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Genetic Diversity and Genetic Structure of Three Sympatric Oak Species in Serbian Landscape of Outstanding Features "Kosmaj" Assessed by Nuclear Microsatellites

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ABSTRACT

Quercus petraea (Matt.) Liebl., *Q. pubescens* Willd., and *Q. frainetto* Ten. grow naturally in sympatry in the Landscape of Outstanding Features "Kosmaj" (Mt. Kosmaj) in Serbia, in the Western Balkans. The levels of genetic diversity and genetic structure in populations of these species (160 trees in total) was assessed by means of 14 nuclear microsatellites. The number of alleles detected in overall sample was 314, with the locus QrZAG90 being the most informative one in all three species (31, 35 and 36 alleles in *Q. frainetto*, *Q. pubescens* and *Q. petraea*, respectively). The levels of genetic diversity of all three species were relatively high (H_E = 0.824, 0.834, and 0.794 in *Q. petraea, Q. pubescens* and *Q. frainetto*, respectively). Low but statistically significant inbreeding was detected in all three species (F = 0.100, 0.131 and 0.065 in *Q. petraea, Q. pubescens* and *Q. frainetto*, respectively), which, however, most likely reflects population substructure, which was observed in the STRUCTURE analysis. The optimal number of genetic groups revealed by Bayesian clustering analysis did not coincide with the number of analyzed species as it amounted to four (one gene pool was observed in each of the two species, *Q. petraea* and *Q. pubescens*, while two gene pools were observed in *Q. pubescens*). Furthermore, a higher level of hierarchical genetic structure, with six gene pools was found, with *Q. frainetto* being the only oak species in which population substructure was not observed. Genetic differentiation of three sympatric oak species was relatively low but statistically significant, with the highest F_{ST} value found among *Q. petraea* and *Q. frainetto* (F_{ST} = 0.047, P = 0.001), and the lowest between *Q. petraea* and *Q. pubescens* (F_{ST} = 0.032, P = 0.001). Our results reveal rather high levels of genetic diversity in all three studied oak species, which retained their genetic integrity despite sympatric distribution, indicating low levels of interspecific hybridization, and pronounced genetic structure of *Q. pubescens* and *Q. petraea.*

Keywords: *Quercus petraea* (Matt.) Liebl.; *Quercus pubescens* Willd.; *Quercus frainetto* Ten.; nuclear SSRs (Simple Sequence Repeats)

INTRODUCTION

Genetic diversity is a key requirement for the long-term survival of species on an evolutionary time scale (Pakkad et al. 2008, Fady et al. 2022) and is usually rather high in long-lived forest tree species (Kremer et al. 2012, Rajora and Zinck 2021). Forests play important roles in ecosystem functioning, species diversity maintenance, climate regulation, and soil and water conservation. Thus, the biodiversity and stability of the entire forest ecosystems are mainly dependent on the genetic diversity of forest trees (Sandurska et al. 2019), commonly shaped by pollen and seed dispersal (Dow and Ashley 1998), demographic changes and evolutionary processes, such as genetic drift and migration, which may account for the occurrence of genetic structure in forest tree populations (Fernández-M and Sork 2006). In forest trees, gene flow is particularly important, as it has the ability to both permit local genetic differentiation and introduce variability in isolated populations (Ducousso et al. 1993) as well as to erase present genetic structure over generations.

Genetic diversity loss became particularly important in the context of climate change, relevant also for the production of well-adapted reproductive material used for reforestation (Ivetić et al. 2016, Katičić Bogdan et al. 2018, Popović et al. 2022). Information on genetic variability, genetic structure, and inbreeding is therefore required for the effective management of forest populations (Craft and Ashley 2007).

Widely known species richness of the Balkan Peninsula is a result of complex geological history, and interactions between populations, species, and ecosystems (Savić 2008). Among many forest tree species, oaks are one of the ecologically and economically most important genera in the northern hemisphere. A total of 12 oak species occur in the Balkans, including six species belonging to the *Quercus* (white oak) group (Nixon 1993). In the genus *Quercus*, several taxonomic groups are characterized by complex patterns of variation, leading to difficulties in the identification of taxa to the species level (Bruschi et al. 2000, Gömöry et al. 2001). Resolving the species boundaries in oaks can be challenging due to the presence of natural hybrids and introgressive forms, especially in contact zones where two or more species occur (Curtu et al. 2007, Viscosi et al. 2009, Yücedağ and Gailing 2013, Lyu et al. 2018). One of such sites, where three oak species, namely *Quercus petraea* (Matt.) Liebl., *Quercus pubescens* Willd., and *Quercus frainetto* Ten., grow in sympatry, is found in Serbia, in the protected zone (protection degree II) within the Landscape of Outstanding Features "Kosmaj" at the Mt. Kosmaj. This site is geographically close to the metropolitan region and it is threatened by habitat loss and degradation, mostly due to the expansion of agricultural and urban areas, illegal cutting of forests for firewood and other wood products, and excessive grazing. These autochthonous oak populations are important both from scientific and economic point of view (Jovanović et al. 2023). Studies of genetic diversity of oaks in Serbia were conducted by several authors, using both chloroplast and nuclear DNA (e.g., Milovanović 2009, Šijačić-Nikolić et al. 2009, Ballian 2010, Neophytou et al. 2010, Kesić et al. 2021, Šijačić-Nikolić et al. 2021, Popović et al. 2022). However, at the region of the Landscape of Outstanding Features "Kosmaj" no such research has been conducted so far.

Due to the overexploitation of forests, together with poorly understood environmental changes, which have resulted in the decline of many oak species, oaks are involved in an increasing number of studies related to gene flow and genetic structure (e.g. Ducousso et al. 1993, Lepais et al. 2009, Fortini et al. 2015, Sandurska et al. 2019, Leroy et al. 2020). Gene flow levels in oaks are high and mostly related to the life history traits of its species, such as phenology, mating system, wind pollination, acorn production, etc. (Ducousso et al. 1993). Due to high levels of intraspecific diversity and hybridization between the species which cause the boundaries of species differentiation to be less distinctive, discerning some oak species at the molecular level and obtaining species-specific diagnostic markers can be challenging (Kelleher et al. 2005). Because of the highly informative and codominant nature, hypervariability, reproducibility, and the possibility of parallel amplification nSSRs are widely used for genotyping in plant and animal species. Although the usage of nSSRs may be challenging (e.g., Kerkez Janković et al. 2019), they have been proven suitable for assessing the levels of genetic diversity in oak species (Kesić et al. 2021). Furthermore, Muir et al. (2000) have demonstrated that nSSRs may be used for differentiation of phylogenetically distant species like *Q. robur* and *Q. petraea* despite the high levels of interspecific gene flow. For phylogenetically close species, genetic diversity in populations in hybrid zones or at the distributional margins of species, EST-SSR markers are useful (Ueno et al. 2008, Aizawa et al. 2018).

The aims of this study were to assess the genetic diversity and structure of *Q. petraea*, *Q. pubescens*, and *Q. frainetto* from the Landscape of Outstanding Features "Kosmaj" (Mt. Kosmaj, Serbia) using nuclear microsatellites. Levels of inter- and intraspecific genetic variability reveled in this study can lay the groundwork for setting the guidelines for the conservation of the available gene pool and prescribing measures for future forest management.

MATERIALS AND METHODS

Study Site and Plant Material

A total of 187 trees found in the protected area of Mt. Kosmaj (44°28'17.68"N, 20°34'32.04"E) were selected for genotyping – 65 of *Q. pubescens*, 60 of *Q. petraea*, and 62 of *Q. frainetto* (Figure 1). The protection zone occupies 3514.50 ha of a forest complex surrounded by predominantly agricultural land and rural settlements. At the lower altitudes (250-400 m a.s.l.) dominant forest type is *Quercetum frainetto-cerris* Rudski, above 400 m a.s.l. *Querco-Carpinetum serbicum* Rudski and from 500 to 626 m a.s.l. *Fagetum montanum* Rudski. At Mt. Kosmaj oaks are abundantly present in sympatry, which can be seen from forest typology previously mentioned, but are negatively impacted by the lack of natural regeneration and the coppice origin.

Samples for molecular genetics analyses comprised of young leaves which were collected in May 2022. The selection of trees was based on phenotypic characteristics (trunk and crown appearance) and general health status (absence of entomological and phytopathological damage). From each selected tree up to five normally developed, healthy leaves were sampled, herbarized, dried in silica gel, and kept in a freezer prior to the analyses. Thus, only adult trees, distant at least 50 m from each other, were sampled.

DNA Extraction, PCR Amplification and Fragment Sizing

Samples were processed in Biotechnology Laboratory at the Faculty of Forestry, University of Belgrade (Serbia). For each selected tree up to 20 mg of dried leaves were homogenized with TissueLyser II (Qiagen, Valencia, CA, USA) and used for extraction of the total genomic DNA with *peqGOLD Plant DNA Mini Kit* (PEQLAB). Genomic DNA was quantified and assessed for purity utilizing NanoVue (GE Healthcare Europe, Freiburg, Germany). DNA solutions were diluted to working concentrations of 50 ng·μl^{-1}.

For genotyping, 20 nuclear microsatellites were selected, 14 of which proved to be very informative and reliable after testing on a panel of 8 samples (Table 1). For the parallel amplification Type-it Microsatellite PCR Kit (Qiagen) was used. Microsatellite loci were grouped into two mixes: OM1 – PIE239, FIR004, QrZAG90, QrZAG108, MSQ13, GOT004,

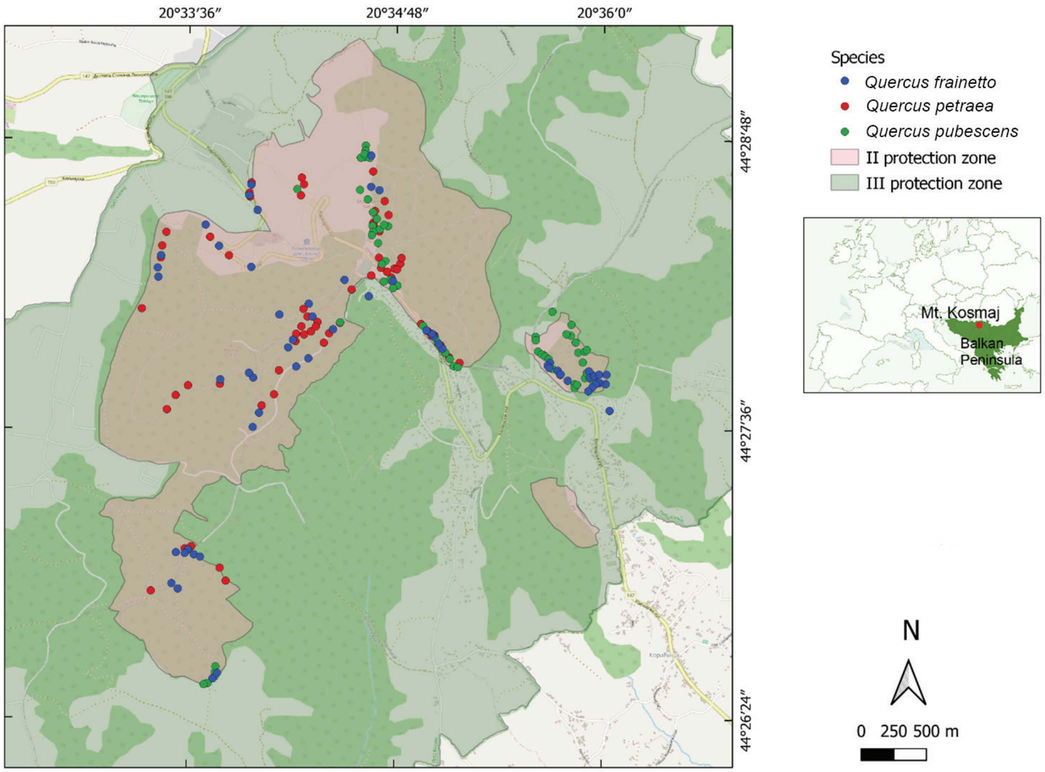

Figure 1. Distribution of the selected oak trees used in the study in the Landscape of Outstanding Features "Kosmaj", with the representation of the protection zones (II and III).

QrZAG87, QpZAG104, QrZAG11, QrZAG103, QrZAG102, and OM2 – QpZAG36, QrZAG101, MAQ4, PIE242, QrZAG20, QpZAG1/2, QpZAG58, QrZAG7, QpZAG110. Forward (F) primers from each primer pair of all selected loci were labelled with one out of the four different fluorescent dyes from the Dye set G5 (DS-33, Applied Biosystems, USA) to enable fragment sizing on an automated DNA fragment analyser. PCRs were carried out using Multigene Opti Max (Labnet International, Inc.). The PCR amplifications were performed as follows: initial denaturation at 95°C for 5 min; then 28 cycles of denaturing for 30 s at 95°C, annealing for 90 s at a 60°C, and extension for 30 s at 72°C; with a final extension at 60°C for 30 min. The PCR products were visualized on 1% agarose gels. PCR amplification products were separated commercially by Center for Forensic and Applied Molecular Genetics at the Faculty of Biology, University of Belgrade via capillary electrophoresis using 96-capillary 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Inc. USA). The lengths of PCR amplification products were assessed using GeneMapper (Applied Biosystems Inc., Foster City, USA), with the GeneScanTM-600LIZTM Size Standard (Applied Biosystems).

Data Analysis

The standard parameters of genetic diversity: number of alleles (A), number of private alleles (PA), average number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (H_O), expected heterozygosity (H_E) and coefficient of inbreeding (F), were determined using the GenAlEx 6.5 software package. The effective population size (Ne) was assessed using the NeEstimator software package (Do et al. 2014) with the rejection of alleles whose frequency was ≤0.02. Allelic richness was estimated using the HP-Rare 1.0 software package (Kalinowski 2005). Genetic structure was determined using two approaches: (1) obtained allele frequencies and genotypes generated for 14 molecular markers were used to determine the parameters of genetic differentiation expressed through the F_{ST} values, and PCoA analysis using the program package GenAlEx 6.5; obtained F_{ST} values were also used to assess levels of gene flow, Nm, according to the formula $Nm = [(1 / F_{ST}) - 1] / 4$; (2) Bayesian method implemented in the STRUCTURE 2.3.4 software package (Pritchard et al. 2000) was used to determine the optimal number of genetic groups (K) using the ΔK Evanno model (Evanno et al. 2005), and to determine the potential substructure of populations. Monte Carlo Markov Chain (MCMC) simulation had burn-in and run lengths of 700,000 iterations each. Ten independent analyzes for each assumed group K = 1-6 were performed. The model of correlated (dependent) frequencies of alleles was used as the allele frequency model, and the admixture model was used as the model of individual kinship.

Table 1. A list of 14 informative nuclear microsatellites used in the study, with primer sequences for their amplification, repeat motif, allele range available in literature, and references.

Locus	Primer sequences	Repeat motif	Allele range size (bp)	Reference
PIE239	CAACAAATGGCTCAACAGTG CCCATTTGGTAGCAAAGAGTC	$(AT)_{12}$	70-83	Durand et al. 2010
FIR004	TCTCTCTCAGGGCAGCTTCT AACCAAACTCAGATCCAGATTCA	$(CT)_{18}$	123-179	Durand et al. 2010
QrZAG90	GGAAGATAGTACC AGCTTGGTGAAT GCCTCATCTCACAGGTCACTCC	(GA)34	220-244	Kampfer et al. 1998
QrZAG108	AAGAGAGCAAATTTAGAGTGATGT GAACCTTGATCATACGTGGAGA	$(GA)_{19}(GGGA)_{3}$	80-108	Kampfer et al. 1998
MSQ13	TGGCTGCACCTATGGCTCTTAG ACACTCAGACCCACCATTTTTCC	(TC)	191-221	Dow et al. 1995
QrZAG87	TCCCACCACTTTGGTCTCTCA GTTGTCAGCAGTGGGATGGGTA	$(TC)_{20}$	110-131	Kampfer et al. 1998
QpZAG104	ATAGGGAGTGAGGACTGAATG GATGGTACAGTAGCAACATTC	$(AG)_{16}AT(GA)_{3}$	176-196	Steinkellner et al. 1997
QrZAG11	CCTTGAACTCGAAGGTGTCCTT GTAGGTCAAAACCATTGGTTGACT	$(TC)_{22}$	238-267	Kampfer et al. 1998
QrZAG101	CCTGCACAATCAAATCCTTCACTT GCCATGAACAACGG AGGTATCTAG	$(TC)_{20}(AC)_{15}$	136-160	Kampfer et al. 1998
MAQ4	TCTCCTCTCCCCATAAACAGG GTTCCTCTATCCAATCAGTAGTGAG	(AG)	203-227	Dow et al. 1995
PIE242	GGAGGGAAAAGAACAATGC TTGCAATCCTCCAAATTTAATG	$(TA)_{10}$	102-128	Durand et al. 2010
QrZAG20	CCATTAAAAGAAGCAGTATTTTGT GCAACACTCAGCCTATATCTAGAA	$(TC)_{18}$	160-200	Kampfer et al. 1998
QrZAG7	CAACTTGGTGTTCGGATCAA GTGCATTTCTTTTATAGCATTCAC	$(TC)_{17}$	115-153	Kampfer et al. 1998
QpZAG110	GGAGGCTTCCTTCAACCTACT GATCTCTTGTGTGCTGTATTT	$(AG)_{15}$	206-262	Steinkellner et al. 1997

RESULTS

Genetic Diversity

The summary of genetic diversity parameters obtained upon analyzing variability at 14 microsatellite loci in the three oak species is shown in Table 2, while the summary of genetic diversity parameters per species and in overall sample is shown in Table 3. A total of 314 alleles were detected in overall sample. The smallest number of alleles in *Q. petraea* was observed at the PIE239 locus (6), in *Q. pubescens* at QrZAG108 (6), and in *Q. frainetto* at QrZAG87 (7). The largest number of alleles for all tree species was observed at the QrZAG90 locus (A = 36, 35, and 31; Ae = 24.69, 20.64, 22.58 in *Q. petraea, Q. pubescens* and *Q. frainetto*, respectively). A total of 249 alleles were detected in *Q. petraea*, 238 alleles in *Q. pubescens*, and 213 alleles in *Q. frainetto* (Table 3). The average number of alleles per locus in the entire sample was 16.67 (SE = 1.07), the average effective number of alleles per locus 8.35 (SE = 0.87), and the average allelic richness obtained by the rarefaction method for 98 gene copies was 16.34. The number of private alleles was 29 in *Q. petraea*, 34 in *Q. pubescens* and 18 in *Q. frainetto*. The effective population size was 305.5 (190.2; 719.2) in *Q. pubescens*, 484.3 (254.8; 3374.5) in *Q. petraea*, and ∞ (1859.9; ∞) in *Q. frainetto*. The observed heterozygosity (Ho) ranged from 0.731 (SE = 0.051) (*Q. pubescens*) to 0.753 (SE = 0.053) (*Q. petraea*), with an average value of 0.745 (SE = 0.030). The expected heterozygosity (He) ranged from 0.794 (SE = 0.033) (*Q. petraea*) to 0.834 (SE = 0.027) (*Q. pubescens*), with an average value of 0.817 (SE = 0.019). A low but statistically significant excess of homozygotes was detected in the populations of all tested oak species.

Genetic Differentiation

To better understand the relationships between the three sympatric oak species, the gene flow (Nm) and genetic differentiation coefficient (F_{ST}) were estimated for all pairs of species (Table 4). The genetic differentiation between species was low but statistically significant ($P \le 0.05$), and ranged from 0.032 among *Q. pubescens* and *Q. petraea*, to 0.047 among *Q. petraea* and *Q. frainetto*. Consequently, the highest gene flow (Nm = 7.563) was observed between *Q. petraea* and *Q. pubescens*. The lowest gene flow (Nm = 5.069) was observed between *Q. petraea* and *Q. frainetto*.

Principal coordinate analysis (PCoA) results, obtained by summarizing the genetic distances between genotypes within each of the populations showed that *Q. frainetto* separated from *Q. pubescens* and *Q. petraea* along the first principal coordinate, while the separation of *Q. pubsecens* and *Q. petraea* was observed along the second principal coordinate (Figure 2). The first principal coordinate (Coord. 1) explained 63.32% of the variability, and the second principal coordinate (Coord. 2) 36.68% of the variability, suggesting high reliability of the obtained results.

Table 2. Summary of the genetic diversity parameters based on genotyping with 14 nuclear microsatellite loci in the three oak species.

Species	***Q. petraea***						***Q. pubescens***						***Q. frainetto***					
Locus	**N**	**A**	**Ae**	**H_O**	**H_E**	**F**	**N**	**A**	**Ae**	**H_O**	**H_E**	**F**	**N**	**A**	**Ae**	**H_O**	**H_E**	**F**
FIR004	52	21	14.383	0.942	0.93	-0.013	53	20	14.707	0.83	0.932	0.109	55	18	11.415	0.891	0.912	0.024
PIE239	52	6	1.633	0.231	0.388	0.405	53	12	2.984	0.509	0.665	0.234	55	9	3.286	0.4	0.696	0.425
PIE242	52	15	7.48	0.865	0.866	0.001	53	13	6.12	0.811	0.837	0.03	55	16	7.544	0.891	0.867	-0.027
MAQ4	52	13	6.216	0.596	0.839	0.29	52	14	6.5	0.5	0.846	0.409	54	12	4.696	0.593	0.787	0.247
MSQ13	52	13	4.189	0.731	0.761	0.04	53	13	4.41	0.66	0.773	0.146	55	16	5.465	0.873	0.817	-0.068
QpZAG11	52	13	4.337	0.538	0.769	0.3	49	17	10.088	0.551	0.901	0.388	54	11	2.508	0.537	0.601	0.107
QpZAG104	52	28	17.731	0.923	0.944	0.022	52	25	11.938	0.846	0.916	0.076	55	27	16.22	0.945	0.938	-0.008
QpZAG110	52	19	7.501	0.865	0.867	0.001	53	18	5.958	0.83	0.832	0.002	55	13	2.307	0.582	0.567	-0.027
QrZAG7	52	19	12.126	0.692	0.918	0.245	53	17	9.753	0.925	0.897	-0.03	55	13	6.335	0.873	0.842	-0.036
QrZAG20	52	18	8.503	0.865	0.882	0.019	53	16	9.571	0.849	0.896	0.052	55	12	6.97	0.873	0.857	-0.019
QrZAG87	52	15	5.307	0.808	0.812	0.005	52	13	3.567	0.769	0.72	-0.069	55	7	4.13	0.745	0.758	0.016
QrZAG90	52	36	24.694	0.885	0.96	0.078	52	35	20.641	0.962	0.952	-0.01	55	31	22.575	0.964	0.956	-0.008
QrZAG101	52	21	11.834	0.942	0.915	-0.029	53	19	7.825	0.868	0.872	0.005	55	18	8.473	0.909	0.882	-0.031
QrZAG108	52	12	3.153	0.654	0.683	0.043	53	6	2.751	0.321	0.637	0.496	55	10	2.789	0.436	0.641	0.32
Total/ Average	52	17.786	9.22	0.753	0.824	0.100	52.429	17	8.344	0.731	0.834	0.131	54.857	15.214	7.48	0.751	0.794	0.065
SE	0	1.984	1.704	0.053	0.04	0.038	0.291	1.825	1.334	0.051	0.027	0.048	0.097	1.795	1.548	0.053	0.033	0.041

N – population size; A – number of alleles; Ae – effective number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; F – coefficient of inbreeding; SE – standard error

Table 3. Standard genetic diversity parameters in populations of the three oak species.

Species	**N**	**A**	**PA**	**Na (SE)**	**Ae (SE)**	**Ar98**	**Ne (95% CI)**	**H_O (SE)**	**H_E (SE)**	**F (SE)**
Q. petraea	52	249	29	17.786	9.220	17.54	484.3	0.753	0.824	0.100
				-1.984	-1.704		(254.8; 3374.5)	(0.053)	(0.040)	(0.038)
Q. pubescens	53	238	34	17.000	8.344	16.71	305.5	0.731	0.834	0.131
				-1.825	-1.334		(190.2; 719.2)	(0.051)	(0.027)	(0.048)
Q. frainetto	55	213	18	15.214	7.480	14.76	∞	0.751	0.794	0.065
				-1.795	-1.548		(1859.9; ∞)	(0.053)	(0.033)	(0.041)
Total / Average	160	314	81	16.667	8.348	16.34	-	0.745	0.817	0.099
				-1.066	(0.872)			(0.030)	(0.019)	(0.024)

N – population size; A – number of alleles; PA – number of private alleles; Na – average number of alleles per locus; Ae – average effective number of alleles per locus; Ar98 – allelic richness according to the rarefaction method for 98 gene copies; Ne – effective population size; 95% CI – 95% confidence intervals; H_O – observed heterozygosity; H_E – expected heterozygosity; F – coefficient of inbreeding; SE – standard error

Table 4. The F_{ST} values and Nm between the pairs of populations of *Q. petraea*, *Q. pubescens* and *Q. frainetto*.

Species	**Q. petraea**	**Q. pubescens**	**Q. frainetto**
Q. petraea	0.000	Nm = 7.563	Nm = 5.069
Q. pubescens	F_{ST} = 0.032	0.000	Nm = 6.160
Q. frainetto	F_{ST} = 0.047	F_{ST} = 0.039	0.000

PCoA results obtained by summarizing the genetic distances between genotypes within each of the individuals of the three species (Figure 3) showed the same grouping pattern as in population analysis (Figure 2) despite the small percentage of the total variability explained by the first two principal coordinates (12.27%, Coord. 1 = 6.84%, Coord. 2 = 5.43%).

Genetic Structure

The optimal number of genetic groups in the study sample, obtained by the ΔK Evanno model, was four (Figure 4). However, a higher level of hierarchical structure, with six genetic groups, was observed as well.

Results of the STRUCTURE analysis with four (K = 4) and six (K = 6) genetic groups in *Q. petraea*, *Q. pubescens*, and *Q. frainetto* are shown in Figures 5 and 6.

The STRUCTURE analysis showed that *Q. frainetto* represents a coherent and distinct genetic group that was not substructured, i.e., in which a large number of individuals had a high proportion of assignment to the unique gene pool. In contrast to *Q. frainetto*, the populations of *Q. petraea* and *Q. pubescens* were substructured, i.e., comprised individuals strongly assigned (qi > 0.80) to distinct gene pools. The population of *Q. petraea* in the area of Mt. Kosmaj consisted of two separate genetic groups. The substructure has also been observed in the population of *Q. pubescens* (Figure 6). The results of the STRUCTURE analysis under the assumption of six genetic groups (K = 6) showed that *Q. pubescens* population comprised individuals strongly assigned (qi > 0.80) to three distinct gene pools.

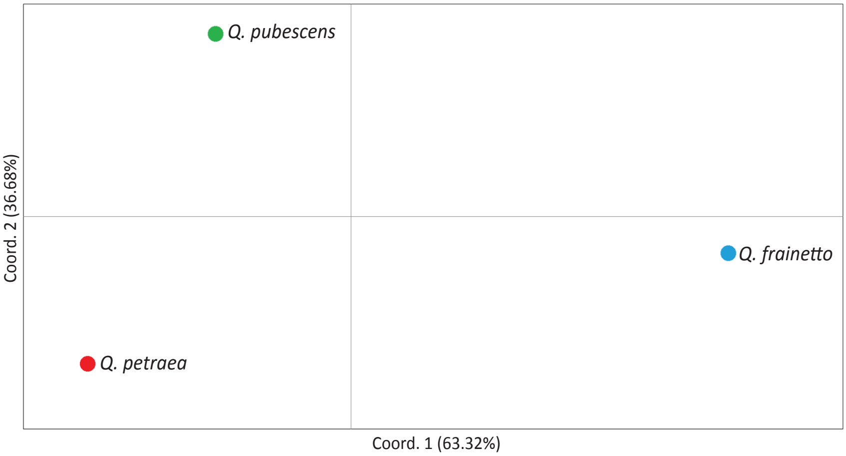

Figure 2. Scatterplot of *Q. petraea*, *Q. pubescens*, and *Q. frainetto* populations based on PCoA analysis obtained by the genetic distances.

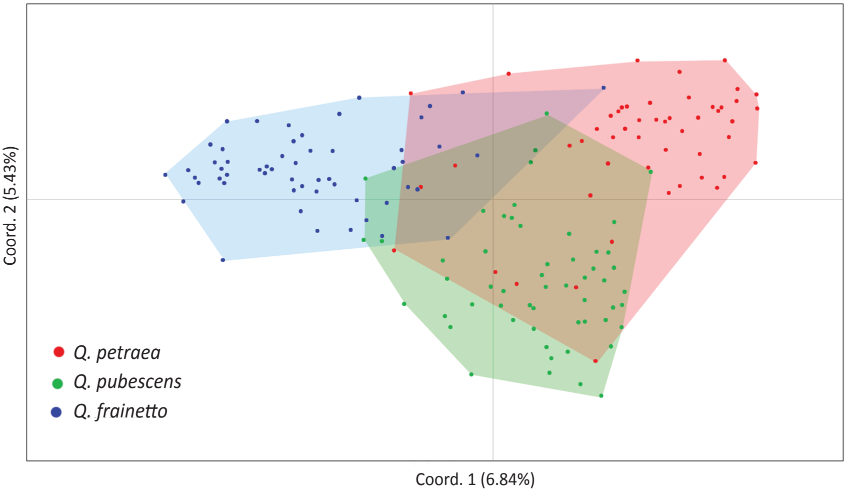

Figure 3. Scatterplot of *Q. petraea*, *Q. pubescens*, and *Q. frainetto* individuals based on PCoA analysis obtained by the genetic distances.

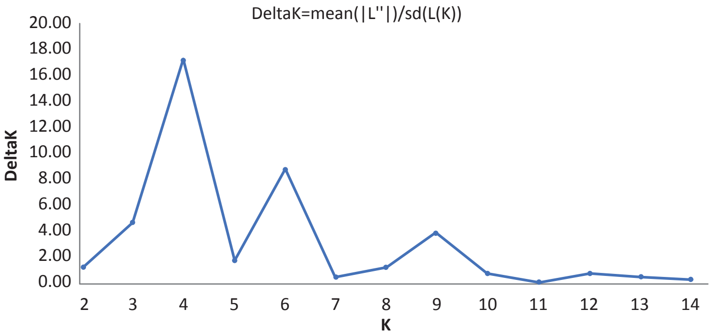

Figure 4. The optimal number of genetic groups in the studied oak populations determined by the ΔK Evanno model.

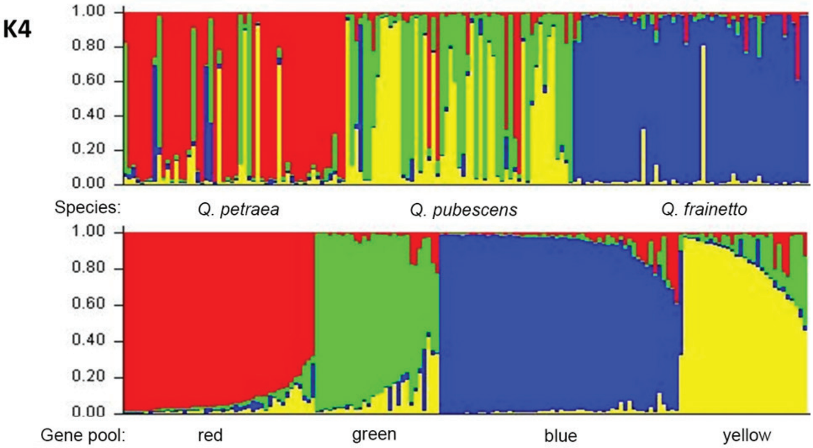

Figure 5. The results of the STRUCTURE analysis under the assumption of four genetic groups (K = 4). The proportion of each cluster group for each individual is shown by the color code.

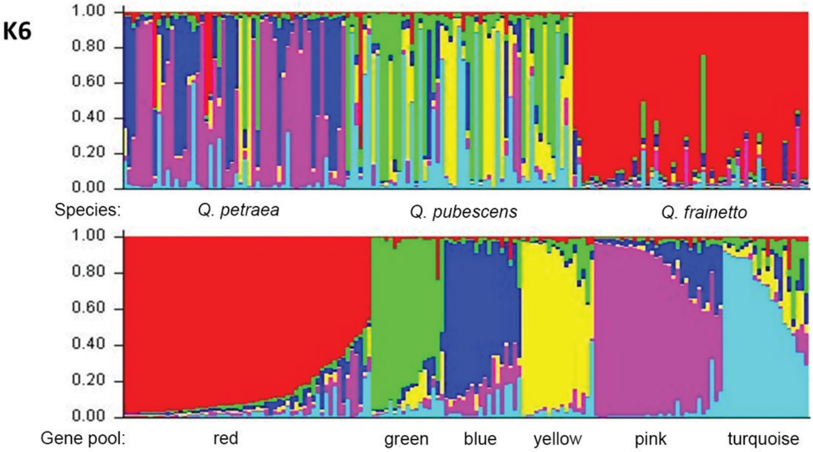

Figure 6. The results of the STRUCTURE analysis under the assumption of six genetic groups (K = 6). The proportion of each cluster group for each individual is shown by the color code.

DISCUSSION

For the conservation of forest genetic resources and sustained forest management, knowledge of the levels and distribution of genetic variation is crucial, especially if it is related to genetic processes taking place during reproduction and stand establishment, i.e. during developmental stages that are under high anthropogenic influence (Liesebach and Zaspel 2004). However, complex patterns of genetic diversity and characteristic phenotypic plasticity of oaks hampered the efforts to manage their gene pools and accurately assess the conservation status of most of the oak taxa (Sullivan et al. 2013). While oaks are capable of high rates of local adaptation, their long generation times and immobility make them vulnerable to rapid environmental changes (Yan et al. 2019). The high levels of genetic diversity favor rapid adaptation, and the result of this local adaptation is the development of a phenotype that optimizes the response to environmental pressures, and is associated with the highest fitness (Kremer 2010).

Q. petraea, *Q. frainetto* and *Q. pubescens* belong to the *Quercus* (white oak) group (Nixon 1993) and their populations are rather common in Serbia, in the Western Balkans, where they occasionally occur in sympatry. This is the case with the site at Mt. Kosmaj, which acquired the status of the Landscape of Outstanding Features, due to its species richness and diversity, in addition to cultural, historical, and geological features. *Q. petraea*, *Q. frainetto* and *Q. pubescens* are autochthonous in this area (Stajić et al. 2019), and have not been studied to date at the molecular level. We analyzed 160 individuals of these three species with 14 nuclear microsatellites in order to assess the levels of genetic diversity in their populations and their genetic structure, needed for the formulation of conservation strategies and management practice.

The parameters of genetic diversity obtained in our study are consistent with previous reports in which oak species were analyzed with the same type of molecular markers (e.g., Muir and Schloetterer 2005, Salvini et al. 2009, Neophytou et al. 2010, Alberto et al. 2010, Katičić Bogdan et al. 2018). The values of expected heterozygosity for the three oak species were also recorded by different authors across Europe – e.g., ranging from 0.781 to 0.815 in *Q. petraea* from central Europe and Balkan Peninsula (Neophytou et al. 2010), from 0.251 to 0.890 in *Q. pubescens* populations from Italy (Di Pietro et al. 2020), or from 0.701 to 0.929 in *Q. pubescens* and from 0.181 to 0.922 in *Q. frainetto* from Romania (Curtu et al. 2011). As our study obtained the values of expected heterozygosity of 0.824 in *Q. petraea*, 0.834 in *Q. pubescens*, and 0.794 in *Q. frainetto*, it can be concluded that the obtained values are in accordance with the values obtained in previous studies. Thus, we found that all three analyzed species are characterized by rather high levels of genetic diversity, which indicates good prospects for their long-term survival, especially in conditions of changing climate and habitat degradation.

Despite living in sympatry, all three species of oaks have retained their genetic integrity, which is a rather important finding relevant for the conservation and management practice. Low but statistically significant genetic differentiation, expressed via F_{ST} values, was observed (0.047 among *Q. petraea* and *Q. frainetto*, 0.039 among *Q. pubescens* and *Q. frainetto*, and 0.032 among *Q. petraea* and *Q. pubescens*). Furthermore, *Q. frainetto* was clearly separated from the other two oak species in PCoA analysis along the first coordinate, while *Q. petraea* and *Q. pubescens* were clearly separated in the PCoA analysis, along the second coordinate. Also, in the case of *Q. frainetto*, almost all individuals were strongly assigned to one gene pool, while individuals belonging to *Q. petraea* and *Q. pubescens* were strongly assigned to two or three distinct gene pools, indicating complex substructure of their populations. Population substructure is commonly associated with limitations to the gene flow (Sork 2016), and introgression, which is rather common in oaks, known for interspecific hybridization (e.g., Curtu et al. 2007, Salvini et al. 2009, Neophytou et al. 2010, Ortego and Bonal 2010). The highest gene flow and the lowest genetic differentiation were observed between *Q. petraea* and *Q. pubescens*, and the lowest gene flow and the highest genetic differentiation between *Q. petraea* and *Q. frainetto*. These results were expected because lower genetic differentiation and higher levels of gene flow are commonly observed among species that are more closely related, such as *Q. petraea* and *Q. pubescens*, than those that are more distantly related, such as *Q. petraea* and *Q. frainetto* (Curtu et al. 2007). A similar pattern has been previously observed in Italian (Salvini et al. 2009, Fortini et al. 2015) and Romanian (Curtu et al. 2007, Curtu et al. 2011) populations of oak species that are more or less related. The substructure in *Q. petraea* and *Q. pubescens* could exist due to higher gene flow and potential presence of introgressive forms in both species.

An important finding is rather high effective population size in all three oak species, which was the highest in *Q. frainetto* (∞, 1859.9; ∞) and the lowest in *Q. pubescens* (305.5, 190.2; 719.2). This finding suggests a rather high number of parent's contribution to the formation of the next generation, which is important for the maintenance of high levels of genetic diversity in next generations. Nevertheless, low but statistically significant inbreeding was observed in all examined oak populations in the study area. The observed excess of homozygotes, however, most likely reflects population substructure, i.e., Wahlund effect (Wahlund 1928). It is well-known that the variation of allelic frequencies among subpopulations may create a heterozygote deficiency at the scale of the whole population (Bacillieri et al. 1994). This is supported by the outcomes of the STRUCTURE analysis which revealed population substructure in populations of *Q. petraea* and *Q. pubescens*, which comprised individuals strongly assigned to two or three distinct gene pools. It is worth mentioning that the lowest F value was observed in the population of *Q. frainetto* (F = 0.065, SE = 0.041), for which population substructure was not found in the STRUCTURE analysis.

CONCLUSIONS

Results of this study showed that the populations of *Quercus fraineto, Q. petraea* and *Q. pubescens* in the area of the Landscape of Outstanding Features "Kosmaj" are characterized by rather high levels of genetic diversity, which is a prerequisite for their long-term survival, especially in terms of climate change. Also, despite the presence in sympatry, all three species of oaks have retained their genetic integrity. However, a pronounced genetic structure, with the existence of a substructure of the populations of *Q. petraea* and *Q. pubescens*, was observed. These findings are essential for the formulation of conservation strategies, which should include the establishment of *in situ* conservation units as a first step towards the conservation and directed utilization of genetic resources of these three native oak species.

Author Contributions

MŠN (project manager) and JM organized the study, MŠN and IKJ sampled the leaves, IKJ and JMA performed laboratory and statistical analyses. IKJ and MJ drafted the manuscript, JMA, JM and MŠN improved the manuscript draft. All authors discussed the results and contributed to the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Exploring the Gene Pool of Silver Fir in Southern Germany on the Search for Climate-Smart Seed Sources

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ABSTRACT

Central European populations of silver fir (*Abies alba* Mill.) grow under a relatively wide amplitude of environmental conditions. Assuming that forest tree stands are locally adapted, the use of forest reproductive material from sites with limited water availability is expected to increase drought tolerance in future forests. At the same time, genetic diversity is of utmost importance as the basis of adaptation to a changing environment. Currently, a selection of potential sources for climate-smart reproductive material of silver fir is under way in Southern Germany. It is based on a multidisciplinary approach elucidating the genetic variation, site conditions, as well as tree resilience based on a dendroecological approach. The analysis encompasses a large number of stands representing the whole range of the species' ecological requirements. The population genetic analysis based on molecular markers (nuclear microsatellites) provided important information concerning the gene pool of the species in Southern Germany. On one hand, it revealed genetic differentiation and transition zones between western and eastern clusters. On the other hand, the results indicated gradients and regional variation of genetic diversity. These patterns can be explained by post glacial recolonization and the peripheral character of the species at the northern limit of its distribution. Together with the outcomes of the site condition and dendroecological approaches, the results of the genetic analysis will be used to inform future provenance recommendations.

Keywords: *Abies alba*; genetic diversity; resilience; microsatellites; forest reproductive material

INTRODUCTION

In Central Europe, silver fir (*Abies alba* Mill.) is expected to cope better with the challenges posed by climate change than the more widely cultivated Norway spruce (*Picea abies* (L.) H. Karst.). Compared to the latter species, silver fir can better tolerate drought events and can also better benefit from mild winters in terms of growth (Vitali et al. 2018). Moreover, its current distribution only covers a limited part of its potential range in this region (Tinner et al. 2013). After a period of decline during the 20th century, which was mainly attributed to sulphur dioxide emissions, it is now generally experiencing a recovery (Elling et al. 2009). Recent results on growth decline and mortality induced by the drought in the summer of 2018 show that Norway spruce might be more vulnerable to such extreme drought events (Schuldt et al. 2020). Therefore, silver fir can be a suitable option for several sites, especially in submontane areas (Ruosch et al. 2016, Vitasse et al. 2019).

Silver fir's growth responses to drought greatly depend on within-species genetic variation (Martínez-Sancho et al. 2021). Common-garden trials have shown that growth performance, drought tolerance and resilience underly a strong ecotypic variation (Commarmot 1995, Dobrowolska et al. 2017, Hansen and Larsen 2004, Martínez-Sancho et al. 2021). Thereby southern origins are not per se better suited to drought events. For example, individuals from the Pyrenees showed reduced growth performance likely pointing to their poor genetic pool due to isolation processes (Martínez-Sancho et al. 2021). On the other hand, provenances from the eastern part of the distribution (Romanian Carpathian Mountains) have demonstrated good growth and tolerance to extreme drought events in Central Europe. Additionally, one southern Italian provenance was among those with the best performances (Martínez-Sancho et al. 2021). However, its relatively early start in the growing season might cause frost damages (Martínez-Sancho et al. 2021). Therefore, certain Central European provenances which have shown a combination of good growth, drought tolerance and recovery after extreme drought events should also be considered in climate change scenarios (Konnert and Schirmer 2011, Martínez-Sancho et al. 2021), including Gschwend, Pfalzgrafenweiler, Alpirsbach and Siegsdorf (Ruetz 2003). Knowledge about adaptive variation, its genetic background and the related environmental factors is important in order to select seed sources for establishing climate resilient forests (Martínez-Sancho et al. 2021). To this end, it is important to consider and unravel complex patterns and interactions, especially with respect to soil and climatic parameters, which may display medium to small-scale differences. For instance, soil water storage capacity influences growth at the small scale (Mellert et al. 2023) and may greatly compensate climatic aridity (Mellert et al. 2018).

The high adaptive genetic variation of silver fir is, at least partly, the result of the species' post-glacial recolonization history. Silver fir reached the full extent of its current distribution range relatively late in the Holocene, only about 3000 years ago (Liepelt et al. 2009). Competition with other species and its late successional character are important factors that might have contributed to this delayed postglacial expansion (Tinner and Lotter, 2006). The gene pools of European silver fir have been greatly shaped by Holocene migration. Genetic evidence based on mitochondrial DNA markers reveals two contact zones between migration routes of Apennine and Balkan origin; one of them in the Northern Carpathians, the other one in Slovenia and Northern Croatia (Liepelt et al. 2002). Thus, all Central European areas northwest of these geographic spots have their maternal origin in Apennine glacial refugia (Liepelt et al. 2002, Ziegenhagen et al. 2005). Yet, silver fir exhibits highly heterogenous gene pools within this region, also due to complex migration routes within and around the Alps. Range-wide and regional population genetic studies have demonstrated a pronounced population genetic structure, decreasing genetic diversity with increasing distance from refugial areas, elevated diversity in suture zones of different migration routes and relatively sharp allele frequency gradients (Konnert and Bergmann 1995, Lewandowski et al. 2001, Liepelt et al. 2009).

