Charles University

Faculty of Science

Study program: Biology

Ondřej Peřina

Polyploidization and hybridization as evolutionary drivers in the Medicago sativa group

Polyploidizace a hybridizace jako hnací síla v evoluci komplexu Medicago sativa agg.

Bachelor's thesis

Supervisor:

RNDr. Filip Kolář, Ph.D.

Prague, 2024

Poděkování

Na tomto místě bych rád poděkoval vedoucímu této práce RNDr. Filipovi Kolářovi, Ph.D. za jeho ochotu a pohotovost a dále všem, kdo mě při psaní této práce podporovali.

Prohlášení:

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

V Praze, 26. 4. 2024

Ondřej Peřina

Abstract

This bachelor's thesis addresses polyploidy induced by whole genome duplication and its consequences to the evolution of *Medicago sativa* species complex, i.e. wild relatives of an important forage crop alfalfa. This complex encompasses several interfertile taxa naturally occurring as diploid (2n = 16) or tetraploid (2n = 32) cytotype. Several recently conducted studies attempted to explain origin and evolution of this complex and relationships among its members using modern molecular methods, however, the results are still fragmentary and inconclusive. Two most widespread members of the complex: purple flowering *Medicago sativa* and yellow flowering *Medicago falcata* are genetically differentiated both at diploid and tetraploid level, what is supporting their recognition as distinct taxa. Furthermore, it has been shown that tetraploid *M. sativa* subsp. *sativa* is an autopolyploid that originated from diploid *M. sativa* subsp. *caerulea* by intraspecific whole genome duplication. On the other hand, the origin of tetraploid *M. falcata* seems to be more complex, presumably involving autopolyploidization followed by past introgression from *Medicago prostrata*. Most of the studies concerning this topic were performed on accessions, which are sometimes of uncertain ploidy and origin, obtained from germplasm databases. This fact leaves our knowledge on the origin and mechanisms of diversification of the *Medicago sativa* complex only fragmentary.

Keywords

polyploidy, evolution, *Medicago sativa* complex, reproductive barriers, hybridization

Abstrakt

Tato bakalářská práce se věnuje tématu polyploidie způsobené celogenomovou duplikací a jejími důsledky v evoluci komplexu *Medicago sativa* agg., tzn. divokých příbuzných významné pěstované pícniny vojtěšky. Tento komplex zahrnuje několik volně křižitelných taxonů přirozeně se vyskytujících v diploidním (2n = 16) nebo tetraploidním (2n = 32) cytotypu. Několik nedávno provedených studií se pokusilo vysvětlit původ a evoluci tohoto komplexu a vztahy mezi jeho členy, ale výsledky jsou stále zlomkovité a neprůkazné. Dva nejrozšířenější členové komplexu: fialově kvetoucí *Medicago sativa* a žlutě kvetoucí *Medicago falcata* jsou jak na diploidním tak na tetraploidním levelu geneticky diferencované, což podporuje jejich uznání jako odlišné taxony. Dále bylo prokázáno, že tetraploidní *M. sativa* subsp. *sativa* je autopolyploid, který vznikl z diploidní *M. sativa* subsp. *caerulea* jednodruhovou celogenomovou duplikací. Na druhou stranu původ tetraploidní *M. falcata* se zdá být komplexnější, pravděpodobně zahrnující autopolyploidizaci následovanou dávnou introgresí z *Medicago prostrata*. Většina studií věnující se tomuto tématu byla provedena na vzorcích občas nejasné ploidie a původu ze semenné databáze. Tento fakt zanechává naše vědomosti o původu a mechanismech diverzifikace komplexu *Medicago sativa* agg. pouze zlomkovité.

Klíčová slova

polyploidie, evoluce, *Medicago sativa* agg., reprodukční bariéry, hybridizace

CONTENT TABLE

1 INTRODUCTION

Polyploidy induced by whole genome duplication is considered one of the most important evolutionary forces driving plant evolution and speciation (Wood et al., 2009). Whole genome duplication typically leads to immediate strong reproductive isolation of polyploids from their ancestors and subsequently can lead to formation of new polyploid species independent on their diploid progenitor (Sémon and Wolfe, 2007). Many studies explaining evolutionary history of polyploid plant species and complexes have been performed in last few decades, significantly deepening our understanding of evolutionary consequences of this widespread and globally important mutation called whole genome duplication. One example of polyploid species complex encompassing several diploid and autotetraploid species interlinked by hybridisation is *Medicago sativa* complex.

Medicago sativa complex encompasses important cultivated forage crop alfalfa and several wild growing taxa, which are perennial outcrossing herbs naturally occurring as diploid (2n=16) or tetraploid (2n=32) cytotype (Lesins and Lesins, 1979). They are able to freely hybridize when they are on the same ploidy level, however, crosses between diploid and tetraploid members are in most cases unsuccessful (Lesins, 1952). Cytology and genetics of alfalfa has been intensively studied and germplasm database including many accessions from various parts of the world is available. Moreover, relatively closely related annual herb *Medicago truncatula* serves as a model organism for legume biology and genetics (Cook, 1999). Also annotated genome sequences of *Medicago sativa* (Chen et al., 2020; Li et al., 2020; Shen et al., 2020; Shi et al., 2024) and *Medicago truncatula* (Krishnakumar et al., 2015) are available.

Thanks to these benefits, the *Medicago sativa* species complex represents a suitable model for evolutionary study of polyploidy. Indeed, several studies exploring mechanisms underlying evolution of *Medicago sativa* complex have been conducted. Molecular methods such as fluorescence in situ hybridization (FISH) (Yu et al., 2017), chloroplast and mitochondrial DNA sequencing (Havananda et al., 2011, 2010) or evaluation of single sequence repeat (SSR) markers (İlhan et al., 2016; Şakiroğlu et al., 2010) and single nucleotide polymorphisms (SNP) (Şakiroğlu and Brummer, 2017) helped to explore genetic diversity of wild populations, explaining the origin of tetraploid members or clarifying the relationships among diploid and tetraploid taxa.

Despite the relatively high number of recently conducted studies, many questions concerning evolution of *Medicago sativa* complex remain unanswered. The aim of this bachelor's thesis is to synthesize current findings about evolutionary history of *Medicago sativa* complex and to propose directions of further study concerning this topic.

2 POLYPLOIDY IN PLANT EVOLUTION

Polyploid organisms (= organisms that possess more than two chromosome sets) can naturally arise by rare macromutation called whole genome duplication (WGD) (Wolfe, 2015). We can divide polyploids according to different way of their formation into autopolyploids and allopolyploids. Autopolyploids emerge from within-species whole genome duplication events, whereas allopolyploids arise from whole genome duplication events involving inter-specific hybridization (Ramsey and Schemske, 2002). They also differ in mode of inheritance, as shown on *Arabidopsis*, with autotetraploid showing tetrasomic inheritance and occasional formation of quadrivalents during meiosis and allotetraploid showing disomic inheritance and formation of bivalents during meiosis, with the two sub-genomes segregating independently (Lloyd and Bomblies, 2016).

Polyploid plants can be formed either by spontaneous doubling of chromosomes in somatic cells or by merging of unreduced gametes, with the latter further divided to unilateral and bilateral polyploidization. Somatic chromosome doubling can lead in plants to formation of polyploid meristematic cells and consequently to polyploid offspring (Upscott, 1939). Unilateral sexual polyploidization involves triploid intermediates that emerged by fusion of reduced with unreduced gametes. Tetraploid individuals are then formed either by selfing of triploid intermediates or by crossing these triploids with other triploid or diploid individuals. On the other hand, bilateral sexual polyploidization happens when two unreduced gametes merge and directly form tetraploid individual with no involvement of triploid intermediates. All three above-described pathways have been observed in various plant species e.g. , somatic chromosome doubling in *Primula* (Upscott, 1939), unilateral sexual polyploidization in *Ranunculus* (Schinkel et al., 2017) and bilateral sexual polyploidization in *Dactylis* (Bretagnolle and Lumaret, 1995).

Whole genome duplication typically leads to immediate strong reproductive isolation of polyploids from their ancestors and is considered as one of the major drivers of plant speciation and the most frequent sympatric speciation mechanism (Otto and Whitton, 2000).

It is responsible for about 35% of all speciation events in vascular plants (Wood et al., 2009) and estimates for incidence of polyploidy in angiosperms are ranging from 30% (Stebbins, 1950) to 70% (Masterson, 1994). Actually, all of present-day seed plants, experienced during their evolution at least one case of whole genome duplication (Jiao et al., 2011). In spite of over 100-years of research in polyploidy, particularly among plant biologists (Ramsey and Ramsey, 2014) and recent increasing interest in genomics of polyploidy, especially the genetic research of polyploid systems is usually lagging behind that of their diploid counterparts, due to taxonomic complexity of polyploid groups as well as methodological challenges associated with complex polyploid genomes. To reveal evolutionary significance of polyploidization, integrative studies of ecology, reproductive systems and genetics of natural systems encompassing close diploid and polyploid populations are needed.

3 MEDICAGO SATIVA COMPLEX

3.1 Taxonomy and morphological species delimitation

With over 60 species, *Medicago* is one of the major genera belonging to the family *Fabaceae*. *Medicago sativa* species complex together with *Medicago prostrata* create subsection *Falcatae* of the section *Falcago* within the genus *Medicago*. This species complex includes important cultivated forage crop *Medicago sativa*, also known as alfalfa or lucerne, and its wild growing relatives, which are perennial mostly outcrossing herbs naturally occurring as diploid (2n=16) or tetraploid (2n=32) cytotypes (Lesins and Lesins, 1979; Quiros and Bauchan, 1988).

Because of their high morphological variability together with frequent hybridization, taxonomical status and nomenclature of taxa included in the *Medicago sativa* complex have been a subject of disputes for many decades (Lesins and Gillies, 1972). Thanks to work of K. A. Lesins, I. Lesins, G. B. Gillies and other authors the enormous number of originally described taxa in this complex has been strongly reduced (Lesins and Gillies, 1972; Lesins and Lesins, 1979). Nevertheless, there still remains a disagreement, whether to recognize members of *Medicago sativa* complex as distinct species or as subspecies of *Medicago sativa*. Despite the fact that some authors recognize them only as subspecies (Quiros and Bauchan, 1988; Small and Jomphe, 1989), I will use taxonomy proposed by Lesins and Lesins, (1979) in their monography and recognize them as distinct species, what is also in agreement with the current taxonomic treatment in the Czech Republic (Kaplan, 2019).

Flower colour, pod shape and presence of glandular hairs on pods are the main morphological characteristics by which taxa included in *Medicago sativa* complex have been traditionally defined (Lesins and Gillies, 1972; Lesins and Lesins, 1979; Quiros and Bauchan, 1988). Purple flowering *Medicago sativa* (s. str.) with spirally coiled pods and yellow flowering *Medicago falcata* with sickle shaped pods are the two morphologically most distinct members of the complex. They both naturally occur as a diploid and tetraploid cytotype with *Medicago sativa* divided according to ploidy level into two subspecies: diploid *M. sativa* subsp. *caerulea* and tetraploid *M. sativa* subsp. *sativa* (Lesins and Lesins, 1979). Hybrids between *M. sativa* and *M. falcata* are on diploid level called *Medicago* ×*hemicycla* and on tetraploid level *Medicago* ×*varia.* Both of these hybrids are morphologically extremely variable, typically possessing greenish or variegated flower colour and loosely coiled pods (see Figure 1).

