Received: 4 November 2021 | Revised: 30 March 2022 | Accepted: 23 April 2022

DOI: 10.1002/edn3.307

Environmental DNA
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WILEY

ORIGINAL ARTICLE

Species-specific DNA distribution in spruce–beech forest soil

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Funding information
Financial support for this study was provided to GI by the University of Udine (www.uniud.it), PRID2017. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Environmental DNA consists of species-specific intracellular and extracellular fractions, whose content and information may not be similar in all environments. In forest soil, in particular, the biogeochemical fate of DNA originated by plant litter input has been extensively reviewed, but species-specific persistence and distribution still await to be quantified. In the present work, based on the purification of extracellular and intracellular DNA fractions from forest soil samples representing 3 soil horizons at 36 randomized locations differing for stand composition (either beech- or spruce-dominated, and mixed), followed by exDNA metabarcoding with the *rbcL* marker, we provide a clear picture of species-specific plant DNA distribution, and explore plant community composition and diversity along the explored gradient and the soil profile. We did not find significant differences in intra- vs. extracellular total DNA distribution, with a progressive depletion with soil depth positively associated with soil organic C and N content and negatively associated with soil pH and mineral content. Species-specific DNA distribution was horizontally dependent on beech and spruce basal area aboveground, while extracellular DNA showed peculiar species-specific vertical patterns. Proportion of *Fagus sylvatica* DNA increased with depth in beech stand soil, and *Picea abies* DNA decreased in spruce stand soil, respectively, possibly linked to species-specific differences in leaf litter decomposition dynamics and root litter contributions. Finally, our approach by metabarcoding provided a faithful, although incomplete, picture of the local plant diversity, suggesting that such technique could positively integrate traditional biodiversity inventory studies based on expert field assessments.

KEYWORDS
extracellular DNA purification, *Fagus sylvatica*, permutational multivariate analysis of variance, *Picea abies*, plant metabarcoding, rbcL

1 | INTRODUCTION

Environmental DNA, mentioned for the first time in Ogram et al. (1987), is defined as the DNA extractable from environmental samples, without first isolating any target organism (Taberlet et al., 2012). It can be found in different environments, including water bodies, sediments, and soils (Nagler et al., 2018), as well as in samples from many different organisms and at any possible degradation level (reviews in Taberlet et al., 2012, and Nagler et al., 2018). The total environmental DNA pool can be divided, and experimentally separated (Ascher et al., 2009), into an intracellular fraction comprising a complex mixture of the genomic DNA located within cell

membranes, and extracellular DNA, which comprises degraded and fragmented DNA molecules (Pietramellara et al., 2009). Although different acronyms have been used in the literature to indicate such DNA fractions, in this paper we use iDNA and exDNA to indicate intracellular and extracellular DNA (Nagler et al., 2018), respectively.

In forest soil, the cycling of exDNA has been extensively reviewed (Nagler et al., 2018; Pietramellara et al., 2009). Most exDNA enters the soil mainly after the lysis of microbial cells, subject to degradation by nucleases, nuclease degradation by proteases, and DNA protection by impurities associated with the released DNA molecules. Plant DNA enters the soil continuously (Levy-Booth et al., 2007) both from aboveground, through pollen dispersal (de Vries et al., 2003) or during litter decomposition (Ceccherini et al., 2003), and from belowground, through the sloughing off of root cap cells (Hawes, 1990; de Vries et al., 2003) or as a result of root colonization by pathogens (Kay et al., 2002). Once into the soil, exDNA pool can be bound to soil minerals (Morrissey et al., 2015) and humic substances (Crecchio and Stotzky, 1998; Pathan et al., 2021), and under specific environmental conditions can persist for years (Nielsen et al., 2007; Pietramellara et al., 2009).

Once unbound and released into the soil, exDNA can diffuse vertically (Potè et al., 2007), either downward through leaching or upward through advection by water capillarity, and horizontally following the soil water flow direction (Ascher et al., 2009; Ceccherini et al., 2007). While diffusing, exDNA may be used as a nutrient source for plant and microbial growth (Ceccherini et al., 2003; Morrissey et al., 2015). Partial DNA breakdown produces nucleotides, nucleosides, ribose, and bases that can be re-assimilated into nucleic acids without further degradation entering a living cell (Levy-Booth et al., 2007). Additionally, soil exDNA can be incorporated into competent bacteria by uptake (de Vries and Wackernagel, 2005; Thomas and Nielsen, 2005) and integration in the prokaryote genome (de Vries and Wackernagel, 2005). Then, transduction and conjugation may spread exDNA genetic information through the horizontal gene transfer (HGT) pathways (de Vries and Wackernagel, 2005; Levy-Booth et al., 2007).

Recently, exDNA was discovered to have a species-specific inhibitory effect, reducing germination and growth of conspecifics in plants (Mazzoleni et al., 2015a) and other organisms (Mazzoleni et al., 2015b). While the underlying mechanisms are not yet fully clarified (but see Chiusano et al., 2021), assessing the relevance of exDNA functional roles at plant community and ecosystem scales (Carteni et al., 2016) requires additional research (Nagler et al., 2018). First, a reliable quantification of species-specific plant exDNA content and horizontal and vertical distribution in soil is a main issue still awaiting to be addressed.

Among the possible methodological approaches to detect species-specific DNA in complex matrices such as soil samples, the use of advanced molecular techniques such as DNA metabarcoding, which allows the simultaneous identification of different species in a complex environmental sample through high-throughput sequencing methods (Bell et al., 2017), is promising. Indeed, environmental DNA metabarcoding has been used to compile biodiversity inventories from environments where the identification of target organisms with traditional approaches is intrinsically complicated (Tsuji et al., 2019), and exDNA and iDNA were successfully processed to assess species-specific fractions, as in the case of bacteria in deadwood environment (Probst et al. 2021). Considering studies targeting plant DNA, metabarcoding has been used to reconstruct past flora (e.g., Jørgensen et al., 2012), and assess taxonomic composition of pollen (Tremblay et al., 2019) or bulk airborne samples (Johnson et al., 2019), studying plant–pollinator interactions (Keller et al., 2015) and tracing invasive species or genetically modified plants (Folloni et al., 2012). In all these cases, eDNA is amplified using primers for plant-specific markers in polymerase chain reaction followed by next-generation sequencing of target amplicons (Ruppert et al., 2019) and taxonomic attribution of the sequences. The standard marker established by the barcode of life for plants (CBOL Plant Working Group, 2009) is the ribulose bisphosphate carboxylase large chain gene (*rbcL*). The recent availability, and progressive update of reference libraries for different genetic markers (e.g., Bell et al., 2017), also allowed to compile plant diversity inventories and assess community composition complementing aboveground investigation (Yoccoz et al., 2012).

However, when metabarcoding application is directed to assessing species-specific exDNA content, rather than occurrence, possible environmental drawbacks and technical issues must be considered (Bohmann et al., 2014). Things are even more complicated when the target metric is not the exDNA, but the species abundance (Deiner et al. 2017). On the one hand, species-specific persistence of DNA in soil, its interaction with the environmental conditions, as well as amplification conditions, different specificity and sensitivity of different markers, and possible sequencing biases should be taken into account (Hollingsworth, 2011; Lamb et al., 2019). On the other hand, recent observations have reported significant relationships between the root biomass of a species in a community and the proportions of that species reads in the mixed exDNA pool resulting from the amplification and sequencing of the selected genetic marker (Matesanz et al., 2019). Therefore, DNA metabarcoding of pooled root samples is increasingly used as a promising tool to track belowground abundance of actively growing plants (e.g., Illuminati et al., 2021; López-Angulo et al., 2020), while the same approach applied to soil samples could allow to assess the content of exDNA from both actively growing and long-dead individuals.

