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Niche expansion of tetraploid *Medicago falcata* at different spatial scales

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Abstract: Whole genome duplication (polyploidization) can lead to changes or expansions of an organism's ecological niche, although these outcomes are highly variable among species and within species across spatial scales. Our study investigated niche variation in *Medicago falcata*, the closest widespread wild relative of the important forage crop alfalfa (*M. sativa*), which is also a valuable source of germplasm for breeding. *M. falcata* exists in nature as both diploid and autotetraploid populations, but their broad distribution patterns and ecological preferences were previously unknown. We used stratified sampling at three spatial scales and analysed 4,919 individuals from 443 populations sampled across a broad latitudinal range from Greece to Norway. Using environmental niche modelling and multivariate analyses, we tested whether polyploidization in *M. falcata* was accompanied by a shift and/or expansion of its ecological niche. We found that the two ploidy levels exhibited subtle but significant niche differentiation at all spatial scales examined: across Europe, within the more intensively sampled central-European area, and within a landscape at one contact zone. Tetraploid niche expansion at the two broader spatial scales was relatively modest (~8–11%), while tetraploids consistently preferred colder, wetter and higher-elevation areas at both broader scales and in one of the two contact zones. The lack of significant differences in a second contact zone suggests that local, idiosyncratic processes, such as land-use history or colonization events, can sometimes obscure these differences, particularly at smaller, local scales. In summary, our results indicate that polyploidy was associated with a subtle but consistent niche shift towards higher elevations and moderate climatic niche expansion at coarse spatial scales in this close alfalfa relative. However, these findings also caution against making broad generalizations about ploidy-related niche differences across a species' entire range, especially when the observed niche shifts are subtle.

Keywords: polyploidy, cytotype distribution, niche expansion, vegetation samples, niche modelling, species distribution models

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Introduction

Whole genome duplication (WGD) is among the leading forces driving plant evolution and lineage diversification (Wood et al. 2009). Most angiosperm lineages have experienced at least one WGD event throughout their evolutionary history (Jiao et al. 2011, Shi & Van de Peer 2026) and WGD is considered to be the most important mechanism underlying sympatric speciation (Otto & Whitton 2000, Soltis & Soltis 2004). Usually, WGD leads to rapid formation of strong reproductive isolation between the newly formed polyploid and its diploid progenitor because interbreeding between different cytotypes produces mainly nonviable offspring (Köhler et al. 2010, Salony et al. 2025). In the initial phase, however, WGD brings multiple challenges for the newly formed polyploids. Decreased fertility due to frequent disruptions in meiosis and pollen tube growth (Ramsey & Schemske 2002, Bomblies 2020, Westermann et al. 2024) and frequency-dependent mating disadvantage in populations dominated by diploids (the minority cytotype exclusion principle, Levin 1975) hamper their establishment.

However, the ubiquity of polyploid plant species found in nature (Rice et al. 2015) along with frequent WGD events that occurred during angiosperm evolution (Wood et al. 2009, Jiao et al. 2011) demonstrates that polyploids sometimes succeed in overcoming the minority cytotype exclusion principle and establish a new polyploid lineage. Several mechanisms such as spatial segregation between the cytotypes (Baack 2005), a perennial life form, clonal growth, an increased rate of selfing (Van Drunen & Friedman 2022) or an incomplete triploid block (Yamauchi et al. 2004) were shown to enhance the ability of newly formed polyploids to overcome the initial challenges. Established polyploids can then benefit from novel morphological and physiological traits, such as larger size (Müntzing 1936, Stebbins 1971) and higher tolerance to environmental stresses (e.g. Deng et al. 2012, Chao et al. 2013, Yang et al. 2024) along with more efficient masking of deleterious recessive alleles (Comai 2005). These traits could enable polyploids to expand into new regions and environments beyond boundaries of their diploid progenitors, thus leading to niche differentiation, one of the key mechanisms underlying long-term coexistence between closely related populations that differ in ploidy (Mortier et al. 2024).

Broad distributional and ecological patterns in polyploids have been discussed since the discovery of the polyploidy phenomenon (e.g. Hagerup 1933, Müntzing 1936, Stebbins 1942). In the last two decades, a broad range of case studies that systematically tested niche differences between ploidies using new tools in niche modelling and vegetation ecology has revealed highly incongruent results across studied taxa. For example, Sonnleitner et al. (2016) found a shift towards warmer, more humid and nutrient-rich conditions with increasing ploidy level in the *Senecio carniolicus* group and Thompson et al. (2014) found a shift towards drier conditions in tetraploid *Chamerion angustifolium* compared to the diploid cytotype. However, Chung et al. (2015) did not find any significant shift in climatic conditions between the two cytotypes of *Lilium lancifolium* though the higher ploidy cytotype exhibited a broader ecological niche. Indeed, a recent meta-analysis of occurrence data from more than a hundred mixed-ploidy species revealed significant niche differentiation between cytotypes in the majority of the studied taxa, but failed to identify any general pattern in the direction and magnitude of ploidy-related shifts across these species (Meirmans & Kolář 2025). In addition, the extent of observed niche differentiation can also depend on the scale of the study, grain of analysis (Kirchheimer

et al. 2016) and evolutionary background of the compared cytotypes (Padilla-García et al. 2023). In sum, although niche shift is a frequent consequence of polyploidization, its magnitude, direction and underlying drivers are hard to predict. Additional detailed studies combining comprehensive sampling of wild populations with a systematic analysis of niche differentiation across spatial scales are thus needed to understand under which circumstances polyploidization results in niche differentiation and expansion.

The genus *Medicago* (*Fabaceae*) is native to most of Eurasia and parts of Africa, with its primary centre of diversity found in the Caucasus, north-western Iran and north-eastern Turkey (Quiros & Bauchan 1988). It contains over 80 species, many of which naturally occur in more than one ploidy cytotype (Lesins & Lesins 1979, Small & Jomphe 1989). One example is the *Medicago sativa* complex, which includes the economically important crop *M. sativa* (alfalfa). *M. sativa* and *M. falcata* (also sometimes treated as *M. sativa* subsp. *falcata*) are the two most widespread taxa in the complex. Both occur naturally at two ploidy levels: diploid (2n = 16) and tetraploid (2n = 32). The tetraploids of both species occasionally form quadrivalents during meiosis (Stanford et al. 1972, McCoy & Bingham 1988), which suggests an autopolyploid origin of both tetraploid *M. sativa* and *M. falcata*. In *M. sativa*, this has been further supported by cpDNA (Havananda et al. 2011) and fluorescence in situ hybridization (Yu et al. 2017). On the other hand, the exact genetic composition of *M. falcata* tetraploids remains unresolved and it is possible that hybridization with other species, including *M. sativa*, may have shaped the genetic composition of some populations (Havananda et al. 2011, Eriksson et al. 2017). Experimental crosses have shown that *M. sativa* and *M. falcata* can hybridize at the same ploidy level and produce highly fertile offspring (Lesins & Lesins 1979). Indeed, artificial introgression of tetraploid *M. falcata* has been widely used in breeding cold-tolerant alfalfa varieties (Fairey et al. 1996, Boe et al. 2020) and there are also efforts to use it to improve drought resistance in alfalfa (Singer et al. 2021, Aili et al. 2023).