Morphometric analysis performed on *Medicago sativa* and *Medicago falcata* plants showed that diploid *M. sativa* subsp. *caerulea* and tetraploid subsp. *sativa* significantly differ in quantitative morphological characteristics. Tetraploid subsp. *sativa* has wider leaves, larger flowers and longer seeds than diploid subsp. *caerulea*. On the other hand, ploidy cytotypes of *Medicago falcata* display overlapping variation in most quantitative morphological characteristics (Small, 1985). However, very low number of tetraploid *M. falcata* specimens examined in this study could negatively affect these results.

In another study, morphological characteristics of unspecified ploidy cytotypes of *M. sativa*, *M. falcata* and their respective hybrids were evaluated. Results of this study suggest that hybrids can possess practically any combination of morphological characteristics from continuum of variation between *M. sativa* and *M. falcata* (Small and Brookes, 1984). Both of these studies were, however, conducted on plants coming from germplasm collections of sometimes unclear origin and karyological status (Şakiroğlu and Brummer, 2011). Therefore, proper quantitative assessment of morphometric analysis performed on a sufficient number of plants collected from natural populations is still missing.

Besides *M. sativa*, *M. falcata* and their hybrids, the complex encompasses another four much rarer taxa. Firstly, diploid *Medicago glomerata* is characterized by yellow flowers and tightly coiled pods covered by glandular hairs (Lesins and Lesins, 1979; Pignatti, 2017). Then, there is tetraploid *Medicago glutinosa*, which is defined by pale yellow or almost milky flower colour and loosely coiled pods also covered by glandular hairs. Its tetraploid hybrid with *Medicago sativa* is called *Medicago* ×*polychroa*. Like hybrids of *M. sativa* and *falcata*, this hybrid is also morphologically very variable, characterized by variegated flower colour

and loosely coiled pods, but it can be distinguished by presence of glandular hairs on the pods (Lesins and Lesins, 1979; Šemdgenelni et al., 1971). Finally, last tetraploid member included in *Medicago sativa* complex is *Medicago ×tunetana*. It has yellow or greenish flower colour and tightly coiled pods, which are again covered by glandular hairs (Lesins and Lesins, 1979; Pottier-Alapetite, 1979). Based on this combination of morphological characteristics, *Medicago sativa* and *Medicago glomerata* are hypothesized to be ancestors of *M. ×tunetana* (Lesins and Lesins, 1979).

Overall, very little is known about these rare members of *Medicago sativa* complex in comparison to *Medicago sativa*, *Medicago falcata* and their hybrids and no quantitative morphometric analysis have been performed on them. All of above-mentioned taxa together with ploidy level and morphological characteristics are summarized in Table 1.

Table 1 – List of taxa belonging to the *Medicago sativa* complex with the major discriminating morphological characters

Taxon	Ploidy	Flower colour	Pod shape
Medicago falcata	2x, 4x	Yellow	Sickle shaped
Medicago sativa subsp. caerulea	2x	Purple	Spirally coiled
Medicago sativa subsp. sativa	4x	Purple	Spirally coiled
Medicago ×hemicycla (M. sativa subsp. caerulea × M. falcata)	2x	Variegated	Loosely coiled
Medicago ×varia (M. sativa subsp. sativa × M. falcata)	4x	Variegated	Loosely coiled
Medicago glomerata	2x	Yellow	Tightly coiled
Medicago glutinosa	4x	Pale yellow	Loosely coiled
Medicago ×tunetana ?*(M. sativa* subsp. *sativa × M. glomerata)*?	4x	Yellow or greenish	Tightly coiled
Medicago ×polychroa (M. sativa subsp. sativa × M. glutinosa)	4x	Variegated	Loosely coiled

Figure 1 – Morphological continuum between tetraploid *Medicago falcata* and *Medicago sativa*: (A) *M. falcata*, (B, C, D) *M.* ×*varia*, (E, F) *M. sativa* (Small and Brookes, 1984)

3.2 Distribution and ecology

Wild growing members of *Medicago sativa* complex naturally occur in vast area extending from Spain to China and from Sweden to North Africa, where they with a few exceptions primarily inhabit sunny and dry habitats in open landscape (Lesins and Lesins, 1979). Cultivated alfalfa (tetraploid *M. sativa* often introgressed by *M. falcata* (Wang et al., 2023)), can be found in roughly the same area, but in addition it has become acclimatized in South Africa, Australia, New Zealand, and both Americas (Michaud et al., 1988). The fact that alfalfa cultivation lasts for several thousands of years (Michaud et al., 1988; Wang and Şakiroğlu, 2021) makes notion about native range of tetraploid *M. sativa* subsp. *sativa* very unclear. Though, it is highly probable that it originated somewhere within the current range of diploid *M. sativa* subsp. *caerulea*, which is distributed only in arid semi-desert regions around Caspian Sea (Lesins and Lesins, 1979; Şakiroğlu et al., 2010).

Medicago falcata is another widespread member of *Medicago sativa* complex. It can be found predominantly in temperate regions of Eurasia, where it prefers to grow in dry steppe conditions (Lesins and Lesins, 1979). Populations of diploid *M. falcata* can be found

scattered in wide range extending from eastern Switzerland to eastern Mongolia (Blanco-Pastor et al., 2021; Şakiroğlu et al., 2010; Savova et al., 1996). Tetraploid *M. falcata* cytotype is distributed in approximately the same area as diploid cytotype (Quiros and Bauchan, 1988), with the exception of Western and Northwest Europe, where diploid cytotype has not been found yet, but tetraploid cytotype is relatively common (Savova et al., 1996).

Medicago ×*hemicycla*, a diploid hybrid between *M. sativa* and *M. falcata*, can be found in regions where distribution area of *M. sativa* subsp. *caerulea* and diploid *M. falcata* overlap. It has been collected in the Caucasian region and in Northwest Kazakhstan (Blanco-Pastor et al., 2021; Şakiroğlu et al., 2010). Similarly, *Medicago* ×*varia*, a tetraploid hybrid of *M. sativa* and *M. falcata*, is common in regions where both *M. sativa* subsp. *sativa* and tetraploid *M. falcata* occur (Kaljund and Leht, 2013; Savova et al., 1996).

All four remaining taxa included in *Medicago sativa* complex have much smaller areas of distribution than *M. sativa* or *M. falcata*. Diploid *Medicago glomerata* can be found just in Maritime Alps (Lesins and Lesins, 1966), tetraploid *Medicago glutinosa* and its hybrid with *M. sativa* (*Medicago* ×*polychroa*) are growing only in the moist subalpine regions of Caucasia (Lesins and Lesins, 1979) and the putative hybrid of *M. sativa* and *M. glomerata* (*Medicago* ×*tunetana*) is endemic to the mountainous regions of Tunisia and Algeria, where it is considered highly endangered due to overgrazing and habitat loss (Ferchichi et al., 2021). Approximate distribution of diploid and tetraploid taxa included in *Medicago sativa* complex is summarized in Figure 2.

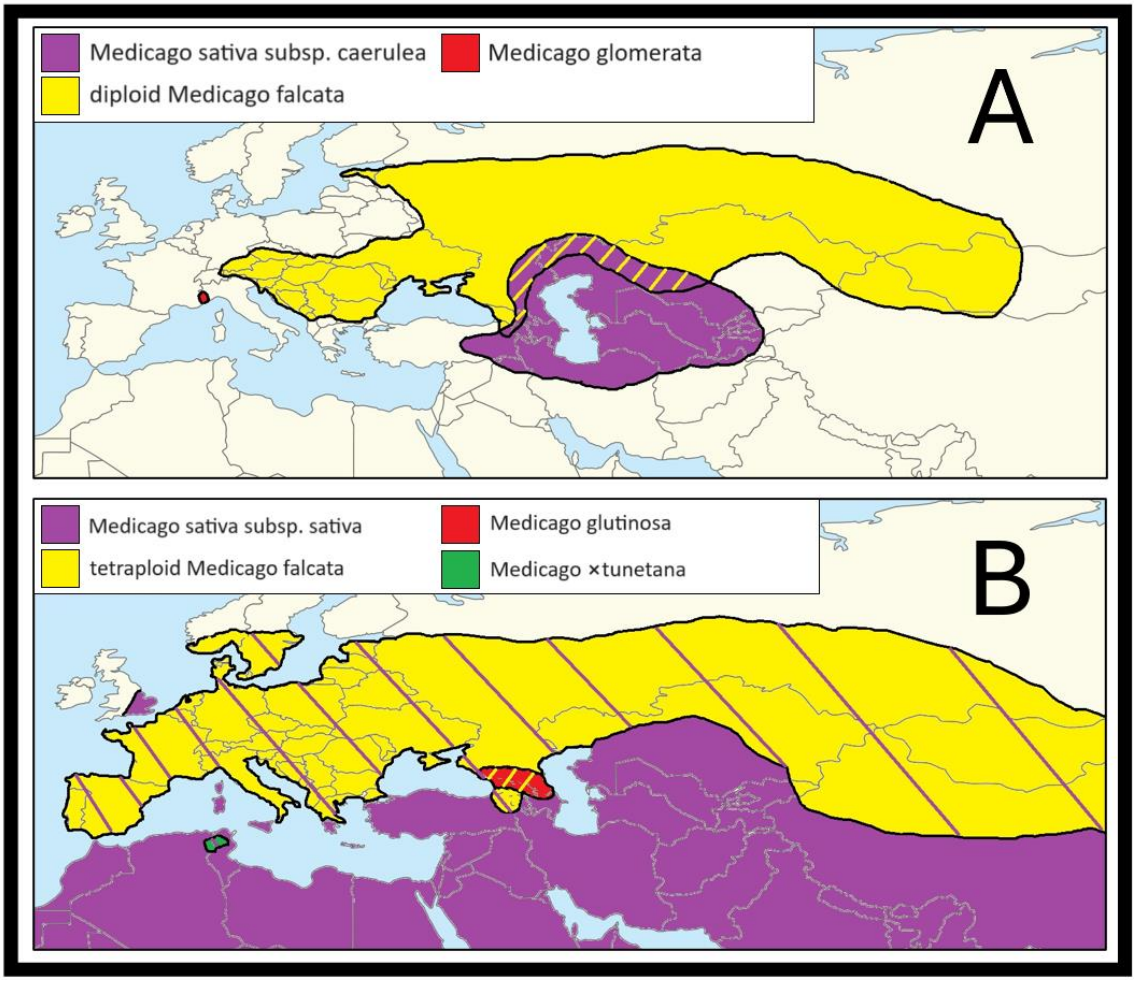

Figure 2 – Approximate distribution of: (A) diploid members of *Medicago sativa* complex, (B) tetraploid members of *Medicago sativa* complex. The distribution of tetraploid *Medicago sativa* includes cultivated and feral populations, the border of native area is unclear.

3.3 Reproductive barriers

3.3.1 Postzygotic reproductive barriers

Experimental crossings (Lesins, 1968, 1957, 1952) demonstrated that members of *Medicago sativa* complex are able to freely hybridize when both partners are on the same ploidy level, with one exception at a diploid level. *Medicago glomerata* is able to cross with both *M. sativa* subsp. *caerulea* and diploid *M. falcata* but resulting F_1 hybrids have low fertility in comparison to those of *M. sativa* with *M. falcata* (Lesins, 1968, 1957).

