Patterns of change in α and β taxonomic and phylogenetic diversity in the secondary succession of semi-natural grasslands in the Northern Apennines

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We studied the secondary succession in semi-natural grasslands (dry grasslands and hay meadows) located in the eastern side of the Tuscan Apennines (Tuscany, Central Italy). We compared these habitats, investigating: i) the changes in species richness, composition and phylogenetic diversity during the succession; ii) whether the trends in species loss and species turnover in taxonomic diversity matched those in phylogenetic diversity. We performed a stratified random sampling, in a full factorial design between habitat type and succession stage (60 sampled plots, 10 x 2 types of habitat x 3 stages of succession). We constructed a phylogenetic tree of the plant communities and compared the differences in taxonomic/phylogenetic α- and β-diversity between these two habitats and during their succession. We identified indicator species for each succession stage and habitat. Looking at α-diversity, both habitats displayed a decrease in species richness, with a random process of species selection in the earlier succession stages from the species regional pool. Nevertheless, in the latter stage of dry grasslands we recorded a shift towards phylogenetic overdispersion at the higher-level groups in the phylogenetic tree. In both habitats, while the richness decreased with succession stage, most species were replaced during the succession. However, the hay meadows were characterized by a higher rate of new species' ingression whereas the dry grasslands became dominated with *J. communis*. Accordingly, the two habitats showed similar features in phylogenetic β-diversity. True phylogenetic turnover resulted the main component, due to replacement of unique lineages along the succession. Nevertheless, in dry grasslands this trend is slightly higher than expected considering the major importance of difference in species richness of dry grasslands sites, and this is due to the presence of a phylogenetically very distant species (*J. communis)*.

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Abstract

We studied the secondary succession in semi-natural grasslands (dry grasslands and hay meadows) located in the eastern side of the Tuscan Apennines (Tuscany, Central Italy). We compared these habitats, investigating: i) the changes in species richness, composition and phylogenetic diversity during the succession; ii) whether the trends in species loss and species turnover in taxonomic diversity matched those in phylogenetic diversity. We performed a stratified random sampling, in a full factorial design between habitat type and succession stage (60 sampled plots, 10 x 2 types of habitat x 3 stages of succession). We constructed a phylogenetic tree of the plant communities and compared the differences in taxonomic/phylogenetic α- and β-diversity between these two habitats and during their succession. We identified indicator species for each succession stage and habitat. Looking at α-diversity, both habitats displayed a decrease in species richness, with a random process of species selection in the earlier succession stages from the species regional pool. Nevertheless, in the latter stage of dry grasslands we recorded a shift towards phylogenetic overdispersion at the higher-level groups in the phylogenetic tree. In both habitats, while the richness decreased with succession stage, most species were replaced during the succession. However, the hay meadows were characterized by a higher rate of new species' ingression whereas the dry grasslands became dominated with *J. communis*. Accordingly, the two habitats showed similar features in phylogenetic β-diversity. True phylogenetic turnover resulted the main component, due to replacement of unique lineages along the succession. Nevertheless, in dry grasslands this trend is slightly higher than expected considering the major importance of difference in species richness of dry grasslands sites, and this is due to the presence of a phylogenetically very distant species (*J. communis)*.

Introduction

Secondary semi-natural grasslands represent important components of European cultural landscapes. They derive from centuries of traditional land use, mainly linked to grazing by livestock (pastures) or hay-making (meadows) (Dengler *et al.* 2014; Janišová *et al.* 2011). Many secondary grassland vegetation types are considered habitats worthy of conservation and are listed in European and national protection directives and laws, such as the Low altitude hay meadows and the *Festuco-Brometea* dry grasslands (respectively codes 6510 and 6210, according to European Council Directive 92/43/EEC). In Europe, because of recent cultural changes, secondary grasslands and meadows have displayed an overall tendency to evolve into shrublands and woodlands through natural secondary successions (Dengler *et al.* 2014; Monteiro *et al.* 2011; Peco *et al.* 2006; Peco *et al.* 2012). Perennial grasslands undergo a vegetation dynamism leading to the gradual transition from herbaceous coenoses belonging to syntaxonomical classes such as *Festuco-Brometea* or *Molinio-Arrhenatheretea* (e.g. Allegrezza and Biondi, 2011; Biondi *et al.* 1995) to shrub coenoses belonging, especially in central Italy, mainly to the *Rhamno-Prunetea* class (e.g. Biondi *et al.* 1988). The shrubland formations are sometimes preceded by intermediate phases dominated by other herbaceous species such as *Brachypodium rupestre* (Assini *et al.* 2014) and mostly originate from the species that form the fringes of woods surrounding grasslands (Biondi *et al.* 1988; Poldini *et al.* 2002).
Such succession, with the consequent loss in species, has been widely studied from a landscape viewpoint (Bracchetti *et al.* 2012; Rocchini *et al.* 2006; Viciani *et al.* 2018). Many authors have also focused on the functionality of the communities, elucidating the central role of competition in the loss of species characterizing the early phases of succession (Csergő *et al.* 2013; Lepš 1999; Peco *et al.* 2012). Nevertheless, less is known regarding the changes occurring in the communities with respect to trends in β-diversity phylogenetic relationships, also concerning their link with changes at the taxonomic level. Indeed, according to Chase and Myers (2011), β-diversity can provide considerable insights into the importance of deterministic and stochastic processes in generating community structure along spatial and ecological gradients. In addition, the use of molecular phylogenies may be helpful in analyzing the forces that influence patterns of biodiversity and biogeography, and in depicting the interactions among co-occurring species (Selvi *et al.* 2016). Indeed, in the last decades, the use of molecular phylogeny has increased widely for ecological purposes, contributing also to the emerging area of community phylogenetics (Webb *et al.* 2002). One of the multiple ways to use the phylogenetic information consists in the measure of the phylogenetic overdispersion or clustering of the community in relation to the variation of the habitat conditions (Erickson *et al.* 2014; Qian *et al.* 2014; Selvi *et al.* 2016). Recently, many authors (Webb 2000; Webb *et al.* 2002; Kembel 2009) have highlighted that the observed patterns of phylogenetic structure of the communities could be used to understand the processes of community assembly, particularly linking patterns of phylogenetic clustering and overdispersion with the processes of habitat filtering and competitive exclusion. Indeed, the use of phylogenetic patterns as proxies for the processes of community assembly is rapidly raising concerns linked to the assumptions underlying such approach, often

only weakly supported. In particular, these assumptions regard the existence of an actual correlation between measures of phylogenetic dispersion and trait dispersion, the idea that trait similarity would enhance competition and that competition necessarily causes species exclusion from the community, and that community assemblages are in status of equilibrium (see Gerhold *et al.* 2015; Prinzing 2016). Furthermore, Kraft *et al.* (2015) reported a misuse of the concept of environmental filtering, considering that most empirical studies hardly distinguish the effects of abiotic factors from those of biotic interactions, and often overestimate the role of the environment in shaping communities.

In our study area, the Tuscan Apennines, the co-occurrence of initial species in secondary grasslands is driven by different agricultural management in different geo-morphological conditions. The areas with low slope inclination and relatively fertile and deep soils are at first subjected to machining and sowing, mainly with plants that increase the nutrient value in the soil (i.e. *Medicago sativa, Onobrychis viciifolia*). Then, herbaceous natural grassland species (many from the families *Poaceae, Fabaceae* and *Asteraceae*) start to colonize these communities, which in a few years become semi-natural hay meadows (Ubaldi 2003). On the other hand, areas on steeper slopes with shallow (sometime even rocky) soils are used as pastures and become dry grasslands. Also in our area of study, the main characteristic differentiating the two habitat types is the stand geomorphology, with significantly steep dry grasslands and almost flat hay meadows: these differences may affect the water capacity, structure and fertility of soils.

We expect that these ecological differences may also affect the secondary succession, leading to different species assemblages. Indeed, as anthropic pressures are relieved, the succession of these habitats is generally characterized by intermediate phases dominated by herbaceous species, leading finally to quite different shrub formations. In particular, while shrubland following hay meadows are dominated by several species of broadleaf shrubs, dry grasslands are dominated by *Juniperus communis*, a distantly related stress-tolerant species (Pierce *et al.* 2017).