The distribution of *M. sativa* is highly influenced by its millennia-long global cultivation, but it probably originated from the semi-desert regions in central and western Asia where its diploid cytotype also occurs (Wang & Şakiroğlu 2021). *Medicago falcata*, on the other hand, naturally grows on steppes and dry grasslands from western Europe to China and both diploid and tetraploid populations have been reported from Europe. These records were, however, based on sporadic chromosome counts and a survey of germplasm accessions that was hampered by imprecise locality information and small sample sizes. Thus, the broad patterns of *M. falcata*'s cytotype distribution, the extent of ploidy coexistence and the underlying drivers of the observed patterns are unknown.

Here we ask the following questions: (i) What are the frequency and distribution of diploid and tetraploid *Medicago falcata* cytotypes across Europe in general, and in mixed-ploidy contact zones in detail? (ii) Do the two cytotypes co-occur within mixed populations and if so, are triploid individuals formed? (iii) Do the ecological niches of diploid and tetraploid cytotypes differ and if so, is the direction of the differences consistent across spatial scales and contact zones, indicating a role of ploidy-related traits in these patterns? In order to answer these questions, we performed an exhaustive ploidy screening of wild *M. falcata* populations using flow cytometry, followed by environmental niche modelling to assess the niche shifts and vegetation surveys to quantify local habitat differences.

Materials and methods

Field sampling

From 2019 to 2025, plant material was collected from 443 wild *Medicago falcata* populations sampled throughout Europe (see Supplementary Table S1 for details). We performed stratified sampling at three spatial scales: (i) coarse Europe-wide sampling covering the entire latitudinal gradient of its occurrence (full dataset); (ii) dense region-level sampling in central Europe, an area rich in steppic habitats (core dataset); (iii) detailed landscape-level sampling in two areas where we had previously identified frequent co-occurrence of both ploidy cytotypes (ploidy contact zones in central Bohemia and southern Moravia). Additionally, we thoroughly sampled three mixed-ploidy populations (MF021, MF022 and MF069), where we recorded the coordinates of individual plants.

In each population we sampled ~10 plants along a transect, at distances >1 m from each other to avoid sampling the same individual. Altogether, we sampled 4,919 individuals with 1–157 (mean 11.1; median 9) individuals per population. The plants were distinguished from closely related taxa (*M. sativa*, *M.* ×*varia*, *M. prostrata*) using known *M. falcata* diagnostic morphological characters: florets in a dense headlike or slender lax raceme, yellow or yellow-orange corolla, straight to sickle-shaped or occasionally corkscrew pods (Lesins & Lesins 1979). Fresh leafy stems from *M. falcata* individuals were collected from each site and kept in a humid plastic bag at a low temperature (4–6 °C) until analysed by flow cytometry. A subset of voucher specimens is deposited in the PRC herbarium (Charles University, Prague). To visualize the sampled populations' geographical distribution, we used Esri: Ocean (Sources: Esri, GEBCO, NOAA, National Geographic, Garmin, HERE, Geonames.org, and other contributors) and Esri: World imagery (Sources: Esri, DigitalGlobe, GeoEye, i-cubed, USDA FSA, USGS, AEX, Getmapping, Aerogrid, IGN, IGP, swisstopo, and the GIS User Community) basemaps.

Flow cytometry

DNA ploidy levels were inferred from relative fluorescence intensities of DAPI-stained nuclei using flow cytometry following the simplified two-step protocol (Doležel et al. 2007). Compared with the original protocol, we reduced the buffer volumes and DAPI concentration and introduced an incubation step before staining. Intact tissue from 1–2 fresh leaves per individual was chopped together with an appropriate volume of the internal reference standard (*Carex acutiformis*, 2C = 799.93 Mbp; Temsch et al. 2022) using a sharp razor blade in a Petri dish containing 0.6 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween-20) for both individual and pooled samples. Coefficients of variation (CVs) were generally low and similar for sample (mean 2.59%, range 0.92%–7.45%) and standard (mean 3.01%, range 1.48%–8.43%) peaks. We did not accept analyses with sample CV above 8%; samples above this limit were re-analysed or discarded if no more material was available. Up to 10 *M. falcata* individuals were pooled in equal proportions into one sample due to overall high quality of the analyses and lack of secondary peaks (actively dividing cells in G2 phase, endoreduplication), making detection of distinct ploidy cytotypes feasible even at low frequencies. Pooling was used for routine ploidy screening but the individuals were re-scored individually whenever assignment to a specific plant was needed. Although this approach may theoretically miss a single individual

of a minority ploidy (~10% of sample nuclei), our individual re-analyses repeatedly confirmed the presence of a single individual of distinct ploidy in a mixed sample. Although this does not provide conclusive evidence for all analyses, the limitation calls for caution when inferring precise cytotype frequencies, especially in mixed-ploidy areas. Nevertheless, our approach provides an alternative to standardized pooling experiments that were not readily feasible in such a large-scale survey. The resulting suspension was filtered through a 42-µm nylon mesh, incubated for ~5–10 minutes and then mixed with 1 ml of a DAPI staining solution (0.4 M $Na_2HPO_4 \cdot 12H_2O$, 2 µg DAPI, 1 µl β-mercaptoethanol). The concentration of DAPI in the final solution was 1.25 µg ml^{-1} and that of β-mercaptoethanol was 0.696 mg ml^{-1}. The solution was incubated for ~2 minutes and then the fluorescence intensity of 1,500 particles was analysed using a Partec PA II flow cytometer equipped with a UV LED chip.

Climatic data acquisition

Differences in climatic niche between diploid and tetraploid *M. falcata* were examined for the two datasets representing the two coarser spatial scales in order to assess consistency of the results over distinct sampling densities and spatial scales: the full dataset (European scale), encompassing all sampled populations, and the core dataset consisting of a densely sampled subset of populations from central Europe. First, we tested whether there was a significant difference in elevation between the ploidies using a Wilcoxon rank-sum test; mixed-ploidy populations were assigned to both ploidy categories for this analysis. Second, for niche differentiation tests and species distribution modelling we used historical climate averages (period 1970–2000) of the sampling locations obtained from the WorldClim database (Fick & Hijmans 2017) and soil pH acquired from the European Soil Data Centre (Panagos et al. 2022). For all analyses in both datasets, we consistently used seven predictors selected to represent different abiotic dimensions of the *M. falcata* niche. These predictors were not strongly correlated ($|r| < 0.7$) and did not exhibit high collinearity (variance inflation factor; $VIF < 5$) in the European dataset. Specifically, they represent the amount of heat and moisture available throughout the year (annual mean temperature – BIO1, annual precipitation – BIO12) and also moisture during the flowering time of the studied species (precipitation of warmest quarter – BIO18). We also included variables describing seasonal variation in temperature and precipitation (isothermality – BIO3, temperature seasonality – BIO4, precipitation seasonality – BIO15). Soil pH was added because this species is moderately calciphilous and acidic soils might represent a strong limiting factor for its distribution.

Testing niche divergence and niche expansion

In order to quantify niche divergence between different *M. falcata* cytotypes and expansion/unfilling of the tetraploid niche, we used a multivariate approach incorporated in the R package ecospat (Broennimann et al. 2026). First, we tested niche similarity and niche equivalency using the functions ecospat.niche.similarity.test and ecospat.niche.equivalency.test with the option overlap.alternative set to “lower” in both cases and using 999 permutations. The amount of niche expansion/unfilling was also calculated with the ecospat.niche.similarity.test function using the same settings. To test overall niche divergence we followed the method of Broennimann et al. (2012), in which a principal component

analysis (PCA) is performed on all selected environmental variables extracted from the raster cells across the entire study area and then the first two PCA axes are divided into 100 bins, creating a two-dimensional rasterized 100 × 100 niche space. The sampled localities are then placed onto this raster according to their values for these same environmental variables. A similar analysis was then performed using two climatic variables (annual mean temperature and average annual precipitation), analysed separately along one-dimensional vectors with 1,000 bins, in order to explore differentiation along individual environmental gradients. Prior to each analysis, the dataset was spatially thinned using the R package dismo (Hijmans et al. 2024) by random subsampling of one population per 30-arc-second grid cell in order to avoid including the same predictor values for multiple spatially close populations.