In addition, closely related species to the complex, *Medicago prostrata*, which can be found in diploid (2n=16) or tetraploid (2n=32) cytotype (Lesins and Lesins, 1960), is also on the same ploidy level also able to hybridize with members of the *Medicago sativa* complex, yet with a striking asymmetry in crossing success depending on which species acts as a pollen donor and which as a pollen recipient. Hybridisation is feasible when *M. prostrata* is used to

pollinate either *M. sativa* or *M. falcata*, but when *M. prostrata* is a pollen recipient, very low seed set is produced. Experimental crosses gave approximately the same results both on diploid and tetraploid level (Lesins, 1962) suggesting ploidy level does not affect the strength of the interspecific barrier, in contrast with other plant polyploid groups such as *Arabidopsis* (Lafon-Placette et al., 2017). Crosses of *M. prostrata* with *M. glomerata* gave similar results to those with *sativa* and *falcata* with small difference in seed morphology. The seeds obtained from crosses with *M. falcata* and *M. sativa* when *M. prostrata* was a pollen recipient were shrivelled (Lesins, 1962). On the other hand, seeds obtained from crosses with *M. glomerata* when *M. prostrata* was a pollen recipient were smooth (Lesins, 1968).

The exact reason, why there is such a big difference in crossing success when *Medicago prostrata* is a pollen donor and when it is a pollen recipient, is, however, not known. The possible explanation could be high hybrid seed lethality due to failure in endosperm development resulting in embryo arrest. The cause of this failure is typically a parental dosage imbalance in the endosperm that could be a consequence of differences in parental taxa "effective ploidy", also known as the endosperm balance number (EBN). Further crossing experiments associated with seed phenotyping and/or transcriptomics may test whether the mechanism underlying the asymmetry in seed lethality observed during *M. prostrata* and *M. sativa* (s. l.) crosses is similar to what has been found in other plant genera such as C*apsella* (Lafon-Placette et al., 2018), *Arabidopsis* (Lafon-Placette et al., 2017) or *Solanum* (Johnston and Hanneman, 1980).

Crosses between *Medicago sativa* complex members of different ploidy level give extremely low viable progeny, only about 1 seed per 100 crosses (Lesins, 1952), and most of these offsprings are tetraploid (Ledingham, 1940). Viable triploid offsprings arise from interploidy crosses only extremely rarely (about 1 viable triploid per 1000 pollinations) (Ledingham, 1940) and according to (McCoy and Bingham, 1988) more often when maternal parent is tetraploid. Vast majority of developing triploid seeds from interploidy crosses, however, abort within few days after fertilization (Ledingham, 1940).

Genomic deviation from 2:1 maternal-to-paternal ratio in the endosperm and subsequent abnormalities in its development, i.e. a process called as "triploid block" is known to be main cause of triploid seeds non-viability in *Arabidopsis* (Scott et al., 1998), *Oryza* (Sekine et al., 2013) or *Zea* (Pennington et al., 2008). Like in the case of above-mentioned emerging reproductive barriers between *M. prostrata* and *M. sativa* (s. l.) if the former is a pollen recipient, further crossing experiments between diploids and tetraploids associated with

seed phenotyping and/or transcriptomics could test, whether "triploid block" is also underlying the triploid seed non-viability in *Medicago sativa* complex.

Diploid *Medicago sativa* complex members can sometimes produce unreduced gametes, both pollen grains and egg cells, due to several possible mechanisms of meiosis failure (Barcaccia et al., 2003). Fusion of unreduced gamete from diploid parent with reduced gamete from tetraploid parent can lead to occasional formation of tetraploid individual in interploidy crosses (Ledingham, 1940; Lesins, 1952). Therefore, some level of gene flow from diploid to tetraploid members of the complex via production of unreduced gametes by diploid parent could be possible in natural populations. Similarly, in genus *Rorippa*, production of unreduced gametes by diploids has been proposed as main driver of diploid-to-tetraploid gene flow (Stift et al., 2010). Gene flow from tetraploids to diploids can be mediated by backcrossing of diploids with triploids, as shown in *Tripleurospermum* (Čertner et al., 2017). This is, however, highly improbable in *Medicago sativa* complex, due to extremely rare formation of triploids (Ledingham, 1940; Lesins, 1952). Their occurrence has not been reported from wild populations of the complex members yet. All gene flow and hybridization possibilities in *Medicago sativa* complex are summarized in Figure 3.

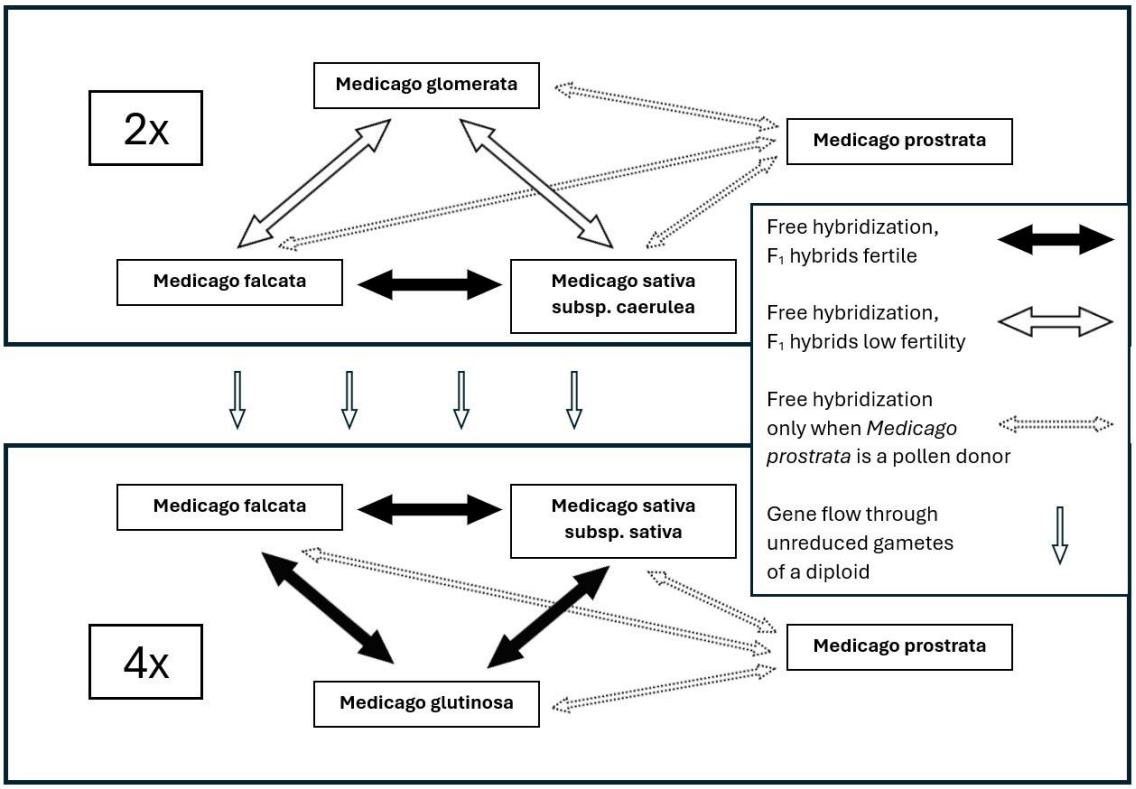

Figure 3 – Overview of hybridization and gene flow possibilities among taxa included in the *Medicago sativa* complex and with closely related *Medicago prostrata*. The scheme summarizes information obtained from crossing experiments (Ledingham, 1940; Lesins, 1968, 1962, 1957, 1952).

3.3.2 Prezygotic reproductive barriers

Vast majority of studies concerning the reproductive barriers in the *Medicago sativa* complex addressed only postzygotic barriers. However, prezygotic barriers can sometimes be even more important in reproductive isolation of taxa as shown for example by genetic isolation due to divergence in style length and pollen size in *Silene* (Brothers and Delph, 2017), increasing reproductive isolation in *Pulmonaria* caused by different ecogeography (Duffy and Jacquemyn, 2019) or pollinator-mediated reproductive isolation in *Mimulus* (Chen, 2013). Actually, several studies that evaluated pollinator preferences were also performed on *Medicago sativa* complex members.

Taxa included in the complex are predominantly outcrossing plants (Dieterich Mabin et al., 2021), which are mostly pollinated by various bee species (Haedo et al., 2022). Both *Medicago sativa* and *Medicago falcata* flower during summer, in the Czech Republic usually from May to October (Kaplan, 2019). At least in Central Europe, cross-pollination between them happens often as suggested by frequent presence of hybrid swarms in regions, where these two taxa grow in sympatry (Mazyad and Ammann, 1999).

Studies evaluating distinct preferences for different flower colour by the most important pollinators of *Medicago sativa* complex, which is alfalfa leafcutter bee (*Megachile rotundata*) and honeybee (*Apis mellifera*) (Haedo et al., 2022), revealed that they preferred purple flowers of *M. sativa* over yellow flowers of *M. falcata* when both taxa were grown together (Goplen and Brandt, 1975; Pankiw, 1967). Moreover, percent of hybrid progeny obtained from experimental fields containing both *M. falcata* and *M. sativa* plants, which were pollinated by alfalfa leafcutter bee, was significantly lower than would be expected under the assumption of random mating (Riday, 2008), however, post-pollination barriers have not been studied, thus leaving the mechanisms underlying these differences unknown. The results suggest that pollinator preferences can lead to a little lower production of *M. sativa-falcata* hybrids on experimental fields, however, it is still very distant from pollinator-mediated reproductive isolation. Also, all of these studies have been performed only on germplasm accessions cultivated in experimental conditions. Data from wild populations regarding pollinators preferences to different flower colours in *Medicago sativa* complex and estimated effects of pollinators for prezygotic isolation of *M. sativa* and *M. falcata* are, however, still missing.

Possible influence of different ecogeography on reproductive isolation of *Medicago sativa* complex members has been practically studied only very little. However, recent

phylogeographic study performed on diploid complex members brought interesting new findings about this topic. There are actually two genetically distinct lineages of diploid *Medicago falcata*, which according to the authors probably correspond to different ecogeography (Şakiroğlu et al., 2010). More detailed analysis of ecological preferences and geographical distribution of these lineages could elucidate whether different ecogeography is truly responsible for their diversification.

Furthermore, practically nothing is known about coexistence of diploid and tetraploid cytotypes of *Medicago sativa* complex members in natural populations, frequency of mixed ploidy populations in contact zones and regions where these contact zones occur. Studies concerning this topic have been conducted e.g. on *Butomus* (Čertner et al., 2022), *Tripleurospermum* (Čertner et al., 2017), *Arabidopsis* (Kolář et al., 2016) or *Knautia* (Kolář et al., 2009). Our aim in currently ongoing study based on exhaustive sampling of natural diploid and tetraploid *Medicago falcata* populations in Central Europe is to bring such insight also into coexistence of *M. falcata* cytotypes.

3.4 Origin and evolution of Medicago sativa complex

3.4.1 Speciation of diploid Medicago sativa and Medicago falcata

Nearly all species included in genus *Medicago* possess yellow flower colour and coiled pods and hypothesized ancestor of the *Medicago sativa* complex was very likely characterized by this morphological combination as well. However, switch from yellow to purple flower colour in *Medicago sativa* and loss of pod coiling in *Medicago falcata* have most likely happened during the divergence of these two taxa (Lesins and Lesins, 1979). Unfortunately, due to lack of ancestral reconstruction analyses and targeted eco-evolutionary experiments, our notion about events that could lead to morphological differentiation and subsequent reproductive isolation of these two taxa represent rather speculative hypotheses so far.

