Charles University

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Microevolutionary processes in selected genera of the Rosaceae family

Mikroevoluční procesy u vybraných zástupců čeledi Rosaceae

Doctoral thesis

Supervisor: Mgr. Tomáš Urfus, Ph.D.

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Content

1 Declaration

I hereby declare that this thesis has been composed by myself using the mentioned references and that it has not been submitted elsewhere, in whole or in part, to obtain the same or other academic degree.

In Prague on 21st April 2020

Prohlašuji, že jsem tuto práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 21. dubna 2020 Lenka Macková

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2 Author contribution statement

I hereby confirm that I have substantially contributed to all papers included, to the following extent:

I. **Macková, L.**, Vít, P., Ďurišová, Ľ., Eliáš, P., & Urfus, T. (2017): Hybridization success is largely limited to homoploid *Prunus* hybrids: a multidisciplinary approach. – Plant Systematics and Evolution 303: 481–495. doi: https://doi.org/10.1007/s00606-016-1385-4
Field sampling, lab work, data analyses, manuscript preparation and review – 80%

II. **Macková, L.**, Vít, P., & Urfus, T. (2018): Crop-to-wild hybridization in cherries – Empirical evidence from *Prunus fruticosa*. – Evolutionary Applications 11:1748–1759. doi: https://doi.org/10.1111/eva.12677
Field sampling, lab work, data analyses, manuscript preparation and review – 80%

III. **Macková, L.**, Nosková, J., Ďurišová, Ľ., & Urfus, T.: Insights into the cytotype and reproductive puzzle of *Cotoneaster integerrimus* in the Western Carpathians. – Plant Systematics and Evolution (preliminary accepted)
Field sampling, lab work, data analyses, manuscript preparation and review – 70%

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4 Abstract

Polyploidization, hybridization and various reproductive strategies significantly contribute to plant evolution and diversity. Their direct influence on plant evolution is especially apparent in the Rosaceae family and is also mirrored in its still partly unclear and reticulate phylogeny. Two model genera were chosen to add a piece of knowledge to the puzzle of polyploidization, hybridization and apomixis in the Rosaceae.

The results demonstrate both the creative and destructive force of hybridization and polyploidization, particularly in the genus *Prunus*. A significant proportion of wild *Prunus fruticosa* populations under examination underwent hybridization and genetic erosion. Crop-to-wild hybridization with both cultivated sour and sweet cherries has resulted in two morphologically indistinguishable hybrids markedly differing in ploidy level and reproductive potential. On the one hand, a triploid block was manifested in sterile triploid hybrids, but, on the other, partial fertility of tetraploid hybrids allowed repeated backcrossing (i.e. introgression). The crop-to-wild phenomenon has significant consequences for both conservation and agriculture.

Polyploidization and hybridization are frequently accompanied by apomixis among the Rosaceae. Apomixis may play a substantial role in the stabilization of newly arisen genotypes (microspecies). Although particular lineages are reflected by a specific genome size/ploidy level and reproductive pattern (e.g. in *Hieracium, Pilosella, Rubus, Sorbus*), *Cotoneaster integerrimus* s.l. in the Western Carpathians did not show any significant differentiation in this respect. The whole group was found to be homogeneously tetraploid and facultatively apomictic. Besides prevailing pseudogamy combined with minor sexuality, different apomictic pathways were identified (e.g. autonomous apomixis or haploid parthenogenesis). The potential for further polyploidization is supported by a minor proportion of B_{III} individuals. By contrast, *Cotoneaster tomentosus* clearly differed in both ploidy level and reproduction mode, being pentaploid and obligately apomictic.

To sum up, the effects of the detected crop-to-wild hybridization in cherries were markedly determined by the ploidy level. Homoploid hybridization represents a gene-flow bridge towards endangered *Prunus fruticosa* whereas heteroploid crosses result in sterile triploid progeny. On the other hand, polyploid and facultatively apomictic *Cotoneaster integerrimus* s.l. exhibited a homogenous cytotype and breeding pattern in the entire study area. The take-home message of the presented case studies emphasizes substantially different consequences of analogous evolutionary drivers in the Rosaceae family (polyploidy, hybridization and reproductive strategies).

5 Abstrakt

Polyploidizace, hybridizace a způsob reprodukce významnou měrou ovlivňují evoluci a diverzitu rostoucích rostlin. Přímý vliv těchto mechanismů na vývoj rostlin je zřejmý zejména v čeledi Rosaceae (růžovité) a odráží se také v jejich doposud částečně nejasné a komplikované fylogenezi. K získání dalších poznatků poodhalujících vliv polyploidizace, hybridizace a apomixie na čeleď Rosaceae byly vybrány dvě modelové skupiny druhů.

Výsledky předkládané práce ukazují, že hybridizace a polyploidizace má, konkrétně v rodu *Prunus*, jak konstruktivní tak destruktivní charakter. U významné části studovaných populací plané *Prunus fruticosa* (třešně křovité) byla prokázána hybridizace a genetická eroze. Jedná se o tzv. „crop-to-wild“ křížení planě rostoucích druhů s druhy pěstovanými člověkem. Třešeň křovitá se kříží s oběma příbuznými pěstovanými druhy, třešní i višní, za vzniku dvou morfologicky neodlišitelných hybridů, které se však jednoznačně odlišují ploidií a reprodukčním potenciálem. Vznikají jak sterilní triploidní hybridi (uplatňuje se triploidní blok), tak částečně fertilní tetraploidní kříženci, kteří se mohou dále zpětně křížit a dochází tak k tzv. introgresi. Křížení tohoto planě rostoucího druhu s druhy pěstovanými má tak významné důsledky pro ochranu přírody i samotné zemědělství, resp. šlechtitelství.

Běžně se spolu s polyploidizací a hybridizací v čeledi Rosaceae vyskytuje také apomixie. Ta může hrát důležitou roli pro stabilizaci nově vzniklých genotypů (mikrospecií). Takové linie bývají charakterizovány určitou ploidií/velikostí genomu a typem reprodukce (např. u rodu *Hieracium, Pilosella, Rubus, Sorbus*). K tomu však v případě komplexu *Cotoneaster integerrimus* s.l. v Západních Karpatech nedošlo, protože žádná taková diferenciace prokázána nebyla. Celý komplex je totiž tetraploidní a fakultativně apomiktický. Vedle převažující pseudogamie kombinované se zbytkovou sexualitou byly identifikovány také další různé typy apomixie (např. autonomní apomixie nebo haploidní partenogeneze). Detekce B_{III} jedinců, i když v malém množství, ukázala na potenciál k další polyploidizaci. Oproti tomu pentaploidní a obligátně apomiktický *Cotoneaster tomentosus* byl jak stupněm ploidie, tak i reprodukčním způsobem jednoznačně definovaný.

Lze tedy shrnout, že míra "crop-to-wild" křížení je u třešní výrazně ovlivněna ploidní úrovní. Zatímco homoploidní hybridizace umožňuje genový tok směrem k ohrožené *Prunus fruticosa*, při heteroploidním křížení vzniká sterilní triploidní potomstvo. Nicméně v případě polyploidního a fakultativně apomiktického komplexu *Cotoneaster integerrimus* s.l. byl zjištěn stejný cytotyp i reprodukční způsob v rámci celé studované oblasti. Na základě výsledků předkládaných dílčích studií je tedy třeba zdůraznit, že podobné evoluční mechanismy v čeledi Rosaceae (polyploidie, hybridizace a různé způsoby reprodukce) vedou k podstatně odlišným důsledkům.

6 Introduction

The family Rosaceae is one of the most heterogeneous, ubiquitous and biosystematically most complex groups of vascular plants. Because of its enormous diversity (92 genera and 2,805 species; Stevens 2020), it ranks among the twenty largest families of vascular plants in the world (Christenhusz and Byng 2016). This family includes important crops and also critical and intricate groups such as *Alchemilla, Crataegus, Potentilla, Rosa, Rubus* or *Sorbus* (e.g. Judd et al. 2016; Herklotz and Ritz 2017; Dickinson 2018). Important sources of this complexity and variability include polyploidization, hybridization and apomixis resulting in reticulate evolution (e.g. Dickinson et al. 2007; Zhang et al. 2017). The enormous complexity of the Rosaceae has defeated attempts to reconstruct the family's phylogeny and devise a comprehensive taxonomic treatment (Potter et al. 2007a; Majeský et al. 2017; Zhang et al. 2017).

6.1 Microevolutionary forces in the Rosaceae family

The family Rosaceae is well known as an evolutionarily dynamic group, which is also mirrored in its complex biosystematics (e.g. Dickinson et al. 2007). The intricate family classification has repeatedly been modified to better reflect the deep relationships among its groups (see the Phylogeny and classification section below). Difficulties are caused mainly by frequent interspecific hybridization, polyploidization, apomixis and resulting rapid radiation (Robertson et al. 1991; Vamosi and Dickinson 2006; Campbell et al. 2007; Whitton et al. 2008).

6.1.1 Hybridization

Hybridization is defined as crossing between different entities, for example two genetically distinct populations (Barton and Hewitt 1985) or species (i.e. interspecific hybridization; Wissemann 2007; Soltis and Soltis 2009). Interspecific hybridization, together with polyploidization, plays an important role in plant evolution. On the one hand, hybridization may be a potent force of speciation (Wissemann 2007; Abbott et al. 2013) and on the other, it can also cause the extinction of species (Todesco et al. 2016). Nevertheless, it is still difficult to predict whether a hybridization event will be favourable for speciation (Abbott et al. 2013).

Hybridization may be considered from various viewpoints. Homoploid hybridization is crossing between species with the same ploidy whereas heteroploid hybridization, frequently resulting in allopolyploidy (see below) is hybridization between species taking place at different ploidy levels (Soltis and Soltis 2009; Yakimowski and Rieseberg 2014). Newly formed hybrids may backcross with their parents or another hybrid and repeated backcrossing can lead to the transfer of genetic material from one species to another (i.e. introgression; Anderson 1948; Soltis and Soltis 2009). Introgressive hybridization may happen symmetrically towards parental taxa or unidirectionally (e.g. Price and Rich 2007; Delplancke et al. 2012). Extensive interbreeding of two (or more) species results in the formation of a hybrid swarm, which is a mix of parental species, F1, F2 and later--generation hybrids, and backcrosses with one or both parental species (Soltis and Soltis 2009). So-called chloroplast capture through hybridization and introgression represents the most extreme case of hybridization and leads to the complete replacement of nuclear

DNA whereas original uniparentally inherited chloroplast DNA remains (Rieseberg and Soltis 1991).

The distribution of spontaneous hybridization among taxa is not random, but some phylogenetic groups are biologically predisposed to the formation and maintenance of hybrids (Ellstrand et al. 1996). It is possible to trace up the traits that these groups share; these are primarily outcrossing, a perennial life cycle and reproductive modes that stabilized hybridity (e.g. apomixis, vegetative spread, permanent odd polyploidy; Ellstrand et al. 1996).

Newly arisen hybrids can be intermediate between their parent species (Kellner et al. 2012; Christensen et al. 2014), almost identical with one of the parents, or show a new combination of characters not possessed by any of the parents (Schanzer and Kutlunina 2010). Although initial hybrids often struggle because of odd ploidy numbers (Comai 2005), a lack of appropriate mating partners (Soltis and Soltis 2009), unsuitable habitat conditions (Schnitzler et al. 2014) or reduced reproductive fitness (Vítová et al. 2015), they are able to stabilize using polyploidization (Hegarty and Hiscock 2005), transition in reproduction (selfing, apomixis; Abbott and Lowe 2004; Lepší et al. 2015) and adaptation to environmental condition (Feurtey et al. 2017). Thus, interspecific hybridization is a mechanism promoting adaptive evolution and speciation (Rieseberg et al. 2003).

Hybrid speciation can be realized at the homoploid level, but the establishment of hybrid progeny is probably facilitated by allopolyploid speciation (Hegarty and Hiscock 2005). Whereas the fitness of homoploid hybrids is strongly reduced due to backcrossing with parental taxa, genome duplication protects hybrid genetic integrity and enables rapid speciation (Rieseberg and Willis 2007).

Nevertheless, interspecific hybridization may also have an adverse effect and promote extinction (Rhymer and Simberloff 1996; Ellstrand et al. 2013; Todesco et al. 2016). If there are no effective reproductive barriers and taxa grow in sympatry, they have an opportunity to partake in almost unrestricted hybridization (Rhymer and Simberloff 1996). If there is a series of repeated backcrosses (introgression), by which genes of one species are transferred into another, this blurs the boundaries between species (Anderson 1948). So, in the resulting hybrid swarms, pure species are overbalanced by various types of hybrids (Omasheva et al. 2017), and in extreme cases they may be completely replaced by them (Boratyński et al. 2003). It may seem that extinction by hybridization directly depends on the fertility of newly arisen hybrids, which are involved in numerous series of spontaneous hybridization (i.e. genetic swamping, Fig. 1; Todesco et al. 2016). However, also sterile F1 hybrids may cause serious difficulties in populations because the wasteful production of maladaptive hybrids reduces the number of appropriate mating partners and promotes undesirable competition for natural resources (i.e. demographic swamping, Fig. 2; Todesco et al. 2016). The risk of hybridization becomes more serious especially in low--abundant (i.e. often rare) species crossing with numerous ones (Levin et al. 1996). Commercial crops, which are often close relatives of wild species, are exactly such ubiquitous, abundant species and often alien at that (Hyams 1971). They can easily hybridize with their wild congeners, and many cases of so-called crop-to-wild hybridization have already been detected (e.g. Ellstrand et al. 1999, 2013).

Fig. 1: Illustration of genetic swamping published in Todesco et al. (2016). Genetic swamping causes the extinction of the rare lineage after hybridization between a rare lineage (red flowers) and a common lineage (yellow flowers). Hybrids are at least partially fertile and viable and replace pure parental genotypes. In contrast to demographic swamping, not all parental alleles themselves are removed. Rare, common and hybrid genotype percentages per generation are represented by the colour-coded bars on the right side.

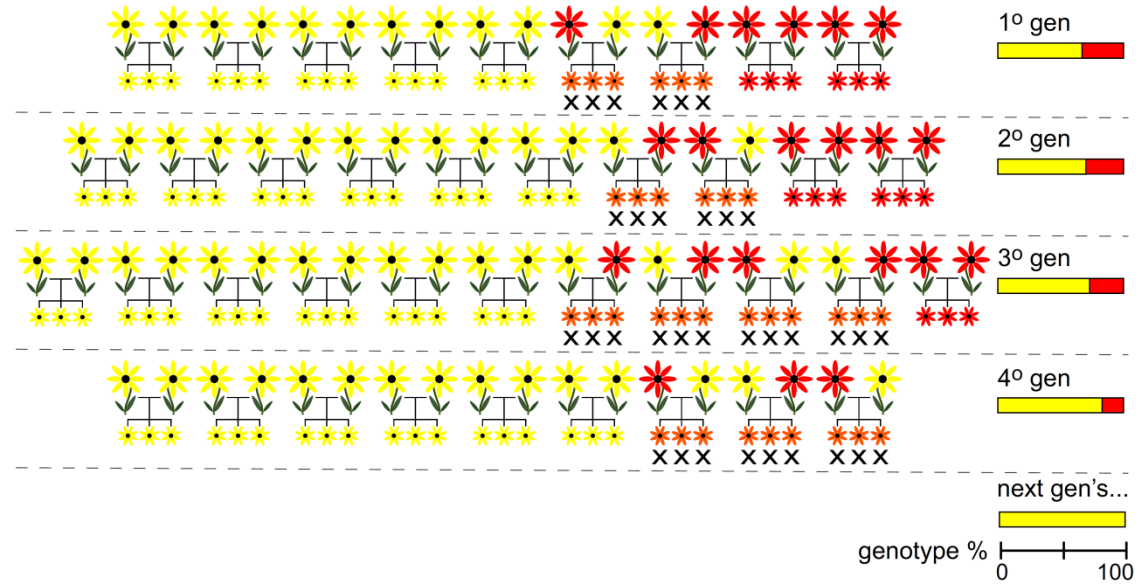

Fig. 2: Illustration of demographic swamping published in Todesco et al. (2016). Demographic swamping results in population extinction of the rare lineage after hybridization between a rare lineage (red flowers) and a common lineage (yellow flowers). Unfit hybrid individuals (light and dark orange flowers) disappear entirely with all rare alleles of their lineage. Rare, common, and hybrid genotype percentages per generation are represented by the colour-coded bars on the right side.

6.1.2 Polyploidization

Polyploidization is currently considered an essential driver of plant biodiversity (Landis et al. 2018). Although previous opinions regarded polyploidization as mere 'evolutionary noise' or 'dead end' (De Wet 1970) of evolution, the recent paradigm emphasizes the key role of polyploidy in the evolution of plants and their speciation (e.g. Rieseberg and Willis 2007;

Soltis et al. 2010). Polyploidization (whole-genome duplication) is the doubling of chromosomal material. This duplication can basically arise in two ways. Autopolyploidization includes genome doubling in a single species whereas allopolyploidization involves hybridization of different species with associated chromosome doubling (Landis et al. 2018). Nevertheless, a continuous spectrum of polyploids besides these two extremes occurs in nature. It is therefore sometimes difficult to recognize between these two categories and, moreover, many polyploids behave cytogenetically as diploids (Leitch and Bennett 1997).

Although it was traditionally assumed that allopolyploids greatly prevail in nature, the current view is that autopolyploid and allopolyploid taxa are similarly frequent (Barker et al. 2016). Autopolyploids have been overlooked in nature (Parisod et al. 2010) because they represent cryptic polyploid species hardly distinguishable by morphological traits (moreover still often without any taxonomic rank; Soltis et al. 2010; Barker et al. 2016). Somatic doubling traditionally represents another possible origin of polyploidy. This process is characterized by an increasing of the chromosome number in somatic tissue because of disorders in mitosis. However, well documented cases of somatic doubling are rare (*Primula* ×*kewensis*; Harlan and deWet 1975), but somatic polyploidization is frequently experimentally induced using the mitotic poison colchicine (e.g. Pavlíková et al. 2017; Wahlang et al. 2019).

Polyploidy has been studied since the beginning of the last century, and over time the percentage of angiosperm species suspected of having undergone a polyploid event has steadily increased (Masterson 1994; Otto and Whitton 2000; Soltis et al. 2009). Finally, it is currently assumed that evolution of all angiosperms included palaeopolyploid events (even in the case of *Amborella*; Jiao et al. 2011). Moreover, about a third of all polyploids were formed recently (e.g. in the genera *Spartina*, *Senecio*, *Cardamine* or *Tragopogon*) and some species arose only within the past 150 years (Soltis and Soltis 2009; Rice et al. 2015).

Unreduced gametes (having the full somatic chromosome number) which arise from errors during cell division are suspected to play an essential role in polyploid formation (Harlan and deWet 1975; Ramsey and Schemske 1998; Barker et al. 2016). Nevertheless, their abundance is generally low in natural populations (Ramsey and Schemske 1998). For this reason, the probability of fusion of two unreduced gametes forming a new polyploid seems to be low. Fusion of an unreduced gamete with a normal reduced gamete, resulting in a triploid individual (i.e. a triploid bridge), is probably more frequent in wild populations (Ramsey and Schemske 1998). Subsequent backcrossing with one of the parents or a triploid leads to polyploid formation. Most triploids are at least partially fertile, producing 1x, 2x or 3x gametes (Harlan and deWet 1975; Husband 2004).

Whole-genome duplication in plants directly affects various substantial characters and traits (incl. ecology, invasiveness; Francis et al. 2008; Herben et al. 2012; Te Beest et al. 2012). Polyploidization causes changes at the cellular and tissue levels via the so-called nucleotypic effect (e.g. cell size, nuclear volume, cell cycle duration; Doyle and Coate 2019). In addition, polyploids differ in physiological and ecological traits from their diploid congeners (e.g. ecological preferences and tolerance, stress resistance, competition or plant/animal interactions; Levin 1983; Soltis et al. 2004; Thompson et al. 2004). Whole--genome duplication also significantly affects the reproductive system (e.g. break-down of self-incompatibility, shift to or greater asexual reproduction; Levin 1983) and may promote the production of viable hybrid seeds due to re-establishment of normal endosperm cellularization (overcoming of an endosperm-based reproductive barrier; Lafon-Placette et al. 2017). Thus, polyploids may immediately survive and adapt to conditions other than those inhabited by their diploid progenitors (Levin 1983).

A current study concluded that ancient polyploidization was tightly linked to changes in the rate of diversification and that polyploidization events were followed by an increase in species richness in some angiosperm clades (Landis et al. 2018). The significant role of polyploidy in the formation of diversity may also be traced at the regional level. For example, a comprehensive study of the Pyrenean flora found the taxonomic diversity of genera with only diploid species to be markedly lower than that of those with polyploid species (Petit and Thompson 1999). Polyploidy has been identified as a direct source of diversity (species richness) even within the family Rosaceae (Vamosi and Dickinson 2006).

6.1.3 Apomixis

Last but not least, apomixis (i.e. agamospermy, asexual production of maternal progeny through seeds or cloning through seeds; Hörandl 2007) is a microevolutionary process effectively involved in the speciation of a significant part of vascular plants (León-Martínez and Vielle-Calzada 2019). Apomixis may promote newly arisen polyploids or hybrids, stabilize their reproduction and facilitate their reproductive isolation from their parents (Wissemann 2007). The definition of apomixis in the wide sense includes all forms of asexuality such as vegetative reproduction by means of bulbs, layers and other vegetative particles or simple fragmentation. In the current restricted sense of the term, apomixis (i.e. agamospermy), as also used here, was defined already by Stebbins (1950) seventy years ago as all types of apomictic reproduction in which embryos and seeds are formed by asexual means. Circumvention of both meiosis and fertilization is an essential feature of apomictic seed production. Therefore, apomictically derived embryos developing in seeds are usually cytologically and genetically identical with their maternal parent (Stebbins 1950).

Pathways by which apomictic seeds can be formed can be divided into three broad categories – adventitious embryony (sporophytic apomixis) and diplospory and apospory (gametophytic apomixis, Fig. 3; Stebbins 1950; Dickinson 2018). Adventitious embryony is the simplest pathway because embryos develop directly from somatic cells (i.e. from sporophytic tissue of the nucellus or ovule integument), so the gametophytic stage is completely omitted (best known in *Citrus*, Fig. 3; Stebbins 1950; Richards 1997; Dickinson 2018). Gametophytic apomixis is characterized by the circumvention of meiosis and the consequent production of unreduced megaspores (i.e. apomeiosis). Diplospory is the situation when meiosis fails (or is abnormal) and the megaspore mother cell develops into an unreduced megagametophyte (Fig. 3). In apospory, the unreduced megagametophyte develops mitotically from a somatic cell of the ovule (usually from a nucellar cell, Fig. 3) instead of the megaspore mother cell (Hörandl 2007; Whitton et al. 2008). Whereas diplospory is more likely to be linked to obligatory apomixis because it directly interferes with sexual gametophyte development, meiosis in the megaspore mother cell of aposporous apomicts usually progresses normally. Thus, apospory often represents a facultative apomictic pathway involving the production of both sexual and apomictic gametophytes (Whitton et al. 2008).

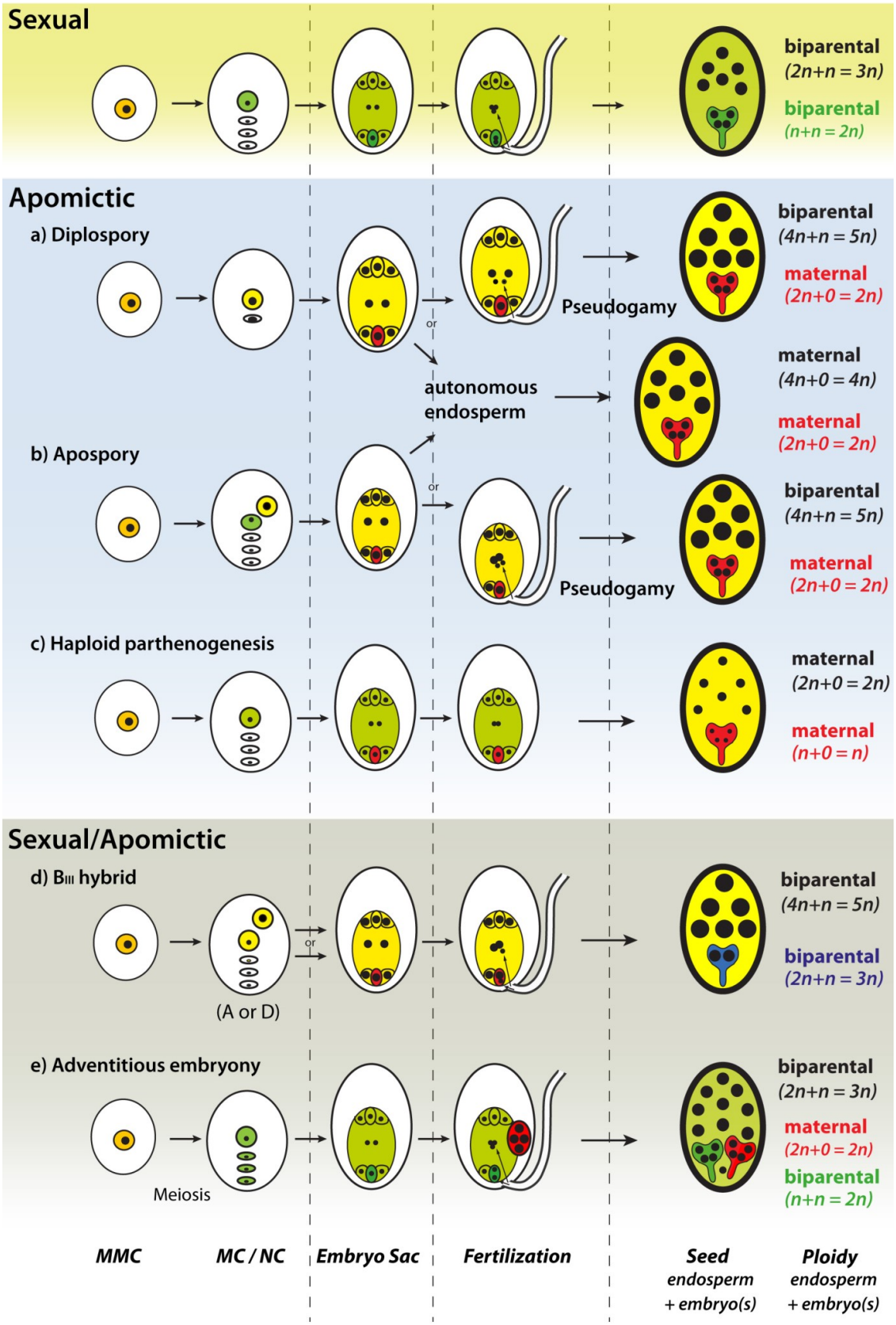

Fig. 3: Main developmental pathways of embryo sac formation in sexual and apomictic flowering plants, published in Hojsgaard and Hörandl (2019). MMC = megaspore mother cell; MC = megaspore; NC = nucellus cell; B_{III} hybrid = offspring produced by fertilization of unreduced egg cells. The size of nuclei corresponds to the relative ploidy level. For details, see the respective paper (pathways C and D are described in the included article on page 128–129).

However, the realization of meiosis does not necessarily mean the successful formation of a sexual gametophyte. In the aposporous apomictic species *Poa pratensis*, the functional megaspore usually experiences normal meiosis but starts to degenerate soon after the end of meiotic division whereas some nucellar cells enlarge to become aposporous initials and prepared to divide (Albertini et al. 2001). Nevertheless, in rare cases, megagametogenesis starts exactly the same but the development of the embryo sac never finishes successfully and the young megagametophyte degenerates, being surrounded by cells resembling aposporous initials (Albertini et al. 2001). Although the production of more than one embryo in a single seed (i.e. polyembryony; Naumova 1993) is a primary characteristic of adventitive embryony (Naumova 1993), it is also indicated in some aposporous apomicts (e.g. *Poa alpina*, *P. pratensis*, *Potentilla argentea*, *P. verna*, *Ranunculus auricomus*; Richards 1997). Several aposporous initials were detected in the ovule of *P. pratensis*, resulting in several aposporous embryo sacs, which reached full development in most of cases. However, only one embryo sac was normally oriented with the egg apparatus toward the micropyle (Albertini et al. 2001). The simultaneous occurrence of sexual and asexual seeds in one inflorescence and an analogous process within the same inflorescence or even the ovule were also detected in *Hieracium* subgenus *Pilosella* (Krahulcová and Krahulec 2000; Bicknell et al. 2003). One to eight aposporous initials in the nucellus of *Crataegus pruinosa* were found to occur simultaneously and two to four of them developed into mature embryo sacs (Muniyamma and Phipps 1979). The above suggests that there is some kind of competition in the embryo sac of aposporous apomicts (as in the case of adventitious embryony; Richards 1997), but the details of the processes still remain unclear (Whitton et al. 2008).

The second essential feature of gametophytic apomixis is parthenogenesis (i.e. embryo development without fertilization; Hörandl 2007). A developed megagametophyte, represented by a mature embryo sac, consists of unreduced genetically maternal cells (formed by diplospory or apospory). One of these cells (most commonly the egg cell) develops parthenogenetically into an embryo (Fig. 3). The resulting embryo is therefore unreduced and genetically maternal (Hörandl 2007).

Endosperm formation nourishing the embryo and enabling its development is essential for both sexual and apomictic seeds. Apomictic plants can be divided into two groups depending on whether they require pollen for proper endosperm development. Whereas the endosperm of autonomous apomicts develops independently without any pollen contribution, so-called pseudogamous apomicts require fusion of a sperm cell with the polar nucleus for successful endosperm development. Pseudogamy (Fig. 3) is closely linked to apospory (almost all aposporous plants are pseudogamous). On the contrary, autonomous endosperm development is associated with diplospory (Czapik 1996; Richards 1997). Moreover, pseudogamy is also linked to self-compatibility and it brings benefits in maintaining at least some viable pollen and increasing fecundity because inbreeding depression can be excluded in apomicts (Noirot et al. 1997). Conversely, autonomous apomixis seems to be associated rather with a decrease of male fertility (e.g. male sterility in autonomous apomictic *Townsendia*; Thompson et al. 2008).

In contrast to widespread polyploidization, only about 1% of angiosperms (vs 3% of pteridophytes) are considered substantially apomictic (Whitton et al. 2008; Liu et al. 2012). Among the 326 apomictic genera, adventitious embryony (148 genera) represents the most frequent apomictic type, mainly in tropical and subtropical woody plants of the families Rutaceae, Celastraceae and Orchidaceae (Naumova 1993; Carman 1997; Richards 1997; Hojsgaard et al. 2014). It is followed by gametophytic apomixis – 110 aposporous and 68 diplosporous predominantly temperate herbaceous genera (Richards 1997; Hojsgaard et al. 2014). Three-quarters of these are restricted just to three families – Rosaceae, Poaceae and

Asteraceae (Hojsgaard et al. 2014). Whereas the Rosaceae and Poaceae are predominantly aposporous, the Asteraceae are more frequently diplosporous (Whitton et al. 2008). The broad taxonomic distribution of apomixis (32 apomictic-containing orders, Hojsgaard et al. 2014) suggests its multiple origins (Whitton et al. 2008). Although searching for predispositions to gametophytic apomixis is complicated, polyploidization, hybridization and production of unreduced gametes in high frequencies are important factors allowing gametophytic apomixis to evolve (Richards 1997; Whitton et al. 2008). The genetic mechanisms underlying both gametophytic and sporophytic apomixis have not yet been sufficiently explained. Various authors have discussed global deregulation of sexual reproductive development, invoking a unique trigger initiating apomictic formation, a few mutations within the reproductive pathway, simple Mendelistic inheritance or complex control involving multiple loci (Garcia et al. 1999; Grimanelli et al. 2001; Ozias-Akins 2006). Research activities are primarily aimed to engineer apomixis in sexual crop species that would enable the production of progeny genotypically identical to the desirable maternal parent (Ozias--Akins 2006).

On the one hand, an apomictic reproductive mode brings several advantages, including assured reproduction (even in the absence of pollination), avoidance of the 'cost of meiosis' and thus the production of all offspring with the same fitness as the mother and, finally, fixing and spreading an extremely fit genotype. In the case of polyploids and hybrids, apomixis represents a unique opportunity to be highly heterozygous (therefore vigorous), fixing this heterozygosity and escape from sterility (Richards 1997). On the other hand, apomictic reproduction also causes the accumulation of disadvantageous mutations (Müller's ratchet) and an inability to recombine new advantageous mutants capable of adapting to environmental change, a very narrow population niche and, finally, a lack of adaptive fine--tuning in hybrids to a particular environmental condition in comparison to their parents (Richards 1997). The tendency of apomicts to be distributed largely at higher latitudes than their sexual relatives and to occupy previously glaciated areas is called geographical parthenogenesis (e.g. Kearney 2005; Hörandl et al. 2008; Mráz et al. 2009). This success of apomicts in colonization is caused by a combination of factors acting together, and these not occur frequently enough to replace their sexual counterparts. The overall predominance of sexuality can therefore be explained by the rare establishment of apomixis (Hörandl 2006).

6.2 Phylogeny and classification of Rosaceae

Although the family Rosaceae is highly economically important and widespread, especially in temperate regions, phylogenetic relationships, particularly at the intrafamiliar level, have long been poorly understood (Potter et al. 2007a). The classification and view of phylogeny changed gradually over time as new progressive methods became available. The numbers of genera and species presented in the literature over the last decades differ depending on the taxonomic treatment used (92 genera / 2,805 species; Stevens 2020; vs 115 genera / 3,000 species; Tachtadžjan 1966). Various approaches have been employed for the reconstruction of phylogenetic relationships within the family. The first phylogenies and classifications were based on morphology (e.g. fruits; Rohrer et al. 1991). Later, molecular phylogenetic analyses began to play major role in the evaluation of family relationships (e.g. Potter et al. 2007a; Xiang et al. 2016; Zhang et al. 2017). Various molecular markers have been used for this purpose, sometimes supplemented by morphological and anatomical data (e.g. Campbell et al. 1995; Xiang et al. 2016). The most significant molecular tools employed in the study of the Rosaceae include the sequencing of different regions of nDNA (18S and 5.8S genes of rDNA, GBSSI 1 and 2 genes, ITS spacer, PGIP gene, PPO gene) and

cpDNA (matK gene, ndhF gene, rbcL gene, trnK exons, trnL intron, trnL-trnF spacer; Morgan et al. 1994; Campbell et al. 1995; Evans et al. 2000; Potter et al. 2002, 2007a). The most current studies employ nuclear and plastid phylogenomics identifying hundreds of nuclear genes (Xiang et al. 2016) and whole-chloroplast DNA (i.e. plastome; Zhang et al. 2017).

6.2.1 Current Rosaceae phylogeny and classification

Rosids, and particularly the order Rosales, are higher taxonomic ranks where the Rosaceae family is currently nested. The circumscription of these groups has been changed to reflect molecular phylogenies based on sequences of both chloroplast and nuclear genes and repeats (Wang et al. 2009; Zhao et al. 2016; Stevens 2020). Strong support has been found that the Rosales order, consisting of nine families (Rosaceae, Barbeyaceae, Dirachmaceae, Rhamnaceae, Elaeagnaceae, Ulmaceae, Cannabaceae, Moraceae, Urticaceae), is monophyletic, and twelve molecular markers well supported all relationships within the family (Zhang et al. 2011).

The former division of the Rosaceae family into four subfamilies (Rosoideae, Spiraeoideae, Prunoideae, Maloideae; e.g. Valentine and Chater 1968) based on fruit types was not supported by the distribution of base chromosome numbers, various chemicals constituents or chloroplast and nuclear DNA sequences (Judd et al. 2016). Recent phylogenies bringing insight into deep relationships within the Rosaceae (Xiang et al. 2016; Zhang et al. 2017, for details see Fig. 4) support the classification into three subfamilies (identically as Potter et al. 2007a) – Dryadoideae, Rosoideae and Amygdaloideae (i.e. Spiraeoideae in Potter et al. 2007a) and their subdivision into sixteen tribes (see Supp. 1). Strong support for the monophyly of all clades was found and the relationships among them were fully resolved (Zhang et al. 2017). Four clades were identified within the tribe Maleae (Zhang et al. 2017). Numerous whole-genome duplications were confirmed in the evolution of the Rosaceae (Xiang et al. 2016). Moreover, molecular clock analysis allowed to estimate the time of divergence of the family and its particular tribes and genera. Crown clades of the Rosaceae diverged probably during the Late Cretaceous around 101.6 Ma (Xiang et al. 2016; Zhang et al. 2017). The origins and history of the multiple fruit types within the Rosaceae were also reconstructed using transcriptomic and genomic datasets. The independent evolution of fleshy fruits from dry fruits has been suggested and it probably happened multiple times (for details see Xiang et al. 2016).

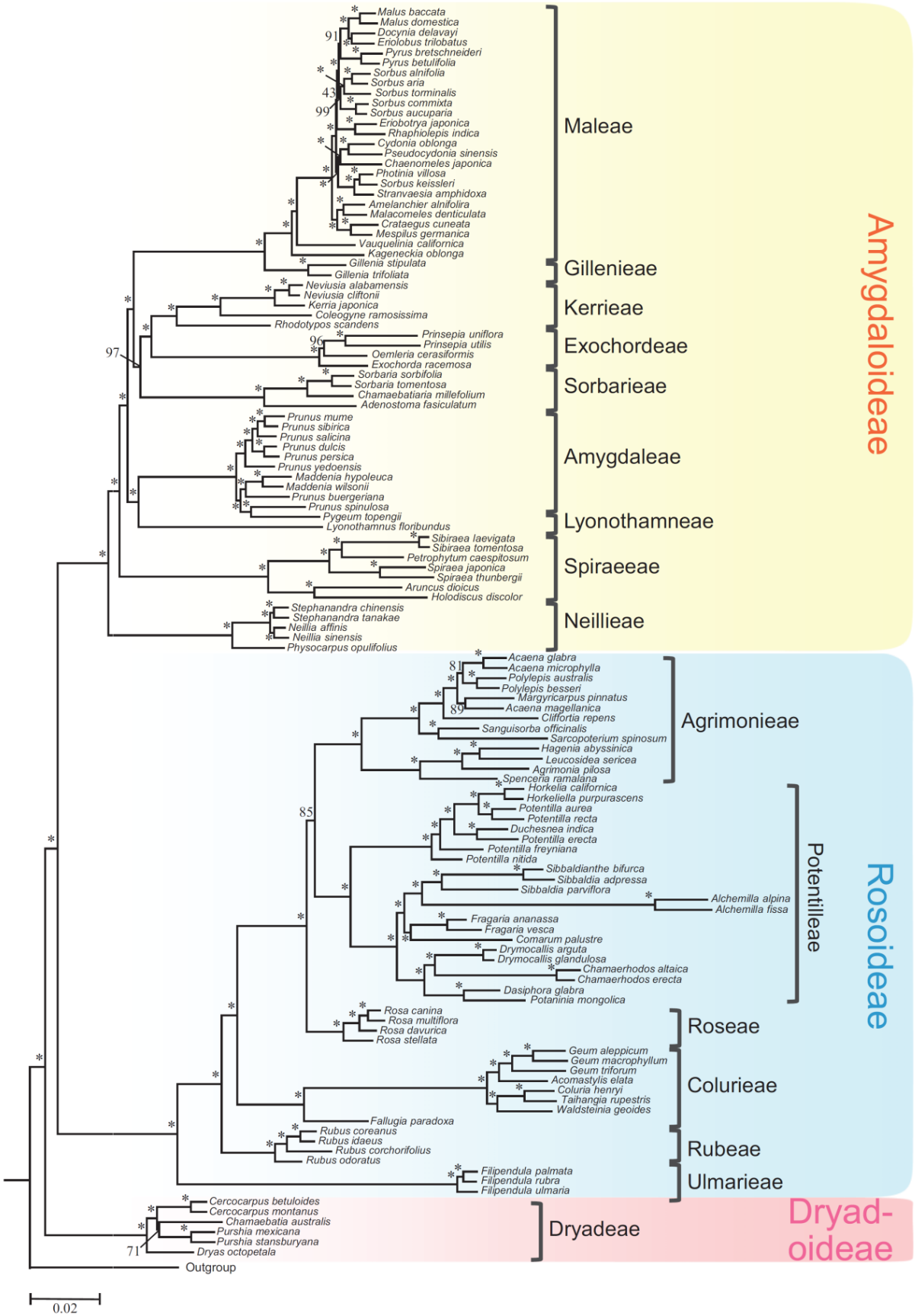

Fig. 4: Phylogenetic reconstruction of the Rosaceae family published in Xiang et al. (2016). The reconstruction was based on maximum likelihood analysis using a dataset of concatenated 113 gene sequences. Numbers associated with nodes indicate bootstrap values obtained by maximum likelihood analyses. Asterisks (*) indicate 100% support.

Thus, the new non-fruit based classification consisting of three subfamilies is widely accepted (Judd et al. 2016; Xiang et al. 2016; Zhang et al. 2017; Stevens 2020; Fig. 4). The Dryadoideae are newly recognized as a subfamily, based on their association with symbiotic nitrogen-fixing actinobacteria of the genus *Frankia*. The Rosoideae are delimited more narrowly (see below). Lastly, the Amygdaloideae are much broader because of the inclusion of the former Spiraeoideae and Maloideae (Judd et al. 2016).

The narrow definition of the Rosoideae is a result of removing the tribe Dryadeae as a distinct subfamily called the Dryadoideae and the tribes Kerrieae and Sorbarieae into the subfamily Amygdaloideae (Judd et al. 2016; Xiang et al. 2016; Zhang et al. 2017; Stevens 2020). The Rosoideae in the current circumscription therefore consist of six tribes (Agrimonieae, Potentilleae, Colurieae, Ulmarieae), two being monotypic (Roseae, Rubeae; for the genera included; see Supp. 1 and 2; Xiang et al. 2016; Zhang et al. 2017; Stevens 2020).

The newly delimited N-fixing Dryadoideae subfamily includes only four genera (*Dryas, Purshia, Cercocarpus, Chamaebatia*; see Supp.1) occurring mostly in western North America (*Dryas* is circumboreal; Judd et al. 2016; Xiang et al. 2016; Zhang et al. 2017; Stevens 2020).

The taxonomic concept of the newly defined broad subfamily Amygdaloideae (former Spiraeoideae in Potter et al. 2007a), including members of the former subfamilies Maloideae and Prunoideae, is not completely uniform (see Supp. 1 and 2). The subfamily consists of nine tribes (Maleae, Gillenieae, Spiraeeae, Sorbarieae, Amygdaleae, Kerrieae, Exochordeae, Neillieae, Lyonothamneae; Xiang et al. 2016; Zhang et al. 2017) but their name and circumscription vary slightly among authors (Stevens 2020). The tribe Amygdaleae, characterized by a base chromosome number of 8 and drupelets (Stevens 2020), contains species that were previously included in the former subfamily Amygdaloideae (i.e. genus *Prunus*). Pome-bearing genera of the former subfamily Maloideae (i.e. *Cotoneaster, Malus, Pyrus, Sorbus*…) are defined by the base chromosome number of 17, and four copies of the GBSSI gene currently form the subtribe Malinae (former Pyrinae in Potter et al. 2007a; see Supp. 2) and, together with *Vauquelinia* and *Kageneckia* (Xiang et al. 2016; Zhang et al. 2017), the tribe Maleae (former Pyreae in Potter et al. 2007a; Judd et al. 2016; see Supp. 2). Moreover, also the supertribe Pyrodae, including the tribe Maleae together with *Gillenia* and *Lindleya,* is strongly supported in current phylogenies (although *Gillenia* may also be classified within the tribe *Gillenieae* in a sister relationship with the Maleae; Potter et al. 2007a; Xiang et al. 2016; Zhang et al. 2017; see Supp. 1 and 2).

6.2.2 History of the phylogeny and classification of the Rosaceae family

Strong molecular evidence for the monophyly of the Rosaceae was reported repeatedly by various authors (e.g. Morgan et al. 1994; Evans et al. 2000; Potter et al. 2002, 2007a), but the intrafamiliar classification changed over the last century, both as to the number and composition of subfamilies. The oldest and for a long time the most widely adopted taxonomic treatment based on fruit morphology used four subfamilies (Spiraeoideae, Rosoideae, Maloideae, Prunoideae; e.g. Valentine and Chater 1968). Moreover, Tachtadžjan (1987) added three more subfamilies (Quillajeoideae, Dichotomanthoideae, Prinsepioideae) and further subdivisions (tribes). Four traditionally recognized subfamilies are characterized by the type of fruit – the Spiraeoideae by follicles, Rosoideae by achenes, drupes or drupelets, Maloideae by fleshy pomes and Prunoideae by drupes (Valentine and Chater 1968). Although further molecular analyses supported some of the traditional groups, they also showed that fruit types cannot correctly elucidate relationships across the Rosaceae (e.g. Morgan et al.

1994; Potter et al. 2007a). Thus, a new intrafamiliar classification substantially influenced by molecular phylogenetic studies was adopted for the Rosaceae.

The first molecular phylogenetic study employing chloroplast DNA sequencing (rbcL gene) supported groups comparable to three traditional subfamilies (Maloideae, Amygdaloideae, Rosoideae), but with some modifications (Morgan et al. 1994). By contrast, the fourth traditional subfamily Spiraeoideae was evaluated as polyphyletic, consisting of several distinct evolutionary lineages. The subfamily Rosoideae was subdivided by base chromosome number (members with x = 9 were well separated from members with x = 8 or 7). The subfamily Maloideae consisted of members with a base chromosome number of either 17 or 15 and also included some taxa from the former subfamily Spiraeoideae. Prunoideae was the subfamily with the lowest support, comprising besides the genus *Prunus* in its traditional sense also three other genera (*Prinsepia, Oemleria, Exochorda*), all with a base chromosome number of 8. The incidence of more than one fruit type in all subfamilies revealed the base chromosome number as a better indicator of phylogenetic relationships among the Rosaceae than the traditionally used type of fruit (Morgan et al. 1994).

Another molecular phylogeny based on the low-copy nuclear gene GBSSI exhibited duplication of this gene predating the evolution of the Rosaceae. The whole family thus possesses at least two loci of this gene (Evans et al. 2000). Moreover, additional duplication occurred in the subfamily Maloideae. So, two copies of this gene (GBSSI 1 and GBSSI 2) were found in diploid taxa with a base chromosome number of 7, 8 or 9 (subfamily Spiraeoideae, Rosoideae, Prunoideae) and four copies (GBSSI 1A, GBSSI 1B, GBSSI 2A, GBSSI 2B) in diploid taxa with a base chromosome number of 15 or 17 (subfamily Maloideae; Evans et al. 2000; Potter et al. 2007a). Analyses of chloroplast matK and trnL--trnF sequences (Potter et al. 2002) showed similar clades as those based on the chloroplast rbcL gene (Morgan et al. 1994), corresponding to traditional subfamilies but with some modifications. Three main lineages diverged in the early evolution of the whole Rosaceae family – Rosoideae s.str. clade (x = 7, occasionally 8, lacking the ability to accumulate sorbitol), actinorhizal Rosaceae clade (x = 9, able to form symbiotic relationships with N--fixing bacteria and prone to sorbitol accumulation, recent Dryadoideae) and the rest of the family (Potter et al. 2002). Whereas the subfamilies Maloideae and Rosoideae were found to be monophyletic, the Prunoideae and Spiraeoideae turned out to be polyphyletic. The strongly supported monophyletic subfamily Maloideae included, besides members with a base chromosome number of 17, also *Vauquelinia* (x = 15, capsule), *Kageneckia* (x = 17, follicle) and other taxa producing pomes (i.e. Maloideae s.l.; Potter et al. 2002).

The first modern and still accepted, more or less without change, classification of the Rosaceae family was proposed based on a compilatory molecular study using ten nucleotide sequence datasets for nuclear and chloroplast regions (many of the data used had already been published; Potter et al. 2007a). Although monophyletic groups closely corresponding with some previously defined subfamilies and tribes were resolved and strongly supported, no previous classifications were confirmed entirely. Three subfamilies were revealed – two large ones, the Rosoideae and Spiraeoideae, and a small one, the Dryadoideae. Thus, the former subfamily Rosoideae was divided into the Rosoideae (s.str.) and the Dryadoideae. The Rosoideae (s.str.) were composed of taxa with a base chromosome number of 7 and 8 and producing achenes, acheneta or drupeta. The remaining taxa with x = 9, traditionally belonging to Rosoideae based on achene production, were found to fall outside it. The subfamily Dryadoideae was characterized by a base chromosome number of 9 and symbiotic nitrogen fixation (via associations with *Frankia* actinobacteria). The subfamily Spiraeoideae (i.e. Amygdaloideae in Xiang et al. 2016; Zhang et al. 2017) was formed as a combination of the former Amygdaloideae, Maloideae and Spiraeoideae, including various types of fruits and chromosome numbers (8, 9, 15, 17). Taxa traditionally belonging to the

Maloideae because they bear pomes (or polypyrenous drupes) were ranked into subtribe Pyrinae (Potter et al. 2007a). The evolution of Rosaceae fruits was complex and resulted in multiple fruit types in each of several clades. The two largest subfamilies were further subdivided into one supertribe, three tribes and three subtribes within the Rosoideae, and two supertribes, seven tribes and one subtribe within the Spiraeoideae (subtribes Rosodeae and Kerriodae were newly described; for details see Supp. 2). Nevertheless, phylogenetic relationships among clades were often weakly supported (Potter et al. 2007a). Worth pointing out is the closer relation of several taxa with x = 15 and 17, which produce follicles (traditionally classified within the Spiraeoideae), to the Maloideae (pome bearing). This again confirms that base chromosome numbers better indicate the phylogenetic relationships across the Rosaceae than fruit type (Potter et al. 2007a). Although phylogenetic studies mentioned above were limited by a low number of loci, their resolution is comparable to that of current studies employing phylogenomics (analysing whole transcriptomic and genomic datasets).

6.2.3 The origin of subtribe Malinae – a model group for studying the complex evolution of the Rosaceae

Various authors have studied in detail particular subclades and tried to evaluate more deeply the relationships within them (e.g. Neillieae, Oh and Potter 2005; Spiraeeae, Potter et al. 2007b; Pyrinae, Campbell et al. 2007; Pyreae, Lo and Donoghue 2012). The subtribe Malinae, grouping taxa producing pomes or polypyrenous drupes, has been of the greatest interest in the last decades (Robertson et al. 1991; Campbell et al. 1995; Aldasoro et al. 2005), especially because of its unusual base chromosome number of 17 (with the sole exception of the early branching genus *Vauquelinia* with x = 15; Goldblatt 1976; Robertson et al. 1991). It has been supposed that hybridization played important role in the formation of this extraordinary number. The hypothesis of the hybrid origin of the subtribe Malinae, holding that the whole subtribe arose by hybridization between primitive or ancestral members of the former Amygdaloideae with x = 8 and former Spiraeoideae with x = 9 (Sax 1931), was suggested since the 1950s (Stebbins 1950). Nevertheless, a phylogenetic analysis based on the GBSSI gene including wide range of taxa across the Rosaceae with different base chromosome numbers brought completely new insight into the evolution of the Malinae (Evans et al. 2000; Evans and Campbell 2002). The long-held hypothesis of hybrid origin was not supported. On the one hand, former genera of the Spiraeoideae (*Kageneckia* and *Vauquelinia*) were found to be the closest relatives of the subtribe Malinae in all four GBSSI clades, but on the other, the study showed that ancestral member of the former Amygdaloideae did not participate in the origin of the subtribe Malinae (Evans et al. 2000). A new alternative hypothesis invoked hybridization and polyploidization in a lineage including the ancestors of *Gillenia* (x = 9; Evans et al. 2000; Evans and Campbell 2002; Velasco et al. 2010) and subsequent aneuploid reduction of x = 18 (Evans and Campbell 2002). Intergeneric hybridization and genome duplication involved in the evolution of the Malinae causes difficulties in reconstructions of its relationships (Campbell et al. 2007). Moreover, the timing and occurrence of whole-genome duplication still remain unclear (Xiang et al. 2016) and rapid ancient radiation is suggested (Campbell et al. 2007). *Gillenia* was resolved as a sister clade based on both the GBSSI 1 and the GBSSI 2 gene (Evans and Campbell 2002; Potter et al. 2002). Based on its distribution in the southeast of the United States, it has been proposed that the subtribe Malinae originated in North America (Evans and Campbell 2002). Thus, the subtribe Malinae contains, besides taxa with a base chromosome number 15 and 17, also genus *Gillenia* (x = 9) and forms a strongly supported monophyletic group (Evans and Campbell 2002; Potter et al. 2007a).