The method of Broennimann et al. (2012) was used to quantify niche differentiation between diploids and tetraploids and the degree of overlap between the cytotypes' niches was then calculated using the D-metric (Schoener 1970), which varies between 0 (no overlap) and 1 (complete overlap). Niche unfilling/expansion was then calculated using the ecospat.niche.similarity.test function. The significance of Schoener's D was tested using a custom permutation scheme following Meirmans (2021) optimized for the presence of mixed-ploidy populations. For each permutation, the dataset was subsampled in the same way as specified above, then the locations of the ploidy-uniform populations were randomized between the diploids and tetraploids (locations of the mixed-ploidy populations were not randomized) and a D-value was calculated for this permuted dataset. We ran 999 permutations and then calculated the P-value by comparing the original D-value to the null distribution obtained from the permuted values.

Species distribution modelling

We also used species distribution models (SDMs) to visualize the patterns of niche differentiation between *M. falcata* cytotypes in space and to identify further potential areas of ploidy overlap. SDMs were calibrated and evaluated separately for the European and central-European datasets using the biomod2 package (Guéguen et al. 2026). Within each region, separate SDMs were built for the diploid and tetraploid *M. falcata* cytotypes (where mixed-ploidy populations were assigned to both cytotypes), yielding four ploidy–dataset combinations that were modelled separately. After spatial thinning, 201 diploid and 279 tetraploid presences were retained in the European dataset and 145 diploid and 217 tetraploid presences in the central-European dataset. For each of these combinations, we selected 1,000 pseudo-absences using the "random" method. Five sets of pseudo-absences were generated independently for each ploidy–dataset combination. The background data for selection of pseudo-absences were, however, similar for both diploid and tetraploid models within each of the regions to ensure comparability of the results. Models were then validated using the "spatial block" strategy, in which the study area was divided into four spatial blocks; three were used for model calibration and one for model validation.

As modelling techniques, we used generalized additive models (GAM), generalized boosted models (GBM), generalized linear models (GLM) and random forests (RF). Projection accuracy of each model was assessed using the true skill statistic (TSS). For each ploidy–dataset combination, four iterations were calibrated for each of the five sets of

pseudo-absences and each of the four modelling techniques, resulting in a total of 80 models per ploidy–dataset combination. Low-quality models (models with a TSS below 0.5; Wang et al. 2025) were removed and all remaining models were then combined into one ensemble model weighted by their TSS for each ploidy–dataset combination (for individual model validation see Supplementary Fig. S1). The two European ensemble models comprised 40 individual models for diploids and 37 for tetraploids, whereas the corresponding central-European ensemble models comprised 28 and 20 individual models, respectively. Based on the ensemble models, binary projections were generated using the TSS-maximizing threshold for each ploidy–dataset combination and mapped at the European and central-European scales. As basemaps we used Natural Earth: Gray Earth with shaded relief (Huffman & Patterson 2013).

Ecological differences at the local scale in contact zones

To test whether possible continent-wide niche differences were recapitulated at the local level, local environmental parameters were inferred from vegetation composition (vegetation relevés) in two intensively sampled ploidy contact zones in Czechia (central Bohemia and southern Moravia). We recorded 81 vegetation plots of 9 m^2 each in wild *M. falcata* populations growing in central Bohemia (43 diploid and 38 tetraploid) and 64 plots in southern Moravia (33 diploid and 31 tetraploid). The ploidy level of five *M. falcata* individuals growing inside each plot (one in each of the four corners and one in the centre) was estimated using flow cytometry and only plots containing a single *M. falcata* ploidy were used for further analyses. All vascular plant species growing inside the plots were identified and their relative abundances scored using the semiquantitative Braun-Blanquet scale. Relative cover of the herb layer was also recorded for each plot and used as a proxy for competitive pressure. All vegetation samples were stored in the database program Turboveg 2 (Hennekens & Schaminée 2001) and further processed in JUICE 7.1 (Tichý 2002; primary data are available in Supplementary Table S1). Species nomenclature follows Pladias.cz (Chytrý et al. 2021).

Local ecological conditions were then assessed in two ways. First, differences in species composition of plots occupied by different *M. falcata* cytotypes were investigated, based on occurrences of all recorded species with the exception of rare species (species with fewer than five occurrences in the total dataset) and *M. sativa* agg. Second, Ellenberg-type indicator values (EIVs) for light, temperature, moisture, soil reaction and nutrients (Chytrý et al. 2018) were calculated for each plot as an abundance-unweighted mean of EIVs of all species found inside the plot. EIVs obtained from the species composition of a community are useful indicators of environmental conditions (Chytrý et al. 2018) and are therefore a suitable alternative to direct measurements of environmental variables for characterization of ecological conditions at the micro-scale.

Differences in species composition and EIVs between plots with diploid and tetraploid *Medicago* were assessed by ordination techniques using the R package vegan (Oksanen et al. 2026) separately for the Bohemian and Moravian contact zones. First, a principal component analysis (PCA) was utilized to visualize the degree of niche differentiation between the cytotypes and the direction of potential niche shift. We investigated the patterns of differentiation in (i) standardized mean EIVs and (ii) species composition (frequencies of each species on an ordinal scale derived from the Braun-Blanquet scale

and transformed using the "Hellinger" method). Then, a redundancy analysis (RDA) with the ploidy of *M. falcata* as an explanatory variable was used to test the significance of differences in EIVs (as an indicator of niche position) between the cytotypes using random assignment of plots to ploidy levels across 9,999 Monte Carlo permutations. We also performed RDA on Hellinger-transformed species-composition data to identify accompanying species that were closely associated with either diploid or tetraploid *M. falcata* cytotype in each contact zone. We used the linear method (RDA) due to the relatively short environmental gradient (the lengths of the first detrended correspondence analysis (DCA) axes were 3.38 and 2.53 for the Bohemian and Moravian contact zones, respectively). Additionally, we also tested for environmental differentiation between the two contact zones (also represented by EIVs) in a separate RDA analysis in order to assess whether the differences in ploidy-related trends could be explained by different ecological preferences of *M. falcata* in the two contact zones.

Local niche breadth was approximated using the multivariate disparity of diploid and tetraploid vegetation plots, calculated with the R package dispRity (Guillerme 2018). Disparity for diploids and tetraploids was calculated separately for the Bohemian and Moravian contact zones from the PCA based on EIVs as the sum of variances. The significance of disparity differences between the two ploidies was tested using a custom permutation scheme. All plots were randomized between the diploids and tetraploids and the difference in disparity was calculated for the permuted dataset. We ran 9,999 permutations and then calculated the P-value by comparing the original difference in disparity to the null distribution obtained from the permuted values.

