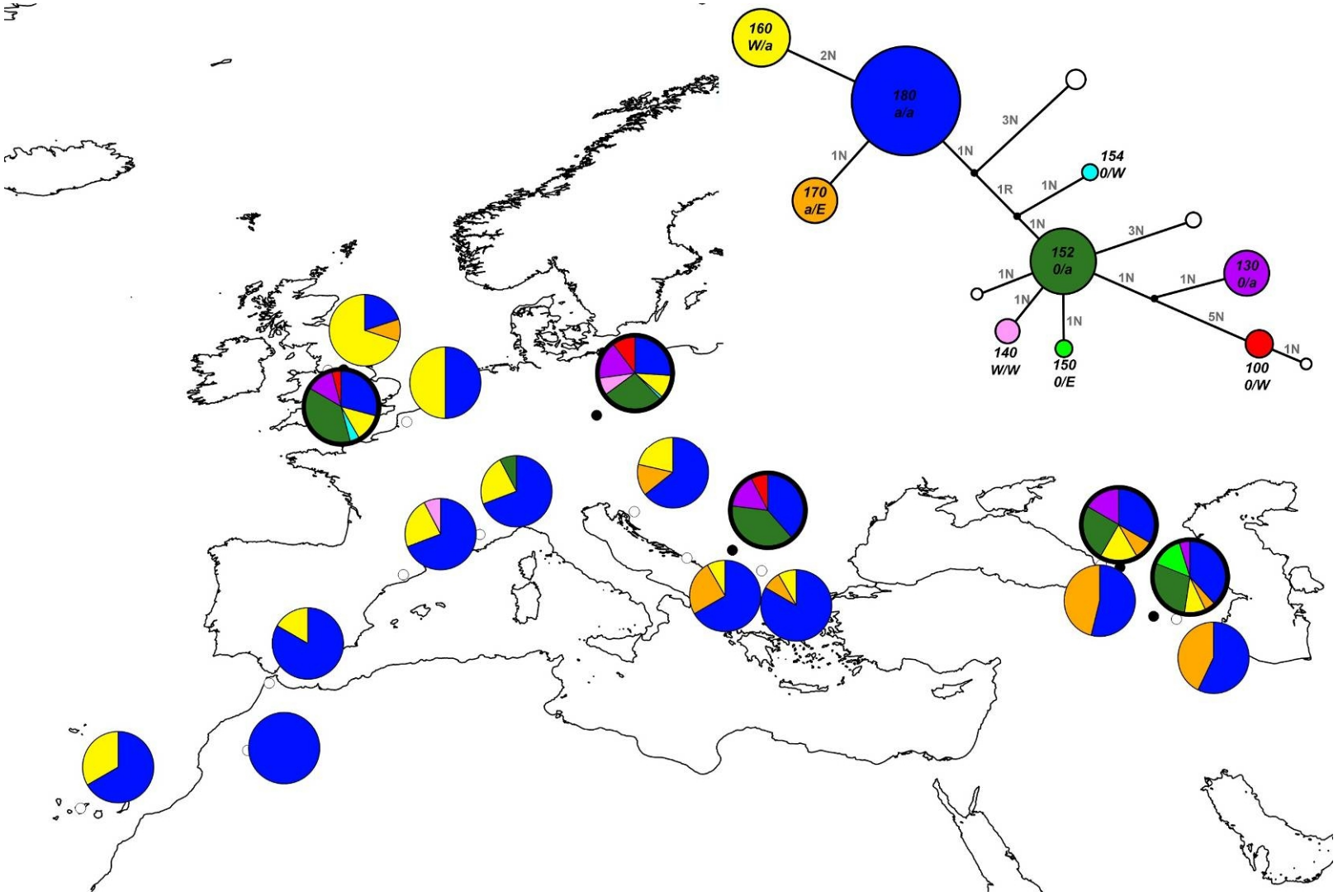
B: Locus 01M20



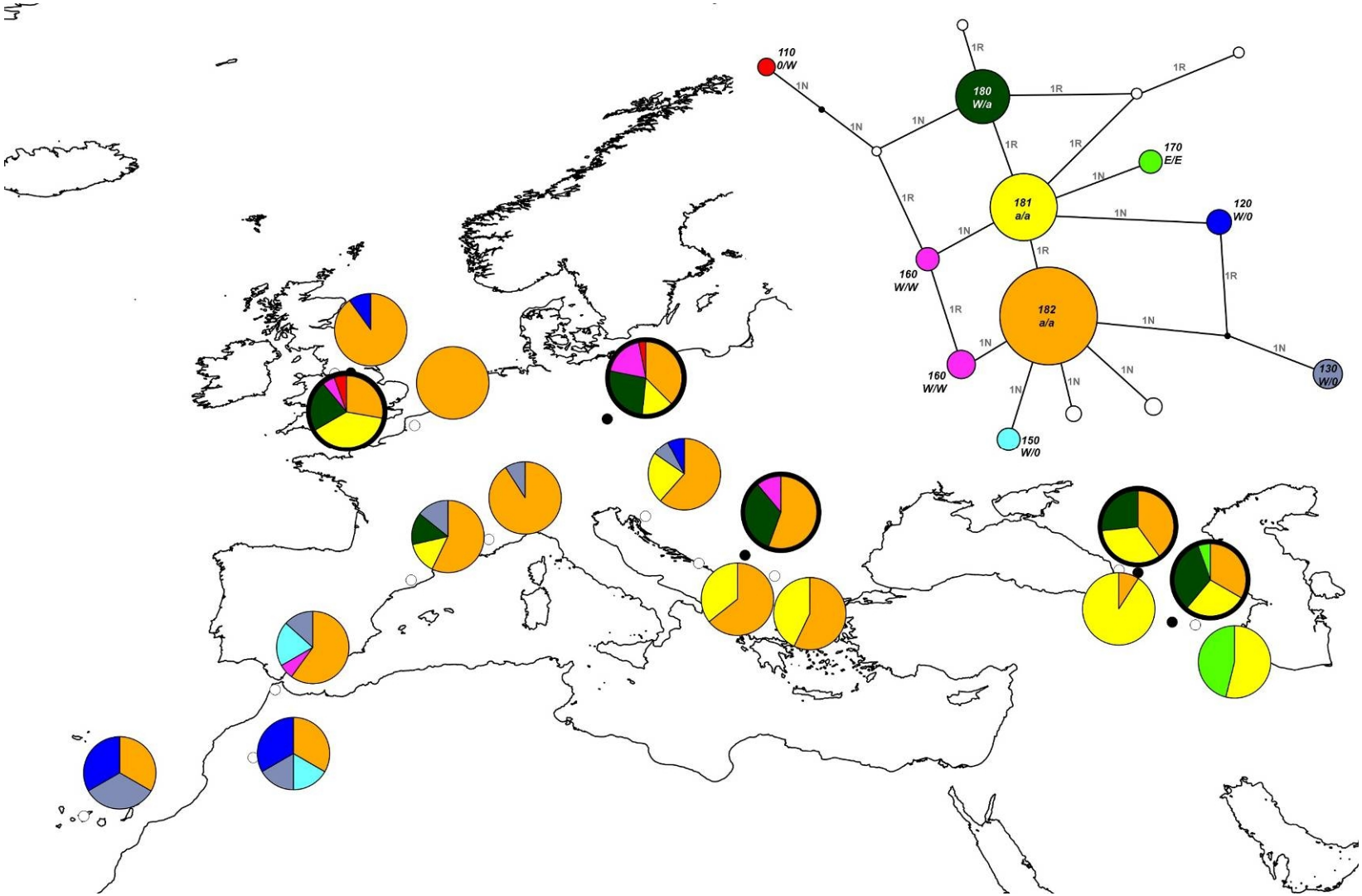
C: Locus 07O09



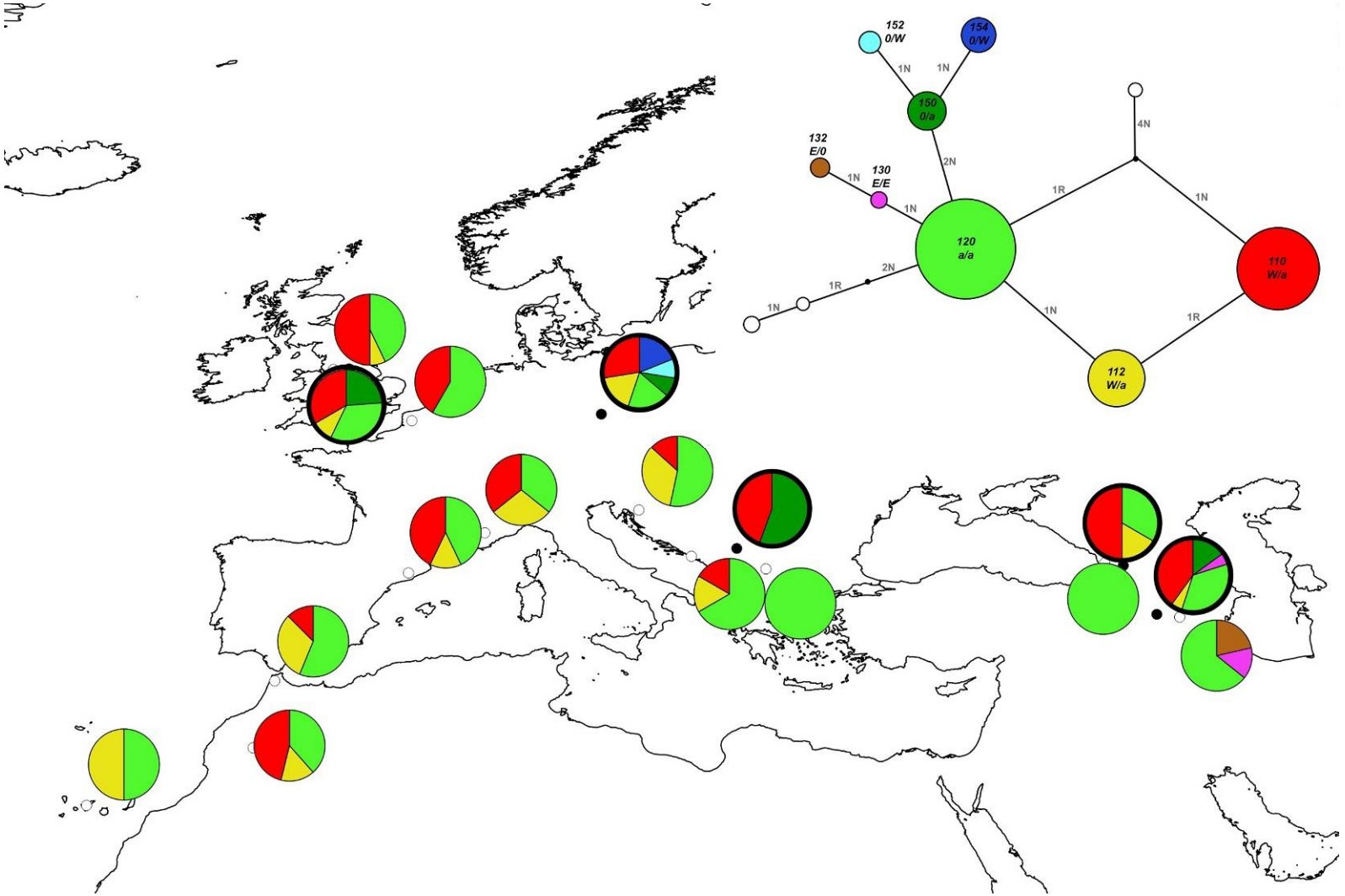