In the present work, we studied the vertical distribution of soil iDNA and exDNA fractions, separated following Ascher et al. (2009) along a mixing gradient of beech and spruce. Where the optimum for these two key species partially overlaps and no disturbance is present (Schütz, 1999; Wilson, 1999), they may coexist alternating each other (Del Favero et al., 1998): For example, where a spruce stand is in its regeneration phase (Oliver and Larson, 1990), there will be a widespread beech regeneration in the understory and vice versa (the so-called “alternation of species”; Schaeffer and Moreau, 1958). In old studies, this switch in species composition in time was related to human management (Giacobbe, 1926), as well as to plant–soil feedback (Lachaussée, 1947; Susmel, 1951). The considered tree species mixing gradient provides an optimal condition to assess whether the

proportion of beech and spruce exDNA reflects the actual species tree density or the effect of past conditions/stand composition can still be detected in relation to exDNA persistence. Based on soil DNA extraction from soil cores collected at 36 locations selected on the base of a stratified random sampling design, and exDNA metabarcoding, the specific aims of this work were: (i) to provide a quantitative overview of iDNA and exDNA content and distribution along the explored gradient and the soil profile; (ii) to assess the relationships between soil DNA content and physicochemical properties; and (iii) to estimate the abundance of beech and spruce exDNA, as well as the whole belowground plant community diversity in the tested conditions. The underlying tested hypotheses were that: (i) iDNA and exDNA are progressively depleted along the soil profile, as a result of the interplay of input by plant litter, persistence by interaction with mineral and humic fractions, and consumption by microbial turnover; and (ii) the proportion in deep horizons is larger for beech vs. spruce exDNA, as related interspecific differences in seasonal litterfall, leaf litter decay rate, and root litter contribution.

2 | MATERIAL AND METHODS

2.1 | Study site and sampling

The study area is located at Fusine lakes (46°30′15″N, 13°38′26″E), in North-East Italian Alps near the border with Slovenia and Austria. Climate shows high annual total precipitations (1520 mm year^{-1}) and low annual temperature (mean 7.3°C, absolute minimum −27°C). The site is characterized by patches of pastures surrounded by forests of spruce (*Picea abies* Karst), often mixed with fir (*Abies alba* Mill.) and beech (*Fagus sylvatica* L.), lying on a Rendzina Leptosol (IUSS, 2006), over a substrate of moraine or alluvial deposits.

Figure 1 shows the experimental setup. Within the forest compartments belonging to the Regional Government, 12 circular plots (radius 13 m) were randomly distributed among the existing pure spruce, pure beech, and mixed stands (Alberti et al., 2013). Within each plot, all standing trees belonging to the two target species were recognized and their diameters at breast height (dbh, 1.30 m) were measured. Trees with dbh < 10 cm were not considered for further analysis (INFC, 2006). Trees were aggregated into four dbh classes as follows: (i) 10–19 cm; (ii) 20–29 cm; (iii) 30–39 cm; and (iv) > 40 cm.

Four soil cores per plot were taken up to 60-cm depth using a percussion drilling set (Cobra TT, Eijkelkamp, the Netherlands) interiorly equipped with a presterilized PE foil liner. Therefore, a total of 48 soil cores were collected. Each soil core, still enveloped into the liner, was brought to the laboratory and subdivided into four homogeneous soil horizons (i.e., Oe, A1, A2, C) using sterilized cutters. A considerable effort was maintained throughout sampling to ensure clean, uncontaminated samples, including the use of gloves during sample collection and decontamination of equipment prior to and during sampling. For subsequent analysis, the homogeneous horizons collected within each plot were pooled together, resulting in 48 pooled soil samples (i.e., 12 plots × 4 horizons). Then, aliquots were collected from each pooled sample with a stainless steel sterilized

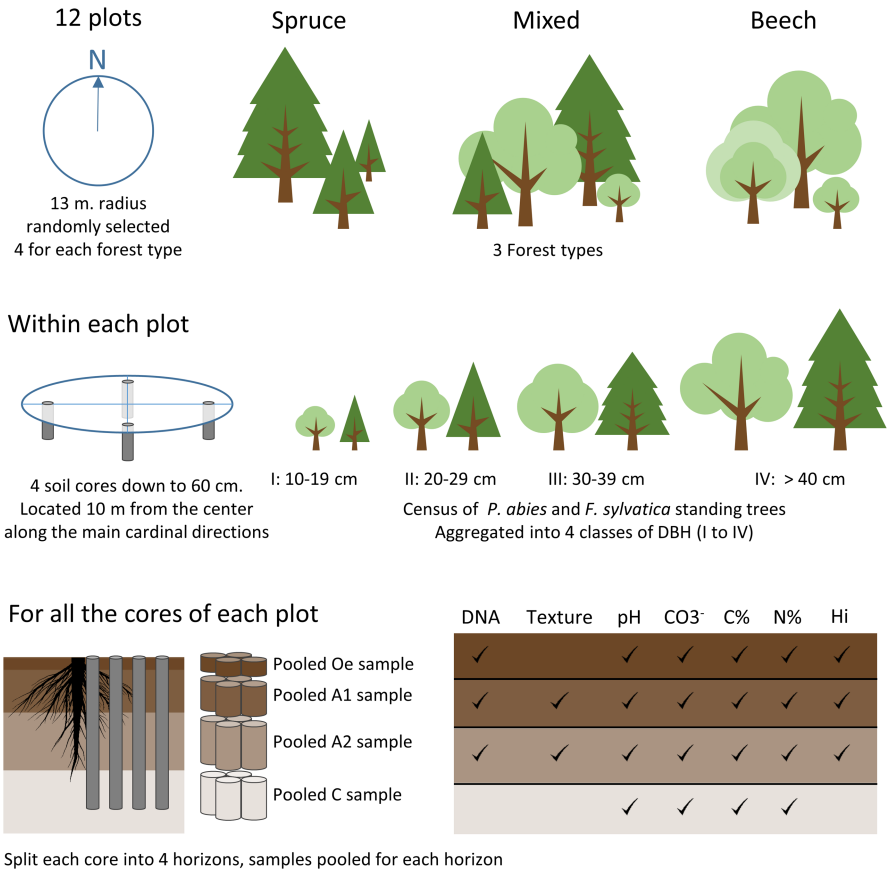

FIGURE 1 Experimental setup and sampling. Three manipulative steps are shown along the experiment timeline: (1) four sampling units consisting of plots of 13-m radius were randomly selected within each of 3 forest types (spruce, mixed, and beech), for a total of 12 plots. (2) Collection of soil cores within each plot. (3) Census of all spruce and beech standing trees with dbh > 10 cm. (4) All cores were split into four soil horizons (Oe, A1, A2 and C). Pooled, homogeneous soil samples were obtained for each horizon by pooling together the four core layers collected at each plot, for a total of 48 pooled samples. (5) Indication by checkmark of the chemical–physical analyses carried out in pooled sample of different horizons

spatula, sieved at 2 mm and stored in 50-ml Falcon tubes at −80°C for subsequent DNA extraction. The remaining soil materials were sieved at 2 mm, dried at 65°C for 48 h, and stored in plastic bags at 4°C for further chemical and physical analyses.

2.2 | Soil chemical–physical analyses

Soil pH was measured potentiometrically with a sureflow combined glass and calomel electrode in H_2O solution in 1:5 solid: liquid ratio (McLean, 1983). Carbonate content was assessed following the volumetric method with the Scheibler apparatus (Williams, 1949). A humification index (HI), calculated as the ratio of nonhumified (nonphenolic) to humified (phenolic) organic carbon after extraction with alkaline sodium pyrophosphate, was determined following the methodology described by De Nobili and Petrussi (1988). Organic carbon (C) and total nitrogen (N) contents and corresponding C-to-N ratio were measured using a vario Micro Cube (Elementar GmbH, Langenselbold, Germany) elemental analyzer in triplicate aliquots of 10 ±0.5 mg of each sample weighed in a silver capsule and treated with HCl to eliminate carbonates (Nieuwenhuize et al., 1994). Particle size analysis (PSA) and the conversion into a texture class were performed according to Bowman and Hutka (2002) by dispersion and pipette subsampling to particle separation into size groups. Soil chemical–physical analyses were carried out on all the 48 pooled samples, with the exceptions of HI, not measured on the samples from C horizons, and PSA, not assessed on Oe and C samples, due to insufficient amount of material from the corresponding soil fine fractions.