6.3 Rosaceae as a focus of applied science

An extraordinarily high number of Rosaceae species are under scientific scrutiny because of their economical/agricultural significance (Fig. 5; Potter et al. 2002; Simpson 2006; Judd et al. 2016). Reflecting the significance of model groups within the Rosaceae, entire genomes of members of six genera have already been sequenced – *Fragaria* (6 species), *Malus* ×*domestica*, *Prunus armeniaca*, *P. avium*, *P. domestica*, *P. dulcis*, *P. persica*, *P. yedoensis*, *Pyrus* (3 species), *Rosa* (2 species) and *Rubus occidentalis* (Fig. 5; GDR database – Jung et al. 2019). Primary research benefits from the intensive focus and the knowledge obtained can be applied back in general plant biosystematics and to less economically important taxa (e.g. *Prunus cerasus*; Horvath et al. 2008 or *Malus* ×*domestica*; Volk et al. 2015).

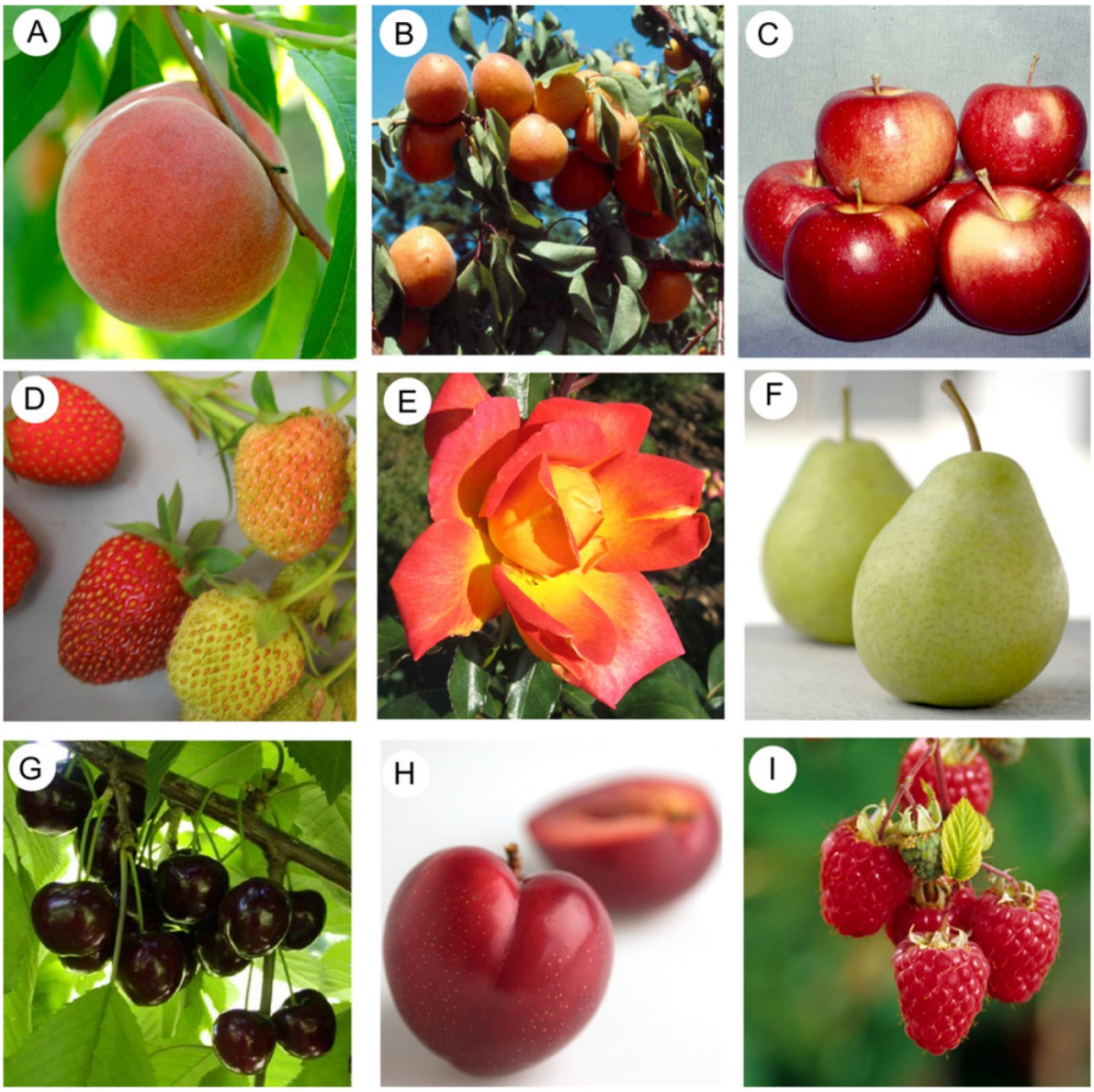

Fig. 5: Rosaceae crops showing variation in fruit types, published in Shulaev et al. (2008). The genomes of all included genera have already been sequenced. A = *Prunus persica* (peach) – fleshy drupe; B = *Prunus armeniaca* (apricot) – fleshy drupe; C = *Malus* ×*domestica* (apple) – pome; D = *Fragaria* ×*ananassa* (strawberry) – achenes; E = *Rosa* ×*hybrida* (rose) – achene; F = *Pyrus communis* (pear) – pome; G = *Prunus avium* (sweet cherry) – fleshy drupe; H = *Prunus domestica* (plum) = fleshy drupe; I = *Rubus idaeus* (raspberry) – drupelets.

These model taxa are predominantly species bearing edible berries (*Fragaria* – strawberry, *Rubus* – raspberry and blackberry) and tree fruit species known as stone fruits (*Prunus armeniaca* – apricot, *P. domestica* – plum, *P. persica* – peach and nectarine, *P. avium* – sweet cherry, *P. cerasus* – sour cherry, *P. dulcis* – almond) and pomiferous fruits (*Malus ×domestica* – apple, *Pyrus communis* – pear, *Cydonia* – quince or *Eriobotrya* – loquat). The world production of five major commercial fruit groups belonging to the family (apple, peach and nectarine, pear, plum, and sloe and strawberry) reached approximately 153 million tons in 2017 (FAO 2020).

The most important ornamental cultivars include herbs, such as *Alchemilla* (lady's mantle), *Fillipendula* (meadowsweet) and *Potentilla* (cinquefoil), and woody plants such as *Chaenomeles* (flowering quince), *Cotoneaster*, *Crataegus* (hawthorn), *Pyracantha* (firethorn), *Rosa* (rose), *Sorbus* (rowan) and *Spiraea* (bridal wreath; Simpson 2006; Judd et al. 2016). Last but not least, *Rosa* is also cultivated for essential oils (Simpson 2006), and wood from *Prunus serotina* is used in the manufacture of furniture (several genera provide timber; Judd et al. 2016).

6.3.1. Importance of wild relatives in breeding programmes

Nevertheless, the overall genetic variation of these crops is restricted compared to their wild related species and their economical yields directly depend on the existence of suitable relative/crossable plants with agronomically important characters. The assessment and maintenance of genetic diversity of wild relatives are crucial for the development of new cultivars (De Andrés et al. 2012). Wild plant genetic resources provide a repository of suitable characters using for the selection of resistant, highly productive varieties and allow the preservation of adaptability to environmental and other changes. Continuous breeding using wild relatives is therefore a way to improve crop genetic resources. Adequate knowledge and evaluation of existing genetic diversity in wild plant populations and the efficient management of crop genetic resources are therefore fundamental for both basic and applied science (Mondini et al. 2009). Cherry breeding may serve as a model example of how wild germplasm may be used as a source of novel genetic diversity. Whereas *Prunus avium* (sweet cherry) cultivars were found to be genetically restricted to Greece, wild *P. avium* populations exhibited genetic variation suggesting that wild germplasm may be useful in cherry breeding programmes (Ganopoulos et al. 2013). Similarly, high diversity levels of wild *Prunus fruticosa* (ground cherry) populations observed in Serbia promise great potential for breeding new cherry varieties and rootstocks for sweet and sour cherry (Barać et al. 2017). The main characters favouring this wild cherry species in breeding programmes is adaptation to abiotic stress in severe climate conditions of steppes and prairies, including resistance to low temperature and drought, late-blooming, a shrubby habit and abundant roots (Iezzoni and Mulinix 1992; Dzhangaliev et al. 2003; Pruski 2007; Iezzoni 2008). Breeding new winter-hardy, drought-resistant and late-blooming cultivars and cultivated shrub cherries that can grow steadily in northern environments is crucially economical important for fruit growers on prairies (e.g. in Canada) and for soil conservation on dry slopes (Pruski 2007).

6.4 Microevolutionary processes of selected model genera of the Rosaceae

The Rosaceae are a family with an enormously high incidence of hybridization and polyploidization (Ellstrand et al. 1996; Dickinson et al. 2007; Stace et al. 2015; Marques et al. 2018). The incidence and frequency of overall hybridization were evaluated in only a few geographical areas that are hard to compare (Iberian Peninsula, Scandinavia, British Isles and Great Plains in North America). For this reason, various features of hybridization are outlined in the text bellow. Half of all hybrids reported from the Iberian Peninsula were found to be restricted to four families, one of them being the Rosaceae (the others were Plumbaginaceae, Lamiaceae and Orchidaceae; Marques et al. 2018). Likewise, the family Rosaceae, along with the Asteraceae, Salicaceae and Poaceae, belonged to the families with the most hybrids in Scandinavia, the British Isles and on the Great Plains (Ellstrand et al. 1996). It has been found that in families where hybridization prevails, polyploidization is also frequent (Marques et al. 2018). Thus, as in the case of hybridization, the frequency of polyploids in the Rosaceae was markedly above average in the Iberian Peninsula (as well as in the families Caryophyllaceae, Poaceae and Liliaceae; Marques et al. 2018). Moreover, polyploidization occurred numerous times in the evolution of the Rosaceae (Evans and Campbell 2002; Vamosi and Dickinson 2006). So, hybridization is a common phenomenon in this family; however, not only crossing between species, but also intergeneric hybridization, was described within it (Campbell et al. 1991; Robertson et al. 1991; Fig. 6). However, a high frequency of intergeneric hybrids is not present throughout the family but is predominantly restricted to the subtribe Malinae, namely to genera such as *Amelanchier*, *Aronia, Chaenomeles, Cotoneaster, Crataegus, Cydonia, Malus*, *Mespilus*, *Pyrus* and *Sorbus*. Their hybrid origin is further illustrated by recent intergeneric hybrids, such as ×*Amelasorbus*, ×*Sorbaronia,* ×*Sorbocotoneaster*, ×*Sorbopyrus*, ×*Pyracomeles*, ×*Pyronia*, ×*Crataegomespilus* and ×*Crataegosorbus*, repeatedly described in the field and also bred in dendrological gardens (e.g. Kovanda 1965; Krügel 1990; Robertson et al. 1991; Hejný and Slavík 1992; Stace et al. 2015). Such intergeneric compatibility in the subtribe Malinae suggests limited genetic divergence (Kovanda 1965; Campbell et al. 1995) and weak overall hybridization barriers which, however, do not necessarily mirror the close relationship among the genera (Robertson et al. 1991). Nevertheless, the fertility of newly arisen intergeneric hybrids differs and some of them are largely sterile or produce few seeds (Campbell et al. 1991).

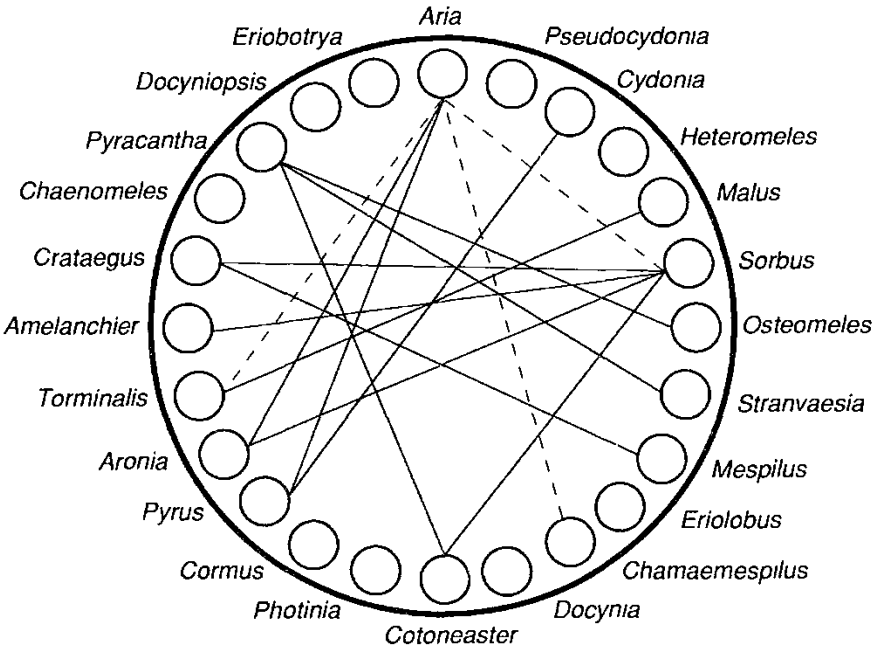

Fig. 6: Published intergeneric hybrids in the subtribe Malinae, summarized in Robertson et al. (1991). Dashed lines represent frequently occurring hybrids within *Sorbus* s.l. For details see the respective paper.

Hybridization in the Rosaceae is concentrated only in a few genera, but interbreeding is all the more frequent within them. For the Mediterranean region, four genera were reported to include a remarkable number of hybrids occurring in the Iberian Peninsula, one of them being *Rosa* including 75% of hybrid taxa (Marques et al. 2018). Other Rosaceae genera with high rates of hybrids in the Iberian Peninsula include *Geum* (50% of hybrid taxa), *Prunus* and *Agrimonia* (both with 33% of hybrid taxa), *Crataegus* (29% of hybrid taxa) and *Potentilla* (9% of hybrid taxa). The genus *Rosa* is the most extensively hybridizing genus of the Rosaceae also in the British Isles and on the Great Plains; moreover, the genera *Sorbus*, *Spiraea*, *Rubus* and *Potentilla* include significant numbers of hybrid taxa in the British Isles (Ellstrand et al. 1996; Stace et al. 2015).

As mentioned above, the great biological significance of polyploidization and hybridization in Rosaceae (and overall in plants) evolution is indisputable, but even in the Rosaceae, these processes are also linked with gametophytic apomixis and fertilization of unreduced female gametes (Dickinson 2018). Apomixis in the Rosaceae family is mostly found in the tribe Pyreae and the subfamily Rosoideae (Dickinson et al. 2007; Dickinson 2018). Particularly in tribe Pyreae, apomictic reproduction has been detected, for example, in the genera *Sorbus*, *Cotoneaster* or *Crataegus* (Dickinson 2018). *Alchemilla*, *Potentilla* and *Rubus* are examples of apomictic genera belonging to the subfamily Rosoideae (moreover, the genus *Rosa* exhibits traits of both apomictic and sexual reproduction; Werlemark et al. 1999; Dickinson et al. 2007). Nevertheless, some genera, such as *Prunus*, *Eriobotrya* and *Fragaria*, represent polyploid genera where spontaneous hybridization occurs without detected apomixis (Dickinson 2018).

From a biosystematic point of view, the Rosaceae family is intricate and includes several levels of complexity. That is why some of the widely studied genera mentioned below serve as model examples exhibiting this complexity. These genera are hierarchically arranged to reflect the influence of different proportions of polyploidization, hybridization and apomixis on the evolution and diversification of the Rosaceae. The evolution of *Prunus*, *Eriobotrya* and *Fragaria* is influenced by polyploidization and hybridization (e.g. Guo et al. 2006; Horvath et al. 2008; Kamneva et al. 2017; Wang et al. 2017). In the genus *Rosa*, these

processes are supplemented by a unique mode of reproduction combining sexual and asexual features (hemisexuality; e.g. Ritz and Wissemann 2003). Asexual reproduction via apomixis plays a certain role in the genera *Malus*, *Crataegus* and *Potentilla*, but sexual reproduction and hybridization still prevail (e.g. Kron and Husband 2009; Lo et al. 2009; Dobeš et al. 2015). The influence of apomixis significantly increases in the genus *Rubus* and *Sorbus* where asexual breeding represents an undisputed force of diversification, manifested in established apomictic lineages, together with ongoing polyploidization and hybridization (e.g. Šarhanová et al. 2012; Lepší et al. 2019). Finally, the genus *Alchemilla* consists of long-standing hybridogenous apomictic species that are higher polyploids and reproduce obligatorily by apomixis (e.g. Czapik 1996; Gehrke et al. 2008).

6.4.1 *Prunus*

The genus *Prunus* includes many crops of high economical importance, but its taxonomy is notoriously problematic and reliable discriminating characters are still missing (Nielsen and Olrik 2001). The circumscription and systematics of the genus differ from author to author. Its definition as one genus is supported by molecular markers (Bortiri et al. 2001), but particular genera are sometimes separated (e.g. *Cerasus, Amygdalus, Padus*; Bertová 1992). The traditional intrageneric classification consists of five subgenera and several sections – subgenera *Prunus* (plums and apricots), *Amygdalus* (almonds and peaches), *Cerasus* (cherries), *Laurocerasus* and *Padus* (Bortiri et al. 2001, 2006). One molecular phylogenetic study (Bortiri et al. 2001) revealed two major lineages, one clade including the subgenera *Padus, Laurocerasus* and *Cerasus*, and the other including *Prunus, Amygdalus, Emplectocladus* and one section of *Cerasus.* Nevertheless, another molecular phylogeny did not support the distinction of the subgenera *Padus* and *Laurocerasus* or the subgeneric classification in general (Wen et al. 2008). However, the subgeneric classification and relationships among groups still remain unclear.

The genus *Prunus* consists of diploid, rather self-incompatible species, besides polyploid, self-compatible species. Whereas almonds, apricots and peaches are diploid, cherries are both diploid and tetraploid. Finally, plums have been reported to be diploid, tetraploid, pentaploid and hexaploid (Darlington and Wylie 1955; Hanelt 1997; Corredor et al. 2004; Verde et al. 2013; Žabka et al. 2018). Both autopolyploidy and allopolyploidy have been described in the genus *Prunus*. Cherries can serve as a model example. Allotetraploid *Prunus cerasus* (sour cherry, 2n = 32; Iezzoni 2008) arose from spontaneous hybridization of diploid *Prunus avium* (sweet cherry, 2n = 16; Iezzoni 2008) and tetraploid *Prunus fruticosa* (2n = 32; Scholz and Scholz 1995). Fusion of a reduced diploid female gamete of *P. fruticosa* and an unreduced diploid male gamete of *P. avium* was proved based on GISH, C-banding (Schuster and Schreibner 2000), AFLP, cpDNA and microsatellites (Horvath et al. 2008). Similarly, the formation of the allotetraploid cultivar *Prunus* ×*gondouinii* (Duke cherry, 2n = 32; Webb 1968) involved the participation of an unreduced gamete of *P. avium* and a reduced gamete of *P. cerasus*. By contrast, *Prunus fruticosa* is thought to be autotetraploid (Tavaud et al. 2004; Fig. 7).

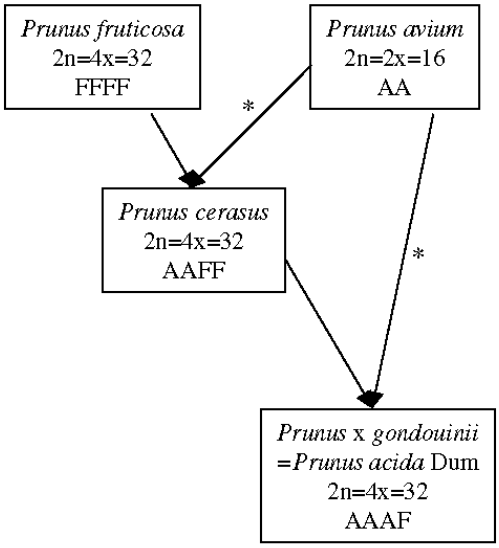

Fig. 7: Hypothesis on the relationships among four *Prunus* species (cherries) published in Tavaud et al. (2004). A = haploid genome from *P. avium*. F = haploid genome from *P. fruticosa*. **P. avium* is thought to produce diploid gametes.

Only a few studies have dealt with crop-to-wild gene flow in *Prunus* species (Delplancke et al. 2012). Wild and cultivated almonds were the subject of one of them. The native wild almond *Prunus orientalis* and its domesticated counterpart, cultivated almond (*Prunus dulcis*), were examined for putative crop-to-wild gene flow in Southwest Asia based on nuclear and chloroplastic microsatellites. In comparison to cherries and apples (discussed below and above), *P. orientalis* is not considered an ancestor of cultivated almond (Zeinalabedini et al. 2010). The two species differ morphologically. Whereas wild *P. orientalis* is a thorny shrub with white tomentose shoots, leaves and fruits, cultivated almond is a non-spiny tree with numerous brachyblasts and relatively large leaves. The study revealed that gene flow between the species occurred commonly, and hybridization was found to be symmetric (bidirectional). Genes of cultivated almond could therefore be spontaneously introgressed into wild *Prunus orientalis,* and, in addition, hybrids showed an intermediate phenotype with large, green and tomentose leaves.

Crop-to-wild gene flow from cultivated plums into wild *Prunus spinosa* (blackthorn or sloe) is also discussed (e.g. Vander Mijnsbrugge et al. 2016; Žabka et al. 2018). Both *Prunus insititia* (damson) and *Prunus domestica* (plum) represent cultivated plums, but their relationship and origin have not yet been sufficiently elucidated and are sometimes considered the same species (Woldring 2000; Nielsen and Olrik 2001). In addition, *Prunus cerasifera* (cherry plum) consists of both wild and cultivated forms (Hanelt 1997). Relationships among cultivated and wild species are far from fully understood. Although AFLP analysis resulted in three genetic clusters (*P. cerasifera*, *P. domestica* + *P. insititia*, *P. spinosa* + *P. ×fruticans*), intra-population coherence was often more obvious than its interspecific counterpart. Thus, the concept including fewer but more diverse species groups has been suggested to be more reliable than distinguishing between several species (Depypere et al. 2009). The great morphological variation observed in *P. spinosa* (Nielsen and Olrik 2001) and its large-fruited forms led to the assumption of hybridization events. The hybrid taxon *Prunus ×fruticans* and even other hybrid taxa resulting from crossing between *P. spinosa* and all three of its cultivated relatives have been supposed (Hanelt 1997; Nielsen and Olrik 2001; Žabka et al. 2018). Nevertheless, distinguishing hybrids with *P. spinosa* was found to be difficult and it has been suggested that *P. ×fruticans is* an old, abandoned fruit crop (Hanelt 1997). Although AFLP-based analysis clustered together *P. spinosa* and *P. ×fruticans,* morphometrics

of leaves and endocarps enabled their separation (Depypere et al. 2009). Results derived from morphological and phenological variation confirmed historical crop-to-wild gene flow between *P. spinosa* and *P. insititia* (including subsequent backcrossing with *P. spinosa*), resulting in *P. ×fruticans*. Nevertheless, hybridization events have occurred already for a long time and probably without adverse effects on abundant wild populations of *P. spinosa* occurring across Europe (Vander Mijnsbrugge et al. 2016).

6.4.2 *Eriobotrya*

One analogous example of a commercial crop substantially affected by polyploidization and hybridization is the genus *Eriobotrya*. This genus is commonly known and also investigated because of *Eriobotrya japonica*, so-called loquat, a horticulturally valuable subtropical plant that is grown for fruit or as an ornamental tree. A study of 21 loquat cultivars revealed prevailing diploidy but also detected minor polyploidy in 0.68% of accessions. Three ploidy levels were identified: prevailing triploids and in a minority also tetraploids and pentaploids (Guo et al. 2006). These results suggest that, occasionally, unreduced gametes may arise and form high ploidy levels in *Eriobotrya*. Interspecific hybridization of two *Eriobotrya japonica* cultivars and wild *Eriobotrya bengalensis* was tested experimentally with the aim to breed a new cold-resistant cultivar. Hybrids were successfully obtained and both parental species exhibited good compatibility in both directions (Wang et al. 2017). For these reasons, possible spontaneous hybridization within wild *Eriobotrya* species or between potentially escaped cultivars and wild congeners cannot be ruled out.

6.4.3 *Fragaria*

The genus *Fragaria* is one of the most important model taxa within the Rosaceae. Its genome has already been sequenced and the genus serves as a model group in studies of introgressive hybridization (Shulaev et al. 2011; Hirakawa et al. 2014; Kamneva et al. 2017). *Fragaria* is generally known as strawberry (cultivated octoploid *Fragaria ×ananassa*), a highly valuable commercial crop. It currently consists of 22 species, ten of which are polyploids, ranging from the tetraploid to the decaploid level (Shulaev et al. 2011; Kamneva et al. 2017). Their evolutionary history, including hybridization events and thus their allopolyploid origin, was revealed based on a current study employing NGS data analysis (Kamneva et al. 2017). Diploid *Fragaria vesca* is used as a versatile experimental plant system, and its genome has already been sequenced. It has the smallest sequenced plant genome besides *Arabidopsis* (Shulaev et al. 2011).

Natural hybridization of *Fragaria* species at a heteroploid level was reported from California (Bringhurst and Khan 1963; Bringhurst and Senanayake 1966). Hybridization involving the participation of unreduced gametes between octoploid *Fragaria chiloensis* and diploid *F. vesca* resulted in the formation of nonaploid, hexaploid and pentaploid hybrids aggressively competing with *F. chiloensis*. The presence of other euploid levels (3x, 4x, 10x, 12x, 16x) in natural conditions was suggested based on at least partial fertility of hybrids, production of unreduced gametes and backcrossing (Bringhurst and Senanayake 1966). That is why researchers are examining the possibility of unintentional crossing between cultivated octoploid strawberry and its wild diploid relatives. A survey done in Switzerland found that wild bee (*Osmia bicornis*), a common flower visitor of *Fragaria* species, did not discriminate between wild and cultivated strawberries and thus represents a potential vector for gene flow (Schulze et al. 2012). Nevertheless, a study of *F. vesca* populations occurring

in the vicinity of strawberry farms in Switzerland and Germany revealed no hybrids (not even among morphologically deviating individuals) using microsatellite markers. By contrast, hand-crosses of the same plant material resulted in clear hybrids with a microsatellite pattern combining traits of both parents (Schulze et al. 2011). The breeding programme developing the hybrid acting as a bridge between cultivated and related wild strawberry (overcoming reproducing barriers preventing desirable gene flow) also brought other insights into crop-to--wild strawberry hybridization. Although low production of achenes was detected in both directional crosses of wild and cultivated strawberry, hybridization between wild *Fragaria* species produces large numbers of achenes. In addition, homoploid crosses had a greater percentage of germination compared to heteroploid crosses, which resulted in no or very few germinating seeds (Luque et al. 2019).

Although it has been confirmed that species of the genus *Fragaria* reproduce sexually (Dobeš et al. 2015), apomixis has also been discussed (Nosrati et al. 2010; Dziadczyk et al. 2011; Leszczuk et al. 2018). Apomictic reproduction has been supposed based on the production of morphologically maternal progeny in experimental interploid crosses of various *Fragaria* taxa. Nevertheless, based on RAPD analysis, the progeny was found to be hybrid, probably due to heterozygosity of the pollen parent (Nosrati et al. 2010). Apomixis as a commercially important trait in crop species is, of course, studied in *Fragaria* ×*ananassa* cultivars and facultative apomixis has been suggested to occur in three of them (Dziadczyk et al. 2011; Leszczuk et al. 2018). In addition, dioecious octoploid *Fragaria* taxa possess sex-chromosomes and, in contrast to the majority of plants, females are heteromorphic (ZW; Charlesworth and Charlesworth 1978; Spigler et al. 2008; Goldberg et al. 2010). Moreover, the ability to repeatedly change the genomic location of its sex region and thus possibly adaptively gather and lock new genes into linkage with sex has been recently identified in this genus (Tennessen et al. 2018).

6.4.4 *Rosa*

The evolution of the polyploid and hybrid genus *Rosa* is complicated by versatile reproduction strategies. Especially the section *Caninae* (dogroses) is notoriously regarded as a biosystematically complex and intricate group, mainly because of its allopolyploid constitution, skewed maternal inheritance and ongoing hybridization (Herklotz and Ritz 2017). Dogroses are mostly pentaploid (base chromosome number 7), but tetraploids, hexaploids and rarely heptaploids or octoploids also occur (Klášterská and Natarajan 1974; Pachl 2011). They are considered complex allopolyploids, as multiple hybridization events have been proved by employing nuclear ribosomal DNA data. The formation of their genome involved crossing between members of different rose sections and now extinct *Protocaninae* (Ritz et al. 2005).

Although dogroses are known for their elusive morphological variation, they are clearly characterized by their unique meiotic behaviour referred to as Canina-type meiosis (Fig. 8). This type of meiosis facilitates sexual reproduction at odd-number ploidy levels and combines sexual and asexual reproduction in one cell (i.e. hemisexuality; Werlemark et al. 1999; Ritz and Wissemann 2003; Nybom et al. 2004). Each somatic cell of a pentaploid dogrose contains 14 homologous chromosomes (normally recombining and forming seven bivalents during meiosis) and 21 additional chromosomes (non-pairing neither recombining and forming 21 univalents during meiosis). Fertile haploid pollen grains produced by unbalanced meiosis contain 7 chromosomes and tetraploid egg cells contain 28 chromosomes (similarly, tetraploid plants produce haploid pollen grains and triploid egg cells, Fig. 8). This heterogamous system leads to a permanently pentaploid organism and

matroclinal inheritance. As a result, the nuclear genome of dogroses consists of 80% of the maternal genome and 20% of the paternal genome (Ritz and Wissemann 2003; Ritz et al. 2005).

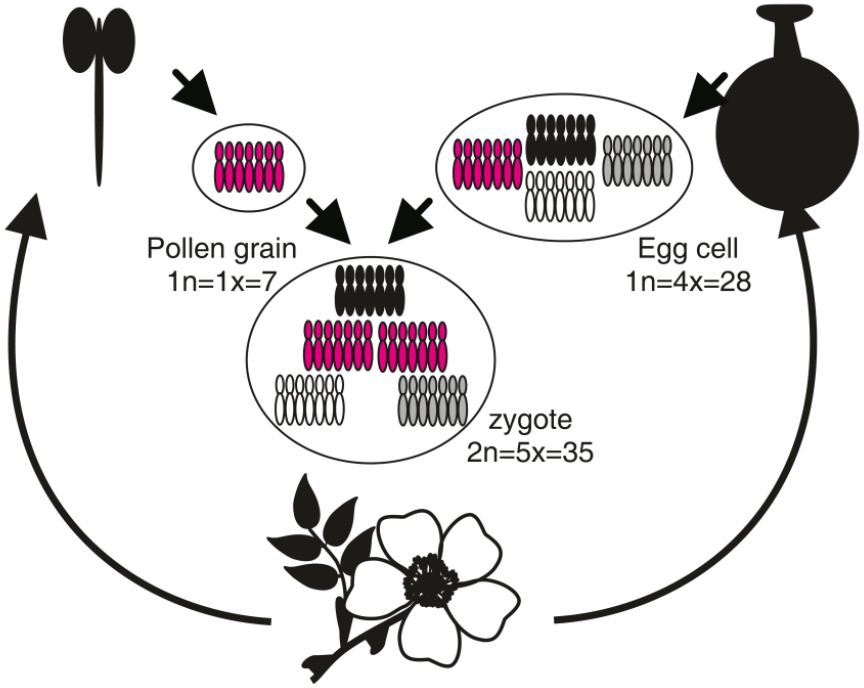

Fig. 8: Diagram of Canina-type meiosis of the genus *Rosa* sect. *Caninae* (dogroses), published in Ritz et al. (2011). Pentaploid dogroses (2n = 5x = 35) produce haploid pollen grains (1n = 1x = 7) and tetraploid egg cells (1n = 4x = 28). Fertilization of haploid pollen grains and tetraploid egg cells restores the pentaploid somatic level of the next generation. Red chromosomes form bivalents. White, grey and black chromosomes represent univalents.

In addition, numerous cases of hybridization were detected among extant dogrose species (e.g. Schanzer and Kutlunina 2010; Ritz and Wissemann 2011). Remarkably, spontaneous gene flow from the invasive neophyte *Rosa rugosa* into the native endangered species *Rosa mollis* was revealed in Germany (Kellner et al. 2012). Despite pentaploid parents, products of hybridization were often hexaploid (Ritz and Wissemann 2003, 2011). It has been hypothesized that hybrids were formed because of the production of unreduced gametes that facilitate meiosis by providing two highly homologous chromosome sets needed for proper bivalent formation in meiosis (Ritz and Wissemann 2011; Herklotz and Ritz 2017). One study of dogroses in Central and Southeastern Europe confirmed this hypothesis by revealing reciprocal spontaneous hybridization between sect. *Caninae* and *Rubigineae* (8% and 32% of hybridogenic individuals, respectively). Unreduced egg cells were detected in subsect. *Rubigineae*. The prevalence of *Rubigineae* hybrids has been explained by the facilitated production of unreduced female gametes, which simplify meiosis in *Rubigineae* plants, and also by the higher abundance of *Caninae* plants (i.e. its greater pollen production; Herklotz and Ritz 2017).

6.4.5 *Malus*

Another example of an intensively studied genus with a fully sequenced genome is the genus *Malus* (Velasco et al. 2010). Compared to the previous genera, apomixis also plays a marginal role, besides polyploidization and hybridization (Kron and Husband 2009). The genus *Malus* is familiar to everyone because of the domestic apple, *Malus* ×*domestica*, a valuable

temperate fruit tree. The origin of domesticated apple turned out to be really complex, involving a number of hybridization events. Four primary progenitor wild diploid species, *M. sylvestris*, *M. sieversii*, *M. orientalis* and *M. prunifolia*, were revealed based on chloroplast markers (Nikiforova et al. 2013; Volk et al. 2015; Fig. 9). Although these wild species possess undesirable fruit and growth habits, their resistance to biotic and abiotic stress makes them beneficial for plant breeders. Thus, knowledge on historical introgression in the domesticated apple and haplotype sharing among *Malus* species can play an important role in introgressive breeding programmes (Volk et al. 2015).

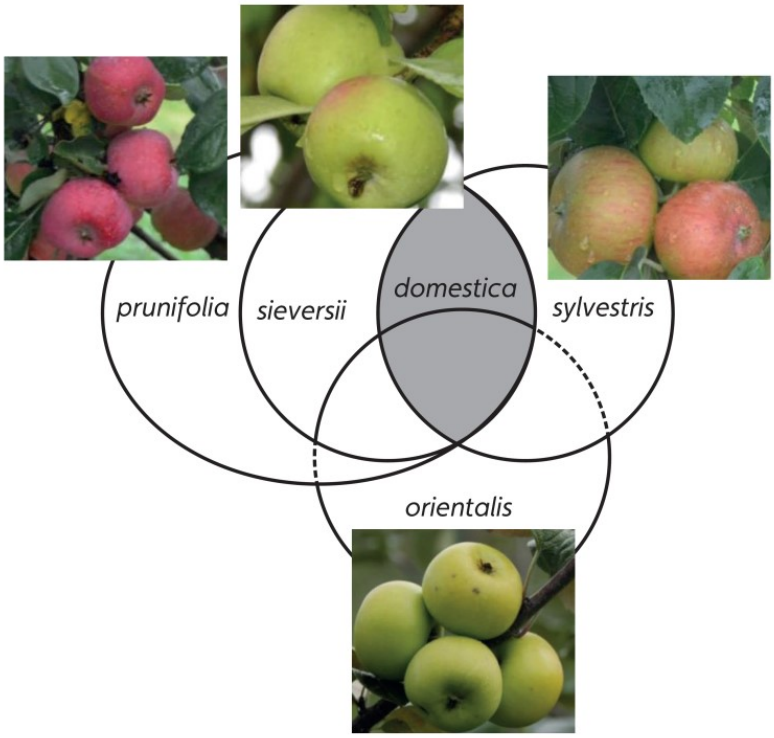

Fig. 9: Relationship between cultivated *Malus* ×*domestica* and its four primary wild relatives based on chloroplast genome sequences published in Volk et al. (2015) and modified by Bramel and Volk (2019).

Crossing between seven diploid *Malus* populations showed 1% incidence of non--diploid hybrids (triploids, tetraploids and aneuploids). Moreover, a unique pattern of gametes was observed based on microsatellites markers. Whereas unreduced eggs exclusively exhibited euploidy (producing triploid and tetraploid offspring), unreduced sperms preserved both euploidy and aneuploidy (producing triploid, tetraploid and aneuploid offspring; Considine et al. 2012). Therefore, the presence of unreduced gametes, albeit at a very low frequency, was able to induce putative autopolyploidization events in the genus *Malus* evolution (Velasco et al. 2010). Besides historical introgression events, hybridization among *Malus* species is still ongoing (e.g. Coart et al. 2006; Kron and Husband 2009; Cornille et al. 2013; Ruhsam et al. 2018). Hybrid seeds were detected in natural population of tetraploid *Malus coronaria* (crab apple) growing in sympatry with introduced diploid *M.* ×*domestica* in Canada, employing ploidy level analysis and isozyme markers (Kron and Husband 2009). It was found that although more than a quarter of all seeds were hybrid (mainly triploid and pentaploid), all growing trees were tetraploid *M. coronaria*. This suggests that hybrid adults are rare or absent in nature. In addition, analysis of reproductive modes revealed besides sexual seeds (3x, 4x, 5x, 6x, 8x) also apomictic seeds (2x, 4x; Kron and Husband 2009). This type of hybridization represents so-called crop-to-wild hybridization, which has been recently repeatedly reported from various areas.

In Europe, recent studies evaluated the risk of hybridization between rare wild *Malus sylvestris* (crab apple) and cultivated domesticated apple (*M.* ×*domestica*) and its implications for conservation strategies and breeding programmes, both at local and Europe--wide level, employing mainly microsatellite markers (e.g. Cornille et al. 2015; Feurtey et al. 2017; Ruhsam et al. 2018). A study examining crop-to-wild hybridization across Europe revealed that 36.7% of *M. sylvestris* samples were of hybrid origin and 37 individuals were misidentified pure *Malus* ×*domestica* (Cornille et al. 2013). Human activities, including apple production and creation of disturbances modifying the diversity of apple pollinators, have been found to be important factors influencing the rate of crop-to-wild interspecific introgression (Cornille et al. 2015). Introgression of domesticated apple into wild *Malus sylvestris* was detected also in Western Europe (mainly Belgian accessions). The study revealed that accessions sampled as *M. sylvestris* included both cultivars and hybrids (11%), based on sharing of rare haplotypes (Coart et al. 2006). Similar results were obtained in northern Britain. Hybridization was detected at a higher frequency: 27% of the samples were classified as hybrids and only 3% were classified as pure *Malus* ×*domestica*. Moreover, 80% of the hybrids backcrossed to *Malus sylvestris*. One-third of trees could not be accurately identified based on traditional morphological characters (leaf size, hairiness, fruit size). Nevertheless, the authors admitted possible overestimation of the hybridization rate caused by the presence of some hybrids in their dataset that probably represented cultivated and escaped trees and not a product of natural hybridization (Ruhsam et al. 2018).

Substantial current crop-to-wild gene flow was also revealed at local scale in populations of wild apples in a French forest (Dourdan forest). Hybrids and domesticated apples showed greater fitness than *Malus sylvestris*. In addition, poor genetic diversity was found in source seeds for the reintroduction of wild apple in agroforestry programmes attempting to support wild populations by promoting genetically genuine wild genotypes. Moreover, some seeds were even found to be introgressed or from misidentified different species. Nature protection should therefore focus on *M. sylvestris* populations with high genetic diversity, free of *M.* ×*domestica* introgressions and occurring far from cultivated apples (pollen dispersal over distances of up to 4 km; Feurtey et al. 2017). By contrast, another local study in a French forest (Rhine valley) found a high level of genetic diversity of pure *M. sylvestris*, only few hybrids and no escaped cultivars. In addition, hybrids and cultivars were found to be clearly disadvantaged in humid conditions of the floodplain forest in comparison to well-adapted *M. sylvestris*. Nevertheless, wild apple populations were faced with regeneration difficulties stemming not from hybridization but from hydrological changes and changes in forestry practices. Therefore, from a genetic point of view, *M. sylvestris* populations in the Rhine valley are a valuable source for wild apple conservation programmes (Schnitzler et al. 2014).

Besides *M. sylvestris*, other ancestral progenitors of cultivated apple, *Malus sieversii* and *M. orientalis* have been examined for putative crop-to-wild gene flow (Cornille et al. 2013; Omasheva et al. 2017). An investigation of native *M. orientalis* populations in the Caucasus and native *M. sieversii* populations in Central Asia revealed crop-to-wild gene flow from cultivated apples, but in lower frequencies (3.2% and 14.8% of hybrids, respectively) in comparison to *M. sylvestris* populations in Europe. However, a study of *M. sieversii* in Kazakhstan found very different frequencies of hybrids in particular populations. Although two populations showed almost no admixture, all remaining populations contained significant proportions of hybrids, ranging from 8 to even 95%. So, together with loss of natural habitats, hybridization with cultivated apples is the reason why rare *M. sieversii* is threatened by extinction in Kazakhstan (Omasheva et al. 2017).

6.4.6 *Crataegus*

In the case of the polyploid and hybridizing genus *Crataegus* (hawthorn), apomixis is vastly important and its incidence varies. The genus *Crataegus* consists of numerous hard to distinguish species (even DNA barcoding provides poor taxonomic resolution; Zarrei et al. 2015) studied mostly in North America (e.g. Lo et al. 2009; Christensen et al. 2014; Zarrei et al. 2014; Coughlan et al. 2017). This complexity is derived from easy crossing among species (incl. introgression; Lo et al. 2009) supplemented by apomictic reproduction and polyploidy (Dickinson 2018). The former tendency to describe newly formed hybrids as new species, even based solely on morphology, and the fact that some species names seem to be synonyms also contribute to the taxonomic problems (Christensen 1992; Dönmez 2004; Christensen and Zieliński 2008). Although various ploidy levels, including diploid, triploid, tetraploid, pentaploid and hexaploid, have been described in the genus, tetraploids are the most frequent, followed by diploids (Talent and Dickinson 2005; Lo et al. 2013). The reproduction differs depending on the ploidy level. Whereas diploids produce sexual seeds, polyploids are facultative apomicts (Lo et al. 2009, 2013). Aposporous gametophytic apomixis requires pollen contribution for proper endosperm formation (pseudogamy; Muniyamma and Phipps 1979; Talent and Dickinson 2007; Kolarčik et al. 2018). Seed analysis of various polyploid *Crataegus* species detected two types of seeds: In the first, which are predominant, a single sperm cell has contributed to the endosperm, and in the second, which are rare, two sperm cells contributed to the endosperm (i.e. polyspermy; Scott 2007; Talent and Dickinson 2007).

Natural hybridization (incl. introgression) was indicated at both the homoploid and the heteroploid level across the whole distribution range of *Crataegus* (e.g. Greece – Christensen 1992; Turkey – Dönmez 2004; Syria – Albarouki and Peterson 2007; North America – Lo et al. 2009; Christensen et al. 2014). Crosses between introduced diploid *Crataegus monogyna* and two diploid species, *Crataegus punctata* and *Crataegus suksdorfii*, native in North America resulted in the formation of two diploid hybrids. Strikingly, the hybrids were relatively easy to recognize because the leaf shape morphology of introduced Old World *C. monogyna* markedly differed that of New World plants and their hybrids were intermediate between them (Christensen et al. 2014). An allopolyploid origin of North American *Crataegus* species, involving repeated hybridization events and the participation of unreduced female gametes, was indicated also based on nuclear ribosomal sequencing (Zarrei et al. 2014). Moreover, a few species were classified as autotriploids, so besides allopolyploidy, autopolyploidy is also demonstrated in *Crataegus* (Lo et al. 2009; Zarrei et al. 2014).

Various studies have dealt with geographical parthenogenesis by comparing sexual diploids and apomictic polyploids, mainly in *Crataegus* series *Douglasianae* in North America (Lo et al. 2009, 2010, 2013, Coughlan et al. 2014, 2017). Relevant studies mostly include the following three species: *Crataegus suksdorfii* comprises sexual self-incompatible diploids and apomictic auto- and allopolyploids (3x, 4x), which are largely allopatric in distribution (Coughlan et al. 2014). *Crataegus douglasii* is self-compatible pseudogamous apomictic allotetraploid, but rarely also pentaploid. *Crataegus gaylussacia* is an apomictic autotriploid. Studies have repeatedly demonstrated geographical parthenogenesis. Polyploid apomicts (*C. douglasii* in particular) have a wider range and broader ecological amplitude compared to sexual diploids, which exhibit the smallest ranges alongside apomictic autotriploids (Lo et al. 2013; Coughlan et al. 2014, 2017). The greatest within-population variation was found in sexual diploids, in contrast to the lowest variation in triploid apomicts. Whereas frequent gene flow was indicated in *C. douglasii* populations, local populations of *C. suksdorfii* were markedly differentiated, leading to allopatric speciation (Lo et al. 2009). Independently arisen polyploid apomictic lineages of *C. suksdorfii* occupy more

environmentally varied habitats than diploids and it has been suggested that they have great potential to expand into new environmental niches (Lo et al. 2013). Moreover, whereas allopolyploids exhibited a more dispersal-oriented strategy and ability to colonize new habitats, sexual diploids and apomictic autotriploids showed a competition-oriented strategy (Coughlan et al. 2014, 2017). Thus, the strong dispersal and colonization ability of apomictic polyploids was manifested by geographically widespread and ecologically generalist (occurring in variable habitats) clones of hybrid origin in the Pacific Northwest (Coughlan et al. 2017). Nevertheless, it has been revealed that the reproduction of tetraploid, predominantly apomictic *Crataegus crus-galli* is accompanied by outcrossing and selfing. Thus, the reproductive assurance of *Crataegus* tetraploids is derived from a combination of pollen fertility, self-compatibility and pseudogamous apomixis (Lo et al. 2010).

6.4.7 *Potentilla*

The same microevolutionary processes involved in the diversification of *Crataegus*, outlined above have resulted in a similar pattern of variation in the genus *Potentilla*. Various ploidy levels and reproduction modes were reported from the *Potentilla* group (Dobeš et al. 2013a, 2015). Whereas diploids reproduced sexually, polyploids were presumed to be apomicts (e.g. *Potentilla argentea* consisted of sexual diploids and hexaploid apomicts; Paule et al. 2011). Moreover, also tetraploids showed sexual reproduction contrasting with apomictic higher polyploids (penta- to octoploids) in *Potentilla puberula* (Dobeš et al. 2013b). Recurrent hybridization events, multiple origin of hybrids and backcrossing gave rise to new hybrid forms, some of which got stabilized by apomixis and thus became established lineages (e.g. three different lineages of hybrid *Potentilla alpicola*; Paule et al. 2012). The resulting extensive morphological variation accompanied by the occurrence of different cytotypes has led to the recognition of species groups or aggregates (e.g. *Potentilla collina*, *P. argentea*), including several forms treated by some authors as species, subspecies, variants or, if apomixis is involved, as microspecies (Tomasz and Kołodziejek 2008). Nevertheless, widely conceived circumscriptions not treating, for example, different cytotypes and genetic lineages or populations inhabiting special types of bedrock as the separate taxa are also common (e.g. *P. argentea* s.l., Paule et al. 2011; *P. crantzii*; Paule et al. 2015).

6.4.8 *Rubus*

The polyploid and hybrid genus *Rubus* exhibits an important shift in reproductive strategies, as apomixis frequently prevails over sexuality in many lineages. The genus *Rubus* is well known for its taxonomic complexity and also a favourite commercial crop (raspberry and blackberry). The contributions of polyploidization, hybridization and apomixis to its evolution have made the genus taxonomically difficult (Majeský et al. 2017). This is also mirrored in extensive morphological variation and the usage of several infrageneric ranks such as subgenera, sections and series (Šarhanová et al. 2017). Thus, the genus *Rubus* is enormously rich in species (763 only in Europe; Kurtto et al. 2010), and many new species are still being described (e.g. Trávníček and Žíla 2011; Velebil et al. 2016). To avoid enormous amounts of descriptions and names, a pragmatic species concept, which considers the size of the distributional area, has gained general adoption (Weber 1996; Kurtto et al. 2010). In this concept, a taxon is considered a species only if it is morphologically stable and has a sufficiently wide distribution area (i.e. at least 50 km wide); and local hybrid morphotypes (biotypes) are ignored (Weber 1996; Kurtto et al. 2010).

The genus *Rubus* is traditionally classified into twelve subgenera, of which *Idaeobatus* (raspberries), *Malachobatus* and *Rubus* (blackberries) are the three largest (Alice and Campbell 1999). Nevertheless, the vast majority of European species belong to the single subgenus *Rubus* (bramble), sect. *Rubus* and sect. *Corylifolii* (Kurtto et al. 2010). Most European brambles are tetraploid, but their ploidy levels range from diploid to hexaploid (Kurtto et al. 2010; Krahulcová et al. 2013). Whereas only four species are sexual diploids in Europe, all remaining species are polyploid apomicts maintaining various degrees of residual sexuality (Kurtto et al. 2010; Šarhanová et al. 2012; Krahulcová et al. 2013).

Moreover, automixis (i.e. the fusion and subsequent parthenogenetic development of two egg nuclei in a reduced embryo sac; Antonius and Nybom 1995), a special type of reproduction combining both sexual and parthenogenetic processes, has been described in the genus *Rubus*. It differs from apomixis in that apomeiosis is absent. Particularly, the sexual process is manifested in the formation of a reduced embryo sac during normal meiosis. However, the reduced unfertilized egg cell then divides into two egg cells which subsequently fuse and thus restore the chromosome number. Then they develop parthenogenetically and form an embryo (Asker and Jerling 1992; Antonius and Nybom 1995). Molecular evidence of automixis was found by crossing experiments with raspberry and blackberry cultivars. Although automictic reproduction inherently brings detrimental homozygotization (e.g. exhibited in reduced vigour and fruiting ability), complete homozygotization has been suggested to be valuable in plant breeding (Antonius and Nybom 1995).

Comparison of the occurrence of apomicts and sexual diploids shows signs of geographical parthenogenesis. On the one hand, diploids prevail in southern warm regions (Mediterranean, Macaronesia) and are rare in temperate Central or Western Europe. On the other, polyploids mostly occur in Central and Western Europe and in the southern Caucasus; however, they are less successful and less spread in warmer regions (Kurtto et al. 2010; Sochor et al. 2017). Analyses of a huge amount of seeds of *Rubus* subgen. *Rubus* employing FCSS revealed high variation in reproductive modes linked to ploidy level (Šarhanová et al. 2012). Although diploids were exclusively sexual, triploids reproduced strictly apomictically. However, tetraploids exhibited the greatest reproductive variation based on pseudogamous facultative apomixis enabling sexual reproduction. However, not only ploidy level, but also external environmental factors, played an important role in the type of *Rubus* reproduction. *Rubus bifrons* was able to switch its reproductive mode in response to environmental conditions (higher temperature and lack of outcrossing increased sexuality). Tetraploid ser. *Glandulosi* exhibit geographical parthenogenesis caused by different incidence of apomicts compared to sexual diploids. Although strictly sexual reproduction was detected in the Western Carpathians (Moravia), partial apomixis was indicated in southwest of the Bohemian Massif. In addition, there are indications of both increases ploidy level, caused by fertilization of unreduced embryo sacs (i.e. B_{III} individuals), and decreases in ploidy level via the phenomenon of polyhaploidy. The study showed that the recent evolution of brambles is connected with preserved sexual reproduction in ser. *Glandulosi*, members of which are nearly fully sexual in some regions and easily hybridize, especially with *R. bifrons*, and enabling the formation of new hybridogenous populations. Species from ser. *Radula* were formed similarly as a result of past hybridization events and subsequent apomictic stabilization (Šarhanová et al. 2012). The origin of apomictic taxa was examined in detail based on microsatellite and chloroplast markers (Šarhanová et al. 2017). The data confirmed the hybrid origin of apomict microspecies of ser. *Radula* resulting from crosses between sexual members of ser. *Glandulosi* and apomictic ser. *Discolores* (pollen donor). Different parental taxa from these series gave rise to the distinct genotypes of individual apomictic microspecies, which were probably further

stabilized by clonal reproduction. Thus, the combination of sexual and apomictic reproduction of *Rubus* species enables both the generation of new, genetically distinct apomictic lineages and the production of clonal offspring, respectively (Šarhanová et al. 2017).