The study of taxonomic, phylogenetic and functional plant (α and β) diversity along secondary succession has already been the object of studies (see Purscke *et al.* 2018), which highlighted that the relative importance of assembly processes had changed over time, but with contrasting patterns of temporal change in the different facets of diversity. Nevertheless, while Purscke *et al.* (2013) observed a general increase in taxonomic, phylogenetic and functional alpha-diversity during succession, we expect a decrease in species taxonomic diversity, not necessarily linked to a decrease in phylogenetic diversity. Moreover, Purscke *et al.* (2013) reported a predominant role of abiotic filtering in community assembly during the early stages of grassland succession, whereas the relative importance of competitive exclusion appears to have increased towards the later succession stage. Conversely, a major role of competition by dominant tall grasses as already been described for the early stages of succession in Apennine grasslands communities (Corazza *et al.,* 2016). According to these differences, we sought to i) assess the relative changes in α- and β-diversity taxonomic and phylogenetic diversity, also identifying the indicator species in the different stages of the succession and ii) assess whether the trends in species loss and species turnover in taxonomic diversity matches those in phylogenetic diversity. Moreover, we

121 further hypothesize that *Juniperus communis* may have a pivotal role, driving a certain degree of
122 overdispersion of the phylogenetic structure of the communities in dry grasslands, considering its
123 distant relatedness.
124 Toward these aims, we conducted a sampling of dry grasslands and hay meadows plant
125 communities in the Tuscan Apennines, adopting a chrono-sequential approach to reconstruct
126 their dynamic changes, assessing the changes in of α- and β-diversity within and between three
127 succession stages of these two habitats.
128

129 ## Materials & Methods

130

131 **Study area**
132 The study area is located in the Adriatic side of the south-eastern Tuscan Apennines (Tuscany,
133 Central Italy, 43.691838°N 12.111936°E). The area is managed by the Raggruppamento
134 Carabinieri Biodiversità of the Reparto Carabinieri Biodiversità Pieve Santo Stefano. The most
135 represented geological units are the clays of the Sillano Formation, deposits of landslide debris
136 and, to a lesser extent, the sandstones of Falterona Mount (Carmignani *et al.* 2013). Climate
137 belongs to the Temperate Oceanic Bioclimate (Pesaresi *et al.* 2017).
138

139 **Sampling design**
140 The study was carried out exploiting a random/stratified sampling design involving two layers:
141 type of habitat (hereafter Habitat) and stage of succession (hereafter Succession). To identify and
142 map these layers correctly, we performed an analysis of orthophotos of the study area using the
143 QGis software (version 2.14.21, QGIS.ORG project). We used the cover percentage of scrub to
144 distinguish: i) dry grasslands and hay meadows (shrub presence not detectable), ii) mixed
145 typologies (dry grasslands/hay meadows with shrub cover < 50%) and iii) shrubland (shrub
146 cover > 50%). Using also the historical knowledge concerning the landscape management
147 provided by the local administrator, we defined the sampling layers as follow: 1) Habitat
148 (corresponding to the land use) distinguishing between dry grasslands and hay meadows and 2)
149 Succession, distinguishing between i) active (hereafter Managed) ii) short-time abandoned
150 (hereafter Transition) and iii) long-time abandoned areas (hereafter Abandoned). The stage of
151 succession is used as a proxy for the time from abandonment of the typical land management
152 practices, considering that an increase of the cover of shrubs corresponds to the increase of time
153 following abandonment. Hence, we performed a random selection of 10 square plots of 2x2
154 meters for each stratum in a full factorial design, leading to 60 sampled plots (10 x 2 types of
155 habitat x 3 stages of succession). In each plot, we performed a floristic sampling, recording the
156 presence/absence of vascular plants. Voucher specimens were collected for identification in the
157 laboratory, and further samples were collected for the genetic analyses.
158

159 **Selection of molecular markers, DNA isolation, sequence alignment and tree reconstruction**

Phylogenetic diversity of the spermatophyte communities was inferred from the analysis of three markers of the nuclear ribosomal DNA, the ITS1-5.8S and ITS2 regions. These markers, widely used for phylogenetic studies both in plants and fungi, have shown a great discriminatory power at low taxonomic levels (Feliner and Rosselló 2007; Hollingsworth *et al.* 2011), supporting this region as a core barcode for spermatophytes (Li *et al.* 2011) and hence potentially usable as a proxy for evolutionary relationships. We assembled a sequence dataset by retrieving accessions of the sampled species from GenBank (http://www.ncbi.nlm.nih.gov/) to construct a tree resolved at the species level. Molecular analyses were performed for 13 species for which no accessions were available in the GenBank. Isolation of genomic DNA followed a modified 2×CTAB protocol successfully adopted in previous studies using molecular tools (see Coppi *et al.* 2008 and Appendix S1 for further details).

The final dataset of the sampled species consisted of 147 accessions (Table S2). Taxa from Gnetales, Ginkgoales, Cycadales, Pinales and Cupressales were added in order to obtain a correct alignment for Gymnosperms, whereas four taxa from Polypodiales and Salviniales were added as outgroups (Table S1). Multiple alignment of the ITS-5.8S dataset was performed with MAFFT (v. 7.0, Katoh and Standley 2013) adopting the parameterization typically used for nucleotide sequences (200PAM/k=2, gap penalty=1.53; offset=0.0), considering that we aligned very distant species. We followed a step by step multi-alignment procedure: (1) taxa were grouped at the order level and aligned using the Q-INS-i strategy, checking each multi-alignment by visual inspection with BioEdit; (2) the multi-alignments were merged at higher ranks using the *merge* option in MAFFT, obtaining separate multi-alignments for Eudicots, Monocots, Gymnosperms and for the outgroup; (3) these four multi-alignments were finally merged again. The alignment was used to build a phylogenetic tree with a maximum likelihood (ML) approach by means of RAxML (Stamatakis, 2006) via the CIPRES supercomputer cluster (http://www.phylo.org/), using 1000 maximum searches. The topology of phylogenetic inference was constrained at the family level using as backbone the tree *slik2015* (Slik *et al.* 2018) available in Phylomatic vers. 3 (http://phylodiversity.net/phylomatic/). This topology is mainly based on the APG III phylogeny, further resolved up to genus level using the species-level phylogeny in Zanne *et al.* (2014), placing at the base of their respective families genera not present in Zanne *et al.* (2014), see Slik *et al.* (2018). The resulting topology is highly consistent with the hypothesis in Magallón *et al.* (2015), which was subsequently used to date the phylogeny, except for the clade involving the *Malpighiales* and the one involving *Boraginales*, *Solanales*, and *Gentianales*. For these clades we followed the tree *slik2015*. The statistical support to the nodes was estimated using the bootstrap method (1000 iterations). Finally, to obtain an ultrametric tree, we calibrated our phylogeny dating the node ages according to Magallón *et al.* (2015), adopting a Molecular Dating approach throughout Penalised Likelihood estimation via the *chronos* function of ape v5.1 R package (Paradis *et al.* 2004).

Evaluation of trends in species richness and phylogenetic relatedness (α-diversity)

We evaluated the changes in taxonomic species richness (SR) and in three indices allowing the assessment of different features of phylogenetic α-diversity. We used the Phylogenetic Diversity (PD) as a measure of the amount of phylogenetic richness in the communities (how much) and the Net Relatedness Index (NRI) and the Nearest Taxon Index (NTI) to provide information regarding the phylogenetic divergence within the communities (how different they are) (see Tucker *et al.* 2017).

Faith's PD (Faith 1992) represents the simplest measure of the cumulative evolutionary age in a community, but it is highly correlated to species richness. Thus, we adopted its Standardized Effect Size index that is generally considered unaffected by species richness (Pavoine *et al.* 2013; Swenson 2014; but see Sandel 2018), and indicates whether the observed PD is different from what would be expected by chance. To allow an interpretation of results comparable with NRI and NTI, we multiplied PD.ses by -1. We defined NTI and NRI as Standardized Effect Size indices of Mean Nearest Taxon Distance (MNTD) for taxa in a community and Mean Pairwise Distance separating taxa in a community (MPD). MNTD is calculated as the mean of the smallest non-diagonal value in the pairwise distance matrix for each species and is a measure of the branch-tip phylogenetic clustering of the species in the community (Webb *et al.* 2002). It describes the phylogenetic relatedness among species, focusing on the distal part of the tree, thus involving lower taxonomical levels. MPD estimates the average phylogenetic relatedness between all possible pairs of taxa in an assemblage, calculated as the mean of the non-diagonal elements in the pairwise distance matrix (Webb 2000). It is a measure of the relationship at the higher-level groups in the phylogenetic tree. NTI and NRI indicate whether the values of MPD and MNTD differ from what would be expected by chance. Positive values of NRI and NTI indicate that observed phylogenetic distances are lower than expected and that phylogenetic clustering of species occurs. Conversely, negative values of such indices indicate phylogenetic over-dispersion or evenness. In general terms, NTI and NRI values higher/lower than 1.96/-1.96 are usually considered indicators of significant patterns. All standardized indices (PD.ses, NRI and NTI). All standardized effect sized indices (PD.ses, NRI and NTI) were calculated using a comparison with *fixed-fixed* null models, which maintain both species richness and species abundance across sites and tend to exhibit low type I and II error rates (see Miller et al., 2017). The null model matrices were randomized using the "independent-swap" algorithm by Gotelli (2000), which is well suited for presence/absence community matrices.