Lesins and Lesins (1979) propose in their monography a theory, that ancestral populations growing in the area between present day Black and Caspian Sea, were separated by some geographical barrier into southern populations, which would later give rise to *Medicago sativa*, and northern populations, that would become *Medicago falcata* by means of allopatric speciation. The southern populations adapted to better pollinator attraction by change of yellow flower colour induced by carotenoids and flavonoids to purple flower colour

caused by anthocyanins. Coiled pods were highly beneficial for seed distribution in arid semi desert regions with low vegetation cover, where rolling of whole pods on the ground was possible. On the other hand, the northern populations remained yellow flowering, however, coiled pods were disadvantageous in steppe regions with high vegetation cover, where rolling of whole pods was not possible. Thus, straightening of pods happened in northern populations. Later, *M. falcata* and *M. sativa* met in secondary contact zones and gave rise to their diploid hybrid *M. ×hemicycla.*

To bring at least some insight into these events, divergence time of *Medicago sativa* and *Medicago falcata* could be estimated using molecular methods. Quantitative assessment of *Medicago falcata* and *Medicago sativa* seed-dispersing abilities in various environments could explain significance of pod shape divergence as an adaptation to their respective natural biotopes. As thoroughly discusses earlier, pollinators seem to indeed prefer purple flower colour of *M. sativa* to *M. falcata* yellow flower colour.

3.4.2 Population structure and genetic differentiation of Medicago sativa, Medicago falcata and their hybrids

Vast majority of molecular studies performed on *Medicago sativa* complex were focused primarily on tetraploid cultivated germplasm accessions. Although, several studies that brought new insights into genetic diversity and differentiation of diploid *Medicago falcata*, *sativa* and ×*hemicycla* have been conducted recently. Chloroplast and mitochondrial DNA sequences, SSR markers, genome-wide SNP´s and fluorescence in situ hybridization (FISH) have been used in order to analyse genetic differences of diploid *Medicago sativa* complex members and infer their population structure.

Chloroplast DNA (cpDNA), which is in *Medicago sativa* complex inherited biparentally with strong paternal bias (Smith et al., 1986), revealed that despite the presence of some shared haplotypes, diploid *M. falcata* and *M. sativa* subsp. *caerulea* are significantly differentiated in chloroplast DNA sequences (Havananda et al., 2010). CpDNA haplotypes of diploid *Medicago prostrata*, a closely related species to *Medicago sativa* complex, were sister to those of *M. sativa* and *M. falcata*. On the other hand, results obtained from mitochondrial DNA (mtDNA), which is in *Medicago sativa* complex inherited strictly maternally (Forsthoefel, 1991), showed no significant differentiation of mtDNA haplotypes in these three taxa (Havananda et al., 2010). Contrasting results obtained from cpDNA and mtDNA

sequences could be explained by past introgression (mitochondrial capture) or by significantly slower mutation rate of plant mtDNA than cpDNA (Wolfe et al., 1987).

Results acquired from 89 SSR markers revealed that diploid *M. falcata* and *M. sativa* subsp. *caerulea* are genetically clearly differentiated taxa and that their putative hybrid *Medicago* ×*hemicycla* indeed showed a hybrid genome pattern. Moreover, each of the two diploid taxa, *M. sativa subsp. caerulea* and *M. falcata*, could be divided into two subgroups (see Figure 4). In *M. sativa*, the group corresponded well with geographical distribution: southern populations fell into one group and northern populations into the other. In contrast, division of *M. falcata* into two subgroups could not be explained only by geography, therefore, some other factor must have been involved. Authors of this study suggested that different ecogeography of diploid *M. falcata* populations could have been this factor and thus, they named the respective subgroups as "lowland ecotype" and "upland ecotype" (Şakiroğlu et al., 2010).

In a follow-up study more than 15 000 single nucleotide polymorphisms (SNP) were used in order to analyse population structure of *M. sativa* subsp. *caerulea*, diploid *M. falcata* and *M.* ×*hemicycla*. Results obtained from this study supported division of both *M. sativa* subsp. *caerulea* and diploid *M. falcata* populations into their two respective subgroups (Şakiroğlu and Brummer, 2017). However, it should be strongly highlighted that subgroups of *M. sativa* subsp. *caerulea* represent rather genetic continuum than two distinct clusters, whereas subgroups of diploid *M. falcata* truly show clear genetic separation (see Figure 5).

In addition, one more molecular study was performed only on *M. sativa* subsp. *caerulea*. This study uncovered that populations in Caucasia have higher mean Fst values, allele diversity and heterozygosity than populations in Central Asia, suggesting the former to be the centre of origin and diversity for *M. sativa* subsp. *caerulea* and the latter to has been colonized later (Şakiroğlu and Brummer, 2013).

Lastly, fluorescence in situ hybridization (FISH) revealed that the mapped repetitive sequences were localized in centromeric, subtelomeric or intersticial regions in chromosomes of *M. sativa* subsp. *caerulea*, whereas in chromosomes of diploid *M. falcata* they were localized mainly in centromeric regions (see Figure 6). These results show that chromosome structure of these two taxa is clearly different (Yu et al., 2017) and support the distinct position of the two taxa.

In sum, these studies demonstrated that *Medicago sativa* subsp. *caerulea* and diploid *Medicago falcata* are genetically clearly differentiated taxa and *Medicago* ×*hemicycla* occurring in relatively narrow area at their contact shows hybrid genome patterns. This pattern fits the scenario of allopatric divergence of both species with later secondary contact and hybridisation leading to the origin of *Medicago* ×*hemicycla*. However, all of these studies were performed on accessions coming from germplasm database and molecular analyses performed on exhaustive sampling from wild populations together with an evaluation of ecological preferences of the species and genetic lineages are still missing.

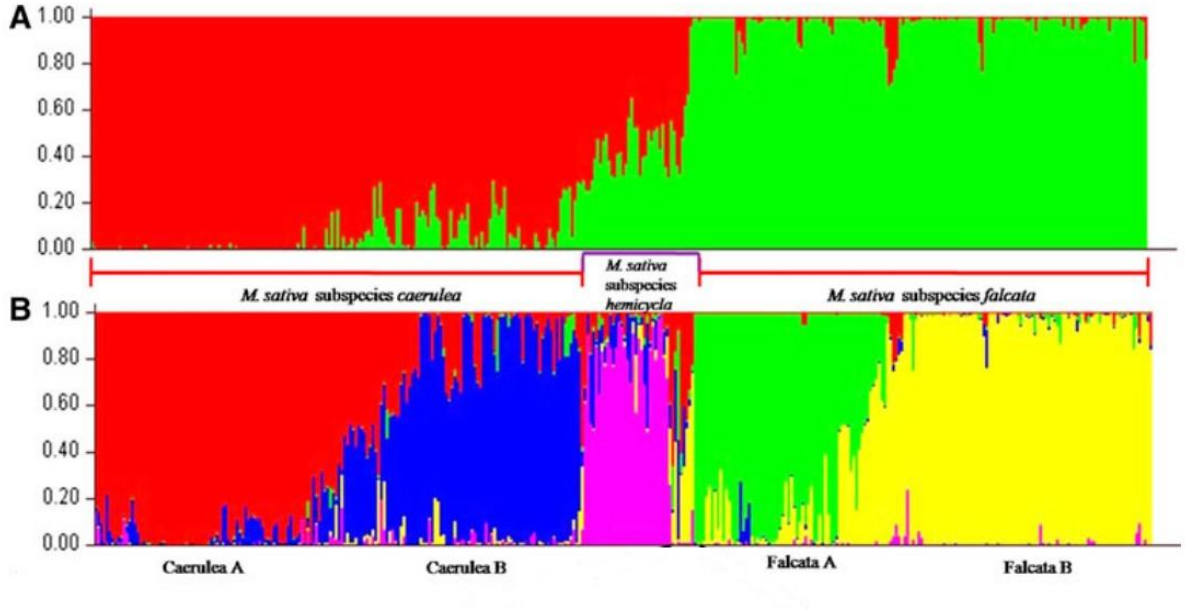

Figure 4 – population structure of diploid *Medicago sativa* complex members based on Bayesian inference among 374 individual genotypes analysed with 89 SSR markers assuming: (A) two clusters (K = 2), (B) five clusters (K = 5) (Şakiroğlu et al., 2010)

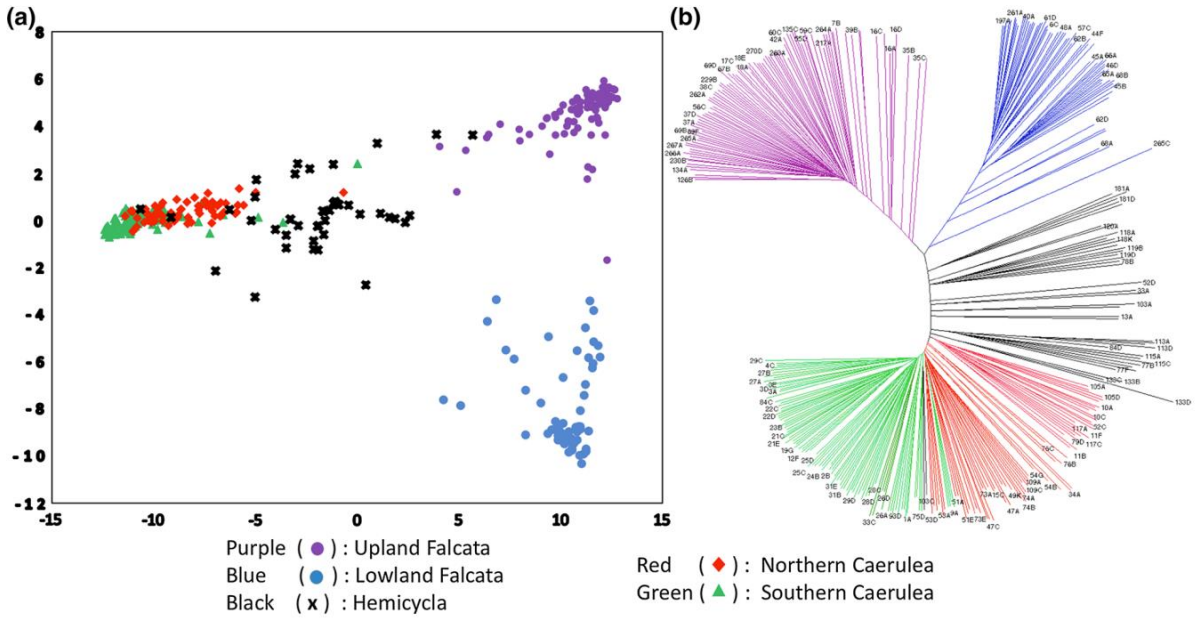

Figure 5 – (a) population structure of diploid *Medicago sativa* complex members based on genome-wide SNPs graphing the first two principal components of a principal components analysis, (b) a neighbour-joining dendrogram (Şakiroğlu and Brummer, 2017)

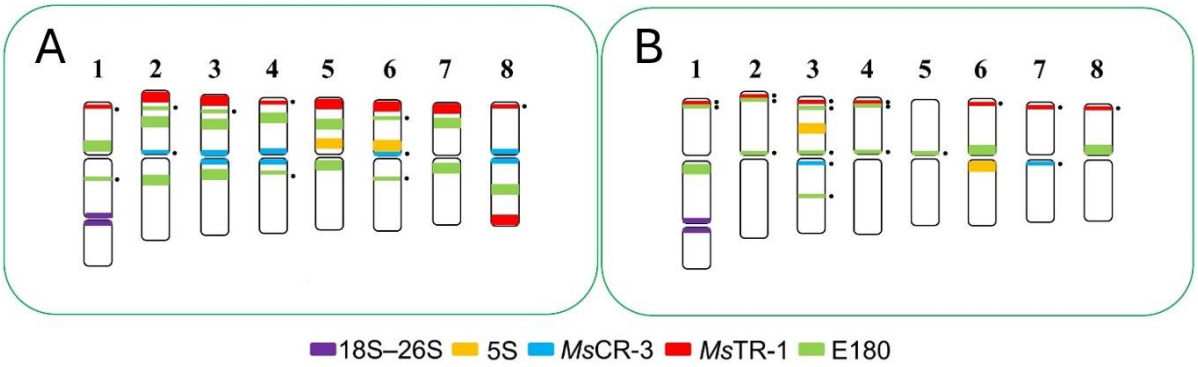

Figure 6 – idiogram of FISH-banded chromosomes of (A) diploid *Medicago falcata*, (B) *Medicago sativa* subsp. *caerulea* (Yu et al., 2017)

As mentioned before, majority of molecular studies focused only on tetraploid cultivated germplasm accessions. Very little is, on the other hand, known about wild growing tetraploid members of *Medicago sativa* complex and only few studies explored genetic diversity and population structure of wild growing tetraploid populations.