The most commonly cited population genetic studies with high coverage of the distribution range of silver fir were conducted based on isozyme markers (Konnert and Bergmann 1995, Liepelt et al. 2009). Although highly variable microsatellite markers (SSRs) were developed or successfully transferred into silver fir (Cremer et al. 2006, Hansen et al. 2005, Postolache et al. 2014), these were either tested in a few populations across the range (Postolache et al. 2014, Teodosiu et al. 2019) or were only rarely applied to explore population genetic structure within parts of central Europe (e.g. Cvrčková et al. 2015). Given the high variability and resolution in detecting population genetic structure (Postolache et al. 2014), more SSR-based population genetic studies would improve the status of knowledge about the gene pool of silver fir. As recent studies highlight the link between post-glacial demographic history and adaptive capacity to drought stress, such studies become highly relevant.

The current study was carried out in the framework of current German regional and national research projects aiming to identify populations of silver fir which can serve as seed sources for climate-smart forests (Schmied et al. 2023). In this context, seed stands from both productive and relatively arid sites are investigated. Given the recently shown link between adaptive responses and (neutral) genetic variation, and the relevance in the framework of assisted gene flow (Martínez-Sancho et al. 2021), a population genetic study based on highly variable SSR markers is an important part of these projects. Here, we present current results of this investigation. In particular, i) we describe the population genetic structure of silver fir in Southern Germany, ii) we explore the spatial variation of genetic diversity across our study populations, and iii) we discuss the results in the context of the species' post-glacial migration history and future assisted gene flow strategies.

MATERIALS AND METHODS

Study Area and Populations

In total, we genotyped 781 silver fir trees from 16 populations in Central Europe. Eight of the populations represented certified seed stands, the other ones represented non-certified stands, distributed over the state of Baden-Württemberg, south-western Germany, the state of Bavaria, south-eastern Germany and the southern state of Carinthia in Austria, situated along the eastern alpine postglacial recolonization pathway (Table 1). The stands were selected along a gradient of water storage capacity of the soil, ranging from 30 mm to over 200 mm, and soil acidity, ranging from acidic soils low in nutrients in the southern Black Forest to basic, nutrient rich soils on the Swabian Jura. Thus, we reached high diversity in stand conditions. Despite the fact that all stands are located in the optimal climatic niche zone (0.7<CMI<1; CMI=climatic marginality index) according to Mellert et al. (2023), they cover a broad climatic spectrum (Table 1). Mean annual temperatures (T_{yr}) range from 6.2°C (Spaichingen) to 9.9°C (Haslach), and annual precipitation from 780 mm (Cham) to 1339 mm (Schrög). The Ellenberg quotient (EQ) allows a differentiation of the beech-dominated forest types in Central Europe including silver fir (Ellenberg 1988). Low values around 10, as in Schrög, are typical for montane beech-fir forests, where fir is co-dominant due to the humid-cold climate. Higher EQ values characterize beech forests where fir dominates only under certain site conditions (e.g., dense soils, waterlogging), as in Cham where boulders cover large areas of the site. The climatic spectrum, which reaches EQ values above 20, shows that the site collective comprises the warm and dry edge of fir distribution in southern Germany.

From each tree, either needles were collected from the tree crown using a shotgun or cambium was sampled from the basis of the trunk using a hollow punch. Samples were put in paper bags, which were then stored in plastic bags filled with silica gel in order to dry the tissue and sent to the lab for genotyping.

Laboratory Procedures

For the 12 populations from Baden-Württemberg, Germany, as well as for the Austrian population from Carinthia, DNA was isolated from the dried tissue samples in the molecular genetics lab of the Institute of Silviculture at BOKU, Vienna, using a commercial extraction kit (DNEasy Plant Minikit, Qiagen, Hilden, Germany). For the three Bavarian populations, DNA was extracted, using an inhouse protocol. DNA quality was assessed by means of 1.0% agarose gels and DNA concentration was quantified using a spectrophotometer (NanoPhototometer®, Implen). Subsequently, the samples were diluted in water to achieve a DNA concentration of 30 ng/µl.

Subsequently, we used the diluted DNA to amplify ten nuclear microsatellite loci by means of polymerase chain reaction (PCR). The following loci, initially developed for *Abies alba* or other fir species, were genotyped: Aag01, Aat01, Aat04, Aat06, Aat11, Aat15 (Postolache et al. 2014); NFF3, NFF7, NFH15 (Hansen et al. 2005); and SF1 (Cremer et al. 2006). The loci were split into two multiplexes. PCR primers were fluorescently labelled in order to allow later scoring by means of capillary electrophoresis. Sequences, fluorescent dyes and other details about the used primers are summarized in Table 2.

PCRs were carried out in a reaction volume of 10µl including: 5µl of 2x Qiagen multiplex PCR master mix

Table 1. List of study populations sampled for the genetic analysis. BW = Baden-Württemberg, BY = Bavaria, A = Austria, D = Germany, T_{yr} = mean annual temperature, P_{yr} = mean annual precipitation, T_{07} = mean monthly temperature for July, EQ = Ellenberg's climate quotient (Ellenberg 1988), CMI = climate marginality index (Mellert et al. 2023), based on WorldClim data (Fick and Hijmans 2017, period 1970-2000), N = sampling size.

Population ID	Location name	Latitude	Longitude	T_{yr}	P_{yr}	T_{07}	EQ	CMI	N
1110	Blumberg (BW, D)	47.78	8.52	7.7	946	17.0	18.0	0.83	50
2112	Lahr (BW, D)	48.30	7.93	9.9	1043	18.7	17.9	0.77	50
2122	Nagold (BW, D)	48.57	8.70	8.3	968	17.3	17.9	0.77	50
2131	Schwäbisch Gmünd (BW, D)	48.83	9.76	8.9	961	17.8	18.5	0.79	50
2212	Hägelberg (BW, D)	47.66	7.74	9.1	1097	18.0	16.4	0.83	50
2221	Eutingen (BW, D)	48.48	8.70	8.0	950	17.0	17.9	0.76	50
2222	Haslach (BW, D)	48.27	8.10	9.9	1146	18.7	16.3	0.83	51
2232	Wutöschingen (BW, D)	47.66	8.39	8.7	1026	18.1	17.6	0.80	50
2312	Schömberg (BW, D)	48.78	8.62	7.5	1336	16.1	12.1	0.90	50
2322	Dunningen (BW, D)	48.20	8.49	7.3	1034	16.3	15.8	0.84	50
2331	Gönningen (BW, D)	48.43	9.11	8.1	798	17.2	21.6	0.80	51
2332	Spaichingen (BW, D)	48.05	8.75	6.2	1105	15.1	13.7	0.95	50
1201	Biberkor (BY, D)	47.94	11.37	8.1	1098	17.0	15.5	0.99	48
1202	Schrög (BY, D)	47.80	12.80	6.8	1339	15.4	11.5	1.00	38
1212	Cham (BY, D)	49.24	12.70	7.7	780	17.2	22.1	0.82	44
AT	Mauthen (K, A)	46.66	12.99	7.7	1170	18.0	15.4	0.95	49

Table 2. Names, primer sequences, fluorescent dyes (Atto550 = yellow, HEX = green, FAM = blue), allele size range (according to the primer notes) in base pairs and PCR multiplexes and primer note for the ten used microsatellite loci.

Locus name	Forward primer sequence	Reverse primer sequence	Allele size range	Dye	Multiplex
NFF3	CCAATGGGTTGTCAGAGTGTT	GGCATTCGAGATTGCTTGAT	105-187	Atto550	1
NFF7	CCCAAACTGGAAGATTGGAC	ATCGCCATCCATCATCAGA	116-174	HEX	1
NFH15	CGCCTCCCTCCATTACTTC	TCGTCTAGAGAGGCGAAATTCT	98-138	FAM	1
SF1	TTGACGTGATTAACAATCCA	AAGAACGACACCATTCTCAC	208-229	FAM	1
Aag01	GCTTATTCTCACTGCTCGCC	ATGACTTGAAGGTGGATGCC	193-250	Atto550	2
Aat01	CCATGTCTCCGATTTCCAGT	GGCCTAACGAAAGCAGAATC	103-127	HEX	2
Aat04	CCATGTATGGTGCTCCTCCT	CCTTCATTGCAGAAAAGCAA	158-191	FAM	2
Aat06	TTATGCGGAGCAGTTCTGTG	TGTTGCTGGCGTACTGGTAG	196-214	HEX	2
Aat11	AGCGTTGATTGGAAGCAGTC	GAAGCATGGTGTCGTTGTTG	255-270	HEX	2
Aat15	AGGAGGAGGTTCAGCATGTC	CTTGCTCTCTGACCCAGTTG	361-373	FAM	2

(Qiagen, Hilden, Germany), 3µl of RNAse-free water, 1µl of template DNA and 1 µl of 10x primer mix which was specific for each multiplex. In multiplex 1, the primer concentration in the 10x primer mix was 2 µM for loci NFH15 and SF1, 4 µM for locus NFF3 and 1 µM for locus NFF7, resulting in 0.2, 0.4 and 0.1 µM in the reaction volume, respectively. In multiplex 2, the primer concentration in the 10x primer mix was 4 µM for locus Aag01, 2 µM for loci Aat01, Aat06 and Aat15; and 1 µM for loci Aat04 and Aat11, resulting in 0.4, 0.2 and 0.1 µM in the reaction volume, respectively. The PCR program for multiplex 1 included an initial denaturation at 95°C for 5 min, followed by 35 cycles with denaturation at 95°C for 30 s, annealing at 57°C for 90 s and elongation at 72°C for 30 s with a final elongation step at 60°C for 30 min. For multiplex 2, the annealing temperature was 62°C with all other program settings being the same. The success of PCR reactions was tested on 1.5% agarose gels, which were also used to select the dilution ratio of the PCR products for subsequent capillary electrophoresis.

The capillary electrophoresis was carried out in a genetic analyser (SeqStudio, Thermo Fischer, Waltham, Massachusetts, USA) in order to determine the length of PCR products (alleles) in base pairs. Allele scoring was performed by applying a fragment length analysis using the GeneMapper v.6.0 (Thermofischer) software. Allele binning was performed manually. The genotype lists were exported for use in population genetic analyses.

Population Genetic Analysis

In order to quantify the genetic diversity within populations, we first calculated the multilocus average and effective number of alleles (n_a and n_e, respectively), observed and expected heterozygosity (H_o and H_e, respectively) and inbreeding coefficient (F_{IS}) per population using the GenAlEx v.6.5 software (Peakall and Smouse 2012, 2006). To assess the genetic differentiation among populations, pairwise F_{ST} values were calculated and their significance was tested by applying 999 permutations of genotypes pairwise between populations, using the G-statistics function of GenAlEx. To visualize the pattern of genetic differentiation, we carried out a Principal Coordinate Analysis (PCoA; Orlóci 1978) with the same software. Mantel tests were carried out with the R-package VEGAN v. 2.5-7 (Oksanen et al. 2017) to address whether genetic differentiation (F_{ST}) and geographic distance are correlated across populations and within subsets (Southern Germany and Baden-Württemberg only). Finally, we applied a Bayesian cluster analysis by means of the STRUCTURE software (Falush et al. 2007, Pritchard et al. 2000), in order to address the population genetic structure. In particular, we used the STRAUTO v1.0 application (Chhatre and Emerson 2017) in conjunction with GNU parallel (Tange 2011) to achieve automation and parallelization of STRUCTURE runs on a 40-core standalone computer. We opted for 50,000 burn-in replications followed by 100,000 MCMC iterations and adopted the admixture model with correlated allele frequencies. We also used the LOCPRIOR model (Hubisz et al. 2009) by setting one location per population (*locispop* option). We set consecutive values of K assumed clusters from 1 to 10, performing 20 independent runs for each K-value. We further processed the data in the CLUMPAK online platform (Kopelman et al. 2015) which implements the CLUMPP method (Jakobsson and Rosenberg 2007) in order to identify different cluster modes (in case of multimodality) and average the membership proportions of individuals to clusters across runs, and with STRUCTURE HARVESTER (Earl & vonHoldt 2012) in order to identify the uppermost level hierarchy of population genetic structure by means of the statistic delta K (ΔK; Evanno et al. 2005).

RESULTS

All used loci were polymorphic, displaying an average number of 5.6 to 6.6 alleles with the numbers varying among populations (Table 3). The highest number of alleles over loci per population (n_a) were observed in populations from the Black Forest in the westernmost part of our study area. With one exception, that of the population 2122-Nagold, these stands exhibited n_a-values of 6.0-6.6 (Table 3). Populations from the Swabian Jura (including 1110-Blumberg, 2322-Dunningen, 2332-Spaichingen, 2331-Gönningen and 2131-Schwäbisch Gmünd) showed n_a-values between 5.5 and 5.8, similar to the three stands from Bavaria and the one stand from Carinthia (Southern Austria). In terms of the effective number of alleles (n_e), the highest values (n_e=3.1-3.31) were found in the three Bavarian populations (1201-Biberkor, 1202-Schrög and 1212-Cham), as well as in the easternmost population of the Swabian Jura (2131-Schwäbisch Gmünd). In the western part of the study area, but also in the southern Austrian stand, n_e-values were generally lower (n_e=2.32-3.0). The genetic diversity in terms of observed (H_o) and expected heterozygosity (H_e) displayed the same trends as the effective number of alleles. However, two populations, 2112-Lahr and 2122-Nagold, showed less heterozygous individuals than expected, which is mirrored in their high inbreeding coefficients (Table 3).

Pairwise F_{ST}-values ranged from 0.004 to 0.089. In general, the highest genetic differentiation was observed between the population AT-Mauthen (Carinthia, southern Austria) and all other populations, with all pairwise comparisons being significant. Also, the three Bavarian populations were significantly differentiated from all other stands (P=0.001 for all comparisons), but also from each other (Table 4). Within Baden-Württemberg, some comparisons were significant with the easternmost population 2131-Schwäbisch Gmünd being significantly differentiated from all other stands. These patterns are also reflected in the scatterplot produced by applying PCoA using the pairwise F_{ST}-distances (Figure 1). The Mantel tests showed a significant correlation between genetic differentiation (in terms of F_{ST}) and geographic distance across all populations (R=0.610, P=0.001), within the subset of all 15 southern German populations (i.e. excluding population AT-Mauthen; R=0.5425, P=0.001), but not within the state of Baden-Württemberg (i.e. also excluding the three Bavarian populations; R=0.1235, P=0.182).

Table 3. Diversity measures averaged over loci ± standard error over loci for each of the study populations. n_a = number of alleles; n_e = effective number of alleles; H_o, H_e = observed and expected heterozygosity, respectively; F = inbreeding coefficient. BW = Baden-Württemberg; BY = Bavaria; A = Austria; D = Germany.

Population ID	n_a	n_e	H_o	H_e	F
1110 - Blumberg (BW, D)	5.80 ± 1.24	2.76 ± 0.48	0.51 ± 0.07	0.54 ± 0.07	0.06 ± 0.03
2112 - Lahr (BW, D)	6.00 ± 1.29	2.81 ± 0.50	0.49 ± 0.07	0.55 ± 0.07	0.15 ± 0.05
2122 - Nagold (BW, D)	5.60 ± 1.13	2.50 ± 0.39	0.47 ± 0.07	0.52 ± 0.06	0.14 ± 0.06
2131 - Schwäbisch G. (BW, D)	5.50 ± 1.23	3.10 ± 0.71	0.53 ± 0.08	0.54 ± 0.08	0.01 ± 0.02
2212 - Hägelberg (BW, D)	6.60 ± 1.71	2.89 ± 0.59	0.52 ± 0.08	0.54 ± 0.07	0.05 ± 0.04
2221 - Eutingen (BW, D)	6.30 ± 1.49	3.00 ± 0.61	0.55 ± 0.07	0.56 ± 0.06	0.04 ± 0.04
2222 - Haslach (BW, D)	6.40 ± 1.32	2.40 ± 0.39	0.47 ± 0.07	0.50 ± 0.06	0.07 ± 0.04
2232 - Wutöschingen (BW, D)	5.60 ± 1.11	2.32 ± 0.39	0.47 ± 0.07	0.48 ± 0.06	0.04 ± 0.05
2312 - Schömberg (BW, D)	6.10 ± 1.26	2.72 ± 0.56	0.47 ± 0.08	0.50 ± 0.08	0.07 ± 0.05
2322 - Dunningen (BW, D)	6.60 ± 1.31	2.44 ± 0.42	0.50 ± 0.08	0.49 ± 0.07	0.03 ± 0.06
2331 - Gönningen (BW, D)	5.80 ± 1.25	2.76 ± 0.52	0.51 ± 0.08	0.52 ± 0.08	0.01 ± 0.02
2332 - Spaichingen (BW, D)	5.70 ± 1.21	2.59 ± 0.38	0.52 ± 0.05	0.55 ± 0.05	0.06 ± 0.03
1201 - Biberkor (BY, D)	5.80 ± 1.19	3.14 ± 0.57	0.59 ± 0.06	0.59 ± 0.07	-0.01 ± 0.02
1202 - Schrög (BY, D)	5.70 ± 1.40	3.31 ± 0.78	0.59 ± 0.06	0.58 ± 0.06	-0.02 ± 0.04
1212 - Cham (BY, D)	5.80 ± 1.35	3.13 ± 0.73	0.51 ± 0.08	0.54 ± 0.08	0.04 ± 0.04
AT - Mauthen (K, A)	5.80 ± 1.22	2.71 ± 0.52	0.51 ± 0.08	0.51 ± 0.08	0.01 ± 0.03

Table 4. Pairwise F_{ST} values for all population pairs (F_{ST} values below diagonal, probability values above diagonal).

	1110	2112	2122	2131	2212	2221	2222	2232	2312	2322	2331	2332	1201	1202	1212	AT
1110		0.141	0.064	0.001	0.753	0.128	0.059	0.001	0.100	0.043	0.484	0.160	0.001	0.001	0.001	0.001
2112	0.007		0.057	0.001	0.492	0.013	0.033	0.001	0.087	0.008	0.432	0.060	0.001	0.001	0.001	0.001
2122	0.009	0.009		0.005	0.341	0.057	0.271	0.002	0.070	0.066	0.171	0.171	0.001	0.001	0.001	0.001
2131	0.015	0.013	0.013		0.002	0.001	0.001	0.001	0.002	0.001	0.018	0.001	0.001	0.001	0.001	0.001
2212	0.004	0.005	0.006	0.013		0.247	0.065	0.002	0.160	0.075	0.637	0.583	0.001	0.001	0.001	0.001
2221	0.007	0.010	0.009	0.015	0.006		0.009	0.001	0.027	0.008	0.028	0.049	0.001	0.001	0.001	0.001
2222	0.008	0.010	0.006	0.018	0.008	0.011		0.001	0.048	0.160	0.048	0.014	0.001	0.001	0.001	0.001
2232	0.017	0.016	0.018	0.027	0.012	0.022	0.017		0.001	0.006	0.001	0.005	0.001	0.001	0.001	0.001
2312	0.008	0.008	0.008	0.014	0.007	0.009	0.009	0.018		0.115	0.462	0.001	0.001	0.001	0.001	0.001
2322	0.009	0.011	0.008	0.018	0.008	0.011	0.007	0.012	0.008		0.073	0.040	0.001	0.001	0.001	0.001
2331	0.005	0.006	0.007	0.009	0.005	0.009	0.009	0.017	0.005	0.008		0.079	0.001	0.001	0.001	0.001
2332	0.007	0.009	0.007	0.015	0.005	0.009	0.010	0.011	0.012	0.009	0.008		0.001	0.001	0.001	0.001
1201	0.030	0.026	0.038	0.023	0.030	0.033	0.042	0.045	0.040	0.041	0.029	0.025		0.007	0.001	0.001
1202	0.023	0.022	0.030	0.018	0.025	0.024	0.032	0.039	0.032	0.034	0.026	0.021	0.012		0.015	0.001
1212	0.043	0.041	0.054	0.031	0.045	0.043	0.054	0.062	0.050	0.055	0.043	0.043	0.024	0.013		0.001
AT	0.077	0.068	0.083	0.061	0.072	0.077	0.089	0.081	0.084	0.086	0.076	0.071	0.046	0.048	0.057	

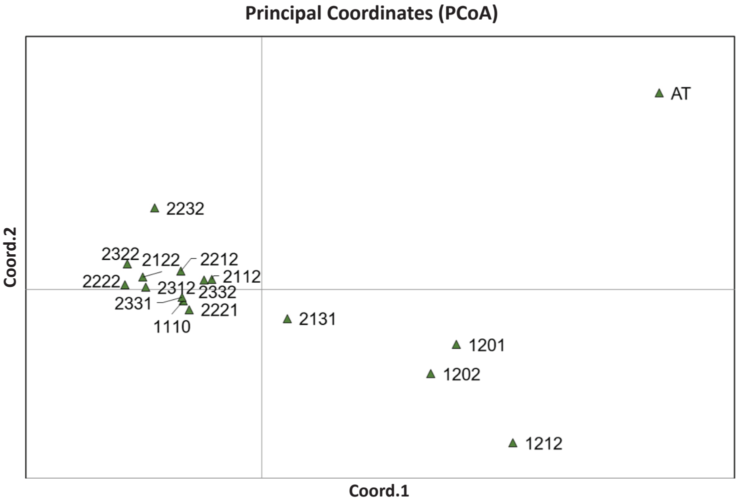

Figure 1. Scatterplot of the Principal Coordinate Analysis (PCoA) based on the pairwise F_{ST} values. The first and second coordinates account for 50.07 and 16.33% of the total variance, respectively.

The STRUCTURE analysis produced meaningful results for an assumed number of K=2 up to K=4 clusters. ΔK was maximum for K=2 and displayed a secondary peak for K=4 (Supplementary Material 1), while all runs within a K-value were unimodal (resulted in the same clustering pattern). To illustrate the spatial distribution of the genetic structure, we plotted the results for K=2 and K=4 on maps (Figure 2, Figure 3). For K=2, a west-east differentiation could be observed. The southern Austrian stand was assigned to an eastern cluster both at the population and individual level. The three Bavarian populations showed mixed affinity to an eastern and a western cluster. The easternmost population of the Swabian Jura (2131-Schwäbisch Gmünd) also showed partial membership (membership proportion q=0.197) to the eastern cluster both at the population and the individual level. All other stands from the western part of our study area showed a high membership proportion (q≥0.926) to the western cluster with the exception of populations 2112-Lahr and 2212-Hägelberg. These populations received somewhat lower membership proportions to the western cluster (q=0.861 and 0.886, respectively) due to heterogeneity at the individual level. In particular, most individuals of these populations were identified as members of the western clusters. However, a small number of trees from these populations received relatively high scores of membership proportion (q>0.6) to the eastern cluster, which was uncommon for other individuals in the same or neighbouring stands (Figure 2).

At K=4, the southern Austrian population significantly differed from all other silver fir stands (not the case at K=3; results for K=3 not shown). The southern German populations showed again a west-east differentiation. A cluster, illustrated with sky blue in Figure 3, was mostly represented in the west. A further cluster, for which we used dark green colour in Figure 3, was more prevalent in the east. The three Bavarian populations presented high membership proportions to this cluster. Along the Swabian Jura we could observe a transition zone with increasing membership coefficients to the eastern cluster from southwest (1110-Blumberg, 2332-Spaichingen) towards northeast (2331-Gönningen, 2131-Schwäbisch Gmünd). Some affinity to this cluster was also observed for stands 2112-Lahr and 2212-Hägelberg at the westernmost part of the Black Forest. However, this was against due to some single individuals in these populations which were assigned to the eastern cluster with high membership proportions, which was unusual for other individuals in the same or neighbouring stands. Finally, a fourth cluster, for which we used dark blue colour, did not show an obvious geographic pattern.

DISCUSSION

The ten used microsatellite markers proved to be useful in order to identify diversity gradients and distinguish among different genetic clusters of silver fir in our study area in Central Europe. Although a sufficient number of nuclear microsatellites for this species have been available for more than a decade (Cremer et al. 2006, Hansen et al. 2005, Postolache et al. 2014), relatively few studies in central Europe have made use of them so far (e.g. Cvrčková et al. 2015). In contrast, several SSR-based studies dealing with population genetics in peripheral populations of silver fir and its related species appeared in this time period (Belletti et al. 2017, Dalmaris et al. 2022, Sancho-Knapik et al. 2014, Teodosiu et al. 2019). Thus, conclusions about diversity gradients and the population genetic structure of silver fir in Central Europe are mostly based on older studies applying isozymes (Breitenbach-Dorfer et al. 1997, Konnert

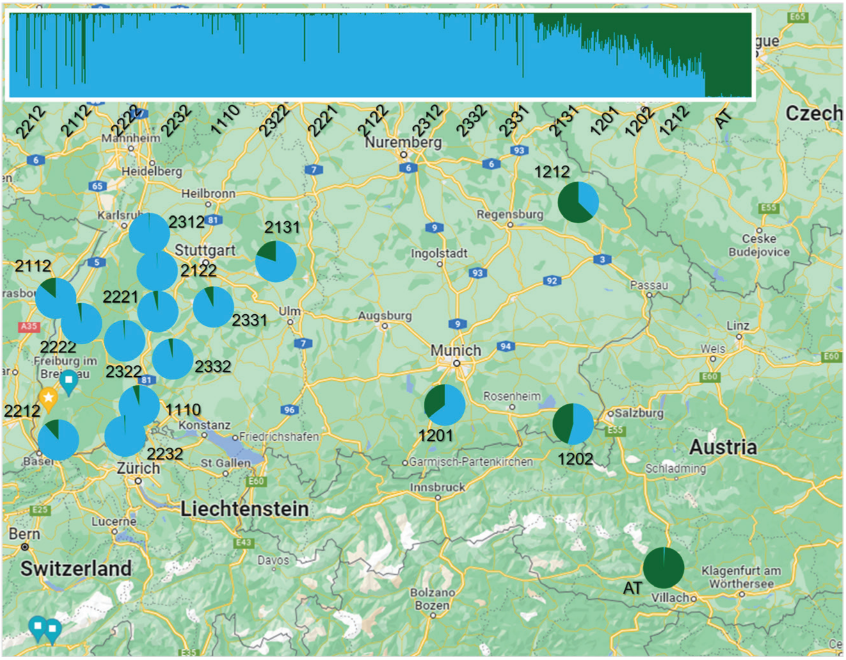

Figure 2. STRUCTURE results for K=2 assumed clusters. The clusters are illustrated with different colours. Individuals in the bar plot are illustrated with one bar each. At the population level, the pie charts depict the membership proportion to the clusters.

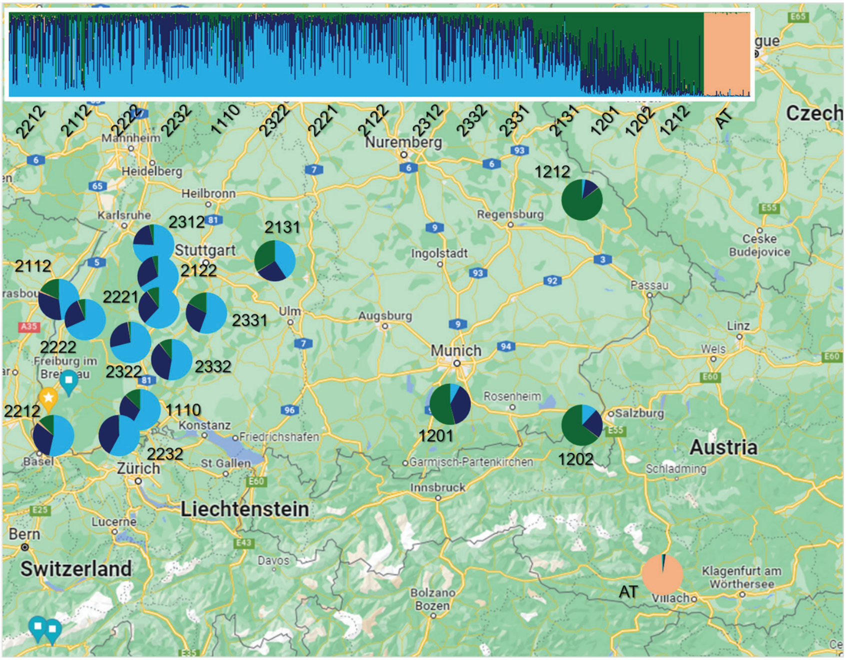

Figure 3. STRUCTURE results for K=4 assumed clusters. The clusters are illustrated with different colours. Individuals in the bar plot are illustrated with one bar each. At the population level, the pie charts depict the membership proportion to the clusters.

and Bergmann 1995, Lewandowski et al. 2001, Liepelt et al. 2009). Given that the microsatellite markers we used are more variable than isozymes, our study complements these previous investigations and refines our knowledge about the genetic diversity and structure of the species in Central Europe.

With respect to genetic variation, one of our main findings is the decreasing trend of the average number of alleles per locus towards the east. This is in contrast with other measures of diversity like the effective number of alleles and heterozygosity, which take the allele frequencies into account. These tend to be higher in the east and especially in Bavaria. In general, the number of alleles is expected to decrease with increasing distance from the glacial refugia, while high genetic diversity may occur in suture zones of different postglacial recolonization pathways (Konnert and Bergmann 1995, Liepelt et al. 2009, Petit et al. 2003). Therefore, we attribute the higher values of this diversity measure in the west to the lower distance of the Black Forest (westernmost part of our study area) from the refugial populations along the relatively short Jura (western alpine) migration route (Liepelt et al. 2009). The lower allele numbers in the three Bavarian and the one Carinthian (southern Austrian) population of our study might have resulted from genetic drift during recolonization. Indeed, the west-east orientation of the Alps at their eastern flank and the occurrence of several parallel mountain chains posed a significant barrier which forced silver fir to expand through narrow valleys and cover a much longer distance during Holocene expansion (Liepelt et al. 2009). This result was not affected by variation in sample size among populations as calculation of rarefied allelic richness (Petit et al. 1998) did not lead to significant changes of the aforementioned pattern (results not shown).

As opposed to the average number of alleles per locus, we found higher values of effective allele number and heterozygosity in the east, namely in Bavaria, but not in Carinthia. We attribute this result to the contact of the western and eastern Holocene migration routes and admixture of the respective gene pools in the region of Bavaria (while Carinthia is situated at a more southern region, completely within the eastern route). A similar observation was made by Konnert and Bergmann (1995), Liepelt et al. (2009) and Breitenbach-Dorfer et al. (1997) based on isozymes. However, in those studies, the maximum values of diversity measures were observed in the Black Forest area, while Bavarian stands were less diverse with a further decrease of genetic diversity eastwards in southeastern Austria and northwards in the Bavarian Forest along the border between Germany and Czechia and further reduction towards the Ore and Beskid Mountains. The genetic diversity values in the population 1212-Cham are comparable with the other two Bavarian populations which is not in line with the hypothesis of a drastic genetic erosion along the eastern migration route towards north. Further recent SSR-based results from Czechia also do not conform with this hypothesis (Cvrčková et al. 2015). Thus, the zone of genetic admixture between the different circum-alpine migration routes might be situated further to the east than as hypothesized by Konnert and Bergmann (1995), while more studies are needed to address whether population at the northernmost margin of silver fir's range has been affected by past population bottlenecks.

The transition from one gene pool to another is also supported by the F_{ST}-based analysis of genetic differentiation, but also by the Bayesian clustering results. The genetic and geographic distances are significantly correlated when observing the entire study area with or without the rather isolated Carinthian AT-Mauthen population. This population appears to be genetically differentiated from the three Bavarian stands, which can be explained by the significant landscape barriers that exist between them. However, it is genetically more similar to the three Bavarian populations than to any other stand of our study, which agrees with the hypothesis that the eastern alpine recolonization pathway has influenced the gene pool of silver fir in Bavaria. On the other hand, populations in the west appear to be relatively homogenous with low pairwise F_{ST}s, lack of isolation by distance (within Baden-Württemberg) and to the same clusters in similar proportions by STRUCTURE analysis. Nevertheless, a gradual differentiation of the eastern populations along the Swabian Jura, and especially the easternmost stand 2131-Schwäbisch Gmünd, can be observed in some of the results (especially STRUCTURE for K=4). These are genetically somewhat closer to the Bavarian populations, which is in line with their geographic location. Furthermore, the range of silver fir in the Swabian Jura is discontinuous so that stands in the centre and southwest of this mountain range (e.g. 2331-Gönningen, 2332-Spaichingen) are separated by more than 30 km (where silver fir is lacking) from occurrences in the northeast (including 2131-Schwäbisch Gmünd). The latter is rather connected to silver fir populations in the northeast, which is in line with its partial affinity to the Bavarian stands. On the contrary, the genetic differentiation of this stand from all other populations in Baden-Württemberg may have resulted through its isolation from further stands towards southwest. These results indicate that genetic structure might be also present at a smaller scale (i.e. within Baden-Württemberg). Increasing the number of loci and populations could better resolve such fine-scale genetic structure.

Finally, in two stands in the Black Forest (2112-Lahr and 2212-Hägelberg), a small number of individuals displayed high membership proportion to an eastern genetic cluster. This suggests transfer of reproductive material from an eastern origin into these two stands. Unfortunately, we could not trace back the origin of these stands. However, trade of reproductive material is known to have taken place for centuries in Europe, which is particularly true for conifers (Hamberger 2011). Also, we assume that the presence of a seed production enterprise (Staatsklenge Nagold) with a history of more than 150 years in short distance from several of our stands in Baden-Württemberg may also have influenced the genetic variation of silver fir in this region. This might be the case, for instance, in stand 2122-Nagold which displayed decreased genetic diversity and elevated inbreeding coefficient. Establishment with reproductive material from a reduced number of trees could be a possible reason behind this. Even if natural regeneration has been more common in silver fir than e.g. in Norway spruce (*Picea abies*), plantation and seed transfer should be considered as another important factor shaping the gene pool of our study species.

CONCLUSIONS

Our study confirms that the gene pool of silver fir in central Europe is structured and characterized by gradients of genetic diversity which have resulted from postglacial migration history of the species in this area, which supports that our populations are mostly autochthonous. Not only molecular markers, but also common garden trials demonstrate the high genetic variation of silver fir in Central Europe (Dobrowolska et al. 2017). Results based on both molecular and quantitative genetic approaches suggest that a rather high resolution of seed zones (i.e. a rather high number of geographically limited seed zones) is meaningful as it accounts for the aforementioned regional genetic differences. At the same time, certain Central European provenances from relatively dry sites may display an increasing significance as seed sources for future climate smart forests (Konnert and Schirmer 2011). While previous common-garden based studies had a rather interregional and range-wide scope, our study focuses on medium (regional) scale adaptation by including both dry and productive sites situated in geographic proximity to each other. A dendroecological approach implemented in the framework of the same project suggests that silver fir on dry sites shows an increased resilience to drought events (Lamprecht 2022) and copes better with persistent climate changes compared to Norway spruce (Schmied et al. 2023). Combination of dendroecological, molecular and quantitative genetic studies will provide a robust basis to define seed sources for climate resilient forests of silver fir.

Author Contributions

BF, HGM, MS und KHM conceived and designed the related project, NL carried out the stand selection and supervised the project activities in Baden-Württemberg, while KHM, MS and BF undertook these tasks in Bavaria. BF and CN designed and supervised the genetic analyses, CN performed the statistical analysis and wrote the manuscript with contributions from all coauthors.

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Conflicts of Interest

The authors declare no conflict of interest.

Supplementary Materials

Supplementary File 1 - Results of the Evanno method. Table and Figures of ln-probability of data, Delta K and further statistics calculated by applying the Evanno method with use of the software STRUCTURE HARVESTER.

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Phenological Variability of *Pinus sylvestris* L. Provenances in the International Provenance Test in Kupres, Bosnia and Herzegovina

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ABSTRACT

Scots pine (*Pinus sylvestris* L.) is an important forest tree species growing on different soils in Bosnia and Herzegovina. Phenological traits are relevant practical indicators of the adaptability and adaptedness of forest tree species. This research aims to determine the dates of the beginning and the end and duration of phases of bud breaking and elongation in *P. sylvestris*. The results will be used in the selection of best provenances for the areas with late frosts. The materials used were *P. sylvestris* plants in the international provenance test Kupres. The test was established in 2012 with 15 provenances (three from Austria, one from Bosnia and Herzegovina, two from Germany, three from Italy, one from Poland, one from Romania, one from Slovakia, two from Norway and one from Ukraine). Six phases were observed for bud breaking and elongation. Phase 1 (start of elongation, scales partly disjoined but still covering the young shoot) was recorded on 10 May in all provenances. Phase 5 (2 needles of the same brachyblasts are clearly distinct) first occurred on 6 June on some plants of every provenances except Bugojno (Bosnia and Herzegovina), Delytayn (Ukraine), and Ca Del Lupo (Italy). Understanding phenological phenomena of Scots pine is important since the occurrence of late spring frost can damage the plants of Scots pine in the environmental conditions of Bosnia and Herzegovina. Research should be continued and include an investigation of the influence of seasonal climate and climate changes.

Keywords: Scots pine; spring phenology; selection; late frost resistance

INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is the most widely distributed pine species in the world, and it can be found all the way across Eurasia (Houston Durrant et al. 2016). It reaches 23-27 m in height on average, but can attain over 40 m and live for 400 years or more (Houston Durrant et al. 2016). It represents one of the most important conifer species in the forests of Bosnia and Herzegovina, where it inhabits large areas and as a pioneer species tolerates poor soils, drought, wind and frost (Pintarić 2002). According to the preliminary results of the Second Forest Inventory in Bosnia and Herzegovina, the area of pure stands of Scots pine, of production character, in Bosnia and Herzegovina amounts to 38,000 ha (Lojo and Balić 2011, Ballian et al. 2019).

Scots pine was investigated in Bosnia and Herzegovina on the morphological level (in clonal plantations, provenance tests, cone and seed morphology) (Daničić 2008, Ballian et al. 2009, 2019, Daničić et al. 2011, Ballian and Šito 2017, Memišević Hodžić et al. 2020a, 2020b), physiological level (Daničić 2008, 2011), phenological level (Daničić et al. 2011, 2015, Ballian and Šito 2017, Ballian et al. 2019), and molecular level using biochemical markers (Ballian and Božič 2004, Ballian et al. 2006).

Phenological traits are relevant practical indicators of the adaptability and adaptedness of forest tree species. According to some authors (Nilson and Walfridsson 1995, Eriksson and Ekberg 2001), due to changing environmental factors caused by global warming, Scots pine is migrating north. Northern and continental populations require a lower temperature sum to complete the phenophases and show a higher level of resistance. Southern and coastal provenances have a longer vegetation period and are less resistant to stressful environmental conditions (Sarvas 1962, Ballian et al. 2019).

This research aims to identify variability in the beginning, the end and the duration of phases of bud breaking and elongation in *Pinus sylvestris* per provenances, to recommend suitable provenances for afforestation in the suitable areas.

MATERIALS AND METHODS

Study Area

The materials used were *Pinus sylvestris* plants in international provenance test Kupres. The test was established in 2012 by planting two-year-old seedlings from 15 different provenances originating from ten European countries (Table 1, Figure 1).

Planting distance was 2×2 m. Thirty-six (36) seedlings in five repetitions per randomized scheme were planted for each provenance (180 plants per provenances). Provenances originated from areas with different climates: provenances from Austria, Slovakia, Poland and Romania are from a temperate continental climate and one from B&H is from a subalpine temperate continental climate. Provenances from Italy are from a continental climate and those from Germany from a moderately warm and humid climate influenced by the Atlantic Ocean. The provenance from Ukraine is from a continental climate, but from one of the coldest regions in Ukraine with an average daily maximum temperature of only 12°C and frequent rainfall and wind.

Provenance test is located on 1140 m of altitude, on terrain characterized by sinkholes and numerous hills

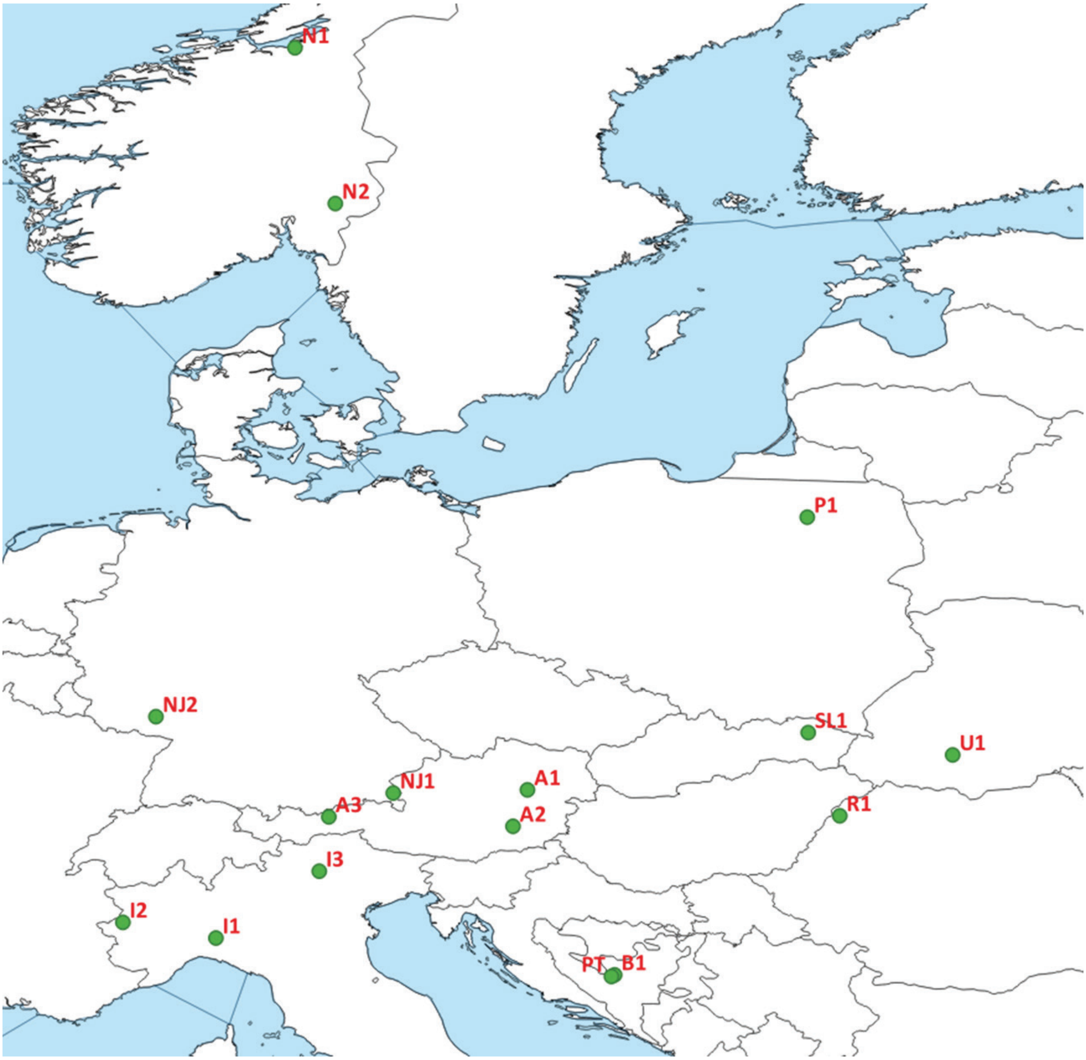

Figure 1. Scots pine provenances included in the provenance test.

Table 1. List of investigated provenances.