Results

Flow cytometry

Flow-cytometric estimates of ploidy level for the 4,919 individuals revealed two clearly distinct groups with respect to relative genome size (RGS) that corresponded to DNA-diploid (mean 1.85±0.028 SD, range 1.76–1.95) and DNA-tetraploid cytotypes (mean 3.69±0.054 SD, range 3.51–3.88). Based on the two-fold difference between these groups and previously published chromosome counts, these two groups were thereafter considered diploids and tetraploids. The remaining samples encompassed one putative triploid individual with approximately intermediate RGS (2.79) and 48 other samples with RGS deviating more than 5% from the diploid or tetraploid average (1.05–1.35 times the diploid average for 36 samples and 0.93–1.06 times the tetraploid average for 12 samples). These 48 samples might have represented aneuploidy or homoploid variation in genome size (Fig. 1). These were individual accessions within populations otherwise dominated by individuals with genome sizes typical of the respective ploidy and their distribution showed no clear geographic or ecological pattern. Apart from the putative triploid, these samples with deviating genome size were thus not considered separate ploidy entities, but were assigned to the closest euploid category for further analyses.

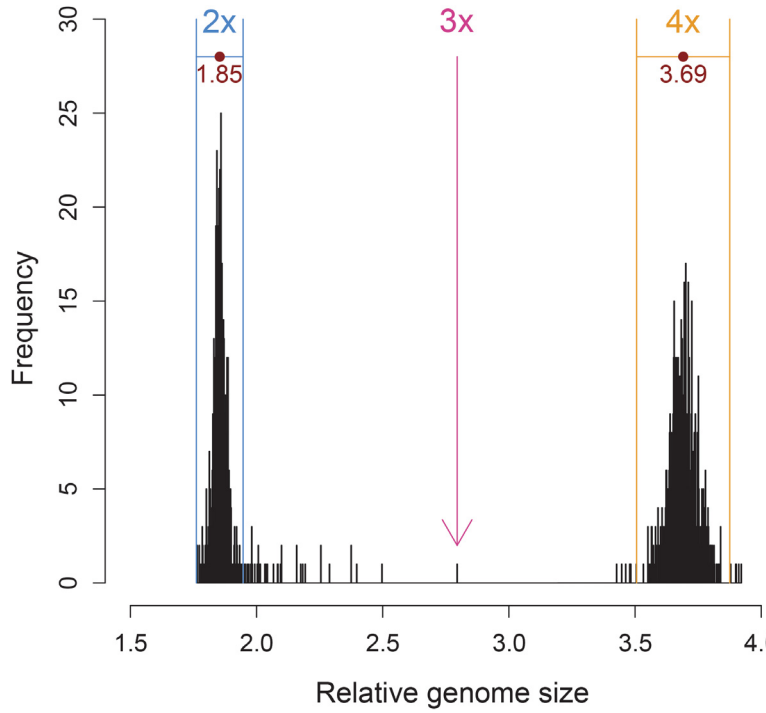

Fig. 1. Frequency of relative genome sizes (RGS) of the 4,919 *Medicago falcata* individuals inferred using flow cytometry. RGS values are expressed as the ratio to an internal reference standard. The two peaks correspond to diploid and tetraploid *M. falcata* cytotypes (mean RGS ± SD: 1.85±0.028 and 3.69±0.054, respectively). A pink arrow denotes the position of one putative triploid individual.

Table 1. Summary of niche differentiation between diploid and tetraploid *Medicago falcata* and expansion of tetraploid niche across distinct spatial scales. [a]Overall niche divergence as inferred by Schoener's D of climate data (ecospat) for European and central-European scales and based on Ellenberg-type indicator values (RDA) for the two contact zones. The RDA axis constrained by ploidy explained 10% of variation in the central Bohemian vegetation data. [b]Tetraploid niche expansion (unfilling in brackets) as inferred by ecospat for European and central-European scales and differences in niche breadth quantified by multivariate disparity for the two contact zones. [c]Tetraploids occurred at higher elevations in all significant cases. [d]Direction of niche shift as inferred by Schoener's D of selected climate variables (ecospat) for European and central-European scales and based on the Ellenberg-type indicator values (loadings on the constrained RDA axis) for the two contact zones.

	Europe	central Europe	central Bohemian contact zone	southern Moravian contact zone
N 2x/4x/2x+4x pops	161/239/43	112/185/34	40/36/4	28/29/7
Data	climatic data	climatic data	local species composition	local species composition
Analysis	ecospat & SDMs	ecospat & SDMs	RDA	RDA
Multivariate niche divergence[a]	yes (D = 0.60; P = 0.013)	yes (D = 0.62; P = 0.001)	yes (P < 0.001)	no (P > 0.1)
Multivariate niche expansion/breadth[b]	8.11% (2.61%)	11.24% (0.20%)	no (P > 0.1)	no (P > 0.1)
Elevation[c]	yes (P = 0.002)	yes (P < 0.001)	yes (P < 0.001)	no (P > 0.1)
Direction of niche shift[d]	tetraploids: colder (D = 0.76; P = 0.001) and wetter (D = 0.69; P = 0.009)	tetraploids: colder (D = 0.61; P = 0.001) and wetter (D = 0.77; P = 0.003)	tetraploids: colder, wetter and more N-rich	no shift

Distribution of Medicago falcata ploidy cytotypes across spatial scales

Of the 443 sampled *M. falcata* populations, 161 populations (36%) comprised only diploid plants, 239 (54%) only tetraploids and 43 populations (10%) were mixed, comprising both diploid and tetraploid individuals. Interestingly, only one triploid plant was found despite intensive sampling in areas of close spatial co-occurrence of both major cytotypes (1,039 individuals were sampled within mixed-ploidy populations). Moreover, this individual was found in an otherwise purely diploid population (MF421) originating from a diploid-dominated region of the Eastern Alps (Fig. 2A) and thus likely represented an outcome of occasional fusion of reduced (n) and unreduced (2n) gametes of two diploid parents.

Several regions were occupied exclusively by one ploidy: only tetraploids were represented among samples from northern Europe, Poland, northern Germany and southern Balkans while only diploids were found in the valleys of the Alps and in the eastern European lowlands. There were multiple areas of marked cytotype intermingling, especially in Bavaria, Czechia, central Slovakia, north-eastern Austria and central Balkans (Fig. 2A and B). Finally, a thorough sampling of three mixed-ploidy populations demonstrated that despite the overall tendency of each cytotype to occur in spatial clusters, there were sites within each population where individuals of different ploidy occurred in very close spatial proximity (Fig. 2C–E).