We studied the variation SR and PD.ses, NTI and NRI in a two-way ANOVA design considering Habitat and Succession as explanatory variables, also taking into account their interaction effect. We further explored the differences in the levels of the significant terms performing a Post-Hoc Tukey Test.

To assess the role of *Junipers communis* in the phylogenetic α-diversity patterns found, we repeated the analyses on PD.ses, NTI and NRI excluding this species, and keeping the same design described above.

Trends of compositional shifts (β-diversity)

To assess the variations in species composition of plots, we ran a comparison among a detrended correspondence analysis (DCA) and a canonical correspondences analysis (CCA) on the plot species composition. Thus, we checked the efficiency of constrained axis to catch the variation explained by unconstrained axis, as provided in the software Canoco 5 (ter Braak and Šmilauer 2012) and following Šmilauer and Lepš (2014). In DCA the axes were detrended by segment, adopting default options in Canoco 5 In the CCA, Habitat and Succession were used as explanatory variables and the significance of the constrained axes was tested with 4999 unrestricted permutations.

We used the methodological framework developed by Podani and Schmera (2011) to evaluate the trends in β-diversity components during the succession. This methodology allows the partition of pairwise gamma diversity into three complementary indices, measuring Similarity, relative Richness Difference and relative Species Replacement (respectively S, D and R), and accordingly is referred to as SDRSimplex approach (see also Appendix S2 for further description). Pairwise-comparisons regarded plots of the same habitat, spanning along the succession. The SDRSimplex results were projected in a ternary plot. Finally, we used a Nonparametric Kruskal–Wallis test to check the significance of the differences among habitats.

To assess the role of particular species in the species turnover, we carried out an Indicator Species Analysis (ISA, Dufrêne and Legendre 1997). The ISA allows computing an indicator value *d* (ranging between 0 and 100) of each species as the product of the relative frequency and relative average abundance of species in clusters. The analysis also produces a significance value, representing the probability of obtaining a *d* value as high as that observed over 1000 iterations. We conducted the analyses considering each stage of succession of the two habitats as a separate cluster.

We used a three dimension Non-Metric Multidimensional Scaling (NMDS) ordination based on the UniFrac index distance matrix, to assess how different were the communities from the phylogenetic point of view. UniFrac is a phylogenetic diversity-based dissimilarity index that measures the proportion of evolutionary history unique to each community and is calculated as the total branch length unique to each community relative to the total branch length linking all species in both communities (Lozupone and Knight 2005).

Furthermore, we studied the evolutionary dissimilarity between communities along the succession (phylogenetic β-diversity), adopting the approach described in Leprieur *et al.* (2012) as an improvement of PhyloSor index. This index expresses to what extent the compared communities are composed by related species rather than by species that share no branch in the phylogeny and can be separated in two components accounting for 'true' phylogenetic turnover ($PhyloSor_{Turn}$) and phylogenetic diversity gradients ($PhyloSor_{PD}$). In addition, we also analyzed the standardized effect size of such indices (i.e. SES.PhyloSor, $SES.PhyloSor_{Turn}$ and $SES.PhyloSor_{PD}$), which describe whether two communities are phylogenetically more or less dissimilar than what is expected given their taxa dissimilarity. These indices are obtained via comparison with a null model in which species are randomized across the tips from the tree while holding constant species richness and compositional beta diversity in 999 simulations.

Again, we used a Nonparametric Kruskal–Wallis test to check the significance of the differences among the two habitat types.
Furthermore, to assess the role of *Junipers communis* in the phylogenetic β-diversity patterns found, we repeated the analyses excluding this species, and keeping the same design described above.
All ordination analyses (DCA, CCA and NMDS) and relative graphs were made using the software Canoco 5 vers. 5.12 (ter Braak and Šmilauer 2012). All other analyses were made using R software (version 3.5, The R Foundation for Statistical Computing, Vienna, Austria, www.R-project.org), and relative graphs were produced with ggplot2 vers. 2.2.1 (Wickham 2009). The ISA was conducted using the package labdsv (R package version 1.8-0, https://CRAN.R-project.org/package=labdsv). Phylogenetic β-diversity indices were calculated exploiting the R functions developed by Leprieur *et al.* (2012). Phylogenetic α-diversity metrics were obtained with the package metricTester vers. 1.3.6 (Miller et al., 2017).

Results

The sampling resulted in 147 species (Table S2), with 69 shared species among the two habitats and 39 species exclusive to each habitat (tot. 108 species in both habitats). Species richness varied from 5 to 29 species per plot, in Abandoned dry grasslands and Managed dry grasslands, respectively. The resulting phylogenetic tree is shown in Figure S1 (see Appendix S3 for the tree in Newick format).
Species richness was comparable between the two habitats and decreased in both during the succession (Table 1, Fig. 1A). Conversely, PD.ses differed significantly among the habitats, with lower values in dry grasslands, and during the succession, with a decrease in the latter stage (Table 1, Fig. 1B). Regarding NRI, the two habitats showed different trends during the succession (significant interaction Succession x Habitat, Table 1). Indeed, while they displayed comparable values at earlier succession stages with values indicating random processes of species selection, dry grasslands shifted in the latter stage to a significant overdispersion of plant composition, with the mean NRI value below the critical threshold of -1.96 and significantly different from the one of hay meadows (Fig. 1C). On the other hand, the two habitats displayed a comparable NTI trend during the succession, with values in hay meadows generally higher (Succession and Habitat both significant, but no significant interaction, Table 1). Notwithstanding a significant drop in the latter stages of succession, in all three stages the mean NTI values remained between +/- 1.96, again indicating random processes of species selection (Fig. 1D).
The analyses concerning phylogenetic α-diversity conduced excluding *J. communis* showed quite a different scenario. PD.ses varied significantly between the two habitats and showed different trends during the succession (Interaction term Succession x Habitat significant, see Table 2), while both NTI and NRI varied significantly only along the succession with comparable trends between the two habitats (Table 2). Indeed, we recorded a steep increase in

PD.ses in abandoned dry grasslands compared to the values obtained considering *J. communis*. As a result, PD.ses in dry grassland is more or less stable along the succession, while it varies significantly in hay meadows (Fig 2A). On the contrary, phylogenetic overdispersion in dry grasslands clearly vanished, with both NTI and NRI higher than those *J. communis* and even closer to 0 than those of hay meadows, thus with a net predominance of random processes of species selection (Fig 2 C, D).

The species composition of two habitats resulted clearly separated along the succession. CCA constrained axes showed a very good efficiency, catching 85.5% and 100% of variation explained by DCA unconstrained axis. Total variation is 4.87, DCA first two axes explained 10.9% and 6.9% of it, while CCA ones 9.3% and 6.9% ($P<0.001$). Plots resulted clearly differentiated in terms of species composition with a clear set of taxa specific to each habitat and each stage, with changes during the succession lying on the horizontal axis and differences among the two habitats on the vertical one (see Fig 3A and B). According to the ordinations, the two habitats show common trends of species replacement, with new species coming in the transition stage and a further differentiation in the last stage.

The pairwise comparisons across the two habitats highlighted significant differences in the trends in species turnover among the succession. Indeed, they shared consistently low values of similarity (S) but were characterized by significantly different values of species replacement (R) and richness difference (D). In particular, dry grasslands displayed a higher D and a lower R than hay meadows (Fig. 4).

The ISA confirmed the presence of different trends in the numbers of species characterizing the succession stages and leading the succession. In the managed stage, the two habitats shared the same number of indicator species, but hay meadows presented a higher number of indicators species than dry grasslands in the following stages (17, 5, and 9 species in hay meadows vs. 17, 3 and 1 in dry grasslands in the Managed, Transitional and Abandoned stages, respectively, see table S3).