No	Provenance label	Country	Locality	Latitude	Longitude	Altitude (m)
1.	A1	Austria	Kobersdorf/Lackenbach	47° 53' 12''	15° 31' 39''	551
2.	A2	Austria	Panholz	47° 07' 14''	15° 17' 14''	424
3.	A3	Austria	Lans/Tirol	47° 13' 49''	11° 26' 12''	1017
4.	B1	Bosnia and Herzegovina	Bugojno	44° 03' 00''	17° 27' 00''	623
5.	NJ2	Germany	Trippstadt	49° 21' 35''	7° 46' 29''	445
6.	I1	Italy	Ca Del Lupo	44° 45' 25''	9° 05' 07''	532
7.	I2	Italy	Fenestrelle (TO)	45° 01' 47''	7° 03' 38''	1182
8.	I3	Italy	Valda (TN)	46° 13' 00''	11° 16' 00''	959
9.	P1	Poland	Ruciane – Nida	53° 37' 00''	21° 29' 00''	149
10.	R1	Romania	Sacueni	47° 21' 09''	22° 05' 29''	104
11.	SL1	Slovakia	Hanušovce	49° 01' 35''	21° 30' 01''	249
12.	NJ1	Germany	Teisendorf	47° 51' 00''	12° 49' 00''	505
13.	N1	Norway	Malvik	63° 22' 22''	10° 45' 03''	201
14.	N2	Norway	Arnes	60° 07' 20''	11° 27' 55''	174
15.	U1	Ukraine	Delytayn	48° 32' 41''	24° 30' 10''	743
Provenance test (PT)		Bosnia and Herzegovina	Kupres	43° 58' 18''	17° 15' 48''	1140

and valleys. The soil type is calcocambisol or brown soil, and geological substrate are limestones and dolomites. According to the classification of climate types in B&H (Milosavljević 1973), this area belongs to the mountain climate, with short summers and long, cold, and very snowy winters. The average annual temperature is 6.2°C. The minimum temperature was measured in January (-26.8°C) and the maximum in September (34.9°C). The annual average of registered frosts is 155 days. The average annual precipitation is 1221 mm, and its distribution varies slightly by season.

Field Observations

Six phases were observed for bud breaking and elongation (Ducci et al. 2012): 0 = dormant bud with joined scales covered by resin; 1 = start of elongation, scales partly disjoined but still covering the young shoot; 2 = significant elongation of terminal bud, scales still present but the green young shoot is visible; 3 = brachyblasts are well visible but still in their envelope; 4 = needles joined in two start to appear; 5 = the two needles of the same brachyblasts are clearly distinct (Figure 2).

Field observations started in March and were conducted every week regularly until the first plant with phase 1 was observed. After that date, the observations were made every third-day, until all plants were in phase 5.

Table 2 provides an overview of the basic climatological data for the nearest meteorological station (Bugojno) for the first half of 2022 (Federalni hidrometeorološki zavod, 2023), which influenced the development of the phenological phases of Scots pine in the researched provenance test.

Score 1 Score 2 Score 3 Score 4 Score 5

Figure 2. Scoring system adopted for bud breaking and elongation in *Pinus sylvestris* (according to Ducci et al. 2012).

Table 2. Climatological data for meteorological station Bugojno for the first half of 2022.

Month	Average air temperature (°C)	Maximum daily temperature (°C)	Date of max temperature	Minimum daily temperature (°C)	Date of min temperature	Sum of precipitation (mm)	Max height of snow cover (cm)
I	-1.1	16.0	05	-18.5	25/04	25.9	10
II	3.9	16.4	10 and 19	-8.2	02	42.5	11
III	3.9	21.3	24	-10.3	12	12.7	15
IV	9.3	25.3	15	-4.0	19	79.8	9
V	16.6	30.8	27	1.6	19	87.8	0
VI	21.8	36.0	29	7.7	15	55.8	0

Statistical Analysis

The observed data were processed in Microsoft Excel to determine the first and last occurrence of phases per provenances, the duration of individual phenophases, and SPSS 20.0 for ANOVA analysis was used to determine the duration of phases and cluster analysis.

RESULTS

The first and last occurrence of phases per provenance are shown in Table 3.

Phase 1 (start of elongation, scales partly disjoined but still covering the young shoot) was recorded on 10 May in all provenances. Phase 5 (the two needles of the same brachyblasts are clearly distinct) first occurred on 5 June in some plants of every provenances except Bugojno (Bosnia and Herzegovina), Delytayn (Ukraine), and Ca Del Lupo (Italy).

Average duration of phases are shown in Table 4.

In Table 4, it is visible that the durations of phases were different per provenance. Phase 1 had the shortest duration, and in the case of some provenances it was not even registered, which means it happened between two observations.

The results of variance analysis for duration of phases are shown in Table 5.

Variance analysis (Table 5) showed statistically significant differences among provenances for all phases (phases 0 and 5 were not included because of incomplete data, i.e. phase 0 started before we began with observations and phase 5 continued after we stopped.)

The results of cluster analysis for duration of phases are shown in Figure 3.

Cluster analysis (between groups linkage, Squared Euclidean distance) showed separation of A1 and I1 in separate group, and in the duration of phenophases (Table 4) it can be seen that these two provenances included phase 1, while in other provenances it was finished between two observations, and other phases started later (Table 3).

Cluster analysis for the duration of phenophases showed that the nearest provenances were the provenance from Ukraine and the provenance from Germany, which are geographically very distant, followed by the provenance from Austria and the provenance from Germany (which are geographically close to each other).

Table 3. First and last occurrence of phases per provenances.

Provenance	Phase 0	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
A1	until 12.5.	10.5.-21.5.	15.5.-24.5.	22.5.-5.6.	30.5.-11.6.	from 5.6.
A2	until 12.5.	10.5.-12.5.	12.5.-24.5.	22.5.-4.6.	30.5.-11.6.	from 5.6.
A3	until 12.5.	10.5.-12.5.	12.5.-24.5.	22.5.-2.6.	27.5.-9.6.	from 5.6.
B1	until 12.5.	10.5.-12.5.	12.5.-24.5.	25.5.-29.9.	30.5.-11.6.	from 8.6.
U1	until 12.5.	10.5.-12.5.	12.5.-24.5.	22.5.-3.6.	30.5.-11.6.	from 8.6.
SL1	until 14.5.	10.5.-14.5.	12.5.-21.5.	22.5.-26.5.	27.5.-7.6.	from 5.6.
R1	until 12.5.	10.5.-12.5.	12.5.-21.5.	22.5.-2.6.	27.5.-11.6.	from 5.6.
N1	until 12.5.	10.5.-12.5.	12.5.-21.5.	18.5.-30.5.	27.5.-7.6.	from 5.6.
N3	until 12.5.	10.5.-12.5.	12.5.-21.5.	22.5.-26.5.	27.5.-4.6.	from 5.6.
NJ1	until 12.5.	10.5.-12.5.	12.5.-21.5.	22.5.-29.5.	30.5-9.6.	from 5.6.
NJ2	until 12.5.	10.5.-12.5.	12.5.-24.5.	22.5.-4.6.	30.5.-11.6.	from 5.6.
P1	until 12.5.	10.5.-14.5.	12.5.-21.5.	22.5.-4.6.	30.5.-11.6.	from 5.6.
I1	until 12.5.	10.5.-17.5.	15.5.-26.5.	25.5.-4.6.	3.6.-11.6.	from 8.6.
I2	until 12.5.	10.5.-12.5.	12.5.-26.5.	25.5.-4.6.	30.5.-11.6.	from 5.6.
I3	until 12.5.	10.5.-12.5.	12.5.-24.5.	22.5.-4.6.	27.5.-9.6.	from 5.6.

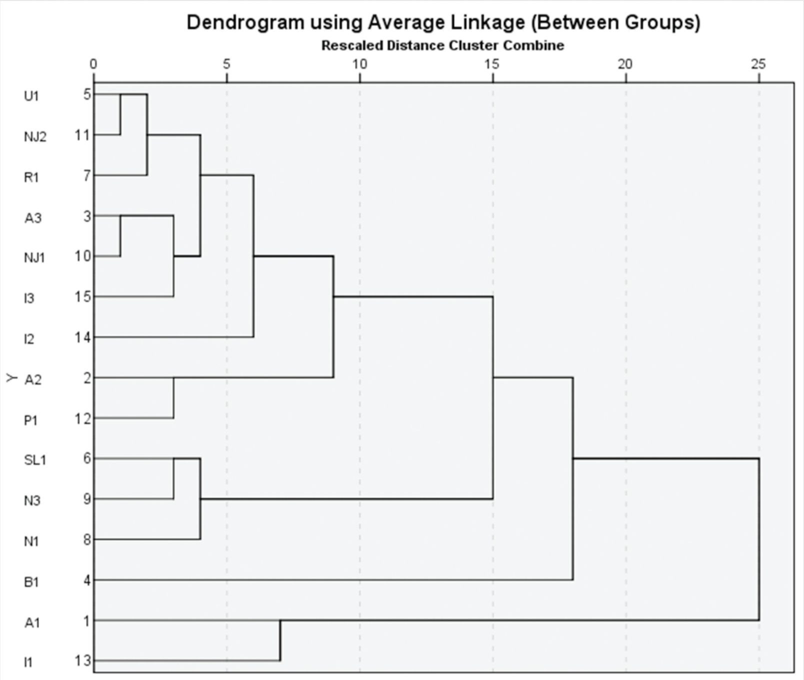

Figure 3. Grouping of provenances according to cluster analysis for duration of phases.

Table 4. Average duration of phases (in days) per provenances.

Provenance	Average duration of phases (days)			
	Phase 1	Phase 2	Phase 3	Phase 4
A1	4.4	7.4	10.2	4.6
A2	0.0	11.2	10.8	4.6
A3	0.0	11.2	7.2	7.8
B1	0.0	13.0	5.0	10.6
U1	0.0	11.2	9.2	7.4
SL1	0.6	9.4	5.0	10.6
R1	0.0	10.0	9.0	8.4
N1	0.0	8.4	7.2	9.0
N3	0.0	10.0	5.0	9.0
NJ1	0.0	10.0	8.0	8.2
NJ2	0.0	11.2	9.6	6.6
P1	0.6	9.4	10.0	5.8
I1	4.2	9.2	9.8	6.2
I2	0.0	13.4	8.2	6.6
I3	0.7	12.4	6.2	8.0
Total	0.6	10.5	8.0	7.6

Table 5. Variance analysis for duration of phases.

Phase	Source of variation	Sum of Squares	df	Mean Square	F	Sig.
1	Between Groups	158.587	14	11.185	11.882	0.000
	Within Groups	64.400	60	1.073		
	Total	220.987	74			
2	Between Groups	195.547	14	13.968	6.021	0.000
	Within Groups	139.200	60	2.320		
	Total	334.747	74			
3	Between Groups	282.347	14	20.168	2.753	0.003
	Within Groups	439.600	60	7.327		
	Total	721.947	74			
4	Between Groups	241.680	14	17.263	3.443	0.000
	Within Groups	300.800	60	5.013		
	Total	542.480	74			

DISCUSSION

This research included observation of phenological phases of Scots pine provenances in the international provenance test in Kupres, Bosnia and Herzegovina. Provenances originated from different parts of Europe, latitudes, longitudes, altitudes, ecological niches and climatic conditions. Observing phenological development of these provenances planted ex-situ in the same conditions could give important results for using Scots pine in afforestation, since late spring frosts can damage the plants of Scots pine in the environmental conditions of Bosnia and Herzegovina.

Ducci et al. (2012) stated that phenological characteristics are relevant practical indicators of the adaptability and adaptedness of forest trees. They are directly related to the growth and to the tree architecture. There is a correlation between them and the frost damage, whose incidence is expected to increase with the climate change. A series of assessments on the same individuals over more years leads to conclusions about the climate development.

In this research, phase 1 first occurred at the beginning of May in most of the provenances, and in the mid-May in other provenances (A1, P1, I1). The dates are in compliance with the results by Salminen and Jalkanen (2015) in Finland, but unlike our results, they recorded buds beginning to extend at the beginning of May in the southernmost stand and in mid-May in the northernmost stands.

In 2017 (Ballian et al. 2019), the latest date when all plants were in phase 0 was 20 April, while in this research it was 10 May. The meteorological data for the nearest meteorological station (Bugojno), shown in Table 2, indicated snow in April 2022, which could cause the later start of bud breaking. Contrary, in this research, all plants were in phase 5 on 5 June, while in 2017 (Ballian et al. 2019) it occurred on 21 June.

In 2012, Ballian and Šito (2017) found that 4 April was the beginning of the growth season (the buds begin to develop) in the international provenance test of Scots pine in Žepče (600 m of altitude), and all plants were in phase 6 on 6 June.

Salminen and Jalkanen (2015) observed bud break phases in periods 2001-2003 and 2008-2010 in two locations in Lapland, Finland. On average, buds began to extend at the beginning of May in the southernmost stand and in mid-May in the northernmost stands, and the variation between years was in the range of 3 weeks (Salminen and Jalkanen 2015).

Although the results of this research showed statistically significant differences in the duration of certain phenophases among provenances, the differences in the first appearance of certain phenophases occur in an interval of no more than seven days. In this sense, we cannot recommend using certain provenances in areas where late frost occurs based on the results of this research, especially considering that the year of observations (2022) was a year with meteorological conditions that deviated from the average. Therefore, it is necessary to carry out research in several consecutive years to be able to give recommendations for the use of certain provenances in areas where late frost occurs.

CONCLUSIONS

Phase 1 (start of growth season) first occurred on 10 May in all provenances. Phase 5 first occurred on 5 June in some plants of every provenances except Bugojno (Bosnia and Herzegovina), Delytayn (Ukraine), and Ca Del Lupo (Italy). Variance analysis has shown statistically significant differences in the duration of phenophases 2 (significant elongation of terminal bud, scales still present but the green young shoot is visible), 3 (brachyblasts are well-visible but still in their envelope) and 4 (needles joined by two start to appear). The first and last appearances of phases are in very short interval for all provenances, but differences between the first and last plant in one provenance (the duration of a phase) are great. Research should be continued and should include an investigation of the influence of seasonal climate and climate changes.

Author Contributions

MMH, DB conceived and designed the research, TM carried out the field measurements, MMH processed the data and performed the statistical analysis, DB supervised the research, MMH, TM and DB wrote the manuscript. The main part of these results was presented at the international IUFRO Conference - Abies&Pinus 2022, “Fir and pine management in a changing environment: Risks and opportunities”, held on 19-22 September 2022 in Sarajevo, Bosnia and Herzegovina.

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Conflicts of Interest

The authors declare no conflict of interest.

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Forest Structure of *Pinus ayacahuite* in Southern Mexico: A Non-Parametric Analysis

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ABSTRACT

Spatial structure refers to the horizontal and vertical arrangement of individual trees, and the most accurate way to describe it within a community is to characterize tree strata in terms of their dimensions. The aim of this study was to determine the horizontal and vertical structure of pure stands of *Pinus ayacahuite* Ehrenb. ex Schltdl., in forests of southern Mexico. Forest measurement data from 24 sample plots were used. For analysis of the horizontal structure, diameters within a range of 0.20 cm to 77 cm were used, while for the vertical structure, heights were from 0.09 m to 40.9 m. Non-parametric histograms and Kernel density methods were used in the analysis, and Fisher and Marron multimodality tests were performed. The homogeneity of the forest stands was determined by the coefficient of homogeneity, and the vertical and horizontal structures were described using the stratification proposed by Pretzsch. The results indicate that the horizontal structure corresponds to a diameter distribution with a reversed "J" shape in 79.2% of the sample plots, while 91.8% of the sites were classified as irregular with coefficients of homogeneity of 1.0 to 3.0. In the vertical structure, it was observed that the lower stratum predominated in 75% of the plots, while 25% had a higher concentration of individuals in the middle stratum. The upper stratum had accumulation percentages ranging from 1.3% to 33.3% but did not predominate in any of the plots. According to the multimodality tests, 50% of the plots present multimodality in the horizontal structure, while in the vertical structure this condition is present in 38% of the plots. Knowledge of the spatial structure of *Pinus ayacahuite* forest stands is essential to define silvicultural strategies that ensure the sustainable functioning of the ecosystem in terms of yield continuity and conservation.

Keywords: *Pinus ayacahuite*; tree stratum; non-parametric methods; irregular forest stands

INTRODUCTION

A managed forest is a biological system that undergoes constant change due to natural processes and applied silvicultural activities (García 1988). Silviculture is a tool for sustainable management of species. Silvicultural practices modify the diversity of forest stands, as well as the spatial mixture of trees, natural regeneration, and size distribution (Del Río et al. 2003, Gadow et al. 2012). In addition to silvicultural treatments, factors such as competition for resources, patterns of regeneration, mortality, differential growth, topographic and climatic variations, soil quality, and interactions between species cause variations in the distribution of tree sizes in forests (Coomes and Allen 2007).

The structure of a forest ecosystem refers to the spatial distribution of the main tree characteristics, and the distribution of species by dimension classes is of special importance (Gadow and Hui 1998, Aguirre et al. 2009, Li et al. 2014). The importance of characterizing and quantitatively measuring the structure of forest stands lies in the fact that it provides an understanding of how the ecosystem functions. This is a fundamental aspect that must be considered to understand productivity and make decisions within sustainable forest management (Jiménez et al. 2001, Aguirre et al. 2003, Araujo et al. 2008).

Spatial structure refers to the horizontal and vertical arrangement of individual trees, and the most appropriate and precise way to describe it within a community is to characterize tree strata from the viewpoint of their dimensions (Gadow and Hui 2001). The most used variables to represent the spatial structure of a forest stand are the heights and diameters of the individuals present in it (Corral-Rivas et al. 2019, Guzmán et al. 2019). Horizontal structure is often evaluated in terms of diameter, although basal area or canopy cover can also be used. The vertical structure is assessed using the heights of the trees that constitute the specific study area (Moret et al. 2008). The methods for describing spatial structure can be parametric or non-parametric, of these methods, density functions, such as frequency histograms and Kernel estimators, are outstanding because of their simplicity and ease of interpretation (Pogoda et al. 2020).

In the Sierra Norte region of Oaxaca, in southern Mexico, studies of diversity and structural composition have been conducted to characterize mixed forest stands and *Pinus patula* Schltdl. *et* Cham. (Castellanos et al. 2008, Castellanos-Bolaños et al. 2010, Vásquez-Cortez et al. 2018, Martin et al. 2021). However, it is important to understand quantitative aspects of the dynamics of other species that hold economic, ecological, and social value within forest management. The aim of this study was to determine the horizontal and vertical structure of *Pinus ayacahuite* Ehrenb. ex Schltdl. stands, considering the diameter and height of the individuals as analysis variables.

MATERIALS AND METHODS

The study was conducted in the communal forests of Ixtlán de Juárez, Oaxaca, southern Mexico. This area is geographically located between the coordinates of 17°23'0.50''–17°23'0.58'' N and 96°28'45''–96°28'53'' W. The region falls within the physiographic province known as the Northern Oaxaca Mountain System (Figure 1). The predominant type of vegetation in this region corresponds to pine-oak forests, which were described as heterogeneous by Castellanos-Bolaños et al. (2010). The natural distribution area of *Pinus ayacahuite*, where the study took place, spans an altitudinal range of 2,600 m to 3,100 m and covers an area of 962.85 hectares. The predominant climates are temperate sub-humid with summer rains and temperate humid with summer rains (STF 2015).

In the establishment of the sampling plots, sub-stands were selected without the presence of forest pests, diseases, traces of fires, or any other disturbances. They were pure stands, meaning that the dominant species was *Pinus ayacahuite*. The sampling plots were squares of 400 m^2 each, divided into four quadrants of 10 m × 10 m, and numbered clockwise. Each plot was established facing north and had five control points: one at each vertex and one in the center. All living trees found within the sampling plot were labeled, starting with the tree closest to the center and continuing until reaching the furthest tree.

The forest variables measured in the field and used for analysis of the structure were the following: diameter at breast height of all the individuals (D, cm) measured with a Haglöf Sweden® tree caliper; diameter at the base (DB, cm) of individuals that did not reach a height of 1.30 m, using a Scala® vernier caliper; total height (H, m) of a representative sample of trees, which included individuals of all diameter classes, obtained with a digital clinometer (Haglöf Sweden®); and the height of the individuals whose diameter at the base was measured with a professional Pretul® flexometer.

With the data obtained, the following stand variables were determined: number of trees per hectare ($N\ ha^{-1}$); basal area per hectare (BA, $m^2 \cdot ha^{-1}$) derived from the individual basal area, which was obtained with the expression $BA = \frac{\pi}{40000} \times D^2$; mean height and diameter of

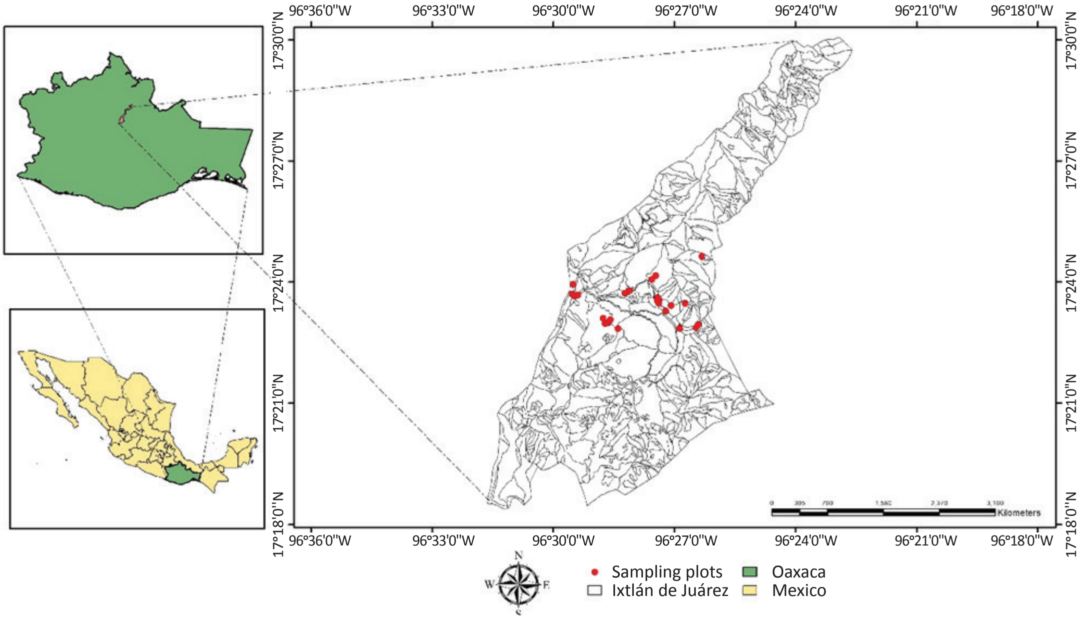

Figure 1. Geographic location of the study area and the sampling plots.

the dominant trees (HD, m; DD, m); and finally, quadratic mean diameter (Dq, cm) $Dq=\sqrt{\frac{40000}{\pi} \times \frac{BA}{N}}$ using the formula (Santiago-García et al. 2013, Pérez-López et al. 2019).

To estimate the heights that were not measured in the field, different documented models for the species were used: *Abies guatemalensis* Rehder, *Alnus acuminata* Kunth, *Arbutus xalapensis* Kunth, *Clethra lanata* M. Martens & Galeotti, *Litsea glaucescens* Kunth, *Pinus ayacahuite*, *P. douglasiana* Martínez, *P. oaxacana* Mirov, *P. patula*, *P. pseudostrobus* Lindl., *P. rudis* Endl, *Quercus* spp., *Clethra mexicana* DC. and other broadleaf species present in the sampling plots (STF 2015, López-Villegas et al. 2017, Santiago-García et al. 2020).

To determine the horizontal structure, the diameters at breast height and diameters at the base were used, and for the vertical structure, the heights observed in the field and those estimated with the height-diameter models were used (Table 1).

In the analysis, histograms and Kernel plots were created for distributions of diameters and heights per sampling unit and for the totality of the data. Pooled and unpooled data by diameter class were used. The statistical programs used were SAS® 9.4 (SAS Institute Inc. 2017) for histograms and Kernel of the grouped data (using the UNIVARIATE and KDE procedures) and RStudio® (R Core Team 2019) for Kernel graphs on non-grouped data (using the DENSITYPLOT function).

To determine the homogeneity of the stands, the homogeneity coefficient (CH) was used, which represents the percentage relationship between the number of trees and the volume, both stratified by diameter classes (De Camino 1976). Authors such as Corral et al. (2005) and Solís et al. (2006) demonstrated that the coefficient of homogeneity can be estimated with equal precision when using the basal area; therefore, due to the number of species present in the plots, this variable was chosen as a volume surrogate for the calculation of CH, through the following expression (Equation 1):

$$CH=\frac{\Sigma_{i=1}^{n} SN\%}{\Sigma_{i=1}^{n} SN\% - SBA\%} \qquad (1)$$

where CH is the coefficient of homogeneity; n is the number of diameter classes; SN% is the sum of the percentages of the number of trees up to diameter class i; and SBA% is the sum of the percentages of the basal area up to diameter class *i*.

The description of the vertical and horizontal structures includes the stratification proposed by Pretzsch (1996), which consists of three strata: stratum I, or superior, with heights and diameters ranging from 80% to 100% of the maximum values of the site; stratum II, or middle, ranging from 50% to 80%; and stratum III, or lower, ranging from 0% to 50%. Furthermore, we analyzed the number of modes for the distribution of heights and diameters using the Fisher and Marron multimodality test with the statistical software RStudio® (R Core Team 2019), under the hypothesis that the height and diameter distribution in the plot is unimodal.

RESULTS AND DISCUSSION

In 79.2% of the plots, a diameter distribution in the form of an inverted "J" or negative exponential was found. In these plots, a greater proportion of individuals with small diameters can be observed; this proportion decreases as the size of the diameter increases (Figure 2a and Figure 2b). Although 20.8% of the remaining plots exhibited a regular distribution, a slight displacement of data towards smaller diameter categories can be observed, resulting in a left-sided distribution (Figure 2c). Gadow et al. (2007) mention that inverted J-shaped curves are typical of diameter distributions of irregular forest stands.

The general irregularity of *Pinus ayacahuite* stands was observed when comparing the 24 sampling plots in Kernel density graphs and a histogram generated from the diameters of all the individuals inventoried (Figure 2). Through graphic analysis, it was possible to determine that the highest densities of individuals were concentrated in diameter classes smaller than 25 cm, with modes ranging from 0.9 cm to 23 cm in the sampling units.

Because of the left-sided asymmetry observed in 79.2% of the plots, we can assume that the *Pinus ayacahuite* stands have a high level of regeneration. Ramirez et al. (2019) characterized the stand structure of a community near Ixtlán de Juárez and found an inverted "J"- shape of the diameter distribution, describing the stands as mature growing systems. It is necessary to consider that the age and size of the trees are not always closely related; this happens more frequently in tolerant species, such as *P. ayacahuite*, which has slower growth than other species of the genus *Pinus* in the study area and incorporates more individuals in lower diameter classes due to the good survival capacity in young stages, leading to the development of an irregular-type structure (Newton 2007, Soto et al. 2010). Restrepo et al. (2012) mention that this structure is the best guarantee of survival in the forest community because taller trees are eliminated and replaced without difficulty by smaller and presumably younger ones.

The horizontal characterization made with histograms and Kernel density graphs confirms the heterogeneity of the

Table 1. Descriptive statistics of data used to determine the horizontal and vertical structure of *Pinus ayacahuite* stands**.**

Variable	n	Mean	Minimum	Maximum	Standard deviation
D	1162	19.610	0.20	77.0	14.706
DB	157	0.842	0.20	2.8	0.526
H	1319	13.204	0.09	40.9	9.142

n - number of trees; D - diameter at breast height (cm); DB - diameter at the base (cm); H - total height (m).

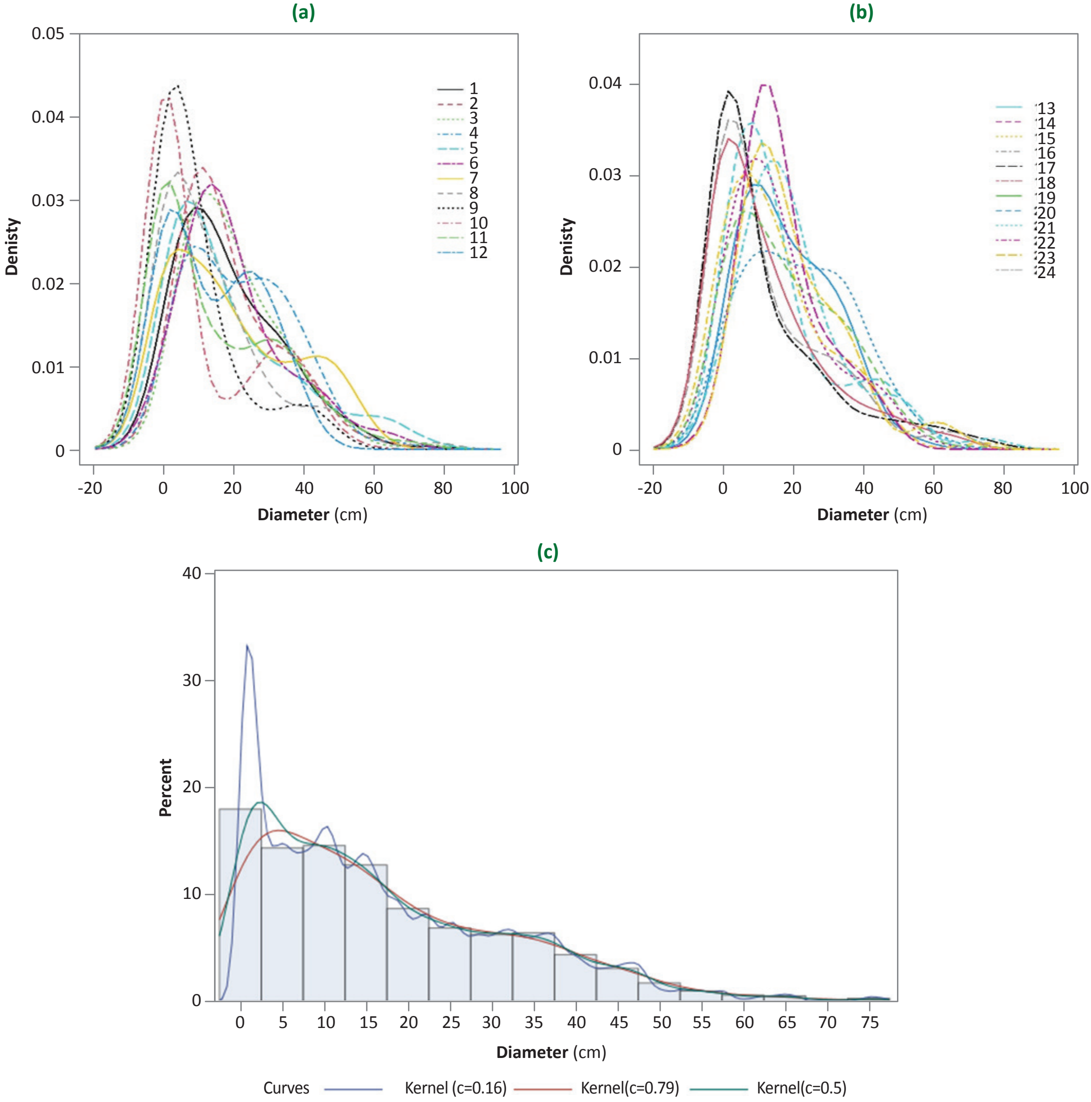

Figure 2. Diameter distributions. **(a)** and **(b)** Diameter distributions of the 24 sampling plots in Kernel density graphs; **(c)** Histogram of the diameter distribution of all the individuals in the 24 sampling plots.

stands of *Pinus ayacahuite*. De Camino (1976) affirms that the coefficient of homogeneity CH varies between one and infinity, with a value of 1.0 indicating complete heterogeneity, and an increase represents an approach towards homogeneity. The CH values obtained fluctuated in the range of 1.51 to 3.55 and were represented with the Lorenz curve, which shows that, as the value of CH decreases, the curve moves further away from the line of maximum homogeneity (Figure 3) (De Camino 1976, Del Río et al. 2003).

Based on the classification established by De Camino (1976), 91.8% of the sites correspond to an irregular forest (CH=1.0-3.0) with values ranging from 1.51 to 2.84. In the transition category (CH=3.1-3.5), only 4.1% of the sites were found with a coefficient of 3.10, and 4.1% were defined as regular forest (CH>3.5) with a value of 3.55.

The stratification for the vertical structure was as follows: the lower stratum (stratum III) predominated in 75% of the plots; the middle stratum (stratum II), present in 25% of the sites, had a concentration of individuals ranging from 41.7% to 57.7%; and the percentages of accumulation in the upper stratum (stratum I) varied from 1.3% to 33.3%, but it did not predominate in any of the plots (Table 2).

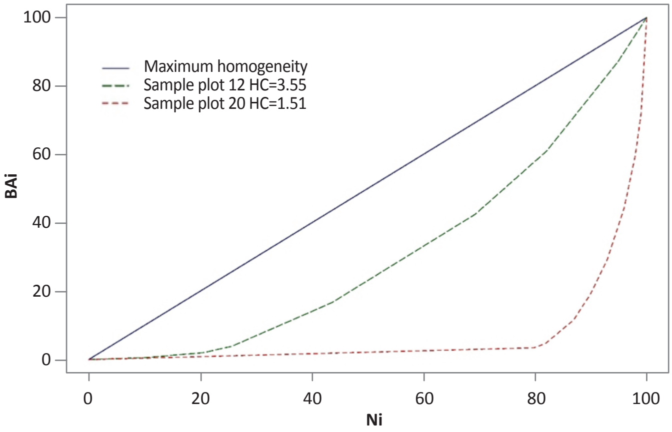

Figure 3. Comparison of the Lorenz curves for the sites with the minimum and maximum values of the coefficient of homogeneity CH. Ni represents the cumulative percentage of the number of trees; and BAi represents the cumulative percentage of basal area.

Table 2. Distribution of individuals by stratum and maximum height of each plot.

Sample plot	Maximum height (m)	Stratum I		Stratum II		Stratum III		Total individuals	
		(Nah)	(%)	(Nah)	(%)	(Nah)	(%)	(Nah)	(%)
1	34.90	6	7.8	17	22.1	54	70.1	77	100
2	30.10	6	9.0	17	25.4	44	65.7	67	100
3	30.80	6	13.0	19	41.3	21	45.7	46	100
4	29.26	13	33.3	11	28.2	15	38.5	39	100
5	40.90	8	18.2	8	18.2	28	63.6	44	100
6	36.87	3	5.8	17	32.7	32	61.5	52	100
7	29.13	8	27.6	13	44.8	8	27.6	29	100
8	32.40	8	19.5	16	39.0	17	41.5	41	100
9	34.51	9	14.8	11	18.0	41	67.2	61	100
10	30.10	7	17.5	14	35.0	19	47.5	40	100
11	30.82	12	30.8	19	48.7	8	20.5	39	100
12	28.43	8	20.5	22	56.4	9	23.1	39	100
13	35.80	7	13.2	12	22.6	34	64.2	53	100
14	33.82	2	4.3	9	19.6	35	76.1	46	100
15	36.26	3	6.3	10	20.8	35	72.9	48	100
16	28.00	11	22.9	20	41.7	17	35.4	48	100
17	29.43	3	6.3	20	41.7	25	52.1	48	100
18	27.15	2	4.9	9	22.0	30	73.2	41	100
19	28.68	11	20.4	21	38.9	22	40.7	54	100
20	28.30	4	1.3	7	3.1	213	95.5	224	100
21	29.35	7	17.1	18	43.9	16	39.0	41	100
22	27.33	8	14.0	17	29.8	32	56.1	57	100
23	25.77	7	26.9	15	57.7	4	15.4	26	100
24	28.87	17	28.8	14	23.7	28	47.5	59	100

Nah - number of trees

In addition to the height stratification, Table 3 presents the results of diameter stratification for each measurement plot.

The lower stratum predominated in 95.8% of the plots, reaffirming the high level of individuals in juvenile and regeneration stages, as can be seen in the inverted "J" shaped diameter distribution (Figure 2). In only one plot was the intermediate stratum dominant, with a 53.8% concentration of individuals. Diameter stratification generally coincides with height stratification.

Through the histograms and Kernel density graphs of the vertical structure, we observed that nine sampling plots have more than one mode, which was verified with the Fisher and Marron statistical multimodality test (R Core Team 2019). According to this test, 62% of the sites exhibit a unimodal height distribution (Table 4). It is important to highlight that these unimodal distributions were found only in the lower stratum, indicating sites where regeneration is occurring.

By plotting the heights of all 24 sample plots and comparing their distributions, we can easily discern the data grouping in the lower and middle strata. The presence of individuals taller than 30 m is rarer (Figure 4) because the upper stratum was not predominant in any plot. The number of modes and the asymmetry in the graphs help to identify the strata and allow us to infer whether the stands are in a juvenile or mature state (Gadow et al. 2007).

The abundance of individuals in the lower stratum is attributed to the tolerance of *Pinus ayacahuite*, as the survival rate in juvenile stages is high despite not receiving direct sunlight, which results in slow growth (Valladares et al. 2004). However, with greater light availability, regeneration may be favored, as observed in one sampling site (plot 20), where the concentration of individuals in the lower stratum was higher compared to the rest of the plots. This is attributed to its proximity to a clearcutting stripe.

Table 3. Distribution of individuals by stratum, minimum and maximum diameter of each plot.

Sample plot	**Maximum diameter** (cm)	**Minimum diameter** (cm)	**Stratum I**		**Stratum II**		**Stratum III**		**Total individuals**	
			(Nah)	(%)	(Nah)	(%)	(Nah)	(%)	(Nah)	(%)
1	57.6	1.6	2	2.6	13	16.9	62	80.5	77	100
2	47.8	1.4	6	9.0	8	11.9	53	79.1	67	100
3	75	4.5	1	2.2	4	8.7	41	89.1	46	100
4	65.5	1.1	1	2.6	12	30.8	26	66.7	39	100
5	74.8	0.2	5	11.4	5	11.4	34	77.3	44	100
6	66.8	5.6	3	5.8	4	7.7	45	86.5	52	100
7	57.2	5.0	7	24.1	6	20.7	16	55.2	29	100
8	77.0	5.0	1	2.4	7	17.1	33	80.5	41	100
9	48.0	1.6	8	13.1	7	11.5	46	75.4	61	100
10	51.7	0.4	4	10.0	14	35.0	22	55.0	40	100
11	57.5	0.8	3	7.7	16	41.0	20	51.3	39	100
12	41.5	3.0	7	17.9	21	53.8	11	28.2	39	100
13	60.5	0.3	6	11.3	10	18.9	37	69.8	53	100
14	76.0	6.2	1	2.2	2	4.3	43	93.5	46	100
15	64.1	1.5	3	6.3	7	14.6	38	79.2	48	100
16	55.0	3.5	1	2.1	12	25.0	35	72.9	48	100
17	51.0	0.4	3	6.3	10	20.8	35	72.9	48	100
18	54.0	4.1	2	4.9	5	12.2	34	82.9	41	100
19	58.7	0.6	3	5.6	13	24.1	38	70.4	54	100
20	70.0	0.2	1	0.4	3	1.3	220	98.2	224	100
21	52.9	3.6	4	9.8	12	29.3	25	61.0	41	100
22	53.2	1.7	2	3.5	11	19.3	44	77.2	57	100
23	52.3	8.3	3	11.5	8	30.8	15	57.7	26	100
24	50.7	3.4	6	10.2	14	23.7	39	66.1	59	100

Nah - number of trees

Table 4. Multimodality in horizontal and vertical structures of the 24 sample plots.

Variable	Number of plots with multimodal distribution	Proportion
Diameter	12	50%
Height	9	38%

Terborgh (1985) points out that forests have multiple vertical strata, which vary in number depending on the latitude of the stand's location, as this determines the angle of penetration of the sun's rays into the understory. According to Parker (1997), the light demand of the species is the reason they are arranged in different positions along the vertical profile of the forest since light intensity decreases as it penetrates towards the lower levels of the canopy. In this way, species that demand more light are positioned in higher canopies, while more shade-tolerant species tend to be positioned at lower heights within the forest (Donoso 1993, Parker and Brown 2000).

Oyarzún et al. (2019) state that vertical heterogeneity corresponds to the degree of dispersion of trees at different canopy heights and determined that, in forests with a temperate climate, functionally tolerant species such as *Amomyrtus luma* and *Myrceugenia planipes* are distributed in the lower canopy with a bimodal distribution, a description that coincides with the characteristics of the species and the vertical structure described in this research.

The graphs of the vertical structure exhibit behavior similar to those of the horizontal structure, demonstrating the efficiency of calculating heights from allometric models that facilitate the data collection phase, as suggested by Gadow et al. (2007).

The structural characterization of *Pinus ayacahuite* allows planning silvicultural treatments to control stand dynamics and achieve objectives of forest management. It is important to consider regeneration and incorporation into different diameter classes to ensure the permanence of the species and continuous yields while protecting the canopy in accord with its tolerance and light demand. The structure of the forest stand is crucial for promoting biodiversity, regulating the water cycle, storing carbon, and resisting disturbances, as well as for providing forest products and services. Therefore, managing, and conserving forests while considering their structure is essential to guarantee their health and sustainable functioning.

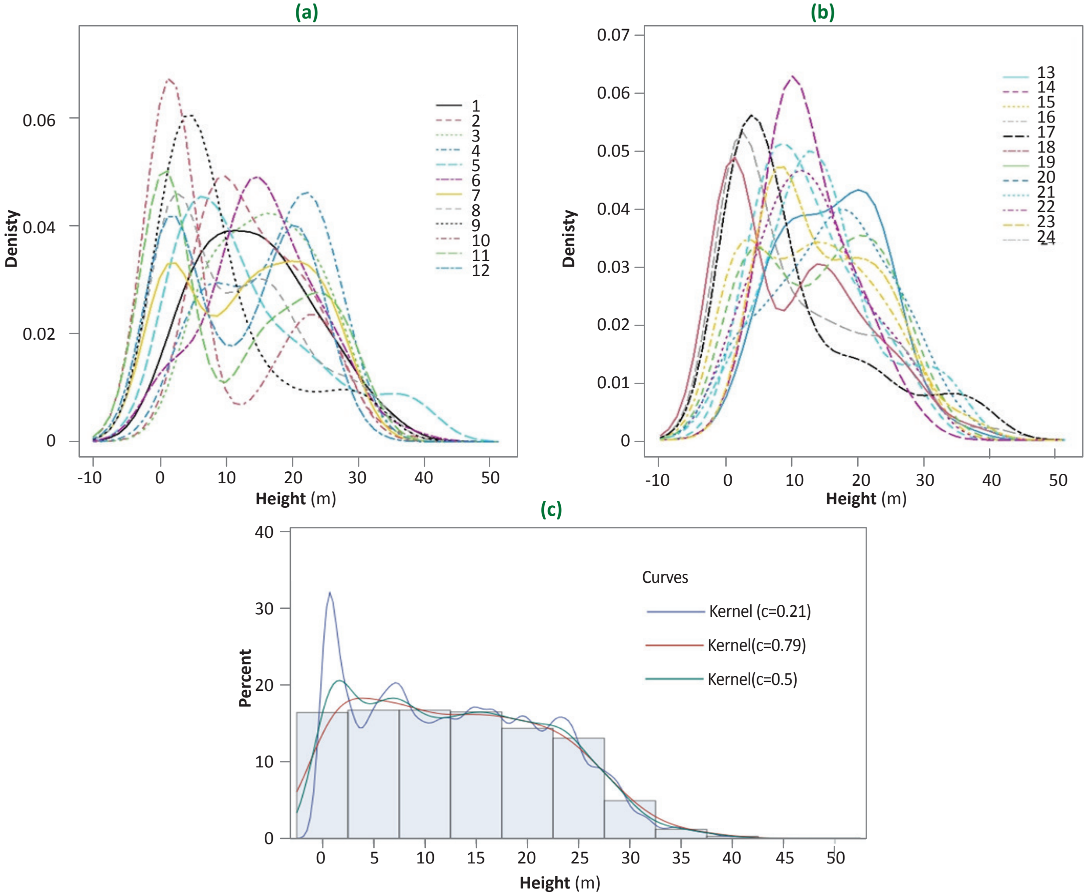

Figure 4. Height distributions: **(a)** and **(b)** Comparison of Kernel density graphs of the heights of the 24 plots; **(c)** Histogram of heights of all individuals.

CONCLUSIONS

The horizontal structure of *Pinus ayacahuite* stands exhibited a reversed "J"-shape in 79.2% of the sampling plots. Therefore, these forest stands can be described as irregular, with a greater number of individuals in lower diameter classes, which decreases as diameter increases. Based on the homogeneity coefficients obtained and following the classification established by De Camino, *Pinus ayacahuite* stands were defined as heterogeneous and irregular, with values ranging from 1.51 to 3.55. In 75% of the sampling plots, the vertical structure showed a higher concentration of trees in the lower stratum, while in the remaining 25%, individuals in the middle stratum predominated. Individuals taller than 30 meters were few; thus, the upper stratum had concentrations that varied from 1.3% to 33.3%. For both the horizontal and vertical structure, the concentration of individuals in lower classes resulted in positive asymmetry, indicating that *Pinus ayacahuite* exhibits a characteristic behavior of a species with a tolerant temperament, especially in the early stages of growth. This data grouping also demonstrates that this species has high potential for regeneration and incorporation of individuals into different diameter classes, resulting in an irregular structure. Frequency histograms and Kernel density graphs are non-parametric methods that provide an alternative for describing spatial structure. They offer advantages such as speed and ease of data interpretation. These tools highlighted the strong relationship between the diameters and heights of the individuals because the data distribution was similar. The results documented in this study regarding the horizontal and vertical structure, contribute to our understanding of size distribution and stratification of the species *Pinus ayacahuite* in southern Mexican forests. This knowledge is relevant for decision-making to achieve sustainable forestry management.