A reconstruction of the evolutionary history of European brambles using nuclear and chloroplast markers has revealed that all European polyploids were derived from six sexual diploids, two of which are already extinct. Extreme reticulate evolution was detected and putative parents of hybridogenous taxa were suggested (Sochor et al. 2015). The first allopolyploidization event in the evolution of the genus *Rubus* has been dated to before the last glaciation and post-glacial gene flow from diploids to polyploids has been detected employing both next generation and Sanger sequencing of nuclear and plastid regions and niche modelling (Sochor et al. 2017). The study indicated that the Iberian Peninsula and Morocco served as refugia during the Last Glacial Maximum. Population bottlenecks were detected in the Eastern Mediterranean and the Caucasus. Northwestern Europe was recolonized from a southern refugium in the post-glacial period (Sochor et al. 2017). Thus, the obvious evolutionary and ecologically success of brambles is manifested by their species richness, widespread distribution in a vast diversity of habitats and high invasive potential (Caplan and Yeakley 2010; Sochor et al. 2015).

6.4.9 *Sorbus*

Analogous processes leading to similar results take place in the species-rich genus *Sorbus* (rowan). The genus consists of five primary diploid sexual species (*S. aria*, *S. aucuparia*, *S. chamaemespilu*s, *S. torminalis*, *S. domestica*) and polyploid apomicts (Liljefors 1953). All primary diploids (excluding *S. domestica*) are able to hybridize with *S. aria* and further backcross with both parents (Kurtto et al. 2018). The origin of hybrids is polytopic and is followed by backcrossing with their parents and stabilization of their reproduction by apomixis (e.g. Nelson-Jones et al. 2002; Robertson et al. 2004; Lepší et al. 2015, 2019). New apomictic hybrids are described as new species (microspecies) based on unique morphology (minute but stable characters), distribution, karyology and genotypic variation (e.g. Lepší et al. 2008, 2009, 2015; Robertson et al. 2010; Vít et al. 2012).

6.4.10 *Alchemilla*

Finally, in the genus *Alchemilla* polyploidization, hybridization and apomixis have resulted in a complete prevalence of long-existing hybrid apomictic species (only a few sexual species in Europe; Gehrke et al. 2008; Majeský et al. 2017). Because the level of polyploidy is very high (diploids are absent), spanning from the lowest chromosome count of 2n = 96 to the highest count of 2n = 152 among European taxa, and because the chromosomes are very small, chromosome numbers are inaccurate and presented rather as ranges (Fröhner 1990; Kurtto et al. 2007; Gehrke et al. 2008). Male meiosis shows signs of disorders resulting in very low pollen fertility and even sterility (Fröhner 1990). Aposporous apomixis with independent (autonomous) endosperm formation (Fröhner 1990; Czapik 1996) is suggested to be almost obligatory, resulting in a lack of current hybridization (Majeský et al. 2017). The systematics of *Alchemilla* are highly difficult and still unresolved because of the microevolutionary processes mentioned above, clonal growth and intricate morphology (e.g. heteroblastic plasticity – differing morphologies of leaves, instability in flower characters; Notov and Kusnetzova 2004; Gehrke et al. 2008). Not surprisingly, a large number

of apomictic microspecies has been described as a separate species based on its putative obligatory apomictic reproduction, distinctive morphological traits, distribution area and ecological niches (Fröhner 1990; Majeský et al. 2017). Nevertheless, a continuum in morphological traits, and an ensuing inability to practically distinguish many described microspecies, has been indicated in Estonia (Sepp and Paal 1998). Nowadays, 433 species are recognized in Europe (Kurtto et al. 2007) and the circumscription of the genus *Alchemilla* in the wide sense (i.e. as the subtribe Alchemillinae, including *Aphanes* and *Lachemilla*; Notov and Kusnetzova 2004) has been confirmed based on morphology and phylogenetic relationships derived from chloroplast and nuclear markers (Gehrke et al. 2008).

6.5 Model species

This thesis deals with the influence of microevolutionary processes on the diversity and evolution of two selected Rosaceae model genera – *Prunus* and *Cotoneaster*. Based on the incidence of evolutionary drivers mentioned above, the two genera represent opposite extremes of a spectrum and thus enable a suitable comparison. The genus *Prunus*, particularly cherries, consists of both diploid and polyploid, strictly sexual species that readily hybridize with each other (Wójcicki 1991; Wójcicki and Marhold 1993; Iezzoni 2008). However, hybridization does not lead to the establishment of distinct separate lineages. Rather, repeated backcrosses result in advanced hybrids and hybrid swarms. Therefore, hybridization as an adverse force disrupting the integrity of species is presumed in this case (Wójcicki 1991; Wójcicki and Marhold 1993). However, hybridization has not yet been examined using a multidisciplinary approach applied across a wider geographic area. By contrast, the evolution of the genus *Cotoneaster* has involved polyploidy, hybridization and apomixis, which together represent a significant diversification force resulting in great diversity of lineages/taxa occurring almost all over the world, albeit of uncertain taxonomic value (Baranec 1992; Kutzelnigg 1994; Dickoré and Kasperek 2010; Kurtto et al. 2013a). Nevertheless, the variation in cytotype and reproductive traits has never been examined across a wider geographic area.

6.5.1 *Prunus fruticosa*, *Prunus cerasus* and *Prunus avium*

Three species of the genus *Prunus*, particularly cherries, were chosen to examine the pattern of presumed interspecific hybridization under natural conditions. On the one hand, *Prunus fruticosa* (ground cherry, Fig. 10) is a rare shrub adapted to the hard conditions of steppes occurring from Central Europe to Central Asia (Meusel et al. 1965a; Bilz et al. 2011). Although this cherry species is not cultivated as a commercial crop, it is being used in breeding programmes for its stress resistance, and its future potential for breeding is undisputable (e.g. Pruski 2007). On the other hand, the remaining two tree-like species, *Prunus avium* (sweet cherry, Fig. 11) and *Prunus cerasus* (sour cherry, Fig. 11), represent ubiquitous cultivated crops of high economic value. Whereas *P. avium* is a native taxon in Europe, *P. cerasus* is an allochthonous species of unclear origin (probably Southwest Asia; Sinskaya 1969; Scholz and Scholz 1995; Kurtto et al. 2013b). Both *P. fruticosa* and *P. cerasus* are tetraploids, in contrast to diploid *P. avium* (e.g. Scholz and Scholz 1995; Iezzoni 2008).

Fig. 10: *Prunus fruticosa* (ground cherry) on Stawska Góra (eastern Poland). Photo by Petr Vít.

The great morphological variation of *P. fruticosa* observed in Central Europe has led to presumptions about interspecific hybridization with cultivated cherries (i.e. crop-to-wild hybridization; Wójcicki 1991; Wójcicki and Marhold 1993). The incidence of tetraploid hybrids (*Prunus* ×*eminens*, Fig. 11) resulting from crossing between *P. fruticosa* and *P. cerasus* was putatively determined based on morphological investigations from Central Europe (Wójcicki 1991; Wójcicki and Marhold 1993; Lepší et al. 2011). By contrast, triploid hybrids (*Prunus* ×*mohacsyana*, Fig. 11) between *P. fruticosa* and *P. avium* were reported only rarely (Wójcicki 1988; Wójcicki and Marhold 1993). Unintentional human influence manifested in ubiquitous cherry cultivation enabling interspecific hybridization and the destruction of suitable habitats have been supposed to be the main factors affecting the decrease of *P. fruticosa* populations in Central Europe (Wójcicki 1988, 1991; Wójcicki and Marhold 1993; Boratyński et al. 2003; Lepší et al. 2011). For detailed information about the cherry species under study, see the Introduction and Methods sections

of the corresponding articles on pages 57–59, 94–96. The present thesis analyses the extent of interspecific hybridization between *P. fruticosa* and cultivated cherries under natural conditions, based on genome size and ploidy level analysis accompanied by multivariate morphometrics supplemented by embryological analysis, to evaluate the conservation implications of crop-to-wild gene flow for wild populations of the rare species *Prunus fruticosa* in Central Europe.

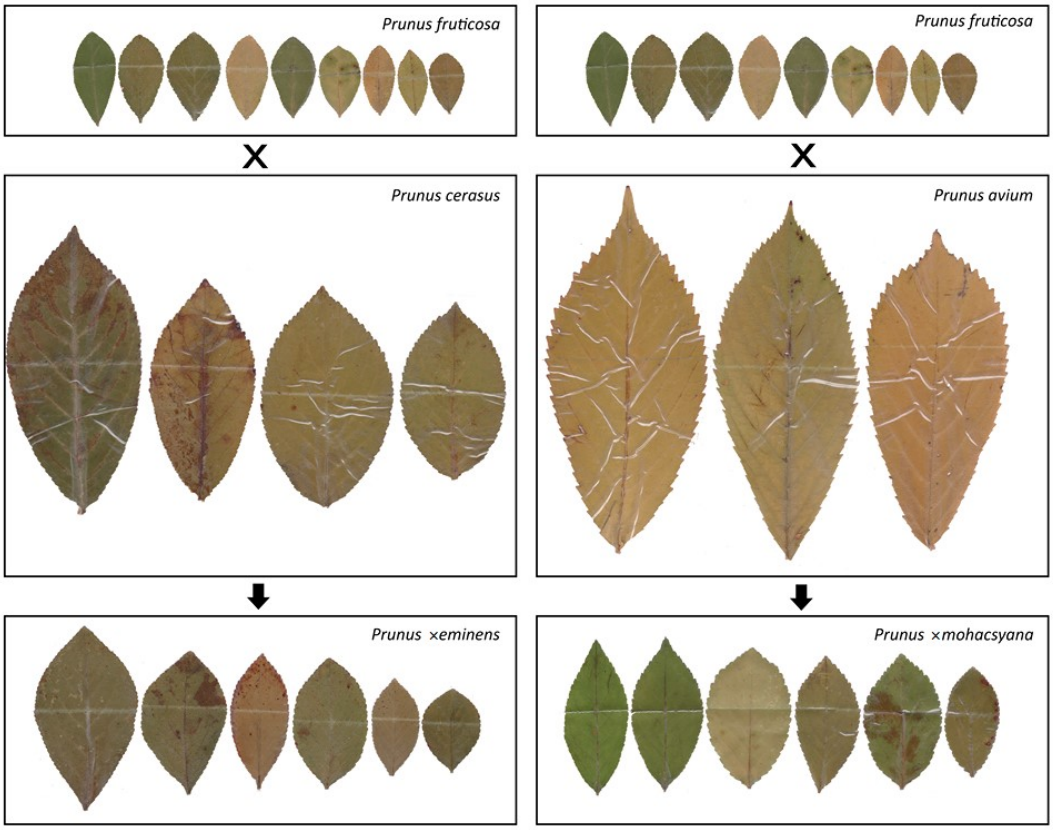

Fig. 11: Variation in the shape of the leaf lamina of the *Prunus* species under study. Depicted are individuals of *P. fruticosa* sampled at Hnanice, Český Krumlov (CZ) and Slanec (SK), *P. cerasus* at the Central Institute for Supervising and Testing in Agriculture at Lysice (CZ), Salka (SK), *P. avium* at Hnanice (CZ), Salka (SK), *P. ×eminens* at Ptáčov, Chvalov, Ústí nad Labem (CZ), *P. ×mohacsyana* at Ptáčov, Český Krumlov (CZ).

6.5.2 *Cotoneaster integerrimus* s.l. and *Cotoneaster tomentosus*

The diversity of European *Cotoneaster* taxa, spineless deciduous shrubs inhabiting dry rocky habitats, is especially linked to mountain ranges – the Alps and the Carpathians (Browicz 1968; Baranec 1992; Kutzelnigg 1994; Fryer and Hylmö 2009; Kurtto et al. 2013a). Two 'basic' closely related species are recognized in Europe: *Cotoneaster integerrimus* s.l. (Fig. 12 and 13) and *Cotoneaster tomentosus* (Fig. 14). Although *Cotoneaster integerrimus* s.l. is a morphologically variable species considered a group of taxa (microspecies) of unclear taxonomical value (Dickoré and Kasperek 2010; Kurtto et al. 2013a), *C. tomentosus* (syn. *C. nebrodensis*) is a morphologically well defined, distinct taxon (Kutzelnigg 1994).

Various distinct species concepts are used to describe the complexity of *C. integerrimus* s.l. in Europe. There are several microspecies concepts (Hrabětová-Uhrová 1961, 1962; Baranec 1992; Fryer and Hylmö 2009) and a few broad concepts (e.g. Dickoré and Kasperek 2010; Sennikov 2010) also used in comprehensive floras (Kurtto et al. 2013a). Using a narrow species complex, various taxa were treated as separate microspecies

from different parts of Europe: *Cotoneaster scandinavicus* and *Cotoneaster kullensis* from Northern Europe, *Cotoneaster pyrenaicus*, *Cotoneaster juranus* and *Cotoneaster raboutensis* from Southwest Europe (the Alps and the Pyrenees), *Cotoneaster laxiflorus*, *Cotoneaster alaunicus* and *Cotoneaster matrensis* from Central Europe (the Western Carpathians). Nevertheless, the circumscriptions of most of the species listed is uncertain, contradict each other and sometimes they are considered synonyms or hybrids (Browicz 1968; Baranec 1992; Kovanda 1992; Kutzelnigg 1994; Dickoré and Kasperek 2010; Kurtto et al. 2013a). Only *C. laxiflorus* (syn. *C. melanocarpus*) seems to be widely accepted, but its native occurrence in Europe has been disputed because its core distribution range spans from Russia (Siberia) to Mongolia and the north of China (Dickoré and Kasperek 2010). For the reasons above, we agree with the broad species concept recently proposed by Dickoré and Kasperek (2010), treating the majority of microspecies within *C. integerrimus* s.l., with the sole exception of *C. tomentosus* in Central Europe. In the present thesis, the narrow concept of *C. integerrimus* s.l. is used for practical reasons to test relevance of microspecies.

Fig. 12: Variation in the colour of pomes of tetraploid *Cotoneaster integerrimus* s.l. The depicted individuals occurred by the town of Moravský Krumlov, Havraníky (vineyards at Šobes, Czechia) and Piatra Neamt (Romania), respectively. Photo by Michael Macek and Filip Kolář.

Fig. 13: Red and blue pomes present on one individual of tetraploid *Cotoneaster integerrimus* s.l. by the town of Moravský Krumlov (saint Florián, Czechia) in October 2017.

Fig. 14: *Cotoneaster tomentosus* with typically elliptical leaves and hairy (tomentose) pomes, sampled in Tomášovský výhľad (Čingov, Slovakia). Photo by Tomáš Urfus.

The distribution of *C. tomentosus* is restricted to mountainous regions of Central, Southeast and Southwest Europe, including the Alps, the Pyrenees, the Apennines and the Carpathians. The range of *C. integerrimus* s.l. in wide sense is much broader, extending from most of Europe to Central and East Asia (Meusel et al. 1965b). Various ploidy levels have been reported from European *Cotoneaster* taxa (2x, 3x, 4x, 5x, 6x), but tetraploids seem to be most common (e.g. Browicz 1968; Baranec 1992; Kovanda 1992; Kutzelnigg 1994; for details see Introduction section of the corresponding article on page 122–123 below). A putative hybrid origin of some European species has been suggested (Browicz 1968; Baranec 1992; Kutzelnigg 1994). Whereas sexual reproduction has been reported for diploids, polyploids, which prevail, have been found to be apomictic (Sax 1954; Hjelmquist 1962). However, reproduction data are restricted only to few pieces of evidence, often indirect (Sax 1954; Hjelmquist 1962; Kroon 1975; Bartish et al. 2001). Moreover, cytotype diversity has never been observed across a wider part of Europe and karyological data are available only from a limited number of individuals (see Online Resource 1 of the corresponding article on page 139–141). For detailed information on the *Cotoneaster* species under study, see the Introduction and Methods sections of the corresponding article on page 121–125. The present thesis examines the cytotypic and reproductive pattern of *Cotoneaster* taxa occurring in the Western Carpathians, discussing the diversity of the genus *Cotoneaster* in Central Europe and its compatibility with recent taxonomic treatments.

6.6 Aims of the thesis

This thesis examines the significance of polyploidization, hybridization and reproductive strategies in the speciation of two model Rosaceae genera, *Prunus* and *Cotoneaster*, using the methodical approaches of flow cytometry (genome size and ploidy level analyses, flow cytometric seed screen), multivariate morphometrics and embryology. Analysis of the data obtained is useful in drawing conclusions and devising hypotheses regarding microevolutionary processes possibly leading to consequences relevant for the biosystematics, conservation and economic utilization of species within the Rosaceae family. The research presented here was commenced with the following questions in mind, answers to which are given in the articles forming the remainder of this thesis:

(1) What is the evolutionary potential of polyploidization in the Rosaceae?

(2) What are the consequences of hybridization in the evolution and diversity of the family?

(3) To what extent do distinct reproductive strategies contribute to the evolution of the Rosaceae and how do they affect diversity?

(4) What are the applicable conservation and economical consequences of such evolutionary forces?

6.7 References

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6.8 Supplements

Supp. 1: Circumscription and taxonomic division of the Rosaceae based on current nuclear and plastid phylogenomics published in Xiang et al. 2016 and Zhang et al. 2017.

Subfamily	**Tribe**	*Example of included genera*
Rosoideae	**Agrimonieae**	*Acaena, Sanguisorba*
	Potentilleae	*Alchemilla, Fragaria, Potentilla*
	Roseae	*Rosa*
	Colurieae	*Geum, Waldsteinia*
	Rubeae	*Rubus*
	Ulmarieae	*Filipendula*
Dryadoideae	**Dryadeae**	*Cercocarpus, Chamaebatia, Purshia, Dryas*
Amygdaloideae	**Maleae**	*Malus, Pyrus, Sorbus, Crataegus, Mespilus, Cotoneaster, Vauquelinia, Kageneckia*
	Gillenieae	*Gillenia*
	Kerrieae	*Kerria, Rhodotypos*
	Exochordeae	*Exochorda, Oemleria, Prinsepia*
	Sorbarieae	*Sorbaria, Adenostoma*
	Amygdaleae	*Prunus, Pygeum, Maddenia*
	Lyonothamneae	*Lyonothamnus*
	Spiraeeae	*Spiraea, Aruncus*
	Neillieae	*Physocarpus, Neillia*

Supp. 2: First modern classification of the Rosaceae based on nucleotide sequence data from nuclear and chloroplast regions published in Potter et al. 2007a. Names of the taxa of different ranks and their circumscription have often been used to this day.

Subfamily	Supertribe	**Tribe**	Subtribe	*Example of included genera*
Rosoideae	Rosodae	**Sanguisorbae**	Sanguisorbinae	*Acaena, Sanguisorba*
			Agrimoniinae	*Agrimonia, Spenceria*
		Potentilleae	Fragariinae	*Alchemilla, Fragaria*
			Potentilla	
		Rosa		
		Colurieae		*Geum, Sieversia*
		Rubus		
	Filipendula			
Dryadoideae				*Cercocarpus, Chamaebatia, Cowania, Purshia, Dryas*
Spiraeoideae (recent Amygdaloideae)	*Lynothamnus*			
		Sorbarieae		*Adenostoma, Sorbaria*
		Spiraeeae		*Aruncus, Spiraea*
		Amygdaleae		*Prunus, Pygeum, Maddenia*
		Neillieae		*Neillia, Physocarpus*
	Pyrodae	**Pyreae (recent Maleae)**	Pyrinae (recent Malinae)	*Amelanchier, Crataegus, Mespilus, Malus, Cotoneaster, Pyrus, Sorbus*
			Vauquelinia	
			Kageneckia	
			Lindleya	
		Gilenia		
	Kerriodae	**Kerrieae**		*Kerria, Rhodotypos*
		Osmaronieae		*Exochorda, Oemleria, Prinsepia*

7 Case studies

7.1 Case study I

Macková, L., Vít, P., Ďurišová, Ľ., Eliáš, P., & Urfus, T. (2017): **Hybridization success is largely limited to homoploid *Prunus* hybrids: a multidisciplinary approach.** – Plant Systematics and Evolution 303: 481–495. doi: https://doi.org/10.1007/s00606-016-1385-4

Hybridization success is largely limited to homoploid *Prunus* hybrids: a multidisciplinary approach

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Abstract

Prunus fruticosa is a rare shrub occurring in Eurasian thermophilous forest-steppe alliances. The species frequently hybridizes with cultivated *Prunus* species in Europe (allochthonous tetraploid *P. cerasus* and partly indigenous diploid *P. avium*). Propidium iodide flow cytometry, distance-based morphometrics, elliptic Fourier analysis and embryology were employed to evaluate the extent of hybridization in six Slovak populations. Flow cytometric analyses revealed three ploidy levels: diploid (*P. avium*), triploid (*P.* ×*mohacsyana*) and tetraploid (*P. fruticosa*, *P.* ×*eminens* and *P. cerasus*). In addition, *P. fruticosa* and *P. cerasus*, at the tetraploid level, were found to differ in absolute genome size. An embryological evaluation suggested the existence of a triploid block in *P.* ×*mohacsyana* and significant potential for hybridization among tetraploid taxa (indicated also by a continuous distribution of genome size data and further mirrored by morphometrics). Although hybrids significantly differ in ploidy level and embryological characteristics, they are almost indistinguishable using morphological characters. Hybridization with *P. cerasus* thus turns out to be a significant threat to wild populations of *P. fruticosa* compared to the relatively weak influence of *P. avium*.

Keywords: absolute genome size, embryology, interspecific hybridization, morphometrics, polyploidy, *Prunus fruticosa*

Introduction

Interspecific hybridization is considered to be a generally frequent phenomenon in angiosperms (e.g. Stace et al. 2015), and its significant role as a major mechanism generating evolutionary novelties (and consequently plant diversity) is widely accepted (Hegarty and Hiscock 2005; Wissemann 2007; Abbott et al. 2013). On the other hand, hybridization can also be markedly disadvantageous. The sole presence of hybrids in a population may decrease its fitness due to competition for abiotic and biotic resources (such as space, nutrition, radiation or pollinators; Buerkle et al. 2000; Bleeker et al. 2007).

A more complex source of danger is repeated backcrossing (introgression), which may cause genetic erosion of particular taxa (Rhymer and Simberloff 1996) and thus blurs differences between species (finally leading to genetic assimilation or extinction of species).

Low-abundant populations (frequent in rare species) may suffer from hybridization with widespread congeners (Levin et al. 1996). Moreover, their hybridization may be significantly influenced by humans. Human-triggered changes in species distribution allowed contact and hybridization between previously allopatric species (e.g. *Pericallis* D. Don species, van Hengstum et al. 2012; *Picris hieracioides* L., Slovák et al. 2012, 2014; *Knautia arvensis* Coult., Rešetnik et al. 2014; *Spartina alterniflora* Loisel., Ainouche and Gray 2016; *Helianthus annuus* L., Owens et al. 2016). Commercially grown species (e.g. cereals, oil crops, fruit trees, often non-indigenous; Hyams 1971) represent common and abundant congeners that may affect pure populations (e.g. *Aegilops peregrina* (Hack.) Maire and Weiller and wheat, Weissmann et al. 2005; *Oryza rufipogon* Griff. and cultivated rice in Asia, Song et al. 2006; *Coffea arabica* L. and coffee cultivars in Ethiopia, Aerts et al. 2013; *Medicago falcata* L. and cultivated alfalfa in Estonia, Kaljund and Leht 2013).

Although numerous cultivated plants originated in Europe (e.g. *Apium graveolens* L., or varieties of *Brassica*; Hyams 1971), there are only a few reported cases of hybridization between cultivars and pure, indigenous populations, for which Wójcicki (1991a) proposed the term anthropohybridization. One scarcely studied example is that of hybridization between cultivated *Prunus cerasus* L. (non-indigenous) and *Prunus avium* (L.) L. (indigenous) with the rare species *Prunus fruticosa* Pall. (Wójcicki 1988, 1991a; Wójcicki and Marhold 1993).

Prunus fruticosa (ground cherry) is a rare member of the native central European fruit tree flora and is included in a number of European red lists (e.g. Bilz et al. 2011). It is a Eurasian steppe or forest-steppe shrub species (Meusel et al. 1965; Jäger and Seidel 1995) of high ornamental, vegetation and horticultural importance. It occurs in relic thermophilous shrub alliances and is a diagnostic species of continental deciduous thickets (Prunion fruticosae), a priority habitat of the Natura 2000 network (Chytrý et al. 2010). Due to its drought and frost resistance, low height, fruit taste and ability to hybridize with other *Prunus* species, *P. fruticosa* is a suitable taxon for breeding new cherry cultivars (Iezzoni and Mulinix 1992; Dzhangaliev et al. 2003; Pruski 2007; Iezzoni 2008). Although *P. fruticosa* is not cultivated in Europe, it has considerable agricultural importance in Russia and Western Canada (Pruski 2007; Iezzoni 2008) and is promoted as a new crop suitable for the inhospitable climatic conditions of steppe areas (Pruski 2007). *Prunus fruticosa* is considered an entomogamous self-incompatible autotetraploid (2n = 32; e.g. Scholz and Scholz 1995; Tavaud et al. 2004; Pruski 2007; Iezzoni 2008). Clonal reproduction probably plays an important role in local spreading (via root sprout shoots; Wójcicki 1991b; Scholz and Scholz 1995). *Prunus fruticosa* represents low completely glabrous shrub with small (1.5–2.5 cm) obovate leaves at short shoots, flowering white flowers in umbels and bearing dark red globose drupes (Webb 1968).

Tetraploid *Prunus cerasus* (sour cherry; 2n = 32; Scholz and Scholz 1995; Iezzoni 2008; Das et al. 2011) and diploid *P. avium* (sweet cherry; 2n = 16; Scholz and Scholz 1995; Iezzoni 2008) are closely related *Prunus* species that are common in nature as well as in cultivation. *Prunus cerasus* probably originated in Southwest Asia (Sinskaya 1969; Kurtto et al. 2013) and is widely cultivated in Europe, whereas *P. avium* is autochthonous (e.g. Scholz and Scholz 1995). Both species are allogamous and selfincompatible (in case of tetraploid *P. cerasus* self-compatibility was observed too; Hauck et al. 2002; Marchese et al. 2010) with ability to clonal reproduction (Dzhangaliev et al. 2003). *Prunus cerasus* and *P. avium* represent trees with well-defined trunk (Wójcicki 1988), bigger and beneath pubescent leaves in contrast to *P. fruticosa*, flowering the similar but bigger flowers as *P. fruticosa* and bearing the same but only bigger red globose drupes various in taste (sweet or bitter; Webb 1968). *Prunus cerasus* is considered allopolyploid resulted from the fusion of a reduced female gamete of *P. fruticosa* and an unreduced male gamete of *P. avium* based

on cpDNA and microsatellites (Horvath et al. 2008), GISH, C-banding (Schuster and Schreibner 2000) and AFLP (Tavaud et al. 2004). Interspecific hybridization is one of the main factors thought to be responsible for the drop in abundance of tetraploid *P. fruticosa* (Wójcicki 1991a). It hybridizes with tetraploid *P. cerasus*, resulting in the tetraploid *Prunus ×eminens* Beck (2n = 32; Webb 1968; Marhold and Wójcicki 1992; Scholz and Scholz 1995). Intermediate morphotypes have been reported at some localities, so hybridization is a suspected cause of local extinction of true *P. fruticosa* (Wójcicki and Marhold 1993; Boratyński et al. 2003; Lepší et al. 2011). A further threat to the genetic integrity of *P. fruticosa* may stem from hybridization with diploid *P. avium*. Their triploid hybrid *Prunus ×mohacsyana* Kárpáti (2n = 24; Oldén and Nybom 1968) has rarely been reported from Slovakia (Wójcicki and Marhold 1993) and Hungary (Wójcicki 1988). Moreover, other extremely rare hybrids have been described (*Prunus ×javorkae* Kárpáti; *P. fruticosa* × *Prunus mahaleb* L.), including the triple hybrid *Prunus ×stacei* Wójcicki (*P. fruticosa* × *P. cerasus* × *P. avium*; Wójcicki 1991b; Hrotkó and Facsar 1996). Some of the other significant threats to *P. fruticosa* in Central Europe apart from hybridization are loss of suitable habitats, landscape fragmentation, natural succession and changes in human landscape use (Ivanišová 2009; Chytrý et al. 2010; Lepší et al. 2011).

Hybridization of *Prunus fruticosa* has scarcely been studied. Existing studies are based mainly on morphological examination of herbarium vouchers (Wójcicki 1988, 1991a; Wójcicki and Marhold 1993; Lepší et al. 2011). Our multidisciplinary approach (distance--based morphometrics, elliptic Fourier analysis, genome size estimation, embryological techniques) allowed us to explicitly discriminate between *Prunus ×eminens* and *Prunus ×mohacsyana*, evaluate the reproductive potential of all the taxa involved and outline conservation concerns.

The specific aims of our study were to: (1) assess the patterns of interspecific hybridization involving *Prunus fruticosa* under natural conditions, (2) reveal morphological characters suitable for the delimitation of species and hybrids, (3) evaluate the conservation consequences of interspecific hybridization involving *P. fruticosa* and (4) compare the reproductive potential of *P. fruticosa* and its hybrids with other *Prunus* species.

Materials and methods

Sampling

Samples of six natural populations of *Prunus fruticosa*, *P. ×eminens* and *P. ×mohacsyana* were collected between 2012 and 2014 in two regions of south-western Slovakia (see Fig. 1, Table 1). Four of them are located near the city of Nitra, and two are near the town of Štúrovo. Our criteria for representative selection of populations were twofold: (1) the occurrence of *P. fruticosa* and its hybrids and (2) the purity of *P. fruticosa* populations. From each population, 2–15 individuals were sampled. Each population sample contained plants which were as distant from each other as possible to cover potential cytotype variability. Each sample from a single individual was represented by a branchlet with vegetative short-shoot leaves. To thoroughly describe the patterns of hybridization and other microevolutionary phenomena, additional samples of *P. avium* and *P. cerasus* were also included in the study. Both comprise 10 accessions sampled in Central Europe (approximately 50% of them directly from the study populations; see Table 1 for locality details) and 10 cultivars provided by the Central Institute for Supervising and Testing in Agriculture, covering the spectrum of the most frequently cultivated cultivars. In the cases of *P. cerasus* and *P. avium*, particular individuals instead of natural population were collected, because

these taxa are scattered in the landscape (allochthonous *P. cerasus*, autochthonous but frequently cultivated *P. avium*). Moreover, the identification of natural populations is highly complicated in fruit trees (e.g. in *Malus*; Gross et al. 2012). The taxa were determined based on the ploidy level (indicating triploid *Prunus* ×*mohacsyana* and diploid *P. avium*), then in the case of tetraploids based on the presence of hairs on the abaxial surface of the lamina (glabrous *P. fruticosa* vs. hairy *P.* ×*eminens* and *P. cerasus*) and growth form (shrubby *P. fruticosa* and *P.* ×*eminens* vs. tree-like *P. cerasus*).

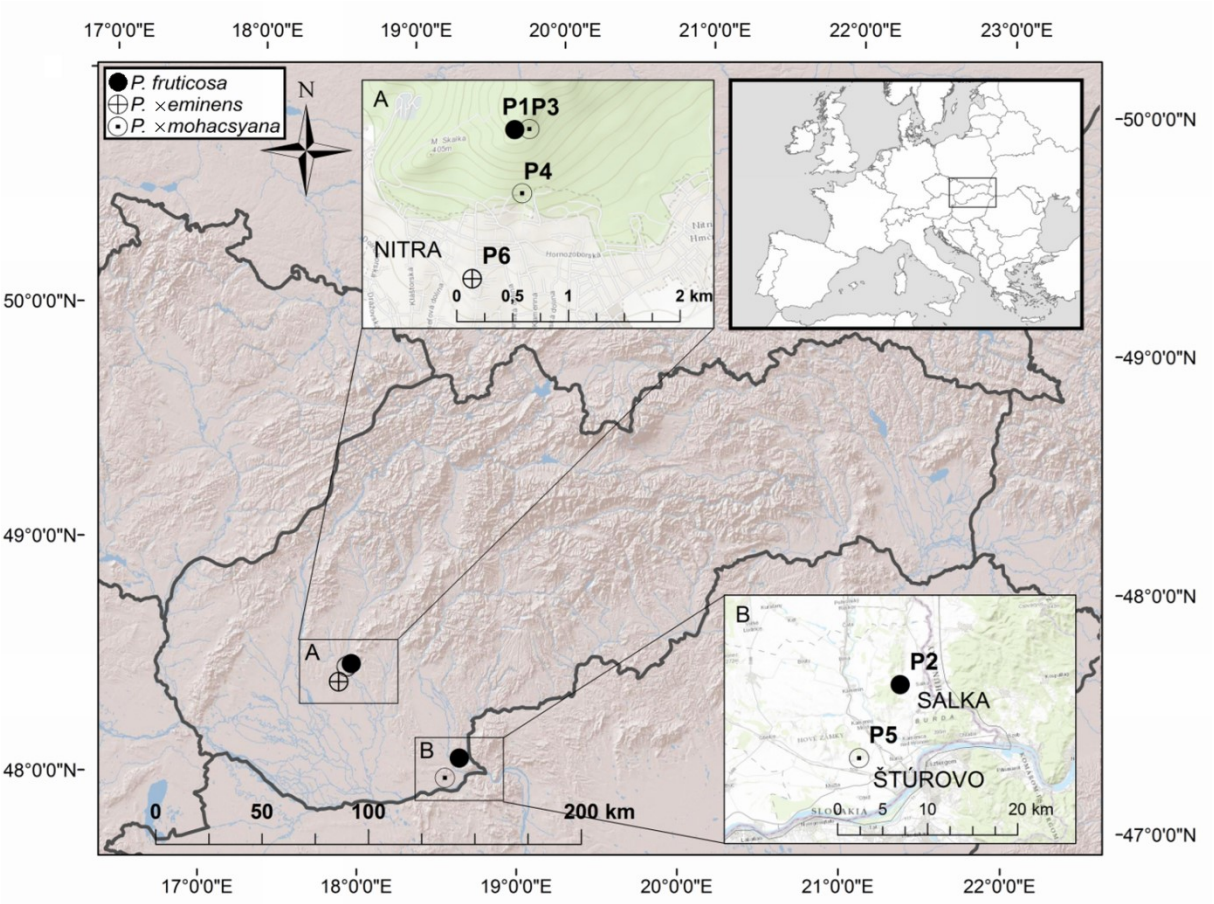

Fig. 1: Sample localities of *Prunus* taxa in south-western Slovakia. P1 = *P. fruticosa*, Nitra Pyramída hill; P2 = *P. fruticosa*, Salka the Sovie Vinohrady; P3 = *P.* ×*mohacsyana*, Nitra Pyramída hill; P4 = *P.* ×*mohacsyana*, Nitra forest edge; P5 = *P.* ×*mohacsyana*, Štúrovo the Vŕšok II hill; P6 = *P.* ×*eminens*, Nitra St. Urban church.

Samples collected from 111 individuals (30 *Prunus fruticosa*, 13 *P.* ×*eminens*, 28 *P.* ×*mohacsyana*, 20 *P. avium* and 20 *P. cerasus*) were analysed using three types of analyses – absolute genome size analysis using flow cytometry (FCM), distance-based morphometrics and elliptic Fourier analysis (see Table 1 for numbers of samples for each analysis). For flow cytometry, fresh plant material was used. For morphometrics, dry plant material was used (short-shoot leaves taped onto sheets of cardboard). Moreover, a subset of 34 individuals (20 *P. fruticosa*, 3 *P.* ×*eminens* and 11 *P.* ×*mohacsyana*) was sampled for the investigation of development stage using embryological techniques.

Table 1: Study sites and numbers of samples used in particular analyses.

Taxon	Number of indiv.	Locality	GPS	FCM	Distance-based morphometrics	Elliptic Fourier analyses	Embryology	Fruit set analyses
P. fruticosa	15	Nitra Pyramída hill (SK)	48°20′32.8′′N, 18°06′15.6′′E	15	30	30	10	3
P. fruticosa	15	Salka the Sovie Vinohrady (SK)	47°53′14.3′′N, 18°43′05.1′′E	15	30	29	10	x
P. ×eminens	13	Nitra St. Urban church (SK)	48°19′50.7′′N, 18°05′49.6′′E	13	26	26	3	1
P. ×mohacsyana	11	Nitra Pyramída hill (SK)	48°20′32.4′′N, 18°06′18.3′′E	11	22	22	9	3
P. ×mohacsyana	2	Nitra forest edge (SK)	48°20′13.9′′N, 18°06′11.9′′E	2	4	4	1	1
P. ×mohacsyana	15	Štúrovo the Vŕšok II hill (SK)	47°49′06.3′′N, 18°38′37.0′′E	15	30	30	1	x
P. cerasus	2	Sedlec (CZ)	48°47′35.9′′N, 16°41′37.3′′E	2	4	4	x	x
P. cerasus	2	Milá (CZ)	50°26′1.50′′N, 13°45′26.58′′E	2	4	2	x	x
P. cerasus	4	Kamýk (CZ)	50°33′49.3′′N, 14°07′08.2′′E	4	8	0	x	x
P. cerasus	2	Salka (SK)	47°53′13.7′′N, 18°43′08.8′′E	2	4	4	x	x
P. cerasus	10	Central Institute for Supervising and Testing in Agriculture – Želešice (CZ)	49°07′09.6′′N, 16°35′40.4′′E	10	20	20	x	x
P. avium	1	Hnanice (CZ)	48°48′06.2′′N, 15°58′59.0′′E	1	2	2	x	x
P. avium	4	Salka (SK)	47°53′16.7′′N, 18°43′08.9′′E	4	8	6	x	x

Taxon	Number of indiv.	Locality	GPS	FCM	Distance-based morpho-metrics	Elliptic Fourier analyses	Embry-ology	Fruit set analy-ses
P. avium	1	Štúrovo (SK)	47°49′03.6′′N, 18°38′34.3′′E	1	2	2	x	x
P. avium	4	Zobor (SK)	48°19′50.8′′N, 18°05′49.6′′E	4	8	8	x	x
P. avium	10	Central Institute for Supervising and Testing in Agriculture – Lysice (CZ)	49°27′24.0′′N, 16°33′23.0′′E	10	20	20	x	x
Total	111	x	x	111	222	209	34	8

Flow cytometry

Absolute genome size was estimated using propidium iodide flow cytometry of 111 individuals (see Table 1). *Bellis perennis* L. (2C = 3.38 pg; Schönswetter et al. 2007) was used as the internal standard. About 1.5 cm^2 of fresh laminar tissue together with 1.8 cm^2 of the internal standard was chopped in 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; Otto 1990) in a Petri dish. The suspension was filtered through a 42-µm nylon mesh and incubated for at least 20 min at room temperature. The suspension was then stained by a solution containing 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$; Otto 1990), β-mercaptoethanol (final concentration of 2 µl/ml), propidium iodide and RNase IIA (both at final concentrations of 50 µg/ml). Finally, each sample was run through a Partec CyFlow flow cytometer (Partec GmbH, Münster, Germany) equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW). Fluorescence intensity of at least 3000 particles was recorded. Most of the samples were analysed twice, and average absolute genome size was calculated from these values. Variation between two different measurements did not exceed 3%. Flow cytometry analyses were calibrated by chromosome counts (at least two counted individuals per ploidy level; standard karyologic methodology with lacto-propionic orcein staining described in Lepší et al. 2008). The resulting histograms were analysed using FloMax version 2.4d. Absolute genome size values were visualized as boxplots in PAST 2.17 (Hammer et al. 2001). One-way ANOVA followed by Tukey's HSD test was used to ascertain the significance of genome size differences between species. Values of genome size were log-transformed before the ANOVA.

The genus *Prunus* belongs to the Rosaceae family, whose members are known to contain significant amounts of secondary metabolites, which can negatively affect analyses (Loureiro et al. 2006). To minimize their negative effect, we optimized the standard procedure by less chopping larger parts of the lamina (1.5 cm^2 of tissue) and extending the incubation time to at least 20 min.

Distance-based morphometrics

Vegetative characters on short-shoot leaves were selected based on the literature (Wójcicki 1988, 1991a; Wójcicki and Marhold 1993; Lepší et al. 2011) and our own field observations. Altogether 8 characters on 222 leaves (two leaves per individual; see Table 1) were measured using a digital calliper (accuracy 0.01 mm; Proteco) and a stereo microscope (Olympus SZ51, Olympus, Tokyo, Japan; magnification 40×). Plant height was measured in the field. Abaxial hairs were measured on at least four leaves per individual and then averaged. Plant height, shape of the laminar tip, adaxial hairs and abaxial hairs were evaluated using semiquantitative scales (see Table 2).

Table 2: Measured characters of *Prunus fruticosa*, *P. ×eminens*, *P. ×mohacsyana*, *P. cerasus* and *P. avium*.

Character	Unit
Plant height	1 = to 50 cm, 2 = 50-100 cm, 3 = over 100 cm, 4 = tree
Laminar length	mm
Laminar width	mm
The widest part of lamina to tip	mm
Laminar length/width (ratio of length and width of lamina)	–
Shape of lamina tip	1 = obtuse, 2 = obovate, 3 = eliptic with aristate apex, 4 = eliptic with broadly acuminate apex
Adaxial hairs (level of hairs on the adaxial surface of lamina)	1 = glabrous, 2 = short hairs, 3 = long hairs
Abaxial hairs (level of hairs on the abaxial surface of lamina)	1 = glabrous, 2 = scattered pubescent, 3 = sparsely pubescent, 4 = densely pubescent

The data matrix was evaluated using multivariate statistical methods in R (version 3.1.2; R Core Team 2013) following procedures (*descr.tax, cormat.s, pca.calc*) described in detail by Koutecký (2015). Basic descriptive statistics including the minimum, maximum, average and the 25 and 75% percentile were calculated for each taxon studied. Correlations of morphological characters were tested using Spearman's correlation coefficient in R. The structure of the data was determined using principal component analysis (PCA). Discriminant analysis was not included, because two essential morphological characters were used to separate tetraploid groups (plant height, abaxial hairs).

Elliptic Fourier analysis

Short-shoot leaves without petioles were taped onto sheets of cardboard paper. A total of 209 leaves were analysed − two leaves from each individual, whenever possible (see Table 1). Only well-developed leaves with an undivided shape were suitable for elliptic Fourier analysis, so 13 partly damaged leaves were excluded. The prepared leaves were scanned at 300 dpi using a desktop scanner CanoScan 8800F (Canon, Tokyo, Japan). The SHAPE 1.3 package (Iwata and Ukai 2002) was then used for leaf shape analysis based on elliptic Fourier descriptors (Kuhl and Giardina 1982). Using the ChainCoder routine, leaf shapes were converted into chain codes, and the CHC2NEF programme converted these chain codes into coefficients of elliptic Fourier descriptors (EFDs, using 20 harmonic axes). These coefficients, representing shape variables (mathematical shape descriptors), were used

to calculate the scores of principal components (PCs) using the PrinComp function, which also reconstructed the leaf shape, corresponding to values of +2 and −2 standard deviations on the first and second component axis and average leaf shape of each taxon (see Lepší et al. 2008 for details).

Embryological analyses

Buds and flowers from 34 individuals (see Table 1) were sampled for embryological analyses in the years 1998–2014. Individual developmental stages were assembled from the collected material, and the whole reproductive cycle was characterized based on the state of reproductive organs and fruit development. Part of the data was extracted from the work of Chudíková et al. (2012); however, the original data set was completely re-evaluated and substantially enlarged.

Earlier developmental stages (buds and flowers) were fixed in the Navashin fixative (Berlyn and Mikshe 1976), and later developmental stages (fruits) were fixed in the FAA (formaldehyde, acetic acid, 96% ethanol and water; 1:0.5:5:3.5) tissue fixative. The fixed material was embedded in paraffin. Series of 5–10-µm thick sections were prepared using an Olympus CUT 4055 rotary microtome (Olympus, Tokyo, Japan). The sections were stained with Heidenhain hematoxylin (Sigma) and differentiated in 2.5% ammonium ferrous sulphate (Erdelská 1986). Microscope slides were examined under an Olympus BX 41 light microscope equipped with an Olympus E 520 digital camera (Olympus, Tokyo, Japan).

Fruit set analyses

The fruit set of *Prunus fruticosa* and its hybrids (estimated in 2014) was analysed for 8 individuals of *P. fruticosa* (3 individuals), *P.* ×*eminens* (1 individual) and *P.* ×*mohacsyana* (4 individuals). Three ramets were marked on each individual. Flowers and fully developed fruits were counted, and the total fruit set was expressed as the percentage of the total number of fruits per population divided by the total number of flowers.

Results

Genome size and DNA ploidy level

Diploid, triploid and tetraploid DNA ploidy level was inferred from absolute genome size values for 111 accessions of *Prunus fruticosa*, *P.* ×*eminens*, *P.* ×*mohacsyana*, *P. cerasus* and *P. avium*. *Prunus avium* was proved to be diploid (mean 2C = 0.73), and the heteroploid hybrid *P.* ×*mohacsyana* was proved to be triploid (mean 2C = 1.02 pg). The homoploid hybrid *P.* ×*eminens*, by contrast, was tetraploid (mean 2C = 1.34 pg), as were *P. fruticosa* (mean 2C = 1.31 pg) and *P. cerasus* (mean 2C = 1.42 pg). The three tetraploid taxa tended to differ in absolute genome size (see Fig. 2, Online Resource 1), although an overlap occurred (constituting a continual series; see Online Resource 2). Absolute genome size significantly differed among the five analysed groups ($F = 2426$, $p<2^{e-16}$). The three tetraploid taxa differed significantly ($F = 73.07$, $p<2^{e-16}$) in the ANOVA. Separate Tukey's HSD tests for all taxa and tetraploid taxa only revealed five and three groups, respectively. Detailed cytometric results (including 2C, SD, CV, variation among repeated measurements and illustrative histograms) are summarized in Online Resource 3.

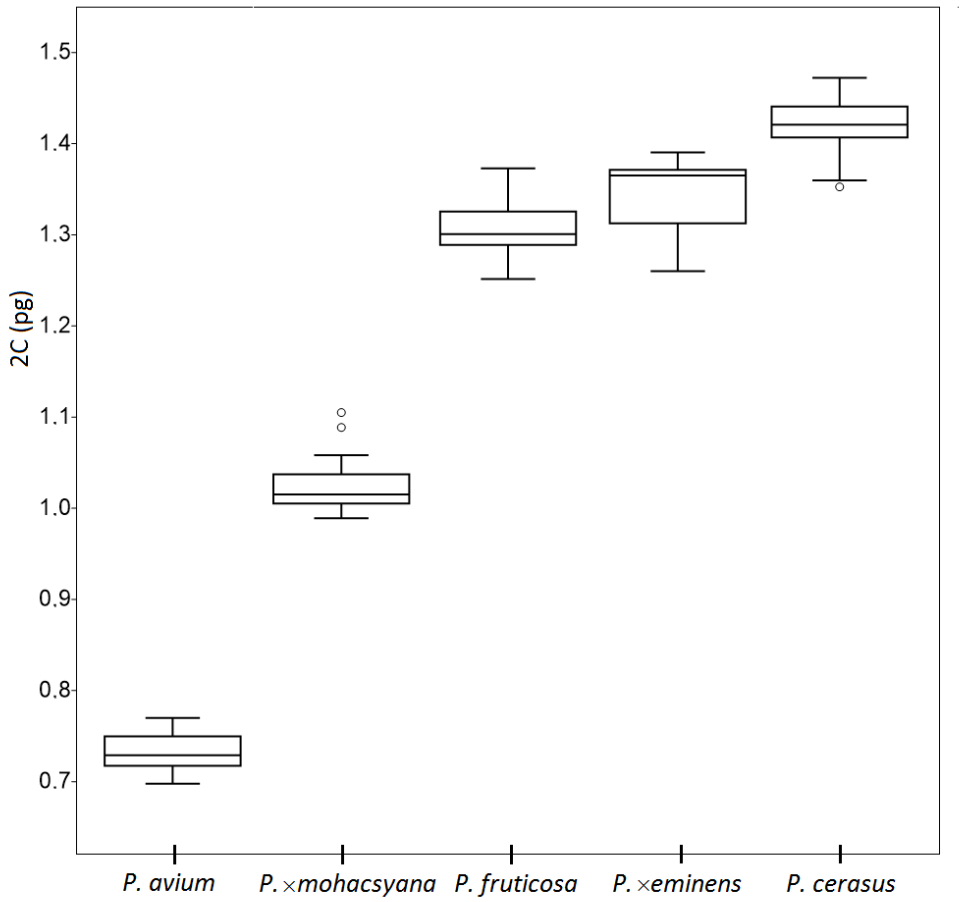

Fig. 2: Comparison of absolute genome size (pg) of *Prunus* taxa under study (diploid *P. avium*, triploid *P. ×mohacsyana*, tetraploid *P. fruticosa*, *P. ×eminens* and *P. cerasus*).

Flow cytometric results (together with morphometrics, see below) revealed the co-occurrence of pure, tetraploid *Prunus fruticosa* and triploid *Prunus ×mohacsyana* in the population on the Pyramída hill in Nitra. Genome size data indicate that hybrids were also able to form recently isolated populations (tetraploid *P. ×eminens* – Nitra St. Urban church, and triploid *P. ×mohacsyana* – Nitra forest edge, Štúrovo, Vŕšok II hill).

Distance-based morphometrics

Eight characters on 222 leaves of *Prunus fruticosa*, *P. ×eminens*, *P. ×mohacsyana*, *P. cerasus* and *P. avium* were included in the evaluation of descriptive statistics (see Online Resource 4). Laminar length and the widest part of lamina to tip were tightly correlated (Spearman's correlation coefficient 0.96), nevertheless, the widest part of lamina to tip describes leaf shape which could be an important additional information, and still PCA is not negatively affected by high levels of correlation values of characters. Three groups of putative parental taxa were well separated along the first component axis in the principal component analysis (the first and the second axis explaining 70.2 and 12.8%, respectively). The two hybrids, by contrast, together formed a compact, overlapping cluster (see Fig. 3). Laminar width, laminar length and the widest part of lamina to tip were the most tightly correlated with the first component axis (see Table 2 for character details; see Online Resource 5 for the table of eigenvectors). Thus, *P. fruticosa* was well differentiated from both hybrids on the basis of 8 morphological characters. Even in the separate analysis excluding *P. avium* and *P. cerasus*, both hybrids

overlapped markedly, and *P. fruticosa* remained well separated (Fig. 4; see Online Resource 5 for the table of eigenvectors).

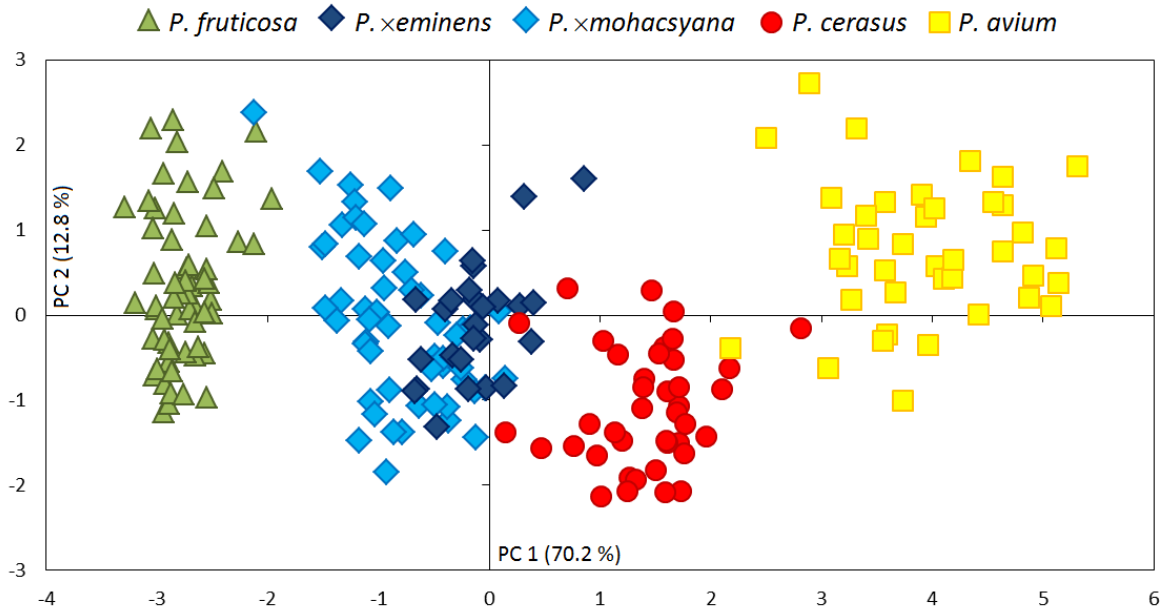

Fig. 3: Principal component analysis (PCA) using 8 morphological characters of 222 leaves *Prunus fruticosa*, *P.* ×*eminens*, *P.* ×*mohacsyana*, *P. cerasus* and *P. avium*. PC 1 explains 70.2% of the variability, PC 2 12.8%.