D: Locus 53E02



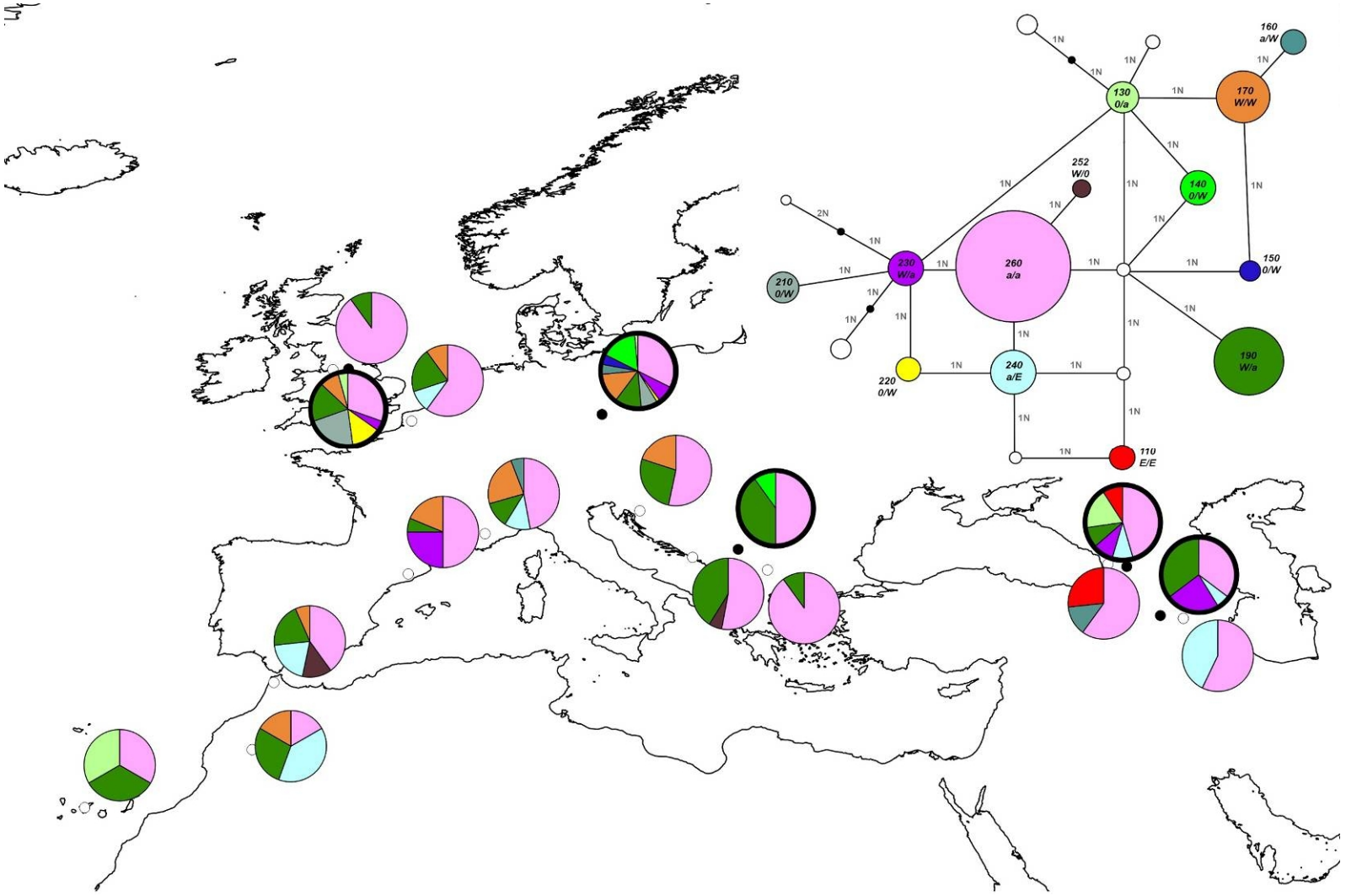
E: Locus 72H02


110
0/W
180
W/a
170
E/E
181
a/a
120
W/0
160
W/W
182
a/a
130
W/0
160
W/W
150
W/0
1R
1N