Author Contributions

WSG conceived and designed the research, KMPV, WSG and ESG carried out the field measurements, KMPV and WSG processed the data and performed the statistical analysis, GAP, FRA and ESG supervised the research and helped to draft the manuscript, KMPV, WSG, GAP, FRA and ESG wrote, reviewed, and edited the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Climate Characteristics of the Illyrian Phytogeographic Area

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ABSTRACT

The first ecological research in the Illyrian phytogeographic area, located on the Balkan peninsula in south-eastern Europe, dates back to the early 20th century. Traditionally, the Illyrian phytogeographic area includes Bosnia and Herzegovina, Montenegro, Croatia and parts of Slovenia. Due to climate change, more available data and new measurement techniques, the Illyrian phytogeographic area may have shifted northwards to the southern Alps and parts of Austria. In this study we have analysed climate as an important ecological variable for delineating the Illyrian phytogeographic area using precipitation and air temperature from 75 climatological stations in Bosnia and Herzegovina, Croatia, Slovenia and Austria (Carinthia). Our statistical analysis suggests, that there are significant differences in the analysed climate parameters across the countries, suggesting that sub-sections may already exist within the extent of the Illyrian phytogeographic area.

Keywords: Dinaric alps; Mediterranean forests; site conditions; climatology; meteorological measurements; Illyrian phytogeographic area

INTRODUCTION

The first analysis of the Illyrian vegetation was done by Beck - Mannagetta (1901). Adamović (1907) then defined the Illyrian area and stated that it more or less follows the area of the central Dinaric Alps. This area covers parts of contemporary Slovenia, Croatia, Bosnia and Herzegovina, Serbia and Montenegro. Adamović (1907) divided the Illyrian zone fittingly into the following subzones: Bosnian, Herzegovinian-Montenegro and Serbian subzones.

The term "Illyrian floral element" introduced by Beck - Mannagetta (1901) represents all plant taxa that are either (1) completely limited in their distribution or (2) have their centre of distribution in the Illyrian area. Illyrian floral elements from the climatic point of view are mainly thermophilic and heliophilic taxa, even when growing in mountains, due to the strong influence of the Mediterranean Sea on the Dinaride mountains (Trinajstić 1992). Similar interpretations of the climate of the Illyrian area are found in Horvatić (1967), Horvat et al. (1974) and Stefanović et al. (1983).

Fukarek (1978) defines the so-called "core" zone of the Illyrian province, which comprises the area between the rivers Kupa, Korana, Una, Sana and Vrbas. More recent references of the Illyrian phytogeographical area also include part of the southern Alps in Carinthia, Austria (Annon. 2006, 2013). Fukarek (1978), similarly to Adamović (1907), has described the wider Illyrian area as a complex area that can be divided into: northern (Slovenia-Croatia), central (Bosnian) and southern (Herzegovina-Montenegro). Mayer (1986) shared this view and published a map of the climate on the Balkan Peninsula. This map shows that the traditional extent of the Illyrian area does not include the Alps, which are assigned to the "alpine" climate and separated from the Illyrian area.

Detailed analysis of the western part of the Illyrian area by Wraber (1960) for Slovenia and Fukarek (1980) for former Yugoslavia suggest that the western boundary of the Illyrian phytogeographical province indeed ends in Slovenia and does not extend into Austria.

Analysing the map by Wraber (1960), Fukarek (1978) states that, at that time under the name *Fagion illyricum* (Horvat 1938) and now under the name *Aremonio-Fagion* (Horvat 1950, Török et al. 1989), the area includes the following sub-regions: Dinaric, pre-Dinaric, pre-Alpine and pre-Pannonian. The Alpine sector starts at the Slovenian-Austrian border, extends into Austria (Mayer 1986), and is separated from the Illyrian Phytogeographical Province. In the classic book "*Vegetation Südosteuropas*" (Horvat et al. 1974), analysing the vegetation of Southeast Europe, the border of the Illyrian province is described in detail: "Tolmin basin in the Soča (Isonzo) valley across the mountains around Cerknog, Idrije and Škofje Loka, and the Sava basin between Kranje and Ljubljana, further along the Sava valley towards Zidan most (Kum, Veliko Kozje). The lower Savinja basin to the valleys of the river Dravinja (Konjiška gora, Boč) and the valley of the river Sotla (Macelj)".

The classic understanding is that the area of the Dinarides coincides geographically and spatially with the phytogeographical area of the Illyrian province. There is evidence for this link in the naming of the neutrophilic beech-fir forests of the Illyrian area. The original name of this forest type was *Abieti-Fagetum dinaricum* by Tregubov (1957). One year later Fukarek and Stefanović (1958) changed this name to *Abieti-Fagetum Illyricum*, suggesting a tight link between the two terms. Currently the valid name of this forest is *Omphalodo – Fagetum* (Tregubov 1957, Marinček et al. 1993).

The above cited authors (Adamović 1907, Horvat et al. 1974, Fukarek 1978, Mayer 1986) individually conclude that the Illyrian area extends to Montenegro, Bosnia and Herzegovina and Croatia, and that the western boundary of the Illyrian area is in Slovenia and the northern boundary in the pre-alpine area of Slovenia, not extending to the Alps. Recent research related to the phytogeographic division of the western part of the Illyrian province in Slovenia was covered by Dakskobler et al. (2000), Dakskobler (2002), and Surina (2002), while Dzwonko et Loster (2000), Marinšek et al. (2013), Stupar et al. (2022), Ugarković et al. (2022) wrote about the ecology, syntaxonomy and phytogeographical differentiation of the southwestern Balkan peninsula.

More recently, Vukelić et al. (2010), Collalti et al. (2014) and Lévesque et al. (2014) highlighted the impacts of climate change (e.g. increase in average temperatures, changes in precipitation amount and seasonal distribution) on forest area, forest productivity or sensitivity to changes in habitat conditions. Climate change may require long-term changes in forest management that will require a reliable understanding of the origin of tree provenances (Lindner et al. 2014), in order to evaluate the future adaptability and adaptability of tree species under various climate change scenarios (Geßler et al. 2006, Taeger et al. 2013). The available literature underpins the necessity of reliable knowledge on the climatic characteristics of phytogeographical areas.

GENERAL CLIMATE CHARACTERISTICS OF THE ILLYRIAN AREA

Historically and for practical reasons, defining the Illyrian area was done almost exclusively based on flora. However, the Illyrian phytogeographic area has some other peculiarities that are often "neglected", such as vegetation syndynamics and vegetation zoning. Both of these characteristics are a consequence of the ecological characteristics of this area and may help in understanding of the Illyrian phytogeographical area. Climate is an important ecological factor and should be considered in attempts defining the Illyrian phytogeographic area.

General descriptions of the climate of the Illyrian area were given by Horvat et al. (1974), who stated that the

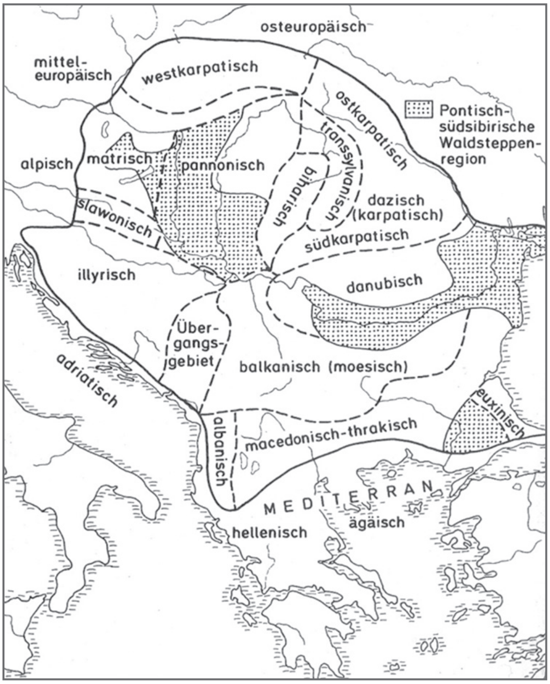

Figure 1. Phytogeographical division of South Eastern Europe (Mayer 1986).

climate of the Illyrian province is different from the climate of other vegetation zones of the Balkan Peninsula, such as the Moesian regions, the Carpathians or the European Alps. In the Illyrian area, there is enough precipitation during the summer and no summer drought, while the Mediterranean climate influence is still present. The seasonal precipitation shows a decrease in the summer months (June-September) for the Illyrian area, while in the Alps in contrast the amount of precipitation increases during summer compared to winter. In the Illyrian area, a usually persistent snow cover lasting from late autumn to March, April or even May protects the soil from frost with an insulating layer from several decimetres up to one meter thick.

The climate in the mountains of the Illyrian area is usually mild, rainy, with a maritime influence. Annual rainfall is usually around 1200-1300 mm, although it can range up to 2000 mm. The middle and upper elevations are often influenced by persistent cloud cover or cloud fog, contributing to the maritime climate character of this area (Horvat et al. 1974). The influence of climate on vegetation is expected to vary along an altitudinal gradient, which is evident from the distinct altitudinal vegetation zoning. As the altitude rises, temperatures drop, late and early frosts shorten the vegetation period, precipitation and the number of foggy days increase and the snow cover is larger and more persistent. More detailed descriptions of climate parameters in the Dinaric Mountains can be found at: https://www.dinarskogorje.com/klima.html.

The objectives of this study are: (1) to analyse regional variations in the Illyrian phytogeographic area using climate station data from Bosnia and Herzegovina, Croatia, Slovenia and Austria (Carinthia), and (2). to check which climatic parameters (air temperature, precipitation) are suitable factors in determining the extent of the Illyrian phytogeographic area.

MATERIAL AND METHODS

All available climate data were collected from Bosnia and Herzegovina (BiH), Croatia, Slovenia, Austria (Carinthia) (mean annual temperature by month, annual average temperature, mean amount of precipitation by month, annual average precipitation sum) for the period 1961-2010 and taken from the Chamber of Agriculture and Forestry in Carinthia (AUSTRIA). In total, data from 75 weather stations were analysed, of which:

- 42 from Slovenia (42 had precipitation data): Illirska Bistrica. Bohinjska Cesnjica, Rateče, Babno Polje, Bovec, Celje, Čepovan, Gornja Radgona, Gornji Lenart, Jareninski vrh, Javorje, Klenik, Kočevje, Kredarica, Krn, Krvavec, Kum, Lesce, Lisca, Ljubljana Bežigrad, Ljubljana JP Airport, Mali Lipoglav, Maribor ER Airport, Maribor Tabor, Miklavški hrib, Mozirje, Murska Sobota, Nanos, Abram, Nova vas, Novo mesto, Planina pri Sevnica, Planina pod Golico, Planina (under Mirno goro), Postojna, Rogaška Slatina, Sevno, Slovenske Konjice, Šmartno near Slovenj Gradac, Topol near Medvodah, Vedrijan, Velenje, Šalovci, Tomaj, Vojsko, Vrhnika, Zgornja Ščavnica;
- 4 from Croatia: Gospić, Knin, Ogulin, Zavižan;
- 13 from Bosnia and Herzegovina: Bihać, Bjelašnica, Bugojno, Butmir, Goražde, Ivan Sedlo, Jajce, Livno, Mostar, Sanski Most, Sarajevo, Tuzla, Zenica;
- 12 from Austria (Carinthia): Bad Bleiberg, Dellach, Bad Eisenkappel, Ferlach, Klagenfurt, Kornat, Reisach, Fresach, Höhenbergen-Tainach, Kanzelhöhe, Loibl-Tunnel, St. Michael ob Bleiburg.

Statistical analysis was conducted using one-factor analysis of variance ANOVA with the StatGraphycs Centurion XVI software. The least significant difference test (LSD test) was used to determine the statistical significance of the average differences for the analysed climate parameters by month between individual countries.

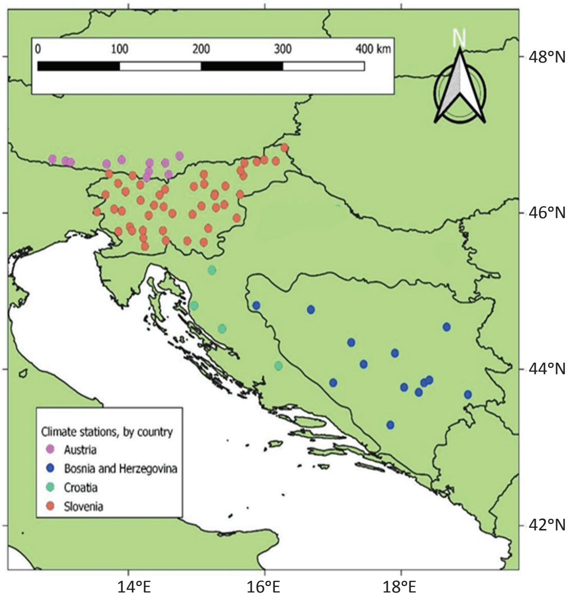

Figure 2. Locations of the used weather stations.

RESULTS

Statistical analyses were conducted for the amount of precipitation and air temperature using data from all weather stations (Table 1).

Table 1 shows that statistically significant differences ($p < 0.05$) occur between October and February for temperature and during the entire year for precipitation except in March, April and November. The climatic elements with significant differences grouped by countries are shown in the following subsections.

Analysis of the Influence of Locality on Precipitation

The results of the conducted one-factor analyses are presented in the graphical (Figure 3-7) and tabular (Table 2-6) form.

Table 1. Presentation of determined values of One-Way ANOVA for the level of significance (p) and the ratio of variances (F) by month, and the average by year for the amount of precipitation (mm) and temperature (°C). Marked (*) are statistically significant differences at p<0.05.

Month	**Factor: Temperature** (°C)		**Factor: Amount of precipitation** (mm)	
	Ratio (F)	**level of significance (p)**	**Ratio (F)**	**level of significance (p)**
I	7.18*	<0.001*	5.22*	0.0027*
II	*	0.0215*	7.20*	0.0003*
III	1.39	0.2536	2.02	0.1190
IV	1.22	0.31	1.85	0.1470
V	0.74	0.5325	3.40*	0.0226*
VI	0.59	0.6231	14.98*	<0.001*
VII	0.73	0.5368	17.70*	<0.001*
VIII	0.98	0.4052	23.52*	<0.001*
IX	1.92	0.1349	11.42*	<0.001*
X	2.88*	0.0421*	4.25*	0.0083*
XI	6.08*	<0.001*	1.65	0.1866
XII	7.52*	<0.001*	2.87*	0.0431*
Average (I-XII)	2.03	0.1175	3.31*	0.0252*

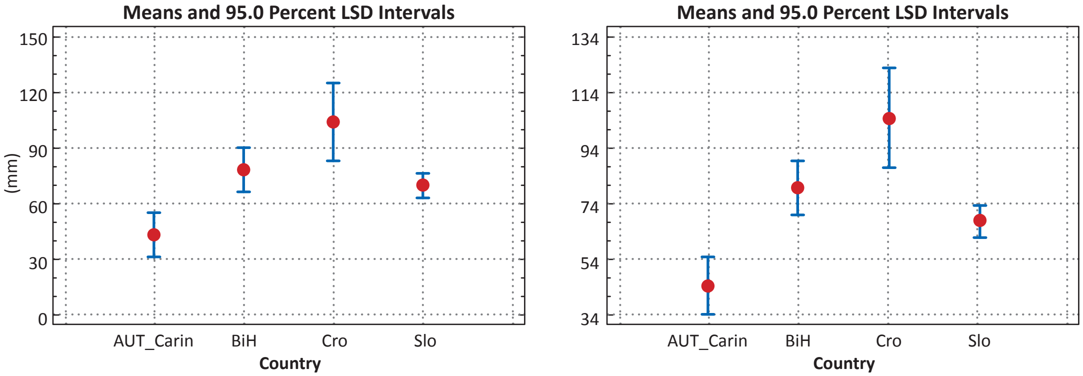

Figure 3. Average values with 95% LSD confidence interval for precipitation (mm) in January **(left)** and in February **(right)** for four different countries.

Table 2. Presentation of the formed homogeneous groups with regard to precipitation (mm) in January **(left)** and in February **(right)**, depending on the analysed countries.

Country	Count	Mean	Homogeneous Groups
AUT_Carin	12	43.50	X
Slo	42	70.12	X
BiH	13	78.89	XX
Cro	4	104.48	X

Country	Count	Mean	Homogeneous Groups
AUT_Carin	12	44.82	X
Slo	42	67.45	XX
BiH	13	79.17	XX
Cro	4	104.20	X

Our results show that for the amount of precipitation in January and February two homogeneous groups were distinguished, namely Austria, as the first group, and the other three countries, as the second group (Figures 1 and 2, Table 1). This means that in these two months the amount of precipitation in Austria is significantly lower compared to Slovenia, Croatia and Bosnia and Herzegovina.

On the other hand, during the vegetation period, i.e. from June to September, two homogeneous groups were evident, the first group including Austria and Slovenia, where on average during these four months there was significantly more precipitation compared to Croatia and BiH, as the second group (Figures 3, 4, 5, 6, and Tables 2, 3).

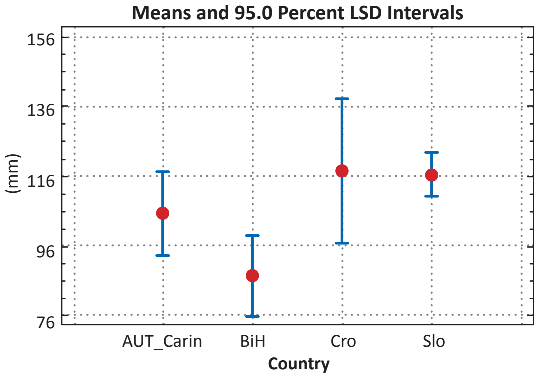

Figure 4. Average values with 95% LSD confidence interval for precipitation (mm) in May **(left)** and in June **(right)** for four different countries.

Table 3. Presentation of the formed homogeneous groups with regard to the average amount of precipitation (mm) in May **(left)** and in June **(right)**, depending on the analysed countries.

Country	Count	Mean	Homogeneous Groups
BiH	13	87.53	X
AUT_Carin	12	105.46	XX
Slo	42	116.52	X
Cro	4	117.73	XX

Country	Count	Mean	Homogeneous Groups
BiH	13	94.22	X
Cro	4	116.65	XX
AUT_Carin	12	128.82	XX
Slo	42	143.52	X

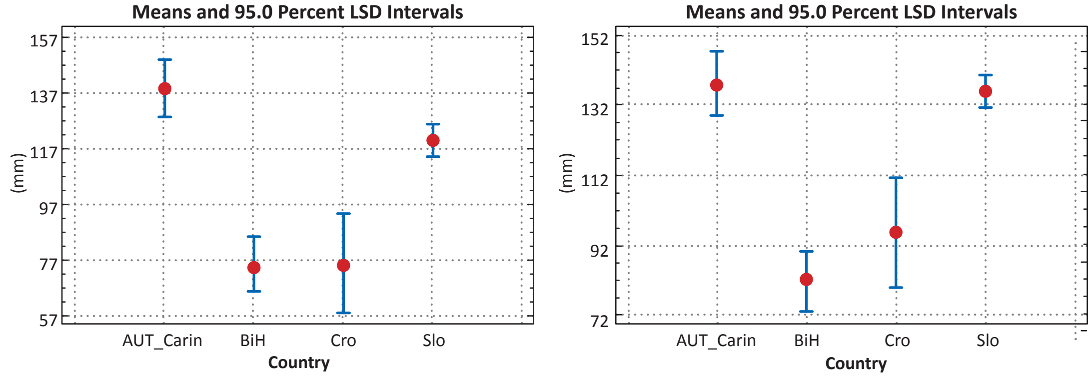

Figure 5. Average values with 95% LSD confidence interval for precipitation (mm) in July **(left)** and in August **(right)** for four different countries.

Table 4. Presentation of the formed homogeneous groups with regard to precipitation (mm) in July **(left)** and in August **(right)** depending on the analysed countries.

Country	Count	Mean	Homogeneous Groups
BiH	13	75.45	X
Cro	4	75,58	X
Slo	42	119.91	X
AUT_Carin	12	138.67	X

Country	Count	Mean	Homogeneous Groups
BiH	13	81.33	X
Cro	4	95.50	X
Slo	42	135.76	X
AUT_Carin	12	138.02	X

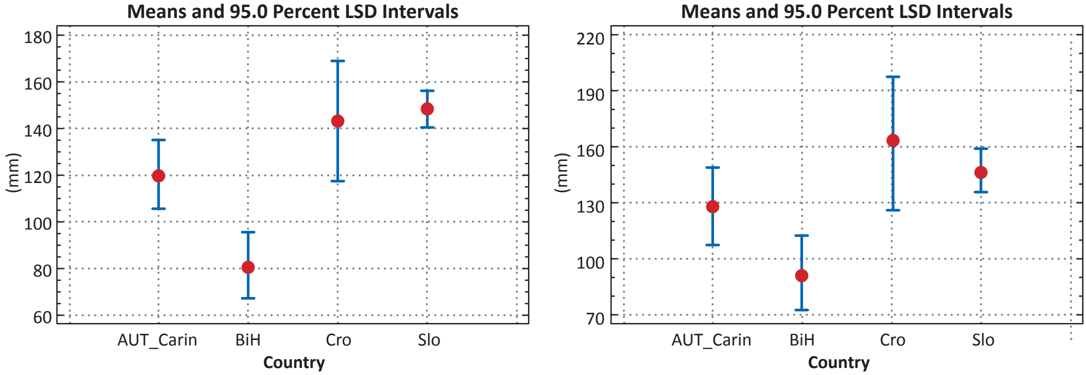

Figure 6. Average values with 95% LSD confidence interval for precipitation (mm) in September **(left)** and in October **(right)** for four different countries.

Table 5. Presentation of the formed homogeneous groups with regard to precipitation (mm) in September **(left)** and in October **(right)** depending on the analysed countries.

Country	Count	Mean	Homogeneous Groups
BiH	13	81.49	X
AUT_Carin	12	120.36	X
Cro	4	143.00	XX
Slo	42	148.31	X

Country	Count	Mean	Homogeneous Groups
BiH	13	92.46	X
AUT_Carin	12	128.62	XX
Slo	42	147.21	X
Cro	4	161.58	X

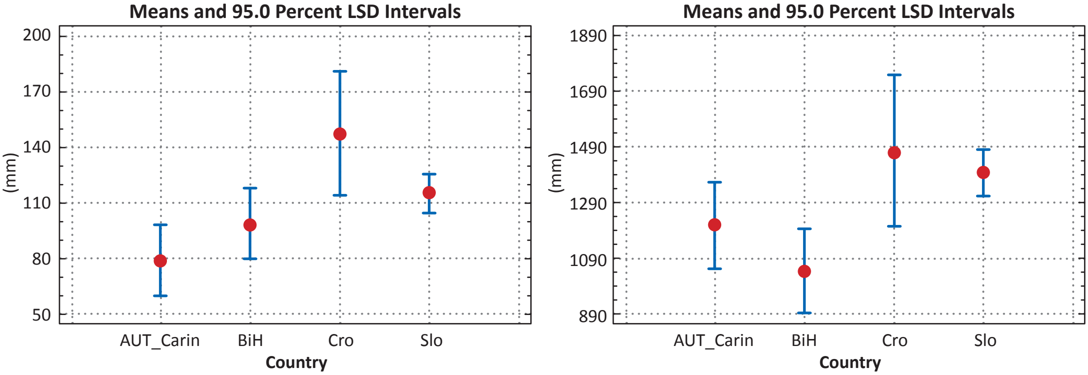

Figure 7. Average values with 95% LSD confidence interval for precipitation (mm) in December **(left)** and for annual precipitation sum **(right)** for four different countries.

Table 6. Presentation of the formed homogeneous groups with regard to precipitation (mm) in November **(left)** and the annual precipitation sum **(right)** depending on the analysed countries

Country	Count	Mean	Homogeneous Groups
AUT_Carin	12	79.31	X
BiH	12	99.01	XX
Slo	42	115.31	X
Cro	4	147.60	X

Country	Count	Mean	Homogeneous Groups
BiH	13	1045.35	X
AUT_Carin	12	1208.01	XX
Slo	42	1397.24	X
Cro	4	1475.08	XX

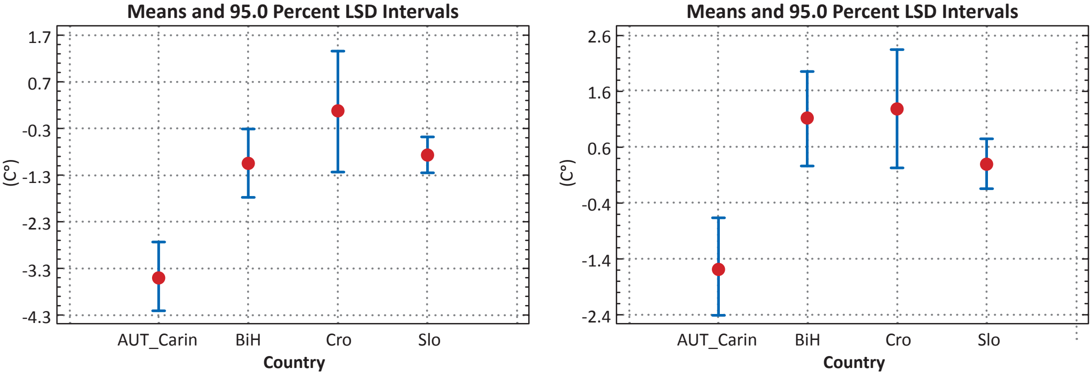

Figure 8. Average values with 95% LSD confidence interval for air temperature (°C) in January **(left)** and in February **(right)** for four different countries.

Table 7. Presentation of the formed homogeneous groups with regard to average air temperatures (°C) in January **(left)** and in February **(right)**, depending on the analysed countries.

Country	Count	Mean	Homogeneous Groups
AUT_Carin	12	-3.48	X
BiH	13	-1.05	X
Slo	46	-0.86	X
Cro	4	0.05	X

Country	Count	Mean	Homogeneous Groups
AUT_Carin	12	-1.53	X
Slo	46	0.30	X
Cro	4	0.78	XX
BiH	13	1.12	X

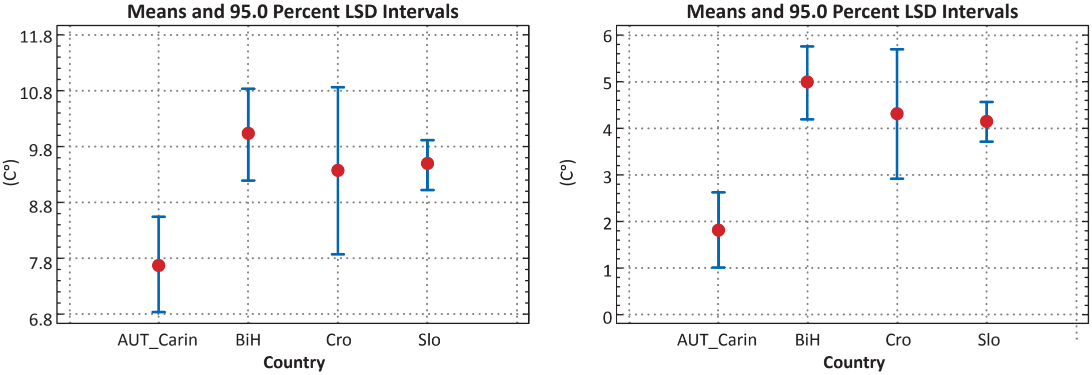

Figure 9. Average values with 95% LSD confidence interval for air temperature (°C) in October **(left)** and in November **(right)** for four different countries.

Table 8. Presentation of the formed homogeneous groups with regard to average air temperatures (°C) in October **(left)** and in November **(right)**, depending on the analysed countries.

Country	Count	Mean	Homogeneous Groups
AUT_Carin	12	7.71	X
Cro	4	9.38	XX
Slo	46	9.47	X
BiH	13	10.02	X

Country	Count	Mean	Homogeneous Groups
AUT_Carin	12	1.80	X
Slo	46	4.12	X
Cro	4	4.30	X
BiH	13	4.9	X

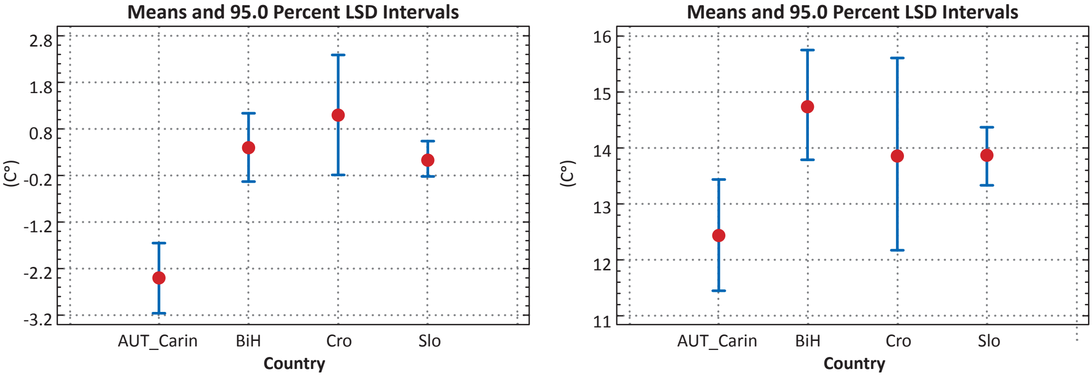

Figure 10. Average values with 95% LSD confidence interval for air temperature (°C) in December **(left)** and during the year **(right)** for four different countries.

Table 9. Presentation of formed homogeneous groups with regard to average air temperatures (°C) in December **(left)** and the average annual temperature **(right)** depending on the analysed countries.

Country	Count	Mean	Homogeneous Groups
AUT_Carin	12	-2.40	X
Slo	46	0.02	X
BiH	13	0.40	X
Cro	4	1.08	X

Country	Count	Mean	Homogeneous Groups
AUT_Carin	12	7.16	X
Cro	4	8.83	XX
Slo	46	8.88	X
BiH	13	9.24	X

This suggests a pronounced summer and winter maritime influence of the Mediterranean climate of the Adriatic Sea on the Dinaride mountains (BiH and Croatia) where the climate is drier, while the Alps (part of Slovenia and Austria) are outside of that influence and are more influenced by the Atlantic climate. It can be concluded that the more humid climate during the summer, i.e. with less precipitation during the winter, distinguishes the Alps from the Dinarides with regards to precipitation.

Analysis of the Influence of Locality on Air Temperature

In a similar way, the monthly results of the ANOVA for temperatures are presented. Statistically significant differences appeared for the following months: January, February, October, November and December (Table 1).

DISCUSSION

Already Horvatić (1967) noted that the entire Illyrian area differs in comparison with the neighbouring eastern Moesian province receiving a higher amount of precipitation. Compared to the southern Mediterranean area with a different precipitation pattern, the summers in the Illyrian area are warm, but have a sufficient amount of precipitation during the summer months. Horvatić (1967) further states that the Mediterranean influence on precipitation prevails in most of the Dinaric mountains, with large amounts of snow in winter creating a thick and long-lasting snow cover, while the continental pluviometric regime is characterized by abundant and frequent rain in summer in comparison to precipitation in winter.

The above implies that in the Illyrian area at the interface of the two climate regimes there are two precipitation peaks: one in autumn-winter and one in spring. This is significantly different compared to seasonal distribution of precipitation in the Alps, where one precipitation maximum during the summer is typical. Our analysis confirms this based on the examples of Bjelašnica (BiH) and Kanzelhöhe (Austria - Carinthia) in Figure 11.

Our results are line with Horvat et al. (1974) and the amount of summer precipitation in Austria (Carinthia) is sufficient without drought, but the Mediterranean influence is visible in summer months (June to September) in the southern parts of the Illyrian area. The distribution of precipitation shows a decrease in the amount of precipitation in these months in the Illyrian area, while in the Alps, on the contrary, the amount of precipitation increases during the summer months.

Similarly, Stefanović et al. (1983) in the ecological-vegetational zoning of BiH indicate that there are significant penetrations of the Mediterranean climate into the continental part of BiH during the summer, describing the area of the inner Dinarides from the mountains of Plješevica in the northwest to the mountains of Maglić and Volujak in the southeast as following: "The entire area is under the influence effects of the mutual conflict between moderate continental and modified Mediterranean climate". Fukarek (1970) decribes the area of the Dinaric mountains of Prenj, Orijen and Čvrsnica as having a climate varying from Mediterranean to continental conditions. This is also supported by Trinjstić (1992), who stated that the climate in the mountains of the Dinarides is strongly influenced by the Mediterranean climate.

Beus (1984) has a somewhat different position on the interface of Mediterranean and temperate continental climates and proposes a division of the Dinarides of Bosnia and Herzegovina into two parts: the outer Dinarides - with a stronger Mediterranean and Pannonian climate influence with prevalence of beech and fir forests without spruce, and the inner Dinarides - without these influences, which is reflected in the appearance of beech and fir forests with spruce. Fukarek (1970) states that the appearance of European and West Asian *Abies* species in the global understanding represents a set of species adapted to the transitional Mediterranean climate, and that *Picea* species (except for *Picea omorika*) show a relation to the boreal, high-elevation climate. The occurrence of larch (*Larix decidua*) is confined to the coldest parts of Slovenia (Bončina et al. 2021).

Elaborating on the association of beech forests and beech-fir forests (*Aremonio – Fagion* Horvat 1950, Török et al. 1989) in the context of understanding the Illyrian area, Vukelić (2010) states that both climate and geological-pedological background play an important role in understanding the extent of this area. In this context, Sukachev (1972) also asks the question: "Is plant cover really a reliable sign for determining the forest type and other habitat conditions do not have to be investigated"?

In a broader context, Vojniković (2015) brings the connection between the occurrence of European larch (*Larix decidua*) in the Alps and Scots pine (*Pinus sylvestris*) in the Dinarides in the context of different directions of succession, which are related to mesophilic habitats. He proposes that the Alps are more mesophilic than the Dinarides and therefore more suitable for the appearance of European larch and the reverse is evident for Scots pine. The occurrence of Scots pine on the dolomite slopes of the alpine area in Slovenia in the plant community *Rodothamno – Pinetum sylvestris* (Rozman et al. 2020) was confirmed by Bončina et al. (2021), who explicitly stated that this type does not include the pioneer succession of Scots pine stands on potential beech sites in the Alps, but rather represents a permanent stage of vegetation, confirming Vojniković's point of view. However, even in the context of construction, Vojniković (2015) understands European larch as a pioneer and gives it a greater importance of belonging to the Alpine area, than the appearance of Illyrian species, which can also be found in the Alpine area.

However, the mentioned border should not be understood "sensu stricto", that is, it should be understood as a diffuse border. Individual Illyrian species, due to their wide ecological amplitude can also enter the alpine sector, but when moving away from the border of the Illyrian province they gradually disappear. The above means that only and exclusively in smaller border areas within the Alpine sector, forms of extra zonal vegetation and fragments of Illyrian forests can appear, which are conditioned by orographic, edaphic and microclimatic factors.

Our analysis provides fresh evidence on a climatic discrepancy in the Illyrian area, which is sometimes considered a homogenous climate region. While BiH and Croatia undoubtedly have a predominantly Illyrian climate, Slovenia and Austria (Carinthia) deviate from the typical

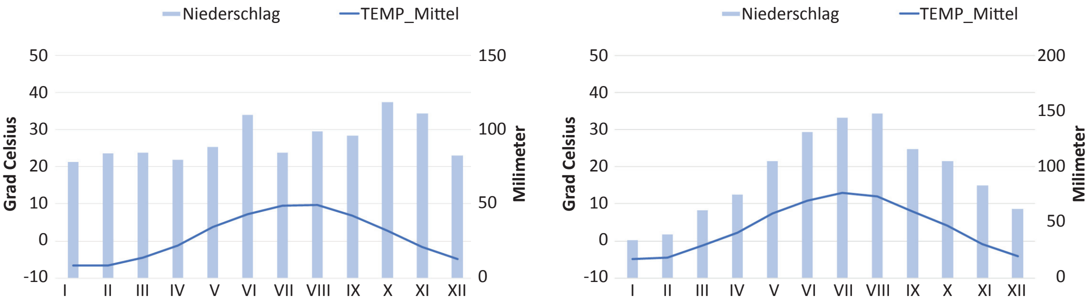

Figure 11. Climate diagrams for: **(a)** Bjelašnica (BiH) and **(b)** Kanzelhöhe (AUT – Carinthia).

Illyrian climate. The Alpine area of Slovenia with regards to temperature and precipitation in winter is similar to BiH and Croatia and with regards to summer precipitation similar to Austria (Carinthia). Austria (Carinthia) deviates clearly from the climatic characteristics of BiH and Croatia in all analysed parameters. The aforementioned climate studies indicate that the Alpine part of Slovenia represents a transitional Illyrian-Alpine area, while Austria (Carinthia) belongs to the Alpine phytogeographical area. This statement does not exclude the fact that Illyrian flora or Illyrian plant communities may occur extra-zonally within the Alpine area.

CONCLUSIONS

Based on the analysis of individual climate parameters by month and annual average/mean (precipitation and temperature), the Alpine area of Slovenia and Austria (Carinthia) differs from the typical Dinaric-Illyrian area (Bosnia and Herzegovina (BiH) and Croatia) in terms of climate. The climate of the typical Illyrian area is mild and rainy in winter and has a distinctly maritime character, especially during the summer, compared to the alpine area, which is influenced by the Atlantic climate during the summer. During the winter months, the amount of precipitation is lower in Austria (Carinthia) and Slovenia, as well as the temperature, while the temperature and amount of precipitation are higher in BiH and Croatia.

Our results suggest that climatic characteristics must be considered as a part of the environmental-habitat factors when defining a specific phytogeographical area. The appearance of "typical floral elements", in this case Illyrian, can also be related to environmental factors, through compensation and replacement of habitat factors. There is uncertainty in using flora to define the Illyrian area and other parameters may be helpful, including climate, geology, syndynamics and vegetation zoning. Utilizing all available evidence (ecological, edaphic, floristic and vegetation) may lead to a better understanding of the phytogeographic nature of an area.

Author Contributions

SV, BB, conceived and designed the research; SV prepared the data and wrote the primary test; BB performed the statistical analyses; BB, ĆV and MN reviewed the research and text; MN prepared map with GIS; SV, BB, ĆV and MN prepared the original draft of the manuscript.

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Influence of Forest Type and Climate Factors on the Number of Caught *Ips typographus* (Coleoptera, Curculionidae) Bark Beetles in Pheromone Traps in Protected Areas of Bosnia and Herzegovina

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ABSTRACT

As part of the research, the population of the eight-toothed spruce bark beetle in different types of forests in five protected areas in Bosnia and Herzegovina was analyzed. The study focused on the protected areas of Sarajevo Canton, specifically the secondary forests of fir and spruce, as well as the mixed forests of beech and fir (containing spruce). Pheromone traps were used as the research sample, and they were placed within PA Bijambara, PA Trebević, and PA Skakavac. The objective was to investigate the influence of forest type and climatological factors on the number of captured *Ips typographus* bark beetles from 2018 to 2021. The average number of captured *I. typographus* bark beetles during that period ranged from 491.39 to 901.68 individuals in secondary fir and spruce forests, and from 201.88 to 701.54 individuals in beech and fir forests (including spruce).

Keywords: eight-toothed spruce bark beetle; spruce; beech; fir; pheromone traps; climatological factors

INTRODUCTION

Currently, around the world, 19 million square kilometers, or approximately 12.5% of terrestrial areas are protected. In Bosnia and Herzegovina (BiH), this percentage is around 1.5% (Annonimus 2012) and protected forest areas cover an area of 18,232.30 hectares, which is approximately 0.7% of the total area of BiH (Beus and Vojniković 2007). There are a total of 7 legally protected areas predominantly characterized by coniferous forests, with spruce (*Picea abies* (L.) H. Karst.) being the dominant species. It is known that various harmful abiotic and biotic factors can significantly affect the health of coniferous forests under certain conditions. The most common abiotic factors are climate and soil-related, while fungal diseases and bark beetles are the primary biotic factors. Bark beetles are of high ecological importance, as the majority of species live in dead or dying plants, thus being the early decomposers in forest ecosystems (Raffa et al. 2015). At the same time, bark beetles are perceived as forest pests that destroy and weaken trees (Gregoire et al. 2004, Raffa et al. 2015, Schebeck et al. 2023), where in specific conditions, like drought periods, they weaken the vitality of whole forests. In coniferous forests, bark beetles directly or indirectly can cause the drying out of more than 50% of trees (Wood 1982). One of the most well-known and dangerous pests for spruce with the potential to cause high economic losses in the forest ecosystems is the spruce bark beetle *Ips typographus* L. (Coleoptera, Curculionidae, Scolytinae) (Wermelinger 2004). Although it is primarily considered as a secondary pest (Wermelinger 2004, Dautbašić et al. 2018, Netherer et al. 2019), in specific conditions it can build high population levels, killing huge numbers of trees in a short time. Such mass outbreaks are usually a consequence of different events like abiotic disturbances, such as wind-throw, snow-break or drought (de Groot et al. 2019), in forests where suitable material for wood production is being build. Climate change and extreme drought along with secondary attacks of bark beetles eventually lead to enlargement of population levels of bark beetles which then attack healthy trees. Such increase of bark beetle population in conifers is very well known and documented in European forestry (Hlasny et al. 2014, Nikolov et al. 2014, Dautbašić et al. 2018, Hlavkova et al. 2022, Hroššo et al. 2020, Vilardo et al. 2022).

In BiH *I.typographus* has two generations per year, with the first occurring in April and the second in July when a single female can deposit between 30 to 100 eggs. Mostly they attack the lower parts where the bark is thicker (Zahirović et al. 2016). Under favourable conditions, it can have a third generation as well. The attack lasts from April to September, after which it burrows under the bark and litter where it overwinters (Tomiczek et al. 2007, Zubrik et al. 2017, Dautbašić et al. 2018). Affected trees die very fast after exit holes appear.

The adult beetle of *I. typographus* is dark brown or black with punctured lines on its wing covers, and on each side of the elytra there are four teeth. It measures approximately 5.5 mm in length. The gallery system beneath the bark is created by the females during egg-laying and by the larvae during their development, and it is usually one- or two-branched, occasionally three-branched. The length of the galleries depends on the intensity of the bark beetle attack, with shorter galleries indicating a stronger infestation and vice versa. The entire gallery system is located within the bark (Tomiczek et al. 2007, Zubrik et al. 2017, Dautbašić et al. 2018). Trees poses a defence mechanism, e.g. resin flow, and after bark beetles overcome and establish a mating chamber in the phloem they start releasing aggregation pheromones, attracting males and females (Francke et al. 1977, Byers et al. 1998). Syntheses for commercial production of the pheromone were developed in order to use the pheromones as bait in traps (Bakke 1983). Pheromone traps are primarily used for monitoring, although there have been attempts at pest control as well (Bakke et al. 1987).

Pheromone traps constitute a system composed of various housing designs that physically capture individuals and contain a chemical attractant - semiochemicals, to lure specific bark beetle species. There is a broad range of semiochemicals (Borden 1977), including pheromones that are released and received by individuals from the same species, and allelochemicals that mediate communication between species (Nordlund and Lewis 1976). The latter are further divided into kairomones, which are released by one species (e.g. host trees) and are to the benefit of the receiver of another species (e.g. bark beetles), allomones, which are beneficial for the emitter of another species, and synomones, which are to the benefit of both the sender and the receiver species (Nordlund and Lewis 1976).

The attractant Pheroprax has been developed for *I. typographus* and is widely used in forestry practices (Zuber and Benz 1992). This study aimed to determine the intensity of *I. typographus* infestation in different protected areas. The study investigated the influence of forest type and climatological factors on the number of caught *I. typographus* bark beetles.