Firstly, differences in repetitive sequences localization on chromosomes of *M. sativa* subsp. *sativa* and tetraploid *M. falcata* have been evaluated using fluorescence in situ hybridization (FISH), which confirmed that just like their diploid progenitors, tetraploid *M. sativa* and *M. falcata* have different localization of mapped sequences on chromosomes (see Figure 7), therefore their chromosomes are also clearly distinct (Yu et al., 2017).

Chloroplast DNA sequences revealed similar patterns of incomplete haplotypes differentiation in tetraploid *Medicago sativa* and *M. falcata* as in their putative diploid progenitors. Despite the presence of some shared haplotypes, most of them were found exclusively either in tetraploid *M. sativa* or tetraploid *M. falcata* (Havananda et al., 2011).

Inferring genetic diversity and population structure of wild growing tetraploids was main purpose of another study. Evaluation of 31 SSR markers revealed that *Medicago sativa* subsp. *sativa* and tetraploid *Medicago falcata* are two extreme forms connected by genetic continuum represented by their tetraploid hybrid *Medicago* ×*varia* (see Figure 8). In contrast to situation on diploid level, no further sub-division within *M. sativa* subsp. *sativa* and/or tetraploid *M. falcata* (see Figure 9) has been revealed (İlhan et al., 2016). Presence of genetic continuum between tetraploid *M. sativa* and *M. falcata*, rather than relatively clear distinction as seen in diploids, could be most likely explained by much more intensive and widespread hybridization of tetraploids due to alfalfa cultivation.

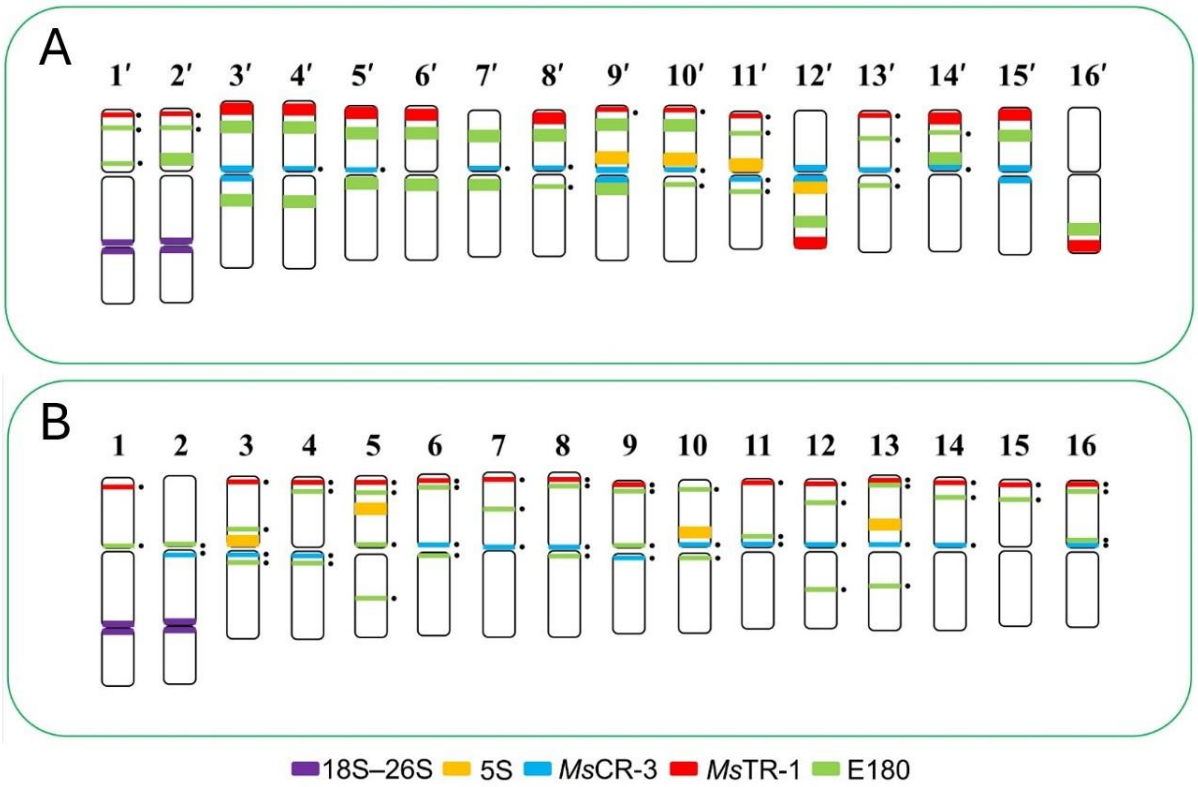

Figure 7 – idiogram of FISH-banded chromosomes of (A) *Medicago sativa* subsp. *sativa*, (B) tetraploid *Medicago falcata* (Yu et al., 2017)

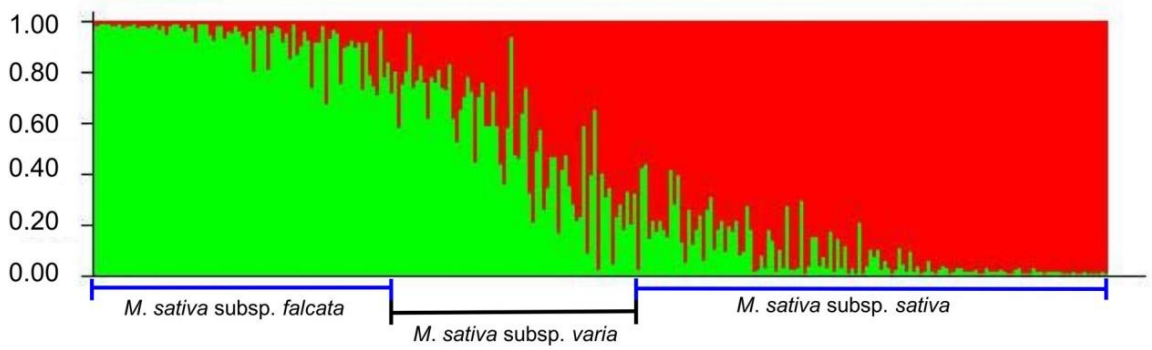

Figure 8 – population structure of tetraploid *Medicago sativa* complex members based on Bayesian inference among 280 individual genotypes analysed with 31 SSR markers assuming two clusters (K = 2) (İlhan et al., 2016)

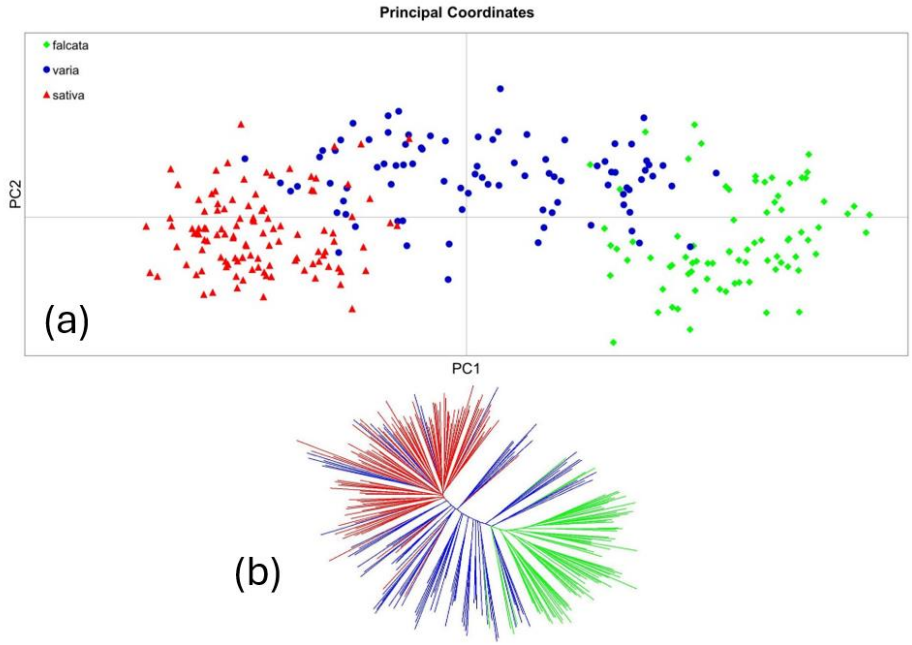

Figure 9 – (a) grouping of tetraploid *Medicago sativa* complex members based on the first two principal components obtained from an analysis of 31 simple sequence repeat markers, (b) a neighbour-joining dendrogram (İlhan et al., 2016)

3.4.3 Origin of tetraploid Medicago sativa and Medicago falcata

Tetraploid members of *Medicago sativa* complex are genetically autotetraploids, because they show tetrasomic inheritance (Quiros, 1982) and occasional production of quadrivalents during meiosis (Armstrong, 1971; Gillies, 1972). Production of unreduced gametes by diploid members of the complex (Barcaccia et al., 1986; Tavoletti, 1994) and formation of viable tetraploid individuals by merging of two unreduced gametes (Barcaccia et al., 1998) were reported from crossing experiments. Triploid individuals emerge only extremely rarely from artificial crosses of diploids with tetraploids (Lesins, 1952) and they

were never reported from natural populations. Therefore, rather than unilateral sexual polyploidization dependent on triploid intermediates, bilateral sexual polyploidization is the most likely way by which tetraploid members of *Medicago sativa* complex originated. Recently, several studies, which examined genetic background of tetraploid *Medicago sativa* and *Medicago falcata* and their relationship to putative diploid progenitors, deepened our understanding of how these tetraploids have been actually formed.

Fluorescence in situ hybridization (FISH) revealed that mapped repetitive sequences in *Medicago sativa* had almost the same chromosomal locations in both diploid and tetraploid subspecies (see Figure 10) (Yu et al., 2017) and evaluating chloroplast DNA sequences showed that tetraploid subsp. *sativa* shared most haplotypes in common with diploid subsp. *caerulea* (Havananda et al., 2011). Both of these findings suggest simple autopolyploid origin of tetraploid *Medicago sativa* subsp. *sativa* from its diploid progenitor subsp. *caerulea*.

On the other hand, origin of tetraploid *Medicago falcata* cannot be that easily explained, in spite of close morphological similarity among the cytotypes suggesting autopolyploidy. FISH revealed that there are several significant differences in locations of some mapped sequences on chromosomes between diploid and tetraploid *Medicago falcata* cytotype (see Figure 10). Moreover, results obtained from chloroplast DNA showed that tetraploid *M. falcata* shared most common haplotype with diploid *M. prostrata* rather than with diploid *M. falcata*. Nearly mutually exclusive sets of haplotypes were found in diploid and tetraploid *M. falcata* (Havananda et al., 2011). Therefore, introgression of chloroplast DNA sequences from diploid *M. prostrata* to genome of tetraploid *M. falcata* might have taken place sometimes in the past. Molecular study on much larger sampling of both *Medicago falcata* and *Medicago prostrata* cytotypes from natural populations rather than from germplasm database could help to clarify involvement of *M. prostrata* in the origin of tetraploid *Medicago falcata* cytotype. However, this investigation was based only on limited sampling of accessions from germplasm collections and the extent of natural variation in both cytotypes remains unknown.