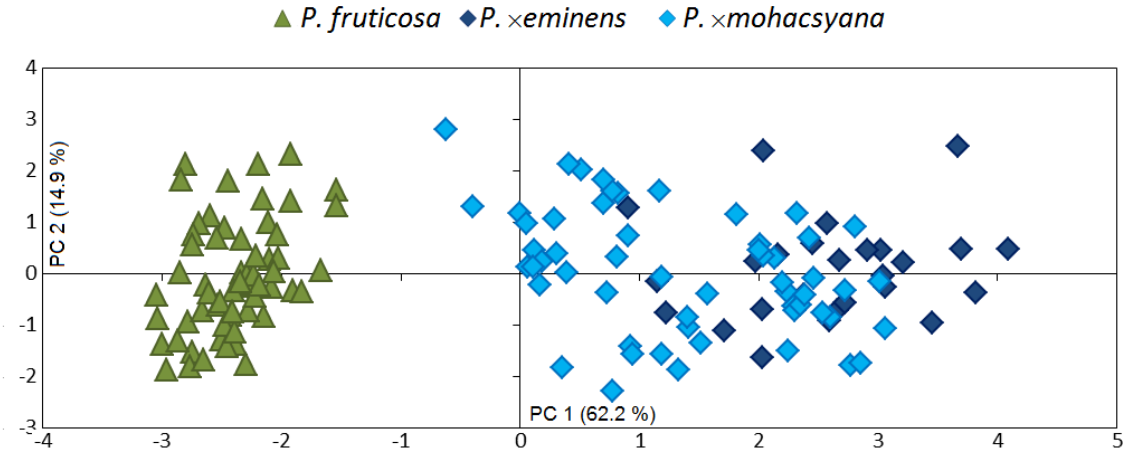

Fig. 4: Ordination diagram of principal component analysis (PCA) based on 142 leaves (8 characters measured) of *Prunus fruticosa*, *P.* ×*eminens* and *P.* ×*mohacsyana*. The first component axis (PC 1) accounts for 62.2% of the variation (PC 2–14.9%).

Elliptic Fourier analysis

Principal component analysis based on standardized elliptic Fourier descriptors of 209 leaves exhibited a similar pattern as distance-based morphometrics. *Prunus fruticosa* formed a mostly compact group that was partly distinct from both hybrids (*P.* ×*eminens* and *P.* ×*mohacsyana*). *Prunus avium* also tended to form a separate group (see Fig. 5), while *P. cerasus* and the hybrids were clustered together. Nevertheless, the separation was not as evident as in the distance-based morphometrics (compare with Fig. 3, 4). The first component axis (explaining 64.2% of the variation) corresponded to relative leaf width, and

the second component axis reflected differences between the two distinct groups (explaining 19.2% of the variation) based on variation in the shape of the leaf base and the shape of the leaf tip.

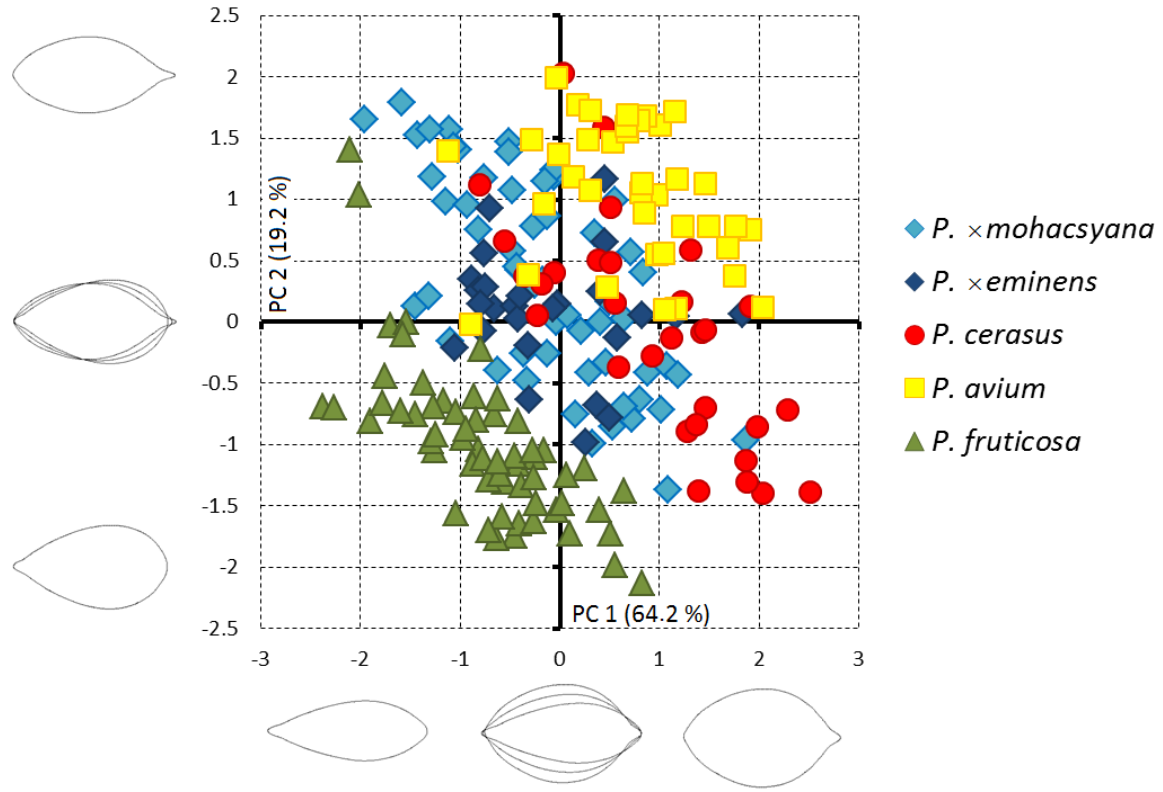

Fig. 5: Principal component analysis (PCA) of Fourier coefficients describing variability in the shape of the lamina of 209 leaves of *Prunus fruticosa*, *P.* ×*eminens*, *P.* ×*mohacsyana*, *P. cerasus* and *P. avium*. PCA scores are standardized by the standard deviation (the scale is in units of standard deviation). Shown along the first two PC axes are leaf shape reconstructions (petiole connection on the left) corresponding to values of -2 SD, 0 and +2 SD.

Thus, leaves of *Prunus fruticosa* tended to be obovate with an obtuse apex, leaves of *Prunus cerasus* formed an elliptic shape with a broadly acuminate apex, and *P. avium* tended to be elliptic with an aristate apex. By contrast, intermediate leaves of the hybrids *P.* ×*eminens* and *P.* ×*mohacsyana* frequently formed an elliptic shape with a broadly acuminate apex, never obtuse or with an aristate apex. Average leaf shapes of the individual taxa are illustrated in Fig. 6. The elliptic Fourier analysis did not find significant differences between the two groups of hybrids (*P.* ×*eminens* and *P.* ×*mohacsyana*).

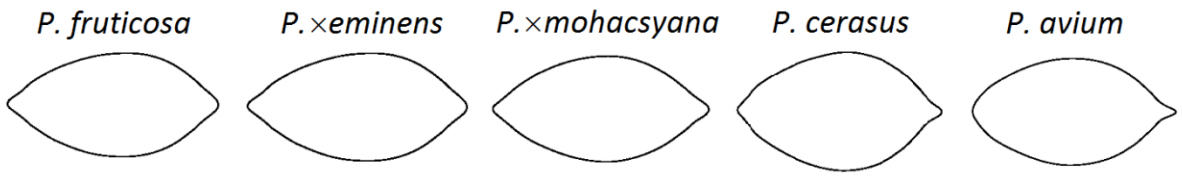

Fig. 6: Average shapes of *Prunus* leaves (petiole connection on the left).

Embryology

Development of the female gametophyte

Functional megaspores were observed in *Prunus fruticosa* and *P.* ×*eminens* (75 and 50%), while degeneration of all megaspore tetrads was frequently recorded in *P.* ×*mohacsyana* (90.0%; see Online Resource 6a), probably due to disturbances of meiosis (for summarized and simplified results see Table 3). Antipodes of the *Prunus* female gametophyte were nearly degenerated, so the mature female gametophyte contained an egg apparatus, which consisted of an egg cell, two synergids and a secondary nucleus that arose by fusion of two polar nuclei before fertilization (see Online Resource 6b). Mature female gametophytes were common in the ovules of *P. fruticosa* and also of *P.* ×*eminens*. Although mature female gametophytes were sporadically present in the ovules of *P.* ×*mohacsyana* (25%), several failures were observed during megagametogenesis, which led to the formation of incomplete or unorganized female gametophytes (three-, four- and six-nucleate with disturbed polarity). Two- and four-nucleate bipolar female gametophytes with degenerated nuclei were also recorded. *Prunus* ×*mohacsyana* ovules (100.0%) did not contain a functional female gametophyte at the time of anthesis as a result of megasporogenesis and megagametogenesis failures.

Development of the male gametophyte

Degeneration of sporogenous cells during differentiation of sporogenous tissue was observed, accompanied by degeneration of the tapetum layer (tetraploid *Prunus fruticosa* and *P.* ×*eminens* – 14.7 and 20.0% vs. triploid *P.* ×*mohacsyana* – 37.5%). Failures in tapetum differentiation in the early developmental stages of *P.* ×*mohacsyana* were also followed by disturbances in the differentiation of sporogenous cells and microsporocytes (50% of disturbed in case of *P.* ×*mohacsyana*, manifested by, e.g. atypical shape; compare Online Resource 6c and 6d).

In *Prunus fruticosa* and *P.* ×*eminens*, a regular course of microsporogenesis was observed (see Online Resource 6e, f, g), but several disorders in microsporogenesis were recorded in *P.* ×*mohacsyana* (reaching 85.7%; see Online Resource 6h, i, j), resulting in a completely undeveloped pollen grain set leading to complete male sterility (up to 100%; see Online Resource 6i). Microspores exhibited considerable shape and size variation after the release of microspores from tetrads and their subsequent growth; the protoplasts were strongly vacuolated (see Online Resource 6h, j). We sporadically observed monads where the size of nuclei showed that they contained a non-reduced number of chromosomes (most likely resulting in further observed giant pollen grains; see Online Resource 6i).

The postfertilization pattern was analogous to traits in male and female reproductive structures in particular taxa (i.e. regular double fertilization and developed embryos in *Prunus fruticosa* and *P.* ×*eminens* vs. degenerated embryos in *P.* ×*mohacsyana* resulting in no mature fruit observed; see Online Resource 6k, l).

Table 3: Ratios of irregularly developed reproductive female and male structures (to the total number of analysed samples) and fruit set (fruits/flowers).

Stages of development	*P. fruticosa*	*P. ×eminens*	*P. ×mohacsyana*
Female			
Percentage of degeneration of all archespore cells	12.5% (1/8)	33.3% (1/3)	66.7% (8/12)
Percentage of degenerated tetrad of megaspores	25.0% (2/8)	50.0% (2/4)	90.0% (9/10)
Percentage of incomplete and degenerated female gametophyte or ovules without female gametophyte	11.4% (0+3+5/70)	36.8% (3+2+2/19)	75.0% (5+11+26/56)
Percentage of completely degenerated ovules (without zygota or embryo)	10.7% (3/28)	25.0% (3/12)	100% (30/30)
Male			
Percentage of damaged sporogenous tissue	14.7% (5/34)	20.0% (2/10)	37.5% (9/24)
Percentage of irregularly formed microsporocytes	0% (0/25)	0% (0/5)	50.0% (10/20)
Percentage of disturbances of microsporogenesis	21.1% (4/19)	50.0% (5/10)	85.7% (30/35)
Percentage of irregularly formed microspores	0% (0/30)	9.1% (1/11)	72.7% (16/22)
Percentage of degenerated pollen grains (entire grains in specimen)	0% (0/30)	0% (0/10)	100% (33/33)
Fruits			
Fruit set	1.8% (3/166)	0.4% (1/251)	0% [0/(58+251)]

Discussion

The applied methodology combines embryology, flow cytometry and morphometrics. Used methods have their limitations and advantages. Flow cytometry usually produces large amounts of data, but the particles passing through the flow cytometer are not under visual control, so it is impossible to differentiate between nuclei and debris, for example. In addition, serious problems may arise due to the influence of secondary metabolites (e.g. reduced fluorescence and measurement accuracy; Doležel et al. 2007). It may also be difficult to interpret the results (e.g. different genome size vs. aneuploidy or evidence of backcrossing of homoploid hybrids in cases of small differences in absolute genome size; Loureiro et al. 2010). Embryology, by contrast, enables direct (visual) observation of particular reproductive stages, but depends on capturing the right ontogenetic stage of the tissue under study and on observing characters visually (e.g. absence of reproductive phases in samples or the presence of secondary metabolites). Moreover, because of the destructive nature of this method and the need for observing different reproductive stages, numerous individual samples of different ontogenetic stage must be analysed. However, as embryology is time-

-consuming and fraught with technical difficulties, only small numbers of individuals are usually analysed (Bhojwani and Bhatnagar 1983; Herr 1984). Traditionally used morphometric approaches are substantially disadvantaged by limited ability of shape description; nevertheless, this may be overcome by combination with geometric morphometrics (including elliptic Fourier analysis; Těšitel et al. 2009; Hanušová et al. 2014; Kabátová et al. 2014). Apparent limitation of morphometric approach is interference of phenotypic plasticity which could blur the discriminative morphological trend of particular species.

Absolute genome size is frequently employed as a neutral marker within groups of closely related taxa (Murray 2005). Small differences in genome size should be interpreted with caution (Doležel and Bartoš 2005; Šmarda and Bureš 2006), especially to avoid methodological artefacts (Greilhuber et al. 2005). We are nevertheless convinced that our results are not negatively influenced by methodological artefacts (due to symmetric peaks of our histograms, relatively high range 49 plant genome sizes – over 17% and proven reproducibility of measurements). The Rosaceae family is challenging to study by flow cytometry due to its higher contents of secondary metabolites, which directly influence coefficients of variation of resulting peaks. To avoid the exclusion of the entire family from cytometric research, higher coefficients of variation are generally accepted (Baird et al. 1994; Jedrzejczyk and Sliwinska 2010; Dobeš et al. 2013). The coefficients of variation of our analyses also reached higher values (1.33–5.95). To exclude the negative influence of secondary metabolites (and higher coefficients of variation), we tested the stability of the analyses by taking repeated measurements.

Differences between obtained genome size values (intra- and interspecific variation) can generally be connected with (1) changes in chromosome sets (aneuploidy, polyploidy, chromosomal heteromorphism, presence of B chromosomes or sex chromosomes; Bennetzen et al. 2005; Šmarda and Bureš 2010) or (2) differential accumulation of transposable elements (Bennetzen et al. 2005; Michael 2014). There are also other processes that should be mentioned in relation to genome size variation, such as sequence length polymorphism, genome duplication, punctual insertions/deletions and irregular recombination (Petrov 2001; Šmarda and Bureš 2010). We can, however, exclude a possible influence of these phenomena based on literature data and our calibration by chromosome counts, which cover the whole interval of measured genome sizes.

Introgressive hybridization

The heteroploid hybrid combination is mirrored by a rather discrete genome size pattern. At the tetraploid level, by contrast, flow cytometry revealed a continuum of genome sizes that can be interpreted as a result of frequent backcrossing. *Prunus ×eminens* is evidently linking the two putative parental taxa. Thus, together with the embryological results, flow cytometry supports the hypothesis of frequent gene flow among tetraploids and possible backcrossing (put forward by Marhold and Wójcicki 1992; Haeupler and Muer 2007), but it has never been proven.

We are aware that *Prunus fruticosa* and *P. ×eminens* were determined based on a single character (presence of abaxial hairs). Although its reliability has been proven by statistical approaches (high values of PCA eigenvectors; see Online Resource 5), three other quantitative characters (laminar width, laminar length and the widest part of lamina to tip) were slightly more correlated with the first component axis in the principal component analysis of all the taxa (see Online Resource 5). We are, however, convinced that the presence of abaxial hair, being a semiquantitative character, is more useful for determining taxa in the field than other mentioned leaf traits.

Both morphometric approaches indicated introgressive hybridization, especially in the direction of *Prunus cerasus* (see Fig. 3, 5). We conclude, based on morphometric data, that the genome size continuum observed in this study indicates introgressive hybridization. The same conclusion was reached, for example, by Suda et al. (2007) and Urfus et al. (2014) in the genus *Pilosella* Hill. or by Hanušová et al. (2014) in *Diphasiastrum* Holub. Of course, for a reliable confirmation of parentage and the direction of hybridization, more sensitive (e.g. AFLP, microsatellites) and uniparentally inherited markers (cpDNA markers) will have to be employed.

Homoploid hybridization (*Prunus fruticosa* × *P. cerasus*) indicated asymmetric type of introgressive hybridization (backcrossing towards *P. fruticosa*; see Fig. 2). Asymmetric tendencies in introgressive hybridization were documented several times (*Quercus* L.; Petit et al. 2004). Potential impact of introgressive hybridization is further enhanced at secondary habitats (especially at anthropogenically disturbed sites), which represent opportunity to establish new hybrid populations with diminished influence of exclusion via competition (e.g. *Viola lutea* subsp. *sudetica* (Willd.) Nyman, Krahulcová et al. 1996; *Banksia* L.f., Lamont et al. 2003; *Argyranthemum frutescens* (L.) Sch. Bip., Fjellheim et al. 2009). In specific cases disturbed habitats enable establishment of highly intricate hybrid swarms (*Pilosella* Hill; Křišťálová et al. 2010; Urfus et al. 2014). In perspective of conservation genetics introgression is frequently linked to secondary habitat whereas the original primary habitat populations remain unspoiled (e.g. *Pinus uncinata* subsp. *uliginosa* (G.E.Neumann ex Wimm.) Businský; Bastl et al. 2008). *Prunus fruticosa* is contrary endangered by introgression even within its primary habitats (shrub alliances and continental deciduous thickets, Chytrý et al. 2010).

Conservation consequences

Based on our data, the cultivation of *Prunus cerasus*, which can introgressively hybridize with *P. fruticosa*, represents a substantially higher conservation risk for *P. fruticosa* than the cultivation of *P. avium*, hybridization with which leads to mostly sterile F1 triploid hybrids. Introgressive homoploid hybridization may cause slow, invisible genetic erosion (Levin et al. 1996). The most efficient conservation strategy is to protect the most isolated pure populations of *P. fruticosa*. Several studies carried out in Central Europe indicate that the risks of introgression should be taken seriously. Hybridization of rare, low-abundant species often threatens small, isolated populations, because they cannot counterbalance the increasing number of hybrids (Vít et al. 2014). The risk of genetic erosion can be further enhanced if the participating taxa are of distant provenances (*P. cerasus* probably came from Southwest Asia; Sinskaya 1969; Kurtto et al. 2013). Although the triploid hybrids most probably do not participate in further backcrossing, they represent a serious complication for the conservation of *P. fruticosa*. They tend to occupy niches of *P. fruticosa*, compete with it for resources and decrease the number of its potential sexual partners, and even tend to overgrow lower *P. fruticosa* shrubs (Lepší et al. 2011).

Embryological evidence

High triploid hybrid sterility was observed during our embryological study. Sterility of *Prunus* ×*mohacsyana* was manifested in both male and female gametophytes. This pattern markedly differs from standard reproductive traits observed in the tetraploid hybrid (*P.* ×*eminens*). Ovules with undeveloped or missing female gametophytes at the time of anthesis occur frequently in cultivated taxa or cultivars of hybrid origin (also among other *Prunus* species; Furukawa and Bukovac 1989; Egea and Burgos 1994). Male sterility

of triploid hybrid individuals is most likely connected with a failure of meiosis caused by an imbalance between the participating genomes. A similar pattern, accompanied by serious disturbances of microsporogenesis manifested by unequal distribution of chromosomes to the poles and the elimination of chromosomes during meiotic division, has been recorded in three triploid species of the genus *Crataegus* L. (manifested also by the presence of morphologically and karyologically diverse or giant pollen grains; Ptak 1989). Moreover, differences in exine morphology have been observed in several cultivars of *P. cerasus* (tetracolpate pollen instead of tricolpate and ''giant'' pollen grains; Miaja et al. 2000) or abnormalities in tapetum development and irregularities in exine development have been reported (e.g. *Prunus salicina* Lindl.; Radice et al. 2008).

Pollen germination is often significantly reduced in hybrids, but the extent of the reduction is highly variable (Ramsey and Schemske 1998). Low pollen germination has been observed in cultivars of tetraploid *Prunus cerasus* (<25%; Miaja et al. 2000). On the other hand, Rybnikárová (2010) reported markedly higher pollen germination (between 29.0 and 58.33%) in the pentaploid (2n = 40; Scholz and Scholz 1995) heteroploid hybrid *Prunus ×fruticans* Weihe (*Prunus insititia* L. × *Prunus spinosa* L.).

All embryological observations confirm that the triploid block is highly efficient in preventing further hybridization events. A similar pattern has been observed also in other sterile triploid hybrids (Ekrt et al. 2009; Ferriol et al. 2012; Duszynska et al. 2013; Samadi et al. 2013; Hanzl et al. 2014; Zozomová-Lihová et al. 2014). On the other hand, triploid hybrids may also be fertile, highly viable, producing 1x, 2x, 3x gametes, and able to contribute significantly to further hybridization events and ploidy diversification (Ramsey and Schemske 1998; Husband 2004; Henry et al. 2005; Hayashi et al. 2009). A tendency towards producing ploidy-variable gametes has also been observed in early stages of microsporogenesis, but it has not been proven among mature gametes of *Prunus ×mohacsyana*. On the other hand, there is a strong potential for introgression (including backcrossing) in tetraploid hybrids (*P. ×eminens*), based on our embryological analyses.

Taxonomical consequences

Our study revealed several morphological characters that are highly efficient for determining the taxa concerned (except hybrids). *Prunus* cerasus and *P. avium* are tree-like in growth form, while the other taxa dealt with in this study are shrubs (*P. fruticosa*, *P. ×eminens* and *P. ×mohacsyana*). The character (abaxial hairs) used for distinguishing *P. fruticosa* (glabrous) from hybrids (hairs) is one of the most useful. Other suitable key characters are laminar size (laminar width, laminar length, the widest part of lamina to tip – see Table 2 for details; *P. fruticosa* has smaller leaves than hybrids), the shape of the leaf apex (*P. fruticosa* – obtuse to obovate, *P. avium* – elliptic with an aristate apex, *P. cerasus* – elliptic with a broadly acuminate apex vs. intermediate hybrids – obovate to elliptic with a broadly acuminate apex, never obtuse or with an aristate apex) and ploidy level (tetraploid *P. fruticosa*, *P. cerasus* and *P. ×eminens*, triploid *P. ×mohacsyana* and diploid *P. avium*). Although the presence of abaxial hairs, leaf size and apex characters are useful for identifying the homoploid hybrid *P. ×eminens*, we cannot assume that they are useful also for identifying introgressants. Thus, populations of *P. fruticosa*, especially those in which *P. ×eminens* occurs, might be better treated as potentially backcrossed, especially with regard to our embryological results (high potential for backcrossing in both male and female reproductive structures).

Conclusions

Our study revealed three ploidy levels: diploid (*Prunus avium*), triploid (*P.* ×*mohacsyana*) and tetraploid (*P. fruticosa*, *P.* ×*eminens* and *P. cerasus*) in six selected populations and additional material from surrounding regions. The tetraploids, moreover, tended to differ in absolute genome size. An embryological evaluation confirmed the existence of a triploid block in *P.* ×*mohacsyana* and significant potential for introgressive hybridization among tetraploid taxa. Morphometrics, flow cytometry and embryology jointly revealed frequent backcrossing. Although hybrids differ in ploidy level and embryological characteristics, they are almost indistinguishable at the morphological level. Hybridization with *P. cerasus* nevertheless turns out to be a significant threat to wild populations of *P. fruticosa* in contrast to the relatively weak danger posed by hybridization with *P. avium*.

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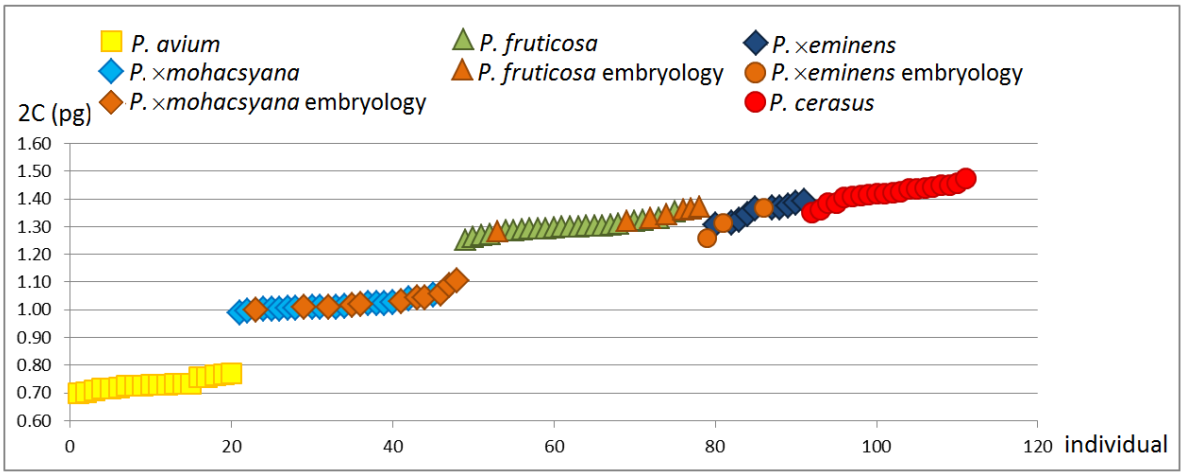
Supplementary material

Online Resource 1: Absolute genome size (pg) of taxa of the genus *Prunus* under study. 2C = nuclear DNA content of somatic cells, SD = standard deviation, CV = coefficient of variance of the sample, N = number of individuals.

Taxa	Average 2C	Range of 2C	Median	SD	Average CV	N
P. avium	0.73	0.70–0.77	0.73	0.02	4.81	20
P. ×mohacsyana	1.02	0.99–1.10	1.01	0.03	5.03	28
P. fruticosa	1.31	1.25–1.37	1.30	0.03	4.45	30
P. ×eminens	1.34	1.26–1.39	1.37	0.04	4.16	13
P. cerasus	1.42	1.35–1.47	1.42	0.03	3.91	20

Online Resource 2: Absolute genome size (pg) variation of 111 individuals of *Prunus fruticosa*, *P. ×eminens*, *P. ×mohacsyana*, *P. cerasus* and *P. avium*. Orange-marked individuals were used in the embryological analysis.



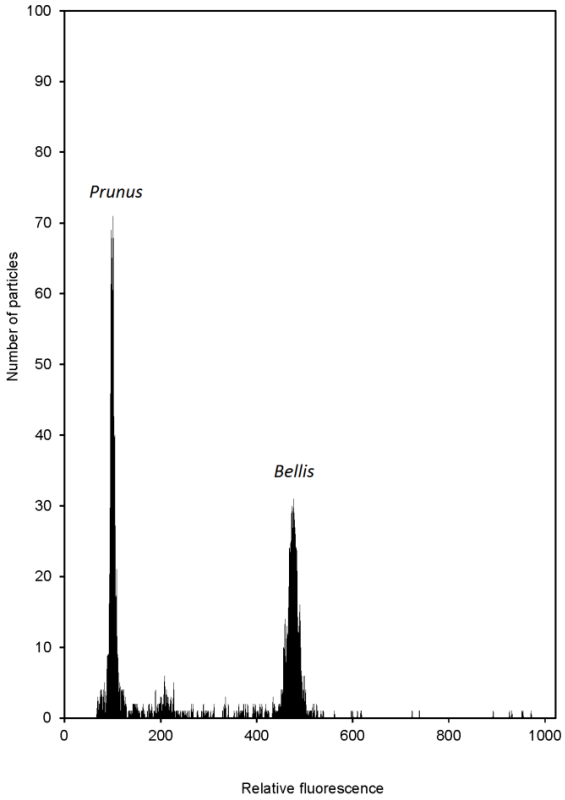
Online Resource 3: Table of ploidy levels and absolute genome size data for *Prunus fruticosa*, *P. ×eminens*, *P. ×mohacsyana*, *P. cerasus* and *P. avium* (index = ratio between sample and internal standard peak, CV = coefficient of variance of the sample, GS = absolute genome size) accompanied by illustrative histograms of each ploidy level (A = diploid *P. avium*, B = triploid *P. ×mohacsyana*, C = tetraploid *P. ×eminens*).

		PI – 1st run			PI – 2nd run					
Sample name	**Sample**	**Index**	**CV sample**	**CV *Bellis***	**Index**	**CV sample**	**CV *Bellis***	**GS [pg]**	**SD**	**Diffe-rence [%]**
P. fruticosa	P1-1	2.658	3.32	2.44	X	X	X	1.27	X	X
P. fruticosa	P1-2	2.677	3.46	2.23	X	X	X	1.26	X	X
P. fruticosa	P1-3	2.604	4.07	2.22	X	X	X	1.30	X	X
P. fruticosa	P1-4	2.631	3.13	2.29	X	X	X	1.28	X	X
P. fruticosa	P1-5	2.592	5.09	2.39	X	X	X	1.30	X	X
P. fruticosa	P1-6	2.621	3.85	2.36	X	X	X	1.29	X	X
P. fruticosa	P1-7	2.701	4.23	2.22	X	X	X	1.25	X	X
P. fruticosa	P1-8	2.611	4.96	2.31	X	X	X	1.29	X	X
P. fruticosa	P1-9	2.563	5.82	3.50	2.513	4.49	2.70	1.33	0.019	1.99
P. fruticosa	P1-10	2.505	1.33	4.56	2.576	3.80	2.36	1.33	0.026	2.83
P. fruticosa	P1-11	2.474	5.29	2.59	2.513	3.11	1.87	1.36	0.015	1.58
P. fruticosa	P1-12	2.523	4.40	2.08	2.507	4.14	2.84	1.34	0.006	0.64
P. fruticosa	P1-13	2.598	4.83	1.94	2.563	3.35	1.94	1.31	0.013	1.37
P. fruticosa	P1-14	2.566	5.16	2.38	2.541	4.60	4.02	1.32	0.009	0.98
P. fruticosa	P1-15	2.602	4.41	2.37	2.585	5.12	3.58	1.30	0.006	0.66
P. fruticosa	P2-1	2.604	4.36	2.75	X	X	X	1.30	X	X
P. fruticosa	P2-2	2.601	4.21	2.57	X	X	X	1.30	X	X
P. fruticosa	P2-3	2.614	5.00	2.65	X	X	X	1.29	X	X
P. fruticosa	P2-4	2.611	5.19	2.79	X	X	X	1.29	X	X
P. fruticosa	P2-5	2.559	5.36	2.95	X	X	X	1.32	X	X
P. fruticosa	P2-6	2.607	5.48	3.01	X	X	X	1.30	X	X
P. fruticosa	P2-7	2.596	3.93	2.62	X	X	X	1.30	X	X
P. fruticosa	P2-8	2.626	4.39	2.80	X	X	X	1.29	X	X
P. fruticosa	P2-9	2.586	4.78	2.47	X	X	X	1.31	X	X
P. fruticosa	P2-10	2.663	4.30	2.83	X	X	X	1.27	X	X
P. fruticosa	P2-11	2.584	4.31	2.43	2.538	3.96	2.88	1.32	0.017	1.81
P. fruticosa	P2-12	2.497	5.77	2.66	2.428	4.24	3.27	1.37	0.027	2.84
P. fruticosa	P2-13	2.491	4.29	2.11	2.463	5.33	2.03	1.36	0.011	1.14
P. fruticosa	P2-14	2.504	5.64	2.51	2.460	5.76	3.04	1.36	0.017	1.79
P. fruticosa	P2-15	2.600	4.95	2.67	2.673	3.60	2.25	1.28	0.025	2.81
P. ×mohacsyana	P3-1	3.367	4.68	2.73	X	X	X	1.00	X	X
P. ×mohacsyana	P3-2	3.307	4.78	2.40	X	X	X	1.02	X	X
P. ×mohacsyana	P3-3	3.301	5.86	2.52	3.308	4.22	1.51	1.02	0.002	0.21
P. ×mohacsyana	P3-4	3.265	5.41	3.55	3.300	4.47	2.72	1.03	0.008	1.07
P. ×mohacsyana	P3-5	3.154	4.99	2.67	3.235	4.24	2.69	1.06	0.019	2.57
P. ×mohacsyana	P3-6	3.246	5.19	2.27	3.257	4.75	2.91	1.04	0.002	0.34

		PI – 1st run			PI – 2nd run					
Sample name	**Sample**	**Index**	**CV sample**	**CV *Bellis***	**Index**	**CV sample**	**CV *Bellis***	**GS [pg]**	**SD**	**Diffe-rence [%]**
P. ×mohacsyana	P3-7	3.217	5.80	2.87	3.190	4.76	2.78	1.06	0.006	0.85
P. ×mohacsyana	P3-8	3.320	5.14	2.63	3.261	5.47	2.78	1.03	0.013	1.81
P. ×mohacsyana	P3-9	3.350	5.87	2.27	3.339	5.74	2.55	1.01	0.002	0.33
P. ×mohacsyana	P3-10	3.342	5.75	2.90	3.280	4.88	2.62	1.02	0.014	1.89
P. ×mohacsyana	P3-11	3.294	5.25	3.06	3.380	5.15	3.00	1.01	0.018	2.61
P. ×mohacsyana	P4-1	3.345	2.74	2.87	3.303	5.72	2.14	1.02	0.009	1.27
P. ×mohacsyana	P4-2	3.213	5.30	1.90	3.269	5.73	2.70	1.04	0.013	1.74
P. ×mohacsyana	P5-1	3.370	5.17	2.53	X	X	X	1.00	X	X
P. ×mohacsyana	P5-2	3.346	4.41	2.50	X	X	X	1.01	X	X
P. ×mohacsyana	P5-3	3.343	5.28	2.76	X	X	X	1.01	X	X
P. ×mohacsyana	P5-4	3.399	5.62	2.13	X	X	X	0.99	X	X
P. ×mohacsyana	P5-5	3.350	4.16	2.96	X	X	X	1.01	X	X
P. ×mohacsyana	P5-6	3.363	4.65	3.08	X	X	X	1.01	X	X
P. ×mohacsyana	P5-7	3.362	5.73	2.34	X	X	X	1.01	X	X
P. ×mohacsyana	P5-8	3.300	5.46	2.71	X	X	X	1.02	X	X
P. ×mohacsyana	P5-9	3.418	3.04	2.10	X	X	X	0.99	X	X
P. ×mohacsyana	P5-10	3.366	5.65	3.00	X	X	X	1.00	X	X
P. ×mohacsyana	P5-11	3.036	5.41	3.11	3.082	5.65	3.63	1.10	0.012	1.52
P. ×mohacsyana	P5-12	3.248	5.17	3.23	3.223	5.58	2.84	1.04	0.006	0.78
P. ×mohacsyana	P5-13	3.339	5.95	2.45	3.364	4.15	2.74	1.01	0.005	0.75
P. ×mohacsyana	P5-14	3.429	4.11	2.05	3.338	4.55	1.76	1.00	0.019	2.73
P. ×mohacsyana	P5-15	3.120	5.09	1.80	3.090	4.58	3.35	1.09	0.007	0.97
P. ×eminens	P6-1	2.431	4.00	2.20	X	X	X	1.39	X	X
P. ×eminens	P6-2	2.460	4.44	2.62	X	X	X	1.37	X	X
P. ×eminens	P6-3	2.476	4.10	2.75	X	X	X	1.37	X	X
P. ×eminens	P6-4	2.470	4.77	2.82	X	X	X	1.37	X	X
P. ×eminens	P6-5	2.472	3.88	2.69	X	X	X	1.37	X	X
P. ×eminens	P6-6	2.514	4.10	2.80	X	X	X	1.34	X	X
P. ×eminens	P6-7	2.585	3.55	2.54	X	X	X	1.31	X	X
P. ×eminens	P6-8	2.442	5.82	3.42	X	X	X	1.38	X	X
P. ×eminens	P6-9	2.575	4.66	2.70	X	X	X	1.31	X	X
P. ×eminens	P6-10	2.557	3.48	2.72	X	X	X	1.32	X	X
P. ×eminens	P6-11	2.609	5.33	2.64	2.542	3.76	3.27	1.31	0.024	2.64
P. ×eminens	P6-12	2.693	4.18	2.71	2.672	2.45	2.01	1.26	0.007	0.79
P. ×eminens	P6-13	2.488	4.15	1.90	2.460	3.89	3.51	1.37	0.011	1.14
P. cerasus	Lys PC1	2.302	3.54	2.32	2.340	4.03	2.48	1.46	0.017	1.65
P. cerasus	Lys PC2	2.451	2.94	1.98	2.425	3.42	1.77	1.39	0.010	1.07
P. cerasus	Lys PC3	2.394	3.56	2.30	2.374	3.89	2.19	1.42	0.008	0.84
P. cerasus	Lys PC6	2.338	4.14	2.36	2.322	4.24	2.90	1.45	0.007	0.69
P. cerasus	Lys PC7	2.355	3.88	2.10	2.355	4.76	2.83	1.44	0.000	0.00
P. cerasus	Lys PC8	2.379	3.07	1.95	2.381	4.81	2.88	1.42	0.001	0.08

		PI – 1st run			PI – 2nd run					
Sample name	**Sample**	**Index**	**CV sample**	**CV *Bellis***	**Index**	**CV sample**	**CV *Bellis***	**GS [pg]**	**SD**	**Diffe-rence [%]**
P. cerasus	Lys PC9	2.404	5.31	3.04	2.404	4.41	2.26	1.41	0.000	0.00
P. cerasus	Lys PC10	2.397	3.17	1.52	2.376	3.87	2.98	1.42	0.009	0.88
P. cerasus	Lys PC11	2.339	2.18	1.91	2.365	4.31	2.59	1.44	0.011	1.11
P. cerasus	Lys PC12	2.374	4.92	2.88	2.414	3.84	1.84	1.41	0.017	1.68
P. cerasus	P33-PC1	2.345	2.83	1.97	X	X	X	1.44	X	X
P. cerasus	P33-PC2	2.372	5.10	2.26	X	X	X	1.42	X	X
P. cerasus	P76-PC1	2.350	4.31	3.10	X	X	X	1.44	X	X
P. cerasus	P76-PC2	2.329	3.72	2.70	X	X	X	1.45	X	X
P. cerasus	P82-PC1	2.499	3.26	2.43	X	X	X	1.35	X	X
P. cerasus	P82-PC2	2.486	5.10	2.71	X	X	X	1.36	X	X
P. cerasus	P82-PC5	2.442	2.99	2.62	X	X	X	1.38	X	X
P. cerasus	P82-PC6	2.399	3.36	2.90	X	X	X	1.41	X	X
P. cerasus	P128-PC1	2.296	4.20	2.50	X	X	X	1.47	X	X
P. cerasus	P128-PC2	2.378	4.28	3.18	X	X	X	1.42	X	X
P. avium	Zel-PA12	4.476	5.46	2.86	X	X	X	0.76	X	X
P. avium	Zel-PA35	4.606	4.61	2.51	X	X	X	0.73	X	X
P. avium	Zel-PA38	4.630	5.52	2.96	X	X	X	0.73	X	X
P. avium	Zel-PA40	4.723	3.91	2.25	X	X	X	0.72	X	X
P. avium	Zel-PA42	4.657	3.50	1.98	X	X	X	0.73	X	X
P. avium	Zel-PA47	4.634	4.63	2.35	X	X	X	0.73	X	X
P. avium	Zel-PA48	4.422	4.34	2.02	X	X	X	0.76	X	X
P. avium	Zel-PA49	4.459	5.52	2.15	X	X	X	0.76	X	X
P. avium	Zel-PA50	4.763	5.60	3.44	X	X	X	0.71	X	X
P. avium	Zel-PA51	4.844	4.55	2.42	X	X	X	0.70	X	X
P. avium	P115-PC1	4.391	5.36	2.74	X	X	X	0.77	X	X
P. avium	P125-PC1	4.659	4.98	2.46	X	X	X	0.73	X	X
P. avium	P125-PC2	4.713	4.88	2.20	X	X	X	0.72	X	X
P. avium	P127-PC1	4.397	4.97	2.47	X	X	X	0.77	X	X
P. avium	P127-PC2	4.611	5.85	2.58	X	X	X	0.73	X	X
P. avium	P130-PC1	4.657	4.41	2.37	X	X	X	0.73	X	X
P. avium	P134-PC1	4.704	3.55	2.05	X	X	X	0.72	X	X
P. avium	P135-PC1	4.802	4.04	2.08	X	X	X	0.70	X	X
P. avium	P140-PC1	4.622	5.68	2.98	X	X	X	0.73	X	X
P. avium	P140-PC2	4.640	4.75	2.19	X	X	X	0.73	X	X

A


Prunus
Bellis
Number of particles
Relative fluorescence
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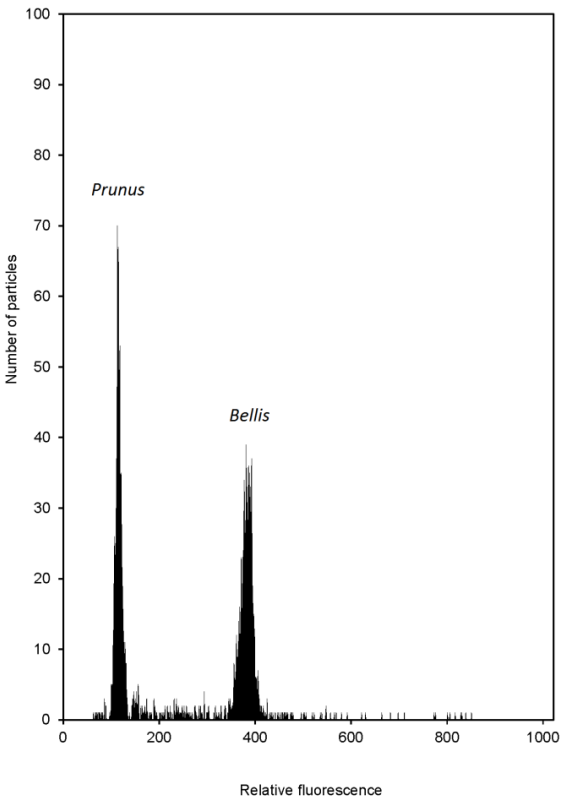
B


Prunus
Bellis
Number of particles
Relative fluorescence
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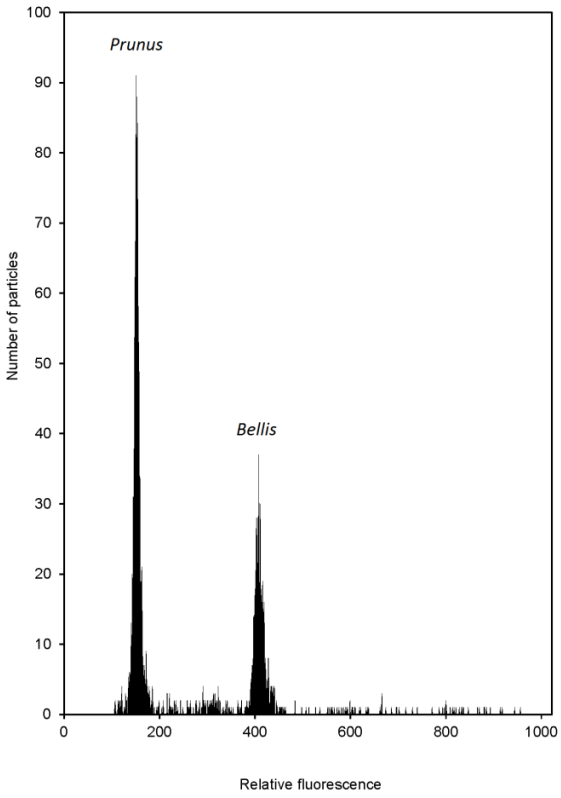
C


Prunus
Bellis
Number of particles
Relative fluorescence
0
10
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Online Resource 4: Basic descriptive statistics for traits of *Prunus fruticosa*, *P. ×eminens*, *P. ×mohacsyana*, *P. cerasus* and *P. avium* (minimum, maximum, average, and 25% and 75% quantile).

Taxa/characters		Plant height	Laminar lenght (mm)	Laminar width (mm)	The widest part of lamina to tip (mm)	Ratio of lenght and width of lamina	Shape of lamina tip	Level of hairs on the adaxial surface of lamina	Level of hairs on the abaxial surface of lamina
Prunus fruticosa	min	1	13.37	6.43	4.84	1.69	1	1	1
	25% quantile	1	16.64	8.12	6.15	1.94	1	1	1
	average	1	18.80	8.86	7.04	2.13	1.53	1.16	1
	75% quantile	1	20.30	9.73	7.87	2.27	2	1.25	1
	max	1	25.87	11.77	10.47	2.82	3	2	1
Prunus ×eminens	min	3	29.66	14.96	10.13	1.70	2	1	1.50
	25% quantile	3	38.81	18.67	14.82	1.89	3	1	2.50
	average	3	41.15	20.02	17.39	2.06	2.88	1.19	2.85
	75% quantile	3	45.13	21.39	19.72	2.21	3	1.25	3.25
	max	3	50.87	26.18	22.94	2.31	3	2	4
Prunus ×mohacsyana	min	1	21.66	8.37	10.73	1.56	2	1	1.25
	25% quantile	2	28.37	12.95	12.58	1.88	3	1	1.75
	average	2.57	33.53	16.29	15.07	2.10	2.88	1.06	2.27
	75% quantile	3	39.26	19.23	17.36	2.32	3	1	2.75
	max	3	50.25	26.07	21.54	2.95	3	1.75	4
Prunus cerasus	min	3	36.82	20.86	17.67	1.45	3	1	2
	25% quantile	4	49.25	29.58	23.01	1.60	3	1	3
	average	3.9	57.72	32.67	26.63	1.77	3.48	1.11	3.23
	75% quantile	4	62.86	35.60	29.12	1.91	4	1	4
	max	4	85.66	46.29	40.18	2.29	4	2.50	4
Prunus avium	min	4	60.20	30.76	29.88	1.55	4	1	4
	25% quantile	4	86.00	43.99	43.09	1.82	4	2	4
	average	4	98.61	51.34	50.50	1.95	4	1.98	3.95
	75% quantile	4	113.77	57.72	57.42	2.07	4	2	4
	max	4	127.11	71.94	69.57	2.63	4	3	3.50

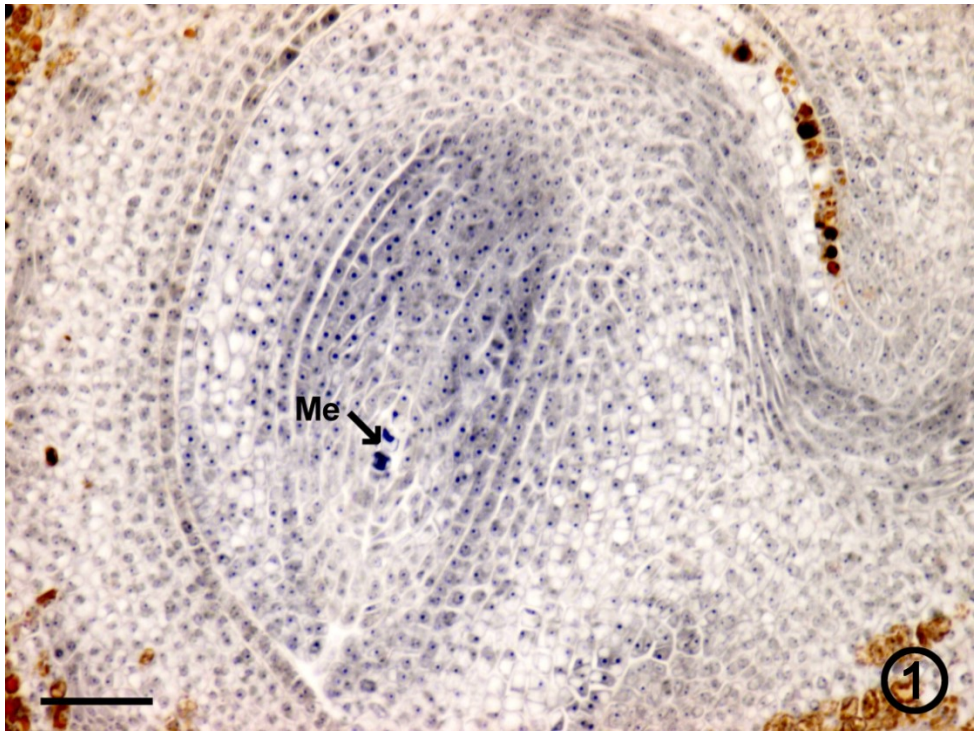
Online Resource 5: Eigenvector values of the first two axes of principal component analysis (8 morphological traits) *Prunus fruticosa*, *P.* ×*eminens*, *P.* ×*mohacsyana*, *P. cerasus* and *P. avium* (natural and cultivated). Highlighted are the signs and values of eigenvectors that contribute most to the distribution of objects along the first axis. See Table 2 for characters abbreviations. PF = *P. fruticosa*, PE = *P.* ×*eminens*, PM = *P.* ×*mohacsyana*, PC = *P. cerasus*, PA = *P. avium*.

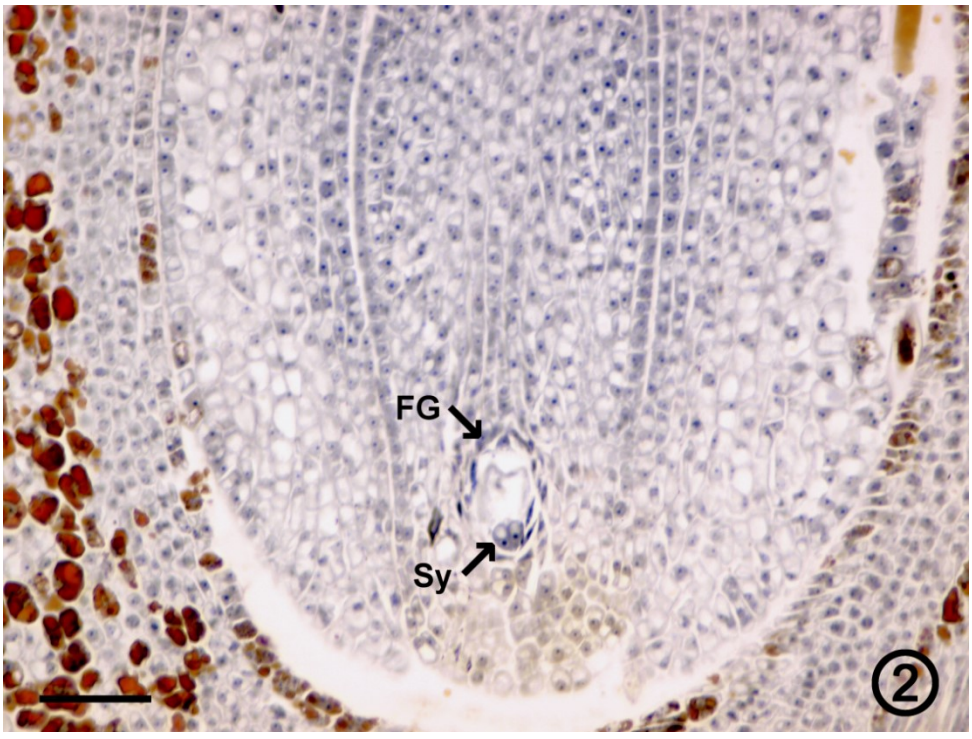
Dataset	Character	Eigenvectors	
PF, PE, PM, PC, PA		PC1	PC2
	plant height	0.377022	-0.246918
	laminar length	**0.404488**	0.136678
	laminar width	**0.406687**	-0.013642
	the widest part of lamina to tip	**0.402547**	0.145780
	lamina length/width	-0.157943	0.803334
	shape of lamina tip	0.368504	0.016545
	adaxial hairs	0.256984	0.502352
	abaxial hairs	0.374197	-0.030586
PF, PE, PM		PC1	PC2
	plant height	0.417087	-0.111662
	laminar length	**0.429537**	0.042236
	laminar width	**0.420463**	-0.207836
	the widest part of lamina to tip	**0.433166**	0.048850
	lamina length/width	-0.042484	0.816209
	shape of lamina tip	0.368942	0.239791
	adaxial hairs	-0.056427	0.430417
	abaxial hairs	0.368792	0.176663

Online Resource 6: Figures of embryological observations.