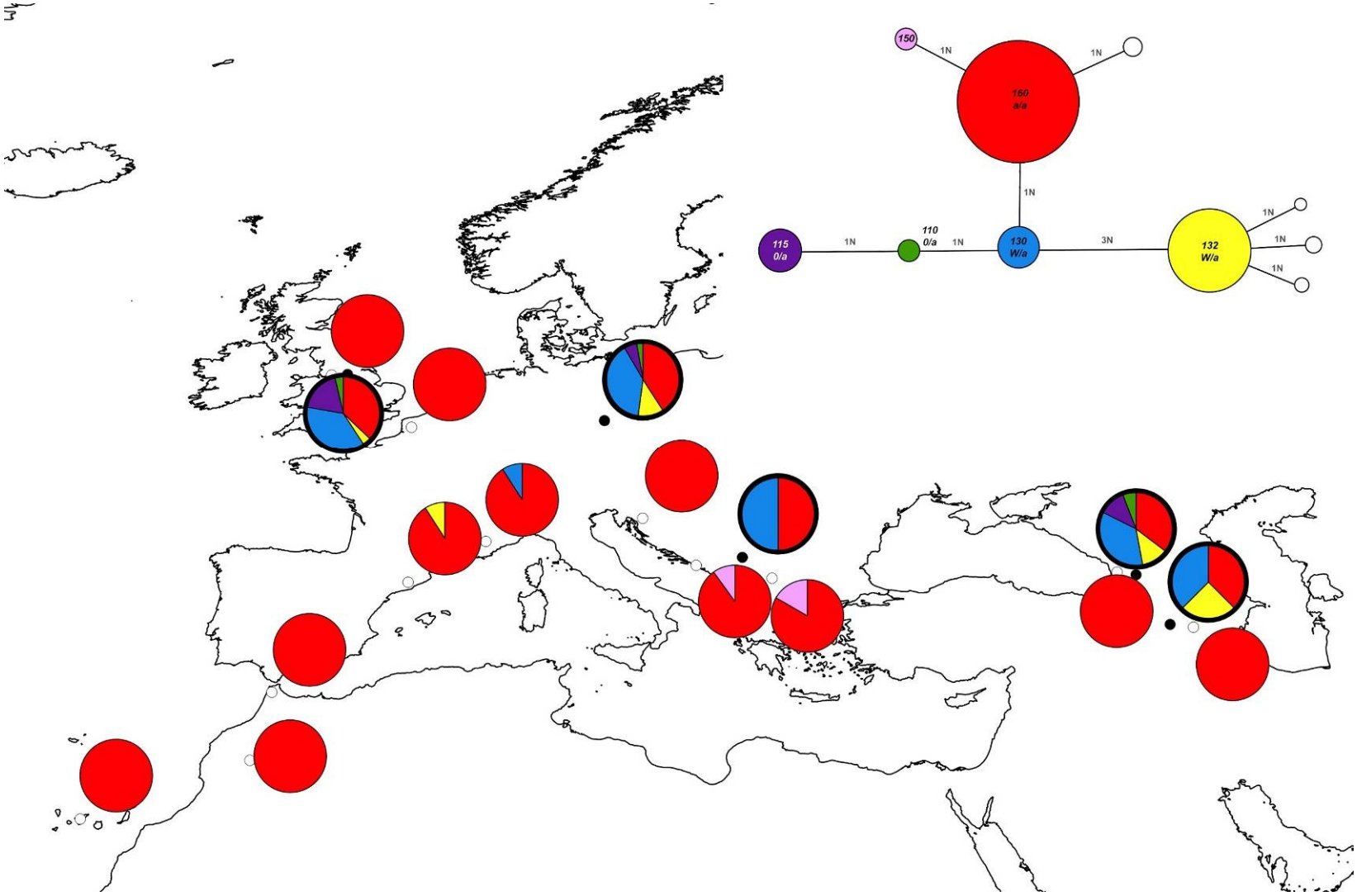
F: Locus 76B



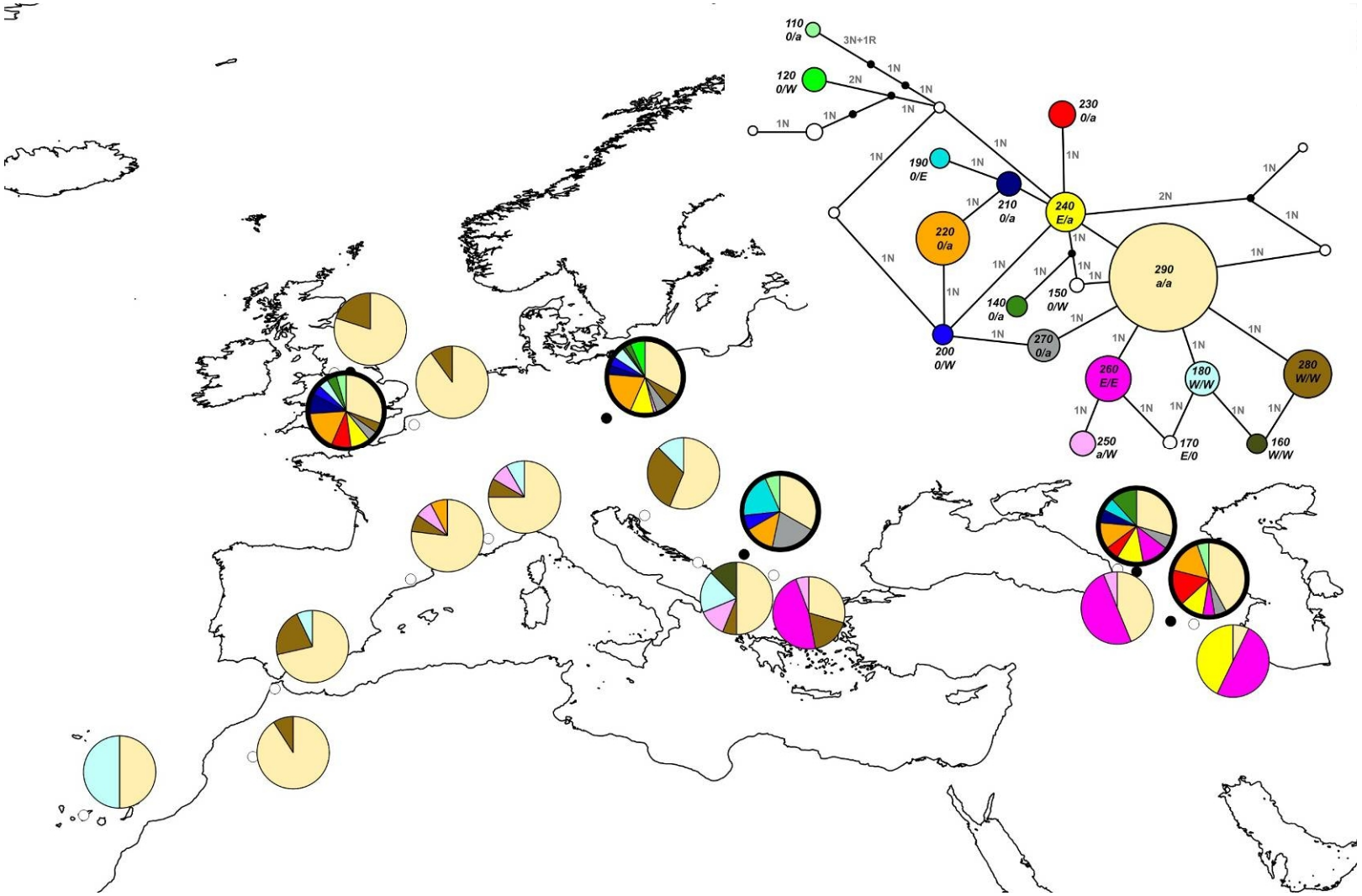
G: Locus 191A05



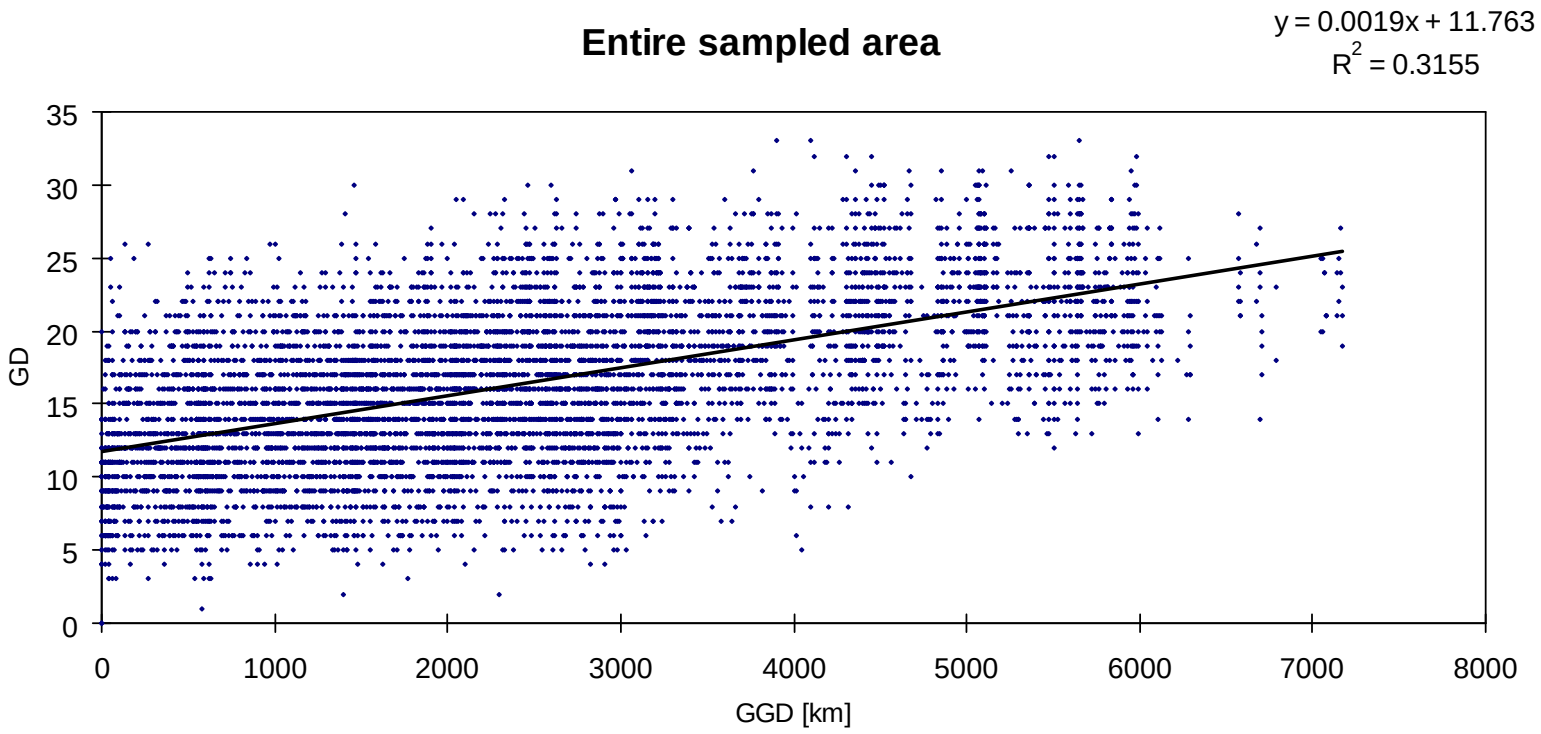
H: Locus FruitC1



I: Locus Rhm23



J: Locus RiM15



Chapter 3, Supplementary figure 3: Mantel test for correlation between genetic (GD) and geographic (GGD; in km) distance for entire sample-set; R = 0.562, P < 0.0001.