The NMDS ordination on phylogenetic distance among plot allowed a good representation of the UniFrac distances (Stress criterion = 0.141). As for DCA and CCA, in the NMDS first axis reported the changes during the succession, while the habitat are separated along the second axis. Here, particularly the transitional stage of hay meadows show a high similarity with managed and transition stages of dry grasslands, while again the two habitats showed a relevant differentiation in the latter stage (Fig. 5).

Phylogenetic β-diversity was comparable among the two habitats and in both communities was mainly composed by phylogenetic turnover (Fig 6A). Only PhyloSorPD was significantly different between the two habitats, being higher in dry grasslands than in hay meadows. None of the phylogenetic β-diversity standardized metrics differed significantly from what is expected given the taxa dissimilarity (all values between +/- 1.96, see fig 6C). Nevertheless, both SES.PhyloSor and the component SES.PhyloSorPD resulted significantly lower in dry grasslands than in hay meadows. The analyses conducted excluding *J. communis* from dry grasslands resulted in higher values of $PhyloSor_{PD}$ and lower ones of PhyloSorPD, both differing

significantly between the two habitats (fig 6B). SES.PhyloSor$_{Turn}$ also showed significantly lower values in dry grasslands (fig 6D).

Discussion

The two habitats showed different features characterizing the changes in species composition during the succession, even if they displayed a comparable tendency in species richness loss. The trend in species loss has a long history of detection in the succession following the abandonment of secondary grasslands (Corazza *et al.* 2016; Csergő *et al.* 2013; Dengler *et al.* 2014; Janišová *et al.* 2011; Rocchini *et al.* 2006). Concerning Apennine hay meadows, Ubaldi (2003) reported that when agricultural activities are abandoned, soil water capacity and structure decreases. Consequently, also hay production decreases and these areas are used as pastures. Meanwhile, trampling and grazing further reduce water capacity and fertility of soils, so that they become dry grasslands, more or less xerophilous depending on the site. However, this general trend is not consistent with our findings, which highlighted a clear divergence of the two habitats during the succession.

The values of PD.ses showed that whether in managed and transition stages the PD is substantially consistent to what should be expected given the taxa richness, in the abandoned ones PD values were higher than expected. This indicate that whether SR decreased, PD do not decreased consistently, because the loss in species seems to be counterweighted by the presence of species with long branches in the phylogeny (and this is particularly true for dry grasslands). Regarding phylogenetic structure, we detected for the first succession stages a predominant role of random processes of species loss in both habitats. These trends may be consistent with a framework in which a random phylogenetic structure of the community is the result of competitive exclusion of species in the case of convergent traits (see Davies 2006). Nevertheless, we detected a net difference of the community structure concerning the deep nodes of phylogeny, linked to the overdispersion in the dry grasslands. This is the result of the reduction of the number of species and the appearance (as a dominant participant) of the stress-tolerant species *J. communis* (Pierce *et al.* 2017). This is in strong agreement with our hypothesis that these environments are dominated by a strong component of ecological stress. Accordingly, species assembling processes may have selected for traits allowing to survive in xeric environments (i.e. traits linked to conservative economics in the leaf economics spectrum, such as small and thick leaves, low growth rate, small specific leaf area and high leaf dry matter content), that in this case were shared between species distant in the phylogeny.

The analysis of β-diversity trends confirmed these differences. In both habitats, we evidenced a strong loss in species, but with a high component of richness difference in dry grasslands, indicating a smaller replacement by new species. These findings match with those of several authors, who showed how short species are outcompeted by dominant tall grasses in the first succession stages after the abandonment (Corazza *et al.* 2016), leading, in agreement with Grime (2001), to the exclusion of subordinate and accidental species. Furthermore, in the latter

succession stages many more species are lost from the community with the dominance of *J. communis*. Also in hay meadows, the loss in species following the abandonment was characterized by a suppression of species, but with a major turnover of species. Accordingly, Csergő *et al.* (2013) demonstrated that the loss in species following the meadows abandonment may be driven by the suppression of dominant grasses by tall forbs, in meadows co-dominated by competitive stress-tolerant ruderals, whereas in meadows dominated by a single stress-tolerant competitor, diversity loss resulted from increased abundance and biomass of the dominant grass.

These trends were confirmed by ISA results, which are consistent with the scenario outlined in the DCA and CCA scatterplots. The higher rate of richness difference, and the lower species replacement of dry grasslands, in particular emerged from the low number of species characterizing transition and abandoned stages. Indeed, in the managed stage, indicator species resulted numerically comparable among the two habitats. In managed hay meadows, the indicator species belong mainly to the families *Poaceae* and *Fabaceae* (*Lolium perenne, Phleum bertolonii, Bromus hordeaceus, Cynosurus cristatus, Trifolium pratense, Vicia sativa* and *Lathyrus pratensis*), strictly linked to the pastoral activities, or consisted of other mesotrophic plants favored by grazing (i.e. *Ranunculus bulbosus*). On the other hand, the indicator species of managed dry grasslands were more typical of shallow and rocky soils, such as *Thymus longicaulis, Acinos alpinus, Trifolium scabrum, Bupleurum baldense* and *Cerastium brachypetalum*. In the Transition, hay meadows were characterized by a higher number of indicator species, with species typical of open habitats (*Centaurea nigrescens, Achillea collina, Poa trivialis, Dactylis glomerata, Cirsium tenoreanum*). These features are consistent with the scenario described above following Csergő *et al.* (2013). Transition dry grasslands were characterized by the dominance of *Brachypodium rupestre*, (together with plants considered precursory of more closed and woody habitats, see e.g. Assini *et al.* 2014). The dominance of *Brachypodium* spp. in successions post-abandonment is a general trend widely demonstrated for Apennine grasslands (Corazza *et al.* 2016). Finally, *J. communis* resulted the sole indicator species of the abandoned dry grasslands, while abandoned hay meadows were characterized by a high number of indicator species, spanning from various woody species of several families (i.e. *Rosaceae, Salicaceae, Aceraceae, Oleaceae*) to some herbaceous plants (for example *Ranunculus lanuginosus* and *Valeriana officinalis*).

The analysis of phylogenetic distances among plots highlighted that while the two habitats were well differentiated in their managed stages (in term of species lineages), a certain degree of phylogenetic similarity could be observed between transition hay meadows and both managed and transition dry grassland, due to the replacement of lineages along the succession. Nevertheless, as the succession proceeded further, the species composition of abandoned grasslands led to a net differentiation of the two habitats. Indeed, the two habitats hosted some species in common (or at least some species sharing common lineages) in the transitional stage (as also highlighted by the CCA). Subsequently they differentiated again in the last stage, with a

higher replacement in hay meadows and dry grassland more or less dominated by a specie (*J. communis*) not present in hay meadows and phylogenetically distant from all other species.
As to phylogenetic β-diversity, both habitats showed a greater contribution of 'true' phylogenetic turnover (reflected in a minor importance of phylogenetic gradient). These results indicated that the difference among plots was due to the replacement of species coming from different lineages, rather than from a simple difference in PD. Nevertheless, in both the cases of the simple and the standardized indices, we recorded differences concerning the amount of beta diversity deriving simply from a difference in PD. This component is numerically higher in dry grasslands (referring to simple Phylosor.PD) but is lower than what could be expected given the taxa dissimilarities when looking at the SES.Philosor$_{PD}$. We can hypothesize that this dependence may be related to the presence in dry grasslands of lower species replacement, with the entrance of a species phylogenetically very distant from the others (*J. communis*), which balances out the importance of simple PD component.
The pivotal role of *J. communis* in dry grasslands emerged on re-running all analyses concerning phylogenetic α- and β-diversity excluding this species. This species resulted responsible for a high amount of PD.ses in dry grasslands, and also the main one responsible for the presence of patterns of overdispersion. Also looking at -diversity, once the balancing effect exerted by *J. communis* had been removed, the PhyloSor$_{PD}$ resulted even higher. In addition, the SES.PhiloSor component rose significantly, showing that the amount of SES.PhiloSor$_{PD}$ was substantially consistent to what could be expected given the taxa dissimilarities and is higher than the one observed in hay meadows. In addition, differences among the two habitats in both PhyloSor$_{Turn}$ and SES.PhyloSor$_{Turn}$ became significant when excluding *J. communis*, indicating firstly a lower replacement of lineages in dry grasslands, balanced by the bigger contribution of PhyloSor$_{PD}$ and secondly that replacement in dry grasslands was driven by species sharing a closer lineage than those in hay meadows. These results highlighted the important role of *J. communis* and pinpointed the importance of including this species in the analyses, also considering that this is a key species in late succession stages of dry grasslands, being the dominant one, but also that its presence may hide phylogenetic differences between habitats driven by other clades. It is also noteworthy that, whereas it is common to remove highly phylogenetic distinct species from phylogenetic analysis, this practice may lead to a partial understanding of the processes at work, and that an in-depth interpretation of phylogenetic patterns should be made both using or not this distantly related species.
In this study, we used presence/absence data, and it should be acknowledged that abundance data may have led to significantly divergent results. Even if in our case, one of the main species responsible for the recorded patterns was a very abundant and dominant one. Nevertheless, further studies including abundance data are necessary to better depict the processes at work.