MATERIALS AND METHODS

Field Work

Out of 7 legally protected areas in Bosnia and Herzegovina (Annonimus 2016), five are located in the Sarajevo Canton: Vrelo Bosne (603 hectares), Skakavac (1,430 hectares), Bijambare (497 hectares), Trebević (400 hectares) and Bentbaša (160 hectares). The determination of catches of *I. typographus* within the protected areas of Sarajevo Canton was conducted over a four-year period between 2018 and 2021 and was carried out in Bijambare (44.09283, 18.50049), Trebević (43.79736, 18.48032), and Skakavac (43.94803, 18.45249). For the catch of *I. typographus* Theysohn® pheromone traps and the Pheroprax® pheromone attractant (BASF Agro B.V Wadenswil, Switzerland) were used. The traps were positioned at a minimum distance of 20 m (± 2 m) from the nearest live coniferous trees. Counting the bark beetles and emptying the traps were carried out every 10-15 days. In the Bijambare protected area, 7 traps were installed during the period of 2018-2020, and 25 traps in 2021. In the Trebević protected area, 6 traps were installed during the period of 2018-2020. In the Skakavac protected area, 9 traps were installed in 2018, 6 traps in 2019, 14 traps in 2020, and 19 traps in 2021 (Figure 1). The traps were placed within two different types of forests: i) secondary forests of fir and spruce, and ii) mixed forests of beech and fir (with spruce). The analysis of trap catches was conducted at the laboratory of the Faculty of Forestry, University of Sarajevo. The distance between traps and healthy standing spruce trees was never under 20 m.

Laboratory Work

For measurement purposes, it was assumed that out of the collected bark beetles in 1 ml tube, there were 40 individuals of *I. typographus* (Hrašovec 1995). The accuracy of such assessment was tested on every twentieth sample, which showed a satisfactory level. The analysis of trap catches was conducted at the laboratory of the Faculty of Forestry, University of Sarajevo. The laboratory processing involved drying of the insects at room temperature and sorting the species under a microscope. The insects were first sorted by taxonomic categories and dried to facilitate counting. Based on the taxonomic categories, the insects were identified using available morphological keys (Pfeffer 1995). All larger insects, such as longhorn beetles, beetles with equally sized wings, and natural enemies were separated.

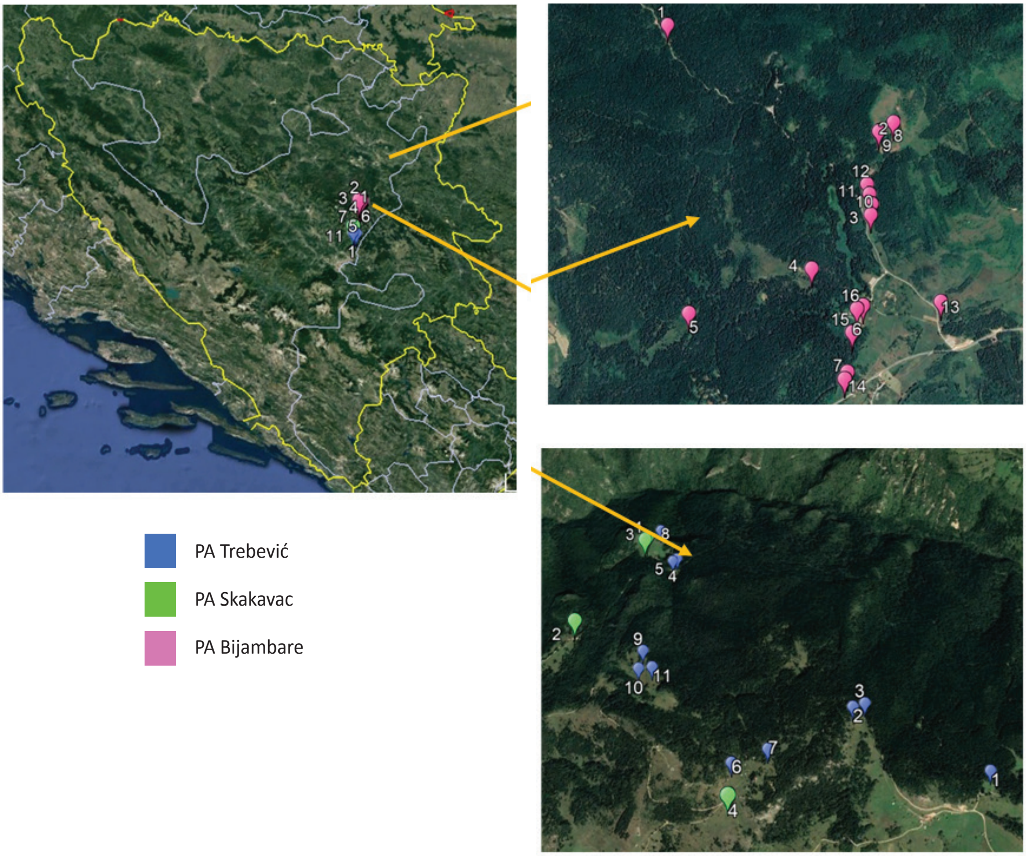

Figure 1. Position of pheromone traps: PA Trebević, PA Skakavac and PA Bijambare (Google Earth Pro).

Counting of the sorted beetles in order to confirm the accuracy of the assessment was done manually. In this work we present only data for *I. typographus*.

Statistical Analysis

The analysis was conducted using the SPSS software (ver. 20), and in addition to descriptive statistics, testing of the mean statistical significance (ANOVA) and Tukey HSD test were performed to determine the strength of the impact of forest type and climatological factors on the number of captured *I. typographus* individuals.

RESULTS

The study revealed that the average number of *I. typographus* individuals in 2018 ranged from 0 to 371.43 (average 266.40) in PA Bijambare, from 0 to 2589.41 (average 1130.79) in PA Trebević, and from 0 to 677.78 (average 474.67) in PA Skakavac. In 2019, the average number of *I. typographus* individuals ranged from 0 to 2511.43 (average 778.57) in PA Bijambare, from 0 to 1748.89 (average 988.40) in PA Trebević, and from 0 to 1173.33 (average 592.38) in PA Skakavac. In 2020, the average number of *I. typographus* individuals ranged from 0 to 268.57 (average 183.21) in PA Bijambare, from 64.44 to 1636.67 (average 758.98) in PA Trebević, and from 0 to 373.33 (average 238.19) in PA Skakavac. In 2021, the average number of *I. typographus* individuals ranged from 0 to 366.51 (average 205.67) in PA Bijambare, from 0 to 958.12 (average 784.62) in PA Trebević, and from 0 to 700.70 (average 484.87) in PA Skakavac. Within this study, a total of 80,470 individuals were caught in the Bijambare protected area, 346,880 individuals in the Trebević protected area, and 137,090 individuals in the Skakavac protected area. Figures 2-5 show the catch of *I. typographus* bark beetles by month for the period 2018-2021 in the respective protected areas.

To analyse the impact of forest type on the number of captured *I. typographus* individuals, a test of statistical significance of mean differences was conducted. Table A1 presents the mean and standard deviation of bark beetle catches for the years 2018-2021 across different protected areas.

To determine the statistical significance of differences in bark beetle catches for the period 2018-2021 across

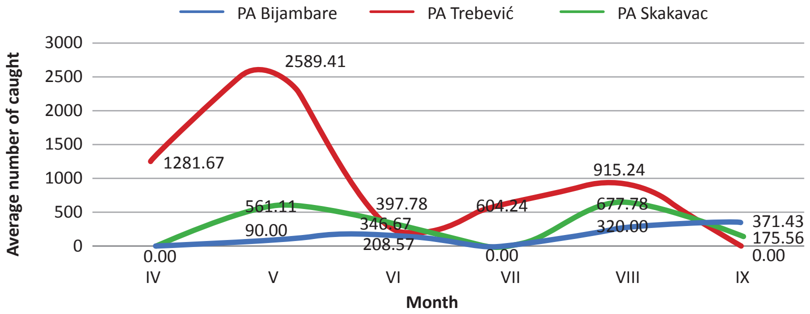

Figure 2. Average number of caught *I. typographus* bark beetles in 2018.

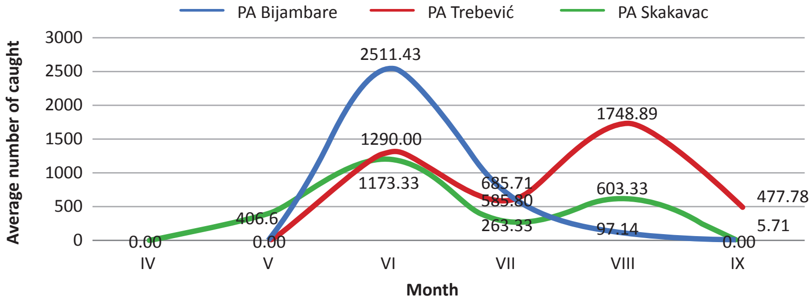

Figure 3. Average number of caught *I. typographus* bark beetles in 2019.

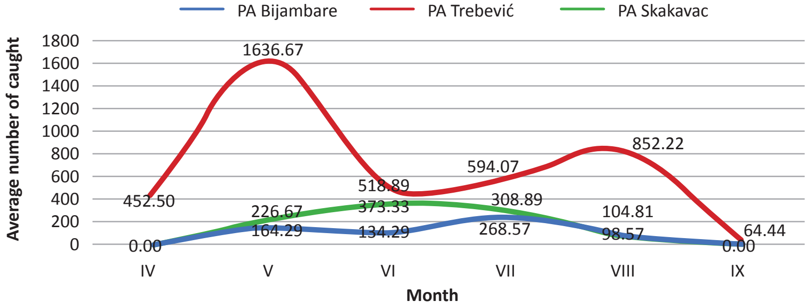

Figure 4. Average number of caught *I. typographus* bark beetles in 2020.

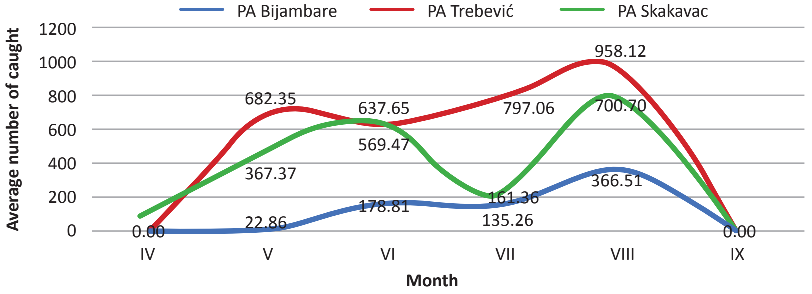

Figure 5. Average number of caught *I. typographus* bark beetles in 2021.

different protected areas, a one-way analysis of variance (ANOVA) was performed. The null hypothesis was set as follows: "There are no statistically significant differences in the average catches of *I. typographus* bark beetles for the period 2018-2021 across different protected areas at a probability of p<0.05." The results of the analysis are presented in Table A2.

The statistical analysis conducted revealed statistically significant differences in the average catches of *I. typographus* bark beetles for the years 2018, 2020, and 2021 across different protected areas, at a probability level of p<0.05. However, no statistical significance was found in the average catches of *I. typographus* bark beetles for the year 2019 across different protected areas (Table A3).

According to the Tukey HSD test, a difference in the average catches of *I. typographus* was found in 2018 between the PA Bijambare and PA Trebević, and between PA Trebević and PA Skakavac. In 2019, no differences in the average catches of *I. typographus* were found. In 2020, a difference in the average catches of *I. typographus* was found between PA Bijambare and PA Trebević, and between PA Trebević and PA Skakavac. In 2021, a difference in the average catches of *I. typographus* was found between PA Bijambare, PA Trebević, and PA Skakavac.

Figures 6-9 depict the catches of *I. typographus* by month and by forest type for the period 2018-2021.

In order to analyse the effect of forest type on the number of captured individuals of *I. typographus*, a test of statistical significance was conducted to examine the differences in means. Table A4 presents the arithmetic mean and standard deviation of the catches of bark beetles for the years 2018-2021, according to different forest types.

To determine the statistical significance of differences in the catch of bark beetles for the period 2018-2021, depending on the forest type, a test of one-way analysis of variance (ANOVA) was conducted. The null hypothesis was set as follows: "There are no statistically significant differences in the average catches of *I. typographus* for the period 2018-2021, depending on the forest type, at a probability level of p<0.05." The results of the analysis are presented in Table A5.

The conducted statistical analysis determined that there are statistically significant differences in the average catches of *I. typographus* for the years 2020 and 2021, depending on the forest type, at a probability level of p<0.05. However, no statistical significance was found between the average catches of *I. typographus* for the years 2018 and 2019, depending on the forest type.

To determine the statistical significance of differences in the catch of bark beetles for the period 2018-2021, depending on climatological factors, a test of one-way analysis of variance (ANOVA) was conducted. The null hypothesis was set as follows: "There are no

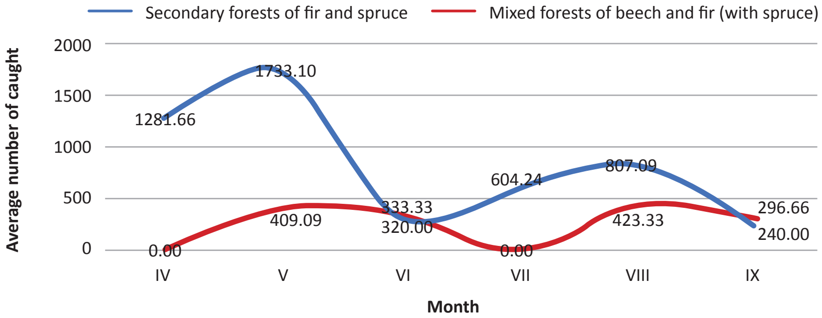

Figure 6. Average number of caught *I. typographus* bark beetles in 2018.

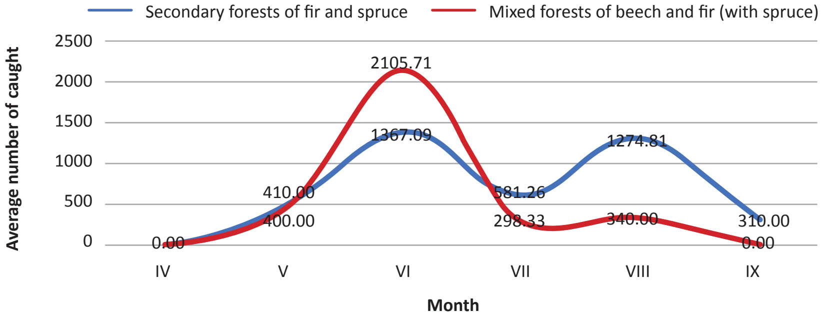

Figure 7. Average number of caught *I. typographus* bark beetles in 2019.

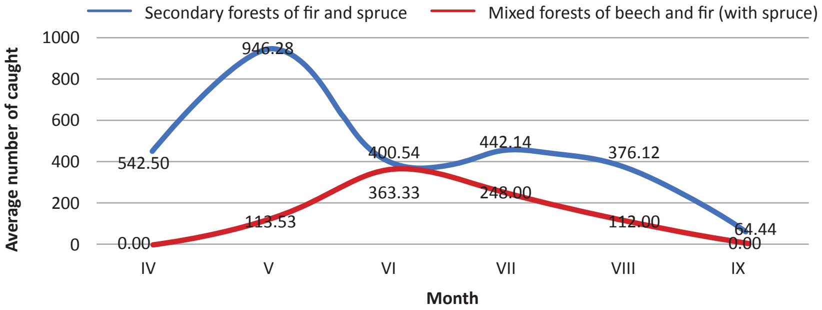

Figure 8. Average number of caught *I. typographus* bark beetles in 2020.

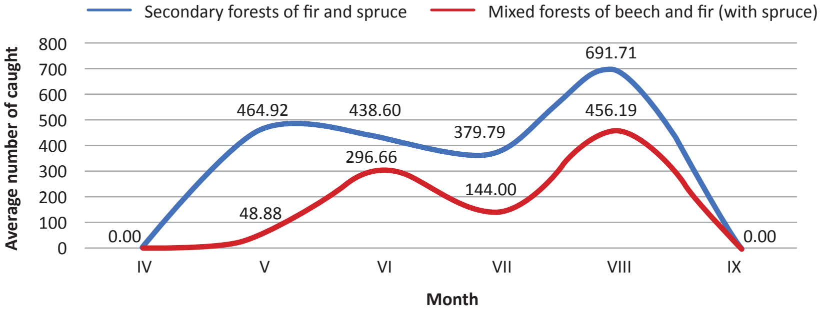

Figure 9. Average number of caught *I. typographus* bark beetles in 2021.

statistically significant differences in the average catches of *I. typographus* for the period 2018-2021, depending on climatological factors, at a probability level of p<0.05." The results of the analysis are presented in Tables A6-A9.

The conducted statistical analysis determined that there are no statistically significant differences in the average catches of *I. typographus* for the years 2018 and 2019, depending on climatological factors, at a probability level of p<0.05. However, statistical significance was found between the average catches of I. *typographus* for the year 2020, depending on the mean annual temperature and the maximum temperature of the warmest month. Statistical significance was also found between the average catches of *I. typographus* for the year 2021, for all parameters.

DISCUSSION

Despite available technologies, so far semiochemical-based tools have been comparatively rarely used in agriculture and forestry, and conventional insecticides as the historically only alternative decreased in acceptance due to their environmental, social, and human health impact, so more sustainable alternatives are urgently needed (Pernek 2002, Gillette and Fettig 2021, Mafra-Neto et al. 2022).

This study encompassed the catch of *I. typographus* as an indicator of infestation in different protected areas of the Sarajevo Canton, as well as in different forest types. The focus of the research was to analyse the influence of year, forest type and climatological factors in the protected areas and forest type on the population abundance of *I. typographus*. The significance of this research lies in the analysis of the catch of *I. typographus* in different protected areas of the Sarajevo Canton, Bosnia and Herzegovina. The Pheroprax pheromone was used to determine the infestation intensity. The same pheromone was used for the catch of *I. typographus* by other researchers as well (Pernek 2002, Gillette and Fettig 2021, Mafra-Neto et al. 2022). Zahirović et al. (2016) found in their research that the average number of *I. typographus* caught on Theysohn® traps ranged from 2.04 to 966.41. The average number of *I. typographus* caught per trap in this study was significantly higher, reaching up to 2332.81 individuals, which can be partially attributed to the population outbreak of this bark beetle during the years of the research.

A one-way analysis of variance was performed to test the difference between the average catches of *I. typographus* depending on the protected area, revealing differences in the years 2018, 2020, and 2021. In 2018 and 2020, differences in catches were found between all protected areas, while in 2021, differences were found between PA Bijambare, PA Trebević, and PA Skakavac. One assumption for these results is that in all three protected areas, a higher number of traps were placed in secondary spruce and fir forests compared to beech and fir (with spruce) forests, resulting in higher catches of the bark beetle.

To determine the statistical significance of differences in bark beetle catches for the period 2018-2021, it was found that there are statistically significant differences in the average catches of *I. typographus* for 2020 and 2021, while no significant differences were found for 2018 and 2019, depending on the forest type. The study revealed that the catch of *I. typographus* was twice as high in secondary spruce and fir forests compared to beech and fir (with spruce) forests. One assumption for such a result is that there is a higher proportion of spruce trees in secondary spruce and fir forests, thereby providing a greater number of hosts for the bark beetle's development. Studies have shown that non-host tree species diversity per se is not the main driver of outbreak risk, but that it strengthens biotic resistance with lower host availability at low altitudes where abiotic conditions are the least favorable to Norway spruce (de Groot et al. 2023).

Furthermore, the catch of bark beetles was investigated in relation to climatological factors, and for the years 2018 and 2019, no influence of climatological factors on the average bark beetle catch was determined. In 2020, the influence of the mean annual temperature and the maximum temperature of the warmest month on the average catch of *I. typographus* was determined. In 2021, the influence of the mean annual temperature, maximum temperature of the warmest month, minimum temperature of the coldest month, mean temperature of the wettest quarter, annual precipitation, and precipitation in the wettest and driest month on the average catch of *I. typographus* was determined. In his research, Faccoli (2009) also did not find an influence of precipitation during the activity period of *I. typographus* and the damage caused by this bark beetle throughout the year (Faccoli 2009). However, he found that increased damage occurred one year later if the precipitation was below the 10-year average. This leads us to the conclusion that precipitation, depending on climatic factors, directly and indirectly affects the population of *I. typographus*. Changes in climate factors can cause a sudden change in the behaviour of bark beetles, i.e. a sudden population growth (Pernek et al. 2019).

An appropriately established monitoring system makes it possible to take timely protective measures to prevent or reduce to the lowest possible level a more severe bark beetle infestation in protected areas. Timely detection of bark beetles gives companies managing protected areas sufficient space and time to respond in time to suppress bark beetle infestations. It should be remembered that the company managing protected areas in the territory of Sarajevo Canton does not have its own employees to perform the tasks of felling and exporting the felled trees, but third parties have to be contracted for these tasks. All this significantly slows down the process of rehabilitation of bark beetle infestation, which is why the monitoring system is the main line of defence when it comes to protecting forests from bark beetle infestation in protected forest areas.

In the future, further research will be needed on bark beetle catches in different protected areas, different forest types, and their relationship with various climatological factors in Bosnia and Herzegovina.

CONCLUSIONS

Based on the conducted research, the following conclusions can be drawn: The study analysed the catch of *I. typographus* in pheromone traps in the protected areas in Bosnia and Herzegovina with respect to different forest types and climatological factors. During the period of 2018-2021, the number of caught *I. typographus* where statistically significant among five studied protected areas. In the period of 2018-2021, a higher average number of caught *I. typographus* individuals were found in secondary forests of fir and spruce compared to beech and fir forests (with spruce). Statistically significant differences in the average catch of *I. typographus* were observed for the years 2020 and 2021, depending on the forest type, while no significant differences were found for 2018 and 2019. There were no statistically significant differences in the average catch of *I. typographus* bark beetles for the years 2018 and 2019 concerning climatological factors, while for 2020, statistical significance was found in relation to the mean annual temperature and maximum temperature of the warmest month. Additionally, for 2021, statistical significance was observed for all parameters. This work is important because it talks about the health condition of forests in protected areas, as well as the monitoring of harmful insects in them. Further research is needed on the catch of bark beetles in protected areas in Bosnia and Herzegovina.

Author Contributions

OM, KZ, AV, DP, SI conceived and designed the research, OM and KZ carried out the field measurements, KZ performed laboratory analysis, KZ, AV and DP processed the data and performed the statistical analysis, MD and MP secured the research funding, supervised the research and helped to draft the manuscript, all authors wrote the manuscript.

Funding

This work was financed from our own resources.

Conflicts of Interest

The authors declare no conflict of interest.

Appendix A

Table A1. Arithmetic mean and standard deviation for *I. typographus* catches from the period 2018 – 2021 for different protected areas.

Protected area		2018	2019	2020	2021
Protected area Bijambare	Mean	266.40	778.57	183.21	205.67
	N	25	42	56	150
	Std. Deviation	211.00	1254.13	202.41	199.06
Protected area Trebević	Mean	1130.79	988.40	758.98	784.62
	N	89	81	98	117
	Std. Deviation	1662.07	1143.64	904.58	599.90
Protected area Skakavac	Mean	474.67	592.38	238.19	484.87
	N	45	42	72	152
	Std. Deviation	437.29	770.58	297.47	497.37
Total	Mean	809.18	834.18	450.40	468.62
	N	159	165	226	419
	Std. Deviation	1317.33	1098.71	681.43	506.18

Table A2. Analysis of variance (ANOVA) of statistically significant differences in *I. typographus* catches depending on the protected area.

		Sum of Squares	df	Mean Square	F	Sig.
2018 * *Protected area*	Between Groups(Combined)	21606052.77	2	10803026.38	6.672	0.002
	Within Groups	252580940.94	156	1619108.59		
	Total	274186993.71	158			
2019 * *Protected area*	Between Groups(Combined)	4511846.99	2	2255923.49	1.889	0.155
	Within Groups	193465767.54	162	1194233.13		
	Total	197977614.54	164			
2020 * *Protected area*	Between Groups(Combined)	16571679.49	2	8285839.74	21.019	0.000
	Within Groups	87908384.66	223	394208.00		
	Total	104480064.15	225			
2021 * *Protected area*	Between Groups(Combined)	22094608.74	2	11047304.37	54.064	0.000
	Within Groups	85005188.39	416	204339.39		
	Total	107099797.13	418	10803026.38		

Table A3. Multiple tests of average differences in the catch of *I. typographus* bark beetles for 2018, 2019, 2020 and 2021 depending on the protected area (Tukey HSD).

Dependent variable	Proceed area (A)	Proceed area (B)	Mean Difference (A-B)	Std. Error	Sig.
2018	Bijambare	Trebević	-864.387*	288.022	.009
		Skakavac	-208.267	317.403	.789
	Trebević	Bijambare	864.387*	288.022	.009
		Skakavac	656.120*	232.750	.015
	Skakavac	Bijambare	208.267	317.403	.789
		Trebević	-656.120*	232.750	.015
2019	Bijambare	Trebević	-209.824	207.793	.572
		Skakavac	186.190	238.471	.715
	Trebević	Bijambare	209.824	207.793	.572
		Skakavac	396.014	207.793	.140
	Skakavac	Bijambare	-186.190	238.471	.715
		Trebević	-396.014	207.793	.140
2020	Bijambare	Trebević	-575.765*	105.176	.000
		Skakavac	-54.980	111.868	.875
	Trebević	Bijambare	575.765*	105.176	.000
		Skakavac	520.785*	97.456	.000
	Skakavac	Bijambare	54.980	111.868	.875
		Trebević	-520.785*	97.456	.000
2021	Bijambare	Trebević	-578.949*	55.756	.000
		Skakavac	-279.202*	52.025	.000
	Trebević	Bijambare	578.949*	55.756	.000
		Skakavac	299.747*	55.595	.000
	Skakavac	Bijambare	279.202*	52.025	.000
		Trebević	-299.747*	55.595	.000

(*) The differences in the catches of I. typographus depending on the protected area are highly statistically significant at a probability of 0.05.

Table A4. Arithmetic mean and standard deviation for the catch of *I. typographus* bark beetle from the period 2018 – 2021 for different forest types.

Type of forest		2018	2019	2020	2021
Secundary forests of fir and spruce	Mean	901.68	858.99	491.39	507.44
	N	131	139	194	351
	Std. Deviation	1424.24	1077.89	721.49	528.72
Mixed forests of beech and fir (with spruce)	Mean	376.43	701.54	201.88	268.24
	N	28	26	32	68
	Std. Deviation	390.67	238.90	234.91	299.17
Total	Mean	809.18	834.18	450.40	468.62
	N	159	165	226	419
	Std. Deviation	1317.33	1098.71	681.43	506.18

Table A5. Analysis of variance (ANOVA) of statistically significant differences in *I. typographus* catches depending on forest type.

		Sum of Squares	df	Mean Square	F	Sig.
2018 * Type of forest	Between Groups(Combined)	6364520.31	1	6364520.31	3.731	0.055
	Within Groups	267822473.40	157	1705875.62		
	Total	274186993.70	158			
2019 * Type of forest	Between Groups(Combined)	543017.09	1	543017.09	0.448	0.504
	Within Groups	197434597.50	163	1211255.19		
	Total	197977614.50	164			
2020 * Type of forest	Between Groups(Combined)	2302452.43	1	2302452.43	5.048	0.026
	Within Groups	102177611.70	224	456150.05		
	Total	104480064.20	225			
2021 * Type of forest	Between Groups(Combined)	3259316.59	1	3259316.59	13.089	0.000
	Within Groups	103840480.50	417	249017.93		
	Total	107099797.10	418			

Table A6. Analysis of variance (ANOVA) of statistically significant differences in *I. typographus* catches depending on climatological factors in 2018.

		Sum of Squares	df	Mean Square	F	Sig.
The average annual temperature	Between Groups(Combined)	32.136	60	.536	.909	.651
	Within Groups	57.723	98	.589		
	Total	89.858	158			
The max temperature of the warmest month	Between Groups(Combined)	98.076	60	1.635	.944	.591
	Within Groups	169.771	98	1.732		
	Total	267.847	158			
The min temperature of the coldest month	Between Groups(Combined)	5.856	60	.098	1.437	.055
	Within Groups	6.655	98	.068		
	Total	12.511	158			
The average temperature of the most humid quarter	Between Groups(Combined)	1838.469	60	30.641	1.005	.484
	Within Groups	2988.304	98	30.493		
	Total	4826.773	158			
The annual rainfall	Between Groups(Combined)	22647.915	60	377.465	1.073	.374
	Within Groups	34489.796	98	351.937		
	Total	57137.711	158			
The rainfall in the most humid month	Between Groups(Combined)	237.150	60	3.953	.918	.636
	Within Groups	421.844	98	4.305		
	Total	658.994	158			
The rainfall in the driest month	Between Groups(Combined)	258.343	60	4.306	.970	.545
	Within Groups	435.141	98	4.440		
	Total	693.484	158			

Table A7. Analysis of variance (ANOVA) of statistically significant differences in *I. typographus* catches depending on climatological factors in 2019.

		Sum of Squares	df	Mean Square	F	Sig.
The average annual temperature	Between Groups(Combined)	34.655	71	.488	.971	.548
	Within Groups	46.746	93	.503		
	Total	81.401	164			
The max temperature of the warmest month	Between Groups(Combined)	90.850	71	1.280	.870	.729
	Within Groups	136.762	93	1.471		
	Total	227.612	164			
The min temperature of the coldest month	Between Groups(Combined)	6.052	71	.085	.963	.562
	Within Groups	8.227	93	.088		
	Total	14.279	164			
The average temperature of the most humid quarter	Between Groups(Combined)	2024.200	71	28.510	.793	.847
	Within Groups	3344.400	93	35.961		
	Total	5368.601	164			
The annual rainfall	Between Groups(Combined)	30090.603	71	423.811	.904	.670
	Within Groups	43601.724	93	468.836		
	Total	73692.327	164			
The rainfall in the most humid month	Between Groups(Combined)	291.415	71	4.104	.914	.652
	Within Groups	417.579	93	4.490		
	Total	708.994	164			
The rainfall in the driest month	Between Groups(Combined)	344.048	71	4.846	.869	.732
	Within Groups	518.800	93	5.578		
	Total	862.848	164			

Table A8. Analysis of variance (ANOVA) of statistically significant differences in *I. typographus* catches depending on climatological factors in 2020.

		Sum of Squares	df	Mean Square	F	Sig.
The average annual temperature	Between Groups(Combined)	51.713	64	.808	1.819	.001
	Within Groups	71.513	161	.444		
	Total	123.225	225			
The max temperature of the warmest month	Between Groups(Combined)	156.821	64	2.450	2.005	.000
	Within Groups	196.722	161	1.222		
	Total	353.543	225			
The min temperature of the coldest month	Between Groups(Combined)	5.505	64	.086	.913	.656
	Within Groups	15.171	161	.094		
	Total	20.676	225			
The average temperature of the most humid quarter	Between Groups(Combined)	2627.769	64	41.059	1.335	.075
	Within Groups	4950.567	161	30.749		
	Total	7578.336	225			
The annual rainfall	Between Groups(Combined)	28590.475	64	446.726	.989	.509
	Within Groups	72721.990	161	451.689		
	Total	101312.465	225			
The rainfall in the most humid month	Between Groups(Combined)	326.302	64	5.098	1.169	.217
	Within Groups	702.198	161	4.361		
	Total	1028.500	225			
The rainfall in the driest month	Between Groups(Combined)	350.155	64	5.471	1.049	.398
	Within Groups	839.774	161	5.216		
	Total	1189.929	225			

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The Community of Fine Root Fungi of Silver Fir (*Abies alba* Mill.) Saplings

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ABSTRACT

This study aimed to assess the biodiversity of fungi colonizing the fine roots (diameter up to 2 mm) of 3-year-old silver fir saplings from areas of Międzylesie Forest District in Poland. It was hypothesized that quantitatively and qualitatively, mycorrhizal fungi would be the dominant fungi in root communities of silver fir. DNA extraction was performed using Plant Genomic DNA purification. The internal transcribed spacer1 (ITS1) rDNA region was amplified using specific primers, and the amplicons were purified and sequenced using sequencing by synthesis (SBS) Illumina technology. The obtained sequences were compared with reference sequences in the UNITE database (https://unite.ut.ee/) using the basic local alignment search tool (BLAST) algorithm to facilitate species identification. A total of 307,511 OTUs was obtained from each sample. There were 246,477 OTUs (80.15%) of fungi known from cultures. The genera *Tuber* spp. (7.51%) and *Acephala* spp. (3.23%) accounted for the largest share of the fungal communities on the fine roots of fir trees. Hence our results indicate the dominance of mycorrhizal fungi in these communities and reflect the excellent quality of the saplings that were assessed. Pathogenic fungi constituted a much smaller share of the fungal communities.

Keywords: Lower Silesia; Międzylesie Forest District; nursery; restoration; Sudeten Mountains

INTRODUCTION

The silver fir tree (*Abies alba* Mill.) used to be one of the most important forest-forming species in the mountainous and upland regions of forest stands in Poland, in which fir is the dominant species, occupying 2% of the forest area and having a 2.7% share in the volume in the forests managed by the State Forests (Bis and Dobrowolska 2012). However, since 1998, the share of fir trees in the forests of the Sudeten Mountains in Poland has fallen below 0.4%, and the species had already lost the possibility of continued existence and self-regeneration in the competitive environment of the forests of the region. For this reason, the State Forests (Poland) undertook a program to restore fir trees to forests of the Sudeten Mountains (Barzdajn 2000, 2012, Barzdajn and Kowalkowski 2012), but with time it was noticed that fungi-related issues should not be overlooked. The studies of fungal communities inhabiting fir roots have focused mainly on mycorrhizae (within and outside their natural occurrence) (Kowalski 1982, Comandini et al. 2001, Laganà et al. 2002, Rudawska et al. 2016) and pathogenic fungi that infect their root systems (Puddu et al. 2003, Oliva and Colinas 2007, Chomicz-Zegar et al. 2016). However, no research has been undertaken in the mountainous regions of Poland. In addition to pathogenic and mycorrhizal fungi, these communities are known to include antagonists of other microorganisms and neutral organisms. Root-associated fungal communities are essential components in ecosystem processes, impacting plant growth and vigour by influencing the quality, direction, and flow of nutrients and water between plants and fungi (Unuk et al. 2019). Hence, the aim of the current study was to assess the diversity of fungi occurring in the rhizosphere of the root systems of small cuttings and fir saplings. We tested the hypothesis that mycorrhizal fungi should dominate these communities

MATERIALS AND METHODS

Fine roots of 6 saplings of silver fir from the forest nursery of Międzylesie Forest District (16°66'23"E, 50°14'86"N, south-west part of Poland) were randomly selected for the study. The samples were collected in June 2017. The research material comprised fine roots (roots up to three rows to identify all types of mycorrhizae) (McCormack et al. 2015). The fine roots were washed on sieves under running tap water and dried on sterile paper. After drying, the roots were ground in a mortar frozen to -70°C. The DNA extracted from dried roots was separated under the microscope. We composited one sample from six trees. Environmental DNA was extracted with Plant Genomic DNA Purification Kit (Thermo Fisher Scientific). The internal transcribed spacer1 (ITS1), 5.8S rDNA region was used to identify the fungal species, and the analysis was carried out with primers ITS1FI2 5′-GAACCWGCGGARGGATCA-3′ (Schmidt et al. 2013) and 5.8S 5`-CGCTGCGTT CTTCATCG-3` (Vilgalys and Gonzalez 1990). Each amplification reaction was carried out in a final volume of 25.0 µl containing 2 ml of DNA, 0.2 ml of each primer, 10.1 ml of deionized water and 12.5 mL of 2X PCR MIX (A&A Biotechnology, Gdynia, Poland). The amplification reaction was carried out in a thermocycler. This included: initial denaturation (94°C, 5 min), 35 cycles of denaturation (94°C, 30 s), annealing (56°C, 30 s), elongation (72°C, 30 s) and final elongation (72°C, 7 min). The product was then checked on a 1% agarose gel stained with Midori Green Advance DNA (Genetics, Dueren, Germany). The obtained product was purified and sequenced using sequencing by synthesis (SBS) technology from Illumina (Genomed S.A. Warsaw, Poland). The results were subjected to bioinformatic and statistical analysis according to Behnke-Borowczyk et al. (2019). The resulting sequences were compared with the reference sequences deposited in the UNITE community database (Nilsson et al. 2018, UNITE community 2020) using the basic local alignment search tool (BLAST) algorithm.

The abundance of fungi was defined as the number of OTUs in a sample. A total amount of OTUs was obtained from six samples collected from each of the 3-year-old small roots of *A. alba*. The frequency of an individual taxon was defined as the percentage (%) of OTUs in the total number of OTUs. Diversity was defined as the number of species in a sample. The trophic role of the detected fungal species in the community was determined based on literature data and listed in Appendix (Table A1).

RESULTS

A total of 307 511 OTUs were obtained. There were 246 477 OTUs (80.15%) of fungi known from cultures, 1 876 OTUs (0.61%) of non-cultured fungi, 47 572 (15.47%) OTUs of non-fungal organisms (mainly plants, including silver fir), and 1 814 OTUs (0.59%) of organisms with no reference sequence in UNITE database. The total number of taxa obtained was 1612.

The community comprised taxa belonging to Ascomycota (46.27%), Basidiomycota (33.64%), Zygomycota (2.52%), Rozellomycota (0.65%), and we also obtained some sequences that are not represented in the UNITE database (0.59%) (Table A1).

The Thelephoraceae (8.22%), *Tuber* spp. (7.51%) and *Acephala* spp. (3.23%) had the largest share of fine roots of common fir trees, therefore accounting for the largest share of the fungal community (Table A1).

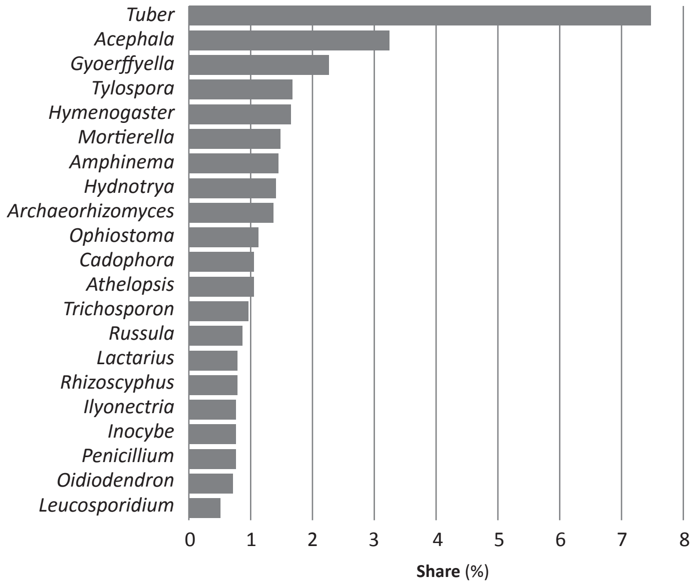


Figure 1. Percentage share (%) of the most abundant types of fungi in the community (share > 0.5%).

Mycorrhizal fungi dominated the fungal community of the fine root (57,62%). The most common taxa identified among mycorrhizal fungi were: *Hydnotrya* spp., *Tuber* spp., *Amphinema* spp., *Hymenogaster* spp., *Tylospora* spp., endophytes including *Acephala* spp., *Cadophora* spp., pathogenic fungi including *Ophiostoma* spp. and saprotrophs *Athelopsis lembospora, Archaeorhizomyces borealis* and *Mortierella* spp. (Table 1, Table A1).

DISCUSSION

The results of our analysis support our hypothesis that mycorrhizal fungi dominate the fungal communities on the fine root systems of silver fir saplings. Taxa, which belong to the fungi, forming mycorrhizal communities of silver fir, include *Tuber* spp. and *Acephala* spp., and these accounted for the largest share of the fungal communities on the fine

Table 1. Genus of ectomycorrhizal (ECM) fungi, whose frequency in the collection in fine roots of silver fir exceeded 0.03%. and which denotes the presence of a taxa in roots or soil in previous scientific reports.

Genus	Root	Soil	Reference
Amanita	+	+	Ważny (2014), Rudawska et al. (2016), Unuk et al. (2019), Behnke-Borowczyk et al. (2020)
Amphinema	+		Ważny (2014), Unuk et al. (2019)
Cenococcum	+	+	Unuk et al. (2019), Ważny (2014), Behnke-Borowczyk et al. (2020)
Cortinarius		+	Ważny (2014), DEEMY (2014-2020), Unuk et al. (2019), Behnke-Borowczyk et al. (2020)
Elaphomyces	+	+	Unuk et al. (2019), Ważny (2014), Behnke-Borowczyk et al. (2020)
Hydnotrya	+	-	Ważny (2014)
Inocybe		+	Ważny (2014), Behnke-Borowczyk et al. (2020)
Lactarius	+	+	Ważny (2014), Rudawska et al. (2016), Unuk et al. (2019), Behnke-Borowczyk et al. (2020)
Meliniomyces	+	+	Unuk et al. (2019), Behnke-Borowczyk et al. (2020)
Piloderma		+	Ważny (2014), Rudawska et al. (2016)
Russula	+	+	DEEMY (2014-2020), Ważny (2014), Rudawska et al. (2016), Unuk et al. (2019), Behnke-Borowczyk et al. (2020)
Tricholoma	+		DEEMY (2014-2020), Rudawska et al. (2016), Behnke-Borowczyk et al. (2020)
Tuber	+	+	Ważny (2014), Rudawska et al. (2016), Behnke-Borowczyk et al. (2020)
Tylospora	+	+	Ważny (2014), Rudawska et al. (2016), Unuk et al. (2019), Behnke-Borowczyk et al. (2020)
Xerocomellus	+		Rudawska et al. (2016)

root systems of fir saplings in our study. These results are slightly different from the studies of soil fungal communities in nurseries producing silver fir seedlings conducted by Behnke-Borowiczyk et al. (2020), where saprotrophic fungi dominated. However, Behnke-Borowczyk et al. (2020) also identified mycorrhizal fungi. We also identified some saprotrophs which accounted for just a small share of the fungal community (not exceeding 1.5%) on the roots of silver fir tree saplings in our study.

Smutek et al. (2010) obtained the following mycorrhizal families and species of fungi on old silver fir trees (90-105 years old): *Cortinarius sertipes, Sebacina* sp., *Amphinema byssoides, Russula puellaris, Clavulina cristata, Cortinarius sp., Tomentella sublilacina, Russula fellea, Laccaria ametistina* and *Tylospora asteropcumum*. Our results concur, confirming the occurrence of fungi belonging to all the taxa recorded by Smutek et al. (2010). Similarly, we have found the presence of some of the taxa detected by Wojewoda (2003) in the fungal community on fir trees, including *Amanita, Amphinema, Boletus, Cenococcum, Clavulina, Cortinarius, Elaphomyces, Hydnotrya, Hydnum, Inocybe, Lactarius, Leotiomycetes, Piloderma, Pseudotomentella, Russula, Sebacina, Tomentella, Tuber*, and *Tylospora*. However, unlike Ważny (2014) and Schirkonyer et al. (2013), the fungi genera *Byssocorticium, Laccaria, Paxillus, Thelephora, Tomentellopsis* and *Xerocomus* were not identified in our study. Ważny (2014) found that the mycorrhizal fungi which dominated on the youngest fir trees examined were: *Clavulina cristata* (25.2%), *Tomentella* sp. (10.5%), *Tuber puberulum* (8.9%), and *Clavulina* sp. (5.1%). While the genus *Tuber* accounted for a similar share (7.51%) of the community of fungi in our work, the presence of the other genera/species did not exceed 1%. Apart from *Geopora* sp. and *Imleria* sp., we identified similar taxa of fungi to those recorded by Rudawska et al. (2016), who studied mycorrhizal fungi of common fir trees outside their natural range, but from mature forest stands. To date, 13 species of ectomycorrhizal fungi (ECM) associated with the genus *Abies* have been included in DEEMY: *Abierhiza fascicularis, A. tomentosa, Cortinarius odorifer, Lactarius caespitosus, L. deliciosus, L. intermedius, L. salmonicolor, L. subsericatus, Polyporoletus sublividus, Russula brevipes, R. ochroleuca, R silvicola*, and *Tricholoma bufonium,* of which only *R. ochroleuca* was found in the sampled fungal community. The root community differs significantly from the fungal community associated with fir analyzed by Behnke-Borowczyk et all (2020), who isolated 13 taxa of ectomycorrhizal fungi (ECM) associated with the genus *Abies.*

The presence of *Cenococcum geophilum* and *Thelephora stuposa* was insignificant in the study, which is in opposition to the research done by Rudawska et al. (2016). The list of mycorrhizal species detected in silver fir roots include *Amphinema byssoides, Clavulina cristata, Lactarius aurantiacus, L. salmonicolor, Piloderma fallax, Tuber puberulum, T. asterophora, T. stuposa Boletus pruinatus, Cenococcum geophilum,* and *Laccaria amethystina*, which have been confirmed in other studies (Eberhardt et al. 2000). In addition, *C. geophilum, A. byssoides, T. stuposa, Amanita, Boletus, Cenococcum, Cortinarius, Inocybe, Laccaria, Lactarius*, *Russula, Sebacina, Tomentella*, and *Tuber* also form mycorrhizal compounds with other fir species (Matsuda and Hijii 1999, 2004, Ishida et al. 2007, Kranabetter et al. 2009).