2.3 | Sequential extraction of soil DNA

DNA was directly extracted from 5 g of fresh, frost fine fraction aliquots of the 36 soil pooled samples from Oe, A1, and A2 horizons. iDNA and exDNA fractions were sequentially purified following the protocol proposed by Ascher et al. (2009), modified as follows: exDNA was extracted by gentle soil washing with 5 ml of 0.12 M Na_2HPO_4 at pH 8 in 50-ml falcon tubes horizontally shaken for 30 min (80 rpm). The tubes were centrifuged (4°C, 30 min, 7500 g), and the supernatant was collected. The same procedure was repeated twice, and the resulting supernatants were pooled together to a final volume of 15 ml of unpurified exDNA. The exDNA solution was purified using a commercial extraction kit (DNeasy® PowerMax® Soil Kit, Qiagen, USA) following the manufacturer's instruction, but avoiding the method step of sample incubation in cell lysis buffer. The soil pellet residual after alkaline washing was used for iDNA extraction. The pellet was transferred into a new 50-ml Falcon tube and processed in the extraction kit according to the manufacturer's instructions, including the method step of sample incubation in the cell lysis buffer. At the end of the purification, all DNA samples were separately suspended in 5 ml of 10 mM Tris solution. Purified DNA samples were quantified by fluorimeter Qubit 3.0 (Life Technology, Carlsbad, California, USA), and the quality was assessed by NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The fragment length distribution was assessed by 0.8% agarose gel electrophoresis.

2.4 | Amplification and sequencing

Amplification of exDNA and iDNA was performed in a final volume of 25 µl for each sample, using 10 µl of DNA extract (concentration of 12 ± 5 ng μl^{-1}), 1X concentrated OneTaq Hot Start Quick-Load, 2X Master Mix with Standard Buffer (New England Biolabs inc.), and 0.5 µM of the forward and reverse *rbcL* primers. This primer set amplifies a 553-bp fragment of the ribulose bisphosphate carboxylase large chain gene (*rbcL*) and is recommended by the CBoL Plant Working Group (2009) for plant metabarcoding as compared to bacterial and fungal ones. The selected sequences were as follows: *rbcLa*_f 5′ ATGTCACCACAAACAGAGACTAAAGC-3′ and *rbcLa*_rev 5′-GTAAAATCAAGTCCACCRCG-3′ (Fahner et al., 2016). The PCR conditions were 94°C for 30 s, followed by 40 cycles of 94°C for 30 s, 64°C for 60 s, 68°C for 30 s, and a final step of 68°C for 5 min. A subsequent amplification run integrating relevant flow-cell binding domains and unique indices was performed with Nextera XT Index Kit (Illumina, San Diego, CA). The amplification products were sequenced on MiSeq instrument platform (Illumina) using 300-bp paired-end and following the manufacturer's instructions.

Taxonomic classification was performed using a database containing 181,133 *rbcL* sequences downloaded from NCBI Nucleotide section on September 9, 2020, using the following key words in search: rubisco [all fields] OR ribulose-1,5-biphosphate carboxylase/oxygenase [all fields]) AND plants[filter] AND biomol_genomic [PROP] AND large subunit [All Fields]. In addition, consensus sequences of *Cyamopsis tetragonoloba* and *Vitis vinifera* were manually inserted.

Bioinformatics pipeline steps were as follows: Reads were trimmed in order to eliminate primer sequences by *cutadapt* (Martin, 2011) with standard parameters (-anywhere, -overlap 5, -times 2, -minimum-length 35, -mask-adapter). Low-quality bases were removed from 3′ with *erne-filter* (Del Fabbro et al., 2013) by applying default parameters, excluding reads <60 bp from further analysis. Reads with an error rate >1% were removed. Chimeric sequences were removed with the algorithm *uchime_denovo* (Edgar et al., 2011) implemented in *usearch* (Edgar, 2010). Reads were clustered to a minimum identity of 97% generating representative sequences with the algorithm *cluster_fast* implemented in *usearch* (Edgar, 2010). Blast against the *rbcL* database was performed without a minimum identity filter, using the lowest unambiguous taxonomic attribution among all the possible blast hits. If there were best hits with the same score indicating different lineage, most common part was reported. Sequences not taxonomically attributed to *Streptophyta* were discarded and not used for further data analysis.

2.5 | Statistical analysis

After testing for normality and homoskedasticity of the data distributions for each dependent variable, using Shapiro–Wilk's and Levene's tests, respectively, we used two-way ANOVA to test main and interactive effect of forest type (three levels: beech, mixed, and spruce) and tree species (two levels: *F. sylvatica* and *P. abies*) on species-specific basal area at the sampling plots ($m^2\ ha^{-1}$). Main and interactive effects of forest type (three levels: beech, spruce, or mixed), soil horizon (three levels: Oe, A1, and A2), and DNA source (two levels: exDNA and iDNA) on soil DNA content were tested using three-way ANOVA. Main and interactive effects of forest type and soil horizon on total read numbers from sequenced *rbcLa* amplicons from soil exDNA samples were tested using two-way ANOVA. After estimating species-specific exDNA abundance in soil as the reads' proportion of a given species on the total reads attributed to Streptophyta in the sample, a further three-way ANOVA model was fitted for the proportion of beech and spruce exDNA in soil including main and interactive effects of soil horizon, forest type, and tree species (either *F. sylvatica* or *P. abies*). For all ANOVA models, pairwise comparisons between combinations of independent factors were tested by Duncan's post hoc test at $\alpha = 0.05$, in all tested comparisons.

The relationships between soil chemical–physical properties and the abundance of species-specific exDNA were assessed separately for the two tree species (*F. sylvatica* and *P. abies*) and for all plants, as well as for all data pooled and for different forest types, by an extensive correlation analysis based on Pearson's *r*. Correlation scores were considered statistically significant at $\alpha = 0.05/N = 0.0019$ after application of the Bonferroni's correction, with *N* being the number of multiple comparisons ($n = 27$).

Based on the data matrix containing the number of reads recorded for all plant species in all soil samples, alpha diversity metrics were calculated including the simple species richness and Shannon's index of evenness H′. A resemblance matrix calculated on Bray–Curtis dissimilarity was used to perform nonmetric multidimensional scaling (nMDS) to assess variation in plant community composition (i.e., species proportion) in soil exDNA across forest types. In association with nMDS, the significance of community composition changes, as well as that of shifts in species richness and H′ in the three forest types, was tested through permutational multivariate analysis of variance (PERMANOVA) with 999 permutations, using the soil horizon and forest types as fixed factors and the replicated plot as random factor. All statistical analyses were performed using the software packages Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA) and Primer-e v. 7 (Primer Ltd, Plymouth, UK).

3 | RESULTS

3.1 | Soil and forest characteristics

Table 1 shows soil horizon properties at the three considered forest types. In general, horizons were rather thin, developed over largely gravelly, and calcareous material (C horizon), with a maximum thickness of A-to-C of 29 cm under mixed forests. At all forest types, pH ranged from acid in Oe horizons (from 4.5 to 5.8) to basic in C horizons (from 8.5 to 8.7). Organic C content significantly and expectedly decreased with soil depth, with a maximum of 30.11 ± 6.80% in the Oe layers, followed by 11.62 ± 7.50% and 2.55 ± 1.42% in the A1 and A2 horizons, respectively, and by a steep decrease in the C horizons, with values lower than 1% at all sites. Total N content followed the same pattern, corresponding substantially to the same values of C/N ratio in Oe and A1 horizons, then progressively decreasing with depth. On the contrary, carbonate content increased with depth, up to values higher than $700\ g\ kg^{-1}$ in the C horizon, while being more variable at the Oe horizons (8.6–49.8 $g\ kg^{-1}$). HI did not vary significantly across the explored vertical and horizontal gradients. Particle size (not performed in Oe and C horizons due to the lack of fine fraction) showed increasing sand and decreasing silt from A1 to A2 layers in spruce forest soil.

As far as forest characteristics are concerned, total stand basal area (SBA) did not substantially vary across the three considered forest types, ranging from 33.8 ± 5.6 $m^2\ ha^{-1}$ in beech plots to 34.5 ± 1.7 $m^2\ ha^{-1}$ in spruce ones with lower value of 28.1 ± 3.5 $m^2\ ha^{-1}$ in mixed plots (Figure 2). In pure stands dominated by either beech or spruce, the dominant tree species accounted for over 80% of SBA, while in mixed stands, species-specific basal area contributions were not significantly different (Figure 2; Tables S1 and S2).

With an insight into contributions to SBA values by different dbh classes, we found that the species-specific SBA pattern across the three forest types was substantially consistent also within each dbh class (Figure S1). Moreover, largest contributions were ascribed to the lowest diametric class (dbh between 10 and 20 cm) in all forest stands, with a consistent progressive decrease for larger diameter classes up to dbh > 40 cm, with the exception of a higher percent frequency of old trees (up to dbh = 54 cm) in spruce-dominated stands (Figure S1).