Conclusions

In conclusion, our data elucidate the differences in the secondary succession of dry grasslands and hay meadows in the Tuscan Apennines. In both cases, we recorded a drop in taxonomic α-diversity during the succession, but the analyses of taxonomic β -diversity highlighted quite different compositional changes, with dry grasslands mainly dominated by richness difference and hay meadows characterized by higher species replacement. As regards the phylogenetic patterns, we were able to verify that they followed a comparable trend in the earlier succession stages of the two habitats, but the entrance of a single species characterized by a deep separation in the phylogeny of the communities (i.e. *J. communis*) raises substantial differences. We propose an important role of the ecological factors in these trends, with the selection of *J. communis* fostered by a dominance of abiotic filters and resulting in the outcompeting of subordinate and accidental species in the latter stage of the habitat succession in dry grasslands, after an initial dominance of competitive exclusion of the species. Nevertheless, as noted in the introduction, the assessment of links among phylogenetic patterns and ecological processes needs more in-depth study. Our proposed scenario could be appropriate in the case of traits not conserved in the phylogeny (and this could be in accordance with the idea that trait conservativism should not be taken for granted, see Gerhold *et al.* 2015), but a certain evaluation of plant traits is necessary to further explore such trends, especially considering the concerns related to the use of phylogeny as proxies for community assembly mechanisms.

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Table 1(on next page)

Analysis of variance table for the effect of Habitat and Succession on indexes of taxonomic and phylogenetic α-diversity.

Species Richness (SR), Standardized Effect Size of Faith's Phylogenetic Diversity (PD.ses), Net Relatedness Index (NRI) and Nearest Taxon Index (NTI). Significance codes: P value < 0.001 "***"; P value < 0.01 "**"; P value < 0.05 "*")

Table 1.

Response	Factors	Df	ResDf	Sum Sq	F Value	P value	
SR	Habitat	1	54	22.82	2.067	0.156	
	Succession	2	54	1930.23	87.458	<0.001	***
	Habitat: Succession	2	54	54.03	2.448	0.096	
PD.ses	Habitat	1	54	9.49	8.34	0.006	**
	Succession	2	54	55.60	24.43	<0.001	***
	Habitat: Succession	2	54	1.87	0.83	0.440	
NRI	Habitat	1	54	21.33	17.77	<0.001	***
	Succession	2	54	50.27	21.33	<0.001	***
	Habitat: Succession	2	54	17.08	7.11	0.002	**
NTI	Habitat	1	54	3.16	2.92	0.093	
	Succession	2	54	48.82	21.19	<0.001	***
	Habitat: Succession	2	54	1.29	0.64	0.554	

Table 2(on next page)

Analysis of variance table for the effect of Habitat and Succession on indexes of phylogenetic α-diversity evaluated excluding from the analyses the species *Juniperus communis.*

Standardized Effect Size of Faith's Phylogenetic Diversity (PD.ses), Net Relatedness Index (NRI) and Nearest Taxon Index (NTI). Significance codes: P value < 0.001 "***"; P value < 0.01 "**"; P value < 0.05 "*".

1 **Table 2.**

2

Response	Factors	Df	ResDf	Sum Sq	F Value	P value	
PD.ses	Habitat	1	54	0.50	0.58	0.451	
	Succession	2	54	28.29	16.30	<0.001	***
	Habitat: Succession	2	54	11.03	6.36	0.003	**
NRI	Habitat	1	54	0.39	2.05	0.158	
	Succession	2	54	7.33	19.03	<0.001	***
	Habitat: Succession	2	54	1.00	2.61	0.083	
NTI	Habitat	1	54	0.01	0.01	0.920	
	Succession	2	54	25.69	17.09	<0.001	***
	Habitat: Succession	2	54	4.18	2.78	0.071	

3

Figure 1

Interaction plots for the variation in taxonomic and phylogenetic α-diversity of the 60 sampled plots according to Habitat and Succession.

(A) Species Richness (SR). (B) Standardized Effect Size of Faith's Phylogenetic Diversity (PD.ses). (C) Net Relatedness Index (NRI). (D) Nearest Taxon Index (NTI) Different letters indicate significant differences ($p<0.05$) after a Post Hoc Tukey's test conducted in A, B and D) between the levels of the factor Succession, and in C) between the levels of the interaction Succession*Habitat, according to the ANOVA results.

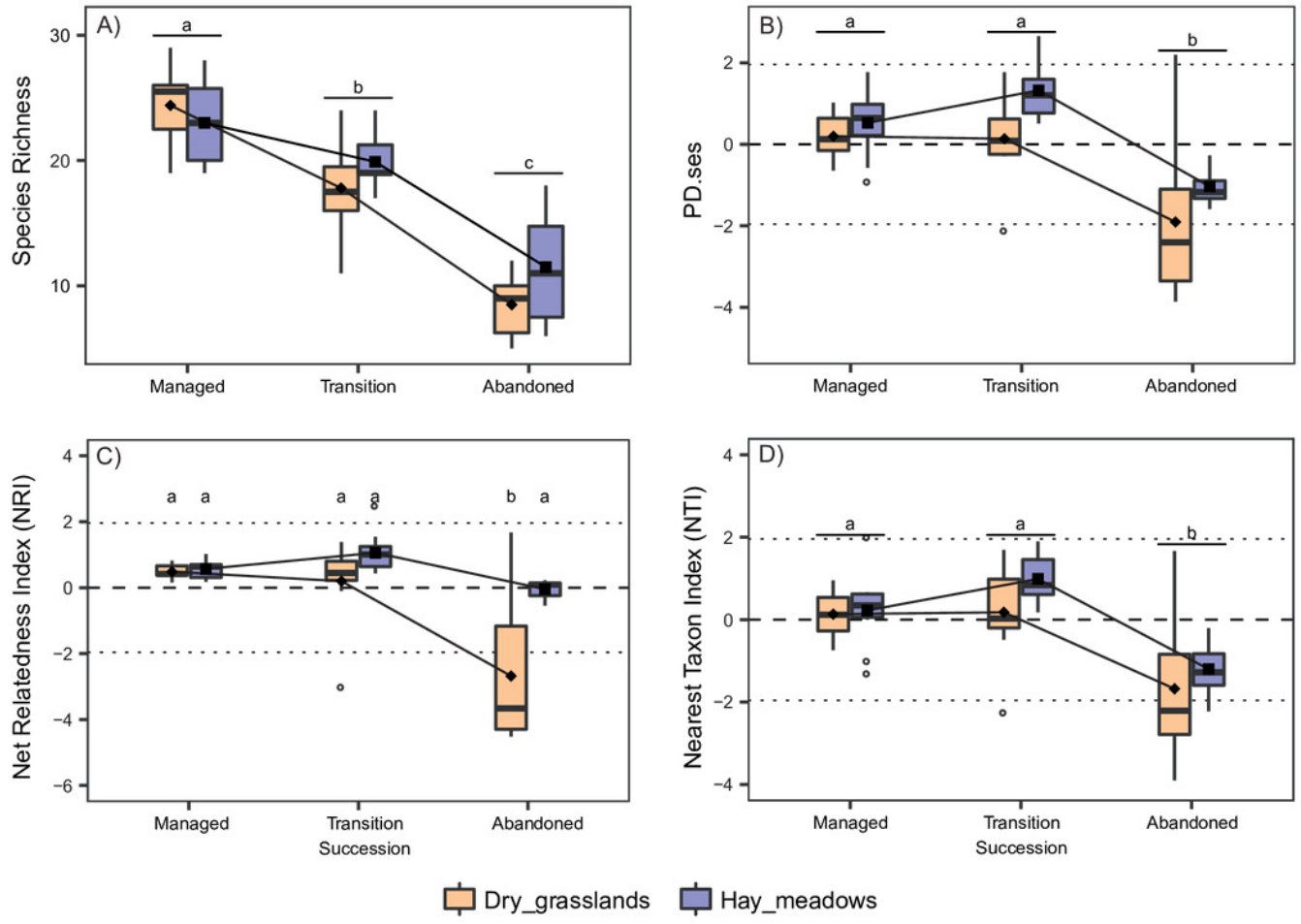


Figure 2

Interaction plots for the variation in phylogenetic α-diversity of the 60 sampled plots according to Habitat and Succession evaluated excluding from the analyses the species *Juniperus communis*.