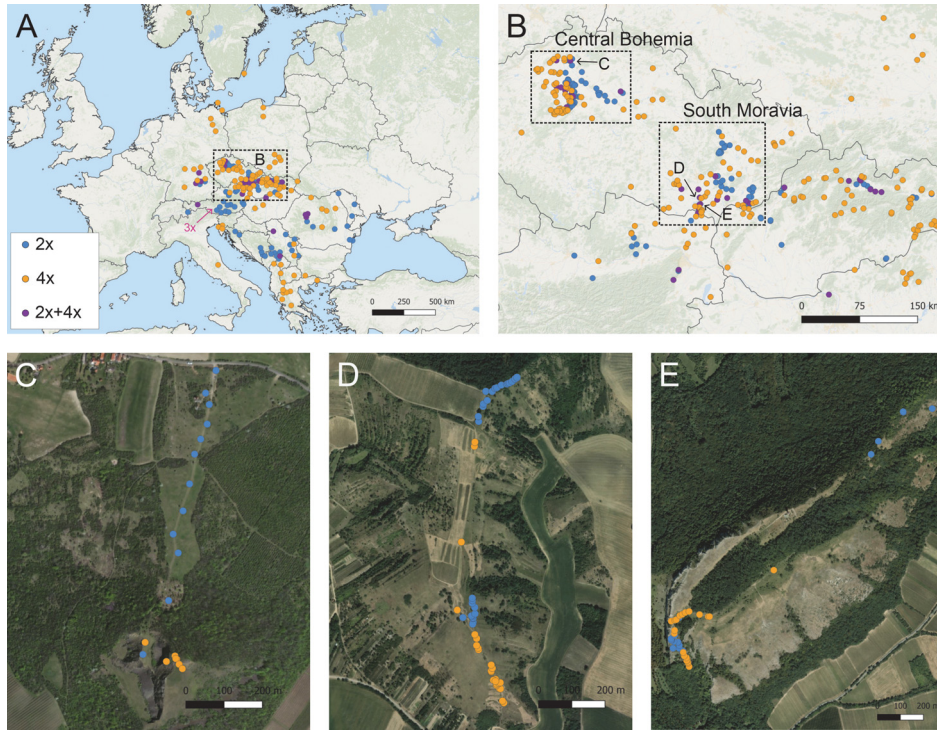

Fig. 2. Distribution of *Medicago falcata* cytotypes across spatial scales. Occurrence of diploid (blue), tetraploid (orange) and mixed (purple) populations (A) across Europe and (B) in central Europe. The two contact zones that were thoroughly sampled at local scale are framed by black rectangles. (C–E) Spatial arrangement of individuals in three mixed-ploidy populations.

Climatic niche differences at two coarse spatial scales

Diploid populations of *M. falcata* were found in localities from central Balkans (42.9°N) to northern Czechia (50.5°N). This was a much narrower latitudinal range than that of tetraploid populations, which were sampled from central Greece (39.5°N) to southern Norway (60.1°N). Tetraploids also extended to higher elevations at both scales (Table 1). However, this difference cannot be uniformly interpreted across the broad latitudinal span of our sampling.

Multivariate analyses of climatic niches performed in ecospat showed very similar results at both the coarse European scale and the finer, densely sampled central-European scale. Niche equivalency was rejected for both the European (P = 0.015) and central-European (P = 0.002) datasets but niche similarity tests did not detect significantly lower overlap than expected from environmental availability in either dataset (P = 0.954 and P = 0.990, respectively). This indicates partial niche differentiation, although the cytotypes' niches still overlapped extensively. In support of slight niche differentiation, overall niche divergence between the ploidies quantified by Schoener's D (Fig. 3A) was relatively minor but statistically significant at both European (D = 0.60; P = 0.013) and central-European scales (D = 0.62; P = 0.001) (Fig. 3D). At both spatial scales, tetraploids exhibited modest (8–11%) expansion of their niche in multidimensional space, while only a negligible proportion of the diploid niche remained unfilled (Table 1). Analyses along

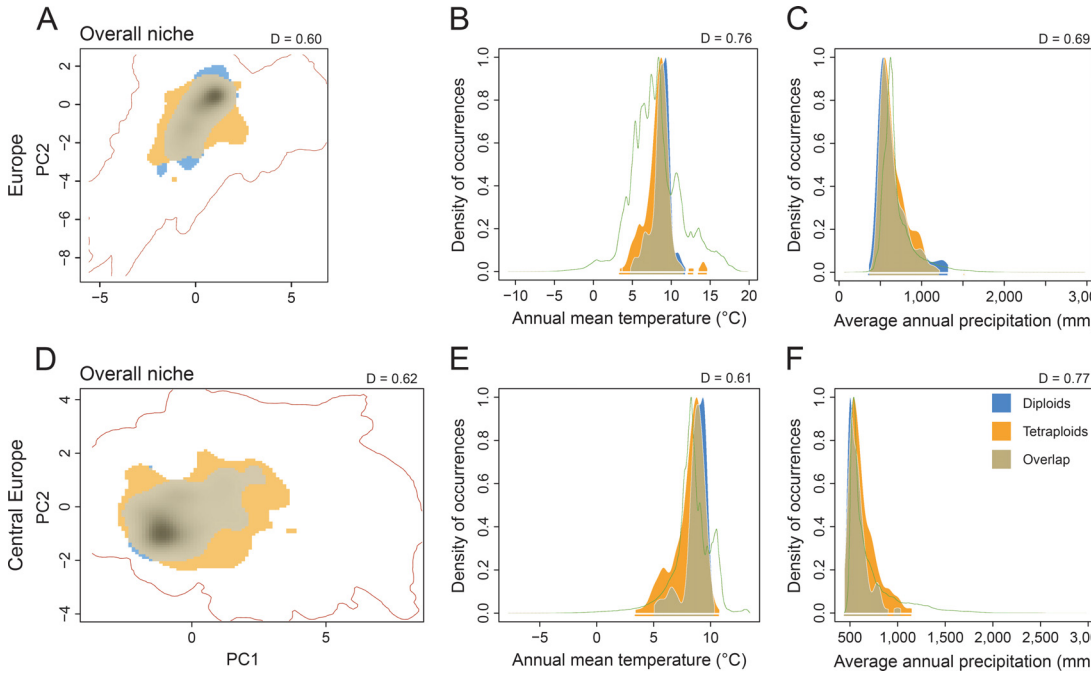

Fig. 3. Climatic niche differences between diploid and tetraploid *Medicago falcata* populations inferred at the two coarse spatial scales. Graphs depicting (A, D) overall niche divergence, (B, E) divergence in annual mean temperature and (C, F) divergence in average annual precipitation as inferred by ecospat for populations sampled across Europe (full dataset, top row) and in central Europe (core dataset, bottom row).

single gradients performed on the two uncorrelated predictors – annual mean temperature and average annual precipitation – also revealed slight but statistically significant differences between the two ploidies in both predictors (Table 1). At both spatial scales, tetraploids occupied areas with lower temperatures and higher precipitation.

Consistent with ecospat analyses, species distribution models showed substantial overlap in areas predicted to be suitable for both cytotypes, but an overall broader potential distribution of tetraploids (Fig. 4A). During evaluation, all ensemble models showed high sensitivity and specificity (between 81% and 99%), supporting their use for assessing potential cytotype distributions. The models' projections based on the complete European dataset revealed large parts of central and south-eastern Europe as potentially suitable for both cytotypes and only a few areas as suitable for only a single cytotype; the most notable of these were the eastern European lowlands and western Balkans that were predicted to be suitable only for diploids and the southern Balkans and Scandinavia only for tetraploids. The areas marked by the model's projections as suitable corresponded closely to the distribution of sampled populations. However, there were some exceptions. Notably, areas in southern and central Poland were predicted to be suitable for both ploidies although no diploids were found there. On the other hand, areas of south-eastern Moldova and Romania were predicted as unsuitable for both cytotypes despite several diploid populations being recorded there. Distribution models covering a smaller central-European region with more representative sampling showed a similar pattern. Their projections identified a larger area with predicted suitable conditions for tetraploids than for diploids (Fig. 4B). Specifically, areas marked as suitable exclusively for tetraploids were those extending towards colline and lower montane regions of the Českomoravská vrchovina highlands and Western Carpathian Mts.