Chapter 4, Supplementary table 1: Geographic origin of studied accessions, taxonomical delimitation, observed haplotypes and ploidy levels (based on FCM or FCSS).

Collection No.	Morphotaxon	Latitude	Longitude	Altitude	Date	Locality	Morphoseries	Haplotype	Ploidy
MS55/13	*R. canescens*	41°50'8"N	3°15'53"E	1809	2013-08-20	Georgia, Samtskhe-Javakheti, Borjomi distr, 7 km WNW of Likani village	*Canescentes*	Can1	-
MS43/14	*R.* cf. *ibericus*	41°41'28"N	41°42'18"E	12	2014-07-10	Georgia, Adjara, Mtsvane Kontskhi, at the entrace to the botanical garden	*Discolores*	Ulm1	tetraploid (FCM)
MS31/14	R. morphotax. dis1	42°39'34"N	42°46'21"E	526	2014-07-04	Georgia, Kvemo Svaneti, Tsageri, open-air theatre on the N edge of the city	*Discolores*	Cau1	tetraploid (FCM)
MS57/13	R. morphotax. dis2	42°23'29"N	41°34'11"E	0	2013-08-17	Georgia, Anaklia, ca 0.5 km SE of the school	*Discolores*	Cau1	tetraploid (FCSS)
MS58/13	R. morphotax. dis3	42°59'15"N	42°15'14"E	770	2013-08-17	Georgia, Svaneti, 8 km NE of Khaishi village	*Discolores*	Ulm1	tetraploid (FCSS)
MS30/14	R. morphotax. dis4	42°39'34"N	42°46'21"E	526	2014-07-04	Georgia, Kvemo Svaneti, Tsageri, open-air theatre on the N edge of the city	*Discolores*	Cau1	tetraploid (FCM)
MS41/14	R. morphotax. dis5	41°43'14"N	41°43'43"E	9	2014-07-09	Georgia, Adjara, Chakvi, at the railway station	*Discolores*	Cau1	tetraploid (FCM)
MS42/14	R. morphotax. dis6	41°43'14"N	41°43'43"E	10	2014-07-09	Georgia, Adjara, Chakvi, at the railway station	*Discolores*	Cau1	tetraploid (FCM)
MS22/14	R. morphotax. dis7	42°34'3.8"N	43°17'02"E	664	2014-06-24	Georgia, Racha, centre of Sori village, 12 km NE of Ambrolauri	*Discolores*	Can3	-
MS01/15	R. morphotax. dis8	43°05'17"N	41°17'55"E	515	2015-07-04	Abkhazia, S bank of lake Amtkel	*Discolores*	San3	tetraploid (FCM)
MS02/15	R. morphotax. dis9	43°05'17"N	41°17'55"E	515	2015-07-05	Abkhazia, S bank of lake Amtkel	*Discolores*	Cau1	tetraploid (FCM)
MS04/15	R. morphotax. dis10	43°02'45"N	41°19'27"E	246	2015-07-05	Abkhazia, near a bridge in Amtkel village, 25 km ENE from Sukhumi	*Discolores*	Cau1	tetraploid (FCM)
MS06/15	R. morphotax. dis11	43°11'48"N	40°43'42"E	244	2015-07-07	Abkhazia, N part of Kvabruta village (near Khabiu)	*Discolores*	Cau1	tetraploid (FCM)
MS07/15	R. morphotax. dis12	43°14'2"N	40°48'14"E	477	2015-07-07	Abkhazia, the Aapsta river valley, 5 km NE of Khabiu	*Discolores*	Cau1	tetraploid (FCM)

MS14/15	R. morphotax. dis13	43°16’35’’N	40°17’53’’E	360	2015-07-16	Abkhazia, 2 km E of Gagra	*Discolores*	Gla3	tetraploid (FCM)
MS17/15	R. morphotax. dis14	43°09’20’’N	40°20’16’’E	1	2015-07-18	Abkhazia, Pitsunda, city center	*Discolores*	Gla3	tetraploid (FCM)
MS18/15	R. morphotax. dis15	43°00’17’’N	41°00’16’’E	5	2015-07-19	Abkhazia, Sukhum, N part of Imam Shamil street	*Discolores*	Can3	tetraploid (FCM)
MS19/15	R. morphotax. dis16	42°10’58’’N	42°27’46’’E	45	2015-07-20	Georgia, Kutaisi international airport	*Discolores*	Cau1	tetraploid (FCM)
MS20/15	R. morphotax. dis17	42°10’58’’N	42°27’46’’E	45	2015-07-20	Georgia, Kutaisi international airport	*Discolores*	Cau1	tetraploid (FCM)
MS05/15	*R. sanctus*	43°11’42’’N	40°43’38’’E	223	2015-07-07	Abkhazia, N part of Kvabruta village (near Khabiu)	*Discolores*	San1	-
MS46B/14	*R. sanctus*	42°11’01’’N	42°28’04’’E	46	2014-07-11	Georgia, Imereti, Kutaisi international airport	*Discolores*	San1	-
MS21/15	*R. sanctus*	43°16’35’’N	40°17’53’’E	360	2015-07-16	Abkhazia, 2 km E of Gagra	*Discolores*	San3	-
MS23/15	*R. sanctus*	43°08’57’’N	40°20’40’’E	7	2015-07-18	Abkhazia, Pitsunda, S of city center	*Discolores*	San3	-
MS24/15	*R. sanctus*	43°00’23’’N	41°00’4’’E	9	2015-07-19	Abkhazia, Sukhum, railway station	*Discolores*	San3	-
MS35/14	*R. sanctus*	41°42’58’’N	41°43’57’’E	7	2014-07-06	Georgia, Adjara, centre of Chakvi	*Discolores*	San3	diploid (FCM)
MS40F/14	*R. sanctus*	41°43’14’’N	41°43’43’’E	8	2014-07-09	Georgia, Adjara, Chakvi, at the railway station	*Discolores*	San3	diploid (FCM)
MS40M/14	*R. sanctus*	41°43’14’’N	41°43’43’’E	8	2014-07-09	Georgia, Adjara, Chakvi, at the railway station	*Discolores*	San3	diploid (FCM)
MS46A/14	*R. sanctus*	42°11’01’’N	42°28’04’’E	46	2014-07-11	Georgia, Imereti, Kutaisi international airport	*Discolores*	San4	-
MS47/13	*R. sanctus*	42°23’18"N	41°33’60"E	0	2013-08-17	Georgia, Anaklia, at the main beach near hotel Golden Fleece	*Discolores*	San3	-