(A) Standardized Effect Size of Faith's Phylogenetic Diversity (PD.ses). (B) Net Relatedness Index (NRI). (C) Nearest Taxon Index (NTI). Different letters indicate significant differences ($p<0.05$) after a Post Hoc Tukey's test conducted in A) between the levels of the interaction Succession*Habitat, and in B and C) between the levels of the factor Succession, according to the ANOVA results.

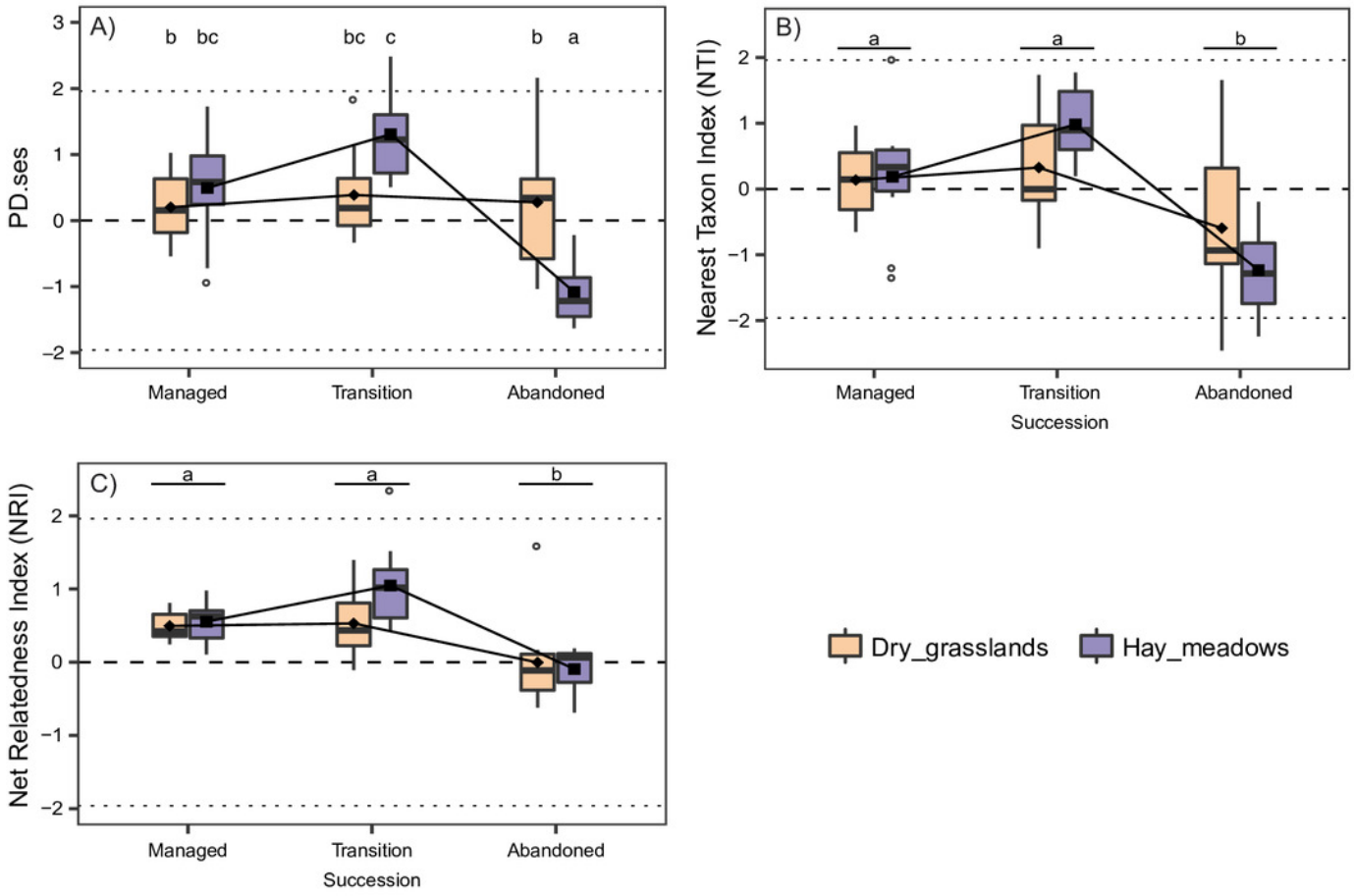
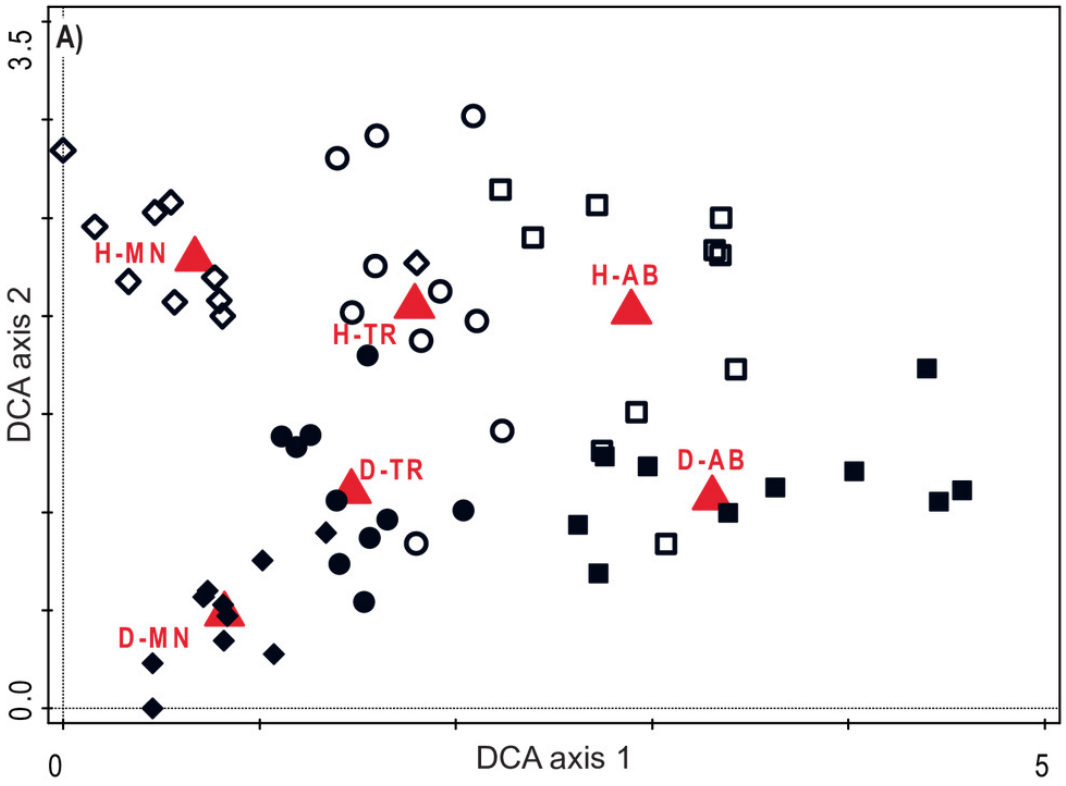


Figure 3

Ordination diagrams considering species composition of plots.

A) Plot distribution according to DCA considering their species composition. Empty symbols represent hay meadows and full symbols represent dry grasslands. Rhombus represent managed plots, circles transition plots and squares abandoned ones. B) Plant species distribution obtained with CCA, only 50 best fitting species are shown, see also table S3 for indicator species. Blue triangles represent the species. In both graphs, red triangles represent plot centroids according to Habitat type and Stage of Succession. H-MN= managed hay meadows, H-TR= transition hay meadows, H-AB= abandoned hay meadows, D-MN= managed dry grasslands D-TR= transition dry grasslands D-AB= abandoned dry grasslands.