Acephala applanata, which we detected in fine roots of *A. alba*, was previously almost exclusively isolated from *Picea abies* (L.) H. Karst (Grünig et al. 2006). While second species from *Acephala* genus *A. macrosclerotiorum* formed ectomycorrhizas on *Pinus sylvestris* (Münzenberger et al. 2009) was detected in a study of the community of fungi on silver fir fine roots. These results are consistent with those obtained by Behnke-Borowczyk et al. (2020) in soil research related to nurseries producing silver fir seedlings. However, the share of *Acephala* spp. in the previously studied soil was lower than in the roots. The greater share of these fungi in the root community is not surprising, because both species inhabit the roots: *A. applanata* is included in the DSE (dark septate endopyhtes) (Stroheker et al. 2021), while *A. macrosclerotiorum* is classified as ectomycorrhizal fungi (Münzenberger et al. al. 2009).

For young silver fir trees, similarly to Unuk et all (2019), we detected endophytic root-associated fungal genera *Oidiodendron, Phialocephala*, and *Rhizoscyphus*. Some consider fungi of these genera to be mycorrhizal, and therefore they are treated as fungi in Appendix A. However, their role in the silver fir root community has not yet been clearly defined.

In fungal community of silver fir fine roots identified cosmopolitan fungi from the genera *Trichoderma* and *Penicillium* as well, which are antagonists of the pathogens *Armillaria* and *Heterobasidion* (Behnke-Borowczyk and Kwaśna 2010; Grantina-Ievina et al. 2013; Baranowska et al. 2023).

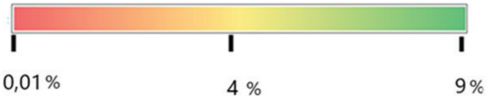
Our study also identified pathogenic fungi in the fine roots of silver fir tree saplings, namely *Ophiostoma nigrocarpum*, which, together with *O. novo-ulmi* and *Ophiostoma* sp., accounted for 1.11% of the fungal community. Fungi of the *Ophiostoma* genus are pathogens whose vectors are bark beetles on older trees (for example, *Pissodes piceae*), which cause white discolouration of fir's wood, thus reducing its economic value (Six and Bentz 2003, Kirisits 2004).

CONCLUSIONS

This study led to recognizing the spectrum of mycorrhizal, saprotrophic, and pathogenic fungi characteristic for fine roots of the 3-year-old *Abies alba.* Relatively low proportion of pathogens in these fungal communities also supports the conclusion that they were healthy trees. To fully confirm the roles and functions of the identified taxa, further identification of communities should be carried out. Parallel to the analysis of the communities of fungi inhabiting silver fir fine roots, it is necessary to study the content of nutrients and soil pH and determine their impact on these communities. In addition, research should be continued at a later stage of tree development, i.e. in young forest stands, to determine the formation of these communities and the spectrum of specific species of fungi associated with young silver fir trees.

Appendix A

Table A1. Taxa occurring in the communities of fungi in the roots of fir trees, whose frequency in the collection exceeded 0.03%. The legend of colours in the "Frequency" column:



Dominant taxa are marked in bold. Symbols of trophic groups are: M - mycorrhizal fungi, S - saprotrophic fungi, A - antagonistic fungi, P - pathogenic fungi, L - lichens, E - entomopathogenic fungi, U - unknown. The "Frequency, %" column contains the collected taxa data in the community, red representing the smallest share, and green representing the largest share of the taxon in the community (the scale is attached above).

Taxon	Order	Frequency (%)	Similarity (%)	Trophic group	Reference
Acephala applanata Grünig & T.N. Sieber + *A. macrosclerotiorum* Münzenb. & Bubner + *Acephala* sp.	Helotiales	3.231	97-100	M	Münzenberger et al. (2009)
Apiognomonia errabunda (Roberge ex Desm.) Höhn.	Diaporthales	0.063	99	P	Mańka (2005)
Archaeorhizomyces borealis Menkis, T.Y. James & Rosling + *Archaeorhizomyces* sp.	Incertae sedis	1.356	98-100		
Ascomycota		1.821	100		
Beauveria pseudobassiana S.A. Rehner & Humber + *Beauveria* sp.	Hypocreales	0.039	98-100	E	Álvarez-Baz et al. (2015)
Cadophora finlandica (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew + *C. orchidicola* (Sigler & Currah) M.J. Day & Currah + *Cadophora* sp.	Incertae sedis	1.049	97-100	P	Yakti et al. (2019)
Capnodiales		0.060	99		
Cenococcum geophilum Fr. + *Cenococcum* sp.	Mytilinidiales	0.355	99-100	M	Spatafora et al. (2012)
Chaetothyriales		0.271	100		
Chalara hyalocuspica Koukol + *Ch. pseudoaffinis* Koukol + *Chalara* sp.	Helotiales	0.482	97-100	S/P	Koukol (2011), Coker et al. (2019)
Cheirosporium triseriale L. Cai & K.D. Hyde	Pleosporales	0.181	99	S	Abdel-Aziz (2016)
Cladophialophora minutissima M.L. Davey & Currah + *C. chaetospira* (Grove) Crous & Arzanlou + ***Cladophialophora*** sp.	Chaetothyriales	0.211	98-100	P/S	Badali et. al. (2008)
Clonostachys rosea (Link) Schroers, Samuels, Seifert & W. Gams + *Clonostachys* sp.	Hypocreales	0.038	98-100	A	Cota et al. (2009)
Coccomyces australis P.R. Johnst.	Rhytismatales	0.088	99	P	Johnson (1986)
Coniochaeta sp.	Coniochaetales	0.042	100	P	Damm et al. (2010)
Coniochaetaceae	Coniochaetales	0.035	100		
Dermateaceae	Helotiales	0.532	99		
Didymella dactylidis (Aveskamp, Gruyter & Verkley) Qian Chen & L. Cai + *D. protuberans* (Lév.) Qian Chen & L. Cai	Pleosporales	0.211	98-100	P/S	Chen et al. (2015)
Dothideomycetes		0.289	100		
Elaphomyces muricatus Fr. + *E. granulatus* Fr.	Eurotiales	0.176	98-99	M	Paz et al. (2017)
Erysiphe euonymicola U. Braun+ *E. hypophylla* (Nevod.) U. Braun & Cunningt.	Erysiphales	0.104	99-100	P	Sepúlveda-Chavera et al. (2013)

Table A1. (countinue) Taxa occurring in the communities of fungi in the roots of fir trees, whose frequency in the collection exceeded 0.03%. The legend of colours in the "Frequency" column:

Taxon	Order	Frequency (%)	Similarity (%)	Trophic group	Reference
Exophiala sp.	Chaetothyriales	0.033	100	S	Feng et al. (2013)
Fimetariella sp.	Sordariales	0.102	100	U	
Fusarium sp. + ***F. oxysporum*** Schltdl.	Hypocreales	0.087	99-100	S/P	Karim et al. (2016)
Geomyces asperulatus Sigler & J.W. Carmich. + *G. auratus* Traaen + *Geomyces* sp.	Helotiales	0.387	98-100		
Gyoerffyella entomobryoides (Boerema & Arx) Marvanová + *Gyoerffyella* sp.	incertae sedis	2.285	99-100	P	Jankowiak et al. (2016)
Halokirschsteiniothelia maritima (Linder) Boonmee & K.D. Hyde		0.046	100	S	Wilson (1951)
Halosphaeriaceae	Microascales	0.067	99		
Helotiaceae	Helotiales	0.685	99		
Helotiales		1.878	100		
Hydnotrya cerebriformis Harkn. + *H. michaelis* (E. Fisch.) Trappe + *H. tulasnei* (Berk.) Berk. & Broome	Pezizales	1.405	99-100	M	Hobbie et al. (2001)
Hymenoscyphus sp.	Helotiales	0.073	100	S	Gumińska and Wojewoda (1985)
Hypocreales		2.001	100		
Ilyonectria morspanacis (A.A. Hildebr.) A. Cabral & Crous + ***I. robusta*** (A.A. Hildebr.) A. Cabral & Crous	Hypocreales	0.744	99-100	P	Farh et al. (2017)
Infundichalara minuta Koukol + *Infundichalara* sp.	Helotiales	0.081	99-100	S	Koukol (2012)
Leotia lubrica (Scop.) Pers.	Helotiales	0.081	100	S	Kuo (2012)
Leotiomycetes		1.099	100		
Leptosphaeria sp.	Pleosporales	0.079	100	P	Brachaczek et. al. (2016)
Lopadostoma polynesium (Berk. & M.A. Curtis) Rappaz	Xylariales	0.033	99	P	Mehrabi and Hemmati (2015)
Lophodermium conigenum (Brunaud) Hilitzer + ***L. pinastri*** (Schrad.) Chevall. + *L. seditiosum* Minter, Staley & Millar	Rhytismatales	0.386	99-100	P	Burdekin and Phillips (1992)
Maasoglossum sp.	Geoglossales	0.182	100		Hustad and Miller (2015)
Mariannaea elegans (Corda) Samson	Hypocreales	0.032	99	U	Wang and Zabel (1990)
Meliniomyces bicolor Hambl. & Sigler + ***M. variabilis*** Hambl. & Sigler + *M. vraolstadiae* Hambl. & Sigler	incertae sedis	0.285	99-100	M	Martino et al. (2018)
Metapochonia bulbillosa (W. Gams & Malla) Kepler, S.A. Rehner & Humber		0.040	100	E	Adachi et al. (2015)
Mycosphaerellaceae	Capnodiales	0.073	99		
Nectriaceae	Hypocreales	0.055	100		
Neobulgaria pura (Pers.) Petr. + *Neobulgaria* sp.	Helotiales	0.113	99-100	S	Gumińska and Wojewoda (1985)
Neonectria sp.	Hypocreales	0.035	100	P	Kryczyński and Weber (2010)
Oidiodendron maius G.L. Barron + *O. rhodogenum* Robak + *Oidiodendron* sp.	Helotiales	0.696	99-100	M/S	Rice and Currah (2006a)
Ophiosphaerella sp.	Pleosporales	0.063	100	P	Dernoeden (2000)

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Taxon	Order	Frequency (%)	Similarity (%)	Trophic group	Reference
Ophiostoma nigrocarpum (R.W. Davidson) de Hoog + *Ophiostoma novo-ulmi* Brasier + ***Ophiostoma*** sp.	Ophiostomatales	1.108	98-100	P/S	Marcinkowska (2012)
Penicillium citreonigrum Dierckx + *P. penicillioides* (Delacr.) Vuill. + *P. subrubescens* Houbraken, Mansouri, Samson & Frisvad + *Penicillium* sp.	Eurotiales	0.727	98-100	A	Pitt et al. (2000)
Petriella sordida (Zukal) G.L. Barron & J.C. Gilman	Microascales	0.033	99	A	Lee and Gloer (1995)
Pezizaceae	Pezizales	0.210	100		
Phaeosphaeriaceae	Pleosporales	0.044	100	S	
Phialocephala fortinii CJK. Wang & H.E. Wilcox + *Phialocephala* sp.	Helotiales	0.414	99-100	M/U	Jumpponen et al. (1998)
Phoma boeremae Gruyter + *Phoma* sp.	Pleosporales	0.070	98-99		Chen et al. (2015)
Pleosporaceae	Pleosporales	0.033	100		
Pleosporales		0.119	100		
Pleotrichocladium opacum (Corda) Hern.0Restr., R.F. Castañeda & Gené	Pleosporales	0.248	99	S	Hernández-Restrepo et al. (2017)
Podospora appendiculata (Auersw. ex Niessl) Niessl + *Podospora* sp.	Sordariales	0.023	99-100	S	Doveri (2008)
Proliferodiscus sp.	Helotiales	0.090	100		Han et al. (2014)
Pseudogymnoascus verrucosus A.V. Rice & Currah	Incertae sedis	0.368	99	S	Rice and Currah (2006b)
Pyronemataceae	Pezizales	1.135	100		
Rhizoscyphus sp.	Helotiales	0.786	100	M	Hambleton and Sigler (2005)
Rhodoveronaea varioseptata Arzanlou, W. Gams & Crous	incertae sedis	0.053	99	M	Tedersoo et al. (2010)
Rhytismataceae	Rhytismatales	0.045	100		
Sporormiaceae	Pleosporales	0.032	100		
Stagonospora pseudovitensis Quaedvl., Verkley & Crous	Pleosporales	0.034	99	P	Quaedvlieg et al. (2013)
Strumella sp.	Pezizales	0.068	100	P/S	Zettur and Kullman (2011)
Sympodiella acicola W.B. Kendr.	incertae sedis	0.065	99	S	Shen et al. (2020)
Tetracladium setigerum (Grove) Ingold + *Tetracladium* sp.	Helotiales	0.097	99-100	S	Anderson and Marvanová (2020)
Trichoderma sp.	Hypocreales	0.120	100	A	Benítez et al. (2004)
Tuber anniae W. Colgan & Trappe + *T. cistophilum* P. Alvarado, G. Moreno, Manjón, Gelpi & Jaime Muñoz + *Tuber* sp.	Pezizales	7.508	99-100	M	(2004-2020)
Umbilicaria americana Poelt & T.H. Nash	Umbilicariales	0.032	100	L	Poelt and Nash (1993)
Venturia hystrioides (Dugan, R.G. Roberts & Hanlin) Crous & U. Braun + *Venturia* sp.	Venturiales	0.146	99-100	P	González-Domínguez et al. (2017)
Venturiaceae	Venturiales	0.076	100		
Wilcoxina sp.	Pezizales	0.049	99	M	DEEMY (2004-2020)
Xenopolyscytalum pinea Crous	Helotiales	0.368	100	P	Koukol (2019)
Frequency of Ascomycota isolates			**46.27**		
Agaricales		2.261	100		

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Taxon	Order	Frequency (%)	Similarity (%)	Trophic group	Reference
Agaricomycetes		0.064	100		
Amanita rubescens Pers. + *Amanita* sp.	Agaricales	0.159	99-100	M	DEEMY (2004-2020)
Amphinema byssoides (Pers.) J. Erikss. + *Amphinema* sp.	Atheliales	1.448	99-100	M	DEEMY (2004-2020)
Amphisterеum leveilleanum (Berk. & M.A. Curtis) Spirin & Malysheva	Auriculariales	0.131	100	U	
Atheliaceae	Atheliales	0.078	100		
Atheliales		0.181	100		
Athelopsis lembospora (Bourdot) Oberw.	Amylocorticiales	1.030	99	U	
Auriculariales		0.444	99		
Basidiodendron caesiocinereum (Höhn. & Litsch.) Luck-Allen	Auriculariales	0.097	100	S	Kotiranta and Saarenoksa (2005)
Basidiomycota		0.565	100		
Cantharellales		0.096	100		
Ceratobasidiaceae	Cantharellales	0.351	100		
Clavariaceae	Agaricales	0.155	99		
Clavulina coralloides (L.) J. Schröt.	Cantharellales	0.245	99	S	Wojewoda (2003)
Clavulinopsis sp.	Agaricales	0.077	100	S	Kautmanová et al. (2012)
Cortinarius croceus (Schaeff.) Gray + *C. semisanguineus* (Fr.) Gillet	Agaricales	0.032	99-100	M	DEEMY (2004-2020)
Cryptococcus sp.	Tremellales	0.056	100	P/S	Springer et al. (2017)
Curvibasidium cygneicollum J.P. Samp.	incertae sedis	0.064	100	P/S	Kaitera et al. (2019)
Deconica phyllogena (Sacc.) Noordel.	Agaricales	0.061	99	S	Noordeloos (2011)
Galerina nana (Petri) Kühner + ***Galerina*** sp.	Agaricales	0.349	99-100	S	Gulden et al. (2005)
Hydnaceae	Cantharellales	0.308	100		
Hydnodontaceae	Trechisporales	0.054	100		
Hygrophoraceae	Agaricales	2.149	100		
Hymenochaetales		0.085	100		
Hymenogaster boozeri Zeller & C.W. Dodge + ***H. huthii*** Stielow, Bratek & Hensel + *H. olivaceus* Vittad.	Agaricales	1.625	99-100	M	Stielow et al. (2011)
Inocybe rufoalba Sacc. + *Inocybe* sp.	Agaricales	0.733	99-100	M	DEEMY (2004-2020)
Itersonilia perplexans Derx + *I. pannonica* (Niwata, Tornai-Leh., T. Deák & Nakase) Xin Zhan Liu, F.Y. Bai, M. Groenew. & Boekhout	Cystofilobasidiales	0.063	99-100	P	McGovern et al. (2006)
Kockovaella sacchari M. Takash. & Nakase	Tremellales	0.032	99	U	
Lactarius sp. + *L. necator* (Bull.) Pers. + ***L. tabidus*** Fr.	Russulales	0.797	98-100	M	DEEMY (2004-2020)
Leucosporidium drummii Yurkov, A.M. Schäfer & Begerow + *Leucosporidium* sp.	Leucosporidiales	0.508	99-100	U	Sampaio et al. (2003)
Malassezia restricta E. Guého, J. Guillot & Midgley + *Malassezia* sp.	Malasseziales	0.068	99	P	Saunders et al. (2012)
Microbotryomycetes		0.050	100		
Mycena sanguinolenta (Alb. & Schwein.) P. Kumm. + *Mycena* sp.	Agaricales	0.244	99-100	S	Perry (2002)

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Taxon	Order	Frequency (%)	Similarity (%)	Trophic group	Reference
Phellinus castanopsidis BK. Cui, Y.C. Dai & Decock	Hymenochaetales	0.022	100	P	Cui and Decock (2012)
Piloderma sp.	Atheliales	0.216	100	M	DEEMY (2004-2020)
Piskurozyma sp.	Filobasidiales	0.016	100	U	
Pterula sp.	Agaricales	0.117	100	S	Leal-Dutra et al. (2020)
Ramariopsis sp.	Agaricales	0.021	99	S	Matouš et al. (2017)
Rhodotorula sp.	Sporidiobolales	0.034	100	A	Akhtyamova and Sattarova (2013)
Rigidoporus sanguinolentus (Alb. & Schwein.) Donk	Polyporales	0.353	100	S	Wojewoda (2003)
Russula puellaris Fr. + *R. fragilis* Fr. + *R. nigricans* Fr. + *R. veternosa* Fr. + *R. badia* Quél. + *R. ionochlora* Romagn. + *Russula* sp.	Russulales	0.859	99-100	M	DEEMY (2004-2020)
Sebacinaceae	Sebacinales	0.174	100		
Sebacinales		0.447	100		
Solicoccozyma terrea (Di Menna) Yurkov + *Solicoccozyma* sp.	Filobasidiales	0.224	99-100	A	Yurkov et al. (2019)
Sporidiobolales		0.049	100		
Stereum sanguinolentum (Alb. & Schwein.) Fr.	Russulales	0.046	100	S	Łakomy and Kwaśna (2008)
Thelephoraceae	Thelephorales	8.220	100		Richard et al. (2011)
Trechispora sp.	Trechisporales	0.069	99	S/M	Gumińska (1985), Vanegas-León et al. (2019)
Trechisporales		0.084	100		
Tremellales		0.044	100		
Tremellomycetes		0.073	100		
Tricholoma fulvum (DC.) Bigeard & H. Guill. + *Tricholoma* sp.	Agaricales	0.064	99-100	M	DEEMY 2004-(2020)
Trichosporon sp.	Tremellales	0.949	100	A	Bosqueiro et al. (2020)
Tylospora asterophora (Bonord.) Donk + *Tylospora* sp.	Atheliales	1.689	98-99	M	DEEMY (2004-2020)
Vishniacozyma victoriae (M.J. Montes, Belloch, Galiana, M.D. García, C. Andrés, S. Ferrer, Torr. -Rodr. & J. Guinea) Xin Zhan Liu, F.Y. Bai, M. Groenew. & Boekhout + *Vishniacozyma* sp.	Tremellales	0.067	99-100	A	Gramisci et al. (2018)
Xerocomellus pruinatus (Fr. & Hök) Šutara + *X. chrysenteron* (Bull.) Šutara n	Boletales	0.032	99-100	M	Šutara (2008)
Frequency of Basidiomycota isolates			**33.64**		
Absidia sp.	Mucorales	0.034	100	S	Alastruey-Izquierdo et al. (2010)
Basidiobolus ranarum Eidam	Entomophthorales	0.064	99	S	Yang (1962)
Endogone sp.	Endogonales	0.072	100	S/M	Warcup (1990)
Jimgerdemannia lactiflua (Berk. & Broome) Trappe, Desirò, M.E. Sm., Bonito & Bidartondo	Endogonales	0.030	99-100	S/M	Desirò et al. (2018)
Mortierella horticola Linnem. + *M. angusta* (Linnem.) Linnem. + ***Mortierella*** sp.		1.484	99-100	S/A	Grantina-Ievina et al. (2014)
Mortierellaceae		0.130	100	S	
Mortierellales	Mortierellales	0.079	100	S	
Umbelopsis dimorpha Mahoney & W. Gams + *U. ramanniana* (Möller) W. Gams + *Umbelopsis* sp.	Umbelopsidales	0.241	99-100	S/A	Crowther et al. (2012), Grantina-Ievina et al. (2014)

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Taxon	Order	Frequency (%)	Similarity (%)	Trophic group	Reference
Frequency of zygomycota isolates			**2.52**		
Frequency of other kingdoms			**15.47**		
Not represented in UNITE database			**0.59**		
Frequency of uncultured fungi			**0.61**		

Author Contributions

WB, WK, JBB, MB conceived and designed the research, MB carried out the field measurements, JB performed laboratory analysis, JB and WB processed the data and performed the statistical analysis, WK, WB secured the research funding, AŁ, RK, JBB supervised the research and helped to draft the manuscript, MB wrote the manuscript. The main part of these results was presented at the international IUFRO Conference - Abies&Pinus 2022, “Fir and pine management in a changing environment: Risks and opportunities” held 19-22 September 2022 in Sarajevo, Bosnia and Herzegovina.

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Conflicts of Interest

The authors declare no conflict of interest.

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Variation of Physical and Mechanical Properties of *Pinus Sylvestris* L. Wood in the Boreal Zone of the European Northeast

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ABSTRACT

The study considers variational aspects of physical and mechanical properties of Scots pine (*Pinus sylvestris* L.) wood depending on conditions and growth areas in the boreal zone of the European Northeast. Anatomical traits of xylem and its strength properties were analyzed. Based on the data received, statistical processing of the material was carried out using correlation and multivariate analysis. The impact of hazardous air emissions on variation of linear dimensions in structural elements of the annual height increment in the trunk diameter was estimated. A varying nature of the influence of environmental factors on the formation of strength properties of *P. sylvestris* wood in growth areas was attested. The variation value of basic wood density decreases as soil and hydrological conditions of growth improve. A significant negative correlation between basic wood density and distance to sources of hazardous air emissions in the areas of concentration of industrial enterprises was established. The results of multivariate analysis make it possible to reveal the weight of the influence of environmental factors and biometric characteristics of wood on volumetric and strength properties of pine wood. Growth in the impact zone of air emissions from large industrial enterprises in the study area does not produce a significant effect on the dynamics of wood strength properties, taking into account various growth conditions. For Scots pine growing in the European Northeast, the study determined a significant impact of the size and structure of the annual height increment of the trunk in terms of diameter, as well as the position of the wood sample in terms of trunk volume, on strength properties of pine wood.

Keywords: pine wood density; xylem macro and microstructure; mechanical properties; boreal forests; air pollution; growth conditions; ANOVA multivariate analysis

INTRODUCTION

The question of the influence of forest growth conditions on wood quality of *Pinus sylvestris* L. in the forestry practice has a long research history (Zobel and van Buijtenen 1988, Saranpää 2003, Ikonen et al. 2008, Pretzsch 2020). So far, a large amount of scholarly literature has been accumulated on the issues of tree increment dynamics and wood structure in connection with changes in growth conditions (Poluboyarinov 1976, Wilhelmson et al. 2002, Schweingruber 2003, Shchekalev and Tarhanov 2006).

The variation of individual quantitative and qualitative parameters of *Pinus sylvestris* L. wood for different geographical areas was explored at different times to one degree or another (Kellomäki et al. 1999, Shchekalev and Tarhanov 2007, Baltunis et al. 2007, Danilov et al. 2020, Beniušienė et al. 2020). The analysis of the accumulated experience makes it possible to determine the extent and nature in the variation of individual indicators of wood depending on environmental factors and anthropogenic impact, including forestry practice.

Growth conditions stimulate a response reaction in ligneous plants. In particular, this is manifested in the formation of a certain type of wood structure. A number of studies have established that changes in the linear dimensions in structural elements of wood and the correlation of tissues are a necessary prerequisite for a tree to adapt to changing environmental conditions (Pazdrowski and Spława-Neyman 1996, Korchagov and Gribov 2009, Eremin and Chavchavadze 2015, Zaytsev et al. 2020, Wieruszewski and Mydlarz 2021). It is obvious that the choice of a growth strategy by a particular tree and changes in its wood structure can be conditioned by the deficit of organic matter and, as a consequence, by the need to place a certain number of water-conducting elements on a smaller area (Arseneva and Chavchavadze 2001, Romanovsky and Shchekalev 2014). Fluctuations in volumetric and strength properties of wood occur depending on forest growth conditions due to the multidirectional action of various factors (Kramer 1964, Poluboyarinov and Fedorov 1982, Babich et al. 2008, Kozlov et al. 2009, Kalbarczyk et al. 2018, Chubinsky et al. 2020).

Within the same growing stock, physical and mechanical properties of the wood of individual trees may be different. Usually, diversity of these properties is at a very low level of variation. Trees of the same age group, similar in development and growing under identical conditions, most often form wood with similar properties (Schimleck et al. 2018). Data on the high variation of physical and mechanical properties of the wood of individual trees, which can be found in scholarly literature, were obtained probably due to significant differences in the methodological basis and research objects, as well as a substantial difference in growth conditions of growing stocks (Baltunis 2007, Wessels et al. 2011, Tomczak et al. 2013).

Taking into account the data published, it becomes possible to speak about the existence of pairs of indicator sets of the anatomical coniferous wood structure correlating with its density (Perelygin 1954, Poluboyarinov 1976, Carlquist 1988, Antonova and Stasova 1993, Auty et al. 2014). Low density wood is partially characterized by thin cell walls, low percentage of latewood content and short fibers, while high density wood has thick cell walls, high percentage of latewood content and long fibers.

Diverse results of the available studies make it possible to argue for a great influence of heredity on the organization of a tree trunk, which sometimes acts independently of environmental factors and the position of a tree in the forest stand (Hannrup et al. 2000, Kisternaya and Aksenenkova 2007, Hong et al. 2014). Factors that have the greatest impact on wood density can include: anatomical structure and structure of the annual ring, age at which wood is formed, and the process of stem core formation (Poluboyarinov 1976, Kramer and Kozlowski 1979, Schweingruber 1993). The position in the stem volume, both vertically and horizontally, also has a significant effect on wood density (Shchekalev and Tarkhanov 2006, Babich et al. 2008).

The question of studying heterogeneity of a tree trunk in terms of wood density is of practical interest. From the perspective of biology, such research helps to better understand the process of wood formation at different age stages of tree growth (Perelygin 1953, Kosichenko 2000, Gao et al. 2017). In combination with the study of increment, density indicators allow the assessment of the intensity of the cambium function in various trunk zones (Antonova 1999, Jordan et al. 2005). Sharp changes in density inside the trunk, as well as along and across the trunk, are an undesirable factor for most commercial varieties of goods. Homogeneity of wood density inside the trunk, on the contrary, is its advantage (Poluboyarinov 1976, Ugolev 2001).

In scholarly literature there is a large volume of publications that allow us to judge the influence of site conditions on the variation of volumetric and technical parameters of pine wood (Melekhov 1949, Vikhrov 1954, Ugolev 2001, Danilov et al. 2018, Schimleck et al. 2018, Wieruszewski and Mydlarz 2021). Under conditions of long-term technogenic impact, Scots pine shows an increase in the share of latewood in the structure of annual growth in terms of diameter (Arseneva and Chavchavadze 2001, Shchekalev and Tarkhanov 2006, Yarmishko 2012). Primarily, these changes occur due to a decrease in the zone width of earlywood. However, a number of authors point to a decrease in the zone width of latewood and radial growth when approaching emission sources. Simultaneity of regression of individual zones and the annual ring as a whole are also noted, and this leads to an increase in the overall wood density (Zobel and van Buijtenen 1988, Tarkhanov 2010). The impact of technogenic load is more pronounced in low-productive forest stands.

The accumulated body of knowledge about the impact of constantly changing environmental conditions in forest stands and in particular on individual trees shows that there are a small number of comprehensive studies on properties of wood formation. As a rule, ongoing research is limited to the analysis of volumetric and technical properties of wood and identification of correlations and patterns with the value of the annual growth (Melekhov 1949, Poluboyarinov 1976, Danilov and Zaytsev 2021, Wieruszewski and Mydlarz 2021). The geography of such studies rarely reaches the regional level (Wilhelmsson et al. 2002, Yarmishko 2012, Kalbarczyk et al. 2018). On the other hand, studies by biologists reveal processes of growth and development of tracheids in most detail, and, as a rule, they do not pay attention to technical properties of wood formation (Yatsenko-Khmelevsky 1954, Barnet 1981).

Taking into account the above analysis of the issues in the variation of the structural elements of the radial growth and physical and mechanical properties of coniferous wood as well as, in particular, Scots pine, the goal of the study was defined as follows: to study patterns of structure and dynamics of strength properties of Scots pine wood for the boreal zone in the Northeast of Europe.

MATERIALS AND METHODS

Study Area

The field work area is located in the north of the Russian Plain, which in turn is located in the north of the Russian and Pechersk Plates that are part of the East European Platform on the territory of Russia. The collection of field material was carried out in the period from 1998 to 2014. The study area covers the territory of the Arkhangelsk region, the Komi Republic and the Nenets Autonomous District within the boundaries from 60° to 68°N and from 38° to 53°E. A significant stretch from north to south and from west to east determines the division of the territory into zones of forest-tundra, northern, middle and southern taiga. This, in turn, determines substantial differences in soil and climate conditions (Figure 1).

Most of the territory has a flat relief with heights up to 200-250 m above sea level and with a general surface slope from south to north. Owing to the significant expanse from north to south, the study area is located in three climate zones: arctic, subarctic and temperate. The above is reflected in different influxes of solar radiation, the value of which, in turn, is the main climate factor influencing wood formation. The annual value of total solar radiation ranges from 1,500 in the south to 700 kW·m^{-2} in the north (Scientific and applied reference guide Climate of Russia 2022).

The study area has a wide spread of *Vaccinium* ssp., *Polytrichum* ssp. and *Sphagnum* spp. groups of forest types, which are characterized by mixed coniferous and coniferous-deciduous forest stands with a dominance of spruce. The study objects are represented by mature and maturing pine phytocenoses located in landscapes with a flat or slightly undulating relief. Pine forests occupy a smaller area than spruce forests (Arkhangelsk region ~27%; Komi Republic ~25% of the total area) and are represented by *Pinus sylvestris- Hylocomium*, *Pinus sylvestris-Sphagnum* spp. and *Pinus sylvestris- Cladonia* ssp. forest types. The average stock of mature and overmature stands amounts to 115-130 m^3·ha^{-1} in the Komi Republic and 130-140 m^3·ha^{-1} in the Arkhangelsk region.

Forest stands in the regions of industrial facilities concentration of the timber industry, thermal power and military complexes, in consideration of the specifics of air pollution from the latter, experience a constant negative impact (Bobkova et al. 1997, Shchekalev and Tarhanov 2004, Martynyuk 2010, Robakidze et al. 2013, Figas et al. 2021). The presence of industrial facilities in the study area, obviously, contributes to the deformation of chemical composition of soil solutions and atmospheric air, which in turn, definitely, affects the growth and development of woody plants. Therefore, when considering such issues as variation of wood structure and properties within certain geographical areas, we took into account the anthropogenic factor of industrial emissions.

The main sources of air pollutant emissions in the study area, which partially covers the territories of the Arkhangelsk region of the Nenets Autonomous District and the Komi Republic, are construction industry facilities, fuel and energy complexes, nuclear shipbuilding industry facilities, infrastructure of the cosmodrome and timber complex concentrated in the vicinity of large cities and administrative centers (the cities of Arkhangelsk, Kotlas, Syktyvkar, Ukhta, Inta, Naryan-Mar, etc.).

Figure 1. Layout of research objects. Numbers in blue (rivers): 1 – Onega; 2 – Severnaya Dvina; 3 – Pinega; 4 – Mezen'; 5 – Pechora; 6 – Vaga; 7 – Vashka; 8 – Izhma; 9 – Vichegda; 10 – Sysola.

When selecting research objects, the principle of representativeness was observed, which ensures conducting comparative assessments in similar forest growth conditions. For the purposes of zoning the technogenic impact from sources of emissions of industrial enterprises, sample plots were located along the transects of all the main wind points (Yarmishko 1997).

The initial selection of plots was made according to forest inventory documents and forest plantation plan. The location of the sample plots was determined in the most typical growth conditions for the study area. For representativeness of the results, the trial plots are located in mature stands. When determining a forest type, we followed the classification proposed by V.N. Sukachev (Sukachev et al. 1957). The number of taxation points by regions and growth conditions are given in Table 1.

Research Methods

Taking into account the goals and objectives of the study, a research methodology was designed to study the anatomical structure and physical and mechanical properties of pine wood, features of tree growth and their dependence on external factors under conditions of the European Northeast.

For a holistic comprehension of how the level of aerotechnogenic pollution impacts the state of coniferous plantings, the factual material on the pollution of snow and vegetation covers with the official data from the hydrometeorological service of the region was collected and analyzed (Scientific and applied reference guide Climate of Russia 2022). As a result, the scale of dispersion of emission products was determined, as well as their fallout with atmospheric precipitation due to activities of industrial enterprises (Tarkhanov 2010). To assess the pollution level, we used maps of dispersion fields for emissions of air pollutants, obtained on the basis of the software products by the “Unified Program for Calculating Air Pollution – Ecologist” and “Maximum Permissible Emissions – Ecologist”, and in accordance with the “A.I. Voeikov Main Geophysical Observatory”, St. Petersburg, Russia.

Based on the materials of field observations, data on forestry and taxation parameters of forest stands were obtained. This made it possible to substantiate the choice of model trees and selected wood samples for subsequent measurements of growth and physical and mechanical properties of wood. For each trial plot, 5-7 models were selected by preliminary measurement of the height and diameter of the trunk (from among commercial trees), close to the average. The models were used to select tilts and wood saw cuts at the height of 1.3 m from the root collar and relative heights of the trunk: 0H, 1/4H, 2/4H, 3/4H.

In laboratory conditions, each tree core was used to measure the width and macrostructure of annual rings, using an MBS-1 stereoscopic microscope. After measuring the width of annual rings for studying linear dimensions of tracheids, 2-4 segments of wood were carved from the cores taken from the model trees. Preparation of wood samples for anatomical study was carried out in accordance with generally acknowledged methods (Carlquist 1988). To work with specimens, a Jenaval Carl Zeiss vertical microscope was used. The photographic survey was made with a Sony DSC-W1 camera through the eyepiece of the microscope. The measurement of linear dimensions of tracheids and the number of resin ducts per cm^2 in the images obtained was carried out with the help of the PhotoM 1.21 program developed at the I.M. Sechenov Institute of Evolutionary Physiology and Biochemistry RAS, St. Petersburg, Russia (Image processing software for cytophotometry PhotoM 1.21). The program is suitable for cytophotometry because it helps calculate the optical density of photographs by processing images in bmp and jpg formats.

The density of the wood samples was determined using the method of maximum humidity of wood samples, which is well suited for samples with a relatively small volume (Smith 1954, Ugolev 1965, Poluboyarinov 1976). The applied method assumes that between the maximum humidity (W_{max}), which a wood sample can have, and density, there is dependence. The formula for calculating wood basic density (ρ_{basic}) in g·cm^{-3} with a known mass of the sample saturated with humidity (m_W) and in an absolutely dry (m_0) state is written as (Equation 1):

$$\rho_{basic} = \frac{1}{\frac{m_w - m_0}{m_0} + \frac{1}{d}} \tag{1}$$

where *d* is the density of woody substance, and the average value is 1.53 g·cm^{-3}. The basic density of wood is independent of moisture content and it is more convenient to use it in wood quality studies (Poluboyarinov 1976).

To determine wood resistance to compression along the fibers, we used wood samples in the form of a rectangular prism, 20x20x30 mm, oriented along the fibers, selected from

Table 1. Distribution of taxation points.

Region	Forest type					
	1	2	3	4	5	Total
Arkhangelsk region	33	11	22	5	6	77
Komi Republic	8	7	6	-	4	25
Nenets Autonomous District	3	-	-	-	-	3
Total	44	18	28	5	10	105

Forest type: 1 – *Pinus sylvestris*–*Vaccinium* spp. –*Sphagnum* spp.; 2 – *Pinus sylvestris*–*Vaccinium* ssp.+ *Ledum palustre*; 3 – *Pinus sylvestris*–*Vaccinium myrtillus*; 4 – *Pinus sylvestris*–*Oxalis acetosella*; 5 – *Pinus sylvestris*–*Cladonia* ssp.

the trunks of the model trees differentiated in terms of height and diameter. Up to the height of 1/4H, 4 samples were carved along the radius: 3 in the area of the wood core, center, middle, and periphery, and 1 in sapwood. As the sampling height increased, the number of samples decreased (Melekhov et al. 2003). The tests were carried out on a universal testing machine SHIMADZU of the AGS-100kNX series. The obtained values of maximum load were given in kgf·m^{-2} and converted into MPa with the coefficient of 10.19716.

The processing of experimental data is based on the methods of correlation, regression, multivariate (Graeco-Latin square design), variance and dispersion analyses (Jayaraman 1999). All measurement results were obtained using software products: Excel version before 2013, Statgraphics, SPSS, MATLAB, and analytical programs developed by us to simplify and accelerate calculations in the C++ language using a specialized package for statistical analysis and forecasting ALGLIB (Gilmore et al. 2009, RCoreTeam 2013). Together with the results of correlation analysis, which describe the dependence of the radial growth on external and internal factors in geographical terms, the data obtained were processed using variance analysis that allows us to estimate the variation value at various levels in the study area (Jayaraman 1999). Student's (*t*) and Fisher's (*F*) criteria were used to compare indicators (James et al. 2013).

The conclusions and patterns obtained are valid at the 5% significance value (Jayaraman 1999). Statistical analysis of mass observations allowed the objective estimation of the findings and drawing valid conclusions with a certain degree of credibility.

RESULTS AND DISCUSSION

Influence of Growth Conditions on Macrostructure and Density of Pine Wood

On the basis of laboratory studies, an array of data was obtained, which allow us to analyze the dynamics of basic density of Scots pine wood in geographical terms, inside the forest stand on a trial plot and inside a separate tree.

The data obtained (Table 2) make it possible to assume that as growth conditions improve, volumetric properties of *P. sylvestris* L. wood decrease in numerical terms. The significance of difference in average values of wood density is noted only when comparing pine wood samples growing under the conditions of *Pinus sylvestris-Vaccinium* spp.-*Sphagnum* spp. forest type (Table 3), in forest stands growing on more drained soils.

Variation in wood basic density within individual study areas is within the framework of a low level (Jayaraman 1999). For shrub-sphagnum forest stands, the variation coefficient (C_V) is 12.3% in the region of Arkhangelsk, 11.7% in the region of Syktyvkar, 9.7% in the region of Kotlas and 7.2% in the region of Naryan-Mar. In more productive myrtillus fresh pine forests, variation of wood basic density is lower and amounts to: Arkhangelsk – 8.3%; Syktyvkar – 8.9% and Kotlas – 9.2%.

Correlation analysis of mature forest stands growing in the area of the Arkhangelsk agglomeration revealed an average connection level of basic wood density with the annual ring width and proportion of latewood therein (Figure 2). The presence of a negative sign (r =-0.549±0.072; t_{fact}=7.62>$t_{0.01}$=2.62) when comparing basic wood density

Table 2. Average values of basic wood density (mean±SE) by forest types and study areas at the trunk height of 1.3 m.

Region	Basic wood density (kg·m^{-3})				
	Forest type				
	1	2	3	4	5
Arkhangelsk region	507±14	473±13	467±14	471±14	480±15
Syktyvkar region	469±12	457±12	442±13	-	453±14
Kotlas region	476±11	448±13	439±14	443±15	431±13
Naryan-Mar region	478±13	-	-	-	-

Forest type: 1 – *Pinus sylvestris–Vaccinium* spp. –*Sphagnum* spp.; 2 – *Pinus sylvestris–Vaccinium* ssp.+ *Ledum palustre*; 3 – *Pinus sylvestris–Vaccinium myrtillus*; 4 – *Pinus sylvestris–Oxalis acetosella*; 5 – *Pinus sylvestris–Cladonia* ssp.

Table 3. Significance of difference in basic density of pine wood by forest types.

Forest type	Arkhangelsk region				Syktyvkar region				Kotlas region			
	2	3	4	5	2	3	4	5	2	3	4	5
1	**1.78**	**2.02**	**1.82**	1.32	0.71	1.53	-	0.87	**1.64**	**2.08**	**1.77**	**2.64**
2	-	0.31	0.11	0.35	-	0.85	-	0.22	-	0.47	0.25	0.93
3	-	-	0.20	0.63	-	-	-	0.58	-	-	0.20	0.42
4	-	-	-	0.44	-	-	-	-	-	-	-	0.61

Forest type: 1– *P. sylvestris–Vaccinium* spp. –*Sphagnum* spp.; 2 – *P. sylvestris–Vaccinium* ssp.+ *Ledum palustre*; 3 – *P. sylvestris–Vaccinium myrtillus*; 4 – *P. sylvestris–Oxalis acetosella*; 5 – *P. sylvestris–Cladonia* ssp.; highlighted in bold are the values exceeding the significance threshold of p = 0.90.

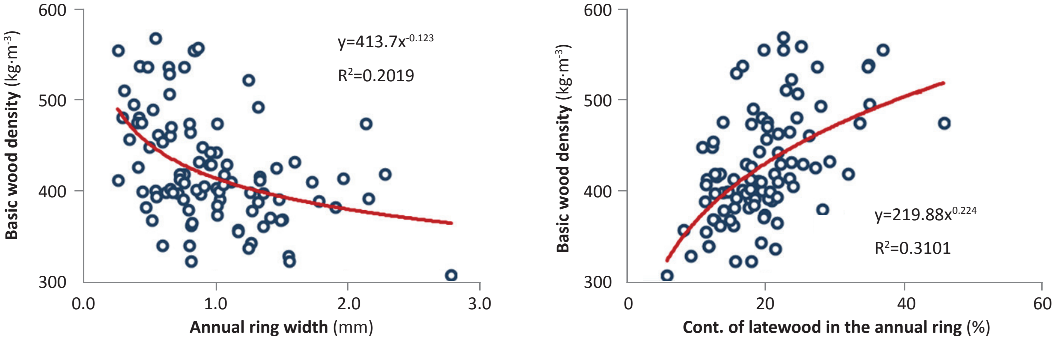
and the annual ring width is logical, since the increase in the ring width is mainly due to an increase in the zone of earlywood. Therefore, our calculations showed that a higher latewood content corresponds to higher wood density values (r=0.592±0.067; $t_{fact}=8.87>t_{0.01}=2.62$), which is comparable with the results of other similar studies in the boreal zone (Melekhov et al. 2003, Kishchenko 2019).