MS48A/13	*R. sanctus*	42°23’41’’N	41°33’57"E	0	2013-08-17	Georgia, Anaklia, near the school	*Discolores*	San3	-
MS12/15	*R. "hirtus"* agg.*	43°28’41’’N	40°39’30’’E	1735	2015-07-14	Abkhazia, 1.5 km NW of peak Anchkho	*Glandulosi*	Cau1	tetraploid (FCM)
MS32/14	*R. "hirtus"* agg.*	41°41’52’’N	41°42’50’’E	60	2014-07-05	Georgia, Adjara, Batumi - Mtsvane Kontskhi botanical garden	*Glandulosi*	Cau1	tetraploid (FCM)
MS33/14	*R. "hirtus"* agg.*	41°41’15’’N	41°49’49’’E	204	2014-07-06	Georgia, Adjara, at the road between Khala and Chakvistavi	*Glandulosi*	Cau1	tetraploid (FCM)
MS60/13	*R. "hirtus"* agg.*	41°51’43"N	43°14’50"E	1900	2013-08-19	Georgia, Samtskhe-Javakheti, 11.7 km WNW of Borjomi, at the Lomis Mt. shelter	*Glandulosi*	Cau1	tetraploid (FCM)
MS16/15	*R. "hirtus"* agg.*	43°19’22’’N	40°18’46’’E	1575	2015-07-16	Abkhazia, Mamdzyshkha range, 6 km NE of Gagra	*Glandulosi*	Gla3	tetraploid (FCM)
MS08/15	*R*. cf. *platyphyllus****	43°16’03’’N	40°47’38’’E	710	2015-07-07	Abkhazia, near the River Makiko, 3.3 km S of the pass Gudautskij pereval	*Glandulosi*	Cau1	tetraploid (FCM)
MS23/14	*R*. cf. *platyphyllus****	42°40’45’’N	43°34’20’’E	1078	2014-06-24	Georgia, Racha, 5 km N of Utsera, 2 km behind Rioni a Chanchakhi confluence	*Glandulosi*	Cau1	tetraploid (FCM)
MS27/14	*R*. cf. *platyphyllus****	42°42’23’’N	43°34’38’’E	1192	2014-06-28	Georgia, Racha, right bank of Rioni, ca 1 km before the confluence with Chanchakhi	*Glandulosi*	Cau1	tetraploid (FCM)
MS43/13	*R*. cf. *platyphyllus****	43°05’23’’N	42°44’34’’E	1551	2013-08-15	Georgia, Svaneti, between Mestia and the Ushba Glacier	*Glandulosi*	Cau1	tetraploid (FCM)
MS45/13	*R*. cf. *platyphyllus****	43° 6’44"N	42°44’51"E	1729	2013-08-15	Georgia, Svaneti, Mestiachala valley, 4 km SSW of the Lekhziri glacier	*Glandulosi*	Cau1	tetraploid (FCM)
MS46/13	*R*. cf. *platyphyllus****	43° 1’ 56’’ N	42°42’43’’E	1559	2013-08-16	Georgia, Svaneti, ca 1.7 km SW of the square in Mestia	*Glandulosi*	Cau1	tetraploid (FCM)
MS52/13	*R*. cf. *platyphyllus****	41°49’46’’N	43°18’5’’E	1079	2013-08-18	Georgia, Samtskhe-Javakheti, Borjomi distr., 2.7 km W of Likani village	*Glandulosi*	Cau1	tetraploid (FCSS)
MS53/13	*R*. cf. *platyphyllus****	41°51’41"N	43°14’45"E	1900	2013-08-19	Georgia, Samtskhe-Javakheti, 11.7 km WNW of Borjomi, at the Lomis Mt. shelter	*Glandulosi*	Cau1	tetraploid (FCSS)
MS56/13	*R*. cf. *platyphyllus****	41°50’35"N	43°15’37"E	1852	2013-08-20	Georgia, Samtskhe-Javakheti, Borjomi distr, 7 km WNW of Likani village	*Glandulosi*	Cau1	tetraploid (FCM)

MS28/14	R. cf. platyphyllus***	42°47’10’’N	43°24’08’’E	1694	2014-06-29	Georgia, Racha, right bank of Rioni 9 km W of Ghebi	*Glandulosi*	Cau2	tetraploid (FCM)
MS13/15	*R*. cf. *platyphyllus***	43°28’41’’N	40°33’48’’E	1040	2015-07-14	Abkhazia, 1 km E of lake Ritsa	*Glandulosi*	Gla3	tetraploid (FCM)
MS24/14	*R*. cf. *platyphyllus***	42°41’14’’N	43°42’02’’E	1729	2014-06-28	Georgia, Racha, at the military highway 2.7 km SE of resort Shovi	*Glandulosi*	-	tetraploid (FCM)
MS26/14	*R*. cf. *platyphyllus***	42°41’53’’N	43°34’57’’E	1143	2014-06-28	Georgia, Racha, at the confluence of Rioni and Chanchakhi, on the right bank of Rioni	*Glandulosi*	-	tetraploid (FCM)
MS42/13	*R*. cf. *platyphyllus***	43°05’23’’N	42°44’34’’E	1551	2013-08-15	Georgia, Svaneti, between Mestia and the Ushba Glacier	*Glandulosi*	-	tetraploid (FCM)
MS44/13	*R*. cf. *platyphyllus***	43°01’56’’ N	42°42’43’’ E	1565	2013-08-15	Georgia, Svaneti, 1.7 km SW of Mestia centre	*Glandulosi*	-	tetraploid (FCSS)
MS61/13	*R*. cf. *platyphyllus***	41°49’46’’N	43°18’50’’E	1079	2013-08-18	Georgia, Samtskhe-Javakheti, Borjomi distr., 2.7 km W of Likani village	*Glandulosi*	-	tetraploid (FCM)
MS34/14	*R. moschus* morphotype 1**	41°40’55’’N	41°50’29’’E	214	2014-07-06	Georgia, Adjara, W edge of Chakvistavi	*Glandulosi*	Gla1	diploid (FCM)
MS49/13	*R. moschus* morphotype 1**	41°49’45,5’’N	43°18’50’’E	1079	2013-08-18	Georgia, Samtskhe-Javakheti, Borjomi, 2.7 km W of Likani village	*Glandulosi*	Gla1	diploid (FCM)
MS51/13	*R. moschus* morphotype 1**	41°51’43"N	43°14’46"E	1919	2013-08-20	Georgia, Samtskhe-Javakheti, 11.7 km WNW of Borjomi, at the Lomis Mt. shelter	*Glandulosi*	Gla1	diploid (FCSS)
MS38/14	*R. moschus* morphotype 1**	41°40’46’’N	41°51’06’’E	247	2014-07-09	Georgia, Adjara, W edge of Chakvistavi	*Glandulosi*	Gla5	diploid (FCM)
MS39/14	*R. moschus* morphotype 2**	41°41’27’’N	41°49’30’’E	192	2014-07-09	Georgia, Adjara, at the road between Khala and Chakvistavi	*Glandulosi*	Gla1	diploid (FCM)
MS36/14	*R. moschus* morphotype 2**	41°40’23’’N	41°50’58’’E	571	2014-07-07	Georgia, Adjara, 1.2 km SW of Chakvistavi	*Glandulosi*	Gla5	diploid (FCM)
MS59/13	*R.* cf. *peruncinatus*	41°49’46’’N	43°18’50’’E	1079	2013-08-18	Georgia, Samtskhe-Javakheti, Borjomi distr., 2.7 km W of Likani village	*Micantes*	Cau1	tetraploid (FCSS)
MS44/14	*R.* morphotax. *mic1*	41°41’28’’N	41°42’18’’E	13	2014-07-11	Georgia, Adjara, Mtsvane Kontskhi, at the entrace to the botanical garden	*Micantes*	Cau1	tetraploid (FCM)

MS29/14	*R.* morphotax. *mic2*	42°39'34''N	42°46'21''E	526	2014-07-04	Georgia, Kvemo Svaneti, Tsageri, open-air theatre on the N edge of the city	*Micantes*	Cau1	tetraploid (FCM)
MS45/14	*R.* morphotax. *mic2*	41°41'28''N	41°42'18''E	14	2014-07-11	Georgia, Adjara, Mtsvane Kontskhi, at the entrace to the botanical garden	*Micantes*	Cau1	tetraploid (FCM)
MS03/15	*R*. morphotax. *mic3*	43°04'54''N	41°18'22''E	530	2015-07-05	Abkhazia, 1 km SE of lake Amtkel	*Micantes*	Gla3	tetraploid (FCM)
MS09/15	*R*. morphotax. *mic4*	43°16'40''N	40°48'02''E	1100	2015-07-08	Abkhazia, 2 km S of the pass Gudautskij pereval	*Micantes*	Gla4	tetraploid (FCM)
MS11/15	*R*. morphotax. *mic5*	43°24'20''N	40°49'05''E	830	2015-07-10	Abkhazia, N margin of Pskhu village	*Micantes*	Cau1	tetraploid (FCM)
MS37/14	*R*. morphotax. *rad1*	41°40'32''N	41°52'44''E	387	2014-07-08	Georgia, Adjara, W edge of Chakvistavi	*Radula*	Cau1	tetraploid (FCM)
MS62/13	*R*. morphotax*. rad2*	41°50'21"N	43°15'47"E	1868	2013-08-20	Georgia, Samtskhe-Javakheti, Borjomi distr, 7 km WNW of Likani village	*Radula*	Cau1	triploid (FCM)
MS15/15	*R*. morphotax*. rad3*	43°16'27''N	40°18'11''E	400	2015-07-16	Abkhazia, 2 km E of Gagra	*Radula*	Gla3	tetraploid (FCM)
MS10/15	*R*. morphotax*. cor1*	43°22'11''N	40°49'01''E	536	2015-07-10	Abkhazia, NW part of Bitaga village near Pskhu	- (sect. *Corylifolii*)	Cae1	tetraploid (FCM)
MS63/13	*R*. cf. *sanctus* x *canescens*	41°50'8"N	43°15'53"E	1808	2013-08-20	Georgia, Samtskhe-Javakheti, Borjomi distr, 7 km WNW of Likani village	- (hybrid)	Can1	diploid (FCM)
MS54/13	*R*. *idaeus*	43°06'29"N	42°44'40"E	1664	2013-08-15	Georgia, Svaneti, Mestiachala valley, 4 km SSW of the Lekhziri glacier	- (subg. *Idaeobatus*)	Ida2	diploid (FCSS)

* We use the name R. "*hirtus*" agg. for all undeterminable members of ser. *Glandulosi*, i.e. unstabilized accessions without leaf tomentum, irrespective of colour of stem glands.