A)
H-MN
H-AB
H-TR
D-TR
D-AB
D-MN
DCA axis 2
DCA axis 1
3.5
0.0
0
5

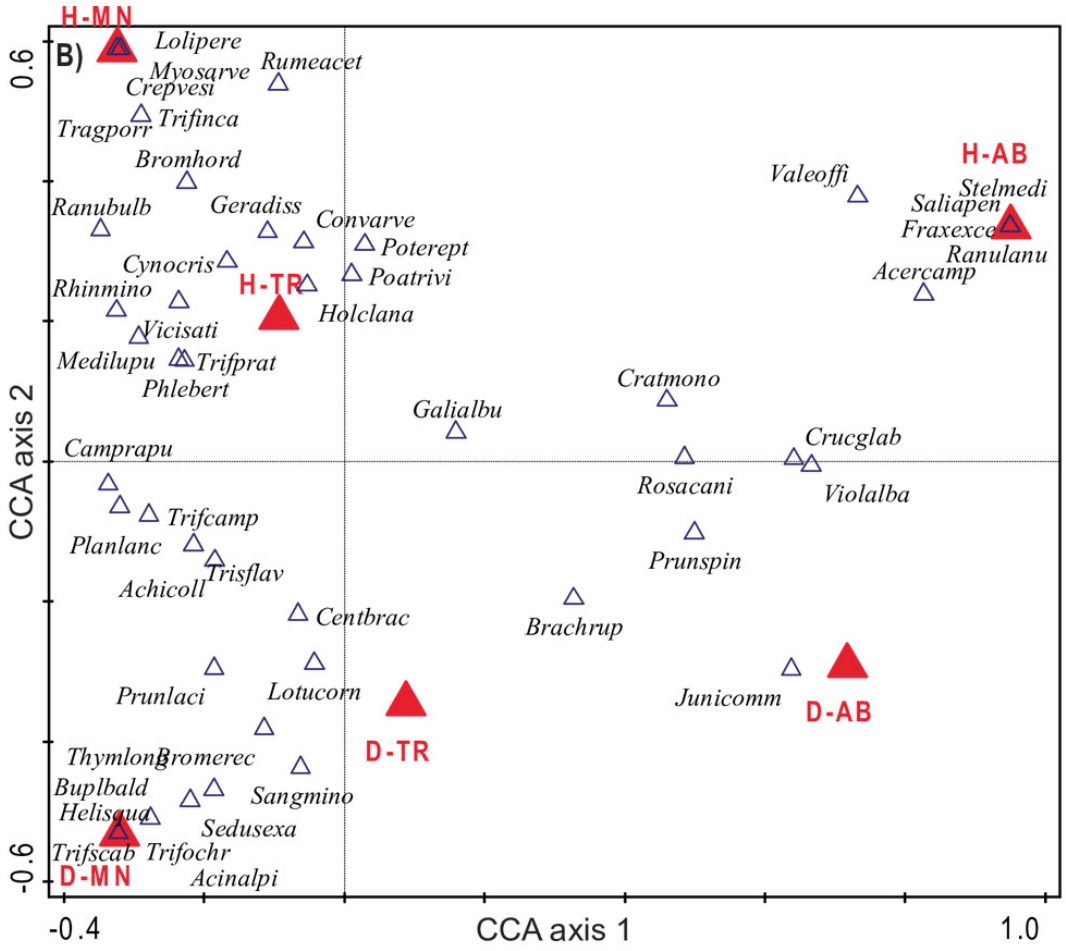

B)
H-MN
Lolipere
Myosarve
Rumeacet
Crepvesi
Tragporr
Trifinca
Bromhord
Ranubulb
Geradiss
Convarve
Poterept
Cynocris
Poatrivi
Rhinmino
H-TR
Holclana
Vicisati
Medilupu
Trifprat
Phlebert
Camprapu
Galialbu
Cratmono
Crucglab
Rosacani
Violalba
Trifcamp
Planlanc
Trisflav
Achicoll
Prunspin
Centbrac
Brachrup
Prunlaci
Lotucorn
Junicomm
D-AB
D-TR
Thymlong
Bromerec
Buplbald
Sangmino
Heliquad
Sedusexa
Trifscab
Trifochr
D-MN
Acinalpi
H-AB
Valeoffi
Stelmedi
Saliapen
Fraxexce
Ranulanu
Acercamp
CCA axis 2
CCA axis 1
0.6
-0.6
-0.4
1.0

Figure 4

SDR simplex ternary plots showing the variation in taxonomic β-diversity along the seconadary succession of the two habitats.

(A) Dry grasslands. (B) Hay meadows. The abbreviations S, D and R refer to similarity, richness difference and species replacement, respectively. Mean values of S, D, and R are reported. Values marked with * are significantly different at $P<0.05$ according to a Kruskal-Wallis test performed between the two habitats.

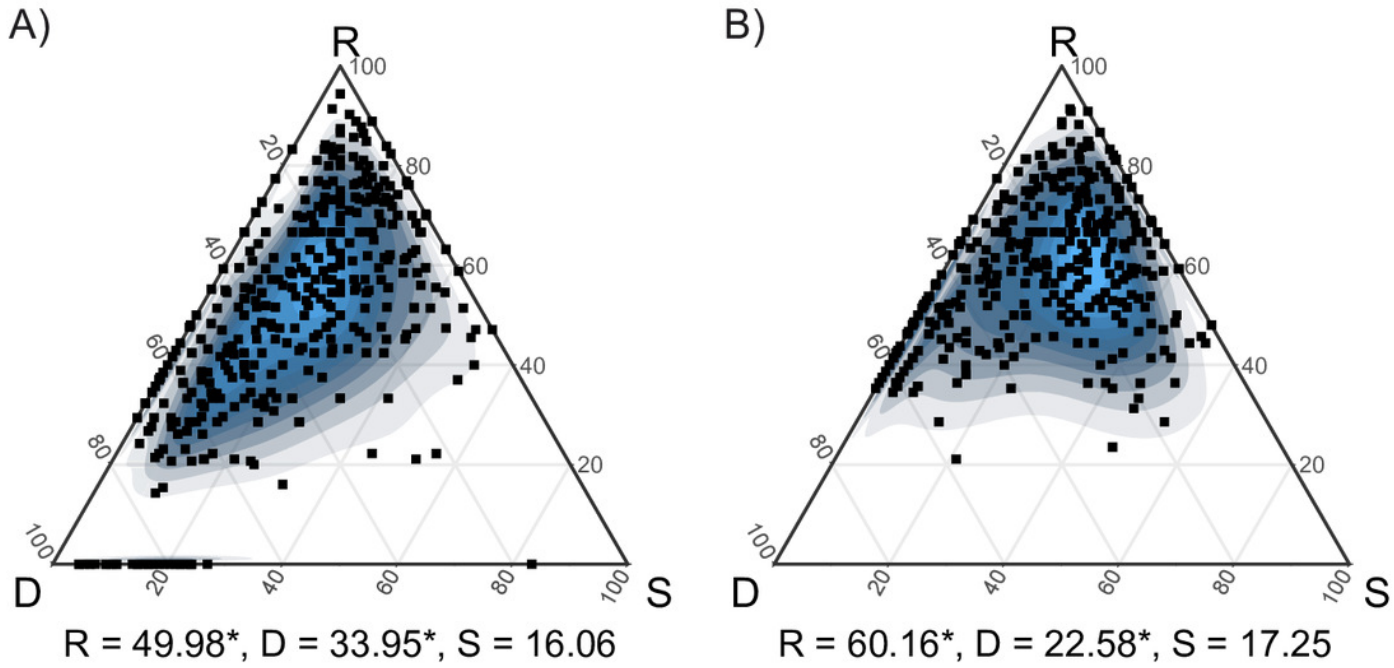


Figure 5

Ordination diagram based on phylogenetic distance of plots.

Plot distribution according to NMDS ordination based on the UniFrac index distance matrix. Empty symbols represent hay meadows and full symbols represent dry grasslands. Rhombus represent managed plots, circles transition plots and squares abandoned ones. Convex hull envelopes enclose plots according to Habitat type and Stage of Succession. H-MN= managed hay meadows, H-TR= transition hay meadows, H-AB= abandoned hay meadows, D-MN= managed dry grasslands D-TR= transition dry grasslands D-AB= abandoned dry grasslands.