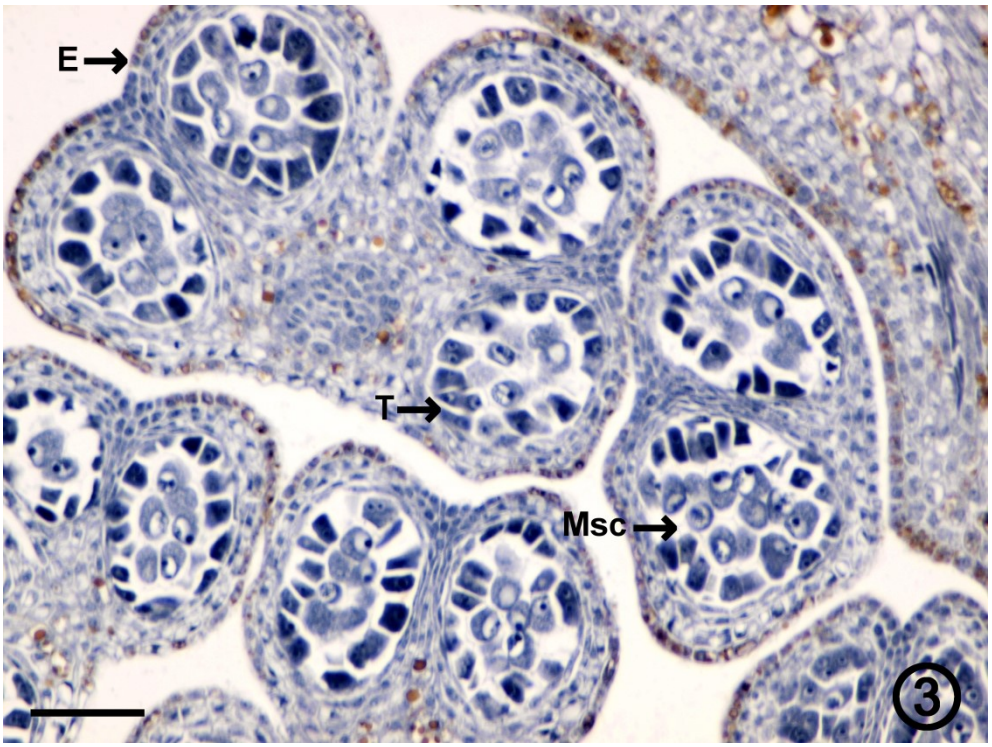
Development of female gametophyte

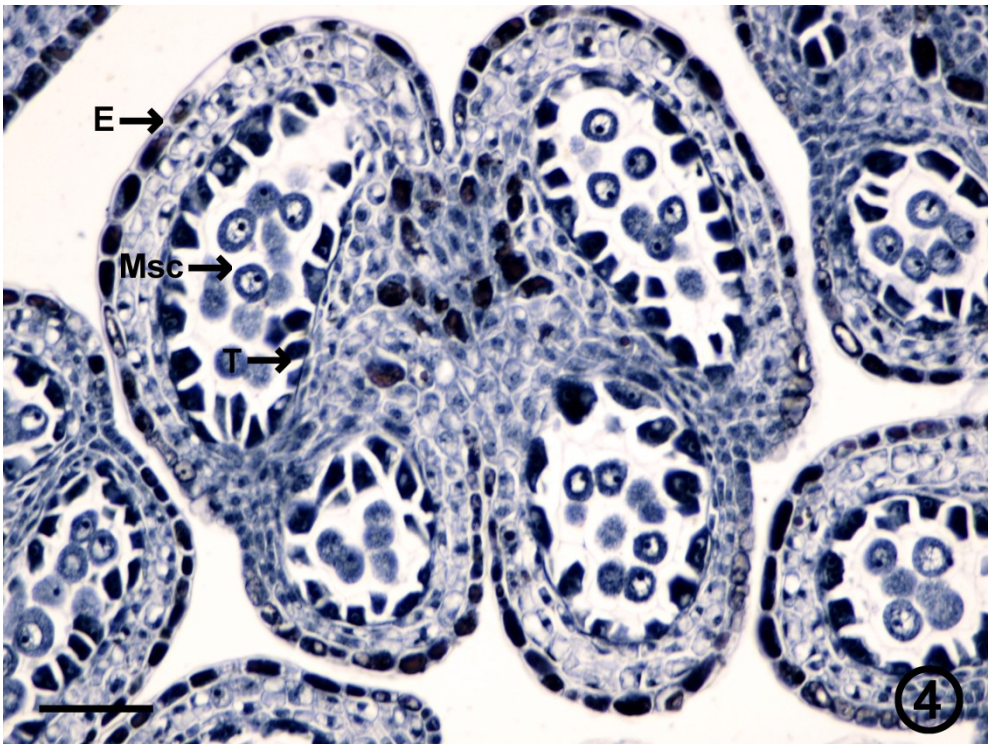
1 – Ovule with degenerate *Prunus* ×*mohacsyana* megaspores, (Nitra Pyramída hill, 16. 4. 2014), Bar = 50 µm
2 – Ovule with mature female *P. fruticosa* gametophyte, (Nitra Pyramída hill, 16. 4. 2014), Bar = 50 µm
FG – female gametophyte, Me – megaspores, Sy – synergids

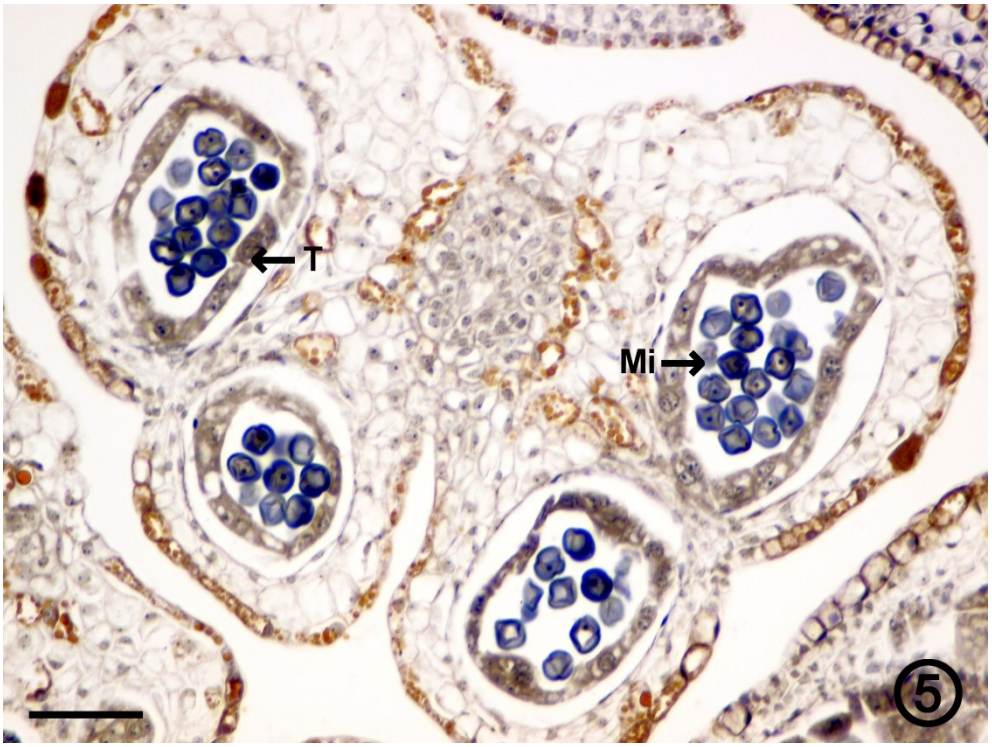


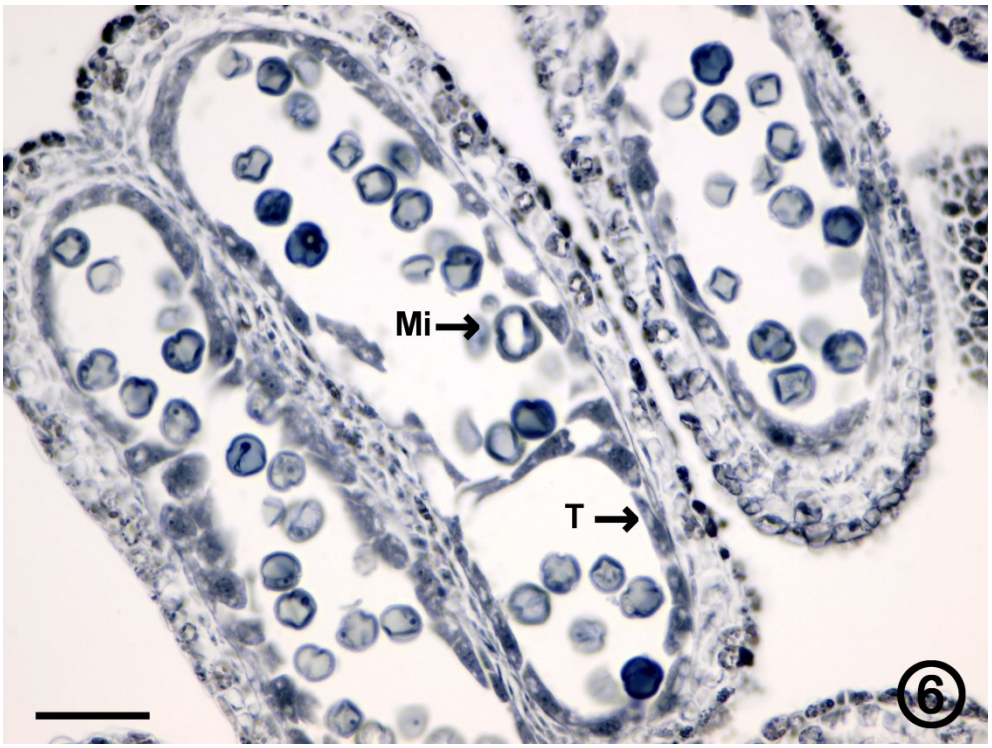


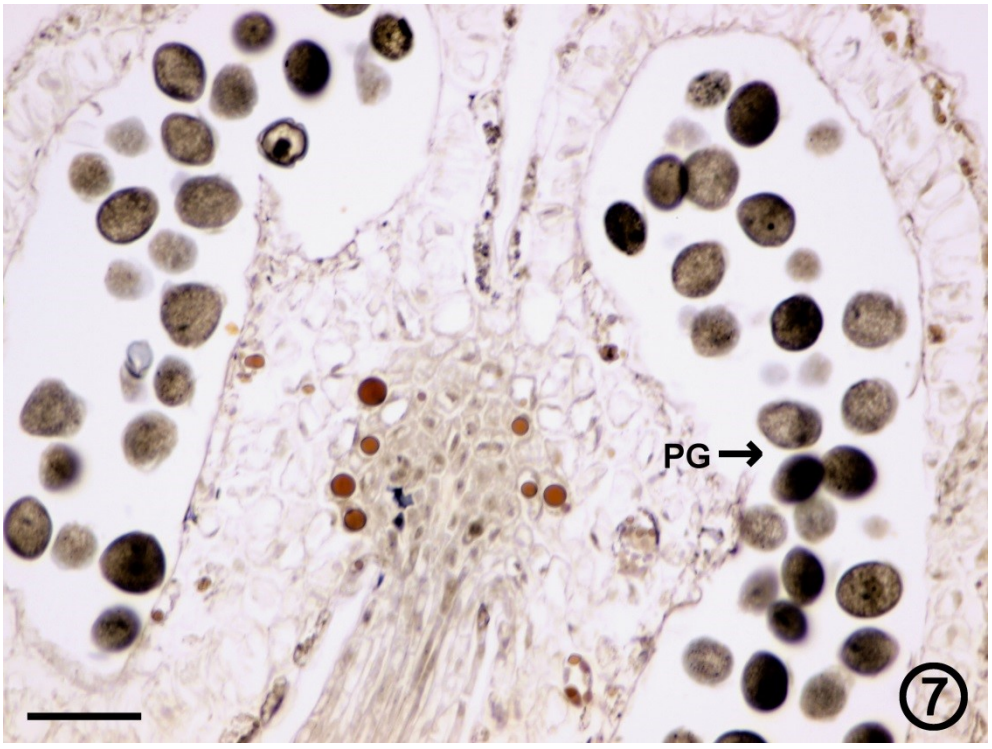
3 – Atypical shape of *Prunus* ×*mohacsyana* microsporocytes, (Nitra Pyramída hill, 20. 3. 2014), Bar = 50 µm
4 – Typical shape and size of *P. fruticosa* microsporocytes, (Nitra Pyramída hill, 20. 3. 2014), Bar = 50 µm
5 – Normally developed microspores in *P. fruticosa* anther (Nitra Pyramída hill, 29. 3. 2014), Bar = 50 µm
6 – One-celled pollen grain in *P.* ×*eminens* (Nitra St. Urban church, 16.4.2004), Bar = 50 µm
7 – Anthers with mature pollen grains of *P. fruticosa* (Salka the Sovie Vinohrady, 10. 4. 1999), Bar = 50 µm
8 – Microspores various size and shape of *P.* ×*mohacsyana*, (Nitra Pyramída hill, 29.3.2014), Bar = 50 µm
9 – Giant pollen grain and undeveloped pollen grains in anther of *P.* ×*mohacsyana*, (Nitra Pyramída hill, 16.4.2014), Bar = 50 µm
10 – Highly vacuolated cells of tapetum of *P.* ×*mohacsyana,* (Štúrovo the Vŕšok II hill, 4. 4. 2003), Bar = 50 µm
E – epidermis, End – endothecium, GPG – giant pollen grains, Mi – microspores, Msc – microsporocytes, PG – pollen grains, T – tapetum.




E
Msc
T
4


T
Mi
5


Mi
T
⑥


PG
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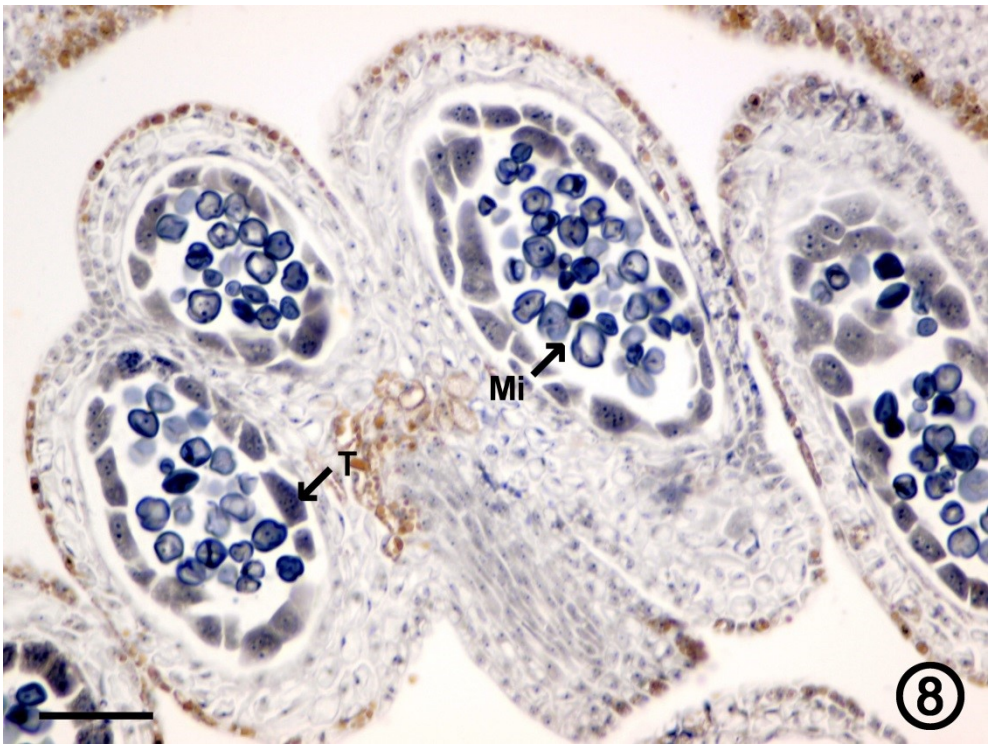
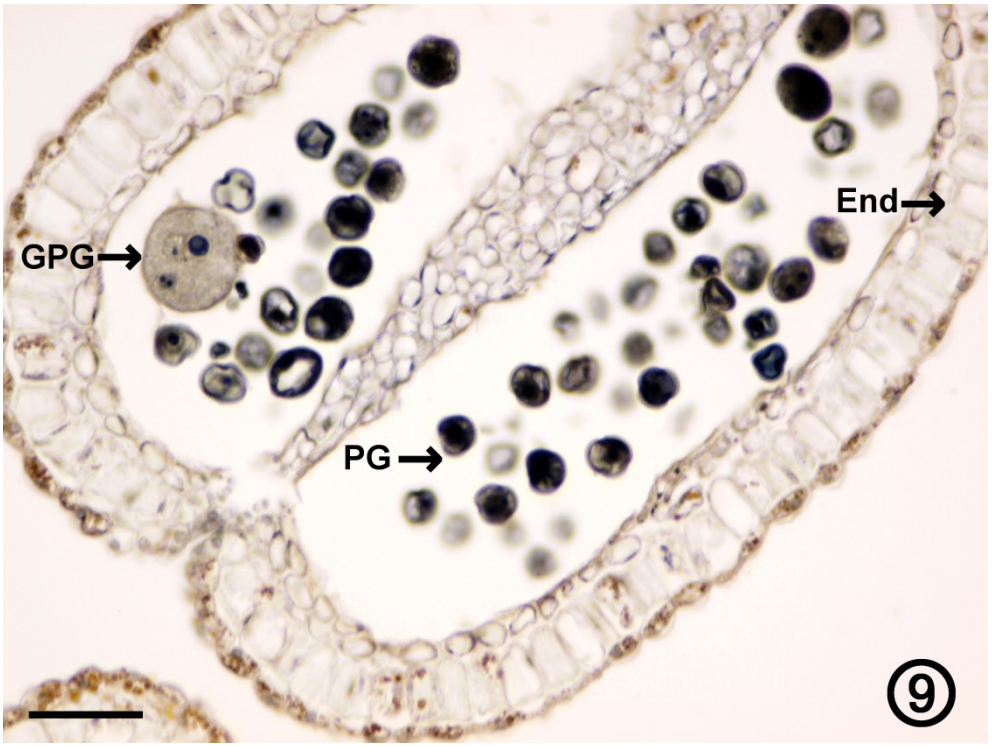
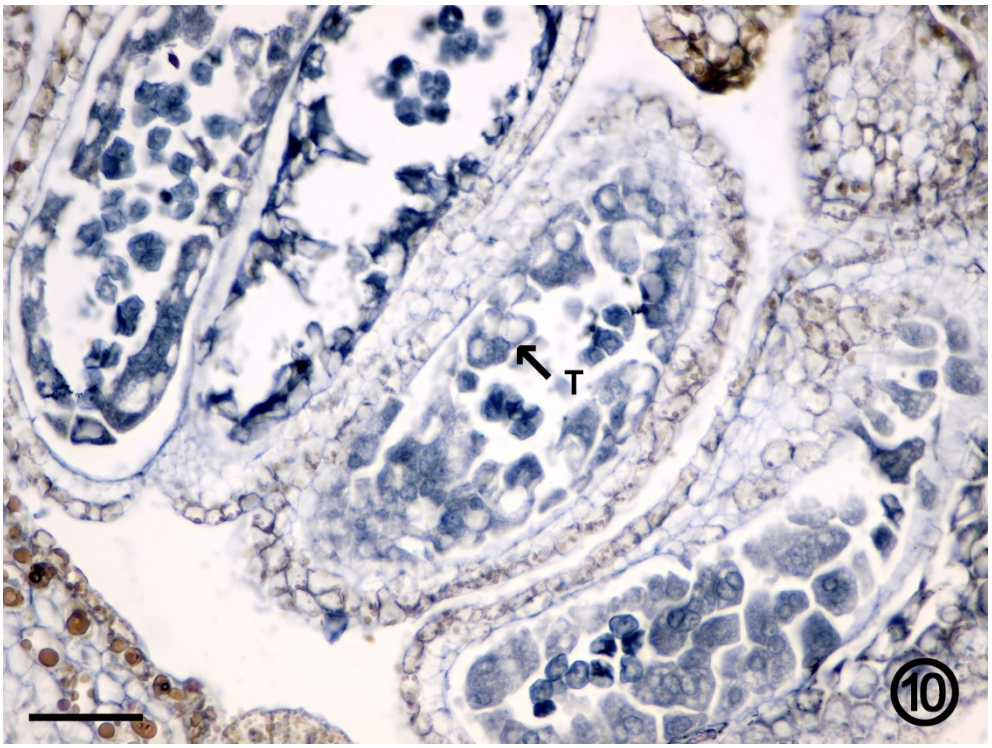
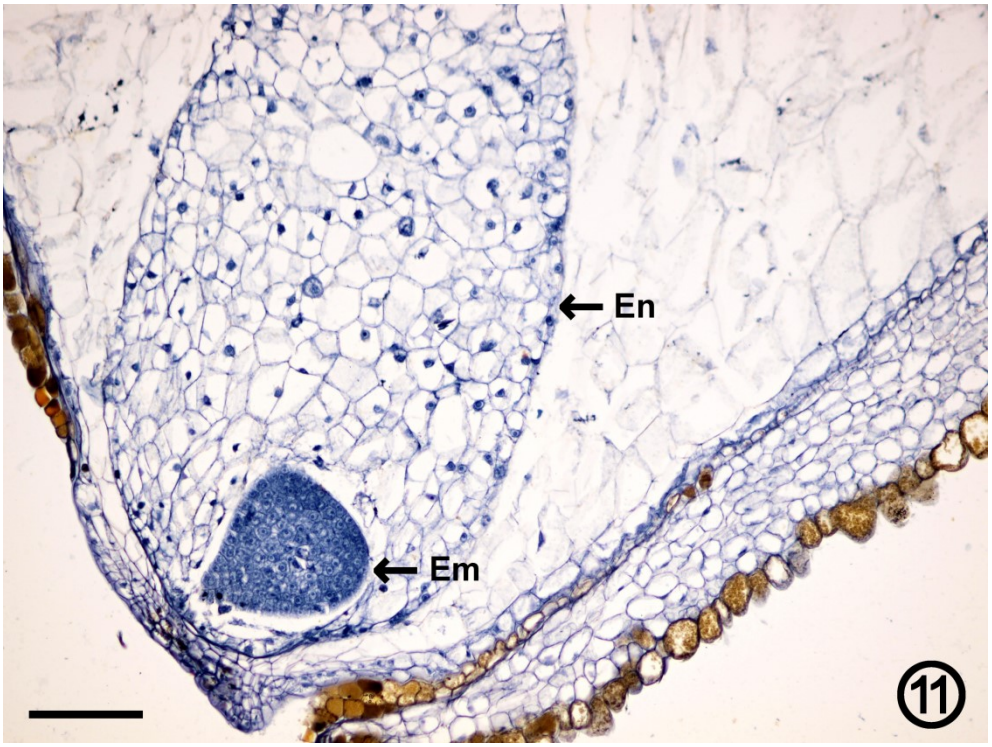

Mi
T
⑧

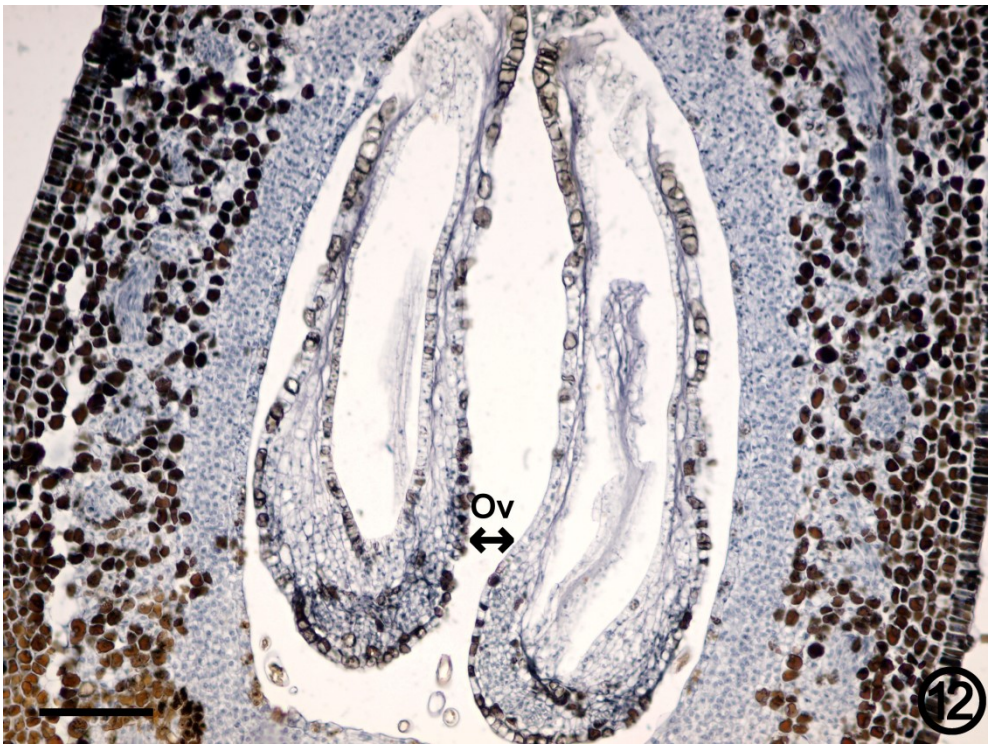

GPG
End
PG
⑨


T
⑩

Fertilization

11 – Late globular embryo of *Prunus fruticosa*, (Nitra Pyramída hill, 9.5.2014), Bar = 100 µm
12 – Ovary with two degenerate unfertilized ovules of *P.* ×*mohacsyana*, (Nitra Pyramída hill, 29. 4. 2014), Bar = 100 µm
Em – embryo, En – endosperm, Ov – ovule.





7.2 Case study II

Macková, L., Vít, P., & Urfus, T. (2018): **Crop-to-wild hybridization in cherries – Empirical evidence from *Prunus fruticosa*.** – Evolutionary Applications 11:1748–1759. doi: https://doi.org/10.1111/eva.12677

Crop-to-wild hybridization in cherries – Empirical evidence from *Prunus fruticosa*

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Abstract

Crop cultivation can lead to genetic swamping of indigenous species and thus pose a serious threat for biodiversity. The rare Eurasian tetraploid shrub *Prunus fruticosa* (ground cherry) is suspected of hybridizing with cultivated allochthonous tetraploid *P. cerasus* and autochthonous diploid *P. avium*. Three *Prunus* taxa (447 individuals of *P. fruticosa*, 43 of *P. cerasus* and 73 of *P. avium*) and their hybrids (198 individuals) were evaluated using analysis of absolute genome size/ploidy level and multivariate morphometrics. Flow cytometry revealed considerable differentiation in absolute genome size at the tetraploid level (average 2C of *P. fruticosa* = 1.30 pg, average 2C of *P. cerasus* = 1.42 pg, i.e., a 9.2% difference). The combination of methods used allowed us to ascertain the frequency of hybrids occurring under natural conditions in Central Europe. The morphological evaluation of leaves was based upon distance-based morphometrics supplemented by elliptic Fourier analysis. The results provided substantial evidence for ongoing hybridization (hybrids occurred in 39.5% of *P. fruticosa* populations). We detected homoploid introgressive hybridization with alien *P. cerasus* at the tetraploid level. We also found previously overlooked but frequent triploid hybrids resulting from heteroploid hybridization with indigenous *P. avium*, which, however, probably represent only the F1 generation. Although both hybrids differ in ploidy, they cannot be distinguished using morphometrics. Hybrids are frequent and may endanger wild populations of genuine *P. fruticosa* via direct niche competition or, alternatively or in addition, via introgression at the homoploid level (i.e., genetic swamping). The cultivation of cherries thus substantially threatens the existence of genuine *P. fruticosa*.

Keywords: absolute genome size, cherry, crop-to-wild gene flow, hybridization, introgression, ploidy level, *Prunus*

Introduction

Human activities significantly contribute to the reduction in global plant diversity (e.g. Frankham et al. 2010). Intensively studied phenomena such as degradation accompanied by fragmentation of natural habitats usually cause changes in the distribution of species, including extinction events or invasions (Corlett 2016). However, the adverse effects of hybridization on plant diversity have scarcely been evaluated (Ellstrand and Elam 1993; Levin et al. 1996; Rhymer and Simberloff 1996; Todesco et al. 2016). Besides invasive taxa

(e.g. Hejda et al. 2009), hybridization with commercial crops poses a significant threat to indigenous species (Ellstrand et al. 1999). The potential repercussions of hybridization have been repeatedly demonstrated (Todesco et al. 2016). However, even though commercial crops are ubiquitous, the topic of crop-to-wild hybridization has been addressed by relatively few empirical studies (e.g. Arrigo et al. 2011; Aerts et al. 2013).

Hybridization as an evolutionary process (together with polyploidization) significantly contributes to the diversity of vascular plants (Soltis and Soltis 2009). It may lead to evolutionary novelties and the establishment of new species. On the other hand, when reproduction barriers leak, hybridization followed by backcrossing may lead to the extinction of parental species (Rhymer and Simberloff 1996). The production of hybrid seeds increases, and the reproduction success of parental species is significantly reduced (Levin et al. 1996). Hybrids with the same or greater fitness as their parental species can significantly affect the populations of their parents (genetic swamping; Todesco et al. 2016). Last but not least, even the mere production of sterile hybrid individuals may lead to the extinction of rare parents through the wasteful production of maladapted hybrids, which decreases the number of potential mating partners, and by competition for resources and suitable niches (i.e., demographic swamping; Todesco et al. 2016).

Some rare (i.e., low abundance) species can hybridize with their widespread congeners (e.g. introgression of *Morus* L., Burgess et al. 2005; *Rumex* L., Ruhsam et al. 2015, which in extreme cases may lead to local extinction as a result of demographic or genetic swamping (Ellstrand and Elam 1993; Todesco et al. 2016). Introgressive hybrid swarms typically occur in transitional or peripheral habitats (e.g. Čertner et al. 2015; Raudnitschka et al. 2007). In addition, anthropogenic activities may promote the formation of hybrid swarms by enhancing secondary contact between species (e.g. Hanušová et al. 2014) or by creating open habitats suitable for the survival and expansion of hybrids (Wójcicki 1991). Hybridization with ubiquitously cultivated commercial, ornamental and consumer plants poses a threat to some indigenous species (Ellstrand et al. 2013).

Crop-to-wild gene flow has been documented in several indigenous plant species and may lead to the establishment of aggressive weeds or even the extinction of rare species (Ellstrand et al. 1999, 2013). So far, only a few human-induced (i.e., with the participation of crop plants) cases of hybridization have been reported. Spontaneous introgression of wild *Prunus orientalis* (Duhamel) by cultivated *Prunus dulcis* (Mill.) D. A. Webb in south-west Asia (Delplancke et al. 2012) and genetic erosion of the rare wild species *Malus sylvestris* (L.) Mill. in Belgium by domesticated apple (*Malus domestica* Borkh.; Coart et al. 2006) often serve as model examples. One extreme case of crop-to-wild gene flow is the genus *Aegilops* L. in the Mediterranean, where more than one quarter of some wild populations bear signs of introgression from wheat (Arrigo et al. 2011). Besides conservation consequences, genetic swamping of wild relatives via hybridization with crops can lead to tremendous economic losses because wild taxa serve as an essential gene pool resource for breeding programmes (Ganopoulos et al. 2013; Barać et al. 2017).

A prime example of a species endangered by human-induced gene flow from cultivated crops is *Prunus fruticosa* Pall. (ground cherry), a rare and morphologically variable relict Eurasian shrub of steppes and forest steppes (Meusel et al. 1965; Jäger and Seidel 1995; Rhodes and Maxted 2016). It is tetraploid (2n = 32 chromosomes; Oldén and Nybom 1968; Scholz and Scholz 1995) and self-incompatible (also propagated by root shoots; Scholz and Scholz 1995; Pruski 2007). *Prunus fruticosa* is of potentially considerable importance in cherry breeding programmes, as it possesses suitable characters for growing in steppe conditions (Iezzoni and Mulinix 1992; Dzhangaliev et al. 2003; Pruski 2007; Iezzoni 2008; Barać et al. 2017). Widely cultivated sour and sweet cherries (*Prunus cerasus* L. and *Prunus avium* (L.) L.) are close relatives of *P. fruticosa* and easily hybridize with it

(e.g. Scholz and Scholz 1995). Whereas diploid *P. avium* is an indigenous European taxon (2n = 16; Webb 1968; Marhold and Wójcicki 1992; Jäger and Seidel 1995), tetraploid *P. cerasus* in Europe is an alien species that occasionally escapes from cultivation (e.g. Webb 1968; Scholz and Scholz 1995). *Prunus cerasus* has been proven to be an allotetraploid that has originated through hybridization of *P. fruticosa* and *P. avium* (2n = 32; Oldén and Nybom 1968; Schuster and Schreibner 2000; Tavaud et al. 2004; Horvath et al. 2008).

The enormous morphological variation of *Prunus fruticosa* has been repeatedly ascribed to interspecific hybridization (e.g. Chrtek 1992; Scholz and Scholz 1995). On the basis of morphology (the purported discriminative characters being plant height and hairs on the abaxial surface of the lamina), two types of hybrids have been described (Wójcicki 1988; Lepší et al. 2011). One of them, *Prunus ×eminens* Beck (*P. fruticosa* × *P. cerasus*; 2n = 4x = 32; Webb 1968; Wójcicki 1991; Scholz and Scholz 1995), has been reported to be abundant (35% of hybrids estimated in the Czech Republic and Slovakia; Wójcicki & Marhold, 1993) and partly fertile (Macková et al. 2017) whereas the other, *Prunus ×mohacsyana* Kárpáti (*P. fruticosa* × *P. avium*; 2n = 3x = 24; Oldén and Nybom 1968; Marhold and Wójcicki 1992), has been recorded only extremely rarely (Wójcicki and Marhold 1993; Scholz and Scholz 1995; Macková et al. 2017) and has been confirmed to be sterile (Macková et al. 2017). Thus, hybridization appears to be a major threat to *P. fruticosa* that is directly connected with human activities such as the cultivation of cherries (Wójcicki 1991; Wójcicki and Marhold 1993; Boratyński et al. 2003).

In contrast to morphology, which has hitherto been used to indicate *P. fruticosa* hybridization, nuclear DNA content represents a highly reproducible species-specific marker (Loureiro et al. 2010) and is convenient for the delimitation of *Prunus* taxa because particular species differ in their ploidy level or absolute genome size (e.g. Baird et al. 1994; Maghuly et al. 2010; García-Verdugo et al. 2013; Macková et al. 2017). Without the use of additional markers (e.g. genome size or ploidy level), it is often difficult to accurately identify hybrids and pure individuals based on morphology only (Ruhsam et al. 2015; Vítová et al. 2015), and this can result in the misled protection of hybrid populations (Kabátová et al. 2014; Vít et al. 2014).

The main goal of this study was to examine the extent of interspecific hybridization of the rare species *Prunus fruticosa* with wild and cultivated cherries (*P. cerasus* and *P. avium*) and to evaluate the impact of hybridization on pure *Prunus fruticosa* populations in Central Europe. To meet this goal, we addressed the following questions: (a) Do ploidy level and absolute genome size correlate with patterns of morphology and delimit *Prunus* taxa on a large spatial scale? (b) What is the frequency of hybrids under natural conditions? and (c) May the presence of hybrids indicate that populations of *P. fruticosa* are under threat from hybridization (incl. introgression)? To find answers to these questions, we collected fresh plant material in natural populations, estimated their nuclear DNA content using flow cytometry and employed distance-based morphometrics together with elliptic Fourier analysis to describe the variation in short-shoot leaves.

Materials and methods

Sampling

Samples from the Central European area (76 populations – 46 *Prunus fruticosa*, 12 *Prunus ×mohacsyana*, 10 *Prunus ×eminens*, eight mixed) were collected in 2010–2013 in the Czech Republic (54 populations), Slovakia (13 populations) and Poland (seven populations; marginally also in Romania – two populations; Figure 1, Supporting information

Table S1). Samples of the putative parents *Prunus cerasus* (43 individuals from 12 locations) and *Prunus*
avium (73 individuals from 38 locations) were also collected in the study area for a better understanding of ongoing microevolutionary processes. Each population sample (usually 5–10 individuals, depending on population size) was represented by a branchlet with vegetative short-shoot leaves. Sampled individuals were as distant from each other as possible to avoid the collection of clonally emerged individuals. Individuals growing together in one place obviously separated from another place were considered a discrete population. As regards *P. cerasus* and *P. avium*, about three individuals were sampled from each location because these cultivated taxa are scattered in the landscape instead of constituting numerous populations.

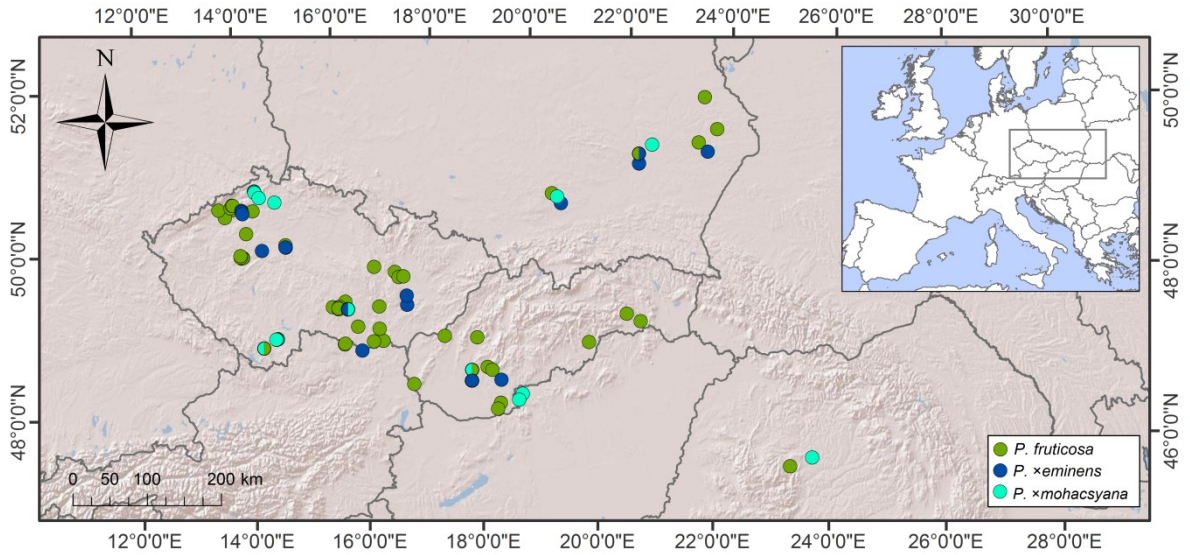

Fig. 1: Sample locations of *Prunus fruticosa* and its hybrids in Central Europe.

The taxa were determined based on their ploidy level (indicating triploid *Prunus ×mohacsyana* and diploid *P. avium*). Tetraploids were differentiated based on the presence of hairs on the abaxial surface of the lamina (glabrous *P. fruticosa* vs hairy *P. ×eminens* and *P. cerasus*) and growth form (shrubby *P. fruticosa* and *P. ×eminens* vs tree--like *P. cerasus*).

In total, plant material from 761 individuals of *Prunus* taxa (447 *P. fruticosa*, 99 *Prunus ×mohacsyana*, 99 *P. ×eminens*, 43 *P. cerasus* and 73 *P. avium*) were used for three types of analyses – absolute genome size analysis using flow cytometry (FCM), distance--based morphometrics and elliptic Fourier analysis. Dry plant material was used (short-shoot leaves taped on to sheets of cardboard) for morphometrics, and fresh plant material was necessary for flow cytometric analysis.

Flow cytometry (FCM)

Ploidy levels/absolute genome sizes of 761 individuals (see Supporting information Table S1 for samples details) were estimated using a Partec CyFlow instrument (Partec GmbH, Münster, Germany) equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW). A slightly modified procedure following (Doležel et al. 2007) was adopted for the isolation and of staining nuclei. *Bellis perennis* L. (2C = 3.38 pg; Schönswetter et al. 2007) was used as the internal standard. About 1.5 cm^2 of fresh laminar tissue together

with 1.8 cm^2 of the internal standard was chopped in 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; Doležel et al. 2007) in a Petri dish. The suspension was filtered through a 42-µm nylon mesh filter and incubated for at least 20 min at room temperature. The suspension was then stained by a solution containing 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$; Doležel et al. 2007), β-mercaptoethanol (final concentration of 2 µl/ml), propidium iodide and RNase IIA (both at the final concentrations of 50 µg/ml). Subsequently, stained samples were run through the flow cytometer. Isolated stained nuclei were excited with a laser beam, and the fluorescence intensity of 3,000 particles was recorded.

Because of the significant amounts of secondary metabolites contained in *Prunus* material (typical of the whole Rosaceae), which complicate FCM analyses, certain optimization steps had to be carried out (for details, see Macková et al. 2017). Although most of the samples were measured at one time point only, we checked the stability of FCM measurements over a long time period (from May to August, 18 individuals from three locations). Variation between two different measurements did not exceed 4% (for information on the stability of FCM measurements over short periods; see Macková et al. 2017). The whole range of measured absolute genome size values was calibrated by chromosome counts (standard karyological methodology; e.g. Lepší et al. 2008).

Resulting FCM histograms were analysed using FloMax (version 2.4d, Partec, Münster, Germany). Absolute genome size values were visualized as boxplots in PAST 2.17c (Hammer et al. 2001) and as scatter plots in Microsoft Excel 2010. One-way ANOVA followed by Tukey's HSD test in PAST 2.17c (Hammer et al. 2001) was used to ascertain the significance of absolute genome size differences between species.

Distance-based morphometrics

To examine morphological variation of the *Prunus* taxa under study, 17 characters (13 primary, four ratio) – eight vegetative and nine generative (see Table 1) – were selected based on the literature (Wójcicki 1988, 1991; Wójcicki and Marhold 1993; Lepší et al. 2011) and own field observations. Well-developed short-shoot leaves (two leaves per individual) and flowers were measured using a digital calliper (accuracy 0.01 mm) and a stereo microscope (Olympus SZ51; magnification 40×). Most of the time, only short-shoot leaves were observed (because of their narrower range of variation; Marhold and Wójcicki 1992). Plant height was measured in the field. Abaxial hairs were measured on at least four leaves per individual and then averaged. Plant height, shape of laminar tip, adaxial hairs and abaxial hairs were evaluated using semiquantitative scales (see Table 1). In total, 1,422 leaves (see Supporting information Table S1 for samples details) and only 84 flowers were measured because the flowering period was very short. Because *P. fruticosa* scarcely bears fruits (Chudíková et al. 2012), no fruits were included in the study.

Tab. 1: List of measured characters on vegetative and generative organs of *Prunus* taxa under study used in distance-based morphometric analysis.

Description of character	Abbreviation	Unit
Plant height	Height	1 = to 50 cm, 2 = 50-100 cm, 3 = over 100 cm, 4 = tree
Laminar length	Length	mm
Laminar width	Width	mm
Distance from the widest part of the lamina to the laminar tip	Widest to tip	mm
Shape of laminar tip	Tip	1 = obtuse, 2 = obovate, 3 = elliptic with aristate apex, 4 = elliptic with broadly acuminate apex
Adaxial hairs (density of hairs on the adaxial surface of lamina)	Adax hairs	1 = glabrous, 2 = short hairs, 3 = long hairs, 4 = long and also short hairs
Abaxial hairs (density of hairs on the abaxial surface of lamina)	Abax hairs	1 = glabrous, 2 = scattered pubescent, 3 = sparsely pubescent, 4 = densely pubescent
Laminar length/width (ratio of length and width of the lamina)	Length/width	–
Petal length	–	mm
Petal width	–	mm
Hypanthium length	–	mm
Sepal length	–	mm
Sepal width	–	mm
Peduncle length	–	mm
Petal length/width (ratio of length and width of the petal)	–	–
Sepal length/width (ratio of length and width of the sepal)	–	–
Hypanthium length/sepal length (ratio of hypanthium and sepal length)	–	–

The data matrix was evaluated using multivariate statistical methods in PAST 2.17c (Hammer et al. 2001). Basic descriptive statistics, including the minimum, maximum, mean and the 25th and 75th percentile, were computed for each of the vegetative characters of all taxa under study. Principal component analysis (PCA) was employed to visualize the basic structure of the data in Canoco 5 (ter Braak and Šmilauer 2012). Absolute genome size was passively projected on to PCA diagrams using a local regression (loess) model in Canoco 5 (ter Braak and Šmilauer 2012). Redundancy analysis (RDA; van den Wollenberg 1977) with a Monte Carlo permutation test (999 permutations) performed in Canoco 5 (ter Braak and Šmilauer 2012) and correlation analysis carried out in R 3.4.3 (R Core Team, 2017; visualized using Microsoft Excel 2010) were used to test for a link between morphological variation (represented by PC1 scores of distance-based PCA) and absolute genome size.

Elliptic Fourier analysis

Shape contours of 1,407 leaves (see Supporting information Table S1 for samples details) were investigated using elliptic Fourier analysis. Only well-developed leaves were included in the analysis (15 partly damaged leaves were excluded). Two leaves of each individual were taped on to a sheet of cardboard paper and scanned (scanner Canon MP270 series Printer; 300 dpi). For leaf shape analysis based on elliptic Fourier descriptors (Kuhl and Giardina 1982), the SHAPE 1.3 package (Iwata and Ukai 2002) was employed. The leaf shapes were converted into chain codes using ChainCoder, and the CHC2NEF programme converted these chain codes into coefficients of elliptic Fourier descriptors (using 20 harmonic axes). These coefficients were used to calculate the scores of principal components using the PrinComp function. The PrinComp routine also allowed the reconstruction of the leaf shape, corresponding to values of +2 and −2 standard deviations on the first and second component axes (see Lepší et al. 2009 and Macková et al. 2017, for details). The first and second component axes were visualized using Microsoft Excel 2010.

Results

Absolute genome size and DNA ploidy level

Ploidy levels and absolute genome sizes of 761 *Prunus* accessions were ascertained by flow cytometry (see Supporting information Table S1 for samples details). Three ploidy levels were detected: diploid (*P. avium*; average 2C = 0.73 pg), triploid (*P.* ×*mohacsyana*; average 2C = 1.01 pg) and tetraploid (*P. fruticosa*, *P.* ×*eminens* and *P. cerasus*; Figure 2, Supporting information Table S2). Moreover, the three tetraploid taxa tended to differ in absolute genome size (*P. fruticosa* – average 2C = 1.30 pg, *P.* ×*eminens* – average 2C = 1.36 pg, *P. cerasus* – average 2C = 1.42 pg, i.e., a 9.2% difference between parental taxa; Supporting information Table S2). Absolute genome size values of tetraploid taxa formed a continuous series of partly overlapping values (Figure 2). Nevertheless, absolute genome size differed significantly between all analysed groups ($F_{4,\ 755} = 8826$, $p < 0.001$) as well as between the three tetraploid taxa ($F_{2,\ 585} = 311.8$, $p < 0.001$) in ANOVA. Separate Tukey's HSD tests revealed five and three groups for all and for the three tetraploid taxa, respectively.

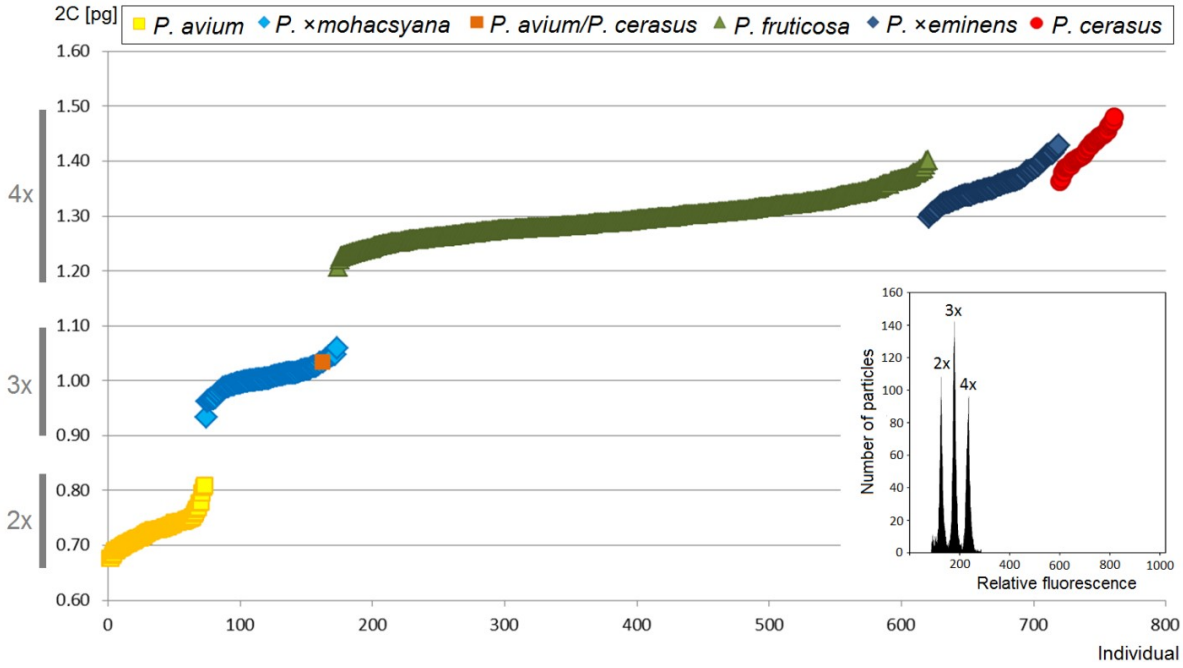

Fig. 2: Absolute genome size variation of the five *Prunus* taxa under study. PI-stained nuclei isolated from 761 leaves. Three ploidy levels were detected: diploid (2x), triploid (3x) and tetraploid (4x). The values are in picograms (pg). Orange-highlighted individual represent triploid with a well-developed trunk. A histogram of simultaneous flow cytometric analyses of three ploidy levels is in the right corner. Peak designations: 2x = diploid *P. avium*, 3x = triploid *Prunus* tree form, 4x = tetraploid *P. cerasus*.

Distance-based morphometrics

Morphometric variation of 1,422 leaves (see Supporting information Table S1 for samples details) was analysed using distance-based morphometrics (for descriptive statistics; see Supplementary Table S3). Principal component analysis (PCA) of all five taxa under study, based on eight vegetative characters of leaves, revealed three obvious groups of putative parental taxa: *P. fruticosa*, *P. cerasus* and *P. avium* (although *P. cerasus* and *P. avium* partly overlapped; Figure 3). The hybrids *P. ×mohacsyana* and *P. ×eminens* formed a compact, overlapping cluster between their putative parents (the first and the second axes explaining 66.5 and 13.7% of the variation, respectively; Figure 3). The distance from the widest part of the lamina to the laminar tip, laminar width and laminar length was the most tightly correlated (see Supporting information Figure S1) with the first component axis. Thus, the eight vegetative characters measured on leaves could not distinguish between the hybrids. The hybrids grouped together even in the case of PCA using characters on generative organs (84 flowers – 42 *P. fruticosa*, 27 *P. ×mohacsyana*, 15 *P. ×eminens*; Supporting information Figure S2). PCA of only tetraploid taxa showed clearly distinguished putative parental taxa (*P. fruticosa* and *P. cerasus*) with the hybrid *P. ×eminens* scattered between them with a partial overlap (the first and the second axes explaining 59.5 and 15.5% of the variation, respectively; Figure 4). It is important that absolute genome size appeared to be well correlated with the first PCA axis; absolute genome size tended to increase from *P. fruticosa* to *P. cerasus* (see the perpendicularly oriented loess curves in Figure 4). The significant association between leaf morphology and absolute genome size of tetraploid taxa was further confirmed by RDA (p = 0.001, 999 permutations); absolute genome size explained 31.9% of the variation (Supporting information Figure S3A). Five morphological characters (Width, Widest to tip, Length, Abax hairs, Height, Tip) exhibited

strong positive correlation with the canonical/genome size axis (see Table 1 for character abbreviations; Supporting information Figure S3B). Moreover, a significant correlation between leaf morphology (represented by PC1 scores) and absolute genome size was found ($r = 0.729$; $t = 35.3$, $df = 1097$, $p < 0.001$), explaining 53% of the overall variation (Figure 5).