** The two examined morphotypes of *R. moschus* do not differ in most characters, except for their overall habitus and prevailing glands coloration. Morphotype 1 (better agreeing with original diagnosis) is usually a low or moderate shrub with conspicuous red glands on its stem. Morphotype 2 forms erect stems up to several meters high having huge leaves and an overall pale appearance caused by white glands and tomentum on its stems. Because morphotype 2 was found only in the Adjara region, which is characterized by extremely humid and warm climate, intraspecific variability or plasticity cannot be rejected as a cause for morphological differentiation without further study, although both morphotypes occurred at the locality without any transition forms.

*** All glandulous brambles with tomentose lower side of leaves; very polymorphic sexual complex which deserves further study.

Olomouc, 2016

Palacký University Olomouc

Faculty of Science

Department of Botany

Diversity, phylogenesis and evolutionary mechanisms in the genus *Rubus*

Mgr. Michal Sochor

Summary of the PhD. Thesis

Olomouc 2016

Ph.D. thesis was carried out at the Department of Botany, Faculty of Science, Palacký University in Olomouc in 2011 – 2016.

PhD. candidate: **Mgr. Michal Sochor**

Supervisor: **doc. RNDr. Bohumil Trávníček, Ph.D.**
Department of Botany, Faculty of Science, Palacký University in Olomouc, Czech Republic

Opponents: **doc. RNDr. Jan Kirschner, CSc.**
Institute of Botany, Czech Academy of Sciences, Průhonice

doc. Mgr. Patrik Mráz, Ph.D.
Department of Botany, Faculty of Science,
Charles University in Prague

Mgr. Tomáš Urfus, Ph.D.
Department of Botany, Faculty of Science,
Charles University in Prague

The PhD. thesis has been approved by Prof. Ing. Aleš Lebeda, DrSc., Department of Botany, Faculty of Science, Palacký University in Olomouc.

The summary of Ph.D. thesis was sent for distribution on ……....

The oral defense will take place on ……..……. in front of the Commission for Ph.D. thesis of the Study Program Botany at the Department of Botany, Šlechtitelů 27, Olomouc.

The Ph.D. thesis is available in the Library of the Biological Departments of Faculty of Science at Palacký University, Šlechtitelů 27, Olomouc.

Content

1 Introduction

Asexual reproduction via seeds (agamospermy or apomixis in the narrow sense) is a widespread phenomenon in plants which has been found in more than 40 plant families. Apomixis is typically associated with both polyploidy and hybridity, since most apomictic taxa are of allopolyploid origin (Bicknell and Koltunow 2004). Asexuality and extensive reticulate evolution make every phylogenetic study on apomictic taxa challenging, not to speak of defining taxonomical concepts that would reflect evolution of the group and, at the same time, be useable in practice. Due to this fact, together with high degree of residual sexuality and a great genotypic diversity, apomictic plants are notoriously known as a nightmare for taxonomists and field botanists, and a delight for evolutionary biologists.

Genus *Rubus*, especially its richest subgenus – *Rubus*, is one of the taxonomically most complicated plant groups in Europe and the Southern Caucasus. In Europe, more than 750 species are recognised, most of which are polyploids with varied degree of apomixis. Only few species are diploid and thus strictly sexual (Kurtto *et al.* 2010). The Caucasian bramble flora is only poorly explored, although the Caucasus undoubtedly represents one of the evolutionary centres of the subgenus. Only a few species has been validly described from the Southern Caucasus, and many of them need critical taxonomical revision (Kutateladze 1980). Almost nothing is known about cytological patterns and reproduction mode in Caucasian brambles. In Europe, the systematics of brambles is more advanced and much is understood regarding microevolutionary processes and morphological differentiation. Nevertheless the mechanisms of diversification, especially with regards to which species were (or still are) involved in polyploid evolution in European *Rubus*, remain unclear.

Furthermore, brambles in Europe an adjacent regions exhibit marked geographical differences among sexual and asexual biotypes (so called geographic parthenogenesis). The highest diversity of polyploid apomicts is concentrated in Northwest Europe and the Southern Caucasus whereas diploids are confounded mainly in the Mediterranean and other warmer regions (Kurtto *et al.* 2010). Advantages of polyploids in the northern, formerly glaciated regions may be related to their reproduction mode (e.g. better colonization ability), polyploidy (e.g. elevated heterozygosity or masking of deleterious mutations) and/or external factors (Hörandl 2006). None of these factors, nevertheless, can satisfactorily explain the observed geographic patterns.

2 Aims of the thesis

This thesis aims to contribute to our understanding of evolution of apomictic complexes in general and uncover relationships and evolutionary mechanisms among different groups of European and Caucasian brambles in particular. The thesis aims to answer these specific questions:

- What is the role (both contemporary and historical) of diploid taxa in the evolution of the polyploid complex?
- What are the driving forces of *Rubus* evolution?
- What is the spatio-temporal framework of *Rubus* evolution and how is it related to the Pleistocene climate fluctuations?
- What are the reasons for differential geographic distributions of sexuals and apomicts and why have apomicts diversified and expanded mainly in Northwest Europe and the Southern Caucasus?
- What are the patterns of cytological, reproductive and molecular diversity in the West Caucasian brambles and how are they related to the European bramble flora?

3 Materials and methods

Plant material

The plants were sampled across the wide geographic range of Europe, Morocco, Macaronesia and the Southern Caucasus. The aim was to cover as large geographic and taxonomic range as possible. From each of the sampled plants, a herbarium specimen was prepared and one leaflet was dried in silica gel. For flow cytometric measurements, fresh leaves were collected and preserved in plastic bags until the analysis. The flow cytometric seed screen was performed on air-dried fruits. Every plant was determined morphologically and the biotypes lacking valid or provisional names were distinguished and numbered as "morphotaxa" or categorized on the level of series.

Molecular methods

DNA was extracted from silica gel-dried leaves, or in few cases from herbarium specimens, following the CTAB (cetyltrimethylammonium bromide) protocol of Doyle and Doyle (1987). Two non-coding regions were selected for the plastid DNA analysis: the *matK* intron amplified with XFA and AST_R primers (Dunning and Savolainen 2010), and the *trnL-trnF* intergenic spacer with e and f primers (Taberlet *et al.* 1991). One universal primer pair, ITS1–ITS4, was used for amplification of the ITS1-5.8S rDNA-ITS2 (internal transcribed spacer) nuclear locus (White *et al.* 1990). ITS PCR products were cloned into a bacterial vector prior to sequencing. Subsequent Templi-Phi reactions and Sanger sequencing of Templi-Phi products (ITS) or polyethylene glycol-purified PCR products (plastid markers; 10% PEG 6000 and 1.25M NaCl in the precipitation mixture) were performed on a 96-capillary ABI 3730 instrument in the IPK (Gatersleben, Germany) central sequencing facility or by Macrogen Europe.

For SSR analysis, a multiplex and barcoding approach was used to amplify and sequence ten SSR loci from at least four linkage groups (see Chapter 3 of the thesis). Only SSR loci with a repeat unit length of at least 3 bp were selected. The 192 individual DNA samples that were used for SSR sequencing were divided into two sample sets, each containing 96 individuals. By appending 8-nucleotide barcodes to the 5' tail of both the forward and reverse primer sequences, we created tagged primers that were specific for each sample set and locus. All ten SSR loci were amplified in two multiplex PCR reactions. The PCR products were then pooled, and a total of 96 libraries were prepared for the paired-end sequencing of the SSR amplicons on an Illumina MiSeq at TraitGenetics GmbH (Gatersleben, Germany).

Molecular data analysis

The sequence data were processed and edited in GENEIOUS (ver. 7.1.7.; created by Biomatters). Further analyses were performed in a variety of specialized software, e.g. NETWORK (Bandelt *et al.* 1999), MAFFT (Katoh and Standley, 2013), MEGA (Tamura *et al.* 2011), GENALEX (Peakall and Smouse 2012), GENEPOP (Raymond and Rousset 1995), STRUCTURE (Pritchard *et al.* 2000).