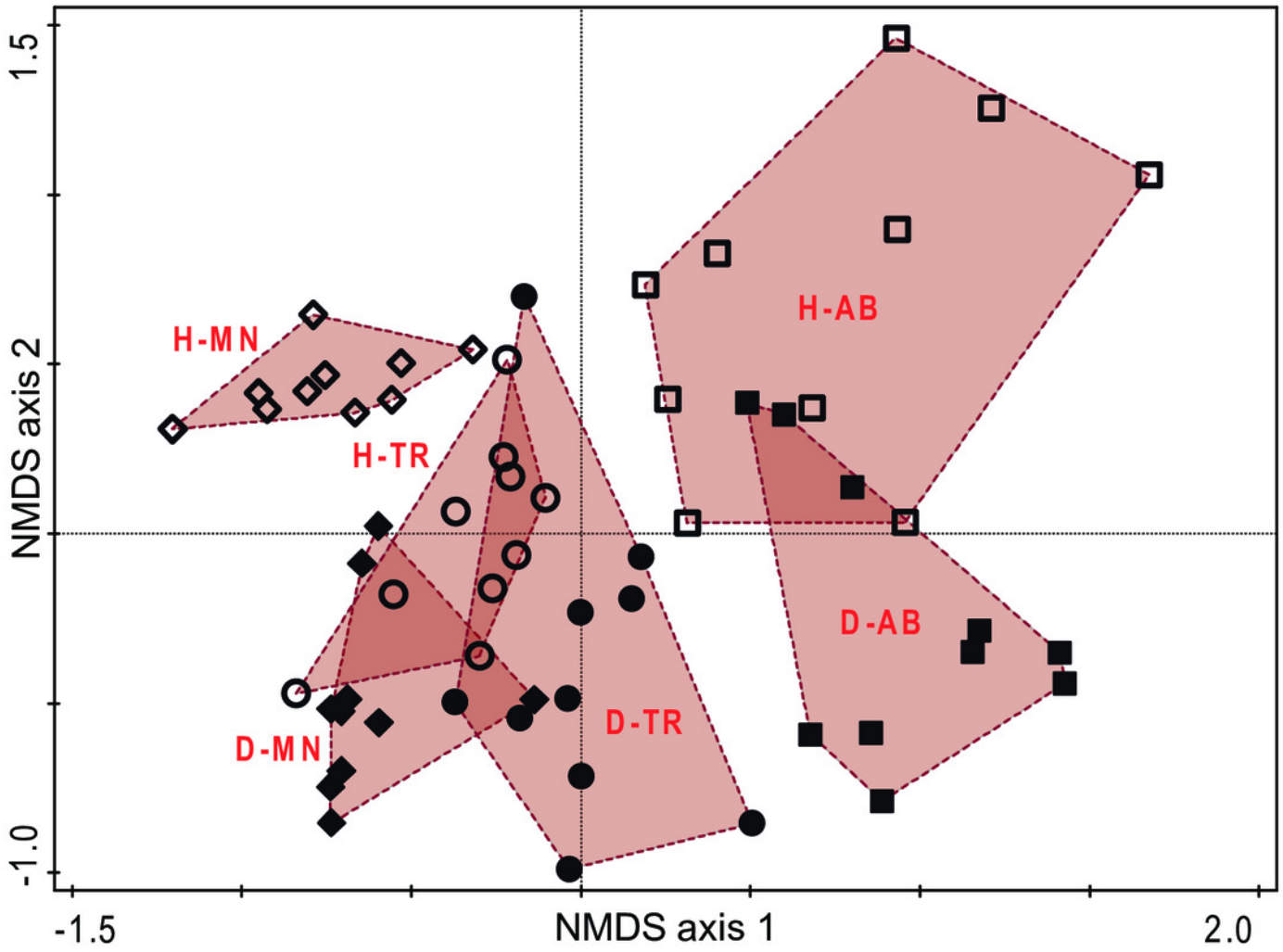
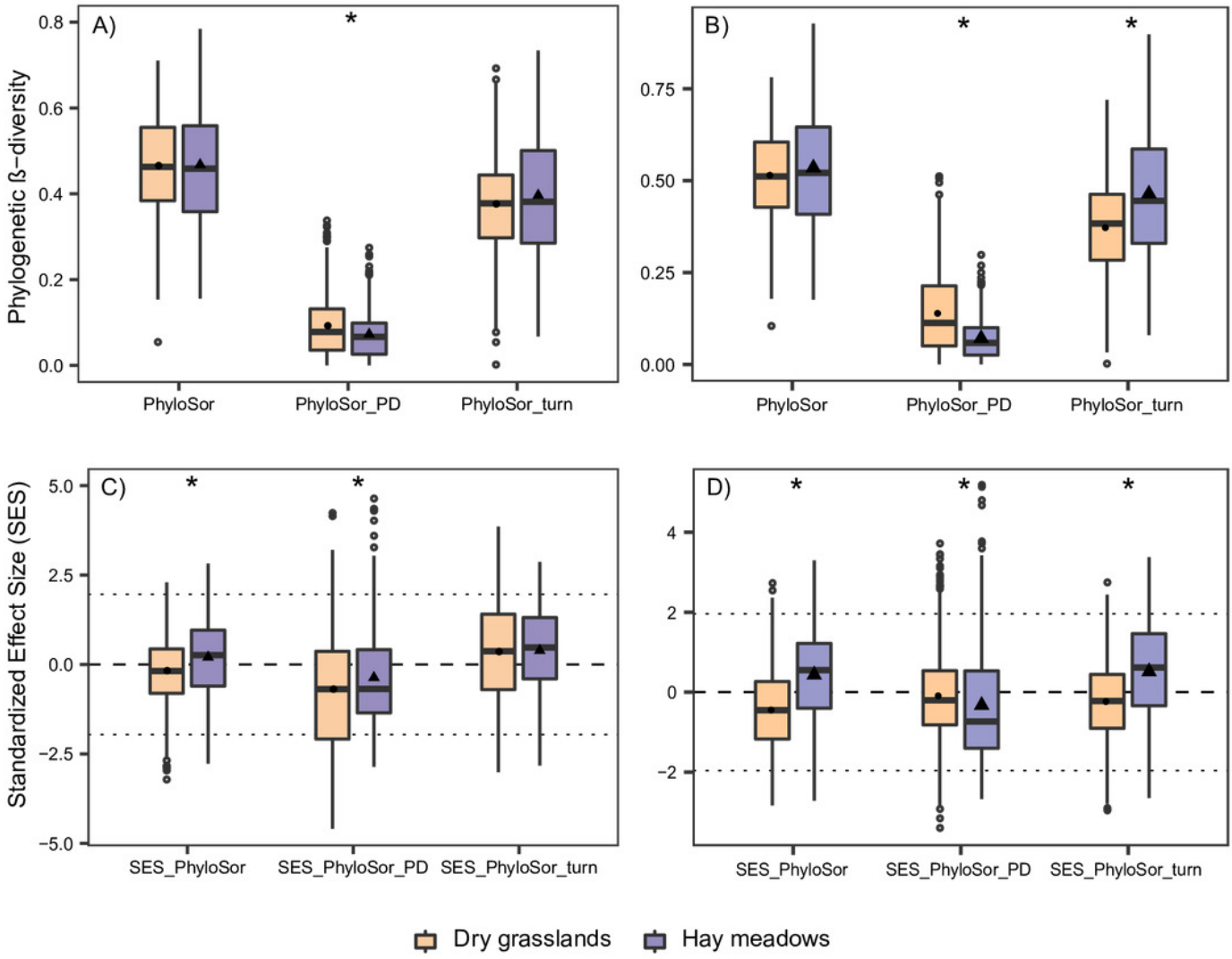


Figure 6

Boxplot graph for the variation in phylogenetic β-diversity according to Habitat and Succession for dry grasslands and hay meadows during the succession, evaluated with and without the species *Juniperus communis*.

A and B) PhyloSor index, and its two separate components accounting for 'true' phylogenetic turnover (PhyloSorTurn) and phylogenetic diversity gradients (PhyloSorPD) evaluated with and without the species *J. communis*, respectively; C and D) their relative Standardized Effect Size (SES.PhyloSor, SES.PhyloSorTurn, SES.PhyloSorPD) again evaluated with and without the species *J. communis*, respectively. Couples of boxplot marked with * show data significantly different at $P < 0.05$ according to a Kruskal-Wallis test performed between the two habitats.


A)
B)
C)
D)
Phylogenetic ß−diversity
Standardized Effect Size (SES)
PhyloSor
PhyloSor_PD
PhyloSor_turn
SES_PhyloSor
SES_PhyloSor_PD
SES_PhyloSor_turn
Dry grasslands
Hay meadows