3.2 | iDNA and eDNA distribution in relation to soil chemical–physical properties

The two fractions of soil DNA were satisfactorily extracted and separated from the soil samples, although with different degree of intactness reflecting the degradation of exDNA, but not of iDNA (Figure S2).

Both fractions persisted along the soil vertical profile, showing a progressive decrease with increasing depth (Figure 3a). Interestingly, such pattern was consistent across the three forest types (Figure 3a), as indicated by the nonsignificant terms T and T × H in the ANOVA model (Table S3). Differences in iDNA and exDNA content between Oe and A2 soil horizons were statistically significant, whereas A1 horizon showed intermediate values for both DNA fractions, in some cases not different from those of the underlying and/or overlying layers (Tables S3 and S4). Finally, within-layer differences between iDNA and exDNA contents were not significant for all soil horizons

TABLE 1 Soil properties by soil horizon according to stand composition

Forest type horizon	Thickness (cm)	pH	C (%)	N (%)	C_{org}/N_{tot}	CO_3^{2-s} (g kg^{-1})	HI	Sand (%)	Silt (%)	Clay (%)
Beech										
Oe	10 ± 1	5.3 ± 0.3 *a*	29.6 ± 1.8 *c*	1.7 ± 0.1 *c*	18.5 ± 0.7 *bc*	49 ± 45 *a*	0.3 ± 0.0 *a*	n.a.	n.a.	n.a.
A1	7 ± 2	6.7 ± 0.3 *b*	13.5 ± 3.6 *b*	0.7 ± 0.2 *a*	18.6 ± 0.9 *c*	137 ± 49 *ab*	0.3 ± 0.1 *a*	32.7 ± 10.6 *a*	53.7 ± 6.5 *a*	13.5 ± 4.3 *a*
A2	10 ± 3	7.7 ± 0.2 *c*	2.9 ± 0.4 *ab*	0.2 ± 0.0 *a*	13.9 ± 0.7 *b*	498 ± 181 *b*	0.3 ± 0.1 *a*	47.2 ± 14.1 *a*	42.0 ± 10.1 *a*	10.7 ± 4.5 *a*
C	15 ± 7	8.5 ± 0.1 *d*	0.6 ± 0.1 *a*	0.1 ± 0.0 *a*	7.7 ± 1.6 *a*	688 ± 54 *c*	n.a.	n.a.	n.a.	n.a.
Mixed										
Oe	11 ± 2	4.8 ± 0.7 *a*	28.6 ± 4.0 *b*	1.4 ± 0.2 *c*	20.7 ± 0.5 *c*	9 ± 5 *a*	0.3 ± 0.1 *a*	n.a.	n.a.	n.a.
A1	12 ± 3	6.8 ± 0.5 *b*	7.0 ± 0.9 *ab*	0.3 ± 0.0 *a*	20.6 ± 2.7 *c*	189 ± 109 *a*	0.2 ± 0.0 *a*	26.2 ± 7.3 *a*	56.0 ± 3.5 *a*	17.7 ± 3.9 *a*
A2	17 ± 3	7.6 ± 0.4 *bc*	3.0 ± 1.1 *ab*	0.2 ± 0.0 *a*	17.1 ± 2.1 *b*	448 ± 187 *b*	0.5 ± 0.1 *b*	42.2 ± 12.5 *a*	45.5 ± 7.6 *a*	12.2 ± 4.9 *a*
C	19 ± 5	8.6 ± 0.1 *c*	0.5 ± 0.1 *a*	0.1 ± 0.0 *a*	5.8 ± 1.1 *a*	765 ± 34 *b*	n.a.	n.a.	n.a.	n.a.
Spruce										
Oe	10 ± 4	5.8 ± 0.3 *a*	32.1 ± 4.6 *c*	1.7 ± 0.3 *c*	18.8 ± 0.8 *c*	15 ± 4 *a*	0.4 ± 0.1 *a*	n.a.	n.a.	n.a.
A1	7 ± 2	7.0 ± 0.4 *b*	14.3 ± 5.2 *b*	0.8 ± 0.3 *b*	17.4 ± 0.4 *bc*	269 ± 130 *ab*	0.3 ± 0.1 *a*	40.7 ± 9.6 *a*	51.7 ± 7.0 *b*	7.5 ± 3.2 *a*
A2	14 ± 3	8.0 ± 0.1 *c*	1.8 ± 0.4 *a*	0.1 ± 0.0 *a*	13.7 ± 0.8 *b*	511 ± 100 *b*	0.5 ± 0.1 *a*	67.0 ± 7.8 *b*	29.5 ± 6.0 *a*	3.5 ± 1.8 *a*
C	16 ± 5	8.7 ± 0.1 *c*	0.5 ± 0.0 *a*	0.1 ± 0.0 *a*	4.8 ± 1.2 *a*	718 ± 71 *b*	n.a.	n.a.	n.a.	n.a.

Note: Data refer to mean ± standard error of 4 replicates (i.e., sampling plots) for each soil property. Different letters indicate pairwise significant differences among soil horizons within each forest type (Duncan's test, $p < 0.05$) for all soil properties, but for horizon thickness.

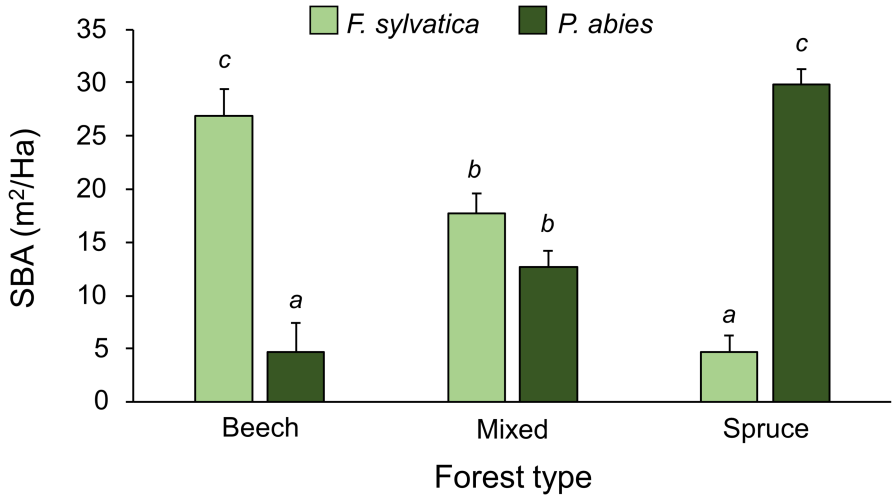

FIGURE 2 *Fagus sylvatica* and *Picea abies* stand basal area (SBA) at the sampling sites. Data refer to mean and standard error of four replicated plots for each forest type. Different letters indicate significant pairwise differences of SBA between different combinations of forest type and tree species ($p < 0.05$, Duncan's post hoc test after two-way ANOVA, detailed statistics in Tables 1 and 2)

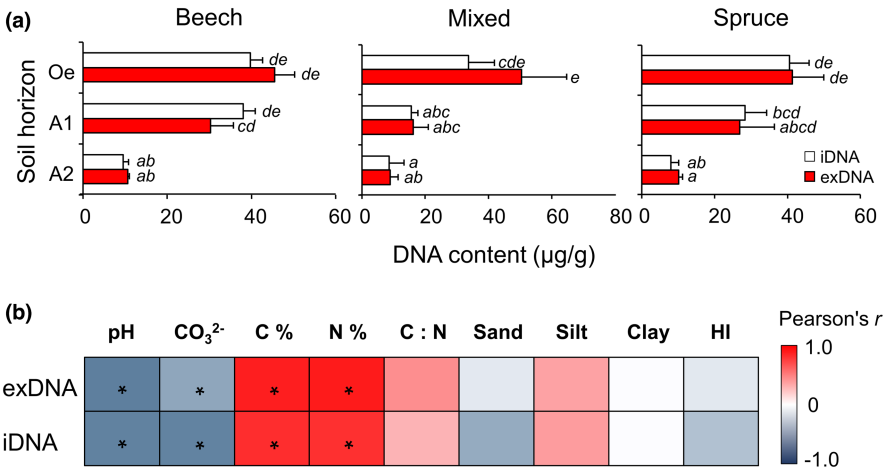

FIGURE 3 Vertical and horizontal distribution of soil intracellular (iDNA) and extracellular (exDNA) DNA fractions and relationships between soil DNA yield and chemical–physical properties at the study sites. (a) Data refer to mean ± standard error of DNA yield ($\mu g\,g^{-1}$) of four replicates for each forest type and soil horizon. Different letters within each panel indicate pairwise significant differences among combinations of DNA fraction and soil horizon ($p < 0.05$, Duncan's post hoc test after three-way ANOVA, detailed statistical results in Tables S3 and S4). (b) Heatmap shows pairwise Pearson's correlation (r) between each chemical–physical variable and either intracellular (iDNA) or extracellular (exDNA) DNA yield ($\mu g\,g^{-1}$) measured in 12 soil samples (three forest types × four replicates). Asterisks indicate significant p-values ($p < 0.05$)

and forest types. Interestingly, the largest difference between iDNA and exDNA contents, though not statistically significant, was observed at the Oe horizon in mixed forests (33.6 ± 8.2 $\mu g\,g^{-1}$ vs. 50.4 ± 14.4 $\mu g\,g^{-1}$ for iDNA and exDNA, respectively).