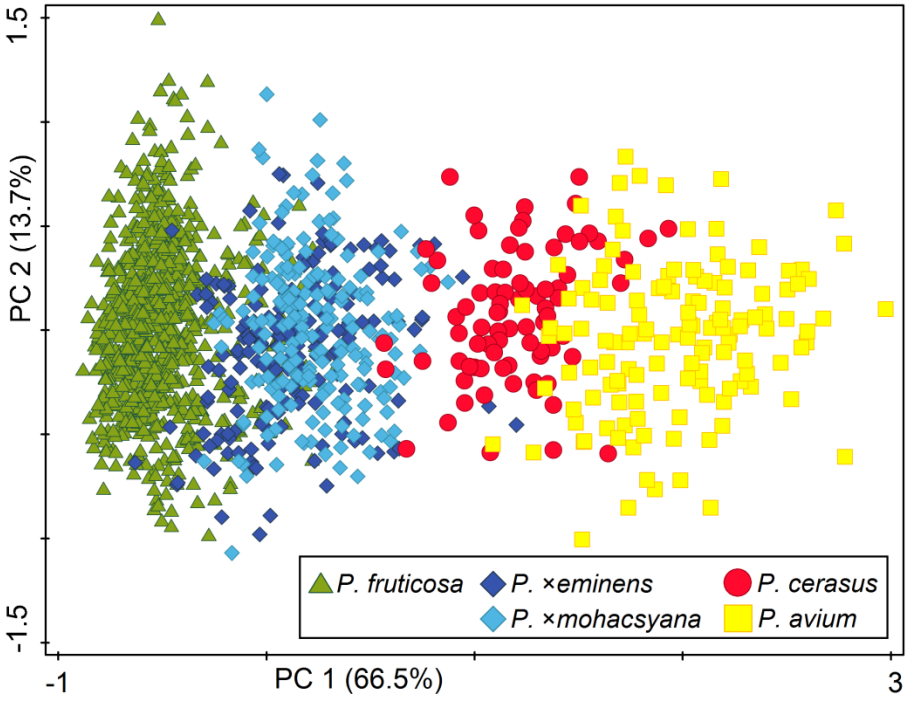

Fig. 3: Ordination diagram of principal component analysis using eight vegetative morphological characters of 1,422 leaves of *Prunus* taxa under study.

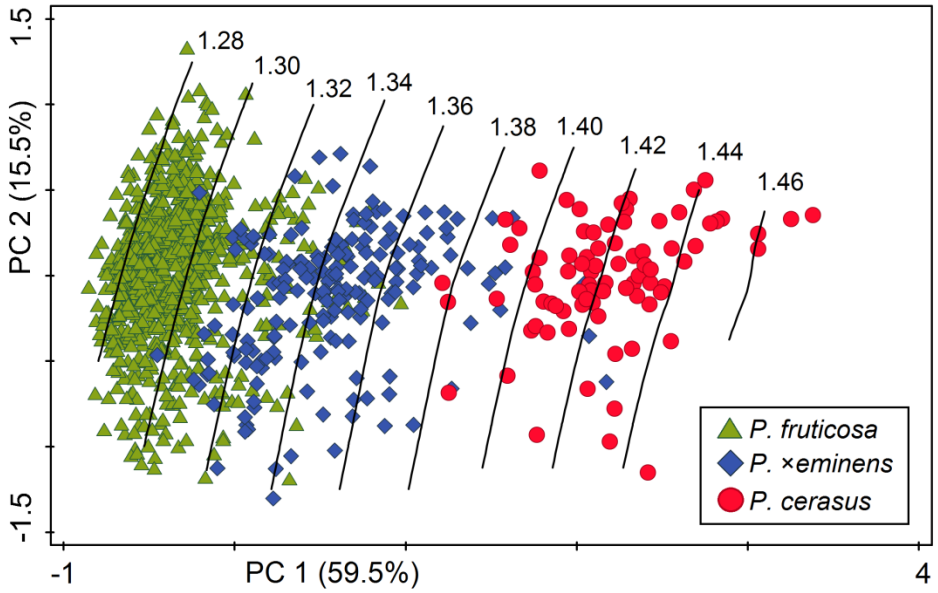

Fig. 4: Correspondence of morphological variation and absolute genome size in three *Prunus* taxa studied. Ordination diagram of principal component analysis based on eight vegetative morphological characters of 1,099 leaves of tetraploid *Prunus* taxa. Absolute genome size (values in pg DNA) is passively projected on to the diagram using a local regression (loess) model.

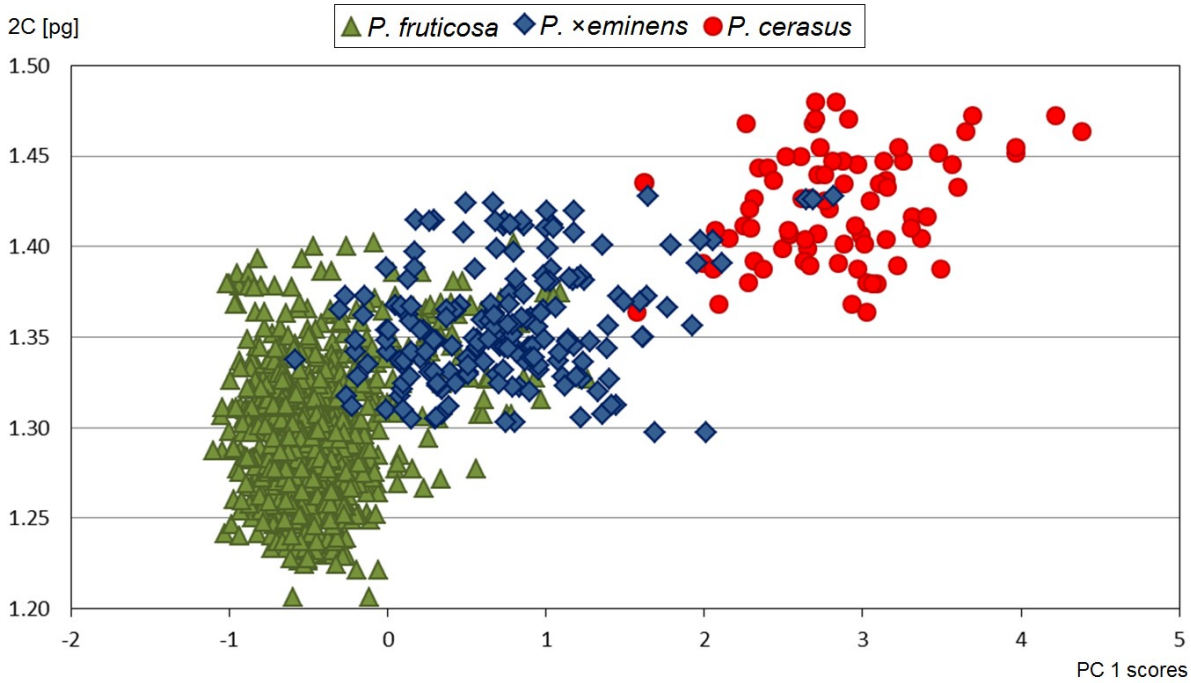

Fig. 5: Correlation analysis of tetraploid *Prunus* taxa (1,099 individuals) under study, showing a link between morphology (represented by the first principal component scores) and absolute genome size, explaining 53% of the overall variation ($r = 0.729$; $t = 35.3$, $df = 1097$, $p < 0.001$).

Elliptic Fourier analysis

Variation in the shape contours of 1,407 leaves (see Supporting information Table S1 for samples details) was evaluated using elliptic Fourier analysis. The groups of *Prunus* taxa under study overlapped more in comparison with distance-based morphometrics (Figure 6). *Prunus avium* formed the most differentiated cluster, while the *P. fruticosa* cluster was distinguished only partly. Nevertheless, both overlapped with other *Prunus* taxa in the principal component analysis. On the contrary, *P. cerasus* and both hybrids were scattered between these two clusters and formed a linked and completely overlapping cluster (Figure 6). The first component axis (68.8%) explained the most variation but was not taxonomic specific (variation in relative leaf width), while the second component axis (13.9%), describing variation in the shape of the leaf base and the shape of the leaf tip, reflecting differences between the taxa studied. The most differentiated groups, *P. avium* and *P. fruticosa*, had elliptic leaves with an aristate apex and obovate leaves with an obtuse apex, respectively. *Prunus cerasus*, *P. ×eminens* and *P. ×mohacsyana* clustered together and tended to form elliptic leaves with a broadly acuminate apex, never obtuse or with an aristate apex (Figure 6). Thus, leaf shape represents a suitable additional character for the determination of parental *Prunus* taxa; however, it fails to distinguish hybrids (similar to distance-based morphometrics).

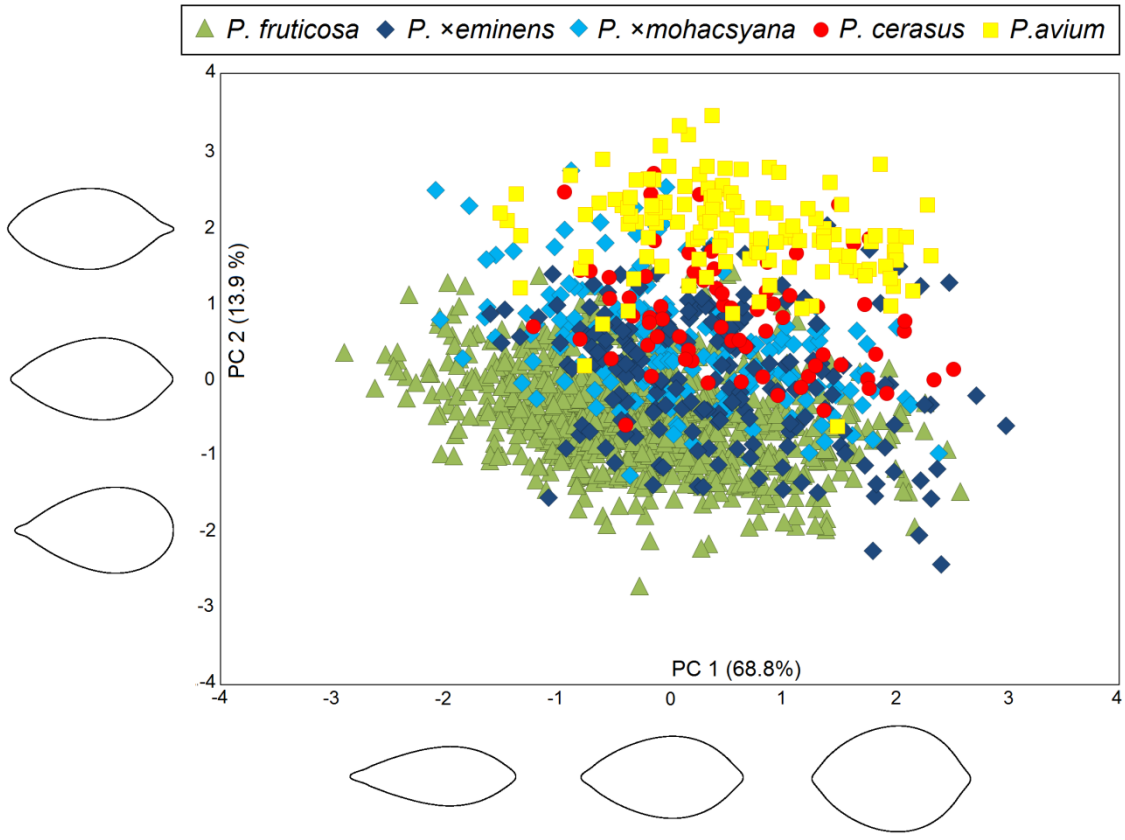

Fig. 6: Ordination diagram of principal component analysis of Fourier coefficients describing variability in laminar shape of 1,407 leaves of the *Prunus* taxa under study. PCA scores are standardized to unit variance (units in standard deviation, SD). Reconstructed leaf contours (petiole connection on the left) corresponding to values of −2 SD, 0 and +2 SD are shown along the PC axes.

Frequency of hybrids under natural conditions

Our multidisciplinary approach has revealed that only 60.5% of populations previously reported to represent genuine *Prunus fruticosa* did not include hybrids; actually, 39.5% of the populations were of hybrid origin (randomly spatially distributed). Most of the hybrid populations under study were composed exclusively of individuals belonging to one of the hybrids; 15.8% of populations consisted solely of *P.* ×*mohacsyana* and 13.2% solely of *P.* ×*eminens*. Only 1.3% of populations included both hybrids. At last, 9.2% of the populations analysed were mixed (i.e., composed of *P. fruticosa* and one of its hybrids).

Discussion

Absolute genome size/ploidy level estimation coupled with morphometrics allowed us to identify the *Prunus* species and hybrids concerned (which occurred in 39.5% of populations under study). Homoploid hybridization between the tetraploid parental taxa *Prunus fruticosa* and *P. cerasus* produces tetraploid hybrids (*P.* ×*eminens*). By contrast, heteroploid hybridization between *P. fruticosa* with *P. avium* generates triploids (*P.* ×*mohacsyana*). The frequencies of the two hybrids turned out to be almost equal in the study area. In contrast to previous attempts to assess the rate of hybridization, which were based solely on morphometrics, our multidisciplinary approach revealed a continuous pattern, pointing to introgression.

Flow cytometry has been employed in several descriptive or local studies of *Prunus* (Dickson et al. 1992; Bennett and Leitch 1995; Macková et al. 2017) and published genome size values fall within the range of measured values presented here. The morphological pattern is also analogous to those found in previous studies (Wójcicki 1991; Wójcicki and Marhold 1993; Lepší et al. 2011). Traditionally used morphological characters (abaxial hairs and plant height; Wójcicki 1988; Lepší et al. 2011) have turned out to be more suitable in the field than the first three characters identified by morphometrics (i.e., distance from the widest part of the lamina to the laminar tip, laminar width and laminar length). However, the morphology-based determinations of hybrid groups used in previous studies were probably not correct (Wójcicki 1991; Wójcicki and Marhold 1993; Lepší et al. 2011; Chudíková et al. 2012). Until now, almost all hybrids had been suggested to be tetraploid (*P.* ×*eminens*; Wójcicki 1991; Wójcicki and Marhold 1993; Lepší et al. 2011), but our data show that the frequency of triploid hybrids, which is roughly 50%, had been considerably underestimated. Leaf shape (elliptic Fourier analysis) seems to be a useful complementary trait for distinguishing between pure *Prunus* species and hybrids, and a similar pattern was also detected in one local study of *P. fruticosa* (Czech Republic; Lepší et al. 2011). Thus, based on DNA ploidy level knowledge, the results of previous studies (Wójcicki 1991; Wójcicki and Marhold 1993; Lepší et al. 2011; Chudíková et al. 2012) might have to be substantially reevaluated.

Identity of hybrids

Due to the broad range of absolute genome sizes possessed by the parental species and their hybrids, it is almost impossible to distinguish cytometrically between F1 hybrids and their more complex backcrossed counterparts at the homoploid level (i.e., 4x). Moreover, an intermediate genome size does not necessarily indicate an F1 hybrid. To draw the conclusion that a plant is an F1 hybrid, one has to rule out the possibility that it is a higher or even backcrossed hybrid. Continuous patterns of absolute genome size are nevertheless

usually accompanied by enormous morphological variation, and a continuous pattern of data distribution in both absolute genome size and morphology is usually indicative of introgressive hybridization (e.g. Šmarda and Bureš 2006; Suda et al. 2007; Hanušová et al. 2014). In addition, our correlation analysis and RDA revealed that hybrids with an absolute genome size similar to that of one of their parental taxa are also morphologically close to that parent, which indicates that they are almost certainly backcrossed. A high probability of backcrossing at the tetraploid level is further supported by the substantial fertility of *P.* ×*eminens* (based on embryology; Macková et al. 2017). By contrast, heteroploid hybridization (i.e., 4x × 2x) produces comparatively straightforward results due to the existence of an effective triploid block, which constrains backcrossing; this has been proved in the case of triploid *P.* ×*mohacsyana* (Macková et al. 2017).

Crop-to-wild studies and their limitations

Human-induced hybridization (or even introgression) affects wild plant species in different ways, and there are several cases that are analogous to that of *Prunus fruticosa*. While hybridization of cultivated *Saccharum* L. or *Brassica* L. with wild counterparts does not pose any risk to their wild relatives, hybridization of cultivated *Oryza* L. and *Gossypium* L. has been implicated in the near extinction of certain wild species of rice and cottonseed (Ellstrand et al. 1999).

Studies dealing with crop-to-wild gene flow rely on the ability to unequivocally distinguish between wild and cultivated plant forms. In most cases, however, this discrimination is not possible based solely on morphological grounds (e.g. *Malus* Mill., Coart et al. 2006; *Vitis* L., de Andrés et al. 2012). Plant sex might serve as another suitable and conspicuous differential trait (e.g. dioecious wild vs mostly hermaphroditic cultivated forms of *Vitis*; de Andrés et al. 2012). Their discrimination is made markedly easier if a wild species and its cultivated counterpart differ in growth form (e.g. shrub vs tree form in *Prunus*; Delplancke et al. 2012; Macková et al. 2017). The combined approach (absolute genome size/ploidy level and morphology) allowed us to distinguish between wild and cultivated *Prunus* plants. Whereas most studies of crop-to-wild introgression deal with rather small datasets (e.g. 237 samples in *Vitis*; de Andrés et al. 2012), our study is based on more than 700 individuals distributed in the Central European region.

Moreover, crop-to-wild gene flow studies are frequently complicated by the existence of naturalized individuals (crop progeny), which can be almost indistinguishable from their wild counterparts or introgressants (e.g. *Malus sylvestris* vs *M. domestica*, Coart et al. 2006; *Vitis vinifera* ssp. *sylvestris* (C. C. Gmel.) Hegi vs *V. vinifera* ssp. *vinifera* L., de Andrés et al. 2012). In cherries, however, it is quite easy to distinguish the progeny of alien *Prunus cerasus* from indigenous *P. fruticosa* and their hybrids or from introgressants based on their growth form (i.e., their tree vs shrub habitus). Heteroploid hybridization of *P. fruticosa* with *P. avium* is analogous to that in the genus *Malus* because *P. avium* in Europe consists of genuine wild individuals and naturalized individuals, which are almost indistinguishable (Webb 1968; Coart et al. 2003; Gross et al. 2012).

Conservation implications

From a species conservation perspective, homoploid hybridization and repeated backcrossing with allochthonous *P. cerasus* accompanied by heteroploid hybridization with autochthonous *P. avium* represent a substantial risk of wild populations of *P. fruticosa*. Plants produced by both types of hybridization may considerably hinder the conservation of wild populations of genuine *P. fruticosa* by competing for resources and suitable niches (analogously

as in *Cerastium* L. or *Dianthus* L.; Vít et al. 2014; Vítová et al. 2015) and by decreasing the number of potential mating partners (i.e., demographic swamping; Todesco et al. 2016). The potential for the displacement of *P. fruticosa* is further enhanced by the fact that the two hybrids tend to outgrow it. In contrast to sterile triploid hybrids (Macková et al. 2017), fertile tetraploid hybrids can directly endanger genuine *P. fruticosa* by introgression (i.e., genetic swamping; Todesco et al. 2016). Still, however, some isolated triploid hybrid populations could represent old, partly fertile, spontaneous hybrids with autochthonous *P. avium* (Lepší et al. 2011). Introgression involving triploid hybrids has also been documented in other genera (e.g. *Betula* L. in Iceland; Thórsson et al. 2007), so the potential risk that triploid F1 hybrid could participate in further backcrossing cannot be ruled out.

The main practical implication of our results is the necessity to limit the cultivation of both sour and sweet cherries in the vicinity of wild populations of genuine *P. fruticosa* (within a perimeter of at least 1.5 km, as recommended by Boratyński et al. (2003). To this end, it is first necessary to select populations to be protected with high priority (i.e., those which are the most genetically variable – see below).

Genome size analysis as a suitable tool for detecting introgression

The continuous absolute genome size values at the homoploid level, together with the wide morphological variation, suggest repeated backcrossing between parents and hybrids (e.g. Šmarda and Bureš 2006; Suda et al. 2007; Hanušová et al. 2014). Nevertheless, the obtained pattern, including the impossibility to unequivocally identify F1 hybrids, constitutes only indirect evidence of introgression. However, all other potential explanations (i.e., aneuploidy, differential accumulation of transposable elements, chromosome recombinations, B chromosomes; Petrov 2001; Bennetzen et al. 2005; Šmarda and Bureš 2010; Michael 2014) are highly unlikely. Our data do not allow us to evaluate population dynamics (changes of hybridization frequency in time) and, particularly, the importance of clonal growth (genetic variation of populations).

The use of molecular markers such as SSRs or RAD-Seq might provide direct evidence for ongoing introgression and help identify the conservational most valuable (i.e., variable) populations of *P. fruticosa* (Barać et al. 2017; Beghe et al. 2017; McVay et al. 2017). However, the complex cytological structure of our data set covering three ploidy levels seriously complicates data analyses. Uncertainty concerning allele dosage in polyploids, an unclear mode of inheritance (Dufresne et al. 2014) and likely asymmetry in strength of gene flow across ploidies (Kolář et al. 2017), precludes the use of standard tools for the detection of hybridisation, such as NewHybrids (Anderson and Thompson 2002).

Conclusions

In the wild, genuine *Prunus fruticosa* frequently hybridizes both at the homoploid level (with cultivated *P. cerasus*) and at the heteroploid level (with *P. avium*). Our direct identification and quantification of interspecific hybridization/introgression under natural conditions has confirmed the serious risk of ongoing demographic and genetic swamping, as 39.5% of the populations we studied are of hybrid origin. Moreover, homoploid introgressive hybridization poses a substantial conservation threat because *P. cerasus* is alien to the European flora. Maintenance of a diverse and heterogeneous *P. fruticosa* gene pool is essential for *Prunus* breeding programmes as well as for the species' protection. A future conservation genetic investigation should focus on the identification of the most valuable (i.e., the most genetically variable) populations of genuine *P. fruticosa*.

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Supporting information

Supplementary Table 1: Study sites and numbers of samples used in particular analyses of *Prunus fruticosa* and its hybrid populations in Central Europe. A: Total number of samples used in particular analyses. B: Number of samples per population. *Prunus avium* and *P. cerasus* scattered in the landscape were also included.
Table notes: PF = *Prunus fruticosa*, PM = *P.* ×*mohacsyana*, PE = *P.* ×*eminens*. PC = *P. cerasus*, PA = *P. avium*. CZ = Czech Republic, SK = Slovakia, PL = Poland, RO = Romania.

A

	Total	PF	PE	PM	PC	PA
FCM	**761**	447	99	99	43	73
Distance-based morphometrics	**1422**	834	184	190	81	133
Elliptic Fourier analysis	**1407**	832	186	186	75	128

B

Taxon	Population	FCM	Distance-based morphome-trics	Elliptic Fourier analysis	State	Location	GPS	Altitude
PF	P24	9	18	18	CZ	Lovoš	N50°31′35.4″, E14°01′02.3″	495
PA	P25-PA	1	2	0	CZ	Dlouhá loučka	N49°42′31.2″, E16°38′39.4″	472
PF	P26	8	10	10	CZ	Dlouhá loučka	N49°42′27.6″, E16°38′39.3″	446
PF	P27	7	14	14	CZ	Cakov	N49°37′25.6″, E17°01′44.8″	299
PA	P28-PA	2	2	2	CZ	Cakov	N49°37′26.0″, E17°01′47.3″	288
PF	P30	6	12	12	CZ	Slatinky – Malý Kosíř	N49°33′17.8″, E17°05′29.3″	311
PF	P31	10	20	20	CZ	Hněvotín – Na Skále	N49°33′21.4″, E17°10′40.9″	245
PF	P33	8	16	16	CZ	Sedlec – Liščí hill	N48°47′35.9″, E16°41′37.3″	254
PC	P33-PC	5	10	10	CZ	Sedlec – Liščí hill	N48°47′35.9″, E16°41′37.3″	254
PF	P34	4	6	6	CZ	Sedlec – Liščí hill	N48°47′41.8″, E16°41′43.5″	252
PF	P36	10	20	20	CZ	Sedlec	N48°47′39.8″, E16°42′13.7″	238
PE	P37	2	4	4	CZ	Praha – Sedlec	N50°08′18.8″, E14°23′26.7″	221
PE	P38	6	12	12	CZ	Drysice	N49°20′20.8″, E17°02′59.0″	357
PA	P38-PA	1	2	2	CZ	Drysice	N49°20′20.8″, E17°02′59.0″	357

Taxon	Population	FCM	Distance-based morphome-trics	Elliptic Fourier analysis	State	Location	GPS	Altitude
PE	P39	4	8	8	CZ	Prostějov – Domamyslice – Dolní vinohrádky	N49°27’07.0’’, E17°03’21.4’’	305
PA	P39-PA	3	6	4	CZ	Prostějov – Domamyslice – Dolní vinohrádky	N49°27’07.0’’, E17°03’21.4’’	305
PF	P40	9	18	18	CZ	Brno – Hády	N49°13’09.5’’, E16°40’30.7’’	387
PA	P40-PA	1	2	2	CZ	Brno – Hády	N49°13’09.5’’, E16°40’30.7’’	387
PF	P41	9	18	16	CZ	Karlštejn – Krupná	N49°55’46.6’’, E14°09’03.4’’	268
PF	P42	10	20	20	CZ	Karlštejn – Budňany rock	N49°56’05.0’’, E14°10’54.0’’	263
PF	P43	10	16	15	CZ	Karlštejn – at the camp	N49°56’03.0’’, E14°10’10.3’’	230
PF	P45	8	16	16	CZ	Srbsko – above the sportground	N49°56’29.3’’, E14°07’58.9’’	253
PF	P46	9	11	11	CZ	Beroun – Hostim	N49°57’35.8’’, E14°08’04.0’’	239
PF	P47	10	20	20	CZ	Vrbčany	N50°03’33.8’’, E15°00’05.8’’	218
PF	P49	9	18	18	CZ	Zeměchy u Kralup n. V. – Zeměchy – loess gulch	N50°13’38.6’’, E14°16’02.4’’	212
PC	P50-PC	5	10	8	RO	Fanatale Clujului	N46°49’38.8’’, E23°37’46.4’’	514
PC	P51 -PC	5	10	10	RO	Badeni	N46°13’07.7’’, E25°20’32.7’’	380
PF	P52	9	18	18	RO	Cheile Turzii (Turga Gorge)	N46°34’10.6’’, E23°40’37.4’’	749
PF	P53 Pol	12	24	24	PL	Stawska Góra	N51°12’22.2’’, E23°24’08.6’’	210
PM	P53 Rum	9	16	16	RO	Cheile Turzii (Turga Gorge)	N46°33’58.6’’, E23°40’41.7’’	739
PA	P54 Pol-PA	5	10	10	PL	Żułów	N50°54’40.4’’, E23°22’46.8’’	273
PA	P54 Rum-PA	1	2	2	RO	Posaga de Sus	N46°28’17.7’’, E23°22’38.8’’	639
PC	P55-PC	4	6	6	PL	Majdan Skierbieszowsky	N50°53’03.2’’, E23°22’57.7’’	241
PA, PC	P56-PA,PC	1, 2	2, 4	2, 4	PL	Iłowiec – Horodyska	N50°49’24.6’’, E23°24’09.0’’	194
PF	P57	11	22	22	PL	Rogów – Świdniki	N50°47’36.5’’, E23°31’28.4’’	210
PF	P58	12	23	24	PL	Kąty	N50°40’21.9’’, E23°07’33.9’’	250
PA	P59-PA	1	2	2	PL	Kąty	N50°40’21.9’’, E23°07’33.9’’	250
PE	P60/a	9	18	18	PL	Sandomierz – Góry Pieprzowe	N50°41’02.9’’, E21°46’48.1’’	154

Taxon	Population	FCM	Distance-based morphome-trics	Elliptic Fourier analysis	State	Location	GPS	Altitude
PE	P60/b	9	18	18	PL	Sandomierz – Góry Pieprzowe – partly separated subpopulation	N50°41’02.9’’, E21°46’48.7’’	159
PF, PE	P60/c	2, 5	4, 9	4, 9	PL	Sandomierz – Góry Pieprzowe – partly separated subpopulation	N50°41’02.9’’, E21°46’50.8’’	160
PA	P62-PA	1	2	2	PL	Sandomierz – Góry Pieprzowe	N50°41’02.9’’, E21°46’48.7’’	159
PA	P64-PA	4	8	8	PL	Sandomierz – Góry Pieprzowe	N50°41’02.9’’, E21°46’51.7’’	160
PA	P66-PA	2	4	4	PL	Sandomierz – Góry Pieprzowe	N50°41’02.4’’, E21°46’55.4’’	156
PM	P67	8	15	15	PL	Sandomierz – Góry Pieprzowe	N50°41’02.7’’, E21°47’09.3’’	147
PA	P68-PA	1	2	2	PL	Sandomierz – Góry Pieprzowe	N50°41’03.0’’, E21°47’12.4’’	149
PF	P69/a	11	22	22	PL	Opalonki	N50°21’00.9’’, E20°10’29.2’’	320
PE	P69/b	7	14	14	PL	Opalonki – partly separated subpopulation	N50°21’00.9’’, E20°10’29.2’’	320
PA	P70-PA	3	4	4	PL	Opalonki	N50°21’00.9’’, E20°10’29.2’’	320
PM	P71	4	8	6	PL	Ojcow	N50°13’43.3’’, E19°49’35.10’’	241
PF	P73	9	18	18	SK	Devínska Kobyla	N48°10’50.0’’, E16°59’08.1’’	216
PF	P74	10	16	16	SK	Hronský Beňadik	N48°20’24.4’’, E18°33’24.9’’	413
PF	P75	8	12	12	CZ	Pouzdřany steppe	N48° 56’49.4’’, E16°38’35,6’’	295
PF	P76	14	28	28	CZ	Milá	N50°26’01.5’’, E13°45’26.6’’	443
PA, PC	P76-PA,PC	1, 2	0, 4	2, 2	CZ	Milá	N50°26’01.5’’, E13°45’26.6’’	443
PC	P77-PC	1	2	0	CZ	Radouň	N50°28’53.0’’, E14°23’47.0’’	203
PF	P78/a	13	26	26	CZ	Křešov	N50°29’57.6’’, E14°24’55.8’’	252
PM	P78/b	2	4	4	CZ	Křešov – partly separated subpopulation	N50°29’56.6’’, E14°24’54.3’’	249
PA	P80-PA	1	2	2	CZ	Křešov	N50°29’52.5’’, E14°24’28.9’’	255
PA	P81-PA	3	6	6	CZ	Vědlice	N50°31’33.0’’, E14°20’25.0’’	182
PA, PC	P82-PA,PC	2, 4	4, 8	4, 8	CZ	Kamýk	N50°33’47.5’’, E14°07’09.1’’	367

Taxon	Population	FCM	Distance-based morphome-trics	Elliptic Fourier analysis	State	Location	GPS	Altitude
PF	P83	11	22	19	CZ	Bořeň	N50°31’55.4’’, E13°45’32.4’’	244
PA	P84-PA	2	4	4	CZ	České Zlatníky – bellow Zlatník hill	N50°30’46.4’’, E13°42’26.3’’	246
PF	P85	7	14	14	CZ	Chotiměř	N50°33’17.8’’, E13°59’43.1’’	329
PA	P86-PA	2	4	4	CZ	Chotiměř	N50°33’16.8’’, E13°59’42.3’’	324
PM	P88	3	6	6	CZ	Ústí nad Labem – Hostovice – Soudný hill	N50°38’55.6’’, E14°02’02.0’’	304
PE	P89	9	16	16	CZ	Ústí nad Labem	N50°38’39.2’’, E14°02’20.3’’	295
PM	P90	10	17	17	CZ	Ústí nad Labem	N50°38’29.8’’, E14°02’21.6’’	288
PM	P91	10	20	20	CZ	Ústí nad Labem – Nad Vaňovem	N50°37’50.5’’, E14°02’23.2’’	296
PE	P92	6	12	12	CZ	Podlešín – Vrkoč	N50°37’33.4’’, E14°02’22.5’’	430
PE	P93	6	9	9	CZ	Chvalov	N50°36’03.5’’, E14°03’18.3’’	340
PA	P94-PA	1	2	2	CZ	Chvalov	N50°36’03.5’’, E14°03’18.3’’	340
PF	P95	6	10	10	CZ	Dubice – Výslunní, Doerell's viewpoint	N50°35’10.5’’, E14°01’31.0’’	271
PA	P96-PA	1	2	2	CZ	Dubice – Výslunní, Doerell's viewpoint	N50°35’10.5’’, E14°01’31.0’’	271
PA	P97-PA	1	2	0	CZ	Dubice – Výslunní	N50°35’16.7’’, E14°01’14.5’’	241
PF	P98	6	12	12	CZ	Církvice	N50°34’58.4’’, E14°02’15.7’’	240
PM	P99	11	22	20	CZ	Kamýk	N50°33’52.2’’, E14°07’02.0’’	449
PF	P100	8	13	13	CZ	Číhalín	N49°15’34.3’’, E15°48’19.9’’	504
PF	P101	4	4	4	CZ	Dolní Heřmanice	N49°18’44.2’’, E16°02’53.7’’	514
PF	P102	6	12	12	CZ	Trnava	N49°15’33.8’’, E15°55’45.0’’	468
PF	P104	5	8	8	CZ	Pocoucov	N49°14’31.5’’, E15°54’39.1’’	472
PF	P105	5	10	10	CZ	Ptáčov	N49°13’57.3’’, E15°55’22.7’’	457
PF	P106	9	18	18	CZ	Ptáčov	N49°13’46.3’’, E15°55’10.5’’	448
PE, PM	P107	1, 3	2, 6	2, 6	CZ	Ptáčov	N49°13’41.7’’, E15°55’02.7’’	446
PF	P108	6	10	12	CZ	Ptáčov	N49°13’41.5’’, E15°54’51.0’’	450
PA	P109-PA	2	2	2	CZ	Vémyslice – Na Kocourkách	N48°59’49.9’’, E16°14’53.1’’	304

Taxon	Population	FCM	Distance-based morphome-trics	Elliptic Fourier analysis	State	Location	GPS	Altitude
PF	P110	13	23	23	CZ	Vémyslice – Na Kocourkách	N48°59'49.9'', E16°14'53.1''	304
PF	P111	3	0	0	CZ	Pocoucov	N49°14'19.3'', E15°54'33.9''	464
PF	P112	11	22	22	CZ	Hnanice (NP Podyjí) – Horecký hill	N48°47'37.2'', E15°58'25.5''	281
PA	P113-PA	1	2	2	CZ	Hnanice (NP Podyjí)	N48°48'12.1'', E15°58'57.2''	284
PF	P114	12	24	24	CZ	Hnanice (NP Podyjí)	N48°48'06.9'', E15°58'59.9''	288
PA	P115-PA	6	12	12	CZ	Hnanice (NP Podyjí)	N48°48'06.2'', E15°58'59.0''	291
PE	P116	9	16	18	CZ	Tasovice	N48°49'50.7'', E16°08'21.9''	227
PA	P117-PA	2	4	4	CZ	Tasovice	N48°49'50.7'', E16°08'21.9''	227
PF	P118	10	20	20	CZ	Nový Přerov – Lange Wart	N48°47'56.8'', E16°31'06.2''	243
PA	P119-PA	2	4	4	CZ	Nový Přerov – Lange Wart	N48°47'56.8'', E16°31'06.2''	243
PF	P120	11	18	20	SK	Nové Mesto nad Váhom – Mnešice – Kobela	N48°46'42.3'', E17°50'11.3''	251
PA	P121-PA	2	4	4	SK	Nové Mesto nad Váhom – Mnešice – Kobela	N48°46'42.3'', E17°50'11.3''	251
PF	P122	6	12	12	SK	Horní Vestenice	N48°42'59.9'', E18°25'42.3''	312
PF	P123/a	8	16	15	SK	Salka – Sovie vinohrady	N47°53'14.1'', E18°43'05.8''	200
PM	P123/b	5	10	10	SK	Salka – Sovie vinohrady – partly separated subpopulation	N47°53'14.8'', E18°43'06.1''	200
PA	P125-PA	2	4	4	SK	Salka – Sovie vinohrady	N47°53'16.7'', E18°43'08.9''	180
PA	P127-PA	1	0	0	SK	Salka – Sovie vinohrady	N47°53'14.4'', E18°43'07.9''	171
PC	P128-PC	3	5	5	SK	Salka – Sovie vinohrady	N47°53'13.7'', E18°43'08.8''	183
PM	P129	9	18	18	SK	Štúrovo – vrch Dank (Vršok II)	N47°49'06.5'', E18°38'36.9''	228
PA	P130-PA	1	2	2	SK	Štúrovo – vrch Dank (Vršok II)	N47°49'03.6'', E18°38'34.3''	201
PF	P131	10	20	20	SK	Štúrovo – vrch Dank (Vršok II)	N47°49'12.8'', E18°39'25.5''	219
PF, PM	P132	8, 2	16, 4	16, 4	SK	Nitra – Zobor – Pyramida	N48°20'32.6'', E18°06'18.9''	561
PE	P133	10	20	20	SK	Nitra – Zobor – St. Urban church	N48°19'50.8'', E18°05'49.6''	273
PA	P134-PA	1	2	2	SK	Nitra – Zobor – St. Urban church	N48°19'50.8'', E18°05'49.6''	273

Taxon	Population	FCM	Distance-based morphometrics	Elliptic Fourier analysis	State	Location	GPS	Altitude
PA	P135-PA	1	2	2	SK	Nitra – Zobor – St. Urban church	N48°19’54.7’’, E18°05’49.3’’	236
PF	P136/a	10	20	20	SK	Nová Dedina – Šándorky	N48°17’58.4’’, E18°38’13.5’’	278
PE	P136/b	7	8	8	SK	Nová Dedina – Šándorky – partly separated subpopulation	N48°17’58.4’’, E18°38’13.5’’	278
PA	P138-PA	4	4	4	SK	Nová Dedina – Šándorky	N48°17’58.4’’, E18°38’13.5’’	278
PF	P139	11	22	22	SK	Dlhá ves – Domické škrapy	N48°28’45.4’’, E20°27’58.9’’	315
PA	P140-PA	2	4	4	SK	Nitra – Zobor	N48°20’23.9’’, E18°06’06.2’’	418
PF	P141	3	6	6	SK	Slanec – castle hill	N48°38’12.6’’, E21°28’14.7’’	407
PF	P142	9	12	12	SK	Košice – Podhradová	N48°45’20.7’’, E21°14’08.3’’	348
PA	P143-PA	3	6	6	SK	Košice – Podhradová	N48°45’20.7’’, E21°14’08.3’’	348
PC	P144-PC	5	10	10	CZ	Přerov nad Labem – Přerovská hůra	N50°09’39.2’’, E14°50’47.2’’	223
PE	P145	1	2	2	CZ	Přerov nad Labem – Přerovská hůra	N50°09’38.7’’, E14°50’43.9’’	221
PE	P146/a	2	4	4	CZ	Přerov nad Labem – Přerovská hůra	N50°09’42.7’’, E14°50’17.8’’	220
PE	P146/b	6	12	12	CZ	Přerov nad Labem – Přerovská hůra – partly separated subpopulation	N50°09’43.3’’, E14°50’16.5’’	227
PC	P149-PC	3	6	4	CZ	Český Krumlov – Nádražní Předměstí	N48°49’22.3’’, E14°19’16.1’’	490
PM	P150	5	10	10	CZ	Český Krumlov – Nádražní Předměstí	N48°49’25.7’’, E14°19’19.9’’	550
PA	P151-PA	2	3	4	CZ	Český Krumlov – Nádražní Předměstí	N48°49’20.9’’, E14°19’23.4’’	490
PM	P152	2	4	4	CZ	Český Krumlov – Nádražní Předměstí	N48°49’19.5’’, E14°19’23.7’’	520
PF, PM	P153/a	2, 3	4, 6	4, 6	CZ	Český Krumlov	N48°49’08.8’’, E14°18’33.5’’	551
PM	P153/b	3	4	4	CZ	Český Krumlov – partly separated subpopulation	N48°49’08.2’’, E14°18’38.5’’	526
PM	P153/c	4	8	8	CZ	Český Krumlov – partly separated subpopulation	N48°49’07.9’’, E14°18’35.3’’	520
PM	P154	3	6	6	CZ	Český Krumlov – Nové Dobrkovice	N48°49’03.1’’, E14°18’05.4’’	540
PC	P155-PC	4	8	8	CZ	Český Krumlov – Nové Dobrkovice	N48°49’01.4’’, E14°18’23.0’’	520

Taxon	Population	FCM	Distance-based morphome-trics	Elliptic Fourier analysis	State	Location	GPS	Altitude
PM	P158	3	6	6	CZ	Český Krumlov – Nové Dobrkovice	N48°49′04.6″, E14°17′46.5″	510

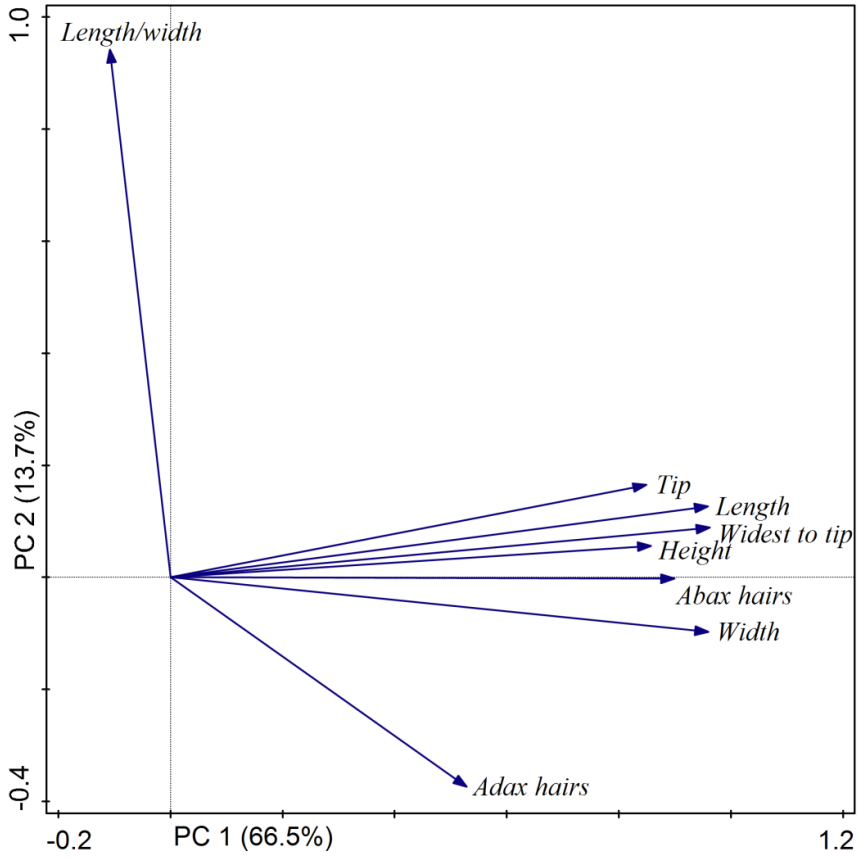
Supplementary Table 2: Detailed absolute genome size results for the five *Prunus* taxa under study.
Table notes: 2C = nuclear DNA content of somatic cells (pg), SD = standard deviation, CV = coefficient of variation of the sample, N = number of individuals.

Taxa	Average 2C	Range of 2C	Median	SD	Average CV	N
P. avium	0.73	0.68–0.81	0.73	0.03	3.83	73
P. ×mohacsyana	1.01	0.93–1.06	1.00	0.02	3.57	99
P. fruticosa	1.30	1.21–1.40	1.29	0.04	3.15	447
P. ×eminens	1.36	1.30–1.43	1.35	0.03	2.86	99
P. cerasus	1.42	1.36–1.48	1.42	0.03	3.29	42

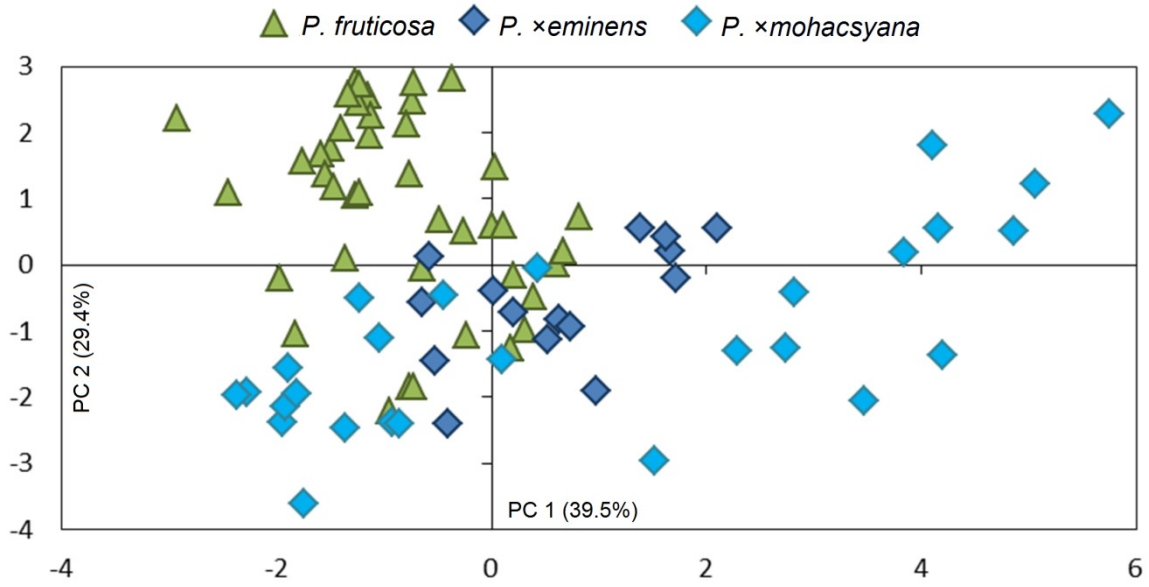
Supplementary Table 3: Basic descriptive statistics for vegetative morphological characters of the five *Prunus* taxa under study.

Taxa/characters		Plant height	Laminar length (mm)	Laminar width (mm)	Distance from the widest part of the lamina from the laminar tip (mm)	Ratio of length and width of the lamina	Shape of laminar tip	Density of hairs on the adaxial surface of lamina	Density of hairs on the abaxial surface of lamina
Prunus fruticosa	Min	1.00	10.77	5.52	4.28	1.30	1.00	1.00	1.00
	25% quantile	1.00	18.53	9.27	7.53	1.80	1.00	1.00	1.00
	Mean	1.19	22.60	11.27	9.14	2.03	1.66	1.15	1.00
	75% quantile	1.00	25.69	12.52	10.23	2.22	2.00	1.00	1.00
	Max	3.00	50.66	27.20	22.09	3.35	3.00	2.75	1.00
Prunus ×eminens	Min	1.00	19.04	10.61	7.99	1.29	1.00	1.00	1.00
	25% quantile	1.00	28.24	15.40	12.04	1.68	2.00	1.00	1.25
	Mean	2.21	34.46	18.59	14.95	1.88	2.36	1.24	1.92
	75% quantile	3.00	39.32	20.62	17.31	2.10	3.00	1.25	2.00
	Max	3.00	62.90	41.84	31.77	2.55	4.00	3.00	4.00
Prunus ×mohacsyana	Min	1.00	19.08	9.64	7.81	1.00	1.00	1.00	1.00
	25% quantile	1.00	30.81	15.86	13.43	1.74	2.00	1.00	1.75
	Mean	2.35	35.77	18.49	15.88	1.97	2.56	1.32	2.06
	75% quantile	3.00	40.94	20.75	18.13	2.14	3.00	1.75	2.00
	Max	3.00	57.00	30.27	24.02	2.91	4.00	2.00	4.00
Prunus cerasus	Min	3.00	36.82	20.86	15.56	1.45	3.00	1.00	2.00
	25% quantile	3.50	51.08	28.53	23.60	1.67	3.00	1.00	3.00
	Mean	3.75	58.97	31.68	27.11	1.87	3.44	1.12	3.38
	75% quantile	4.00	65.63	35.26	29.37	2.06	4.00	1.00	4.00
	Max	4.00	88.32	46.29	42.21	2.48	4.00	2.50	4.00
Prunus avium	Min	3.00	31.83	21.06	19.88	1.48	3.00	1.00	2.00
	25% quantile	4.00	63.89	35.26	31.94	1.74	4.00	2.00	4.00
	Mean	3.83	78.97	40.92	37.70	1.94	3.98	2.10	3.95
	75% quantile	4.00	93.27	47.07	41.70	2.11	4.00	2.00	4.00
	Max	4.00	127.11	58.41	70.13	2.72	4.00	4.00	4.00

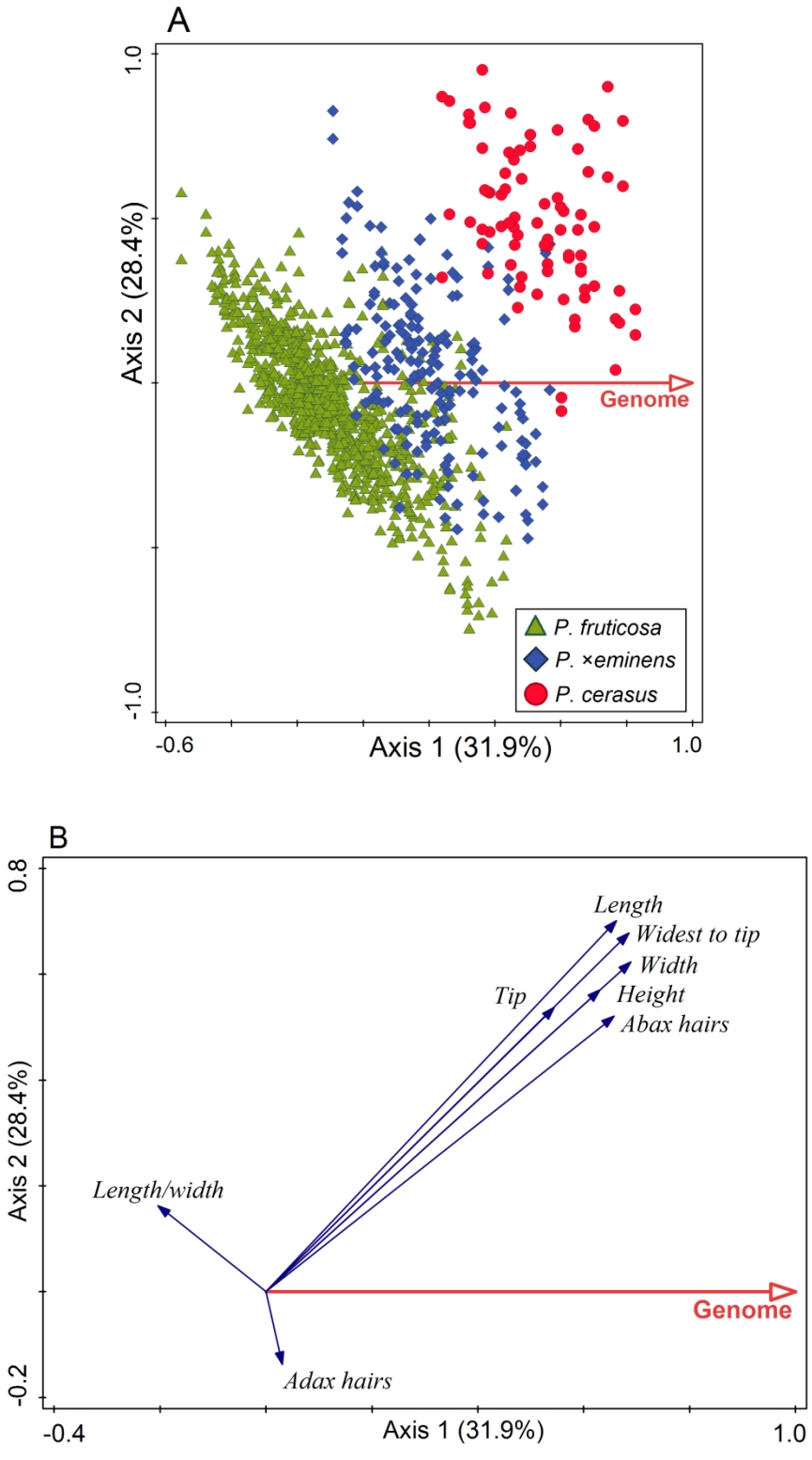
Supplementary Figure 1: Ordination diagram of PCA using eight vegetative morphological characters of 1,422 leaves of *Prunus* taxa under study showing directions of changes in morphological characters displayed in relation to the first two component axes (see Table 1 for character abbreviations).