However, two oppositely directed processes take place. On the one hand, the relationship between wood density and latewood content in the annual ring increases from north to south (Figure 3). On the other hand, the dependence of wood basic density on the annual ring width also decreases from north to south, also decreasing when soil and hydrological conditions of growth improve. The multiple correlation coefficient of wood basic density with the width of annual growth and percentage of latewood is 0.589±0.066 for shrub-sphagnum pine forests located in the region of Arkhangelsk, while the results for other regions are as follows: Syktyvkar – 0.561±0.082; Kotlas – 0.517±0.095 and Naryan-Mar – 0.544±0.113. For myrtillus fresh pine forests, the following multiple correlation values were obtained for the study areas: 0.541±0.082 for Arkhangelsk; 0.522±0.092 for Syktyvkar and 0.507±0.094 for Kotlas. When comparing identical growth conditions from different regions, and when comparing different conditions within a particular region, no differences in correlations were revealed ($t_{fact}<t_{table}$). It should be noted that the developed samples of basic wood density, differentiated by growth conditions and study areas, differ significantly according to the results of dispersion analysis conducted with the use of the Kolmogorov-Smirnov criterion ($\lambda_{fact}>\lambda_{0.05}$).

Influence of Pollutants on Density and Annual Growth of Pine Wood

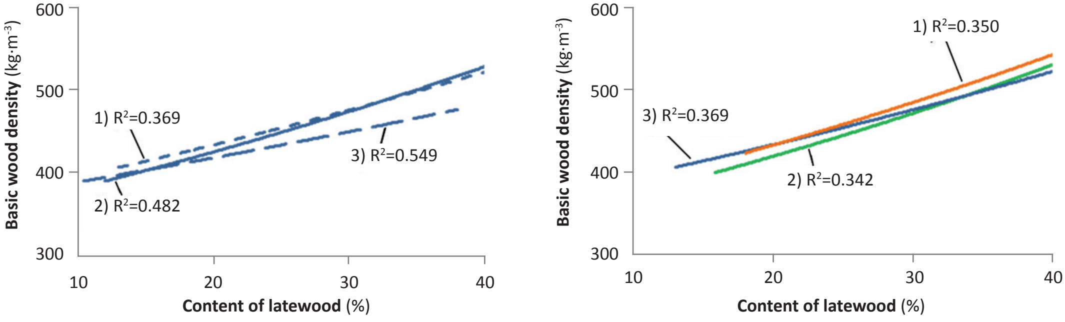
An additional criterion of growth conditions in the study is the degree of technogenic load. The findings show that the degree of aerotechnogenic load corrects the dependence of basic density on the value and structure of the annual ring. In particular, when approaching the source of emissions, the relationship between wood density and the annual ring width weakens (Figure 4). This situation may be associated with a reduction in variation of tree growth strategies with deterioration of growth conditions.

The location of the trial plots differentiated by distance from industrial enterprises makes it possible to evaluate the degree of influence of air pollution on the absolute values of basic wood density. The constructed dynamics sets of basic wood density (average over the radius, at the trunk height of 1.3 m) show that it has a tendency to rise as the



a) b)

Figure 2. Dependence of basic density of pine wood on **(a)** the annual ring width, and **(b)** content of latewood in the annual ring.



a) b)

Figure 3. Dependence of basic density of Scots pine wood on latewood content in the annual ring by study areas based on the sample from myrtillus fresh pine forest **(a)** 1 – Arkhangelsk; 2 – Syktyvkar; 3 – Kotlas, and by forest types; **(b)** 1 – *P. sylvestris–Vaccinium* spp. –*Sphagnum* spp. ; 2 – *P.sylvestris–Vaccinium* ssp.+ *Ledum palustre;* 3 – *P. sylvestris–Vaccinium myrtillus.*

technogenic load increases on the territory of the regions under consideration (Figure 5).

It is worth noting that an increase in values of basic wood density, calculated as the average over the radius, in the course of distance reduction to the emission source is observed at all trunk heights considered from 0H to 3/4H (Figure 6).

Having summarizing the data on the dynamics of basic wood density along and across the axis of the trunk in different forest types and at different distances from the source of technogenic emissions, we designed maps of conditional distribution of basic wood density inside the trunk separately for the trial plots and for the study area as a whole. One of the variants (averaged representation) is shown in Figure 7.

Considering the cartograms obtained, a matrix of parameters affecting basic density of pine wood was developed for conducting multivariate analysis (Table 4): (*A*) spatial arrangement of the wood sample inside the trunk along and across the axis; (*B*) integral indicator of the annual ring width and content of latewood therein; (*C*) distance to the source of emissions; (*D*) crown diameter; (*E*) average annual precipitation rate and temperature mode of the vegetation season (over the period included in linear dimensions of the samples). From the above group of factors, the influence of the size and structure of annual growth and the position of the wood samples by trunk volume were highlighted in terms of their significance. It is also worth noting that there is a trend towards an increase in the significance of unidentified factors (Z). Note that the data on the dependence and direction of structural changes in the annual growth of wood, which objectively affect the indicators of wood density, and which indirectly confirm our conclusions, have been mentioned by other authors (Fox et al. 1986, Stolte et al. 1992).

Variation of Compressive Strength along Pine Wood Fibres

Mechanical properties of wood are determined by the stress values it can withstand while being under load before destruction. The most indicative parameter characterizing mechanical properties of wood is its compressive strength along the fibers.

The calculation of compression limits along the fibers of pine wood was carried out. The limit of this indicator (P_{max}) was the moment when the load curve passed through the extremum point. The angle of deviation of the load curve became negative. Visually, at this moment and during the further course of compression, microcracks appear and become larger on the surface of the sample, and some

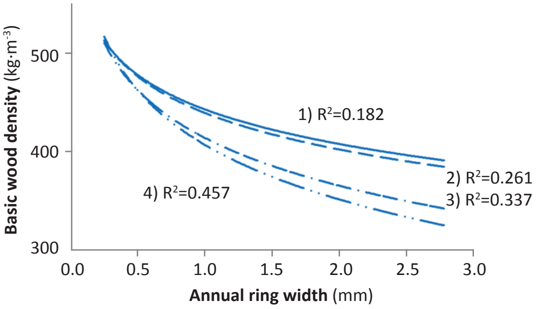


Figure 4. Tightness of the relationship between basic density of pine wood and the annual ring width when moving away from the source of aerotechnogenic emissions: 1 – <5 km; 2 – from 5 to 10 km; 3 – from 10 to 20 km; 4 – >20 km.

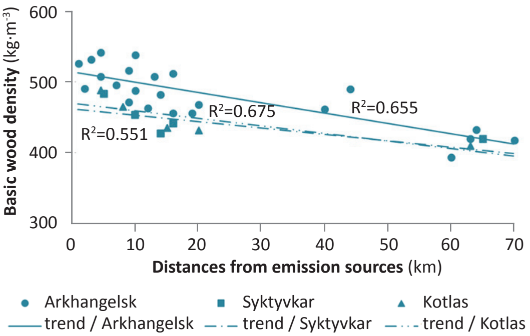


Figure 5. Dynamics of change in basic density of pine wood at different distances from emission sources (forest type: *P. sylvestris–Vaccinium* spp. –*Sphagnum* spp.).

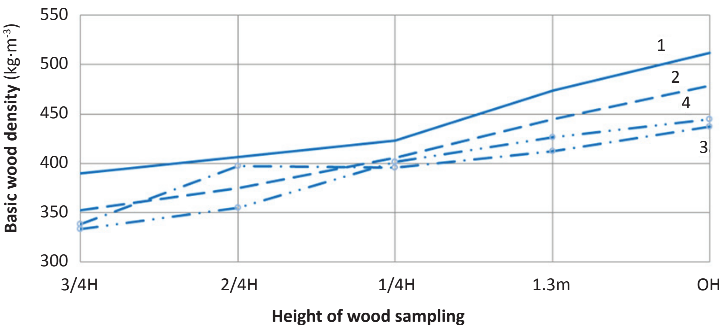


Figure 6. Dynamics of basic wood density inside a pine trunk at different distances from the emission source: 1 – <5 km; 2 – from 5 to 10 km; 3 – from 10 to 20 km; 4 – >20 km (forest type: *P. sylvestris–Vaccinium* spp. –*Sphagnum* spp.).

Table 4. Evaluation of the influence power of factors on basic density of pine wood.

Value	Factors considered					Z	X (total)
	A	B	C	D	E		
Arkhangelsk region							
S	62	79	40	27	14	197	419
v	4	4	4	4	4	59	79
σ^2	15.50	19.75	10.00	6.75	3.50	3.34	-
t-value	4.64	5.91	2.99	2.02	1.05	-	-
Syktyvkar region							
S	57	88	29	22	9	216	421
v	4	4	4	4	4	59	79
σ^2	14.25	22.00	7.25	5.50	2.25	3.66	-
t-value	3.89	6.01	1.98	1.50	0.61	-	-
Kotlas region							
S	66	71	37	39	19	225	457
v	4	4	4	4	4	59	79
σ^2	16.50	17.75	9.25	9.75	4.75	3.81	-
t-value	4.33	4.65	2.43	2.56	1.25	-	-

A – spatial arrangement of the wood sample inside the trunk along and across the axis; B – integral indicator of the annual ring width and content of latewood therein; C – distance to the source of emissions; D – crown diameter; E – average annual precipitation rate and temperature mode of the vegetation season (over the period included in linear dimensions of the samples); Z – unidentified factors; S – variation sum; v – the number of degrees of freedom: t – estimated t-statistic.

sections of the sample begin to “slide” relative to each other. Further, the load curve levels off and for the most part becomes perpendicular to its axis. At this moment, deep cracks are formed on the surface of the sample, flaking it along the fibers. The performed statistical analysis of the dependence of the change moment in the slide angle of the load curve at a given point relative to growth conditions of the standing wood did not allow us to identify valid values of the gravitation momentum of the onset of irreversible deformations to the listed factors (Table 5).

When conducting correlation analysis, dependences of pine wood resistance to compression along the fibers (hereinafter referred to as resistance) on structural elements of the annual ring were revealed (Figure 8).

In particular, we noted the presence of a significant negative relationship between the average force of the resistance limit of wood compression along the fibers with the annual ring width ($r \pm m_r$ is -0.522±0.133, $t_{fact}>t_{0.05}$), as well as a positive relationship between the average force and a specific content of latewood in the annual ring, expressed as a percentage ($r \pm m_r$ is 0.547±0.128, $t_{fact}>t_{0.05}$).

An insignificant positive relationship ($r \pm m_r$ is 0.311±0.136, $t_{fact}>t_{0.05}$) of physical and mechanical properties of wood with an increase in the number of vertical resin ducts per unit area on a radial cut was revealed. The dependence established seems to be logical, taking into account that the main number of resin ducts is located in the latewood zone and directly depends on the length of the latewood zone and entire annual growth as a whole.

Among linear dimensions of tracheids of earlywood and latewood, the thickness of cell walls of tracheids in the late zone of the annual ring along and across the trunk radius

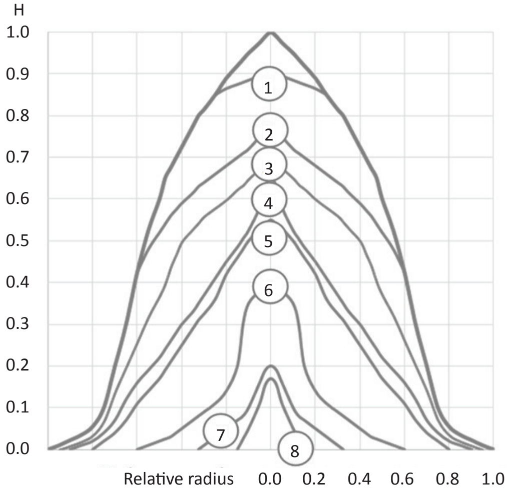


Figure 7. Distribution scheme of basic wood density inside a conditional pine trunk. Isoline: 1 – 350; 2 – 375; 3 – 400; 4 – 425; 5 – 450; 6 – 475; 7 – 500; 8 – 525 kg·m^{-3} (forest type: *P. sylvestris*–*Vaccinium* spp. –*Sphagnum* spp., region – Arkhangelsk).

Table 5. Difference between the onset moment of irreversible deformations of pine wood (mean±SE) relative to growth conditions.

Factors	Moment of irreversible deformations, part of P_{max}	C (%)	*t*-statistic	*F*-test
Growing region factor				
Arkhangelsk	0.71±0.02	16.1	0.675*	1.226
Kotlas	0.68±0.02	15.1		
Arkhangelsk	0.71±0.02	16.1	1.028	1.056
Naryan-Mar	0.67±0.04	16.5		
Forest type factor				
Pinus sylvestris–*Vaccinium* spp.–*Sphagnum* spp.	0.68±0.02	14.8	1.022	1.025
Pinus sylvestris–*Vaccinium* ssp.+ *Ledum palustre*	0.65±0.02	15.7		

Note: * – $t_{fact} < t_{0.05} = 2.021$; $F_{fact} < F_{0.05} = 1.73$; C – variation level.

produces a more significant effect on the ultimate strength of wood along the fibers ($r \pm m_r$ respectively 0.373±0.133 and 0.352±0.142 with $t_{fact} > t_{0.05}$).

Differentiation of data by study areas (Table 6) shows a slight fluctuation of the compression strength limit indicator along the fibers in forest stands growing in different forest types and study areas. The validity of the comparative average values of physical properties exceeds the tabular values only when comparing the results of laboratory analyses of the samples taken in shrub-sphagnum pine forests and lichen pine forests ($t_{fact}=2.097$ with $t_{0.10}=1.697$; $t_{0.05}=2.142$; $t_{0.01}=2.75$).

The variation level (*C*, %) of strength properties within the European Northeast is at a low level and increases slightly from east to west and from south to north. For pine forest stands of the sphagnum group, the variation index in the southern part of the study area (Kotlas) is 11.9%, whereas in the northern part (Naryan-Mar) it equals 12.5%; in the east (Syktyvkar) it is 12.2%, and in the western part (Arkhangelsk) it amounts to 12.6%. Improvement of soil and hydrological conditions of growth within individual locations leads to a decrease in the variation level of the limit values of pine wood resistance to compression along the fibers. In particular, for plantations growing in the area of Arkhangelsk, variation in strength properties of Scots pine wood is as follows: shrub-sphagnum – 12.6%; myrtillus wet – 12.5%; myrtillus fresh – 11.5%; sorrel – 11.2%. Similar dynamics were also noted for pine stands growing in the areas of Syktyvkar and Kotlas.

It can be argued that mechanical properties of wood are a species-specific indicator, and all fluctuations in their value occur within a single range, with a significant deviation beyond its boundaries being fatal for an individual tree. For pine wood, the average resistance value of 46 MPa is given (Ugolev 2001).

To assess goodness of fit between the distribution sets of wood resistance values in the study areas, Pearson's chi-squared test (χ^2), was used, the value of which during the pairwise comparison of wood resistance distribution in the study areas did not exceed the critical values of c^2_{table} with $p_{0.05}$ ($c^2_{0.05}=9.49$; $c^2_{0.01}=13.28$).

The revealed feedback strength of the limit of wood resistance to compression along the fibers and radial growth increases from west to east within the study area (Figure 9), $r \pm m_r$ for the Arkhangelsk region is -0.495±0.118 ($t_{fact} > t_{0.05}$); for Kotlas it is -0.606±0.125 ($t_{fact} > t_{0.05}$); for Syktyvkar it is -0.661±0.143 ($t_{fact} > t_{0.05}$) and decreases to the more northern regions of Naryan-Mar – -0.473±0.141 ($t_{fact} > t_{0.05}$).

Table 6. Limit values of pine wood resistance to compression (mean±SE) along the fibers by research areas and forest types.

Forest type	Resistance limit of pine wood to compression (MPa)			
	Research area			
	Naryan-Mar	Arkhangelsk	Syktyvkar	Kotlas
P. sylvestris–*Vaccinium* spp. –*Sphagnum* spp.	48.3±1.2	49.5±0.9	47.2±1.1	47.0±1.0
P. sylvestris–*Vaccinium* ssp.+ *Ledum palustre*	-	48.3±1.1	45.8±1.2	46.2±1.1
P. sylvestris–*Vaccinium myrtillus*	-	48.0±1.0	44.9±1.1	45.8±0.8
P. sylvestris–*Oxalis acetosella*	-	46.3±1.2	-	46.0±1.2
P. sylvestris–*Cladonia* ssp.	-	47.9±1.4	46.3±1.4	46.4±1.3

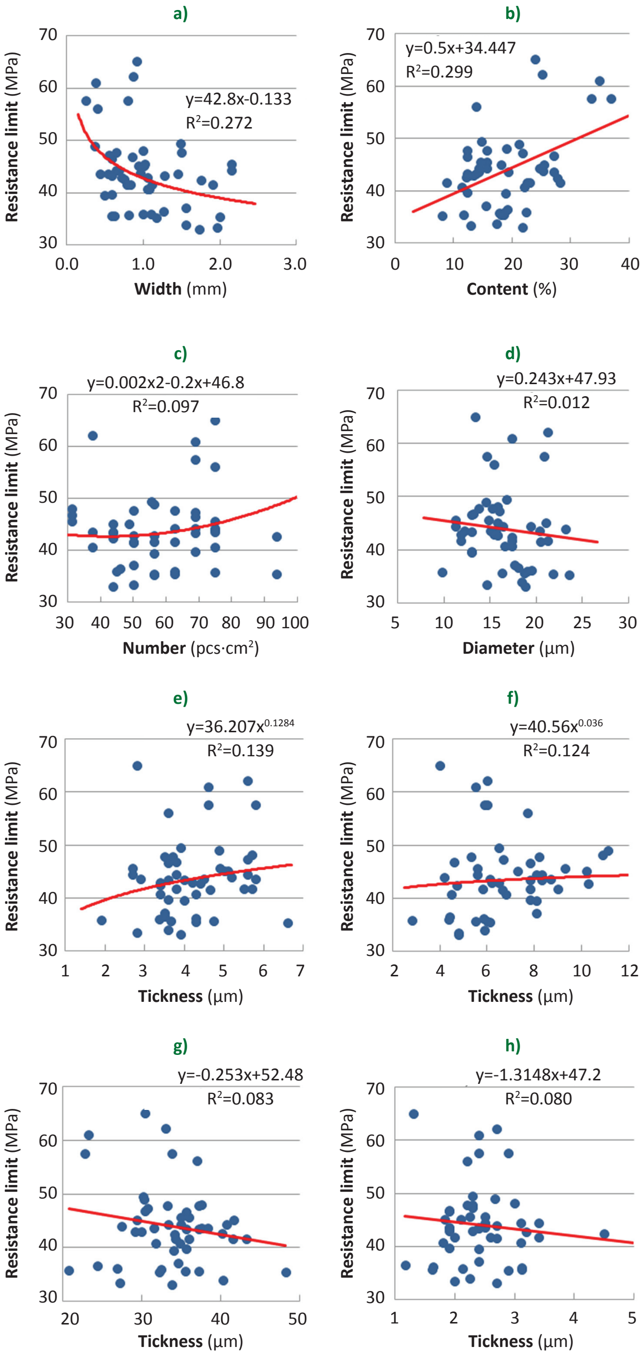


Figure 8. Correlation of the resistance limit of pine wood to compression along the fibers with: **(a)** annual ring width; **(b)** content of latewood in the annual ring; **(c)** number of vertical resin ducts; **(d)** latewood tracheid diameter; **(e)** latewood tracheid cell wall thickness along the radius; **(f)** cell wall thickness of latewood tracheids in the tangential direction; **(g)** cell wall thickness of latewood tracheids along the radius; **(h)** cell wall thickness of earlywood tracheids along the radius.

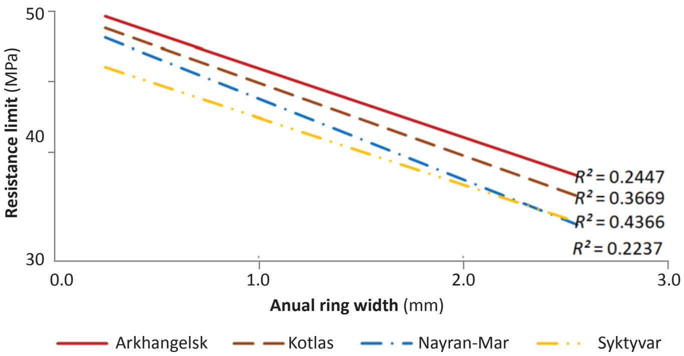


Figure 9. Correlation of the resistance limit of pine wood to compression along the fibers with the annual ring width by study areas (*P. sylvestris–Vaccinium* spp. –*Sphagnum* spp. forest type).

Pollutants and Physical and Mechanical Properties of Pine Wood

The conducted dispersion analysis showed that we can accept the null hypothesis that the samples formed in accordance with the value of pine wood resistance to compression along the fibers in the study areas can be perceived as part of the general population (Table 7). An exception can be considered a significant difference in dispersion of wood resistance values in sphagnum pine forests of the southern taiga and polar regions.

The nature of the relationship between physical and mechanical properties of pine wood and the value of radial growth is influenced not only by the geographical aspect and soil and hydrological conditions of growth, expressed through the forest type. The configuration of the dependence under consideration is transformed when the technogenic impact factor is introduced into the analysis perimeter, expressed, as we indicated earlier, through the distance gradient from sources of the aerotechnogenic impact. In the “closer than 5 km” and “from 5 to 10 km” zones, the strength of the described relationship tends to increase (Figure 10). We also noted a similar trend when decomposing the dependence of wood resistance on content of latewood in the annual ring, depending on the distance from sources of aerotechnogenic pollution. This fact is quite logical, given that an increase in technogenic load, i.e. a decrease in the distance to the source of emissions leads to a reduction in the annual radial increment value and an increase in the late part of wood relative to the total increment value.

We should note a slight overrun in the binding strength of the limit of wood resistance to compression along the fibers and content of latewood in the annual ring in the region of Arkhangelsk of 0.745±0.149 (t_{fact}=4.99) relative to the other regions. We have observed the minimum value for pine samples in the region of Naryan-Mar of 0.426±0.153 (t_{fact}=2.88). For the region of Kotlas, the correlation value was 0.676±0.142 (t_{fact}=4.76), and for Syktyvkar it was 0.574±0.138 (t_{fact}=4.16).

The obtained values of the relationship between the pine wood resistance and the value of the annual increment and content of latewood therein are consistent along the direction of the relationship, but differ in numerical terms from the data obtained by other authors for neighboring regions (Poluboyarinov 1976, Ugolev 2001). Taking into account our results concerning the value and significance of the relationships under consideration for plantations growing in the area of Naryan-Mar, it can be assumed that the significance degree of macrostructural indicators of the annual ring decreases by the value of wood resistance to compression along the fibers in the polar regions of the pine area.

Table 7. The value of Fisher’s criterion (*F*) for pine wood resistance to compression along the fibers.

Research area	Arkhangelsk	Kotlas	Syktyvkar
Kotlas	1.07 1.09	-	-
Syktyvkar	2.06 1.69	2.18 1.55	-
Naryan-Mar	2.13 -	2.27* -	0.97 -

Numerator – *P. sylvestris–Vaccinium* spp. –*Sphagnum* spp. forest type, denominator – *P. sylvestris–Vaccinium* ssp.+ *Ledum palustre* forest type; $F_{0.05}$ = 2.19; * – the value significant at $p_{0.05}$.

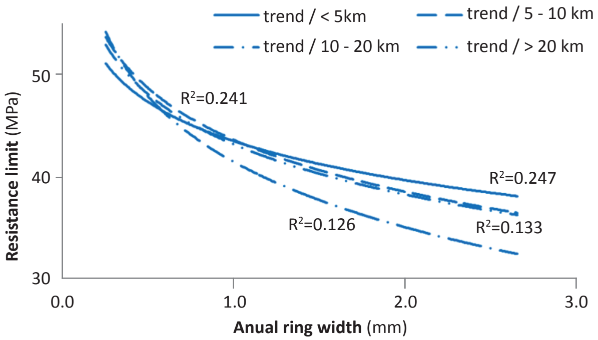


Figure 10. Correlation of the limit of pine wood resistance to compression along the fibers and the annual ring width, including distance to the source of emissions (forest type: *P. sylvestris*–*Vaccinium* spp. –*Sphagnum* spp.; region – Arkhangelsk).

The array of data on physical and mechanical properties of Scots pine wood available in scientific literature, differentiated or segmented in terms of geography, growth conditions and origin of pine plantations, makes it possible to argue that an increase in basic wood density entails an increase in the limit values of wood resistance to compression (Melekhov 1949, Perelygin 1953, Poluboyarinov 1976, Shchekalev and Tarkhanov 2006). This has also been confirmed in our study with the significance of the relationship under consideration according to the results of univariate dispersion analysis amounting to 4.43, with tabular $t_{0.10}$=3.49; $t_{0.05}$=5.85; and $t_{0.01}$=9.95. However, this relationship cannot always be described by a straight line and extended to the entire spectrum of values. As a rule, the data published fall within the value range of 35-55 MPa. Yet, partly due to data fragmentation, partly due to a too high level of generalization, it is not possible to form an unambiguous judgment about the nature of the variation factor of wood resistance to compression along the fibers within the distribution area, in particular, in the north of the European part.

During the study, it was found that a change in volumetric properties of pine wood leads to a disproportionate change in wood resistance to compression along the fibers (Figure 11a). A possible division of the sample into three groups, separated by the curve's extrema, can be considered. The resulting decomposition of test data (Figure 11a), along the basic wood density axis, can be divided into three ranges: less than 400; from 400 to 500 and over 500 kg·m^{-3}. Graphically, the result can be represented as follows (Figure 11b). On Figure 11a correlation trend is superimposed on the data, corresponding to the 5th degree polynomial, reaching the maximum value of determination (R^2=0.728). Figure 11b is decomposed into three regions with the highest value frequency in the central part, which corresponds to a normal distribution.

The selected ranges (Figure 11b) are statistically different (F_{fact}>$F_{0.05}$=2.09) and also show different strengths of the relationship between basic density and wood compression resistance and its decrease from the left to the right: 0.567±0.119 (t_{fact}=4.88); 0.476±0.117 (t_{fact}=4.06) and 0.389±0.113 (t_{fact}=3.44).

The comparison of average values of wood resistance to compression along the fibers and the value of its dispersion, within a single variant for basic wood density (Table 8), indicates that a change in basic density of pine wood by

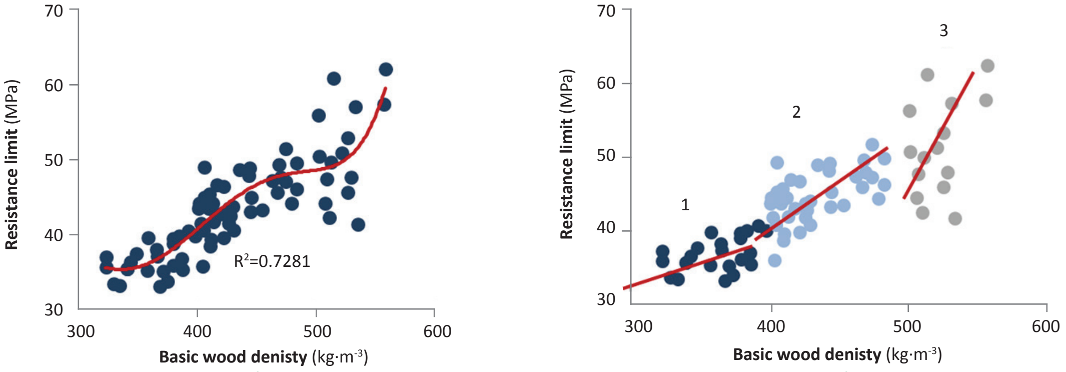


Figure 11. Dynamics dependence of the resistance limit of Scots pine wood to compression along the fibers on the basic wood density value **(a)** – in complex; **(b)** – in decomposition: 1 – <400 kg·m^{-3}; 2 – 400–500 kg·m^{-3}; 3 – >500 kg·m^{-3}.

60 kg·m^{-3} entails a significant change in wood strength properties. Neighboring variants are comparable and, as a rule, do not differ statistically by dispersion and by the average value of wood technical properties.

The analysis of multiple correlation of pine wood strength properties in separate study areas from a group of factors (radial increment value, content of latewood in the annual ring, basic wood density) does not reveal a trend in the relationship dynamics in terms of geography. The increase in the strength of relationship with this factor occurs fragmentarily from plantations growing in the regions of Naryan-Mar, then Kotlas, Syktyvkar, and the maximum value was obtained for plantations in the region of Arkhangelsk (respectively: 0.491±0.098; 0.549±0.088; 0.574±0.092; 0.623±0.086).

The assessment of the dependence of pine wood strength properties (as an average value for the trunk) on the height of the tree as a whole for the study area shows a weak level of negative correlation -0.219±0.104. The upper limits of the range correspond to plantations of middle taiga, whereas the lower ones correspond to the polar regions.

Elaboration of correlation sets (Figure 12) of wood strength properties and the position of the test specimen inside the trunk (differentiated by the sampling height and location along the radius) shows that at the base of the trunk (0H) and at the height of 1.3 m, pine wood resistance to compression along the fibers decreases from the center of the trunk to the bark. With the further advance up the trunk from the height of 1/2H to 3/4H (in fact, in the crown zone), strength properties increase in the same direction.

Growth in the impact zone of emissions from large industrial enterprises in the region leaves an insignificant imprint on the dynamics structure of wood strength properties, depending on growth conditions.

The maximum level of correlation between strength properties and the degree of remoteness from emission sources was found for pine forests growing in the region of Arkhangelsk urban agglomeration (-0.382±0.112; t_{fact}=3.40, at $t_{0.05}$=4.0), whereas the minimum level for pine forests was in the area of the Kotlas forestry industry complex (-0.319±0.124; t_{fact}=2.56, at $t_{0.05}$=4.0). The value of *F*-criterion when comparing pine forests growing in the region of Arkhangelsk and more southern regions amounts to 1.90 and 1.89 ($F_{0.05}$=1.35). The low level of correlation with the presence of fragmentary deviations in the value of wood strength properties confirms our assumption about the mosaic nature of the distribution of pine wood technical properties across the study area.

To conduct multivariate analysis for three basic study areas, a matrix of parameters affecting strength properties of pine wood was formed (Table 9): (*A*) crown projection diameter; (*B*) trunk diameter at the height of 1.3 m; (*C*) integral indicator of the annual ring width and content of latewood; (*D*) basic wood density; (*E*) distance to emission sources; (*F*) place of specimen collection inside the tree trunk. A valid result was obtained concerning a high degree of the influence exerted on strength properties of pine wood by the parameters of structural elements of the annual increment and basic wood density. We should note a slight decrease in the impact of the technogenic factor on the properties of pine wood from the northern taiga zone to the middle one. In the same direction, a tendency was observed towards an increase in the significance of unidentified factors (*Z*).

Table 8. Interval comparison of the dynamics of the wood resistance limit to compression along the fibers relative to changes in wood basic density.

	Intervals (kg·m^{-3})						
	320-350	350-380	380-410	410-440	440-470	470-500	500-530
350-380	1.19*	-	-	-	-	-	-
	2.65						
380-410	4.81	3.31	-	-	-	-	-
	7.23	2.73*					
410-440	8.11	5.74	1.78*	-	-	-	-
	3.54	1.34	2,04*				
440-470	11.58	8.64	4.37	1.97*	-	-	-
	4.35	3.13	3.08	1.51*			
470-500	8.69	7.18	4.13	2.98	0.81*	-	-
	3.26	3.23	2.22*	1.09*	1.39*		
500-530	8.40	7.25	4.69	3.71	1.89*	1.05*	-
	12.81	4.83	3.77	3.61	5.46	3.93	
530-560	11.79	10.32	7.48	6.64	4.73	3.44	2.11*
	29.93	11.28	4.14	8.45	12.75	9.19	2.34*

Note: * – difference is not valid at $p_{0.05}$; numerator – *t-test* ($t_{0.05}$=2.20; $t_{0.01}$=3.11); denominator – *F-test* ($F_{0.05}$=2.97; $F_{0.01}$=4.85).

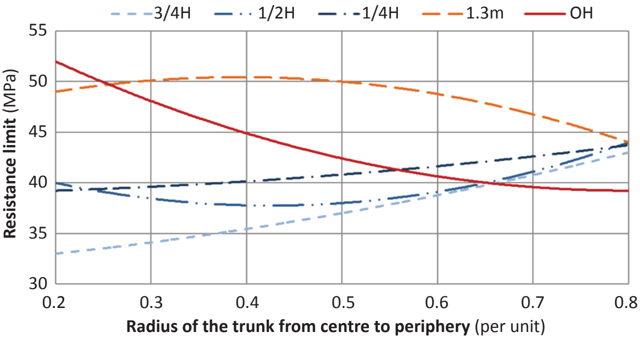


Figure 12. Dynamics of wood resistance to compression (MPa) along the trunk axis.

Table 9. Evaluation of the influence strength of various factors on pine wood resistance to compression along the fibers.

Value	Factors considered						Z	X (total)
	A	B	C	D	E	F		
				Arkhangelsk region				
S	25	21	57	68	29	35	234	469
v	5	5	5	5	5	5	119	149
σ^2	5.00	4.20	11.40	13.60	5.80	7.00	1.97	-
t-value	2.54	2.14	5.80	6.92	2.95	3.56	-	-
				Syktyvkar region				
S	27	22	59	64	21	23	249	465
v	5	5	5	5	5	5	119	149
σ^2	5.40	4.40	11.80	12.80	4.20	4.60	2.09	-
t-value	2.58	2.10	5.64	6.12	2.01	2.20	-	-
				Kotlas region				
S	23	25	66	58	17	29	252	470
v	5	5	5	5	5	5	119	149
σ^2	4.60	5.00	13.20	11.60	3.40	5.80	2.12	-
t-value	2.17	2.36	6.23	5.48	1.61	2.74	-	-

A – crown projection diameter; B – trunk diameter at the height of 1.3 m; C – integral indicator of the annual ring width and content of latewood; D – basic wood density; E – distance to emission sources; F – place of specimen collection inside the tree trunk, Z – random factors; *S* – variation sum; *v* – the number of degrees of freedom; *t* – conclusion validity (t_{table} at v_1=4 and v_2=v_z =79: $t_{0.10}$=2.78; $t_{0.05}$=4.60; $t_{0.01}$=8.61).

CONCLUSIONS

Based on the results of the study, the following conclusions can be drawn:

Basic density of Scots pine wood in mature forest stands in the European Northeast ranges from 430 to 510 kg·m^{-3}. The upper limit of the range corresponds to forest stands of the sphagnum group of forest types growing in conditions of the northern taiga in the northwestern part of the study area (Arkhangelsk region). The minimum values of basic wood density were obtained for lichen pine forests of the middle taiga in the southeastern part of the study area (Kotlas area). Fluctuations in basic density of pine wood within individual locations in the European Northeast are sketchy.

The variation value of basic wood density decreases as soil and hydrological conditions of growth improve. At the same time, for pine forests of the sphagnum group from northwest to southeast, the variation value decreases from 12.3% in the region of Arkhangelsk to 11.7% in Syktyvkar and 9.7% in Kotlas, with the exception of 7.2% in the region of Naryan-Mar, whereas in pleurocarpous moss pine forests it increases from 8.3% in the area of Arkhangelsk to 8.9% and 9.2% in the regions of Syktyvkar and Kotlas, respectively.

A significant negative correlation was found between the value of basic wood density and the distance to sources of emissions of harmful substances into the atmosphere in the areas where industrial enterprises are concentrated. For shrub-sphagnum pine forests growing in the region of the Arkhangelsk urban agglomeration, *r* is -0.745±0.092.

On the territory of the European Northeast, a slight fluctuation in strength properties of Scots pine wood was determined. The range of average values of the ultimate resistance of pine wood to compression along the fibers, regardless of growth type conditions, amounts to 44.9-49.5 MPa. This indicates that mechanical properties of Scots pine wood are a species-specific indicator with a low level of variation within the boundaries of individual locations.

Growth in the impact zone of atmospheric emissions from large industrial enterprises in the study area does not significantly affect the dynamics of wood strength properties, depending on growth conditions. The revealed negative correlation of strength properties and the degree of remoteness from sources of aerotechnogenic emissions is -0.382±0.112 for pine forest stands growing in the region of Arkhangelsk and -0.319±0.124 for the region of Kotlas, which is not significant.

The study established a significant effect of the size and structure of trunk annual growth in diameter and the position of the wood sample in terms of trunk volume on volumetric and strength properties of wood for Scots pine.

Author Contributions

RVS, DAD and SAK conceived and designed the research, RVS and SAK carried out the field measurements, RVS performed laboratory analysis, DAD and DAZ processed the data and performed the statistical analysis, RVS and SAK supervised the research and helped to draft the manuscript, VIM helped to draft the manuscript, DAD and DAZ wrote the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chemical and Energetic Properties of Seven Species of the Fabaceae Family

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ABSTRACT

In this work, the chemical compositions and energetic properties of the wood and bark of seven Fabaceae species were determined to evaluate their dendroenergetic potential. Chemical composition, elemental, proximate and heating value analyses were conducted. In addition, an ash microanalysis was performed. The obtained results varied as follows: cellulose (from 20.21% in *Parkinsonia aculeate* bark to 58.83% in *Albizia plurijuga* sapwood), hemicelluloses (from 8.81% in *Eysenhardia polystacya* heartwood to 23.71% in *Pakinsonia aculeate* wood), lignin (from 12.88% in wood to 26.53% in bark of *Parkinsonia aculeate*), extractives (from 11.68% in sapwood to 36.17% in bark of *Eysenhardia polystacya*), carbon (from 42.4% in *Albizia plurijuga* bark to 49.5% in *Eysenhardtia polystacya* heartwood), hydrogen (from 6.4% in *Eysenhardtia polystacya* bark to 7.3% in *Albizia plurijuga* sapwood), oxygen (from 42.3% in *Prosopis laevigata* bark to 50.5% in *Acacia pennatula* bark), nitrogen (from 0.11% in *Albizia plurijuga* heartwood to 1.64% in *Prosopis laevigata* bark), sulfur (from 0.04% in *Prosopis laevigata* heartwood to 0.14% in *Acacia farnesiana* wood, *Erythina caralloides* bark, and *Prosopis* laevigata bark), ash (from 0.76% in *Eysenhardtia polystacya* heartwood to 11.49% in *Acacia plurijuga* bark), volatile material (from 70.08% in *Eysenhardtia polystacya* bark to 91.75% in *Albizia plurijuga* sapwood), fixed carbon (from 6.97% in *Albizia plurijuga* sapwood to 23.44% in *Prosopis laevigata* bark), and calorific value (from 17.36 MJ·kg^{-1} in *Acacia pennatula* bark to 21.23 MJ·kg^{-1} in *Prosopis laevigata* bark). The most abundant chemical elements in wood ash and bark ash are listed here: Ca>K>P>Mg>Na. According to the obtained results, the wood and bark of the seven Fabaceae species could be used to produce solid biofuels for local use. Additionally, highlighting the high concentrations of extractives was important, especially in the bark samples, which could be a potential source of phytochemicals.

Keywords: wood; bark; ash microanalysis; ultimate analysis; proximal analysis; chemical composition; calorific value

INTRODUCTION

The family Fabaceae (Leguminosae) is in the order Fabales, which comprises trees, shrubs, and annual or perennial herbs. There are approximately 730 to 750 genera and 19,400 to 20,000 species, making it the third largest angiosperm plant family (Llamas and Acedo 2016, Stevens 2017). Many plants of this family are known because they serve as food for humans and domestic animals; others are used for ornamental and forage purposes. The genera *Acacia*, *Bahuinia*, *Erytrina*, or *Peltogyne* include species that have interesting wood due to their high flavonoid content (Llamas and Acedo 2016). Wood of other genera (e.g., *Prosopis*, *Ebenopsis*, *Acacia*, *Pakinsonia*, and *Eysenhardtia*) is used to make handicrafts, tool handles, saddles, construction materials, fence posts, charcoal, and firewood (Estrada-Castillón et al. 2005).

In Mexico, Fabaceae are the second most diverse group of plants, and their species are spread throughout the country (Sousa and Delgado 1993, Estrada-Castillón et al. 2005). In Lake Cuitzeo basin located in the state of Michoacán, Mexico, these species are abundant and develop in the vegetation of temperate forests, scrublands, and low deciduous forests; additionally, aquatic and underwater vegetation exists in this region (Bravo-Espinosa et al. 2008, Maza-Villalobos et al. 2014).

Seven species of the Fabaceae family (*Acacia farnesiana* (L.) Willd, *A. pennatula* (Schl. et Cham) Benth, *Albizia* *plurijuga* (Standl.) Britton & Rose, *Erythina caralloides* DC., *Eysenhardtia polystacya* (Ortega) Sarg., *Pakinsonia aculeate* L., and *Prosopis laevigata* (Humb. & Bonpl.) Jonhst.) stand out in Lake Cuitzeo basin; some research on them is available. For *Acacia farnesiana,* in a chemical study of its fruit, 23% protein and a high concentration of amino acids, such as histidine, valine, threonine, leucine and isoleucine, were found, and it was concluded that it is a resource potentially usable as a low-cost food option for sheep (Barrientos-Ramírez et al. 2012). A phytochemical study of its bark reports the presence of steroidal, terpene-type compounds, sulfur compounds and tannins (Daza-Bareño 2014) and a study of its wood for pulp and paper applications have been proposed (Ramírez-Casillas et al. 2019). These researchers report excellent bleachability in the cellulose pulp obtained through the ASAM process, in addition to the fact that this wood could be considered as raw material for obtaining dissolving grade cellulose. In relation to *Acacia pennatula* wood, data on energetic properties have been collected, such as calorific value (18.54 KJ·g^{-1}), volatile matter (86.56%), ash (1.07%) and fixed carbon (12.37%), and it has been concluded that this wood could be used for energy production (Apolinar-Hidalgo et al. 2017). For *Erythina caralloides* wood, the antimicrobial activities of its extracts have been studied, and the results obtained show the antimicrobial potential and justify its traditional use for the treatment of some diseases of bacterial or fungal origin (Mata-González 2015). For *Eysenhardtia polystacya* bark, a study reported six new flavoniods, and it was concluded that their antioxidant properties are a promising strategy to improve therapeutic effects and could alleviate diabetes complications (Pérez-Gutiérrez et al. 2016). Regarding *Pakinsonia aculeata*, the essential oils of the air-dried aerial parts were studied and their antimicrobial and antioxidant activities were tested. It has been concluded that the essential oils showed moderate antimicrobial effect against bacteria and fungi (Al-Youssef and Hassan 2015). Finally, for *Prosopis laevigata*, the following data on the chemical composition have been reported: holocellulose (61.5% to 64.7%), lignin (29.8% to 31.4%) and total extractives content (14% to 16%) (Carrillo et al. 2008). Likewise, the following data have been reported in wood and bark: calorific value 16.7 MJ·kg^{-1} and 15.0 MJ·kg^{-1}, and ash content 2.4% and 3.6%, respectively (Martínez-Pérez et al. 2015).

The wood of some of the Fabaceae species that grow in the region of Lake Cuitzeo—*Acacia farnesiana* and *Prosopis laevigata*—is used locally as fuel; the wood of the latter is used to make rustic furniture, doors, windows and floors. *Erythina caralloides* wood is used locally to make handicrafts. When wood is processed, the residue lignocellulosic biomass (e.g. bark, sawdust, chips and trimmings) is generated, which generally has no use and is usually deposited in the open air, which can cause environmental problems (Saval 2012).

The use of this type of biomass for energy purposes can help decrease carbon emissions and ensure environmental sustainability (Ferrandez-Villena et al. 2019, Reid et al. 2020). In addition, the growing demand for energy has led to the sustained use and depletion of fossil fuels; to maintain a sustainable and environmentally friendly energy level, renewable energy sources have been sought after (EIA 2022), including biomass. Therefore, it is relevant to determine the chemical and energetic properties of different biomasses, which can be used as energy sources. Recently, scholars on this topic have focused on studying lignocellulosic residues from some woods of the *Quercus* genus (Herrera-Fernández et al. 2017, Cárdenas-Gutiérrez et al. 2018) used for energy and of the *Pinus* genus (Pintor-Ibarra et al. 2017, Morales-Máximo et al. 2020, Rutiaga-Quiñones et al. 2020) used for wood. However, little information is available related to the wood species selected herein. Thus, for this work, seven Fabaceae species were chosen, which grow in the basin of Lake Cuitzeo, Michoacán. The objective is to determine the basic chemical compositional and energetic properties of their wood and bark to contribute to scientific knowledge and to determine the species' viability as biofuels.