The observed distribution of the two soil DNA fractions was associated to the soil chemical–physical properties. When such relationships were tested on all data pooled for the three forest types (Figure 3b), we found a negative association of soil iDNA and exDNA abundance with pH ($r = -0.652$, $p = 0.001$ and $r = -0.735$, $p < 0.001$, respectively) and carbonate content ($r = -0.646$, $p < 0.001$ and $r = -0.529$, $p = 0.001$, respectively), and a positive correlation with organic C ($r = 0.841$, $p < 0.001$ and $r = 0.872$, $p < 0.001$, respectively) and total N percent ($r = 0.845$, $p < 0.001$ and $r = 0.872$, $p < 0.001$, respectively) contents.

Considering data separately for each forest type (Figure S3A), the general pattern of association between the content of DNA fractions and the soil chemical–physical properties still held for spruce forest soil, while the negative association with pH and carbonates did not emerge for beech and mixed forest soils, and the positive correlation of DNA with organic C and total N was not found for beech soil. On the contrary, when data were separately analyzed for each soil horizon, the pattern of association between soil quality parameters and DNA fractions was mostly released (Figure S3B). Finally, we found that soil DNA content was unrelated to C: N ratio, texture, particle size, and HI, both when the association was tested on all data pooled and when the analysis was limited at single forest types or soil horizons (Figure 3 and Figure S3).

3.3 | Plant exDNA species-specific distribution

Of a total of 4.6 million of unique sequenced *rbcLa* amplicons from exDNA, the taxonomic assignment produced a total of 386,702 reads attributed at Streptophyta clade and 261,082 reads assigned at plant order or lower rank taxa, out of which 223,629 were taxonomically attributed at species level. Total read numbers did not vary significantly neither among forest types ($F_{2,27}$ =0.343; p = 0.713), nor among soil horizon ($F_{2,27}$ =0.008; p = 0.991) or their interaction ($F_{4,27}$ =0.598; p = 0.667), with high within-group data variability (Figure S4).

The distribution of *F. sylvatica* and *P. abies* exDNA showed an interesting pattern (Figure 4), significantly different among forest types and between the two species, also showing, for each of them, significant shifts with soil depth, as indicated by the significant terms forest type, species, forest x species, and species x horizon in the three-way ANOVA model (Table S5). In detail, the contribution of the two tree species to the total DNA pool reflected the level of the tree species cover above ground (Figure 4), with beech and spruce DNA fractions largely predominating in the soil DNA pool collected in beech and spruce forest stands, respectively. DNA samples from mixed forest showed higher content of beech DNA at all depths. Beech vs. spruce differences in exDNA proportion were significant in most cases, with beech DNA predominating at all depths in beech and mixed forest soils (with the exception of Oe in mixed forest), while spruce DNA was higher only at the superficial layer of spruce forest soil (Table S6). Interestingly, along the soil profile the persistence of exDNA from the two tree species followed opposite patterns: The proportion of *F. sylvatica* DNA showed a progressive increase along the vertical profile in beech forest (from 44.7 ±8.8% in Oe to 82.6 ±5.2% in A2) and nonsignificant variation in mixed and spruce forests, although in the latter case, a large within-group variability could have masked a possible enrichment along the soil profile (Figure 4, Table S6); spruce DNA proportion did not change significantly in mixed stands, but showed a remarkable decrease along the soil profile in pure stands (Figure 4, Table S6), passing from 44.6 ±3.1% to 13.5 ±2.0% at the Oe and A2 horizons, respectively.

Considering the relationships between the abundance of species-specific exDNA and the chemical–physical properties of the forest soils samples, we did not find any significant association (Table S7) after controlling for multiple comparisons. However, the magnitude of some of the correlation scores and the associated p-values were very close to the corrected significance threshold, as in the cases of pure spruce stands where the spruce exDNA abundance pattern was negatively related to soil pH (r =−0.781, p = 0.008) and positively related to organic C (r = 0.821, p = 0.004) and total N (r = 0.844, p = 0.002) contents.

3.4 | Plant community composition by metabarcoding

Metabarcoding analysis identified 47 species belonging to 30 families (Figure S5). Plant community composition, as assessed by exDNA metabarcoding, was significantly different among the three forest types, as indicated by the PERMANOVA results (Table 2).

Such compositional differences were well represented by the nMDS plot (Figure 5a), where the plant communities of beech and spruce stands were clearly separated. Indeed, beech vs. spruce forest compositional differences were statistically significant, both considering exDNA information separately for each soil horizon and all data pooled (Table 3). Mixed forest community expectedly showed intermediate composition, mostly closer to that of the beech forest (Figure 5a), although also showing significant compositional differences with respect to the spruce forest, when data pooled for the three soil horizons were considered (Table 3). The minor spreading of beech forest data points along the two ordination axes, compared with the larger spreading of mixed and spruce ones, indicated more homogeneous composition of beech stand community, while a much higher taxonomic variability occurred in mixed and spruce stands (Figure 5a). Consistently, the Shannon's index H′ showed effects of forest type at the limit of statistical significance (Table 2), with significant differences between beech (H′ = 1.20±0.73) and spruce (H′ = 1.95±0.58) forest soils (Figure 5b).

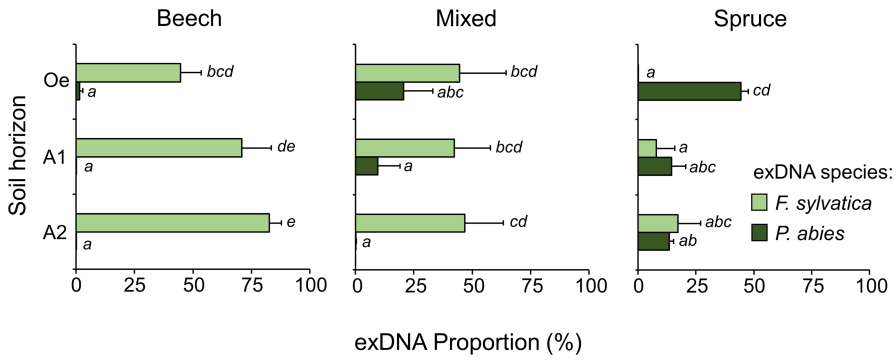

FIGURE 4 Proportion of species-specific exDNA from *F. sylvatica* and *P. abies* across three different forest types (beech stands, spruce stands, or mixed forest) and along the vertical soil profile (Oe, A1, and A2 horizons). Data refer to mean and standard error of four replicates for each combination of dependent variables. Lettering above bars indicate significant pairwise differences between different combinations of exDNA species, forest types, and soil horizon (p <0.05, Duncan's post hoc test after GLM, detailed statistical results in Tables S5 and S6)

Species richness as assessed by exDNA metabarcoding was not significantly affected by forest type and soil depth, as indicated by the PERMANOVA results (Table 2) and by pairwise comparisons (Table 3). Finally, by considering the composition of plant communities, as assessed by exDNA metabarcoding, a clear-cut shift was observed passing from beech to spruce forest, both at level of families (Figure 6) and at species (Table 3, Figure S5).