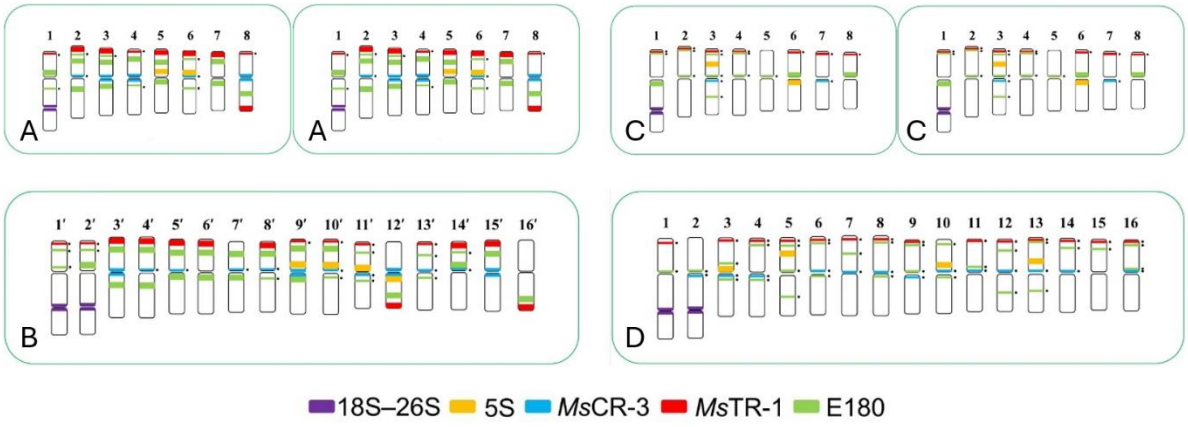

Figure 10 – idiogram of FISH-banded chromosomes of (A) *Medicago sativa* subsp. *caerulea*, (B) *Medicago sativa* subsp. *sativa*, (C) diploid *Medicago falcata*, (D) tetraploid *Medicago falcata* (Yu et al., 2017)

3.4.4 Origin and population structure of other complex members and Medicago prostrata

In contrast to *Medicago sativa*, *M. falcata* and their hybrids, which are now at least partially explored using molecular methods, other members of *Medicago sativa* complex have been studied very little or not at all. Only one individual of *Medicago glomerata*, four *M.* ×*tunetana* and four *M. glutinosa* individuals were analysed using chloroplast DNA (Havananda et al., 2011, 2010), therefore the results addressing these taxa cannot be considered conclusive. Population structure of diploid *Medicago glomerata* and tetraploid *Medicago glutinosa*, *M.* ×*polychroa* and *M.* ×*tunetana* remains unknown, as well as origin of these taxa and their relationship to other *Medicago sativa* complex members.

Medicago prostrata, which is closely related to *Medicago sativa* complex, has been recently analysed by sampling eight nuclear loci. Results of this study showed that diploid *Medicago prostrata* alleles were clearly distinct from those of *Medicago sativa* complex members, however several tetraploid individuals of *M. prostrata* shared some alleles with the complex members (Eriksson et al., 2017). Therefore, most likely origin of tetraploid *M. prostrata* is autotetraploid origin from diploid *M. prostrata* followed by subsequent introgression from *Medicago sativa* complex. Current notion about origin and evolutionary history of *Medicago sativa* complex members and *Medicago prostrata* is graphically depicted in Figure 11.

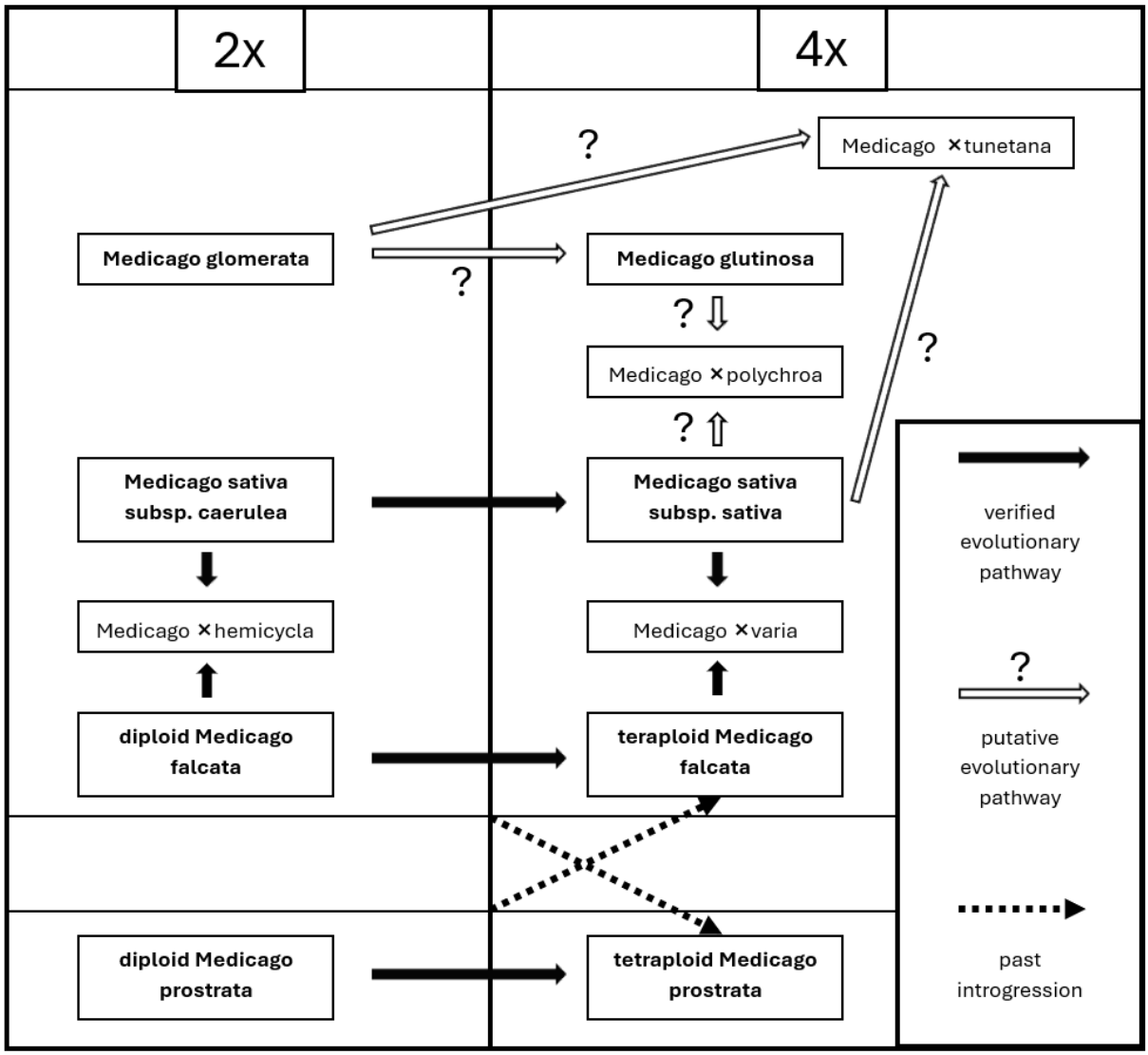

Figure 11 – The most likely scenario depicting the origin and evolutionary history of *Medicago sativa* complex members and *Medicago prostrata*, synthesizing information from (Eriksson et al., 2017; Havananda et al., 2011, 2010; İlhan et al., 2016; Lesins and Lesins, 1979; Şakiroğlu et al., 2010; Şakiroğlu and Brummer, 2017; Yu et al., 2017)

4 CONCLUSION

Alfalfa (*Medicago sativa*) in one of the most important forage crops cultivated across the globe and belongs among the most valuable autopolyploid crops. At the same time, the entire *Medicago sativa* complex represents example of reticulate polyploid complex that serves as a prominent example of polyploid speciation, hybridisation and evolution of floral colour and pod shape, demonstrating its value for evolutionary research. Knowledge of the patterns and drivers of natural diversity of alfalfa and its allies is thus important prerequisite for our detailed understanding of the biology of this important crop potential design of further breeding strategy as well as for better understanding of general evolutionary processes in plant speciation and adaptation.

Difference in ploidy level represents the most important postzygotic reproductive barrier preventing gene flow among members of *Medicago sativa* complex, given the fact that same-ploidy-level members of the complex are able to hybridize freely but crosses between different-ploidy-level members are in most cases unsuccessful due to triploid seed abortion. Exact mechanism underlying triploid seeds non-viability has not been described in *Medicago* yet, however, this mechanism would likely be genomic deviation from 2:1 maternal-to-paternal ratio in the endosperm leading to subsequent abnormalities in its development (so called "triploid block"), which is known, e.g. from *Arabidopsis*, *Zea* and *Oryza*.

Prezygotic reproductive barriers such as different pollinator preferences or distinct ecological preferences have been only marginally studied in *Medicago sativa* complex, in spite of striking floral colour differentiation and distinct distribution areas in the complex. It seems that main pollinators of *Medicago sativa* complex members slightly prefer purple flower colour of *Medicago sativa* to yellow flower colour of *Medicago falcata*. Differences in ecological preferences very likely play a significant role in reproductive isolation of some *Medicago sativa* complex members as shown by two genetically distinct lineages of diploid *Medicago falcata* presumably corresponding to different ecogeography. Nevertheless, whether the distinct ecogeography is indeed the primary mechanism underlying genetic differentiation within diploid *M. falcata* needs to be verified.

Furthermore, very little is known about coexistence of diploid and tetraploid complex members in nature, frequency of mixed-ploidy populations and overall geographical distribution pattern of different ploidy populations. We are currently addressing these topics in natural diploid and tetraploid *Medicago falcata* populations occurring in Central Europe.

Medicago sativa and *Medicago falcata* are genetically differentiated both at diploid and tetraploid level, which is supporting their recognition as distinct taxa. Their putative hybrids, diploid *Medicago* ×*hemicycla* and tetraploid *Medicago* ×*varia*, indeed possess hybrid genome pattern between *M. sativa* and *M. falcata*, confirming their hybrid origin that has been originally hypothesized based on morphology. Tetraploid *Medicago sativa* subsp. *sativa* was shown to be of an autopolyploid origin from diploid *Medicago sativa* subsp. *caerulea*. On the other hand, the origin of tetraploid *Medicago falcata* seems to be more complex. Based on limited sampling, an autopolyploid origin from diploid *M. falcata* is likely, however, it has been presumably followed by an introgression of chloroplast DNA from *Medicago prostrata*, a species closely related to *Medicago sativa* complex. In turn, tetraploid *M. prostrata* originated from diploid *M. prostrata*, but with likely additional pulses, further underlining complex genetic reticulations between polyploid cytotypes of both species (complexes).

Most of the studies were, however, performed on a limited sampling, exclusively relying on accessions from germplasm collection of sometimes unclear origin and ploidy. Molecular analyses performed on representative sampling from wild populations together with an evaluation of ecological preferences of the species and genetic lineages are still missing.

Moreover, the origin and population structure of other *Medicago sativa* complex members remain unknown. Such a big information gap leaves open the question about the timing and place of origin of the entire *Medicago sativa* complex. Especially diploid *Medicago glomerata*, an endemic of Maritime Alps, might have played a significant role in the complex origin and it could have been involved in tetraploid *Medicago glutinosa*, and *Medicago* ×*tunetana* formation. Comprehensive molecular analysis focusing on these less studied complex members could bring interesting insights into evolutionary processes underlying formation of new taxa, as well as clarify the origin and further diversification of the *Medicago sativa* complex members.