Flow cytometric analyses

Ploidy levels were assessed based on the relative fluorescence of propidium iodide-stained nuclei, as determined by flow cytometric measurements (FCM) of fresh leaves or, in a few cases, by the flow cytometry seed screen (FCSS) of dried fruits, using a BD Accuri C6 (BD Biosciences, Franklin Lakes, NJ, USA) or a Partec CyFlow ML (Sysmex Partec, Görlitz, Germany) flow cytometer. Reproduction mode was assessed from the relative position of the peaks for embryo, endosperm, and an internal standard by FCSS (Matzk *et al.* 2000). As internal standards, *Solanum lycopersicum* (2C = 1.96 pg; Doležel *et al.* 1989) or *Glycine max* (2C = 2.5 pg; Doležel *et al.* 1994) were used for FCM and *Zea mays* (2C=5.43 pg; Lysák and Doležel 1998) for FCSS. For ploidy level calibration, genotypes of *R. moschus* (2n = 14; chromosomes counted by Krahulcová and Holub 1997) and *R. bifrons* [2n = 28; counted by Tesařová (2012)] were also measured.

Ecological niche modeling

Ecological niche modelling was performed for *R. ulmifolius* agg. using the maximum entropy approach as implemented in MAXENT (Phillips *et al.* 2006). Current and past maps of habitat suitability were constructed based on 19 biologically relevant climatic variables compiled in the WorldClim database (Hijmans *et al.* 2005; http://www.worldclim.org). As projection input data, bioclimatic layers from three paleoclimate models (CCSM, MIROC-ESM and MPI-ESM-P) of the Last Glacial Maximum (LGM; 22 ky BP) and the mid-Holocene (6 ky BP) and one model of the last interglacial period (LIG; 120–140 ky BP) were used (Brady *et al.* 2012; Sueyoshi *et al.* 2013; Otto-Bliesner *et al.* 2006; Giorgetta *et al.* 2013).

4 Survey of results

ITS sequences formed only six ingroup clusters (fig. 1), each characterized by one or a few sexual species (including *R. idaeus*), whereas apomicts contained ITS ribotypes clustering to two or three of these clusters. Plastid sequences provided a very similar pattern with haplotypes of European polyploids being restricted to five clusters (*R. idaeus* haplotypes not shared). Furthermore, West Caucasian polyploids often bore a unique haplotype not shared by any known diploid species. Survey of ribosomal DNA evolution provided evidence for intragenomic homogenization which prevented exact determination of all diploid ancestors in every single polyploid accession. Nevertheless, the homogenization is slow enough to enable elucidation of overall patterns.

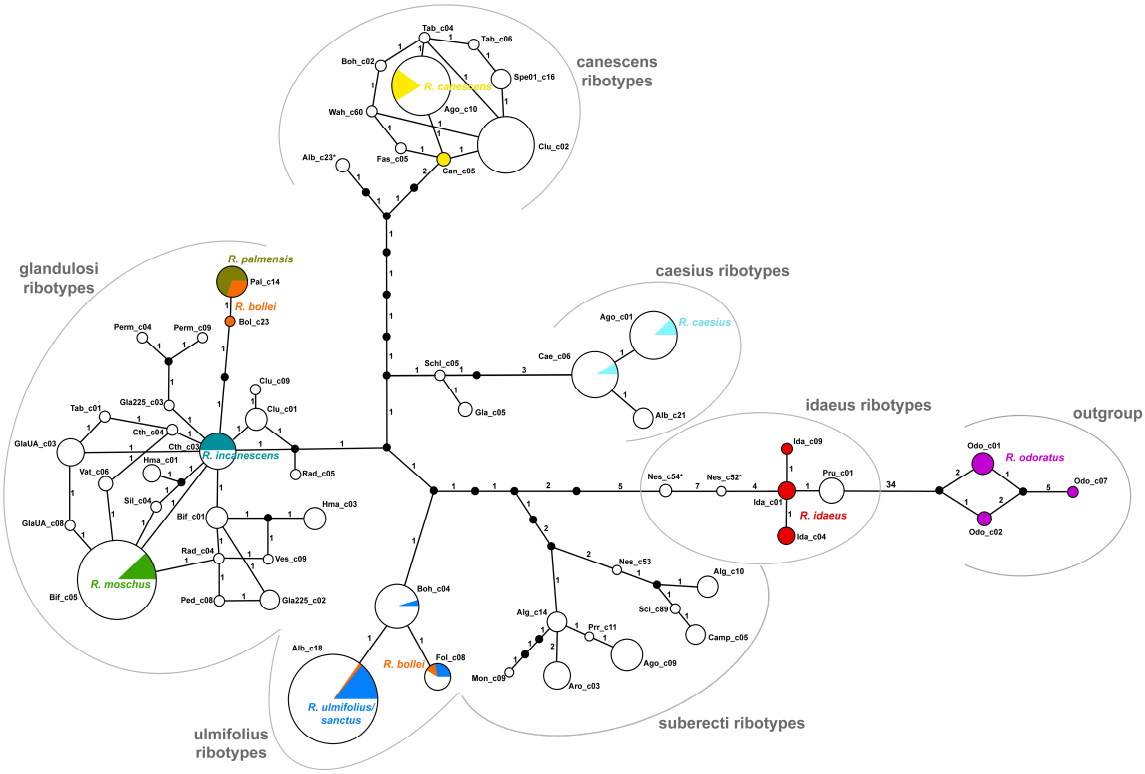


Figure 1: Median-joining network of cloned ITS sequences (503 sequences from 63 species or hybrids: each circle denote a distinct ribotype or group of similar ribotypes (circle size corresponds to the number of sequenced bacterial colonies), colonies from diploid taxa and *R. caesius* are plotted in color whereas other colonies are white. The number of mutations between ribotypes or median vectors (black dots) is shown above branches, possible recombinant ribotypes marked with an asterisk.

Comparison of distributions of sexuals and apomicts sharing the same plastid haplotype revealed high diversity of polyploids on the northern margins of the sexuals' distribution. Accidentally, these areas were either glaciated or vastly affected by glaciation during the Last glacial maximum. A more detailed study focusing on sexual diploid *R. ulmifolius* agg.

(incl. *R. sancus*) revealed three groups of populations differing in allelic (*Hd*) and nucleotide (*Pi*) diversity. First, the Caucasian and Balkan populations exhibited low values of both *Hd* and *Pi*. Second, Northwest European populations exhibited high *Pi* and low *Hd*. Third, all of the other populations were characterized by both high *Pi* and high *Hd*. This indication of bottlenecks in the East and recolonization of Northwest Europe was further supported by ecological niche models. Polyploid descendants shared alleles with the diploid populations from their respective region. Eastern polyploids furthermore shared many alleles with the western (both diploid and polyploid) populations indicating possible pre-glacial origin of these alleles. Similarly, two plesiomorphic plastid haplotypes were shared among western diploids and eastern polyploids, but no haplotypes of the eastern diploids were found in the west of the distribution area and they were only rarely shared with the eastern polyploids.

The last part of the thesis focused on diversity of the West Caucasian (more specifically Colchic) bramble flora. It turned out that this group was strongly understudied. Patterns of cytological variability deviated slightly from European brambles, with most accessions being tetraploid (85 %), followed by diploid (12 %) and rarely triploid (only one accession). Morphoseries *Radula* and *Glandulosi* exhibited obligate sexuality and other taxa were mostly apomictic with a low degree of residual sexuality. A few exceptions were observed that deserve further attention, for example, sexuality induced hypothetically by haploid pollen or by environmental conditions, a high proportion of triploid embryos, or polyspermy. Plastid haplotype variability revealed specific, ancient evolutionary patterns with limited involvement of extant diploid taxa, and recent isolation from European brambles.

5 Conclusions

The results show that the rich and diverse polyploid complex of Euro-Caucasian brambles originated from only seven diploid species or species aggregates, of which three are probably extinct today. On contrary, some South European and Macaronesian diploids probably did not contribute to the evolution of polyploids. One of the diploid ancestors, *R. ulmifolius/sancus* agg., experienced a reduction in its distribution and significant bottlenecks in the eastern parts of its distribution area in the Last glacial maximum. This led to the decrease of genetic diversity and subsequently possibly to lower competition abilities which may have enabled an expansion of newly arisen polyploids in Northwest Europe and the Southern Caucasus. It is further shown that apomicts combine pre-glacial gene-pools of the diploid ancestors and genetic diversity of recent sexuals from their region. Although most of the recent apomicts were formed in the Holocene, the whole agamic complex is much older, its history stretching at least to the last interglacial period. Therefore, apomixis should not be seen as an evolutionary dead end, but as a way of preservation and spread of genetic diversity in space and time. In addition to the advantages of asexuality and (allo)polyploidy, contemporary apomicts can use both genetic diversity of their extinct (or markedly changed) ancestors and the locally adapted gene complexes of recent diploids.