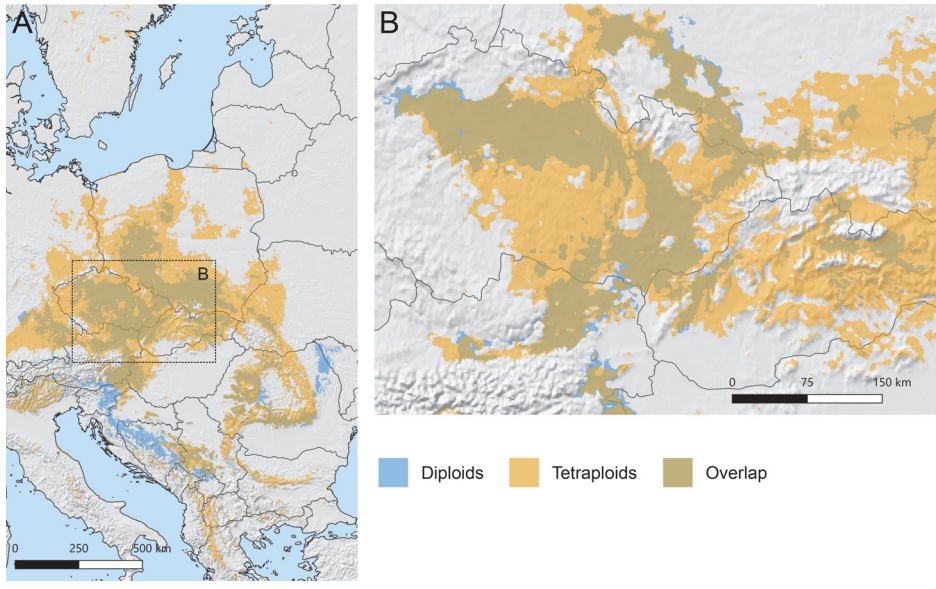

Fig. 4. Habitat suitability for diploid and tetraploid *Medicago falcata* in Europe. The maps depict suitable conditions for the cytotypes as predicted by species distribution modelling based on data sampled (A) across Europe and (B) only in densely sampled central Europe.

Ecological differences at the local scale in contact zones

Local environmental parameters inferred from vegetation plots revealed contrasting results in the two contact zones. In the central Bohemian contact zone, PCA indicated a slight shift in species composition between habitats occupied by diploid and tetraploid *M. falcata* populations (Supplementary Fig. S2). Tetraploids were associated with plant species characteristic of less xerothermous but more competitive (tall grass species) and ruderal habitats such as *Convolvulus arvensis* and *Elymus repens*, while diploids showed the strongest association with species typical of less competitive steppic grasslands (Table 2). On the other hand, in central Bohemia there was no significant difference in cover of the herb layer, which can be considered an additional crude proxy of overall habitat competitiveness ($P > 0.05$). Local habitat parameters inferred from the Ellenberg-type indicator values (EIVs) revealed that tetraploid populations grew in colder, more nutrient-rich and moister conditions than diploid populations (Fig. 5A). Direct ordination by

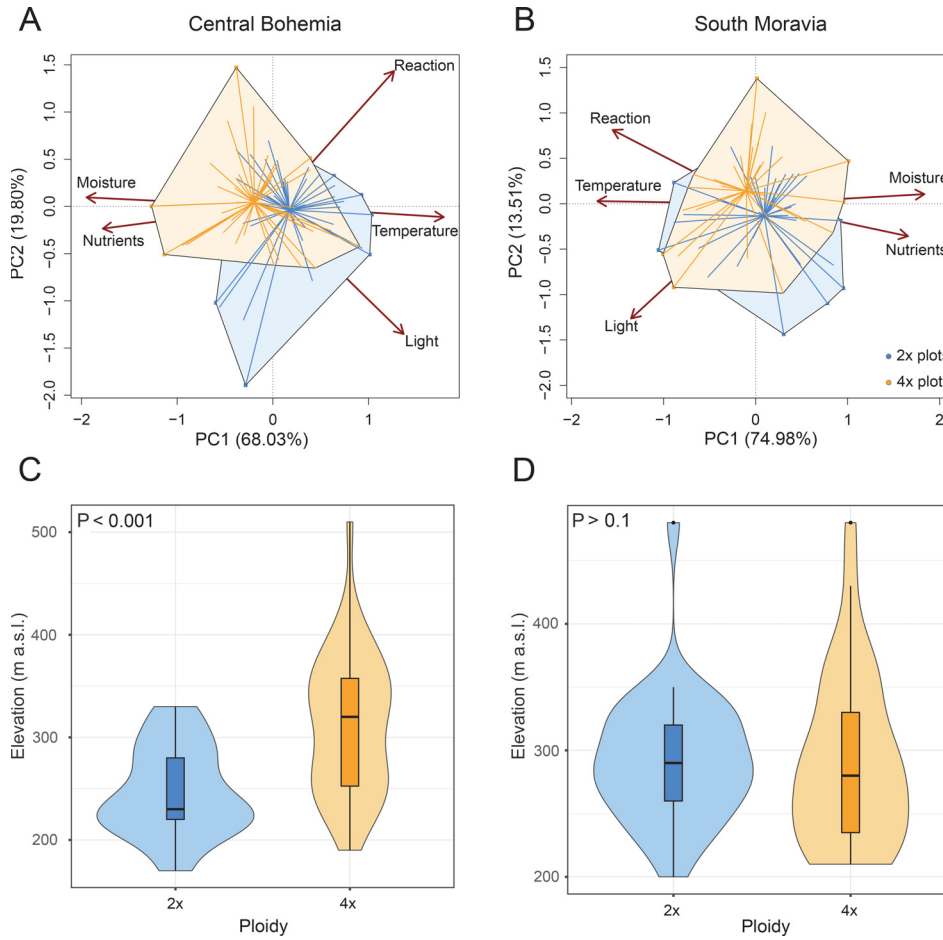

Fig. 5. Ecological differences at the local scale in the two contact zones based on local floristic composition. (A, B) PCA biplots showing differences in mean Ellenberg-type indicator values between plots occupied by diploid and tetraploid *Medicago falcata* cytotypes (A) in the central Bohemian contact zone and (B) in the southern Moravian contact zone. (C, D) Differences in elevation between diploid and tetraploid *M. falcata* cytotypes in the (C) central Bohemian and (D) southern Moravian contact zones.

Table 2. Plant species most strongly associated with diploid and tetraploid *Medicago falcata* in the central Bohemian contact zone. The table lists the 10 species with the highest loadings on the RDA axis constrained by ploidy in the direction of diploid- and tetraploid-occupied plots, respectively.

Diploids	*Carex humilis*, *Brachypodium pinnatum*, *Salvia pratensis*, *Potentilla incana*, *Koeleria macrantha*, *Festuca valesiaca*, *Sanguisorba minor*, *Filipendula vulgaris*, *Scabiosa ochroleuca*, *Thymus pannonicus* agg.
Tetraploids	*Bromus erectus*, *Elymus repens*, *Arrhenatherum elatius*, *Lolium perenne*, *Festuca rubra*, *Convolvulus arvensis*, *Verbascum lychnitis*, *Falcaria vulgaris*, *Dactylis glomerata*, *Securigera varia*

means of redundancy analysis (RDA) demonstrated that the differences in EIVs between *M. falcata* cytotypes were statistically significant ($P < 0.001$, Table 1). Unlike the larger scales, fine-scale habitat shift was not reflected in an increase in multidimensional niche breadth as indicated by a non-significant difference in disparity between diploid and tetraploid populations in the multidimensional space of the EIVs (Table 1).