4 | DISCUSSION

4.1 | Progress and problems on the use of the rbcL barcode

The use of metabarcoding is revolutionizing biodiversity studies, especially the detection of so-called hidden species (Taberlet et al., 2018). However, floristic studies based on the use of soil DNA metabarcoding are still rare (but see Yoccoz et al., 2012), mostly because of the difficulty of finding a unique, robust, and effective barcode allowing to correctly detect and discriminate amplicons from DNA fragments of all plant species occurring in a given environmental sample (Hollingsworth, 2011). In 2009, the Consortium for the Barcode of Life (CBOL) Plant Working Group proposed the use of two specific portions of two coding regions from the chloroplast genome—*rbcL* and matK—as a source for barcode for plants, which have been integrated with a great numbers of specific markers, belonging to ITS regions (e.g., China Plant BOL Group, 2011) or trnL P6 loop (Taberlet et al., 2007, Yoccoz et al., 2012). In our study, we obtained a satisfactory result using only the *rbcLa* marker: We detected a total of 47 plant species (Figure 6), all known for the study area (Nimis and Martellos, 2006). However, for a large fraction (32.5%) of the 386,702 unique amplicons attributed to *Streptophyta*, it was not possible to find a satisfactory taxonomic match, since the identification was limited to supra-generic ranks. Then, following Fahner et al. (2016), the use of multiple markers should be recommended, especially considering the availability of nonplastidial markers such as those belonging to the ITS region. In our case, as shown in other studies (e.g., Abdullah, 2017), the combined use of *rbcLa* and ITS markers may have offered a significant increase in the discriminatory power at the infra-generic taxonomic level. Finally, considering possible intrinsic biases of the selected barcode, which has been previously criticized as showing good recovery and sequence quality but low species discrimination (Hollingsworth, 2011), our result possibly indicates that the reference database for the target barcode in plants is largely incomplete, at least for alpine and beech–spruce forest stands. Therefore, further investigation and sequencing of *rbcL* barcode are needed, especially in alpine forest ecosystems, in order to increase database completeness and then improve our knowledge of the barcode performance.

It is noteworthy that 91.6% of the 4.6 million amplicons, though high-quality sequences, were not attributed to taxa within the plant kingdom. Such large predominance of unknown or nontarget sequences resulting from the amplification of a barcode specific for plants should not be surprising. Indeed, the length of *rbcLa* sequence is negligible in comparison with the length and sequence diversity of all the possible exDNA fragments occurring in the soil environment. Therefore, it is reasonable that the *rbcLa* primers, although specifically selected to amplify plant DNA and discriminate among different plant species, could also amplify by chance exDNA fragments of nonplant origin and hence not included in the reference database.

The possibility of using DNA metabarcoding to estimate species abundance is a subject of debate since the first studies based on this methodology (Matesanz et al., 2019). Translating the amplicon copy numbers to abundance is challenging, as the number of copies in the original sample can vary across cells, tissues, individual, species, and probably time (Taberlet et al., 2018). Additionally, the efficiency of amplification for the same primers can change for different target sequences, as well as a function of the experimental conditions (Taberlet et al., 2018). However, such approach has been successfully adopted in previous studies showing that in appropriate conditions, it is even possible to find a significant positive association between paired data of raw reads number and species-specific biomass (Matesanz et al., 2019; Yoccoz et al., 2012). In our study, we cannot show the same level of correspondence between the cause (plant species biomass) and the effect (abundance of plant exDNA), although our result of read numbers at the sampling plot for beech and spruce exDNA clearly reflects these expectations, considering that the highest read proportion of *F. sylvatica* and *P. abies* exDNA was consistently found in beech and spruce pure stands, respectively, while in mixed forest soils, we found intermediate values for the exDNA of the two tree species (Figure 3).

4.2 | Soil DNA distribution along the vertical profile

Our results showed that iDNA and exDNA contents in soil progressively decrease with depth. This is fully consistent with previous observations, based on the same approach to separate the two soil DNA fractions (Ascher et al., 2009) even though in different forest ecosystems, including silver fir pure forests (Agnelli et al., 2004), mixed black pine and silver fir forests (Ascher et al., 2009), and downy oak and maritime pine mixed forests (Borgogni et al., 2019). We can explain such consistent observations across different ecosystems considering that the content of exDNA in soil dynamically changes as a function of inputs in the upper layer by litterfall and decay, which feeds the soil microbiome turnover, and its persistence due to the interaction with the soil organic and mineral fractions (Levy-Booth et al., 2007, Morrissey et al., 2015). The pattern of decreasing content with depth corresponds to that of the soil organic matter, which in turns interplays with soil microbiome (Bonanomi et al., 2017). In other words, both fractions of soil DNA mostly correspond to microbial DNA, which is more abundant in the upper layers where the large availability of organic compounds sustains the growth and development of microbial living cells (Agnelli et al., 2004), hence enhancing iDNA content and the microbial turnover (Carini et al., 2016), which

in turn increases exDNA input in the soil by cell death and lysis. As the process is recursive, larger microbial mass also make available larger amounts of organic compounds, with positive feedback effects limited by the overall soil nutrient resources. Accordingly, the amount of exDNA has been used as a proxy of microbial activity (Nagler et al., 2018), based on the evidence that when the microbial turnover is rapid, exDNA is abundant in soil (Ascher et al., 2009), but when the soil is subjected to destructive events, the microbial community is rapidly degraded and its activity is not detectable (Borgogni et al., 2019).

Moreover, it is known that the soil microbiome undergoes a rapid turnover and variation with depth (Sirois & Buckley, 2019), mainly in association with the availability of organic carbon and nitrogen (Andersson et al., 2004). Consistently, we found a significant positive association between iDNA and exDNA content and soil acidity and carbonate content, which in local forest soils frequently vary along the vertical profile (Abramo & Michelutti, 1998), with a progressive increase in pH and carbonates with soil depth in relation to the calcareous nature of the rock substrate. Finally, our results did not confirm previous indications that DNA persistence in the soil could be enhanced by its interaction with the mineral soil fraction, particularly with the clay minerals, and with the recalcitrant humic organic fraction (Pietramellara et al., 2009; Ranjard and Richaume, 2001). Indeed, the content of both iDNA and exDNA was unrelated to the total clay content, as well as to the humification index in our samples. In the first case, our result could be simply explained by considering the broad range of effects of different clay minerals on exDNA persistence in soil (Morrissey et al., 2015). In the case of the humification index, our result could depend on co-occurring conditions at our sampling sites: i) a relative humification stability along the vertical profile (Table 1); and ii) the predominance of soil biological processes over its chemical properties in controlling DNA dynamics and persistence, as previously shown under homogeneous chemical–physical conditions (Ranjard and Richaume, 2001).

4.3 | Plant exDNA horizontal and vertical distribution

We found that the content of beech and spruce exDNA in the organic soil horizon reflected the species-specific tree density above ground. This result, which quantitatively depends on the species-specific DNA input by litterfall and decay, indirectly confirms the reliability of our methodological approach, as discussed in the previous section.

More interestingly, we found a progressive increase with depth of *F. sylvatica* exDNA under pure beech stands and, contrarily, a decrease with depth of *P. abies* exDNA proportion in pure spruce forests. Considering exDNA input by litterfall and decomposition, as we measured the same basal area levels for the two species at the pure stands (Figure 2), beech and spruce can release yearly comparable amounts of leaf litter (Pedersen & Bille-Hansen, 1999). However, although in this study we did not directly measure the rate of species-specific litterfall, it is reasonable to assume that, in the case of *P. abies*, litterfall is more evenly distributed throughout the year, while for *F. sylvatica*, it is mostly limited to the fall period. On the contrary, it is clear that the two opposite species-specific patterns of vertical exDNA distribution cannot be uniquely related to exDNA input by litterfall, but litter decomposition dynamics must be also taken into account. Once on the forest floor, litter is subjected to physical, chemical, and biological degradation (Hattenschwiler & Gasse, 2005), releasing organic

TABLE 2 Results of PERMANOVA testing for the effects of forest type (F), soil horizon (H), and their interaction between plant community composition, species richness, and Shannon's index

Effect	*df*	SS	MS	Pseudo-*F*	*p*
Community composition					
Forest type (F)	2	23870.0	11935.0	5.711	***0.001***
Soil horizon (H)	2	5574.1	2787.1	1.334	0.201
F×H	4	4806.6	1201.7	0.575	0.963
Error	27	56425.0	2089.8		
Species richness					
Forest type (F)	2	36.167	18.083	1.752	0.184
Soil horizon (H)	2	1.167	0.583	0.056	0.936
F×H	4	26.667	6.667	0.646	0.610
Error	27	278.75	10.324		
Shannon's index (H′)					
Forest type (F)	2	3.407	1.703	3.280	***0.049***
Soil horizon (H)	2	0.293	0.146	0.282	0.764
F×H	4	2.025	0.506	0.975	0.438
Error	27	14.022	0.519		

Note: Community metrics were calculated based on the exDNA metabarcoding results, and filtered considering only amplicons taxonomically attributed at species level ($N = 223{,}629$). Significant effects ($p < 0.05$) are marked in italic bold.