Supplementary Figure 2: Ordination diagram of principal component analysis based on nine generative morphological characters of 84 flowers of *Prunus fruticosa* and its hybrids.



Supplementary Figure 3: Redundancy analysis of three *Prunus* taxa under study, showing the morphological variation of eight vegetative characters measured on 1,099 leaves of tetraploid *Prunus* taxa along a gradient of absolute genome size. A: Individuals. B: Loadings of individual morphological characters (see Table 1 for character abbreviations). The canonical (constrained) axis (axis 1) corresponds to the effect of absolute genome size and the first unconstrained axis (axis 2) shows the remaining major trend in morphological variation not explained by absolute genome size.



7.3 Case study III

Macková, L., Nosková, J., Ďurišová, Ľ., & Urfus, T.: **Insights into the cytotype and reproductive puzzle of *Cotoneaster integerrimus* in the Western Carpathians.** – Plant Systematics and Evolution (preliminary accepted)

Insights into the cytotype and reproductive puzzle of *Cotoneaster integerrimus* in the Western Carpathians

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Abstract

The Western Carpathians are traditionally recognized as one of the hotspots of temperate European biodiversity. The polyploid and apomictic group of *Cotoneaster integerrimus* s.l. is supposed to be particularly variable there, and this is also mirrored by taxonomy. We therefore examined the ploidal and reproductive pattern of *C. integerrimus* s.l. and its close relative *Cotoneaster tomentosus* in the Western Carpathians and compared it to that in the Bohemian Massif.

Using flow cytometry, we detected tetraploid (468 individuals, 100 populations) and pentaploid (35 individuals, 11 populations) cytotypes, and eight additional mixed populations. The pentaploid cytotype was found exclusively in *C. tomentosus*, which only occurs in the Western Carpathians. A further flow cytometric seed screen (1114 seeds) revealed facultative apomixis (10.1% of sexual progeny) of tetraploid *C. integerrimus* s.l. whereas the pentaploid *C. tomentosus* was almost obligatorily apomictic. In addition, 3.8% of sexual progeny was formed with the contribution of an unreduced female gamete. Moreover, apomixis in tetraploids was further structured into distinct subtypes: pseudogamy (77.2%), autonomous apomixis (3.7%) and haploid parthenogenesis (0.3%). The reproductive pattern among the study taxa and between the two model regions was significantly uniform. Furthermore, our comparative dataset from the Western Alps also included sexual diploids. For this reason, greater ploidal and reproductive variation may be expected in that region.

The Western Carpathians therefore do not represent a centre of cytotype and reproductive variation of *C. integerrimus* s.l. and facultative apomixis is a universal reproductive strategy in both the Western Carpathians and the Bohemian Massif.

Keywords: *Cotoneaster integerrimus* s.l., flow cytometric seed screen, polyploidy, reproductive mode, Western Carpathians

Introduction

Despite being a subject of research for more than a century, apomixis (agamospermy in the strict sense – clonal reproduction through seeds) is still surrounded by a significant number of unresolved questions (Whitton et al. 2008). Apomixis is a rather rare phenomenon among the angiosperms, being present in less than 1% of species (Mogie 1992; Whitton et al. 2008) and ca 75% of apomictic species belong to three families: Asteraceae, Poaceae and Rosaceae (Hojsgaard et al. 2014). On the other hand, the prevalence of apomictic groups might be underestimated because the number of newly identified apomictic taxa keeps

increasing every year (e.g. Kissling et al. 2006; Lepší et al. 2009; Hajrudinović et al. 2015a; Vašut and Majeský 2015).

Regarding the distribution of apomictic plants, several patterns have been described. First of all, apomictic taxa tend to have larger distribution ranges than their diploid and sexual relatives (geographic parthenogenesis; Hörandl et al. 2008). Moreover, the incidence of apomixis seems to increase with latitude and elevation (Schinkel et al. 2016). Contradictory results, however, have been reported from the Alps where apomixis does not prevail in alpine (subnival) plants (Hörandl et al. 2011).

Within Central Europe, the Carpathians are a particularly significant hotspot of plant diversity (Ronikier 2011; Kliment et al. 2016; Mráz et al. 2016). That they also constitute a hotspot of apomictic taxa is suggested by studies on particular genera (e.g. *Sorbus*, Uhrinová et al. 2017; *Hieracium*, Štorchová et al. 2002; Chrtek et al. 2007) and by their great number of apomictic endemics (Kliment et al. 2016).

One possible example of a species-rich group in the Carpathians is the genus *Cotoneaster* (based on hitherto described microspecies; Hrabětová-Uhrová 1962; Baranec 1992). European taxa of *Cotoneaster* (Rosaceae) are deciduous spineless shrubs bearing small pomes (or polypyrenous drupes; Rohrer et al. 1991) and are usually linked to dry rocky habitats (Browicz 1968). European mountain ranges, particularly the Alps and the Western Carpathians, are considered the diversity centres of the genus (Browicz 1968; Baranec 1992; Kutzelnigg 1994; Fryer and Hylmö 2009; Kurtto et al. 2013). Several ploidy levels and cases of hybridization have been reported from the Western Carpathians (Baranec 1992; Kutzelnigg 1994; Kurtto et al. 2013). Apomixis is the supposed reproductive mode of polyploid *Cotoneaster* taxa whereas diploids are expected to be sexual (Sax 1954; Hjelmquist 1962). Nevertheless, apomictic reproduction in *Cotoneaster* has scarcely been confirmed experimentally (i.e. the single direct piece of embryological evidence has been presented by Hjelmquist (1962). Uncertain results of a flow cytometric seed screen (FCSS) have been published in a conference abstract (Mahmutović-Dizdarević et al. 2015) and the occurrence of apomixis was indirectly deduced from the pattern of morphological variation, high ploidy level diversity and a partially clonal phylogenetic pattern (Sax 1954; Kroon 1975; Bartish et al. 2001). Probably because of their great number of small chromosomes and high content of secondary metabolites, chromosomes have been counted only rarely. The majority of published chromosome counts originated from nurseries and arboreta or lack proper localization. After critical revision we identified only ten relevant counts (see Online Resource 1).

In this study we focused on *Cotoneaster integerrimus* s.l., a complex taxon consisting of numerous microspecies in Central Europe and the closely related species *C. tomentosus* (syn. *C. nebrodensis*; Bartish et al. 2001) in the Western Carpathians. There are several distinct species concepts for *C. integerrimus* s.l. taxa in Central Europe – several different microspecies concepts (Hrabětová-Uhrová 1961, 1962; Baranec 1992; Fryer and Hylmö 2009) vs broad concepts in comprehensive floras (Dickoré and Kasperek 2010; Sennikov 2010; Kurtto et al. 2013). The number of different species concepts is naturally reflected by different numbers of taxa described in various senses (Kurtto et al. 2013). Because several of the taxonomic concepts used for Europe contradict each other (Dickoré and Kasperek 2010) and some authors tend to give up on discriminating microspecies of the group, a broader concept was recently proposed by Dickoré and Kasperek (2010), in which majority of microspecies are treated within *C. integerrimus* s.l., with the sole exception of *C. tomentosus* in Central Europe. Whereas *C. tomentosus* is a well differentiated and probably uniform taxon (Kutzelnigg 1994), *C. integerrimus* s.l. has repeatedly been considered variable and suspected of being composed of hybridizing microspecies (Browicz 1968; Baranec 1992; Kutzelnigg 1994). Moreover, hybridization of *C. tomentosus* and

C. integerrimus s.l. has been reported (Browicz 1968; Kutzelnigg 1994). One of the most important sources of their variation is probably polyploidy (cytotypes reported from Europe: 2x, 3x, 4x, 5x, 6x; x=17; e.g. Favarger in Löve 1969, 1975, Česchmedjiev in Löve 1983; Baranec 1992; Měsíček and Javůrková-Jarolímová 1992; Murín and Májovský 1992; see also Table 1 and Online Resource 1).

Reported within *Cotoneaster integerrimus* s.l. in the Western Carpathians were the following microspecies: *Cotoneaster laxiflorus* (syn. *C. melanocarpus*, *C. niger*), *Cotoneaster alaunicus*, *Cotoneaster matrensis* and *C. integerrimus* s.str. (Baranec 1992; Bölöni 2012); for ploidy and distribution details, see Table 1 and Online Resource 1. Nevertheless, microspecies are accepted only locally, their names are sometimes considered synonyms or pertaining to hybrids of basic species, and different authors state the need for further study (e.g. Browicz 1968; Kovanda 1992; Kutzelnigg 1994; Kurtto et al. 2013). The only somewhat more widely accepted microspecies is *C. laxiflorus* (Browicz 1968; Kovanda 1992; Kutzelnigg 1994; Kurtto et al. 2013). But still, Dickoré and Kasperek (2010) considered adventive records of *C. laxiflorus* in Central Europe doubtful and placed its supposed distribution range far towards the east.

Table 1: Published chromosome counts and distribution of European *Cotoneaster* taxa studied: *C. integerrimus* s.l. and *C. tomentosus*. Microspecies are named if they are specified by the source; remaining records are labelled as *C. integerrimus* s.l.

Taxon	W. Carpathians, B. Massif	References	Other parts of Europe	References	Distribution (Meusel et al. 1965)
C. integerrimus s.l.	3x, 4x	Baranec 1992, Kovanda 1992, Měsíček and Javůrková--Jarolímová 1992	2x, 3x, 4x, 6x	Gladkova 1968, Favarger in Löve 1969, Favarger in Löve 1975, Česchmedjiev in Löve 1983, Kovanda 1992, Fryer and Hylmö 1994, Kutzelnigg 1994, Lauber and Wagner 1996, Kurtto et al. 2013	Central, Southern and Southeastern Europe, scattered in Scandinavia, the Pyrenees and the Alps
C. tomentosus	4x	Baranec 1992, Murín and Májovský 1992	3x, 4x, 5x	Favarger in Löve 1969, Kutzelnigg 1994, Lauber and Wagner 1996, Goranova 2007, Kurtto et al. 2013	Central and Southeast Europe, the Alps, isolated areas in the Pyrenees and the Apennines

Taxon	W. Carpathians, B. Massif	References	Other parts of Europe	References	Distribution (Meusel et al. 1965)
C. laxiflorus	4x	Baranec 1992, Kovanda 1992	4x	Kutzelnigg 1994, Kurtto et al. 2013	Central Europe to Central Asia (according to Dickoré and Kasperek 2010 only Eastern Europe to Asia)
C. alaunicus	3x, 4x	Baranec 1992	4x	Krügel 1990, Kurtto et al. 2013	S Russia, NW Caucasus (according to Baranec 1992 additionaly Slovakia, Western Carpathians)
C. matrensis	NO		NO		Slovakia, Western Carpathians (Baranec 1992)

Microspecies of *C. integerrimus* s.l. are delimited based on minute, overlapping and difficult-to-assess characteristics including the colour of the pome, the number of seeds (pyrenes) in the pome or the number of fruits in infructescence (Baranec and Eliáš 2004). One characteristic especially frequently adopted in floras is the colour of fruit (e.g. Browicz 1968; Baranec 1992; Kovanda 1992; Kutzelnigg 1994).

Our study was targeted at Central Europe, where the Western Carpathians are supposed to be a hotspot of *Cotoneaster* diversity (5 taxa; Baranec 1992) compared to the putatively less diverse Bohemian Massif (Kovanda 1992). We have arbitrarily chosen the Bohemian Massif as a well investigated comparative region with only two taxa described.

We employed DNA ploidy level analysis and reproductive mode testing (flow cytometric seed screen) to elucidate the complex pattern of *Cotoneaster* taxa in the area of interest. To meet this goal, we addressed the following questions: (1) Do spatial ploidal and genome size patterns in Central Europe agree with any of the recent taxonomic concepts? (2) Is the pattern of reproductive modes within and among the *Cotoneaster* taxa under study congruent with any of taxonomic concept? and Do reproductive modes in the Western Carpathians differ from those in the Bohemian Massif?

Materials and methods

Sampling

Our study is based on 503 individuals of *Cotoneaster* from 119 populations collected between 2012 and 2018 in Czechia, Slovakia and adjacent countries (Fig. 1, Online Resource 2). Besides *Cotoneaster tomentosus* (35 individuals, 11 populations), which is a sister species to *C. integerrimus* s.l. (Bartish et al. 2001), we included populations treated as microspecies of *C. integerrimus* s.l. in the Western Carpathians and neighbouring regions, namely *C. laxiflorus* (114 individuals, 13 populations), *C. alaunicus* (39 individuals, 8 populations), *C. matrensis* (31 individuals, 7 populations) and *C. integerrimus* s.str. (284 individuals, 70 populations). Moreover, ten populations were mixed (see Online Resource 2). Localities were searched based on the Pladias database of the Czech flora and vegetation (Wild et al. 2019), the occurrence of rock habitats and information from local botanists. In addition, we preferentially focused on sites reported to host microspecies: *C. laxiflorus*, *C. alaunicus* and *C. matrensis* (Hrabětová-Uhrová 1961, 1962; Baranec 1992; Baranec and Eliáš 2004; Ďurišová et al. 2015). Although Dickoré and Kasperek (2010) proposed the broader concept accepting only *C. integerrimus* s.l. for Central Europe, we decided to distinguish also the above-mentioned microspecies (*C. laxiflorus*, *C. alaunicus*, *C. matrensis, C. integerrimus* s.str.; Baranec 1992) to test their cytological and reproductive features.

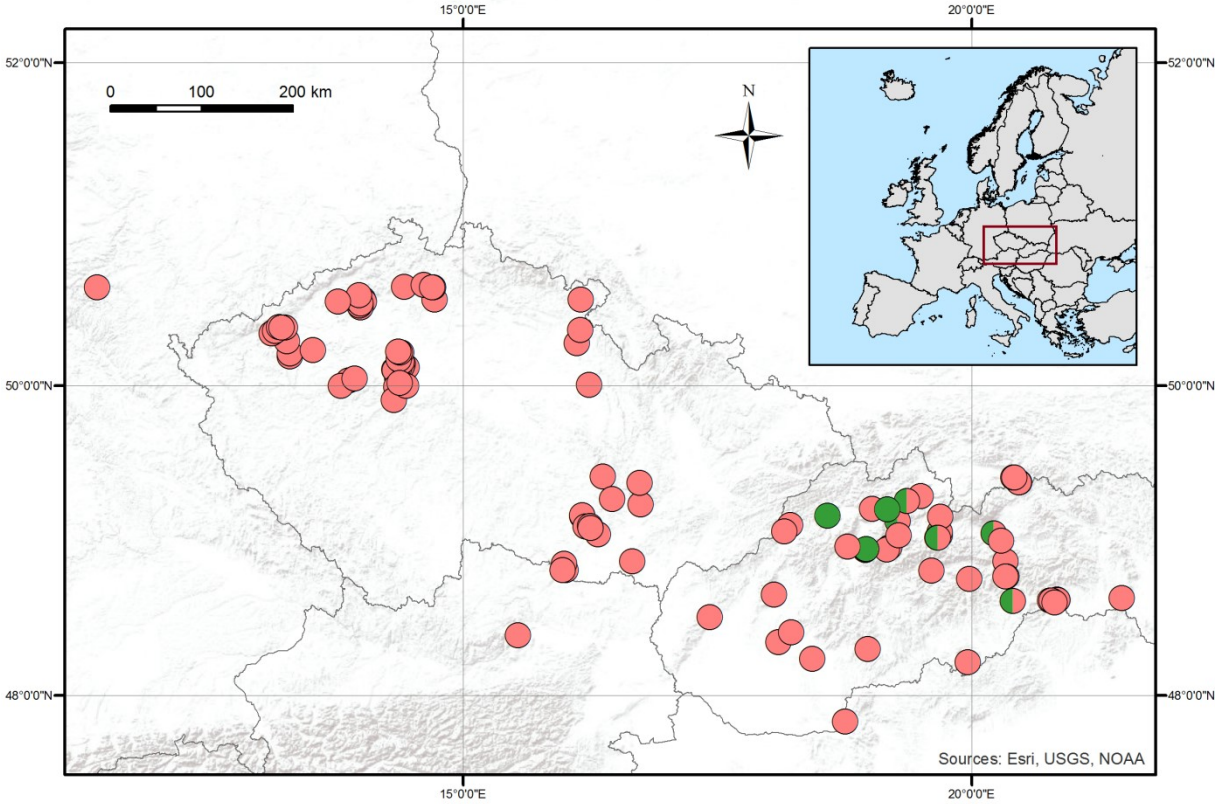

Fig. 1: Sample locations of tetraploid *Cotoneaster integerrimus* s.l. (red) and pentaploid *C. tomentosus* (green) in Czechia, Slovakia and adjacent countries.

We treated the sampling area as two major regions: the Bohemian Massif and the Western Carpathians together with adjacent Pannonia (precisely defined units based on biogeography, physical geography and geology (Kaplan 2012; see Online Resource 2). Due to the low abundance of *Cotoneaster tomentosus*, we extended its dataset by populations from the Alps (France, Austria; 6 populations, 7 individuals, 23 seeds), the Dinaric Alps

(Croatia; 1 population, 2 individuals, 14 seeds) and Macedonia (1 population, 3 individuals, no seeds). In addition, we also sampled six populations (20 individuals) of *C. integerrimus* s.l. in the French Alps for comparison.

Cotoneaster tomentosus was determined based on its laminar shape and whitish tomentum of fruits (Browicz 1968). By contrast, microspecies of *C. integerrimus* s.l. were determined based on literature information (*C. laxiflorus*, *C. alaunicus*, *C. matrensis*; Hrabětová-Uhrová 1961, 1962; Baranec 1992; Kovanda 1992; Baranec and Eliáš 2004; Ďurišová et al. 2015) and knowledge of local botanists. The microspecific classification of *C. integerrimus* s.l. is therefore mainly tentative and will require further testing. Each population sample (usually 1–10 individuals, depending on population size) was represented by a branchlet with leaves. From fertile individuals, pomes were also taken. In total, 1114 seeds from 339 individuals (*C. integerrimus* s.str. – 504 seeds from 165 individuals, *C. laxiflorus* – 326 seeds from 90 individuals, *C. alaunicus* – 165 seeds from 38 individuals, *C. matrensis* – 44 seeds from 23 individuals, *C. tomentosus* – 75 seeds from 23 individuals) were collected (see Online Resource 2). Because of frequent vegetative reproduction by root shoots (e.g. Ďurišová et al. 2015) individuals were sampled as far from each other as possible to avoid the collection of ramets of the same individual. Individuals growing together in one place were considered a discrete population (obviously spatially separated individuals were regarded as a subpopulation marked by the letter *a, b* or *c*; see Online Resource 2). Representative voucher specimens will be deposited in the Herbarium of the Charles University (PRC).

Estimation of somatic DNA ploidy level

Flow cytometry (FCM) with DAPI (4',6-diamidino-2-phenylindole) as the fluorescent stain was employed to estimate the DNA ploidy level of 503 *Cotoneaster* individuals. The standard protocol for the isolation and staining nuclei followed (Doležel et al. 2007). *Carex acutiformis* (2C = 800 Mpb; Veselý et al. 2012) was used as the internal standard. An approximately 0.5 cm long part of a fresh leaf petiole together with an appropriate amount of the internal standard was chopped in 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid monohydrate, 0.5% Tween 20; Doležel et al. 2007) in a Petri dish. The suspension was filtered through a 42--µm nylon mesh filter and the samples were incubated at least for 10 min at room temperature. Then a staining solution consisting of 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$; Doležel et al. 2007), β-mercaptoethanol (2 µl/ml; Fluka, Buchs, Germany) and DAPI (final concentration 4 µg/ml; Sigma, Steinheim, Germany) was added to the samples. Finally, after 5 min of incubation at room temperature, the stained samples were run through a CyFlow ML instrument (Partec GmbH, Münster, Germany) equipped with a 365-nm LED UV light source, and the fluorescence intensity of 3000 particles was recorded. Means and coefficients of variation (CV) were obtained from the resulting fluorescent histograms in FloMax version 2.4d software (Partec GmbH, Germany). The sample:standard fluorescence ratio was calculated. Ploidy levels were determined based on this index and calibration by chromosome counts (a standard karyological methodology with lacto-propionic orcein staining described in Lepší et al. (2008). Relative equivalent of Cx values (AT bases amount per monoploid genome – further in the text referred to as relative Cx values, i.e. peak index/ploidy level) were visualized as boxplots in PAST 2.17c (Hammer et al. 2001). One-way ANOVA followed by Tukey's HSD test in PAST 2.17c (Hammer et al. 2001) was used to test the significance of relative Cx values differences between *Cotoneaster integerrimus* s.l. and *C. tomentosus*. Relative Cx values were log--transformed before the ANOVA.

Members of the Rosaceae family (including *Cotoneaster*) contain significant amounts of secondary metabolites that increase the coefficients of variation of flow cytometric peaks (Loureiro et al. 2006), but tissue from the leaf petiole (not the leaf lamina) provided peaks of adequate quality (lower CV values).

Flow cytometric seed screen (FCSS)

All *Cotoneaster* seeds (pyrenes) were first manually extracted from pomes, counted and cut in half. Subsequently, the numbers of sterile (i.e. aborted or empty) and well developed seeds were recorded. Only these well-developed seeds were used for the detection of reproductive modes based on a flow cytometric seed screen (Matzk et al. 2000). Each sample consisted of one seed with the internal standard. The procedure of sample preparation generally copied the procedure of leaf petiole analysis described above. Nevertheless, a slight modification consisting of using a larger amount of the Otto I buffer (0.7 ml) and prolonging the incubation time to 15 min. was adopted.

The ploidy levels of the embryo and the endosperm were calculated from the peaks of fluorescence histograms following the original methodology of Matzk et al. (2000) and Dobeš et al. (2013). The contributions of female (F) and male (M) gametes to seed formation were subsequently inferred from the ploidy levels of the embryo (Emb) and the endosperm (End). For sexual seeds: $F = End - Emb$, $M = Emb - F$; for apomictic (pseudogamous) seeds: $F = Emb$, $M = End - 2 \times Emb$. The last mentioned equation was modified if more than two polar nuclei were involved in endosperm formation in pseudogamous seeds: $M = End - N \times Emb$, where N = number of polar nuclei.

The resulting frequencies of reproductive pathways are presented as a scatter plot and a bar chart in Microsoft Excel 2010. The proportional incidence of reproductive patterns (primarily the proportion of sexuality) is shown in pie chart form to visualize differences between particular microspecies and geographic regions. Differences in the proportion of sexuality between particular taxa and geographic regions were also tested statistically using generalized mixed-effect linear models (GLMM) with binomial distribution carried out in R 3.4.3 (R Core team 2017).

Results

DNA ploidy level

Using flow cytometry, we identified tetraploids (468 individuals, 100 populations) and pentaploids (35 individuals, 11 populations), and eight mixed populations composed of plants of both ploidy levels in the study area in Czechia, Slovakia and adjacent countries (Fig. 1, Online Resource 2). In addition to our model regions, we also recorded diploids in four comparative populations (15 individuals) in the Alps (see representative histogram in Online Resource 3).

Although the Rosaceae family has repeatedly been reported to interfere with fluorescent staining because of high levels of secondary metabolites (Jedrzejczyk and Sliwinska 2010; Macková et al. 2017, 2018), the coefficients of variation achieved in our study did not exceed 3% (the average CVs of tetraploid and pentaploid individuals were 2.32% and 1.92%, respectively; see also the representative histogram of a simultaneous FCM analysis in Online Resource 3). The DNA ploidy levels (i.e. 2 individuals of tetraploid *C. integerrimus* s.str., 2 individuals of tetraploid *C. laxiflorus*, 1 individual of pentaploid *C. tomentosus*) estimated by flow cytometry were calibrated by chromosome counts.

The entire dataset of *Cotoneaster integerrimus* s.l. (incl. all involved microspecies) was revealed to be tetraploid (468 individuals) and included plants occurring in both study regions (the Bohemian Massif and the Western Carpathians) whereas the pentaploid cytotype was exclusively restricted to *C. tomentosus* (23 individuals) occurring in the Western Carpathians (supplemented by 12 accessions from the extended area – the Alps, the Dinaric Alps and Macedonia).

Although ANOVA did not produce a statistically significant result ($F_{1, 394} = 262.2$, $p > 0.001$), *Cotoneaster tomentosus* tended to differ from *C. integerrimus* s.l. in relative Cx values. The difference between median values reached 3.4% (*C. integerrimus* s.l. 0.56±0.007 and *C. tomentosus* 0.58±0.006; Fig. 2). Based on relative Cx values, none of the *C. integerrimus* s.l. accessions formed any distinguishable group or spatial pattern in the whole study area (and even within *C. tomentosus*).

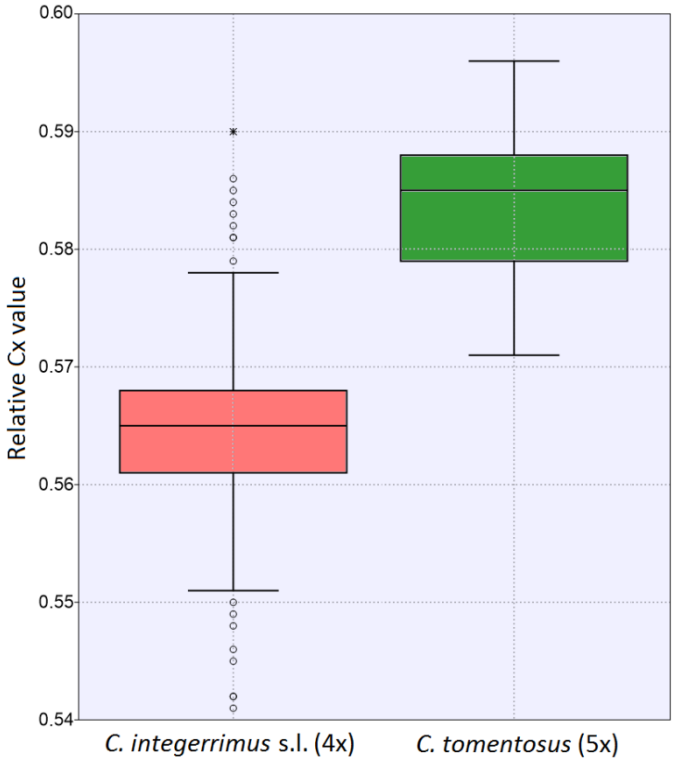

Fig. 2: Difference (3.4%) in relative equivalent Cx values (amount of AT bases per monoploid genome) between tetraploid *Cotoneaster integerrimus* s.l. and pentaploid *C. tomentosus*.

Reproductive modes

Altogether 5440 seeds were prepared from *Cotoneaster* pomes, but 66% (3595 seeds) of them were empty or aborted. The remaining 34% of seeds (1845 seeds) were used for our flow cytometric seed screen (FCSS). Still, however, in 40% of them (731 seeds) only the embryo was detected. The ploidy level of both the embryo and the endosperm was estimated for a total of 1114 seeds of *Cotoneaster integerrimus* s.l. and *C. tomentosus* (average CV 2.67%; see representative FCSS histograms in Online Resource 4). Moreover, in 20 seeds from 12 populations of all taxa under study (except *C. matrensis*), multiple embryo and endosperm were detected in single seeds (i.e. altogether, 1134 reproductive mode determinations were made for 1114 seeds).

As a side result of our FCSS analysis, we found tetraploid *C. integerrimus* s.l. and pentaploid *C. tomentosus* to differ in the number of seeds per pome. The modus values

for tetraploid *C. integerrimus* s.l. and pentaploid *C. tomentosus* were 3 and 4 seeds per pome, respectively. The numbers of seeds in the pome did not differ between tetraploid taxa belonging to *C. integerrimus* s.l.

Various types of apomixis were captured in 90.5% of seeds (the remaining seeds were sexual). Seven major reproductive pathways were detected: sexual reproduction (involving a reduced or unreduced female gamete), haploid parthenogenesis (involving a reduced or unreduced male gamete), autonomous apomixis and pseudogamy (involving a reduced or unreduced male gamete; Fig. 3, Table 2, 3, Online Resource 4). Pseudogamy was the most frequent reproductive mode (78.0% of all *Cotoneaster* seeds, the frequency of unreduced and reduced male gamete being almost equal). A surprisingly high rate of putative unreduced gamete participation (for another possible interpretation see also the Discussion) could be deduced from the obtained FCSS endosperm ploidy pattern (unreduced male gametes participated in 39.8% of pseudogamous endosperms). As regards sexuality, we revealed only 3.6% of B_{III} plants (an unreduced gamete participated in embryo formation – 2n+n; for more details see the Discussion). Moreover, 8.5% (i.e. 96 seeds) of all *Cotoneaster* analyses were characterized by endosperm/embryo ratios that cannot be exactly linked to any reproductive pathway (4:9, 11, 13, 15, 17; 5:9, 16). Nevertheless, these ratios are probably products of an irregular pseudogamous process (for details see Online Resource 5).

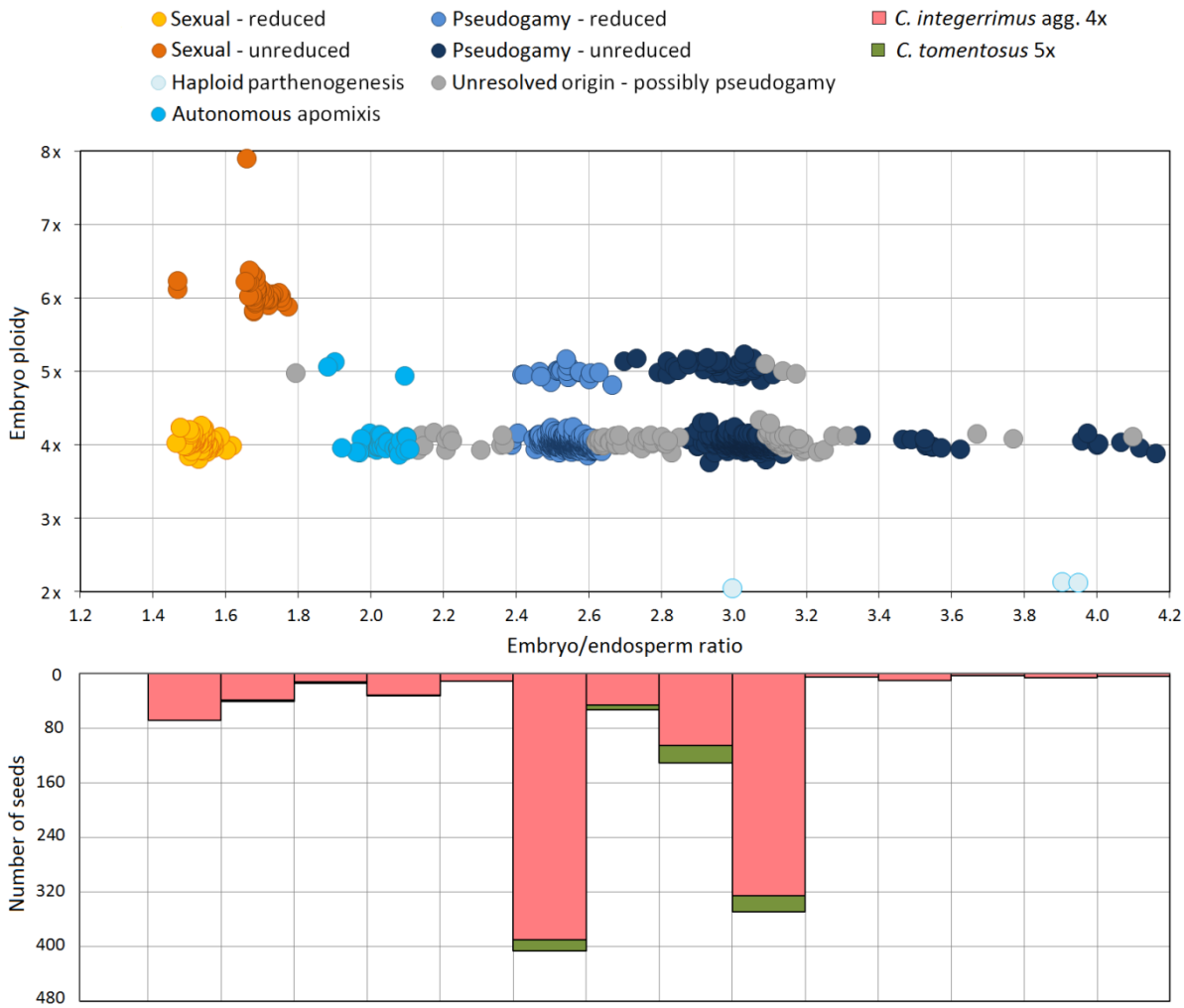

Fig. 3: Association between the endosperm/embryo ploidy ratio to ploidy of the embryo in 1114 *Cotoneaster* seeds (pyrenes) showing variation in reproductive modes in tetraploid *C. integerrimus* s.l. and pentaploid *C. tomentosus* (based on FCSS).

Table 2: Variation in breeding systems of tetraploid *Cotoneaster integerrimus* s.l. based on ploidy of embryo and endosperm ratios detected by FCSS. Only major reproductive modes are included.

Type of reproduction	Ploidy of embryo	Ploidy of endosperm	Female gamete	Male gamete	Embryo formation	Endosperm formation
Sexual - reduced	4	6	reduced 2C	reduced 2C	fertilized 2+2=4C	fertilized (2+2)+2=6C
Sexual - unreduced	6	10	unreduced 4C	reduced 2C	fertilized 4+2=6C	fertilized (4+4)+2=10C
Haploid parthenogenesis	2	6	reduced 2C	reduced 2C	parthenogenetic 2C	fertilized (2+2)+2=6C
Haploid parthenogenesis	2	8	reduced 2C	unreduced 4C	parthenogenetic 2C	fertilized (2+2)+4=8C
Autonomous apomixis	4	8	unreduced 4C	0	parthenogenetic 4C	autonomous (4+4)=8C
Pseudogamy - reduced	4	10	unreduced 4C	reduced 2C	parthenogenetic 4C	fertilized (4+4)+2=10C
Pseudogamy - unreduced	4	12	unreduced 4C	unreduced 4C	parthenogenetic 4C	fertilized (4+4)+4=12C

The FCSS pattern of the progeny of tetraploid *C. integerrimus* s.l. differed substantially from that of pentaploid *C. tomentosus* (significantly mirrored also by a generalized mixed-effect linear model: $N = 1133$, $\chi^2 = 6.13$, $p = 0.013$). Whereas *C. tomentosus* reproduced almost exclusively apomictically, progeny of tetraploid *C. integerrimus* s.l. always retained a certain degree of sexuality (Fig. 4). Out of 75 analysed seeds of pentaploid *C. tomentosus*, only a single seed indisputably resulted from sexual reproduction. Most *C. tomentosus* seeds (89.5%) were formed by pseudogamy – predominantly with the participation of a putative unreduced male gamete (emb:end = 5:15; Fig. 3, 4, Table 3). In three cases (3.9%), autonomous apomixis was detected.

Table 3: Variation in breeding systems of pentaploid *Cotoneaster tomentosus* based on ploidy of embryo and endosperm ratios detected by FCSS. Only major reproductive modes are included.

Type of reproduction	Ploidy of embryo	Ploidy of endosperm	Female gamete	Male gamete	Embryo formation	Endosperm formation
Sexual - unreduced	8	13	unreduced 5C	reduced 3C	fertilized 5+3=8C	fertilized (5+5)+3=13C
Autonomous apomixis	5	10	unreduced 5C	0	parthenogenetic 5C	autonomous (5+5)=10C
Pseudogamy - reduced	5	12	unreduced 5C	reduced 2C	parthenogenetic 5C	fertilized (5+5)+2=12C
Pseudogamy - reduced	5	12.5	unreduced 5C	reduced 2.5C	parthenogenetic 5C	fertilized (5+5)+2.5=12.5C
Pseudogamy - reduced	5	13	unreduced 5C	reduced 3C	parthenogenetic 5C	fertilized (5+5)+3=13C
Pseudogamy - unreduced	5	14	unreduced 5C	unreduced 4C	parthenogenetic 5C	fertilized (5+5)+4=14C
Pseudogamy - unreduced	5	15	unreduced 5C	unreduced 5C	parthenogenetic 5C	fertilized (5+5)+5=15C

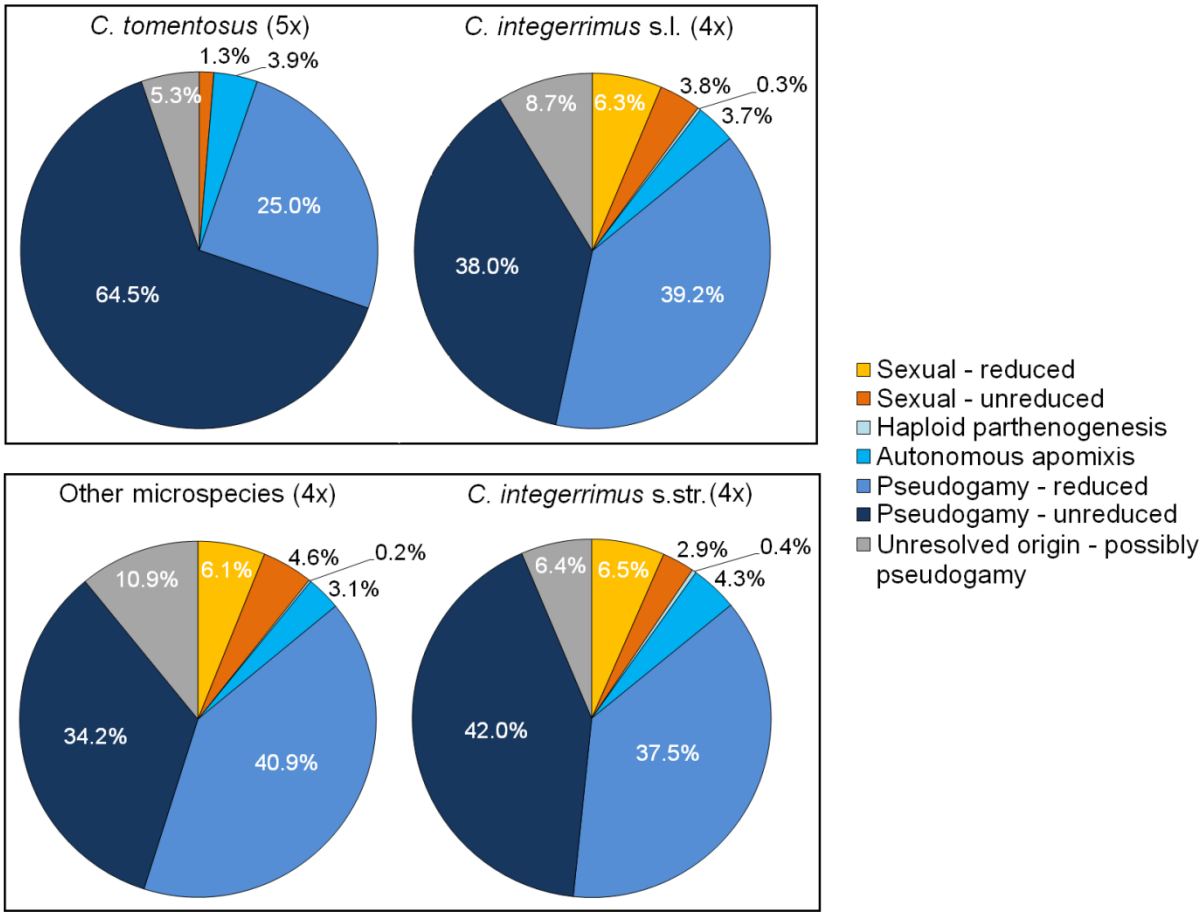

Fig. 4: Proportions of sexual vs apomictic seed formation (based on FCSS) in pentaploid *Cotoneaster tomentosus* (75 seeds) vs tetraploid *C. integerrimus* s.l. (1039 seeds) and tetraploid *Cotoneaster* microspecies (*C. laxiflorus* – 326 seeds, *C. alaunicus* – 165 seeds, *C. matrensis* – 44 seeds) vs *C. integerrimus* s.str. (504 seeds).

By contrast, tetraploid *C. integerrimus* s.l. turned out to be partly sexual (10.1% of seeds; Fig. 3, 4, Table 2). Besides a typical sexual FCSS profile, we also detected B_{III} individuals (participation of an unreduced gamete) in 40 seeds (3.8%). The majority of seeds was formed via pseudogamy – emb:end = 4:10 (39.2%) and emb:end = 4:12 (38.0%; corresponding to the contribution of a reduced and an unreduced male gamete, respectively). Autonomous apomixis (emb:end = 4:8) was detected in a minority of cases (3.7% of all seeds). Moreover, also haploid parthenogenesis was proven in three cases (0.3%) which involved both reduced and unreduced male gametes – emb:end = 2:6 and emb:end = 2:8; respectively. The entire tetraploid group of *C. integerrimus* s.l. microspecies (*C. integerrimus* s.str., *C. laxiflorus*, *C. alaunicus* and *C. matrensis*) exhibited no significant difference in the proportion of sexuality (i.e. from 4.6% to 13.8%; see Online Resource 6 or Fig. 4). A generalized mixed-effect linear model did not reveal any significant difference in the rate of sexuality between *C. integerrimus* s.str. and the group of other microspecies (N = 1057, $\chi^2 = 0.45$, p = 0.502) or between the groups of microspecies tested (N = 1057, χ^2 = 2.81, $p = 0.094$). Moreover, even within the geographically grouped dataset (i.e. separating plants from the Bohemian Massif and the Western Carpathians) we found no obvious difference or trend (see Online Resource 7; $N = 1096$, $\chi^2 < 0.001$, $p = 0.926$).

The reproductive modes of seeds from several populations from the Alps were more complex because of the occurrence three ploidy levels (2x and 4x *C. integerrimus* s.l. and 5x *C. tomentosus*). Whereas all diploids (28 seeds/3 pop.) were proved to be exclusively

sexual (emb:end = 2:3), tetraploid seeds again indicated facultative apomixis (5 seeds/1 pop.) and pentaploid *C. tomentosus* (23 seeds/3 pop.) were also proven to be dominantly apomictic.

Discussion

Two ploidy levels of *Cotoneaster* taxa were revealed in Central Europe. *Cotoneaster integerrimus* s.l. was found to be exclusively tetraploid in both study regions, the Western Carpathians and the Bohemian Massif whereas the pentaploid species *Cotoneaster tomentosus* was restricted to the Western Carpathians. In addition, the two taxa differed by 3.4% in relative Cx value and in the number of seeds per pome. Reproductive mode analysis detected, on the one hand, various apomictic types supplemented by a significant degree of sexuality (10.1% of sexual seeds) in *C. integerrimus* s.l. and, on the other, almost entirely apomictic reproduction in *C. tomentosus*. Besides the anticipated complete predominance of regular pseudogamy, we also identified haploid parthenogenesis and autonomous apomixis. In addition, we found 3.8% of hexaploid B_{III} embryos among the progeny of *C. integerrimus* s.l., indicating a potential of further polyploidization via unreduced gametes. The distribution of the detected types of reproduction was significantly homogenous across the whole of Central Europe, and we have not found any differences between the Bohemian Massif and the Western Carpathians (as well as among *C. integerrimus* s.l. microspecies).

Discrepancies between reported and detected ploidy levels

Various ploidy levels (3x, 4x, 5x) have been reported for *Cotoneaster* in Central Europe (Baranec 1992; Měsíček and Javůrková-Jarolímová 1992; Murín and Májovský 1992). However, we have confirmed (using flow cytometry calibrated by chromosome counts) only two of them: tetraploid (for *C. integerrimus* s.l.) and pentaploid (for *C. tomentosus*). This allows us to avoid complications arising from classical karyology of *Cotoneaster* species, including the high basic chromosome number, common occurrence of polyploidy, and presence of small and crowded somatic cells (Zeilinga 1964). Moreover, flow cytometry allowed us to analyse a large number of samples and also critically evaluate published karyological data (reported by Rothleutner et al. 2016). Some ploidy reports (e.g. triploid counts by Sax (1954) have already been repeatedly doubted; Zeilinga 1964; Kroon 1975). Hitherto published ploidal data on *C. integerrimus* s.l. in the Western Carpathians (Baranec 1992) indicate 4x *C. laxiflorus*, 3x *C. integerrimus* and both 3x and 4x *C. alaunicus*. Western Carpathian *C. tomentosus* has been found to be tetraploid (Baranec 1992; Murín and Májovský 1992). Our findings, however, differ tremendously (exclusively 4x *C. integerrimus* s.l. and 5x *C. tomentosus*). Whereas triploid counts from the Western Alps (Lauber and Wagner 1996) are congruent with our ploidy results (a potential hybrid between a diploid and a tetraploid cytotype within our Alpine dataset), reports of triploids from the Western Carpathians (Baranec 1992) might be doubtful and our extensive sampling has not confirmed any indication of either triploids or diploids. At the same time, the tetraploid count for *C. tomentosus* from the Western Carpathians (Murín and Májovský 1992) might be interpreted as a case of misidentification (probably confusion with *C. integerrimus* s.l.). Thus, based on published data and our comparative dataset, the Western Alps (rather than the Western Carpathians) remain a putative cytotype diversity hotspot (e.g. 2x, 3x, 4x, 5x; Favarger 1969; Löve 1969; Lauber and Wagner 1996; see also Online Resource 1).

Comparison of reproductive modes

That apomictic reproduction takes place in *C. integerrimus* s.l. and *C. tomentosus* has repeatedly been speculated on (Sax 1954; Kroon 1975; Bartish et al. 2001). However, only Hjelmquist (1962) and Li et al. (2017) published direct embryological and molecular evidence (for different *Cotoneaster* species). Detecting apomixis using other morphological (Sax 1954; Kroon 1975) and molecular (RAPD data by Bartish et al. 2001) means has already been deemed doubtful (Campbell et al. 1991; Dickoré and Kasperek 2010) because of the obscure horticultural origin and uncertain RAPD pattern of the plant material concerned. Moreover, our results (total prevalence of pseudogamy) and findings for the entire subtribe Malinae (Campbell et al. 1991; Dickinson et al. 2007) contradict evidence of apomixis from emasculation and style removal tests (Sax 1954).

The results of our reproductive mode analyses indicate an unexpectedly high proportion of endosperm formed with the participation of putative unreduced male gametes (one-half of pseudogamous *C. integerrimus* s.l. progeny and almost three-quarters of *C. tomentosus* pseudogamous progeny). We, however, have not found any evidence for the production of unreduced male gametes in sexual progeny. On the contrary, B_{III} individuals clearly demonstrate the formation of unreduced female gametes (37.4% of sexual progeny).

The putative involvement of unreduced male gametes in pseudogamous endosperm formation has been interpreted as the participation of two reduced sperm cells (i.e. dispermy) in *Crataegus* (Scott 2007; Talent and Dickinson 2007), *Sorbus* (Hajrudinović et al. 2015a, b), *Potentilla* (Dobeš et al. 2013) and *Rubus* (Šarhanová et al. 2012) so our result indicating pseudogamy may be interpreted in the same way (at least in part). The substantial proportion of sexual B_{III} progeny is not mirrored by our results on the ploidal structure in the field (no record of a hexaploid individual). We are aware, however, that our seeds screen data represent only potential progeny and that hexaploid B_{III} individuals might be getting excluded at the level of germination or competition. A significantly high proportion of the progeny (8.7% of *C. integerrimus* s.l. and 5.3% *C. tomentosus*) exhibited dubious embryo:endosperm ratios (4:9, 11, 13, 15, 17 and 5:9, 16). These probably come down to pseudogamy, odd endosperm ploidies and a partially continuous pattern of our FCSS results, so they may be simply explained by meiotic irregularities (Dobeš et al. 2013; Kolarčik et al. 2018) or fertilization by other Malinae genera (potential for intergeneric hybridization common in the subtribe; Robertson et al. 1991). Moreover, we detected autonomous apomixis, which may be interpreted as G2 phases of the embryo. Nevertheless, we did not detect any other putative G2 peaks of embryo tissue within the entire FCSS dataset (analogously to Šarhanová et al. 2012). Last but not least, we detected three seeds of *C. integerrimus* s.l. formed by haploid parthenogenesis, so our findings expand the small group of taxa in which this rare and obscure reproductive pathway has been identified – *Malus* (Kron and Husband 2009), *Pilosella* (Krahulcová and Krahulec 2000; Krahulec et al. 2011), *Potentilla* (Dobeš et al. 2013), *Sorbus* (Jankun and Kovanda 1986), *Rubus* (Šarhanová et al. 2012), the *Botriochloa-Dichanthium* complex (De Wet 1968), *Panicum* (Savidan and Pernès 1982), *Agropyron* (Hair 1956) and *Ranunculus* (Nogler 1984).

Among our results on reproductive modes there were 20 single-seed analyses in which two different endosperm peaks were detected. Such a pattern is probably reflecting the occurrence of twin embryos (overlapping peaks). It follows that we probably detected both sexually and apomictically developed embryos as well as two different apomictically developed twin embryos (e.g. 4:6 + 4:10, 12 and 4:8 + 4:10, 12). Surprisingly analogous results on reproductive behaviour have been obtained for the genus *Rubus* (including very similar frequencies; Šarhanová et al. 2012)

Our data on ploidy and reproductive modes correspond to the pattern observed in *Rubus* subg. *Rubus* published by Šarhanová et al. (2012). Both *Cotoneaster* and *Rubus* include sexual diploids, facultative apomictic tetraploids and obligatorily apomictic cytotypes with odd numbers of chromosomes. Šarhanová et al. (2012) also revealed a comparable ratio of B_{III} individuals (*Cotoneaster* 3.6% and *Rubus* 2.9%) and haploid parthenogenesis (*Cotoneaster* 0.3% and *Rubus* 2%). Although both systems are predominantly pseudogamous, a minor proportion of autonomous endosperm development has been reported (*Cotoneaster* 3.7% and *Rubus* 1.8%). In contrast to the genus *Rubus*, in which a regular pseudogamous ratio (4:10) is prevalent and higher and odd ploidies of endosperm are in a minority (from 9x up to 20x), apomictic *Cotoneaster integerrimus* s.l. seeds consist of more or less equal proportions of 4:10 and 4:12 endosperm ploidies as well as of a significant proportion (8.7%) of odd endosperm ploidies.

A comparable pattern with a predominant 4:12 ratio was also observed in *Sorbus bosniaca* (Hajrudinović et al. 2015a, b). Extensive parallels can be drawn between our FCSS results and those for the genus *Potentilla* s.l. (Dobeš et al. 2013), even though the sampling coverage of our data is hardly comparable. Nevertheless, reported reproductive pathways mostly match our results. In contrast to our results, however, Dobeš et al. (2013) detected also endosperm formed with the participation of a single polar nucleus while at the same time they found no evidence of autonomous apomixis.

Taxonomic implications

Our results do not provide a solid base for any taxonomic conclusions (in line with our original intentions), but they are relevant to future taxonomic revisions. The pattern of ploidy levels, relative genome size and reproductive behaviour that we have observed in *Cotoneaster integerrimus* s.l. shows no correlation with previously described microspecies of *Cotoneaster integerrimus* s.l. On the contrary, our data undoubtedly support the distinct status of *C. tomentosus* based on differences in ploidy, reproductive pathways and morphology (in concordance with e.g. Kutzelnigg 1994). We admit that additional differences within *C. integerrimus* s.l. might be found by means of molecular analyses. However, our data on reproduction confirm a significant proportion of sexuality (10.1%). Facultative apomixis is generally considered a serious obstacle to the taxonomical treatment of microspecies (Majeský et al. 2017). We therefore find the current concept of microspecies in the Western Carpathian region worthy of further discussion (Hrabětová-Uhrová 1961, 1962; Baranec 1992; Baranec and Eliáš 2004), as we have not found any evidence of a different cytological pattern or any reproductive isolation for *C. alaunicus, C. matrensis* and even *C. laxiflorus.* The taxonomic status of *C. alaunicus* remains obscure because its type material is from Western Russia (Orjol District; Fryer and Hylmö 2009) and therefore its Carpathian populations would form an enormous disjunction. Moreover, the type material of *C. laxiflorus* and *C. melanocarpus* is based on cultivated plants of unclear origin (Fryer and Hylmö 2009). The status of *C. matrensis,* even within the frame of the taxonomic concept splitting species into microspecies, is considered unclear (Baranec 1992; Bartha 2009; Fryer and Hylmö 2009). Moreover, the ultimate discrimination character between Western Carpathian microspecies, the colour of pomes (Baranec 1992; Baranec and Eliáš 2004), is highly variable, even within the fruit set of a single individual (it changes during maturation from dark orange to bluish tones; Macková et al. unpublished data; Kovanda 1992).