5 LITERATURE

Armstrong, K.C., 1971. CHROMOSOME ASSOCIATIONS AT PACHYTENE AND METAPHASE IN *MEDICAGO SATIVA*. Can. J. Genet. Cytol. 13, 697–702. https://doi.org/10.1139/g71-099

Barcaccia, G., Rosellini, D., Falcinelli, M., Veronesi, F., 1998. Reproductive behaviour of tetraploid alfalfa plants obtained by unilateral and bilateral sexual polyploidization. Euphytica 99, 199–203. https://doi.org/10.1023/A:1018359310706

Barcaccia, G., Tavoletti, S., Mariani, A., Veronesi, F., 2003. Occurrence, inheritance and use of reproductive mutants in alfalfa improvement. Euphytica 133, 37–56. https://doi.org/10.1023/A:1025646523940

Barcaccia, G., Tavoletti, S., Mariani, A., Veronesi, F., 1986. Unreduced gametes in diploid Medicago and their importance in alfalfa breeding. Euphytica 133, 37–56. https://doi.org/10.1023/A:1025646523940

Blanco-Pastor, J.L., Liberal, I.M., Şakiroğlu, M., Wei, Y., Brummer, E.C., Andrew, R.L., Pfeil, B.E., 2021. Annual and perennial *Medicago* show signatures of parallel adaptation to climate and soil in highly conserved genes. Molecular Ecology 30, 4448–4465. https://doi.org/10.1111/mec.16061

Bretagnolle, F., Lumaret, R., 1995. Bilateral polyploidization in Dactylis glomerata L. subsp. lusitanica: occurrence, morphological and genetic characteristics of first polyploids. Euphytica 84, 197–207. https://doi.org/10.1007/BF01681812

Brothers, A.N., Delph, L.F., 2017. Divergence in style length and pollen size leads to a postmating-prezygotic reproductive barrier among populations of *Silene latifolia*. Evolution 71, 1532–1540. https://doi.org/10.1111/evo.13248

Čertner, M., Fenclová, E., Kúr, P., Kolář, F., Koutecký, P., Krahulcová, A., Suda, J., 2017. Evolutionary dynamics of mixed-ploidy populations in an annual herb: dispersal, local persistence and recurrent origins of polyploids. Annals of Botany 120, 303–315. https://doi.org/10.1093/aob/mcx032

Čertner, M., Rydlo, J., Dudáš, M., Hroudová, Z., 2022. A unique diploid – triploid contact zone provides insights into the evolutionary mechanisms of cytotype coexistence in flowering rush (Butomus umbellatus). Perspectives in Plant Ecology, Evolution and Systematics 54, 125659. https://doi.org/10.1016/j.ppees.2022.125659

Chen, G.F., 2013. Sexual isolation in two bee-pollinated Costus (Costaceae). Plant Reprod 26, 3–16. https://doi.org/10.1007/s00497-012-0203-6

Chen, H., Zeng, Y., Yang, Y., Huang, L., Tang, B., Zhang, H., Hao, F., Liu, W., Li, Youhan, Liu, Y., Zhang, X., Zhang, R., Zhang, Y., Li, Yongxin, Wang, K., He, H., Wang, Z., Fan, G., Yang, H., Bao, A., Shang, Z., Chen, J., Wang, W., Qiu, Q., 2020. Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa. Nat Commun 11, 2494. https://doi.org/10.1038/s41467-020-16338-x

Cook, D.R., 1999. Medicago truncatula — a model in the making! Current Opinion in Plant Biology 2, 301–304. https://doi.org/10.1016/S1369-5266(99)80053-3

Dieterich Mabin, M.E., Brunet, J., Riday, H., Lehmann, L., 2021. Self-Fertilization, Inbreeding, and Yield in Alfalfa Seed Production. Front. Plant Sci. 12, 700708. https://doi.org/10.3389/fpls.2021.700708

Duffy, K.J., Jacquemyn, H., 2019. Climate change increases ecogeographical isolation between closely related plants. Journal of Ecology 107, 167–177. https://doi.org/10.1111/1365-2745.13032

Eriksson, J.S., Blanco-Pastor, J.L., Sousa, F., Bertrand, Y.J.K., Pfeil, B.E., 2017. A cryptic species produced by autopolyploidy and subsequent introgression involving Medicago prostrata (Fabaceae). Molecular Phylogenetics and Evolution 107, 367–381. https://doi.org/10.1016/j.ympev.2016.11.020

Ferchichi, Y., Sakhraoui, A., Ltaeif, H.B., Ben Mhara, Y., Elimem, M., Ben Naceur, M., Ghrabi-Gammar, Z., Rouz, S., 2021. Eco-Geographical, Morphological and Molecular Characterization of a Collection of the Perennial Endemic Species Medicago tunetana (Murb.) A.W. Hill (Fabaceae) from Tunisia. Plants 10, 1923. https://doi.org/10.3390/plants10091923

Forsthoefel, N.R., 1991. Mitochondrial inheritance in Medicago sativa (Master's thesis). The University of Arizona.

Gillies, C.B., 1972. Pachytene chromosomes of perennial Medicago species: I. Species closely related to M. sativa. Hereditas 72, 277–288. https://doi.org/10.1111/j.1601-5223.1972.tb01052.x

Goplen, B.P., Brandt, S.A., 1975. Alfalfa Flower Color Associated with Differential Seed Set by Leaf-cutter Bees. Agronomy Journal 67, 804–806. https://doi.org/10.2134/agronj1975.00021962006700060019x

Haedo, J.P., Martínez, L.C., Graffigna, S., Marrero, H.J., Torretta, J.P., 2022. Managed and wild bees contribute to alfalfa (Medicago sativa) pollination. Agriculture, Ecosystems & Environment 324, 107711. https://doi.org/10.1016/j.agee.2021.107711

Havananda, T., Brummer, E.C., Doyle, J.J., 2011. Complex patterns of autopolyploid evolution in alfalfa and allies (*Medicago sativa* ; Leguminosae). American J of Botany 98, 1633–1646. https://doi.org/10.3732/ajb.1000318

Havananda, T., Brummer, E.C., Maureira-Butler, I.J., Doyle, J.J., 2010. Relationships among Diploid Members of the *Medicago sativa* (Fabaceae) Species Complex Based on Chloroplast and Mitochondrial DNA Sequences. Systematic Botany 35, 140–150. https://doi.org/10.1600/036364410790862506

İlhan, D., Li, X., Brummer, E.C., Şakiroğlu, M., 2016. Genetic Diversity and Population Structure of Tetraploid Accessions of the *Medicago sativa–falcata* Complex. Crop Science 56, 1146–1156. https://doi.org/10.2135/cropsci2015.12.0750

Jiao, Y., Wickett, N.J., Ayyampalayam, S., Chanderbali, A.S., Landherr, L., Ralph, P.E., Tomsho, L.P., Hu, Y., Liang, H., Soltis, P.S., Soltis, D.E., Clifton, S.W., Schlarbaum, S.E., Schuster, S.C., Ma, H., Leebens-Mack, J., dePamphilis, C.W., 2011. Ancestral polyploidy in seed plants and angiosperms. Nature 473, 97–100. https://doi.org/10.1038/nature09916

Johnston, S.A., Hanneman, R.E., 1980. Support of the endosperm balance number hypothesis utilizing some tuber-bearing Solanum species. American Potato Journal 57, 7–14. https://doi.org/10.1007/BF02852750

Kaljund, K., Leht, M., 2013. Extensive Introgressive Hybridization between Cultivated Lucerne and the Native Sickle Medic (*Medicago sativa* ssp. *Falcata*) in Estonia. Annales Botanici Fennici 50, 23–31. https://doi.org/10.5735/085.050.0103

Kaplan, Z. (Ed.), 2019. Klíč ke květeně České republiky, 2nd ed. Academia, Praha.

Kolář, F., Lučanová, M., Záveská, E., Fuxová, G., Mandáková, T., Španiel, S., Senko, D., Svitok, M., Kolník, M., Gudžinskas, Z., Marhold, K., 2016. Ecological segregation does not drive the intricate parapatric distribution of diploid and tetraploid cytotypes of the *Arabidopsis arenosa* group (Brassicaceae): Cytogeography of *Arabidopsis arenosa*. Biol. J. Linn. Soc. 119, 673–688. https://doi.org/10.1111/bij.12479

Kolář, F., Štech, M., Trávníček, P., Rauchová, J., Urfus, T., Vít, P., Kubešová, M., Suda, J., 2009. Towards resolving the Knautia arvensis agg. (Dipsacaceae) puzzle: primary and secondary contact zones and ploidy segregation at landscape and microgeographic scales. Annals of Botany 103, 963–974. https://doi.org/10.1093/aob/mcp016

Krishnakumar, V., Kim, M., Rosen, B.D., Karamycheva, S., Bidwell, S.L., Tang, H., Town, C.D., 2015. MTGD: The Medicago truncatula Genome Database. Plant and Cell Physiology 56, e1–e1. https://doi.org/10.1093/pcp/pcu179

Lafon-Placette, C., Hatorangan, M.R., Steige, K.A., Cornille, A., Lascoux, M., Slotte, T., Köhler, C., 2018. Paternally expressed imprinted genes associate with hybridization barriers in Capsella. Nature Plants 4, 352–357. https://doi.org/10.1038/s41477-018-0161-6

Lafon-Placette, C., Johannessen, I.M., Hornslien, K.S., Ali, M.F., Bjerkan, K.N., Bramsiepe, J., Glöckle, B.M., Rebernig, C.A., Brysting, A.K., Grini, P.E., Köhler, C., 2017. Endosperm-based hybridization barriers explain the pattern of gene flow between *Arabidopsis lyrata* and *Arabidopsis arenosa* in Central Europe. Proc. Natl. Acad. Sci. U.S.A. 114. https://doi.org/10.1073/pnas.1615123114

Ledingham, G.F., 1940. CYTOLOGICAL AND DEVELOPMENTAL STUDIES OF HYBRIDS BETWEEN MEDICAGO SATIVA AND A DIPLOID FORM OF M. FALCATA. Genetics 25, 1–15. https://doi.org/10.1093/genetics/25.1.1

Lesins, K., 1968. INTERSPECIFIC CROSSES INVOLVING ALFALFA. IV. *Medicago glomerata* × *M* . *sativa* with reference to *M* . *prostrata*. Can. J. Genet. Cytol. 10, 536–544. https://doi.org/10.1139/g68-072

Lesins, K., 1962. INTERSPECIFIC CROSSES INVOLVING ALFALFA. III. *MEDICAGO SATIVA* L. × *M* . *PROSTRATA* JACQ. Can. J. Genet. Cytol. 4, 14–23. https://doi.org/10.1139/g62-003

Lesins, K., 1957. CYTOGENETIC STUDY ON A TETRAPLOID PLANT AT THE DIPLOID CHROMOSOME LEVEL. Can. J. Bot. 35, 181–196. https://doi.org/10.1139/b57-018

Lesins, K., 1952. SOME DATA ON THE CYTOGENETICS OF ALFALFA. Journal of Heredity 43, 287–292. https://doi.org/10.1093/oxfordjournals.jhered.a106333

Lesins, K., Gillies, C.B., 1972. Taxonomy and Cytogenetics of *Medicago*, in: Hanson, C.H. (Ed.), Agronomy Monographs. American Society of Agronomy, Madison, WI, USA, pp. 53–86. https://doi.org/10.2134/agronmonogr15.c3