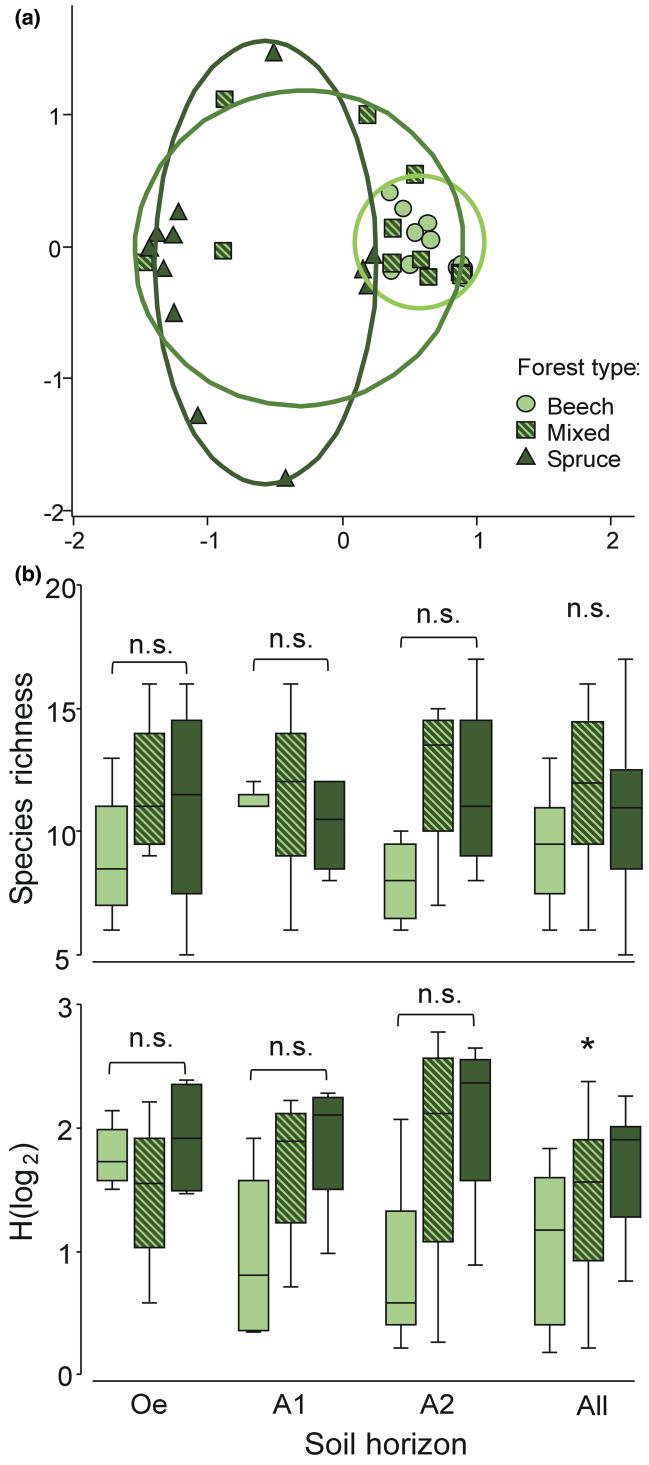

FIGURE 5 Plant community metrics as assessed by exDNA metabarcoding. (a) nMDS ordination of exDNA samples based on Bray–Curtis similarity calculated on species abundance data, showing changes of plant community composition according to stand composition. 2D stress score for the ordination is 0.13. (b) Box-and-whisker plots of species richness and Shannon's index H′ across the forest types, separately calculated for each soil horizon and for all data pooled. Data refer to median, quartiles (boxes), and extremes (whiskers). Statistical significance of beech vs. spruce differences within each soil horizon is also reported (n.s.: not significant, *: $p < 0.05$, pairwise comparisons after PERMANOVA analysis, detailed results in Table 2)

compounds that, if hydrophilic, can diffuse both horizontally and vertically following water movements through leaching and percolation (Laskowski and Berg, 2006). The accumulation of soil organic matter depends on decomposition rates and progressive preservation of recalcitrant compounds, which include not only hydrophobic biomolecules (Attiwill and Adams, 1993) but also resistant hydrophilic molecules physically and chemically protected by the interaction with the soil mineral fraction, as well as hydrophobic domains (Incerti et al., 2017). Among these, exDNA under suitable environmental conditions can persist for a long time in the soil (Levy-Booth et al., 2007), ranging from weeks to even years (Carini et al., 2016). The major determinants of decomposition dynamics include climatic variables and plant litter molecular properties, which interact and selectively prevail at regional and local spatial scales, respectively (Bradford et al., 2016). This also applies in our study, where species-specific exDNA input in the upper soil layer is strictly controlled by spruce vs. beech litter decomposition rates, which in turn depends on species-specific litter quality. Although we did not measure directly litter decay rates, some previous studies reported that *P. abies* litter decomposes at a fairly lower rate compared with *F. sylvatica* one (Bonanomi et al., 2013), as related to the spruce needles' relatively high content of recalcitrant hydrophobic compounds such as resins, cutins, and waxes (Incerti et al., 2011). Other more recent studies clearly showed that, more than an intrinsically different decay rate linked to litter chemistry, spruce and beech litter decompose significantly more slowly in pure spruce stands when compared to either mixed or pure beech stands because of changes in soil acidity, soil structure, and humus form (Albers et al., 2004; Berger and Berger, 2014). Therefore, the novel, stand-specific vertical distribution pattern of plant exDNA highlighted by our study could be easily explained considering that a more rapid decomposition in beech forest floor is likely to favor the plant exDNA vertical migration by percolation following water flow, while, under spruce stands, a slower litter decay may limit the release of exDNA and its vertical migration. However, the mechanism of exDNA transport along the soil column is complex and not yet fully clarified (Ascher et al., 2009; Ceccherini et al., 2007; Potè et al., 2007, 2010) and further investigation is required to fully clarify the vertical distribution pattern we observed. A possible, promising approach to study species-specific plant DNA dynamics in soil, and quantitatively trace exDNA fate and persistence in the soil environment, could be based on the use of isotopically labeled DNA in manipulative field studies, as recently suggested by Foscari et al. (2021) in a study proposing a protocol to maximize heavy ^{15}N-labeled DNA extraction yield from plant material. Finally, the higher beech exDNA proportion at the deepest horizon could rely on the nucleic acid release by root exudates and turnover (Pietramellara et al., 2009). Although the role of such processes and their contribution to the nutrient cycle in forests have not yet been fully evaluated (Lukac, 2012), it is suggestively consistent with the different rooting depth ranges of beech vs. spruce (Schmid and Kazda, 2001), with the latter showing much lower exDNA content in the soil layers where its roots are less abundant, and vice versa.

TABLE 3 Result of PERMANOVA pairwise significance test across forest types at the field sites, on plant community metrics based on exDNA diversity

	Composition		Species richness		Shannon's index (H′)	
Comparison	***t***	***p***	***t***	***p***	***t***	***p***
Soil horizon Oe						
Beech vs. mixed	1.020	0.389	1.288	0.254	0.836	0.423
Beech vs. spruce	2.399	***0.010***	0.722	0.525	0.501	0.614
Mixed vs. spruce	1.161	0.271	0.267	0.800	1.067	0.300
Soil horizon A1						
Beech vs. mixed	1.028	0.371	0.120	0.895	1.405	0.221
Beech vs. spruce	2.109	***0.017***	0.943	0.365	1.870	0.119
Mixed vs. spruce	1.205	0.243	0.542	0.612	0.425	0.669
Soil horizon A2						
Beech vs. mixed	0.933	0.469	2.109	0.076	1.391	0.225
Beech vs. spruce	1.881	***0.041***	1.756	0.134	2.096	0.082
Mixed vs. spruce	1.431	0.089	0.190	0.850	0.3627	0.730
All horizons						
Beech vs. mixed	1.331	0.122	2.016	0.060	1.469	0.159
Beech vs. spruce	3.466	***0.001***	1.300	0.237	2.802	***0.013***
Mixed vs. spruce	1.975	***0.009***	0.557	0.602	0.965	0.357

Note: Analysis based on the exDNA metabarcoding results, and filtered considering only amplicons taxonomically attributed at species level ($N = 223{,}629$). In the PERMANOVA analysis, forest types and soil horizons were used as fixed factors, and replicated plots as random factor. Significant values ($p < 0.05$) are marked in italic bold.