In agreement with our findings, Dickoré and Kasperek (2010) do not support the splitting of Western Carpathian taxa, commenting that 'the record for Central Europe seems doubtful'. To sum up, the Western Carpathian region is definitely a hotspot of European diversity of vascular plants (Kliment et al. 2016), but this does not apply

to *Cotoneaster integerrimus* s.l. Based on the literature and our comparative dataset, the actual centre of *C. integerrimus* s.l. diversity is probably located in the mountain ranges of Southwestern Europe (the southwestern Alps and the Pyrenees; Fryer and Hylmö 2009; Kurtto et al. 2013).

Conclusions

Flow cytometry of wild representatives of the genus *Cotoneaster* in our two model Central European regions (the Bohemian Massif and the Western Carpathians) has revealed the presence of two cytotypes: tetraploid *C. integerrimus* s.l. (incl. all subordinate microspecies) and pentaploid *C. tomentosus* (both without exceptions). Whereas *C. integerrimus* s.l. occurs throughout the study area, *C. tomentosus* is restricted to the Western Carpathians. The two taxa also differ in relative Cx values (3.4% lower in *C. integerrimus* s.l.).

Analysis of reproductive modes using the flow cytometric seed screen approach indicates almost obligatory apomictic reproduction in *C. tomentosus* (only one sexual seed detected) contrasting the facultative apomictic pattern of *C. integerrimus* s.l. (10.1% of sexual seeds). Moreover, apomixis in *C. integerrimus* s.l. takes place via several pathways of embryo and endosperm formation – pseudogamy, autonomous apomixis and haploid parthenogenesis. Pseudogamous apomixis (with the participation of both reduced and unreduced male gametes) appears to be the most frequent reproductive mode. In addition, unreduced female gametes contribute to the formation of more than one third of the sexual progeny of *C. integerrimus* s.l. (i.e. B_{III} individuals). Nevertheless, within tetraploid *C. integerrimus* s.l., we have not found any significant difference in reproductive mode between microspecies or between geographic regions, because the proportion of residual sexuality was significantly equal in each of the groups tested. To sum up, our results support the rather broad taxonomical concept of Dickoré and Kasperek (2010), over all narrow ones (Baranec 1992; Fryer and Hylmö 2009).

Thus, the Western Carpathians do not seem to be the hotspot of cytotype variation or diversity in reproductive modes of *C. integerrimus* s.l. On the contrary, we detected significantly greater variation (incl. diploids) within our small comparative dataset from the Western Alps, a region on which future investigations should be focused.

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Supplementary material

Online Resource 1 – Published chromosome counts of *Cotoneaster integerrimus* s.l. and *Cotoneaster tomentosus* in Europe.

Taxonomically relevant and properly located:

Taxon	Chromo-some count (2n)	Ploidy	State	Location	Reference
C. integerrimus	34	2x	France	Hautes Alps: Ceillac 1660 m a.s.l.	Favarger in Löve 1969
C. alaunicus	68	4x	Russia	Voronezh region: nature preserve Galitschja Gora	Krügel 1990
C. cambricus (*C. integerrimus* s.l.)	68	4x	Great Britain	North Wales: Great Ormes Head, Llandudno, Caernarfonshire	Fryer and Hylmö 1994
C. integerrimus	68	4x	Czech Republic	Prague: S part of the forest Kunratický les, SW. slope above the brook, 290 m a.s.l.	Měsíček and Javůrková-Jarolímová 1992
C. integerrimus	68	4x	Bulgaria	Rila Mountains: near Rila Monastery	Češchmedjiev in Löve 1983
C. integerrimus	68	4x	Russia	Caucasus (Eastern Elbrus): Schelda gorge	Gladkova 1968
C. integerrimus	102±2	6x	Sweden	Ölmevalla	Favarger in Löve 1975
C. tomentosus	68	4x	Slovakia	Sulov rocks: Sulov castle ruins	Murín and Májovský 1992
C. tomentosus	68	4x	Bulgaria	Rhodopi Mountains (Central): Trigrad gorge	Goranova 2007
C. tomentosus	c. 85	5x	France	Hautes Alps: Ceillac 1700 m a.s.l.	Favarger in Löve 1969

Uncertain location or taxonomy:

Taxon	Chromo-some count (2n)	Ploidy	State	Location	Reference
C. integerrimus	34	2x	Switzerland	Neufchâtel	Kroon 1975
C. integerrimus	34	2x	France	western Alps, Reculet (southern Jura)	Favarger 1969
C. integerrimus	34	2x	France	Dyon	Kroon 1975
C. integerrimus	51	3x	Ukraine	Crimea	Gladkova 1967

Taxon	Chromo-some count (2n)	Ploidy	State	Location	Reference
C. integerrimus	51	3x	Ukraine	Crimea	Gladkova 1968
C. integerrimus	62	?	Germany	Kunitz (Jena)	Krügel 1992
C. integerrimus	68	4x	Austria	Vienna	Kroon 1975
C. integerrimus	68	4x	Sweden	Lund	Kroon 1975
C. integerrimus	68	4x	Switzerland	central Jura	Favarger 1969
C. integerrimus	68	4x	France	Alps: Drôme, Isère, Savoy, Hautes-Alpes	Flinck et al. 1998
C. integerrimus	68	4x	Scandinavia, south--central Europe	NO	Hensen 1966
C. juranus (*C. integerrimus* s.l.)	68	4x	France	Vosges, Jura, Massif-Central, Pyrenees and Alps	Flinck et al. 1998
C. melanocarpus (*C. laxiflorus*)	68	4x	Ukraine	Odessa	Kroon 1975
C. melanocarpus (C. laxiflorus)	68	4x	Russia	Rostov	Kroon 1975
C. melanocarpus (C. laxiflorus)	68	4x	Romania	Bucharest	Kroon 1975
C. soczavianus/ C. tomentosus	68	4x	Russia	Northern Caucasus: Malaja Laba	Gladkova 1968
C. tomentosus	68	4x	France	Massif Central, Jura, Pyrenees, Alps: Haute-Savoie, Savoie, Isère, Drôme, Hautes-Alps, Alps de Haute--Provence, Alps Maritimes and Vaucluse	Flinck et al. 1998
C. tomentosus	68	4x	southern Europe	NO	Hensen 1966
C. tomentosus	85	5x	Austria, Slovenia	Karawanken 1600 m	Krügel 1992

Horticultural origin:

Taxon	Chromo-some count (2n)	Ploidy	State	Location	Reference
C. integerrimus	51	3x	USA	Arnold Arboretum	Sax 1954
C. alaunicus	62+B	4x	Russia	Kirovsk – botanic garden	Krügel 1992
C. integerrimus	68	4x	Armenia	Jerevan – botanical garden	Gladkova 1968
C. integerrimus	68	4x	Russia	Saint Petersburg – Park botanical institute	Gladkova 1968
C. integerrimus	68	4x	United Kingdom	Royal Botanical Garden Kew	Moffett 1931
C. melanocarpus var. *laxiflorus*	51	3x	USA	Arnold Arboretum	Sax 1954
C. melanocarpus (C. laxiflorus)	68	4x	USA	Arnold Arboretum	Sax 1954
C. melanocarpus (C. laxiflorus)	68	4x	Russia	Saint Petersburg – Park botanical institute	Gladkova 1968
C. melanocarpus (C. laxiflorus)	68	4x	USA	New York, Geneva	Dickson 1992
C. tomentosus	51	3x	USA	Arnold Arboretum	Sax 1954
C. tomentosus	68	4x	Russia	Saint Petersburg – Park botanical institute	Gladkova 1968
C. tomentosus	68	4x	Netherlands	Wageningen – arboretum, Boskoop – experimental station	Zeilinga 1964
C. tomentosus	85	5x	Austria	Klagenfurt – botanical garden	Krügel 1992

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Online Resource 2 – Study sites and sampling details used for the estimation of ploidy level (FCM) and analysis of reproductive modes (FCSS). Mixed populations are labelled by asterisks.

Population	Taxon	Ploidy	Individuals (N)	Seeds (N)	Location	State	Region	GPS	Altitude (m a.s.l.)
CI1*a,b*	*C. integerrimus* s.str.	4	24	86	Prokopské údolí – Albrechtův vrch (Albrecht hill)	Czech Republic	Bohemian Massif	50.044222, 14.346222	256
CI3	*C. integerrimus* s.str.	4	7	31	Prokopské údolí – Butovické hradiště (Butovice hill-fort)	Czech Republic	Bohemian Massif	50.040333, 14.354917	252
CI6*a,b,c*	*C. integerrimus* s.str.	4	3	0	Praha – Radotínské skály (Radotín rocks)	Czech Republic	Bohemian Massif	49.990194, 14.357444	255
CI10	*C. integerrimus* s.str.	4	2	0	Praha – Jabloňka	Czech Republic	Bohemian Massif	50.116056, 14.439111	196
CI12	*C. integerrimus* s.str.	4	2	0	Praha – Bohnické údolí (Bohnice valley)	Czech Republic	Bohemian Massif	50.137056, 14.40225	268
CI13	*C. integerrimus* s.str.	4	1	0	Praha – Baba	Czech Republic	Bohemian Massif	50.117817, 14.390753	217
CI14	*C. integerrimus* s.str.	4	1	0	Praha – Nad Mlýnem	Czech Republic	Bohemian Massif	50.112446, 14.368443	247
CI16	*C. integerrimus* s.str.	4	1	0	Praha – Jenerálka	Czech Republic	Bohemian Massif	50.103776, 14.348924	259
CI17	*C. integerrimus* s.str.	4	1	0	Praha – Dívčí skok	Czech Republic	Bohemian Massif	50.099829, 14.319419	294
CI18	*C. integerrimus* s.str.	4	7	13	Divoká Šárka – Kozákova skála (Kozák rock)	Czech Republic	Bohemian Massif	50.095173, 14.321414	330
CI20	*C. integerrimus* s.str.	4	1	0	Praha – Roztocký háj (Roztoky grove)	Czech Republic	Bohemian Massif	50.149942, 14.390485	244
CI23	*C. integerrimus* s.str.	4	3	0	Muráňská planina	Slovakia	Western Carpathians	48.760248, 19.971524	1184
CI26	*C. integerrimus* s.str.	4	1	0	Oparno – zřícenina hradu (castle ruins)	Czech Republic	Bohemian Massif	50.542278, 14.009861	271

Population	Taxon	Ploidy	Individuals (N)	Seeds (N)	Location	State	Region	GPS	Altitude (m a.s.l.)
CI28	*C. integerrimus* s.str.	4	1	0	Lovoš	Czech Republic	Bohemian Massif	50.528250, 14.018861	545
CI29	*C. integerrimus* s.str.	4	1	0	Košťálov	Czech Republic	Bohemian Massif	50.489806, 13.984917	461
CI30	*C. integerrimus* s.str.	4	3	0	Holý vrch (Holý hill)	Czech Republic	Bohemian Massif	50.502556, 13.978306	460
CI36	*C. integerrimus* s.str.	4	8	14	Havraníky	Czech Republic	Bohemian Massif	48.8170000, 16.0013056	332
CI37	*C. integerrimus* s.str.	4	1	0	Ostrý	Czech Republic	Bohemian Massif	50.531748, 13.951981	518
CM1	*C. laxiflorus*	4	3	0	Borač – Prudká: Sokolí skála	Czech Republic	Bohemian Massif	49.420583, 16.366056	364
CM2	*C. laxiflorus*	4	2	0	Chudčice – Břenčák	Czech Republic	Bohemian Massif	49.274233, 16.457833	268
CI42	*C. integerrimus* s.str.	4	1	0	Kaulsdorf	Germany	Bohemian Massif	50.617337, 11.394243	240
CI44*a,b,c*	*C. integerrimus* s.str.	4	5	0	Roztoky – Na Babě	Czech Republic	Bohemian Massif	50.031394,13.868653	277
CI47	*C. integerrimus* s.str.	4	1	0	Máslovická stráň 1	Czech Republic	Bohemian Massif	50.206667, 14.388333	250
CI49	*C. integerrimus* s.str.	4	1	0	Praha – Černá rokle (Black gulch)	Czech Republic	Bohemian Massif	49.990184, 14.338088	286
CI50*a,b,c*	*C. integerrimus* s.str.	4	7	11	Malý Bezděz	Czech Republic	Bohemian Massif	50.5391003, 14.7129103	510
CI51*a,b,c*	*C. integerrimus* s.str.	4	10	5	Hostěnice – Údolí říčky	Czech Republic	Bohemian Massif	49.241083, 16.738306	411
CI54	*C. integerrimus* s.str.	4	1	4	Mašovice – Mašovický lom	Czech Republic	Bohemian Massif	48.858694, 15.984917	357
CI55	*C. integerrimus* s.str.	4	4	6	Koňský spád	Czech Republic	Bohemian Massif	49.377889, 16.728972	492
CI56*a,b*	*C. integerrimus* s.str.	4	2	4	Boreč	Czech Republic	Bohemian Massif	50.515083, 13.987361	395
CI60	*C. integerrimus* s.str.	4	1	3	Vrbička	Czech Republic	Bohemian Massif	50.183194, 13.289194	518
CI61	*C. integerrimus* s.str.	4	1	3	Nová ves	Czech Republic	Bohemian Massif	50.201361, 13.289222	512
CI62	*C. integerrimus* s.str.	4	1	0	Soběchleby	Czech Republic	Bohemian Massif	50.222417, 13.51815	305
CI63	*C. integerrimus* s.str.	4	2	0	Radechovské skály	Czech Republic	Bohemian Massif	50.278056, 13.264	426
CI64	*C. integerrimus* s.str.	4	5	5	Choceň – Peliny	Czech Republic	Bohemian Massif	50.0034167, 16.2316667	326
CI65	*C. integerrimus* s.str.	4	3	0	Hracholusky – Čertova skála (Devil rock)	Czech Republic	Bohemian Massif	49.997861, 13.791111	342

Population	Taxon	Ploidy	Individuals (N)	Seeds (N)	Location	State	Region	GPS	Altitude (m a.s.l.)
CI66	*C. integerrimus* s.str.	4	1	0	Zbečno	Czech Republic	Bohemian Massif	50.043278, 13.927028	395
CA74*a,b*	*C. alaunicus*	4	2	1	Haligovské skály (Haligov rocks)	Slovakia	Western Carpathians	49.381944, 20.462111	700
CA76	*C. alaunicus*	4	6	53	Haligovské skály (Haligov rocks) – viewpoint	Slovakia	Western Carpathians	49.3827503, 20.4556942	770
CM77	*C. laxiflorus*	4	1	0	Trzy Korony (Three Crowns)	Poland	Western Carpathians	49.412472, 20.405917	580
CM78	*C. laxiflorus*	4	6	27	Trzy Korony (Three Crowns) – peak	Poland	Western Carpathians	49.4140278, 20.4143333	971
CM79	*C. laxiflorus*	4	6	0	Náměšť n. Oslavou – viewpoint	Czech Republic	Bohemian Massif	49.166389, 16.165444	424
CM80	*C. laxiflorus*	4	9	0	Náměšť n. Oslavou – Údolí Oslavy a Chvojnice	Czech Republic	Bohemian Massif	49.1727222, 16.1633056	320
CI82	*C. integerrimus* s.str.	4	1	0	Řivnáč	Czech Republic	Bohemian Massif	50.165222, 14.361806	290
CI83	*C. integerrimus* s.str.	4	1	0	Děvín – Pálava	Czech Republic	Bohemian Massif	48.874, 16.659269	405
CI84	*C. integerrimus* s.str.	4	1	0	Kletečná	Czech Republic	Bohemian Massif	50.566761, 13.971611	648
CI85	*C. integerrimus* s.str.	4	2	0	Černolice – Hřebeny	Czech Republic	Bohemian Massif	49.906431, 14.314556	343
CMat88*a,b*	*C. matrensis*	4	2	0	Rakytov	Slovakia	Western Carpathians	48.96425, 19.182278	1400
CMat90	*C. matrensis*	4	1	0	Minčol	Slovakia	Western Carpathians	48.950472, 19.158583	1340
CI91	*C. integerrimus* s.str.	4	1	6	Blatnica – Pekárová	Slovakia	Western Carpathians	48.9477500, 18.9475278	592
CT92*	*C. tomentosus*	5	1	0	Blatnica – Pekárová	Slovakia	Western Carpathians	48.9504444, 18.9536667	626
CI92*	*C. integerrimus* s.str.	4	1	11	Blatnica – Pekárová	Slovakia	Western Carpathians	48.9504444, 18.9536667	626
CT94	*C. tomentosus*	5	1	0	Blatnica – towards Pekárová	Slovakia	Western Carpathians	48.9521944, 18.9569444	680

Population	Taxon	Ploidy	Individuals (N)	Seeds (N)	Location	State	Region	GPS	Altitude (m a.s.l.)
CA95	*C. alaunicus*	4	1	1	Biela skala	Slovakia	Western Carpathians	49.293972, 19.49525	674
CT96*a,b*	*C. tomentosus*	5	2	14	Paklenica	Croatia	Dinara Mountains	44.3638800, 15.4605100	1091
CI100	*C. integerrimus* s.str.	4	2	12	Vojenský újezd Hradiště (Military area of Hradiště)	Czech Republic	Bohemian Massif	50.327336, 13.114111	771
CI101	*C. integerrimus* s.str.	4	5	12	Vojenský újezd Hradiště (Military area of Hradiště) – Humnický vrch	Czech Republic	Bohemian Massif	50.3438778, 13.1471778	696
CI102	*C. integerrimus* s.str.	4	2	6	Pokutice – Úhošť	Czech Republic	Bohemian Massif	50.365103, 13.241672	519
CM103	*C. laxiflorus*	4	10	17	Hontianske Nemce	Slovakia	Western Carpathians – Pannonia	48.304139, 18.974722	235
CA104	*C. alaunicus*	4	6	31	Valaská – Horné lazy	Slovakia	Western Carpathians	48.813361, 19.598139	570
CI105	*C. integerrimus* s.str.	4	5	25	Stratená	Slovakia	Western Carpathians	48.875389, 20.325417	835
CM106*	*C. laxiflorus*	4	5	27	Svit	Slovakia	Western Carpathians	49.0528356, 20.2112636	735
CT106*	*C. tomentosus*	5	5	15	Svit	Slovakia	Western Carpathians	49.0528356, 20.2112636	735
CM108*	*C. laxiflorus*	4	8	49	Liptovský Ján – cintorín (cemetary)	Slovakia	Western Carpathians	49.043028, 19.679028	660
CT108*	*C. tomentosus*	5	1	7	Liptovský Ján – cintorín (cemetary)	Slovakia	Western Carpathians	49.043028, 19.679028	660
CM110*	*C. laxiflorus*	4	5	28	Ružomberok	Slovakia	Western Carpathians	49.0631944, 19.3085278	500
CT110*	*C. tomentosus*	5	2	1	Ružomberok	Slovakia	Western Carpathians	49.0631944, 19.3085278	500
CI112*	*C. integerrimus* s.str.	4	10	60	Studničná – Sedem kostolov	Slovakia	Western Carpathians	49.134528, 19.265194	770
CT112*	*C. tomentosus*	5	1	6	Studničná – Sedem kostolov	Slovakia	Western Carpathians	49.134528, 19.265194	770

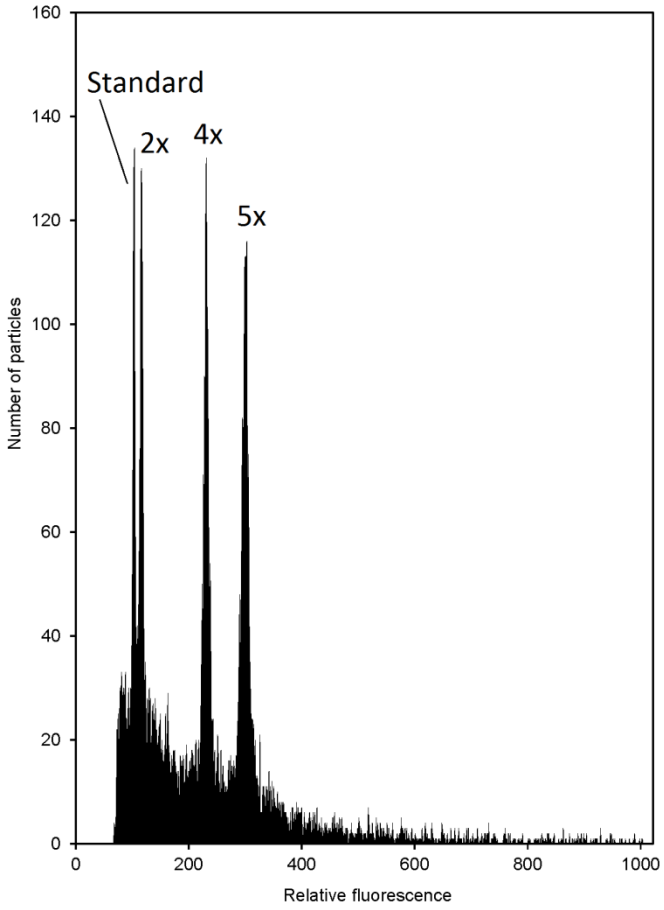
Population	Taxon	Ploidy	Individuals (N)	Seeds (N)	Location	State	Region	GPS	Altitude (m a.s.l.)
CM113	*C. laxiflorus*	4	5	22	Lietava – Lietavský hrad (Lietava Castle)	Slovakia	Western Carpathians	49.1606389, 18.6845556	590
CMat114	*C. matrensis*	4	5	14	Nitra – Zobor	Slovakia	Western Carpathians	48.348861, 18.095481	400
CM115	*C. laxiflorus*	4	13	59	Kvetnica	Slovakia	Western Carpathians	49.007806, 20.285528	720
CA117	*C. alaunicus*	4	2	12	Liptovský Ján – Javorovica 1	Slovakia	Western Carpathians	49.0265, 19.655483	1047
CA118*	*C. alaunicus*	4	4	16	Liptovský Ján – Javorovica 2	Slovakia	Western Carpathians	49.0274500, 19.6618167	968
CT118*	*C. tomentosus*	5	2	0	Liptovský Ján – Javorovica 2	Slovakia	Western Carpathians	49.0274500, 19.6618167	968
CI119	*C. integerrimus* s.str.	4	4	1	Máslovice 2	Czech Republic	Bohemian Massif	50.207611, 14.370917	220
CI120	*C. integerrimus* s.str.	4	3	0	Dolánky – Hlaváčková stráň	Czech Republic	Bohemian Massif	50.21303, 14.357418	229
CI121	*C. integerrimus* s.str.	4	2	13	Vlkolínec	Slovakia	Western Carpathians	49.041694, 19.27675	805
CI124	*C. integerrimus* s.str.	4	5	26	Kraviarske	Slovakia	Western Carpathians	49.211039, 19.016619	1330
CM125*	*C. laxiflorus*	4	4	1	Oravský Podzámok	Slovakia	Western Carpathians	49.263122, 19.359567	520
CT125*	*C. tomentosus*	5	3	7	Oravský Podzámok	Slovakia	Western Carpathians	49.263122, 19.359567	520
CT126	*C. tomentosus*	5	5	2	Súľov	Slovakia	Western Carpathians	49.168528, 18.578808	460
CA127	*C. alaunicus*	4	6	15	Lednica	Slovakia	Western Carpathians	49.109703, 18.209361	485
CI128*	*C. integerrimus* s.str.	4	4	14	Vršatské Podhradie	Slovakia	Western Carpathians	49.065967, 18.151081	730
CA128*	*C. alaunicus*	4	4	13	Vršatské Podhradie	Slovakia	Western Carpathians	49.065967, 18.151081	730
CM129	*C. laxiflorus*	4	6	7	Čifáre	Slovakia	Western Carpathians – Pannonia	48.240247, 18.429231	185
CMat130	*C. matrensis*	4	3	4	Kamenica nad Hronom	Slovakia	Western Carpathians – Pannonia	47.826111, 18.748244	192
CMat131	*C. matrensis*	4	5	1	Hajnáčka	Slovakia	Western Carpathians – Pannonia	48.218036, 19.955542	356
CI132*	*C. integerrimus* s.str.	4	2	4	Slanec	Slovakia	Western Carpathians	48.636942, 21.471017	460

Population	Taxon	Ploidy	Individuals (N)	Seeds (N)	Location	State	Region	GPS	Altitude (m a.s.l.)
CA132*	*C. alaunicus*	4	6	13	Slanec	Slovakia	Western Carpathians	48.636942, 21.471017	460
CT133*	*C. tomentosus*	5	1	0	Plešivec – Ostrý vŕšok	Slovakia	Western Carpathians – Pannonia	48.615206, 20.400197	695
CMat133*	*C. matrensis*	4	5	0	Plešivec – Ostrý vŕšok	Slovakia	Western Carpathians – Pannonia	48.615206, 20.400197	695
CMat134	*C. matrensis*	4	5	15	Topoľčiansky hrad (Topoľčany Castle)	Slovakia	Western Carpathians	48.658208, 18.050211	470
CMat135	*C. matrensis*	4	5	10	Jelenec – Dúň	Slovakia	Western Carpathians	48.411042, 18.222178	490
CT136	*C. tomentosus*	5	1	0	Lučivná	Slovakia	Western Carpathians	49.209106, 19.165361	500
CM137	*C. laxiflorus*	4	18	43	Moravský Krumlov – saint Florián	Czech Republic	Bohemian Massif	49.047884, 16.319888	311
CI138	*C. integerrimus* s.str.	4	4	18	Havraníky – vineyards Šobes	Czech Republic	Bohemian Massif	48.818236, 15.97435	324
CI139	*C. integerrimus* s.str.	4	2	4	Praha – Na Beránku	Czech Republic	Bohemian Massif	49.997696, 14.432302	285
CA140	*C. alaunicus*	4	1	5	Zádielská tiesňava	Slovakia	Western Carpathians – Pannonia	48.626879, 20.833634	535
CA141	*C. alaunicus*	4	1	5	Zádielská tiesňava – educational trail	Slovakia	Western Carpathians – Pannonia	48.621624, 20.838893	601
CM142	*C. laxiflorus*	4	8	41	Vdovčíkovo křeslo	Slovakia	Western Carpathians	48.77479, 20.338623	875
CM143*a,b*	*C. laxiflorus*	4	5	5	Brdárka – Malý Radzim 1	Slovakia	Western Carpathians	48.776006, 20.325863	971
CI145	*C. integerrimus* s.str.	4	1	0	Hrhovský amfiteatr – Kresadlo	Slovakia	Western Carpathians – Pannonia	48.618514, 20.764585	771
CI146	*C. integerrimus* s.str.	4	6	9	Hrhovský amfiteatr	Slovakia	Western Carpathians – Pannonia	48.618337, 20.780072	689

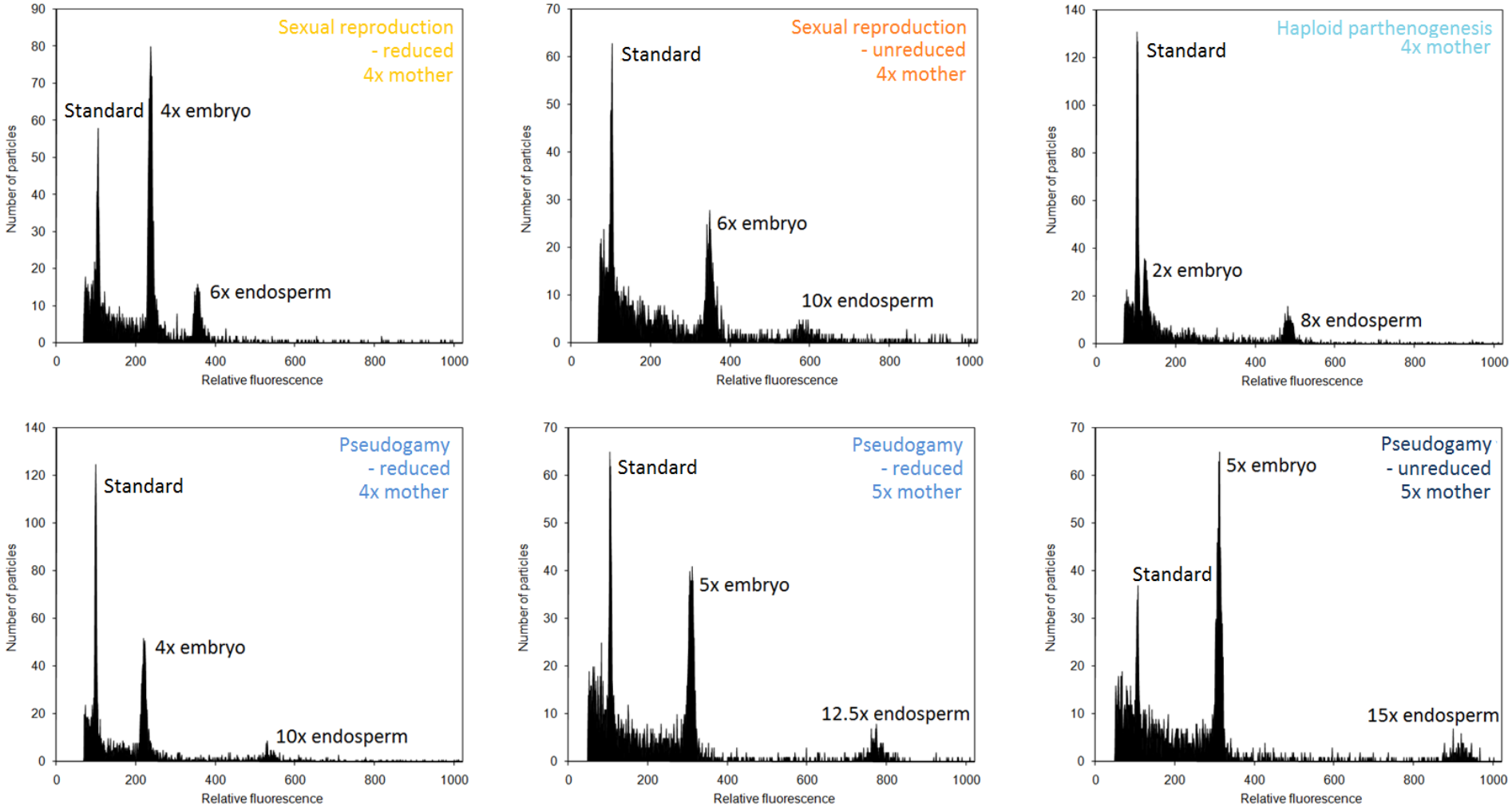
Population	Taxon	Ploidy	Individuals (N)	Seeds (N)	Location	State	Region	GPS	Altitude (m a.s.l.)
CI147	*C. integerrimus* s.str.	4	1	5	Zádiel – Hradisko	Slovakia	Western Carpathians – Pannonia	48.6079722, 20.8095556	273
CI148	*C. integerrimus* s.str.	4	1	5	Smolenice – Hlboča	Slovakia	Western Carpathians	48.511417, 17.420475	300
CI149	*C. integerrimus* s.str.	4	1	3	Kláštor pod Znievom	Slovakia	Western Carpathians	48.968592, 18.773086	940
CT154	*C. tomentosus*	5	2	12	Launsdorf	Austria	Alps	46.77631, 14.46047	628
CT155	*C. tomentosus*	5	1	0	Bad Mitterndorf	Austria	Alps	47.52575, 13.930278	883
CI159	*C. integerrimus* s.str.	4	6	0	Opočno – castle park	Czech Republic	Bohemian Massif	50.2644722, 16.1114722	279
CI160	*C. integerrimus* s.str.	4	1	0	Nové Město nad Metují	Czech Republic	Bohemian Massif	50.3500556, 16.1481667	297
CI161	*C. integerrimus* s.str.	4	11	31	Stárkov	Czech Republic	Bohemian Massif	50.5380556, 16.1513611	470
CI162	*C. integerrimus* s.str.	4	1	0	Blíževedly – Ronov	Czech Republic	Bohemian Massif	50.6200975, 14.4136522	516
CI163	*C. integerrimus* s.str.	4	11	11	Provodín – Provodínské kameny (Provodín stones)	Czech Republic	Bohemian Massif	50.6302772, 14.6086167	406
CI165	*C. integerrimus* s.str.	4	5	0	Praha – Homolka	Czech Republic	Bohemian Massif	50.0146767, 14.3736817	270
CI166	*C. integerrimus* s.str.	4	8	0	Lestkov	Czech Republic	Bohemian Massif	50.3671322, 13.1843611	529
CI167	*C. integerrimus* s.str.	4	29	0	Rašovické skály (Rašovice rocks)	Czech Republic	Bohemian Massif	50.3628997, 13.2078294	524
CI168	*C. integerrimus* s.str.	4	4	2	Mohelno – Fiolka	Czech Republic	Bohemian Massif	49.0976750, 16.2013567	276
CI169	*C. integerrimus* s.str.	4	9	0	Lhánice – Velká skála	Czech Republic	Bohemian Massif	49.1022911, 16.2408678	340
CI170	*C. integerrimus* s.str.	4	11	0	Templštejn	Czech Republic	Bohemian Massif	49.0901047, 16.2481306	358
CT171	*C. tomentosus*	5	1	0	Berndorf	Austria	Alps	47.9418389, 16.1150528	322
CT174	*C. tomentosus*	5	3	0	Orlovo Brdo – Pepelishte	Macedoina	Macedonia	41.53937, 22.14046	160
CI175	*C. integerrimus* s.str.	4	2	19	Wach Dürnstein – Höhereck	Austria	Bohemian Massif	48.3925200, 15.5322219	247
CT178	*C. tomentosus*	5	1	5	Schottwien	Austria	Alps	47.6593056, 15.8742778	573

Population	Taxon	Ploidy	Individuals (N)	Seeds (N)	Location	State	Region	GPS	Altitude (m a.s.l.)
CI179	*C. integerrimus* s.str.	4	1	2	Bílina – Bořeň	Czech Republic	Bohemian Massif	50.5276575,13.7638942	530
CI180	*C. integerrimus* s.str.	4	2	10	Hradčanské stěny (Hradčany Walls)	Czech Republic	Bohemian Massif	50.61536,14.70044	324
CI181	*C. integerrimus* s.str.	4	1	5	Hradčanské stěny (Hradčany Walls)	Czech Republic	Bohemian Massif	50.61552,14.69806	336
CI182	*C. integerrimus* s.str.	4	1	5	Hradčanské stěny (Hradčany Walls)	Czech Republic	Bohemian Massif	50.61733,14.68945	320
CT189	*C. tomentosus*	5	1	6	Grenoble – Fort du Saint--Eynard	France	Alps	45.2366392,5.7701097	1232
CT192	*C. tomentosus*	5	1	0	Grenoble – Aiguille de Quaix	France	Alps	45.2665661, 5.7202411	1065

Online Resource 3 – Histogram of a simultaneous flow cytometric analysis of *Cotoneaster* leaves showing three detected ploidy levels (2x – diploid *C. integerrimus* s.l., 4x – tetraploid *C. integerrimus* s.l. and 5x – pentaploid *C. tomentosus;* internal standard – *Carex acutiformis*; 2C = 800 Mpb).



Online Resource 4 – Representative histograms of flow cytometric analyses of *Cotoneaster* seeds showing six prevailing reproductive modes defined by the ratio between the ploidy of the embryo and that of the endosperm.

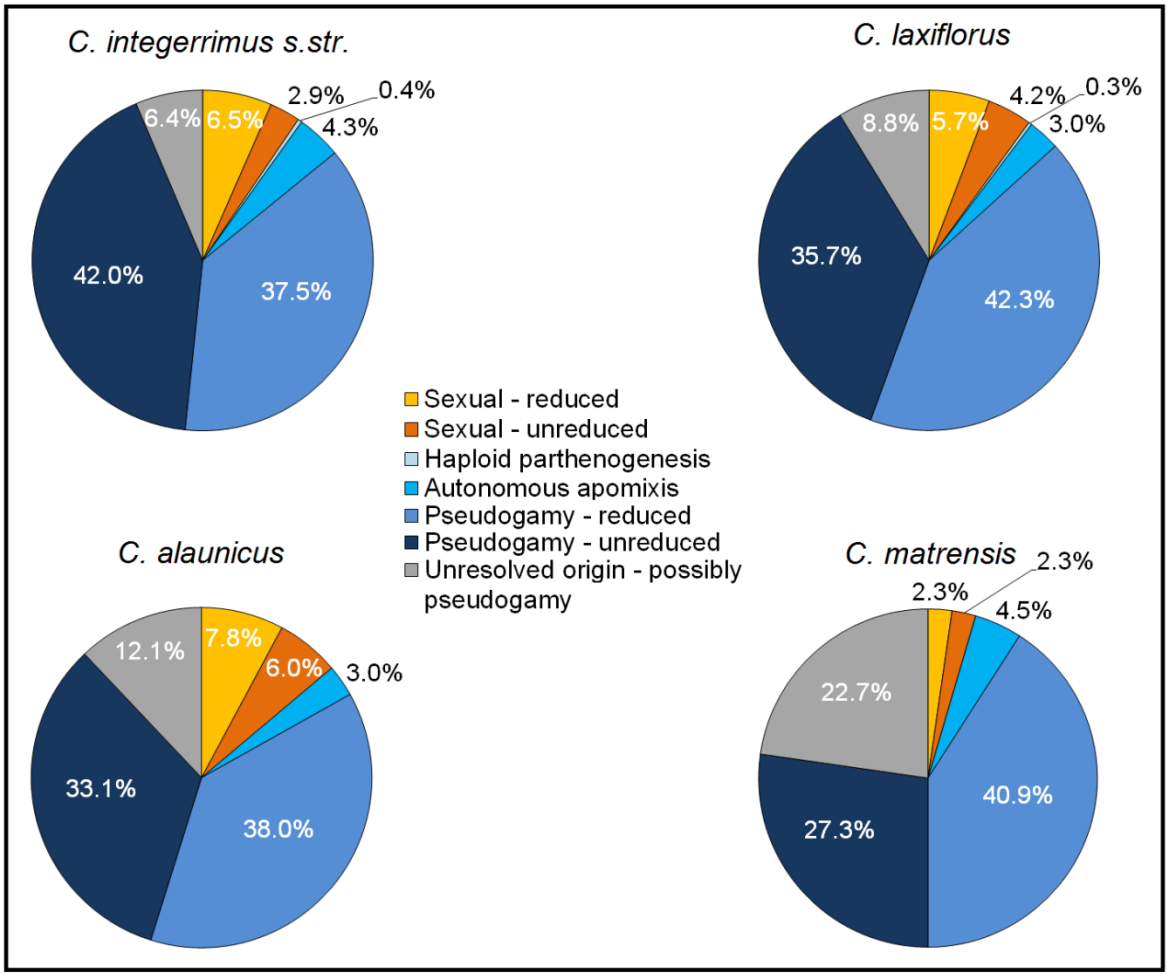


Online Resource 5 – All detected ratios between the ploidy of the embryo and that of the endosperm (including their abundance) showing pathways of seed formation in *Cotoneaster* taxa under study (based on FCSS).

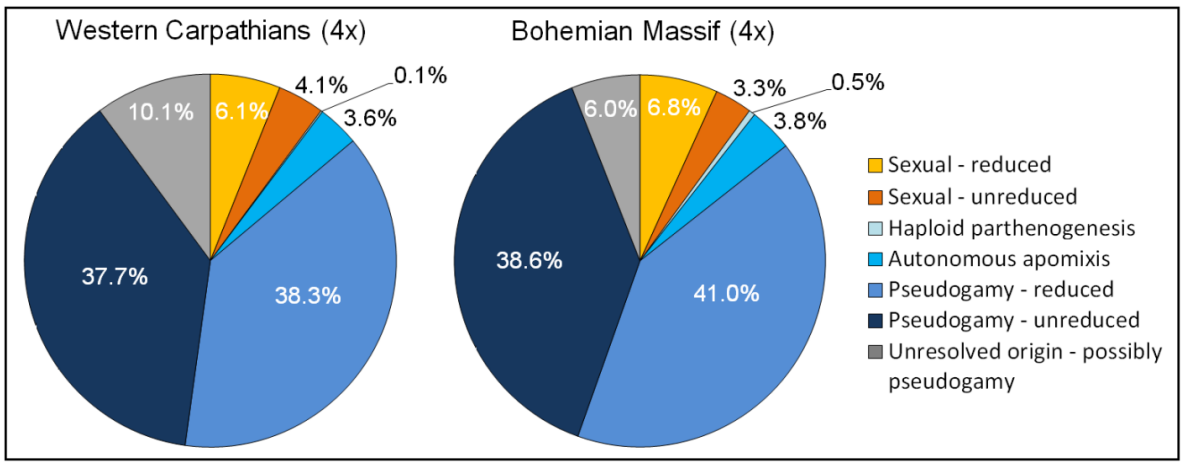
Type of reproduction	Ploidy of embryo	Ploidy of endosperm	Female gamete	Male gamete	Embryo formation	Endosperm formation	Seeds (N)	taxon
Sexual – reduced	4	6	reduced 2C	reduced 2C	fertilized 2+2=4C	fertilized (2+2)+2=6C	67	*C. integerrimus* agg.
Sexual – unreduced	6	9	unreduced 4C	unresolved	fertilized 4+2=6C	unresolved	2	*C. integerrimus* agg.
Sexual – unreduced	6	10	unreduced 4C	reduced 2C	fertilized 4+2=6C	fertilized (4+4)+2=10C	38	*C. integerrimus* agg.
Sexual – unreduced	8	13	unreduced 5C	reduced 3C	fertilized 5+3=8C	fertilized (5+5)+3=13C	1	*C. tomentosus*
Haploid parthenogenesis	2	6	reduced 2C	reduced 2C	parthenogenetic 2C	fertilized (2+2)+2=6C	1	*C. integerrimus* agg.
Haploid parthenogenesis	2	8	reduced 2C	unreduced 4C	parthenogenetic 2C	fertilized (2+2)+4=8C	2	*C. integerrimus* agg.
Autonomous apomixis	4	8	unreduced 4C	0	parthenogenetic 4C	autonomous (4+4)=8C	39	*C. integerrimus* agg.
Autonomous apomixis	5	10	unreduced 5C	0	parthenogenetic 5C	autonomous (5+5)=10C	3	*C. tomentosus*
Pseudogamy – reduced	4	10	unreduced 4C	reduced 2C	parthenogenetic 4C	fertilized (4+4)+2=10C	415	*C. integerrimus* agg.
Pseudogamy – reduced	5	12	unreduced 5C	reduced 2C	parthenogenetic 5C	fertilized (5+5)+2=12C	3	*C. tomentosus*
Pseudogamy – reduced	5	12.5	unreduced 5C	reduced 2.5C	parthenogenetic 5C	fertilized (5+5)+2.5=12.5C	6	*C. tomentosus*
Pseudogamy – reduced	5	13	unreduced 5C	reduced 3C	parthenogenetic 5C	fertilized (5+5)+3=13C	10	*C. tomentosus*
Pseudogamy – unreduced	4	12	unreduced 4C	unreduced 4C	parthenogenetic 4C	fertilized (4+4)+4=12C	383	*C. integerrimus* agg.
Pseudogamy – unreduced	4	14	unreduced 4C	reduced 2C	parthenogenetic 4C	fertilized (4+4+4)+2=14C	12	*C. integerrimus* agg.
Pseudogamy – unreduced	4	16	unreduced 4C	unreduced 4C	parthenogenetic 4C	fertilized (4+4+4)+4=16C	7	*C. integerrimus* agg.
Pseudogamy – unreduced	5	14	unreduced 5C	unreduced 4C	parthenogenetic 5C	fertilized (5+5)+4=14C	9	*C. tomentosus*
Pseudogamy – unreduced	5	15	unreduced 5C	unreduced 5C	parthenogenetic 5C	fertilized (5+5)+5=15C	40	*C. tomentosus*
Unresolved origin – possibly pseudogamy	4	9	unreduced 4C	unresolved	parthenogenetic 4C	unresolved	12	*C. integerrimus* agg.

Type of reproduction	Ploidy of embryo	Ploidy of endosperm	Female gamete	Male gamete	Embryo formation	Endosperm formation	Seeds (N)	taxon
Unresolved origin – possibly pseudogamy	4	11	unreduced 4C	unresolved	parthenogenetic 4C	unresolved	31	*C. integerrimus* agg.
Unresolved origin – possibly pseudogamy	4	13	unreduced 4C	unresolved	parthenogenetic 4C	unresolved	46	*C. integerrimus* agg.
Unresolved origin – possibly pseudogamy	4	15	unreduced 4C	unresolved	parthenogenetic 4C	unresolved	2	*C. integerrimus* agg.
Unresolved origin – possibly pseudogamy	4	17	unreduced 4C	unresolved	parthenogenetic 4C	unresolved	1	*C. integerrimus* agg.
Unresolved origin – possibly pseudogamy	5	9	unresolved	unresolved	unresolved	unresolved	1	*C. tomentosus*
Unresolved origin – possibly pseudogamy	5	16	unreduced 5C	unresolved	unresolved	unresolved	3	*C. tomentosus*

Online Resource 6 – Proportions of sexual vs apomictic seed formation (based on FCSS) in tetraploid *Cotoneaster integerrimus* s.l. – *C. integerrimus* s.str. (504 seeds), *C. laxiflorus* (326 seeds), *C. alaunicus* (165 seeds) and *C. matrensis* (44 seeds).



Online Resource 7 – Proportions of sexual vs apomictic seed formation (based on FCSS) in tetraploid *Cotoneaster integerrimus* s.l. in the Bohemian Massif (366 seeds) vs the Western Carpathians (711 seeds).



8 Conclusions

Polyploidization is a significant speciation force in the Rosaceae family, manifested in the cytotype variation of both model genera under study. Moreover, both model systems exhibited a potential for further polyploidization (triploid individuals and unreduced gametes). Individual *Prunus* species (incl. hybrids) are distinguished by different ploidy levels (diploid, triploid, or tetraploid) and by different absolute genome size (at the tetraploid level). Different ploidy levels also clearly determine *Cotoneaster* species (diploid and tetraploid *C. integerrimus* s.l., and pentaploid *C. tomentosus*) and their reproductive strategies (see below).

Hybridization plays a substantial role in the evolution and diversification of the Rosaceae. Besides its significance for speciation, a reverse effect of eroding species was exhibited by model genera studied. Hybridization markedly contributed to the speciation of both model groups, especially when combined with polyploidization (allopolyploid origin of *Prunus* tetraploid species and potential allopolyploidy in *Cotoneaster* species). On the other hand, our data revealed an adverse effect of hybridization manifested by disruption of the gene pool of wild *Prunus fruticosa* caused by crop-to-wild gene flow from cultivated cherries.

Reproductive strategies markedly shape the evolution of the Rosaceae. Heteroploid crossing of sexual *Prunus* species produces sterile triploid plants, which significantly contribute to the isolation of particular ploidy levels within heteroploid complexes, whereas homoploid hybridization results in fertile hybrids. However, reproduction determines especially the evolution of partially agamic complex of the genus *Cotoneaster*. Reproductive modes include both apomixis and residual sexuality. Apomixis involves various pathways of embryo and endosperm formation with prevailing pseudogamy. The ratio of the two reproductive strategies was found to be equal in all *Cotoneaster integerrimus* s.l. taxa under study (in contrast to *C. tomentosus*, which is almost obligatorily apomictic).

Results of the study of microevolutionary processes are indisputably applicable in agriculture (breeding programmes) and efficient conservation. Firstly, understanding microevolutionary processes facilitates effective species conservation. *Cotoneaster tomentosus* was found to occur only in the Western Carpathians in low abundance. By contrast, the putative diversity of *C. integerrimus* s.l. in the Western Carpathians was not confirmed based on the methods used. Revealing facultative apomixis has led to suggestions that reported microspecies may be of uncertain taxonomical value. Therefore, nature conservation management plans should mirror these findings. Secondly, direct nature conservation of *Prunus fruticosa* can be more effective based on this study. The methods used facilitate the clear identification of main threats (hybridization with sour cherry) and also highlight genuine wild populations. These populations, which are free of hybridization with cultivated cherries, should be targeted by nature conservation. Last but not least, solely economical implications are worthy of attention. Wild relatives of valuable crops represent an important source of genetic diversity for breeding programmes and their genetic erosion will lead to significant economic losses.

9 Curriculum Vitae

Lenka Macková (née Musilová)
*26. 4. 1988 in Opočno, the Czech Republic

Education

Since 2013: Ph.D. study of Botany, Department of Botany, Faculty of Science, Charles University
2010 – 2013: M.Sc. study of Botany, Department of Botany, Faculty of Science, Charles University
2010 – 2012: study of Education of Biology, Faculty of Science and Faculty of Arts, Charles University
2007 – 2010: Bc. study of Biology, Faculty of Science, Charles University
1999 – 2007: Grammar School Dobruška

Ph.D. courses and workshops

2016 – 2019: English courses (level B2, B2+, C1) – Professional English for Biology, Conversation Advanced (Faculty of Science, Charles University)
2016: Practical Rhetoric and Presentation (Faculty of Science, Charles University)
2015 – 2016: Scientific Presentations, Scientific Writing (Faculty of Science, Charles University)
2015 – 2016: University Pedagogy (Faculty of Science, Charles University)

Employment

Since 2018: teaching assistant of a practical course in Plant morphology and Systematic Botany (Department of Botany, Faculty of Science, Charles University) – part time job
Since 2017: researcher and lab technician at Department of Botany, Faculty of Science, Charles University (Laboratory of flow cytometry, field work, morphometrics – part time job for projects of prof. RNDr. Karol Marhold, CSc. and RNDr. Filip Kolář, Ph.D.)
2015 – 2016: teaching of a practical course in Systematic Botany for the Institute for Environmental Studies (Faculty of Science, Charles University) – part time job
2013 – 2016: teacher at basic school Břečťanová in Prague – part time job

Publications

Macková, L., Nosková, J., Ďurišová, Ľ., & Urfus, T.: Insights into the cytotype and reproductive puzzle of *Cotoneaster integerrimus* in the Western Carpathians. – Plant Systematics and Evolution (preliminary accepted)

Macková, L., Vít, P., & Urfus, T. (2018): Crop-to-wild hybridization in cherries – Empirical evidence from *Prunus fruticosa*. – Evolutionary Applications 11:1748–1759. doi: https://doi.org/10.1111/eva.12677

Macková, L., Vít, P., Ďurišová, Ľ., Eliáš, P., & Urfus, T. (2017): Hybridization success is largely limited to homoploid *Prunus* hybrids: a multidisciplinary approach. – Plant Systematics and Evolution 303: 481–495. doi: https://doi.org/10.1007/s00606-016-1385-4

Conference participation

Macková, L., Bílá, J., Ďurišová, Ľ., Eliáš, P. jun. & Urfus, T.: Dusk of sexual reproduction? Endless possibilities of plant breeding systems – insight into the genus *Cotoneaster*

(2017) – Biogeography of the Carpathians (Ecological and evolutionary facets of biodiversity). Cluj-Napoca, Romania

Macková, L., Vít, P., Ďurišová, Ľ., Eliáš, P., & Urfus, T.: Dusk of cherries? Hybridization success is largely limited to homoploid *Prunus* hybrids (2016) – ICPHB (International Conference on Polyploidy, Hybridization and Biodiversity). Rovinj, Croatia

Awards

Student poster awards:

Dusk of sexual reproduction? Endless possibilities of plant breeding systems – insight into the genus *Cotoneaster* – scientific conference Biogeography of the Carpathians 2017 (Ecological and evolutionary facets of biodiversity). Cluj-Napoca, Romania

Dusk of cherries? Hybridization success is largely limited to homoploid *Prunus* hybrids – ICPHB 2016 (International Conference on Polyploidy, Hybridization and Biodiversity). Rovinj, Croatia

Grant projects

2012–2014: Důsledky antropohybridizace *Prunus fruticosa* (třešně křovité) s pěstovanými zástupci rodu *Prunus* (Consequences of anthropohybridization of *Prunus fruticosa* with cultivated relatives) – GA UK 669812 (main student investigator)

Professional activities

2018: reviewing of a bachelor's thesis at the Department of Botany, Faculty of Science, Charles University (Anna Tesařová: Ochranářské aspekty endemismu ve střední Evropě se zvláštním přihlédnutí k modelové skupině rodu *Sorbus* – Conservation aspects of endemism in Central Europe with special respect to genus *Sorbus*)

2016–2018: co-supervision of a master's thesis at Department of Botany, Faculty of Science, Charles University (Bc. Klára Ondříčková: Význam hybridizace v evoluci rodu *Sorbus* – Importance of hybridization in genus *Sorbus* evolution)

Organization membership

Since 2012: Czech Botanical Society