Lesins, K., Lesins, I., 1966. LITTLE-KNOWN MEDICAGOS AND THEIR CHROMOSOME COMPLEMENTS. IV. SOME MOUNTAIN SPECIES. Can. J. Genet. Cytol. 8, 8–13. https://doi.org/10.1139/g66-002

Lesins, K., Lesins, I., 1960. SIBLING SPECIES IN *MEDICAGO PROSTRATA* JACQ. Can. J. Genet. Cytol. 2, 416–417. https://doi.org/10.1139/g60-043

Lesins, K.A., Lesins, I., 1979. Genus Medicago (Leguminosae). Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-94-009-9634-2

Li, A., Liu, A., Du, X., Chen, J.-Y., Yin, M., Hu, H.-Y., Shrestha, N., Wu, S.-D., Wang, H.-Q., Dou, Q.-W., Liu, Z.-P., Liu, J.-Q., Yang, Y.-Z., Ren, G.-P., 2020. A chromosome-scale genome assembly of a diploid alfalfa, the progenitor of autotetraploid alfalfa. Hortic Res 7, 194. https://doi.org/10.1038/s41438-020-00417-7

Lloyd, A., Bomblies, K., 2016. Meiosis in autopolyploid and allopolyploid Arabidopsis. Current Opinion in Plant Biology 30, 116–122. https://doi.org/10.1016/j.pbi.2016.02.004

Masterson, J., 1994. Stomatal Size in Fossil Plants: Evidence for Polyploidy in Majority of Angiosperms. Science 264, 421–424. https://doi.org/10.1126/science.264.5157.421

Mazyad, P.R.A., Ammann, K., 1999. Biogeographical assay and natural gene flow, in: Ammann, K., Jacot, Y., Kjellsson, G., Simonsen, V. (Eds.), Methods for Risk Assessment of Transgenic Plants. Birkhäuser Basel, Basel, pp. 95–98. https://doi.org/10.1007/978-3-0348-8700-7_12

McCoy, T.J., Bingham, E.T., 1988. Cytology and Cytogenetics of Alfalfa, in: Hanson, A.A., Barnes, D.K., Hill, R.R. (Eds.), Agronomy Monographs. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, USA, pp. 737–776. https://doi.org/10.2134/agronmonogr29.c24

Michaud, R., Lehman, W.F., Rumbaugh, M.D., 1988. World Distribution and Historical Development, in: Hanson, A.A., Barnes, D.K., Hill, R.R. (Eds.), Agronomy Monographs. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, USA, pp. 25–91. https://doi.org/10.2134/agronmonogr29.c2

Otto, S.P., Whitton, J., 2000. POLYPLOID INCIDENCE AND EVOLUTION. Annu. Rev. Genet. 34, 401–437. https://doi.org/10.1146/annurev.genet.34.1.401

Pankiw, P., 1967. Studies of Honeybee Preference and Behaviour on Normal and Standardless Alfalfa Flowers. Journal of Apicultural Research 6, 105–112. https://doi.org/10.1080/00218839.1967.11100167

Pennington, P.D., Costa, L.M., Gutierrez-Marcos, J.F., Greenland, A.J., Dickinson, H.G., 2008. When Genomes Collide: Aberrant Seed Development Following Maize Interploidy Crosses. Annals of Botany 101, 833–843. https://doi.org/10.1093/aob/mcn017

Pignatti, S., 2017. Flora d'Italia. Volume 1, Seconda edizione in 4 volumi. ed. Edagricole, Milano.

Pottier-Alapetite, G., 1979. Flore de la Tunisie: Angiospermes-dicotylédones. Apétales-dialypétales. Ministère de l'Enseignement Supérieur et de la Recherche Scientifique et le Ministère de l'Agriculture, Tunis.

Quiros, C.F., 1982. TETRASOMIC SEGREGATION FOR MULTIPLE ALLELES IN ALFALFA. Genetics 101, 117–127. https://doi.org/10.1093/genetics/101.1.117

Quiros, C.F., Bauchan, G.R., 1988. The Genus *Medicago* and the Origin of the *Medicago sativa* Comp, in: Hanson, A.A., Barnes, D.K., Hill, R.R. (Eds.), Agronomy Monographs. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, USA, pp. 93–124. https://doi.org/10.2134/agronmonogr29.c3

Ramsey, J., Ramsey, T.S., 2014. Ecological studies of polyploidy in the 100 years following its discovery. Phil. Trans. R. Soc. B 369, 20130352. https://doi.org/10.1098/rstb.2013.0352

Ramsey, J., Schemske, D.W., 2002. Neopolyploidy in Flowering Plants. Annu. Rev. Ecol. Syst. 33, 589–639. https://doi.org/10.1146/annurev.ecolsys.33.010802.150437

Riday, H., 2008. Alfalfa Subsp. *Sativa* by *Falcata* Intersubspecific Semi-Hybrid Seed Production Using Alfalfa Leafcutter Bees. Journal of New Seeds 9, 19–31. https://doi.org/10.1080/15228860701879083

Şakiroğlu, M., Brummer, E.C., 2017. Identification of loci controlling forage yield and nutritive value in diploid alfalfa using GBS-GWAS. Theor Appl Genet 130, 261–268. https://doi.org/10.1007/s00122-016-2782-3

Şakiroğlu, M., Brummer, E.C., 2013. Presence of phylogeographic structure among wild diploid alfalfa accessions (Medicago sativa L. subsp. microcarpa Urb.) with evidence of the center of origin. Genet Resour Crop Evol 60, 23–31. https://doi.org/10.1007/s10722-012-9811-0

Şakiroğlu, M., Brummer, E.C., 2011. Clarifying the ploidy of some accessions in the USDA alfalfa germplasm collection. Turkish Journal of Botany. https://doi.org/10.3906/bot-1002-47

Şakiroğlu, M., Doyle, J.J., Brummer, E.C., 2010. Inferring population structure and genetic diversity of broad range of wild diploid alfalfa (Medicago sativa L.) accessions using SSR markers. Theor Appl Genet 121, 403–415. https://doi.org/10.1007/s00122-010-1319-4

Savova, D., Rufener Al Mazyad, P., Felber, F., 1996. Cytogeography of Medicago falcata L. and M. sativa L. in Switzerland. https://doi.org/10.5169/SEALS-72199

Schinkel, C.C.F., Kirchheimer, B., Dullinger, S., Geelen, D., De Storme, N., Hörandl, E., 2017. Pathways to polyploidy: indications of a female triploid bridge in the alpine species Ranunculus kuepferi (Ranunculaceae). Plant Syst Evol 303, 1093–1108. https://doi.org/10.1007/s00606-017-1435-6

Scott, R.J., Spielman, M., Bailey, J., Dickinson, H.G., 1998. Parent-of-origin effects on seed development in *Arabidopsis thaliana*. Development 125, 3329–3341. https://doi.org/10.1242/dev.125.17.3329

Sekine, D., Ohnishi, T., Furuumi, H., Ono, A., Yamada, T., Kurata, N., Kinoshita, T., 2013. Dissection of two major components of the post-zygotic hybridization barrier in rice endosperm. The Plant Journal 76, 792–799. https://doi.org/10.1111/tpj.12333

Šemdgenelni, Кецховели, Н., Долуханов, Армен Георгиевич, 1971. Sakʿartʿvelos pʿlora = Флора Грузии = Flora Georgiae, 2 izdanie. ed. Мецниереба, Tbilisi.

Sémon, M., Wolfe, K.H., 2007. Consequences of genome duplication. Current Opinion in Genetics & Development 17, 505–512. https://doi.org/10.1016/j.gde.2007.09.007

Shen, C., Du, H., Chen, Z., Lu, H., Zhu, F., Chen, H., Meng, X., Liu, Q., Liu, P., Zheng, L., Li, X., Dong, J., Liang, C., Wang, T., 2020. The Chromosome-Level Genome Sequence of the Autotetraploid Alfalfa and Resequencing of Core Germplasms Provide Genomic Resources for Alfalfa Research. Molecular Plant 13, 1250–1261. https://doi.org/10.1016/j.molp.2020.07.003

Shi, K., Dong, H., Du, H., Li, Y., Zhou, L., Liang, C., Şakiroğlu, M., Wang, Z., 2024. The chromosome-level assembly of the wild diploid alfalfa genome provides insights into the full landscape of genomic variations between cultivated and wild alfalfa. Plant Biotechnology Journal pbi.14300. https://doi.org/10.1111/pbi.14300

Small, E., 1985. Morphological differentiation in *Medicago sativa* s.l. in relation to ploidy. Can. J. Bot. 63, 1747–1752. https://doi.org/10.1139/b85-245

Small, E., Brookes, B.S., 1984. Taxonomic circumscription and identification in the Medicago sativa-falcata (alfalfa) continuum. Econ Bot 38, 83–96. https://doi.org/10.1007/BF02904419

Small, E., Jomphe, M., 1989. A synopsis of the genus *Medicago* (Leguminosae). Can. J. Bot. 67, 3260–3294. https://doi.org/10.1139/b89-405

Smith, S.E., Bingham, E.T., Fulton, R.W., 1986. Transmission of chlorophyll deficiencies in Medicago sativa. Journal of Heredity 77, 35–38. https://doi.org/10.1093/oxfordjournals.jhered.a110163

Stebbins, G.L., 1950. Chapter VI. Isolation and the Origin of Species, in: Variation and Evolution in Plants. Columbia University Press, pp. 189–250. https://doi.org/10.7312/steb94536-007

Stift, M., Bregman, R., Oostermeijer, J.G.B., Van Tienderen, P.H., 2010. Other tetraploid species and conspecific diploids as sources of genetic variation for an autotetraploid. American J of Botany 97, 1858–1866. https://doi.org/10.3732/ajb.1000048

Tavoletti, S., 1994. Cytological mechanisms of 2n egg formation in a diploid genotype of Medicago sativa subsp. falcata. Euphytica 75, 1–8. https://doi.org/10.1007/BF00024525

Upscott, M., 1939. The nature of tetraploidy in Primula kewensis. Journal of Genetics. Journal of Genetics 39, 79–100.

Wang, H., Coulman, B., Bai, Y., Tarʿan, B., Biligetu, B., 2023. Genetic diversity and local adaption of alfalfa populations (Medicago sativa L.) under long-term grazing. Sci Rep 13, 1632. https://doi.org/10.1038/s41598-023-28521-3

Wang, Z., Şakiroğlu, M., 2021. The Origin, Evolution, and Genetic Diversity of Alfalfa, in: Yu, L.-X., Kole, C. (Eds.), The Alfalfa Genome, Compendium of Plant Genomes. Springer International Publishing, Cham, pp. 29–42. https://doi.org/10.1007/978-3-030-74466-3_3

Wolfe, K.H., 2015. Origin of the Yeast Whole-Genome Duplication. PLoS Biol 13, e1002221. https://doi.org/10.1371/journal.pbio.1002221

Wolfe, K.H., Li, W.H., Sharp, P.M., 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc. Natl. Acad. Sci. U.S.A. 84, 9054–9058. https://doi.org/10.1073/pnas.84.24.9054

Wood, T.E., Takebayashi, N., Barker, M.S., Mayrose, I., Greenspoon, P.B., Rieseberg, L.H., 2009. The frequency of polyploid speciation in vascular plants. Proc. Natl. Acad. Sci. U.S.A. 106, 13875–13879. https://doi.org/10.1073/pnas.0811575106

Yu, F., Wang, H., Zhao, Y., Liu, R., Dou, Q., Dong, J., Wang, T., 2017. Karyotypic evolution of the Medicago complex: sativa-caerulea-falcata inferred from comparative cytogenetic analysis. BMC Evol Biol 17, 104. https://doi.org/10.1186/s12862-017-0951-x