6 Souhrn (Summary, in Czech)

Název práce: Diverzita, fylogeneze a evoluční mechanismy v rodu *Rubus*

Autor: Michal Sochor

Evropské a kavkazské ostružiníky patří mezi přední zástupce kategorie „noční můra taxonoma, rozkoš evolucionisty“. Práce ukazuje, že je to velmi dynamická skupina poznamenaná častou hybridizací. Všechny původní ostružiníky Evropy a Kavkazu jsou totiž potomky pouhých čtyř recentních diploidních druhů nebo druhových komplexů (*R. ulmifolius–sanctus* agg., *R. canescens*, *R. moschus*, *R. idaeus*) a tří pravděpodobně vyhynulých (diploidi z *R.* subsect. *Rubus*, předchůdce *R. caesius* a neznámý diploid ze západu Jižního Kavkazu) a mnoho apomiktických mikrospecií je odvozeno ze dvou tetraploidních sexuálních taxonů (*R. caesius* a *R.* ser. *Glandulosi*). Úplná nebo převládající sexualita *R. caesius* a *R.* ser. *Glandulosi* a reziduální sexualita jiných biotypů vedla k extrémní morfologické a genetické diverzitě, zejména v územích dříve postižených zaledněním a v oblastech se sníženou kompeticí ze strany sexuálních předků, tedy v severozápadní Evropě a na Jižním Kavkazu.

Čtvrtohorní klimatické oscilace a anthropogenní změny v krajině byly pravděpodobně nejsilnějšími evolučními tlaky, jelikož většina apomiktických linií vznikla až v holocénu jako důsledek postglaciálních migrací. Z tohoto pohledu se apomixe opravdu může jevit jen jako dočasný fenomén – slepá ulička evoluce. Na druhou stranu, v práci je předložena řada nepřímých důkazů, že apomiktické ostružiníky existovaly již před posledním glaciálním maximem. Z nich velmi pravděpodobně mnoho vyhynulo, ale alespoň některé přežily až do holocénu a zachovaly tak část ancestrálních genofondů, které by jinak vymizely. Navíc, apomiktické linie reprezentují nové, nezávislé evoluční jednotky jen příležitostně přijímající genetický materiál recentních diploidů. Jsou tak schopni využívat jak adaptivní variabilitu svých dávných předků, tak i nové, lokálně výhodné alely recentních diploidních populací. Apomixe tak spíše než slepou uličku evoluce představuje způsob šíření genetické variability v prostoru a čase a její uchování pro následné „evoluční využití“.

Ačkoliv tato disertační práce přináší řadu nových poznatků o evolučních mechanismech a trendech apomiktických ostružiníků, zůstává v této oblasti stále mnoho nezodpovězených otázek. Kromě taxonomických problémů kolem příbuznosti konkrétních taxonů je to zejména genetika a regulace apomixe, která je u ostružiníků jen velmi málo prozkoumaná. Batologie a další vědní obory zabývající se ostružiníky tak zdaleka nejsou vyčerpané a v budoucnu jistě přinesou řadu zajímavých poznatků.

7 References

Bandelt HJ, Forster P, Rohl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37–48.

Bicknell R, Koltunow A. 2004. Understanding apomixis: recent advances and remaining conundrums. *The Plant Cell Online* **16**: 228–246.

Brady EC, Otto-Bliesner BL, Kay JE, Rosenbloom N. 2012. Sensitivity to glacial forcing in the CCSM4. *Journal of Climate* **26**: 1901–1925.

Doležel J, Doleželová M, Novák FJ. 1994. Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*). *Biologia Plantarum* **36**: 351–357.

Doležel J, Binarová P, Lucretti S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. *Biologia Plantarum* **31**: 113–120.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Dunning LT, Savolainen V. 2010. Broad-scale amplification of *matK* for DNA barcoding plants, a technical note. *Botanical Journal of the Linnean Society* **164**: 1–9.

Giorgetta MA, Jungclaus J, Reick CH, Legutke S, Bader J, Böttinger M, Brovkin V, Crueger T, Esch M, Fieg K, *et al.* 2013. Climate and carbon cycle changes from 1850 to 2100 in MPI-ESM simulations for the Coupled Model Intercomparison Project phase 5. *Journal of Advances in Modeling Earth Systems* **5**: 572–597.

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. 2005. Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* **25**: 1965–1978.

Hörandl E. 2006. The complex causality of geographical parthenogenesis. *New Phytologist* **171**: 525–538.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.

Krahulcová A, Holub J. 1997. Chromosome number variation in the genus *Rubus* in the Czech Republic. I. *Preslia* **68**: 241–255.

Kurtto A, Weber HE, Lampinen R, Sennikov AN (Eds.). 2010. *Atlas florae Europaeae. Distribution of vascular plants in Europe, vol. 15: Rosaceae (Rubus)*. Helsinki: Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo.

Kutateladze SI. 1980. Genus 12. Rubus L. In: Ketskhoveli NN, ed. Flora of Georgia, vol. 6. 95–135.

Lysák MA, Doležel J. 1998. Estimation of nuclear DNA content in *Sesleria* (Poaceae). *Caryologia* **51**: 123–132.

Matzk F, Meister A, Schubert I. 2000. An efficient screen for reproductive pathways using mature seeds of monocots and dicots. *The Plant Journal* **21**: 97–108.

Otto-Bliesner BL, Marshall SJ, Overpeck JT, Miller GH, Hu A, members CLIP. 2006. Simulating arctic climate warmth and icefield retreat in the Last Interglaciation. *Science* **311**: 1751–1753.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research - an update. *Bioinformatics* **28**: 2537–2539.

Phillips S, Anderson R, Schapire R. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling* **190**: 231–259.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Raymond M, Rousset F. 1995. GENEPOP (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. *Journal of Heredity* **86**: 248–249.

Sueyoshi T, Ohgaito R, Yamamoto A, Chikamoto MO, Hajima T, Okajima H, Yoshimori M, Abe M, O'ishi R, Saito F, *et al.* 2013. Set-up of the PMIP3 paleoclimate experiments conducted using an Earth system model, MIROC-ESM. *Geoscientific Model Development* **6**: 819–836.

Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of 3 noncoding regions of chloroplast DNA. *Plant Molecular Biology* **17**: 1105–1109.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution* **28**: 2731–2739.

Tesařová M. 2012. Stanovení velikosti genomu u vybraných druhů rodu *Rubus* metodou průtokové cytometrie. Master thesis, depon. in library of Palacky University in Olomouc.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR Protocols: A guide to methods and applications. New York: Academic Press, Inc., 315–322.

8 List of author's publications

Publications related to the PhD. thesis

Sochor M, Vašut RJ, Sharbel TF, Trávníček B (2015) How just a few makes a lot: Speciation via reticulation and apomixis on example of European brambles (Rubus subgen. Rubus, Rosaceae). *Molecular Phylogenetics and Evolution*, **89**, 13–27.

Sochor M, Trávníček B (2016) Melting pot of biodiversity: first insights into the evolutionary patterns of the Colchic bramble flora (*Rubus* subgenus *Rubus*, Rosaceae). *Botanical Journal of the Linnean Society* (early view).

Velebil J, Trávníček B, **Sochor M**, Havlíček P (2016) Five new bramble species (*Rubus*, Rosaceae) in the flora of the Czech Republic. *Dendrobiology*, **75**, 141–155.

Other publications:

Sochor M, Vašut RJ, Bártová E, Majeský Ľ, Mráček J (2013) Can gene flow among populations counteract the habitat loss of extremely fragile biotopes? An example from the population genetic structure in *Salix daphnoides*. *Tree Genetics and Genomes*, **9**, 1193–1205.

Dančák M, Hroneš M, **Sochor M** *et al*. (2013) A new species of *Thismia* (Thismiaceae) from Brunei Darussalam, Borneo. *Phytotaxa*, **125**, 33–39.

Vašut RJ, **Sochor M**, Hroneš M [eds.] (2013) *Vrby České republiky*. Univerzita Palackého v Olomouci, Olomouc.

Skálová D, Navrátilová B, Richterová L, Knitl M, **Sochor M**, Vašut RJ (2012) Biotechnological methods of in vitro propagation in willows (Salix spp.). *Central European Journal of Biology*, **7**, 931–940.

Egertová Z, Hairaud M, **Sochor M** (2016) *Belonioscyphella hypnorum* (Helotiales), a rarely reported bryoparasitic ascomycete new for the Czech Republic. *Ascomycete.org*, **8**, 91–95.

Sochor M, Egertová Z (2015) Bioluminiscence hub - odvěký a stále záhadný fenomén. *Živa*, **2015** (6), 282–284.

Manuscripts under review:

Sochor M, Šarhanová P, Pfanzelt S, Trávníček B (under review) Origin of apomicts as a result of the sexual ancestor's phylogeography: a model case of European and Caucasian brambles (*Rubus*, Rosaceae). *Submitted to Molecular Ecology*.

Sochor M, Sukri RS, Metali F, Dančák M. (under review) *Thismia inconspicua* (Thismiaceae), a new mycoheterotrophic species from Borneo. *Submitted to Phytotaxa.*