4.4 | Plant community composition and diversity by exDNA metabarcoding

Our analysis by exDNA metabarcoding provided consistent information about the vegetation in the survey area as compared to the local phytosociology (Mucina, 1997). Indeed, in addition to the two dominant tree species, we found exDNA attributed to tree species occurring also at higher (*Pinus mugo*) or lower (*Pinus sylvestris*, *Abies alba*) altitude, scattered in some of our samples, mostly in mixed forest stands. Considering shrubs and herbaceous species, we found some characteristic entities of the association, such as *Vaccinium myrtillus*, *Carex alba*, *Anemone trifolia*, *Polystichum aculeatum*, and *Erica carnea*, although unequally distributed among plots and sporadically in some cases. Other species such as *Hedera helix*, *Hypericum perforatum*, and *Urtica dioica* were widespread in many of our samples, though being not characteristic of beech–spruce forests, as showing a cosmopolitan distribution.

Considering compositional and diversity differences among the plant communities at the three forest types, we found that species richness was not significantly different among the forest types, as being highly variable among the plots of each forest type (Figure 5). This latter result could trivially reflect the variability of exDNA taxonomy at small spatial scale, considering the scattered within-plot distribution of most plant species (with the exception of the ubiquitous dominant tree species). This could also explain our finding of a significantly higher compositional heterogeneity in spruce forests as compared to mixed and beech ones, as shown by both the NMDS graph and the Shannon index H′ (Figure 5), which contrasts with previous reports of higher biodiversity in mixed stands in spruce–beech systems (Vacek et al., 2021). It is reasonable assuming that the small-scale variability intrinsic in our exDNA assessment may have masked the general pattern, rather than considering our study plots a remarkable exception to a generalized phenomenon.

5 | CONCLUSIONS

In this study, for the first time, we provided a clear picture of total iDNA and exDNA, as well as species-specific plant exDNA proportion and distribution, in spruce or beech pure and mixed forest soils. About total iDNA and exDNA, our study confirms previous observations from other different ecosystems, showing a progressive depletion with soil depth, independent of forest type, in relation to the interplay among inputs, soil chemical–physical features, and microbial turnover.

Our results clearly show the possibility to successfully use the metabarcoding approach to obtain a quantitative overview of species-specific exDNA proportion and distribution. While not producing direct mass balance evidence, our results demonstrate clear-cut different patterns of vertical distribution for beech exDNA vs. spruce exDNA, as likely related to species-specific differences in litter quality and microenvironmental conditions, both decisively affecting litter decomposition dynamics in the different forest types.

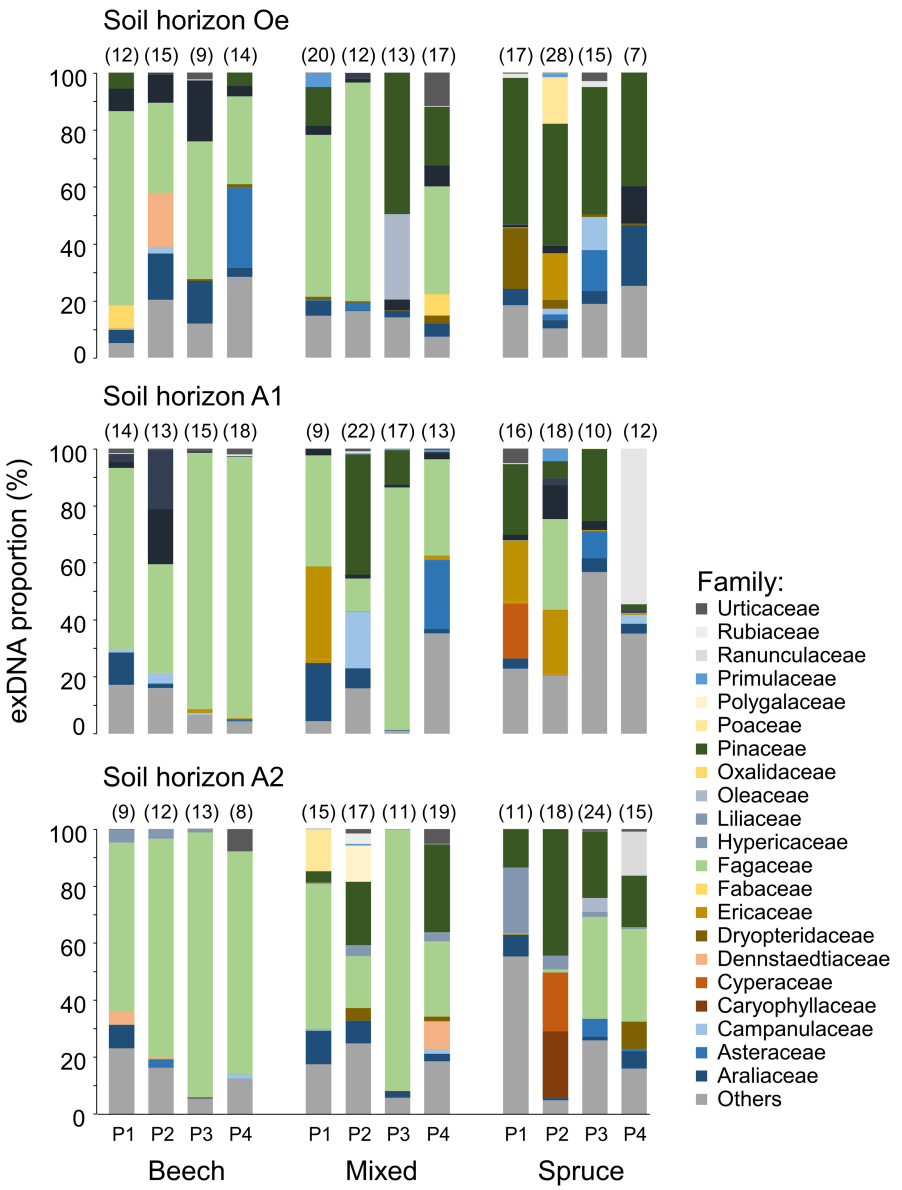

FIGURE 6 Plant community diversity assessed by exDNA metabarcoding at family level. Stacked bar chart of percentage proportion of plant families exDNA in each sampling site stratified for forest type (pure beech, and mixed and pure spruce) and soil horizon (Oe, A1, and A2). Numbers in brackets refer to the total number of species at each site

Finally, exDNA metabarcoding also provided a faithful, although incomplete, picture of the local plant diversity, indicating that such technique could positively integrate traditional biodiversity inventory studies based on expert field assessments.

AUTHOR CONTRIBUTIONS

GI, AF, and GA designed the study. AF did the sampling under GI supervision. AF carried out the DNA extraction and quality controls. AF and MZ performed the analyses and interpreted the data under the supervision of GI and GA. AF and GI led the writing. GI was responsible for funding of this study. All authors contributed to the manuscript and gave final approval for publication.

ACKNOWLEDGMENT

We are grateful to Nicoletta Felice for technical assistance during laboratory work, Giusi Zaina for critical discussion about the methodology, Marco Pontello for support during laboratory work, and Diego Chiabà for fieldwork assistance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study, when not provided in the manuscript or in the Supplementary Material, are openly available in Sequence Read Archive (SRA) of NCBI databases, with accession number PRJNA767787.

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How to cite this article: Foscari, A., Alberti, G., Zotti, M. & Incerti, G. (2022). Species-specific DNA distribution in spruce–beech forest soil. *Environmental DNA*, *00*, 1–16. https://doi.org/10.1002/edn3.307