In contrast, in the second contact zone, in southern Moravia, diploids and tetraploids exhibited largely similar local habitat preferences, as characterized by species composition (Supplementary Fig. S2) and derived EIVs (Fig. 5B). RDA showed non-significant ploidy-related differences in local environmental conditions as characterized by EIVs ($P > 0.05$) and the difference in herb layer coverage was also not significant ($P > 0.05$). Tetraploids in southern Moravia also did not show significantly higher multivariate disparity than diploids (Table 1). The contrasting cytotype patterns were unlikely to reflect differences in the environmental conditions sampled, as RDA based on EIVs detected no significant differences between the two contact zones ($P > 0.05$).

Discussion

Distribution of Medicago falcata cytotypes and factors promoting their coexistence

Our analysis presents the first large-scale evaluation of ploidy distribution in *Medicago falcata*, a close relative of the major fodder crop *M. sativa* (alfalfa). The occurrence of two cytotypes has previously been reported based on chromosome counts (Savova et al. 1996, Kaljund & Leht 2010) and a flow-cytometric survey across germplasm collections (Şakiroğlu & Brummer 2011), but their exact distribution and frequencies have remained unknown. We found that both diploids and tetraploids occur frequently in Europe and identified areas with high frequencies of both ploidy levels. While diploids dominate across several areas in central Europe (Eastern Alps foothills) and south-eastern Europe (northern Balkans, steppic regions of eastern Romania and Moldova), tetraploids were the only cytotype found at both latitudinal extremes of the species' range. Specifically, they were the exclusive ploidy in northern Europe, roughly from Poland and central Germany to Scandinavia and in the southern half of the Balkan Peninsula (Fig. 2A). Preference of polyploids for higher latitudes is a broadly recognized trend, at least at the level of species and higher taxa (Brochmann et al. 2004, Rice et al. 2019), yet prevalence of polyploids in southern Europe has also occasionally been reported, e.g. in *Minuartia verna* agg. (Nunvářová Kabátová et al. 2019) and *Alnus glutinosa* agg. (Mandák et al. 2016).

In addition, we found several diffuse contact zones where both cytotypes spatially overlapped at a landscape scale. Diploid and tetraploid populations intermingled particularly in central Bohemia (Czechia), at the western border of the Pannonian Basin (Austria and Czechia), in the Western and Southern Carpathians (Slovakia and Romania) and the central Balkans (southern Serbia), i.e. areas representing well-known biogeographic crossroads where ploidy contacts were previously reported for other species, e.g. *Senecio jacobaea* (Hodálová et al. 2015), *Vicia cracca* (Trávníček et al. 2010), *Knautia arvensis* (Kolář et al. 2009), *Arabidopsis arenosa* (Kolář et al. 2016) or the *Pilosella alpicola* group (Šingliarová et al. 2011). Ploidy mixing was also apparent at the finest spatial scale. Mixed-ploidy populations constituted a considerable fraction of populations in all the aforementioned contact zones and represented 10% of all populations sampled. Surprisingly, we found no triploids within any of these mixed populations, including three finely sampled populations where diploid and tetraploid individuals grew next to each other (Fig. 2C–E). This is consistent with previous reports of unsuccessful crosses between diploid and tetraploid individuals in the closely related *M. sativa* (Ledingham 1940, Lesins 1952), suggesting the existence of a strong postzygotic interploidy barrier to F1 hybrid seed formation (i.e. triploid block; Köhler et al. 2010, Salony et al. 2025) in this group. Altogether, our findings indicate reduced potential for interploidy hybridization between diploid and tetraploid cytotypes of *M. falcata*.

Additionally, our finding of frequent ploidy sympatry in spite of a likely strong triploid block also raises questions about additional isolation mechanisms promoting local ploidy coexistence. This is because under the minority cytotype exclusion principle (Levin 1975), a strong triploid block should lead to the disappearance of the ploidy level that has the lowest frequency in a mixed population, because it suffers the greatest fitness reduction due to the triploid block. However, when there are additional isolation mechanisms, prezygotic barriers may prevent interploidy hybridization and thus reduce the fitness costs of the triploid block (Rodríguez 1996, Kolář et al. 2017). Although we have not specifically recorded microhabitat differences at the level of individuals, extensive ecological overlap between ploidies within contact zones (Fig. 5), further supported by our field observations of homogeneous vegetation in sampled mixed populations, indicates that microsite ecological segregation is unlikely to be the primary mechanism promoting cytotype coexistence. On the other hand, it should be noted that our results are too coarse for a detailed assessment of divergence in biotic interactions such as nitrogen-fixing bacterial symbioses, which are known to interact with ploidy in other legumes (Forrester & Ashman 2018), including *Medicago sativa* (Forrester & Ashman 2020), and may underlie finer-scale microniche differentiation. Additional mechanisms of assortative mating such as phenological shifts, pollinator preferences or breeding-system shifts may also promote prezygotic isolation (Husband & Sabara 2004, Kolář et al. 2017). However, the reported similarity in floral morphology between the two cytotypes of *M. falcata* (Small 1985) suggests that differences in flower morphology are unlikely to drive assortative mating. Furthermore, although a quantitative assessment of phenology in *Medicago* is lacking, we sampled flowering individuals of both ploidies during the same field surveys, which suggests at least partial phenological overlap. An alternative non-exclusive explanation for frequent ploidy sympatry may involve non-adaptive processes such as migration and colonization history (Hanzl et al. 2014). Transport of fodder and movement of grazing animals among managed grasslands may promote seed dispersal and migration,

especially for tetraploids, taking into account their broader niche and their preference for more ruderal habitats. Thus, the observed ploidy mixtures may at least in some cases represent only recently formed, transient states. This would resemble cases reported, for example, in another grassland legume *Vicia cracca* (Trávníček et al. 2010) and in the rapidly cycling weed *Tripleurospermum inodorum* (Čertner et al. 2019). Further research is thus needed to directly test the hypothesis of prezygotic barriers and to uncover the relative contribution of distinct mechanisms that allow cytotype coexistence in *M. falcata*.

Slight but consistent niche shift of tetraploids

Polyploidization is often viewed as a process providing evolutionary opportunities (Van de Peer et al. 2017, 2021), and this notion has been supported by multiple reports of niche expansion associated with WGD in ploidy-variable plant taxa, e.g. *Urtica dioica* (Urfus et al. 2021), *Allium oleraceum* (Duchoslav et al. 2020) or the *Salix retusa* complex (Pittet et al. 2025). In line with this expectation, autotetraploids of *M. falcata* showed evidence of niche expansion based on climatic data at the two broader spatial scales studied. Although the overall breadth of the multivariate niche characterized by vegetation data was similar at the finest spatial scale, tetraploids exhibited a shift towards higher elevations and preferences for wetter, nutrient-rich habitats in one contact zone. Thus, the niche differentiation, although generally slight and quantitatively variable across scales, was consistently associated with a tetraploid niche shift towards cooler, wetter, higher-elevation areas. This supports the interpretation that the observed differences are associated with polyploidy rather than with stochastic or historical processes that would leave different signatures at different spatial scales. Indeed, a comparative assessment over spatial scales is a recommended strategy for distinguishing consistent ploidy-driven niche differences expressed at both the macroclimatic level and in local habitat preferences assessed through vegetation surveys (e.g. Kirchheimer et al. 2016, Čertner et al. 2019).

The exact mechanisms underlying ploidy-associated niche shifts are poorly known and perhaps species-specific. On one hand, WGD *per se* is expected to cause predictable phenotypic changes, resulting from the doubling of DNA content, which increases nuclear and cell size and can in turn lead to larger organs (gigas effect; Stebbins 1971, Bomblies 2020). The environment can interact with these changes, leading to niche expansion at macro- and microscales (e.g. Thompson et al. 2014, Čertner et al. 2019). On the other hand, responses of different species and sometimes even populations to WGD are not uniform (Pacey et al. 2020), an observation that has been supported by a recent meta-analysis of mixed-ploidy species' niches that documented frequent habitat shifts, yet unpredictable outcomes with respect to both the direction of niche divergence and niche expansion (Meirmans & Kolář 2025). The causes of the niche shift in *M. falcata* are likely multifaceted. During our fieldwork we observed slight morphological differences (particularly in leaf width and seed size) between the ploidy levels. Although these observations are inconclusive, broader leaves and larger flowers and fruits have been documented in tetraploid *M. sativa* compared with diploids (Small 1985), suggesting some form of gigas effect might be manifested in the *M. sativa* group. Alternatively, introgression from co-occurring *M. sativa* may further broaden the niche of *M. falcata* tetraploids, which, unlike diploids, are cross-compatible with tetraploid cultivated alfalfa

(Ledingham 1940, Lesins 1952). Although our sampling was designed to avoid hybrids that are usually well-recognisable in the field based on flower colour and fruit morphology (Kaplan et al. 2019), we acknowledge that weakly introgressed individuals might have been missed as they are difficult to distinguish based solely on morphology. Introgression from more robust and fast-growing *M. sativa* might then have promoted transfer of traits linked to greater competitive ability and stress tolerance. In addition, introgression between *M. falcata* and a tetraploid cytotype of the closely related species *M. prostrata* has been suggested (Havananda et al. 2011, Eriksson et al. 2017), further highlighting the potential of tetraploid *Medicago* to acquire novel genetic variation through interspecific introgression. The deviating genome size detected in some of the *M. falcata* individuals might actually have been caused by *M. prostrata* introgression because *M. prostrata*, unlike *M. sativa*, has a considerably smaller genome than *M. falcata*. However, because *M. falcata* samples with deviating genome sizes showed no clear geographical pattern, we do not consider the involvement of introgression to be the sole explanation of the pattern. Additionally, non-adaptive processes such as different colonization routes, historical range oscillations and/or differences in dispersal abilities might have affected distribution patterns at specific scales, possibly contributing to the observed quantitative niche variation across scales and contact zones. In sum, further genetic and phenotypic investigations are needed to determine to what extent introgression or WGD-associated phenotypic changes are responsible for the observed ecological associations. Intriguingly, an interaction between the two processes might have played a role. It has been shown that polyploidization unlocks hybridization potential between previously isolated species, and tetraploids are thus more likely to experience (adaptive) introgression (e.g. Marburger et al. 2019; reviewed in Schmickl & Yant 2021).

The lack of ecological niche divergence in one contact zone in southern Moravia provides an exception to the overall trend otherwise observed across all spatial scales. Here, both diploids and tetraploids were sampled in similar low-elevation thermophilous grassland habitats. This may partly reflect the higher frequency of ploidy sympatry in this region (frequency of mixed-ploidy populations was 11% in southern Moravia while only 5% in central Bohemia) as well as idiosyncratic effects of local colonization history. In addition, for logistical reasons our surveys avoided higher elevations along the Pannonian Basin border, especially in the adjacent areas of the Bohemian Massif in south-western Moravia and in the Moravian Carpathians, where the species is also reported (Pladias.cz; Chytrý et al. 2021). Further sampling in these areas may help determine whether the lack of ploidy differentiation in southern Moravia partly reflects sampling bias.

Conclusion

In summary, our results describe a novel case of polyploidy-associated niche shift and expansion within a plant species, supported by largely consistent patterns across spatial scales. Specifically, the tetraploid cytotype exhibited niche expansion at both broader spatial scales and a slight yet consistent niche shift to wetter and colder conditions at these scales and in one of the two contact zones. Furthermore, given the importance of *M. falcata* in alfalfa breeding, our results provide detailed information on natural ploidy

diversity across Europe that may be relevant for future fodder crop improvement programmes. The near absence of triploid individuals despite frequent spatial intermingling of cytotypes is consistent with the reported strong postzygotic isolation between ploidies in *Medicago*. However, it also suggests the presence of additional prezygotic isolation mechanisms, beyond the relatively modest niche shift, and/or the potential role of non-adaptive processes in maintaining the frequent coexistence of cytotypes.

Supplementary materials

Fig. S1. Validation scores of individual species distribution models.
Fig. S2. Differences in accompanying species composition at the local scale in contact zones.
Table S1. Locality details of the sampled *Medicago falcata* populations and vegetation sample data from the Bohemian and Moravian contact zones.

Supplementary materials are available at https://www.preslia.cz

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Expanze niky tetraploidní tolice srpovité napříč různými prostorovými škálami

Celogenomová duplikace (tzv. polyploidizace) může vést ke změnám nebo k expanzi ekologické niky organismu, velikost a směr těchto změn jsou však velmi variabilní napříč různými prostorovými škálami, a to jak napříč různými druhy, tak i v rámci jednoho druhu. Naše studie se zabývala rozšířením a rozdíly v ekologické nice ploidních cytotypů tolice srpovité (*Medicago falcata*), nejbližšího široce rozšířeného příbuzného významné pícniny tolice vojtěšky (*Medicago sativa*), který také představuje významný zdroj jejího šlechtitelského materiálu. Tolice srpovitá se v přírodě vyskytuje ve dvou cytotypech, diploidním a autotetraploidním, jejichž geografické rozšíření a ekologické nároky však byly dosud neznámé. V rámci této studie jsme provedli stratifikovaný sběr napříč třemi prostorovými škálami a analyzovali 4 919 jedinců z 443 populací vyskytujících se na gradientu zeměpisné šířky od Řecka až do Norska. Pomocí modelování environmentálních nik a mnohorozměrných analýz jsme testovali, zda je polyploidizace u tolice srpovité doprovázena posuny či rozšířením ekologické niky. Zjistili jsme, že tyto dva ploidní cytotypy vykazovaly drobné, ale signifikantní rozdíly v nice v rámci všech studovaných škál: napříč Evropou, v intenzivněji prosbírané střední Evropě i v jedné kontaktní zóně. Tetraploidy vykazovaly na dvou širších prostorových škálách poměrně mírnou expanzi ekologické niky (přibližně 8–11 %) a na obou těchto škálách i v jedné ze dvou kontaktních zón konzistentně preferovaly chladnější a vlhčí stanoviště ve vyšších nadmořských výškách. Absence signifikantní diferenciace niky v druhé kontaktní zóně ukazuje, že lokální procesy, jako jsou rozdíly ve využívání krajiny a v kolonizační historii, mohou tyto rozdíly občas zakrývat, především na lokálních škálách. V souhrnu naše výsledky ukazují, že polyploidie byla spojena s mírným, ale konzistentním posunem niky směrem k vyšším nadmořským výškám a s mírnou expanzí klimatické niky na širších prostorových škálách. Zároveň ale naše výsledky varují před vyvozováním obecných závěrů o rozdílech v nice spjatých s polyploidií napříč celým areálem druhu, obzvláště v případech, kdy jsou pozorované rozdíly malé.

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