MATERIALS AND METHODS

Collection Area and Preparation of Study Materials

The lignocellulosic materials were collected in the region of Lake Cuitzeo in the State of Michoacán, Mexico. In the Lake Cuitzeo basin, the average annual temperature and pluvial precipitation are 20°C to 22°C and 890 mm, respectively (Carlón-Allende and Mendoza 2007). The names of the tree species and some general data are shown in Table 1, and three individuals were collected from each species. From each tree, at a height of 1.30 m from the ground, a slice 10 cm in length was procured. Subsequently, wood and bark were separated, and when possible, the wood was separated into sapwood and heartwood. The chips obtained manually using a knife were air dried in the shade to a moisture content of approximately 12%. Finally, the material was ground in a mill (Model K20F, series 236, Micron S.A. de C.V., Mexico City, Mexico) and sieved in a Ro-Tap machine (Model RX-29, W.S. Tyler, Mentor, OH, USA); 40-mesh (425 μm) wood meal was used for chemical and energetic characterization. The 40 mesh fraction was the one that passed the same sieve and remained at the 60 mesh.

Basic Chemical Compositions

Cellulose, hemicelluloses and lignin contents were determined once using α-amylase in fibre analysis equipment (ANKOM Fibre Analyser, model AMKON200, ANKOM Technology, Macedon, New York, USA) according to the methodology described by Van Soest et al. (1991). The extractives content was determined according to the differences and by ash correction.

Ultimate Analysis

Carbon, hydrogen and nitrogen contents were determined once by the modified Dumas method using Perkin-Elmer, Model 2400 CHNS-O analyzer (Rotz and Giazzi 2012), and sulfur quantification was performed by the turbidimetric method with gum arabic. Oxygen content was calculated by the differences.

Proximate Analysis

For each dry lignocellulosic sample, the ash percentage was determined in triplicate based on UNE-EN ISO 18122 (2016), and the volatile material content was determined according to ASTM E872-82 (2013). Fixed carbon was calculated by the differences. The mean value and standard deviation were reported.

Table 1. Geographical location and general information on the seven Fabaceae species.

Scientific name	Common name	Coordinates	Diameter at 1.3 m (cm)	Total height (m)	Altitude (m)
Acacia farnesiana	Huizache	Tree 1: 19°57´49´´N 101°6´21’’W	10	3.40	1,870
		Tree 2: 19°57´50´´N 101°6´23´´W	14	2.80	1,880
		Tree 3: 19°58´29´´N 101°9´20´´W	16	4.20	1,840
A. pennatula	Tepame	Tree 1: 19°58´53´´N 101°7´16´´W	20	5.10	1,840
		Tree 2: 19°57´53´´N 101°6´19´´W	21	3.80	1,880
		Tree 3: 19°58´32´´N 101°5´47´´W	13	4.30	1,860
Albizia plurijuga	Palo blanco	Tree 1: 19°58´34´´N 101°5´40´´W	22	8.30	1,860
		Tree 2: 19°58´28´´N 101°15´51´´W	34	9.10	1,860
		Tree 3: 19°59´3´´N 101°8´45´´W	36	10.50	1,920
Erythina caralloides	Colorin	Tree 1:19°58´8´´N 101°6´28´´W	14	3.50	1,910
		Tree 2: 19°58´7´´N 101°6´24´´W	12	5.40	1,910
		Tree 3: 19°58´19´´N 101°6´3´´W	13	3.70	1,920
Eysenhardtia polystacya	Palo dulce	Tree 1: 19°58´32´´N 101°5´45´´W	16	5.30	1,860
		Tree 2: 19°58´38´´N 101°5´42´´W	15	4.80	1,850
		Tree 3: 19°58´13´´N 101°6´11´´W	24	4.60	1,930
Pakinsonia aculeate	Palo verde	Tree 1: 19°58´31´´N 101°8´55´´W	14	5.30	1,840
		Tree 2: 19° 58´27´´N 101°8´45´´W	21	6.10	1,840
		Tree 3: 19°58´13´´N 101°8´50´´W	10	3.60	1,840
Prosopis laevigata	Mezquite	Tree 1: 19°58´51´´N 101°7´15´´W	76	9.10	1,850
		Tree 2: 19°58´17´´N 101°7´30´´W	82	9.80	1,880
		Tree 3: 19°58´21´´N 101°7´28´´W	71	7.40	1,880

Calorific Value

The high heating value was determined in triplicate in a LECO calorimetric pump (LECO AC 600, LECCO Corporation, St. Joseph, USA) based on the UNE-EN ISO 18125 (2018) standard. The mean value and standard deviation were reported.

Ash Microanalysis

Ash microanalysis was determined once by using inductively coupled plasma optical emission spectrophotometer (ICP–AES) (VARIAN 730-ES, Varian Inc., (Agilent), Mulgrave, Australia) according to the procedure described by Arcibar-Orozco et al. (2014).

RESULTS AND DISCUSSION

Basic Chemical Composition

Table 2 features a summary of the results of the basic chemical analysis. The cellulose results ranged from 20.21% (*Parkinsonia aculeate* bark) to 58.83% (*Albizia plurijuga* sapwood); the values were in general agreement with data reported in the literature, e.g. 23.8% for *Fagus sylvatica* L. bark and 56.2% for *Cayra tomentosa* Sarg. wood (Fengel and Wegener 1984). Higher cellulose concentrations were observed in wood than in bark; these results were in general agreement with previous reports for hardwoods (Fengel and Wegener 1984, Honorato-Salazar and Hernández-

Pérez 1998, Bautista-Hernández and Honorato-Salazar 2005, Herrera-Hernández et al. 2017). Specifically, for *Acacia farnesiana* wood, 51.48% cellulose was reported (Ramírez-Casillas et al. 2019); this value is close to the one found herein. Conversely, 45.7% cellulose was found in the heartwood of *Prosopis leavigata* (Carrillo et al. (2008); this value is close to the one found herein.

For hemicelluloses, the results ranged from 8.81% (*Eysenhardia polystacya* heartwood) to 23.71% (*Pakinsonia aculeate* wood) (Table 2). The obtained results were generally close to values reported for some hardwood species: wood (from 21.2% to 36.0%), bark (from 9.3% to 23.1%) (Fengel and Wegener 1984), heartwood (from 12.88% to 24.38%) and sapwood (from 11.75% to 19.82%) (Ruiz-Aquino et al. 2019). Specifically, for *Acacia farnesiana* wood, Ramírez-Casillas et al. (2019) found 11.4% hemicelluloses, and Carrillo et al. (2008) reported 15.1% hemicelluloses in the heartwood of *Prosopis laevigata*; in both cases, the reported values were near the values obtained herein.

Regarding lignin content, the results ranged from 12.88% (wood) to 26.53% (bark) for *Parkinsonia aculeate* (Table 2). Lignin concentration is higher in bark than in wood (Sjöström 1981, Fengel and Wegener 1984), and this trend was generally observed in the results obtained in this research. Previous studies on *Acacia farnesiana* wood reported 17.40% lignin (Ramírez-Casillas et al. 2019), and for the heartwood of *Prosopis laevigata,* 29.8% lignin was reported (Carrillo et al. 2008); in both cases, the reported values were higher than those found herein, which could be due to the extraction method applied.

The extractives content ranged from 11.68% (sapwood) to 36.17% (bark) in *Eysenhardia polystacya* (Table 2). Clearly, a higher concentration of extractives was observed in bark than in wood; this finding agreed with literature data (Hillis 1971, Sjöström 1981, Fengel and Wegener 1984). Additionally, the extractives content was higher in heartwood than in sapwood, which agreed with previous reports (Fengel and Wegener 1984, Bautista-Hernández and Honorato-Salazar 2005, Herrera-Hernández et al. 2017). The bark extractives values obtained in this work were within the range reported for different wood species: from 20% to 40% (Sjöström 1981). Specifically, for the heartwood of *Prosopis laevigata*, extractives contents of 14.1% to 16.0% were found (Carrillo et al. 2008); these values were close to those obtained herein.

Due to the relatively high values of polysaccharides and the relatively low values of lignin (Table 2), the woods studied could be advantageous for the pulp and paper industry or for obtaining high-yield pulps (Casey 1990), in order to seek different applications in the field of nanocellulose. Regarding the bark, due to its high extractive content (Table 2), it could have potential in the search for chemical applications with different purposes (Fengel and Wegener 1984).

Table 2. Basic chemical compositions of lignocellulosic materials by species and samples.

Id	Species	Sample	Cellulose	Hemicelluloses	Lignin	Extractives
			(%)			
1	*Acacia farnesiana*	Wood	52.86	16.26	14.30	14.21
2		Bark	36.09	11.01	19.23	24.73
3	*A. pennatula*	Wood	45.58	20.85	13.39	17.29
4		Bark	37.03	11.98	16.95	23.00
5	*Albizia plurijuga*	Heartwood	49.07	19.83	10.60	19.43
6		Sapwood	58.83	12.83	13.67	13.44
7		Bark	29.26	17.69	13.12	28.44
8	*Erythina caralloides*	Wood	47.09	9.41	22.66	17.02
9		Bark	23.51	11.54	21.73	34.19
10	*Eysenhardtia polystacya*	Heartwood	48.97	8.81	20.55	20.91
11		Sapwood	53.05	12.34	21.63	11.68
12		Bark	22.34	10.20	21.25	36.17
13	*Pakinsonia aculeate*	Wood	37.41	23.71	12.88	24.27
14		Bark	20.21	15.47	26.53	27.13
15	*Prosopis leavigata*	Heartwood	45.35	14.09	22.14	16.72
16		Sapwood	41.32	18.50	23.39	14.93
17		Bark	23.92	9.70	25.57	34.96

Ultimate Analysis

Table 3 shows the results of the elemental analysis. The values found varied as follows: carbon (C) from 42.4% (*Albizia plurijuga* bark) to 49.5% (*Eysenhardtia polystacya* heartwood), hydrogen (H) from 6.4% (*Eysenhardtia polystacya* bark) to 7.3% (*Albizia plurijuga* sapwood), oxygen (O) from 42.3% (*Prosopis laevigata* bark) to 50. 5% (*Acacia pennatula* bark), nitrogen (N) from 0.11% (*Albizia plurijuga* heartwood) to 1.64% (*Prosopis laevigata* bark), and sulfur (S) from 0.04% (*Prosopis laevigata* heartwood) to 0.14% (*Acacia farnesiana* wood, *Erythina caralloides* bark, *Prosopis laevigata* bark). The average values for wood/xylem (bark) were as follows: carbon 47.2% (45.9%), hydrogen 7.1% (6.7%), oxygen 45.4% (46.2%), nitrogen 0.26% (1.13%), and sulfur 0.07% (0.09%). In general, the obtained results were close to the values reported for some hardwood species and other lignocellulosic biomasses (Vassilev et al. 2010, García et al. 2012, UNE-EN ISO 17225-2 2014, Rutiaga-Quiñones et al. 2020).

Bark samples contained higher concentrations of nitrogen, which could limit their use as solid biofuels due to the environmental problems of biomass combustion (Demirbaş 2005, Obernberger et al. 2006). Analysed samples with nitrogen concentrations of ≤0.5% could be used to make class A2 pellets, and those with concentrations of ≤1.0% could be used to make class B pellets (ENplus 2015). Conversely, the analysed samples had low sulfur contents (<1.0%), which was favorable because this chemical element could damage human health and foul combustion equipment (Obernberger et al. 2006, García et al. 2012). Low concentrations of these two chemical elements would be desirable in biomasses for combustion (Hartmann 2012, UNE-EN ISO 17225-2 2014).

For the C/N ratio, the results ranged from 30 to 435 (Table 3); this range was within the reported span (24 for lime leaves to 5,025 for pine sawdust) for various biomasses (Rutiaga-Quiñones et al. 2020). For fermentation processes, low biomass values (between 20 and 30) were adequate, since high values indicated low nitrogen availability (Velázquez-Martí 2018); thus, only the bark studied herein could be used for this purpose.

Proximate Analysis

The results of the proximal analysis are shown in Table 4. The lowest amount of ash (0.76%) was found in the *Eysenhardtia polystacya* heartwood sample, while the highest concentration (11.49%) was in the *Acacia plurijuga* bark sample. Clearly, the bark samples contained more inorganic substances than the wood samples, which was in agreement with previous reports (Fengel and Wegener 1984, Martínez-Pérez et al. 2015). The concentration of ash was higher in sapwood than in heartwood, and this trend coincided with other investigations in different woods (Rutiaga-Quiñones 2001, Ávila-Calderón and Rutiaga-Quiñones et al. 2014).

Ash evaluation is an important parameter for determining the qualities of solid biofuels (Demirbaş and

Table 3. Ultimate analysis and C/N ratio by species and samples.

Id	Species	Sample	C	H	O	N	S	C/N
			(%)					
1	*Acacia farnesiana*	Wood	46.5	6.8	46.2	0.32	0.14	145
2		Bark	46.8	6.5	45.5	1.16	0.08	40
3	*A. pennatula*	Wood	45.9	7.1	46.7	0.21	0.08	219
4		Bark	42.5	6.4	50.5	0.58	0.07	73
5	*Albizia plurijuga*	Heartwood	47.9	7.2	44.7	0.11	0.06	435
6		Sapwood	46.5	7.3	46.0	0.15	0.06	310
7		Bark	42.4	6.6	50.0	0.96	0.06	44
8	*Erythina caralloides*	Wood	46.3	6.9	46.3	0.41	0.08	113
9		Bark	48.7	7.2	43.1	0.91	0.14	54
10	*Eysenhardtia polystacya*	Heartwood	49.5	7.1	43.2	0.14	0.05	354
11		Sapwood	47.4	7.2	45.0	0.29	0.07	163
12		Bark	45.6	6.4	46.4	1.52	0.08	30
13	*Pakinsonia aculeate*	Wood	46.2	7.2	46.1	0.41	0.05	113
14		Bark	46.5	6.8	45.5	1.12	0.12	42
15	*Prosopis leavigata*	Heartwood	48.7	6.9	44.2	0.21	0.04	232
16		Sapwood	46.9	7.1	45.6	0.34	0.06	138
17		Bark	49.1	6.8	42.3	1.64	0.14	30

Demirbaş 2004). High concentrations negatively affected calorific value (Martínez-Pérez et al. 2012, Martínez-Pérez et al. 2015, Ngangyo-Heya et al. 2016, Carrillo-Parra et al. 2018) and caused problems in combustion and emission of polluting particles into the environment (Obernberger and Thek 2006, Tumuluru et al. 2010, Werkelin et al. 2011). The lignocellulosic samples analysed, with ash contents ≤2.0% (Table 4), could be used to produce class B pellets for marketing or local application purposes, according to international standards (ENplus 2015).

Regarding volatile material, the obtained results varied from 70.08% for *Eysenhardtia polystacya* bark to 91.75% for *Albizia plurijuga* sapwood (Table 4). Except for *Erythina caralloides*, less volatile matter was found in the bark samples, and more was found in sapwood than in heartwood (Table 4). The obtained results were generally in agreement with previous reports for different lignocellulosic biomasses (Vassilev et al. 2010, García et al. 2012, Rutiaga-Quiñones et al. 2020). Specifically, the result obtained for *Acacia pennatula* wood was close to the reported value (86.56%) for the same species (Apolinar-Hidalgo et al. 2017). Considering that biomass with a high concentration of volatile matter was suitable for thermochemical conversion, such as biogas or pyrolysis (Holt et al. 2006), the samples studied could be a source of biofuels derived from these processes.

Table 4 shows the results of the fixed carbon content, which varied from 6.97% for sapwood (*Albizia plurijuga*) to 23.44% for bark (*Prosopis laevigata*), and they were in the range reported for different biomasses (Vassilev et al. 2010, García et al. 2012, Rutiaga-Quiñones et al. 2020). Except for *Albizia plurijuga* and *Erythina caralloides*, the bark contained more fixed carbon than the wood (xylem). In particular, Apolinar-Hidalgo et al. (2017) reported 12.37% fixed carbon for *Acacia pennatula* wood; this value was similar to that found herein for the same species.

Calorific Value

The results of the high heating value (HHV) are shown in Table 4. The calorific values were obtained for each material analysed by averaging, and they were ordered from lowest to highest: 18.7 MJ·kg^{-1} (bark), 19.2 MJ·kg^{-1} (wood), 19.9 MJ·kg^{-1} (sapwood), and 20.3 MJ·kg^{-1} (heartwood). The calorific value obtained for the wood samples was at the lower limit of the reported range (19.5 MJ·kg^{-1} to 20.0 MJ·kg^{-1}) for hardwoods (UNE-EN-14961-1 2011). Except for the bark of *Prosopis laevigata*, the obtained results were within the reported range (15.0 MJ·kg^{-1} to 18.9 MJ·kg^{-1}) for bark of different hardwoods (Martínez-Pérez et al. 2015). The results of the sapwood and heartwood samples were similar to the values reported for some hardwoods (Ruiz-Aquino et al. 2019). The calorific values of the heartwood samples were slightly higher than those of the sapwood samples, which coincided with previous research (Martínez-Pérez et al. 2015, Ruiz-Aquino et al. 2019). Finally, the calorific value results obtained herein were within the reported range (17.1 MJ·kg^{-1} to 23.0 MJ·kg^{-1}) for wood in general (FAO 1991).

Table 4. Results of the proximate analysis and high heating value (HHV) by species and samples.

Id	Species	Sample	Ash (%)	Volatile matter (%)	Fixed carbon (%)	HHV (MJ·kg^{-1})
1	*Acacia farnesiana*	Wood	2.37	83.29	14.34	19.17
2		Bark	8.94	72.15	18.94	18.72
3	*A. pennatula*	Wood	2.89	85.08	12.01	18.32
4		Bark	11.04	71.95	17.00	17.36
5	*Albizia plurijuga*	Heartwood	1.07	86.59	12.32	20.33
6		Sapwood	1.23	91.75	6.97	20.01
7		Bark	11.49	78.76	9.77	18.03
8	*Erythina caralloides*	Wood	3.82	78.35	17.84	19.39
9		Bark	9.03	78.47	12.47	18.02
10	*Eysenhardtia polystacya*	Heartwood	0.76	81.76	17.47	20.53
11		Sapwood	1.30	85.71	12.98	19.95
12		Bark	10.04	70.08	19.87	17.86
13	*Pakinsonia aculeate*	Wood	1.73	85.62	12.63	19.83
14		Bark	10.66	74.24	15.07	19.63
15	*Prosopis leavigata*	Heartwood	1.70	78.46	19.83	20.16
16		Sapwood	1.86	81.49	16.62	19.81
17		Bark	5.85	70.72	23.44	21.23

Table 5. Ash microanalysis results.

Id	Al	B	Ba	Ca	Cr	Cu	Fe	K	Li	Mg	Mn	Na	Ni	P	Pb	Si	Sn	Sr	V	Zn
	(ppm)																			
1	29.69	86.07	352.01	5,857.73	ND	28.14	165.58	17,816.19	20.15	1,741.02	27.95	1,112.67	3.26	4,931.83	ND	13.46	ND	455.12	ND	15.88
2	106.51	55.94	145.80	1,148.42	ND	10.52	35.35	4,819.37	12.67	355.89	10.54	288.11	1.48	1,408.56	ND	142.96	0.26	120.63	0.13	10.77
3	10.45	45.34	245.41	3,608.25	ND	18.83	21.95	12,151.00	24.98	1,233.74	25.59	975.55	35.92	3,131.79	ND	ND	ND	329.02	ND	15.62
4	32.15	53.28	136.38	995.38	ND	6.92	ND	4,618.75	19.39	347.39	9.75	173.17	12.09	1,415.00	ND	48.48	0.12	115.94	ND	9.23
5	34.14	345.07	116.19	5,808.86	ND	39.72	86.76	19,958.07	137.36	2,075.61	11.92	2,773.95	13.55	340.06	ND	93.20	ND	517.85	ND	7.51
6	59.97	114.08	123.87	8,341.36	ND	30.83	11.51	23,627.18	59.55	2,442.03	47.34	2,076.00	4,094.75	5,393.37	ND	51.94	ND	600.06	ND	45.76
7	18.81	44.87	122.69	786.60	ND	3.53	ND	3,911.98	6.52	316.04	24.38	260.89	0.47	1,076.34	ND	7.43	0.16	104.66	ND	6.61
8	15.25	54.69	191.80	3,538.31	ND	23.70	ND	9,697.37	24.06	915.15	81.39	736.64	52.10	5,540.06	ND	456.42	ND	259.82	ND	29.33
9	27.18	72.23	143.92	1,179.51	0.18	10.91	1.11	4,802.26	25.69	334.18	70.02	265.17	127.39	1,546.80	ND	205.24	0.20	129.15	ND	21.44
10	52.07	262.23	886.69	11,972.38	ND	292.09	ND	26,465.17	13.19	3,414.15	134.04	601.81	177.20	637.67	ND	77.73	ND	861.95	ND	123.31
11	42.64	162.86	406.16	6,107.08	ND	85.84	0.41	17,414.83	153.77	1,720.43	124.44	1,856.34	8.52	11,380.60	ND	30.34	ND	447.32	ND	102.78
12	87.38	102.96	172.74	1,473.15	ND	26.72	7.04	5,679.15	22.92	486.36	35.26	302.65	1.13	1,807.69	ND	95.17	0.25	151.75	0.10	45.95
13	7.31	84.78	129.07	6,884.34	ND	34.02	ND	20,791.99	127.23	2,038.47	72.06	4,160.62	156.10	7,164.49	ND	251.43	1.00	503.97	ND	199.13
14	284.94	95.80	170.34	5,934.58	ND	15.10	1.30	19,482.88	5.38	2,042.74	48.40	1,822.37	118.08	1,453.56	0.32	616.68	5.04	512.88	0.09	34.38
15	154.87	60.27	100.90	6,933.19	ND	28.13	ND	20,249.06	59.68	648.53	16.33	2,818.17	0.02	349.69	ND	ND	0.18	523.49	ND	12.19
16	12.88	52.62	83.89	5,938.52	ND	31.66	ND	16,946.85	6.28	1,468.00	59.77	1,985.28	0.96	3,555.77	ND	ND	11.42	444.57	ND	25.03
17	83.02	109.07	116.78	2,204.21	ND	13.57	ND	7,611.24	12.01	526.32	56.53	498.99	24.14	636.42	ND	91.57	ND	192.89	0.11	10.97

ND = not detected.

Ash Microanalysis

Twenty inorganic elements were identified in the biomass ash of the seven Fabaceae species (Tables 5). The UNE-EN 14961-1 (2011) standard mentions that the most prevalent minerals in ash are aluminium, calcium, iron, potassium, magnesium, manganese, sodium, phosphorous, and silicon; these previous minerals were detected in the wood and in the bark of the seven species studied herein. The results obtained herein indicated that the most abundant elements in wood ash and bark ash of the seven Fabaceae species, from highest to lowest concentration, were calcium > potassium > phosphorous > magnesium > sodium. These minerals were found in a higher proportion in wood ash than in bark ash (Table 5). Some scholars have indicated that calcium, potassium, phosphorous, and magnesium were the main chemical elements in wood (Fengel and Wegener 1984, Ngangyo-Heya et al. 2016, Ruiz-Aquino et al. 2020, Rutiaga-Quiñones et al. 2020), agreeing with what was found herein. Conversely, the results obtained herein showed that calcium and potassium were the most abundant elements in the bark, coinciding with Sjöström (1981). Higher concentrations of potassium, phosphorus and magnesium were observed in the sapwood than in the bark, which was consistent with previous reports for some woods (Rowell 2005, Ávila-Calderón and Rutiaga-Quiñones 2014). Boron, copper, manganese, silicon, and zinc could be found (Sjöström 1981, Fengel and Wegener 1984, Rutiaga-Quiñones et al. 2020); in this work, these elements were detected in the wood and in the bark. In another ash microanalysis, calcium, potassium, magnesium, phosphorus, silicon, and aluminium were detected in the wood and bark of *Prosopis laevigata* (Martínez-Pérez et al. 2015).

The UNE-EN 14961-1 (2011) standard indicates that the chemical elements present in a lower proportion in the ash are: astatine, cadmium, chromium, cobalt, copper, mercury, nickel, lead, vanadium, and zinc. In this work, astatine, cadmium, cobalt, and mercury were not detected, and only chromium and lead (Table 5) were identified in low concentrations in the barks of *Erythina caralloides* and *Pakinsonia acuelate*, respectively. The microanalysis results detected strontium and barium and, to lesser extents, lithium and tin in some samples; another study with Mexican woods reported the presence of barium and lithium (Rutiaga-Quiñones et al. 2020).

Calcium, potassium, phosphorus and magnesium, which were found in greater proportion in this research, were important, since they could limit the applications of these biomasses as solid biofuels; according to various scholars, these minerals could challenge the melting point of ash and cause slag, corrosion, fine particle emission and scale formation in furnaces and boilers (Obernberger and Thek 2004, Van Lith et al. 2006, Obernberger and Thek 2010, Telmo et al. 2010). Calcium and magnesium could be favorable in combustion because they increase the melting point of the ash, reduce its amount in the combustion equipment and favor the safety of residues when dispersed in the environment (Van Lith et al. 2006). Finally, sodium, iron, and silicon could cause ash melting, scale, and corrosion problems (Obernberger and Thek 2004, Obernberger and Thek 2010).

CONCLUSIONS

The chemical compositions and energetic properties of wood and bark of seven Fabaceae species were determined to evaluate their applicability as biofuels. This study provides valuable insights into the wood and bark chemical composition shedding light on its potential applications in different wood industries and its suitability for various purposes. It is important to highlight that the bark samples have high concentration of extractives; thus, they could be an important source of phytochemicals. Due to the nitrogen content, the wood of the studied species could be used to produce class A2 pellets. By considering the ash concentration, the wood of the studied species with a value ≤2.0% could be used to make class B pellets. The sulfur content was relatively low, which would not limit the use of these materials to produce solid biofuels. The studied bark samples could be useful in fermentation processes due to their low C/N ratios. A high content of volatile material was found in the studied samples; thus, they could be suitable for thermochemical conversion. The microanalysis of the ash revealed the typical presence of inorganic substances. In general, no heavy chemical elements were detected. Based on the obtained results regarding the wood and bark of the seven Fabaceae species, solid biofuels could be made and used locally.

Author Contributions

FSH, LFPI, JGRQ conceived and designed the research, FSH, LFPI and NGO carried out the field measurements, FSH, RM, CANB performed laboratory analysis, FSH and JJAF processed the data, JGRQ secured the research funding, supervised the research and helped to draft the manuscript, JGRQ, JJAF and FSH wrote the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Study of the Effects of Polypropylene Tree Shelters and Hydrophilic Polymers on Growth, Survival, Health and Physiological Condition of Pedunculate Oak Seedlings (*Quercus robur* L.)

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ABSTRACT

This paper presents the results of eight years of scientific research on the effect of polypropylene shelters (Tully tubes) and hydrophilic polymers on growth, survival, health and physiological condition of pedunculate oak seedlings (*Quercus robur* L.). The experiment was established in 2014 on relative forest soil, on partially forested land in subcompartment 35a, forest management unit Kragujna, which is managed by the Forest Administration Vinkovci, Forest Office Županja. In the autumn of 2014, one-year-old bare-rooted pedunculate oak seedlings were planted as a randomized block design experiment - four blocks with four repetitions. Four types of planting were tested: seedlings without a polypropylene shelter, without the addition of Zeba hydrophilic polymer granules (S variant), seedlings without a polypropylene shelter, with the addition of Zeba hydrophilic polymer granules (SP variant), seedlings with a polypropylene shelter, without the addition of Zeba hydrophilic polymer granules (SS variant), and seedlings with a polypropylene shelter, with the addition of Zeba hydrophilic polymer granules (SSP variant). The experiment was established as a result of considering the long-term problem of difficulties in natural regeneration. Disturbances in natural regeneration are certainly caused by increasing climate changes (floods, long-term droughts, storms, hailstorms, etc.) and other unfavourable biotic and abiotic factors that cause a significant reduction in quantities and/or an almost complete absence of pedunculate oak acorn yield. Due to the aforementioned circumstances, in the very near future we will be forced to resort more often to different forms of artificial forest regeneration, such as regeneration by planting sheltered seedlings that are less exposed to risks and challenges during survival, and which have greater competitiveness in relation to other vegetation on the regeneration surface (higher growth). Nowadays, this method of regeneration is used mostly for filling in places where, for various reasons, natural regeneration has been unsuccessful on several occasions and over a long period of time, and/or where there are great difficulties in carrying out natural regeneration (floods, areas damaged by fires, game damages and competition of weeds). This paper presents the results of scientific research after five periodic measurements, i.e. after the first, second, fifth, sixth and eighth growing season. The results show that even after the eighth growing season, seedlings protected with a polypropylene shelter have greater survival rate and greater height growth than unprotected seedlings. The highest survival rate was found in seedlings protected with a polypropylene shelter, with the addition of Zeba hydrophilic polymer of 82.14% (SSP variant), while unprotected seedlings with the addition of Zeba hydrophilic polymer (SP variant) had the lowest survival rate of 62.63%. The highest growth was recorded on seedlings protected with a polypropylene shelter, with the addition of Zeba hydrophilic polymer (SSP variant) amounting to 202.75 cm, while on average the smallest seedlings were those without polypropylene shelter protection and without the addition of Zeba hydrophilic polymer (S variant), amounting to 129.02 cm.

Keywords: forest regeneration; polypropylene shelter; Zeba hydrophilic polymer granules; seedlings; height growth; survival; pedunculate oak

INTRODUCTION

Degradation of pedunculate oak lowland forests, i.e. the dieback of pedunculate oak (*Quercus robur* L.) and narrow-leaved ash (*Fraxinus excelsior* Vahl.) as a result of climate change and the influence of various biotic and abiotic factors that cause variability in yield, the yield reduction or almost complete absence of pedunculate oak acorn yield (Gradečki-Poštenjak et al. 2011) would as a result certainly require a more or less intense artificial forest regeneration by planting seedlings, in exchange of natural regeneration, which is still prevailing today. Furthermore, increasing climate changes with their various extreme weather events (floods, long-term droughts, storms, hailstorms, etc.) and calamities of autochthonous and especially invasive species will force us to rationalize the management of collected seeds (acorns) to a much greater extent (Garcia-Barreda et al. 2023). This implies a reduction in the required amount of seeds per regeneration area, and thus a lower need for work force to perform these works (Liović et al 2013). Planting seedlings without a polypropylene shelter (hereinafter: shelter) is financially more expensive than planting seedlings with a shelter (Liović et al. 2013) since it requires a larger number of seedlings per area, as well as more frequent and extensive cultivation work to care for seedlings after planting. In 1979, English forester Graham Tuley introduced the first shelters for seedling protection (Tuley 1983, Tuley 1985). The advantages of shelters are rapid growth and height growth and better seedling survival, which have been reported by numerous authors (Potter 1988, Bainbridge 1991, Kerr 1992, Lantagne 1995, Liović 1997, Conner et al 2000, Liović et al 2001, Liović et al 2013, Liović et al 2019, Benko 2020). The use of shelters positively affects growth and survival as well as transplant stress that usually occur after seedlings is moved from the nursery to the forest habitat, a condition also known as "transplant shock". Such a condition is associated with the loss of a significant part of the root system when taking out seedlings, as well as stress caused by insufficient moisture and nutrients, which can often lead to higher mortality in the first growing season (Rietveld 1989, Struve and Joly 1992, Jacobs et al. 2005). Also, dense weeds quickly overgrow the seedlings, overshade them, and thereby affect the process of photosynthesis, which affects their survival and height growth (Myers 1988). Oak seedlings are particularly sensitive to competition from weeds, shrubs and other competitive species that reduce available sunlight, moisture and nutrients in the soil (Matić 1996). Pedunculate oak seedlings protected with a shelter achieved a three times higher average height compared to seedlings without shelter protection, and such height growth would enable them to quickly leave the zone of competition with weeds and achieve better survival rate (Liović et al. 2019). In addition to protection from weeds, shelters provide shade for the seedlings, i.e. reduce solar radiation, slow down the evaporation of moisture, protect seedlings from sudden changes in temperature, and increase the humidity inside the shelter by condensing moisture on the walls (Kjelgren and Rupp 1997, Del Campo et al. 2006, Bergez and Dupraz 2009, Ghazian et al. 2020). Also, game has a detrimental effect on the growth of seedlings because animals bite off the end buds and thus reduce the seedlings competitiveness in relation to the weeds that surround them (Jeffrey 1995, Gill 2001, Watt et al. 2003). The influence of game on the natural regeneration in pedunculate oak stands during shelterwood cutting was research by Krejči et al. (1997), Krejči et al. (2001), Krejči and Dubravac (2004). The use of shelters also reduces the attack of powdery mildew on oak (*Microsphaerae alphitoides* Griff et Maubl) because of the specific climate inside the shelter (Liović 1997, Liović et al. 2019, Benko 2020).

Although shelters have been in use for more than 40 years, disadvantages such as necessity of removing the old shelters after usage and not using biodegradable materials for their construction are the reasons why public may not support these modern forestry technologies (Graf 2022). Since European environmental regulations on plastics are becoming stricter, it will be necessary to use environmentally friendly, biodegradable shelters (European Commission 2021).

The main goal of this study is to determine whether and how the application of polypropylene tree shelters and the addition of hydrophilic granules (polymers) affect the growth, survival rate and overall health of planted pedunculate oak seedlings for the purpose of restoring the oak stand.

Although, the importance and applicability of the results will become more pronounced in the upcoming inevitable and increasingly frequent extreme climate changes In addition to climate changes, numerous biotic and abiotic factors also contribute to the ever-increasing pressure on forest ecosystems, which, with their unfavourable effects, also affects the (ir)regularity of the acorn yield, thus impeding the natural regeneration of our most valuable stands of pedunculate oak as well as sessile oak (Harris RMB et al. 2018).

MATERIALS AND METHODS

The experiment is a continuation of a multi-annual scientific research, whose results of monitoring survival and height growth were published in scientific journal SEEFOR (Liović et al. 2019).

Experimental area is located in the lowland continental part of the Republic of Croatia and according to the Köppen climate classification it belongs to the Cfwbx climate. The average annual air temperature is 12.4°C, and the average annual air temperature during the growing season is 18.9°C. The average annual level of precipitation is 686 mm, while the average annual level of precipitation during the growing season is 382 mm.

The experiment was established on relative forest soil, partially forested only with scarce shrubby and grassy vegetation, in Forest Administration Vinkovci, Forest Office Županja, forest management unit Kragujna 35a. The area of the subcompartment is 2.77 ha and the altitude is 80–83 m (from the forest management plan for forest management unit Kragujna). The experimental area is a light zone along the forest road, and next to the road there is also a canal with an additionally widened belt for the purpose of its maintenance. The experimental plots were located on the edge of the micro-depression area along the edge of old forest which was naturally regenerated by shelterwood cutting and it belonged to the forest community of pedunculate oak and common hornbeam (*Carpino betuli - Quercetum roboris* Rauš 1969).

In the autumn of 2014, one-year-old bare-rooted pedunculate oak seedlings were planted: without shelter protection and without the addition of polymers (hereinafter: S variant), without shelter protection and with the addition of polymers (hereinafter: SP variant), with shelter protection and without the addition of polymers (hereinafter: SS variant) and with shelter protection and with the addition of polymers (hereinafter: SSP variant). The scheme of the experiment is shown in detail in Figure 2.

To protect the seedlings, 1.2-m-high double-layer polypropylene shelters resistant to UV rays were bolted by a wooden stake and attached with two plastic ties. When planting the SP variant and the SSP variant, polymer was added to the soil in the amount of 4 g per planting hole. A polymer that was used is based on corn starch that can absorb water up to 500 times its volume, creating a hydrogel around the roots of the plant, thus retaining and if necessary releasing the water for the plant. Due to their composition (glucose molecules), the granules are non-toxic, biodegradable, and pH-neutral, and they are food for microorganisms in the soil as well (Vizitiu et al. 2012).

Variants of protection and planting of seedlings, with plant spacing, and the total number of seedlings are shown in Table 1. Seedlings were planted in pits with a spacing of 1.5x1 m in the variants without a shelter, and 3x2 m in the variants with a shelter. The experiment was conducted as a randomized block design, including four blocks with four repetitions. A total of 2,160 seedlings were planted.

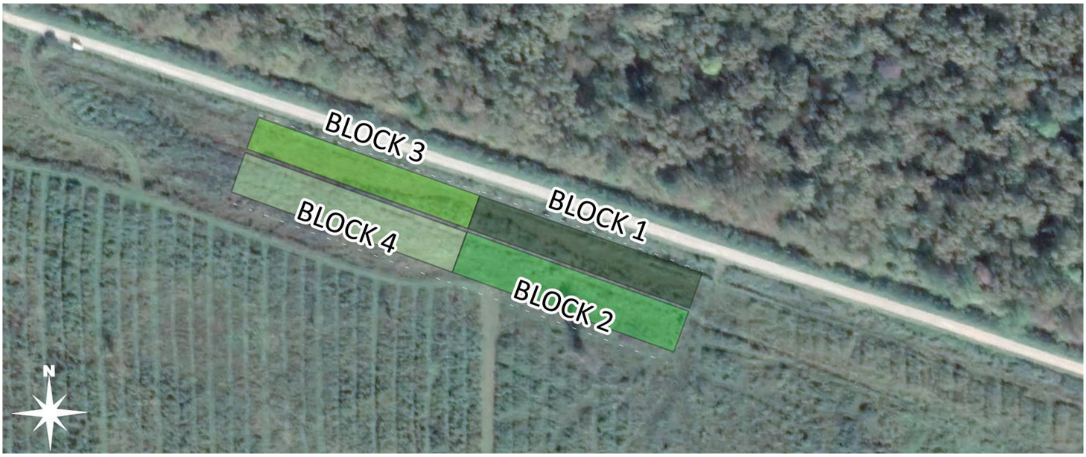

Figure 1. Location of the experiment Kragujna 35a, Forest Office Županja, Forestry Administration Vinkovci (682613 E, 4992867 N (HTRS96/TM)).

Figure 2. Experiment scheme - randomized block arrangement of experiment, four blocks with four repetitions.

Table 1. Variants of protection and planting of seedlings, plant spacing and the number of seedlings (N).

Variant	Protection	Polymer	Plant spacing (m)	N (total)	N (per ha)
S	Without shelter	Without polymers	1.5x1	800	6,667
SP	Without shelter	With polymers	1.5x1	800	6,667
SS	With shelter	Without polymers	3x2	280	1,667
SSP	With shelter	With polymers	3x2	280	1,667

During May 2015 and 2016, in a circle with a diameter of 1 meter around each shelter, shrubby and grassy vegetation was sprayed with herbicide based on the active substance glyphosate in order to reduce the negative impact of weeds (Liović et al. 2019). In the following years, there was no treatment with herbicides, nor with fungicides and insecticides.

During the growing season of 2020 and 2022, manual care of the seedling was carried out in order to free the end buds of plants and accelerate their height growth.

In the period from 2015 to 2022, at the end of the growing season, in five periodic measurements (2015, 2016, 2019, 2020, and 2022), survival rate and plant heights were measured. Seedling heights were measured with a measuring stick with an accuracy of 1 cm. Recorded data of survival and measured heights were analysed in MS Excel and include average value of survival rate and quantitative properties of height by standard indicators: mean, median, minimum and maximum, standard deviation (SD) and analysis of variance (ANOVA) for the quantitative property of height, as well as the Tukey-Kramer post-hoc test (α=0.05) to compare the differences between the varieties.

RESULTS AND DISCUSSION

Survival

The results in Table 2 and Figure 3 show that, after eight growing seasons and five measurements, i.e. after the first, second, fifth, sixth and eighth growing season, the planting of seedlings protected by shelters (SS and SSP variants) provides better conditions for survival, while seedlings planted without shelters (S and SP variants) have lower survival rates due to the fact that they are more exposed to harmful factors.

Seedlings of SSP variant have the best survival rate of 82.14%, while seedlings of SP variant have the lowest survival rate of 62.63%. Seedlings of SS variant have a survival rate of 80.00%, while seedlings of S variant have a survival rate of 66.75%. Potter (1998), Liović et al (2013, 2019) and Benko (2020) have also found that the survival rate of seedlings protected by shelters is higher than of those without shelters.

During the measurement all living seedlings were considered and measured. In 2019 some of seedlings seemed to be dry; however in 2020 they grow again from the roots of the plant also evident from number of survived seedlings and minimum height of seedlings in Table 6 and survival rate in Table 2.

The differences in survival between variants of seedlings with and without shelters are shown in Table 3. The variation in survival rates in favour of seedlings of SS variant compared to the seedlings of S variant increases with the number of growing seasons: 5.99% in 2015, 10.42% in 2016, 17.10% in 2019, 15.43% in 2020, and 16.56% in 2022. After eight growing seasons, the seedlings of SS variant had 16.56% better survival rate compared to the seedlings of S variant.

Also, the difference in survival rates increases in favour of the seedlings of SSP variant compared to the seedlings

Table 2. Total number (N) and survival of seedlings during measurements in S and SP variants (without shelters), and SS and SSP variants (with shelters) after the first, second, fifth, sixth and eighth growing season.

Variant	N	2015	2016	2019	2020	2022
				(%)		
S	800	86.46	79.50	69.88	70.38	66.75
SS	280	91.97	88.75	84.29	83.21	80.00
SP	800	85.54	84.75	69.75	69.75	62.63
SSP	280	95.57	87.00	86.07	86.43	82.14

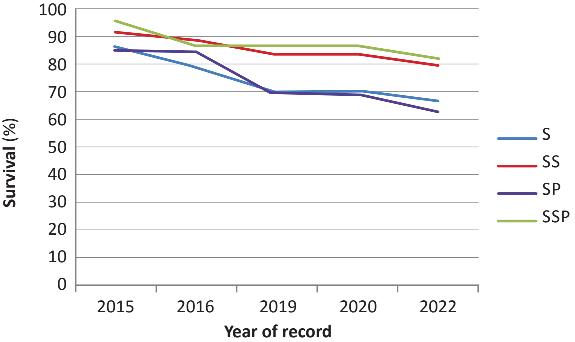

Figure 3. Survival of seedlings without shelters (S and SP variants) and seedlings with shelters (SS and SSP variants) after the first, second, fifth, sixth and eighth growing season.

of SP variant with the number of growing seasons: 10.49% in 2015, 2.59% in 2016, 18.96% in 2019, 19.30% in 2020, 23.76% in 2022. After eight growing seasons, the seedlings of SSP variant had 23.76% better survival rate than the seedlings of SP variant.

In the eight-year research period, the average annual level of precipitation (684 mm) corresponds to the average level of precipitation for the period 1991–2020 (Vučetić and Anić 2021). Table 4 shows the total monthly and annual precipitation according to data from the nearest meteorological station Gradište, of the Croatian Meteorological and Hydrological Service (DHMZ), which is located 15 km in aerial distance from the experiment location. The first year after planting had below average annual precipitation (2015, 644 mm), the second year was above average (2016, 746 mm), while the third year (2017) was the driest, with 581 mm. Most precipitation was recorded in 2018 (852 mm), while 2019 was above average (717 mm), and 2020 and 2021 were close to average (657 mm and 671 mm, respectively). The year 2022 was dry below average (605 mm).

However, Table 5 shows the average annual level of precipitation during the growing season (382 mm) of the research period (2015–2022) also corresponds to the average level of precipitation for the period 1991–2020 (Vučetić and Anić 2021).

Nevertheless, if the data of the average levels of precipitation during the growing season for the research period are analysed, a significant deviation from the average levels of precipitation can be observed, especially in 2015, 2017, 2021 and 2022. While 2016 and 2020 had values closest to the average, 2018 and 2019 were above average in terms of precipitation during the growing season.

Given the same conditions regarding moisture, the planting of seedlings protected by shelters (SS and SSP variants) provided better conditions for survival, while seedlings not protected by shelters (S and SP variants) had a lower survival rate. Other authors (Kjelgren and Rupp 1997, Del Campo et al. 2006, Bergez and Dupraz 2009, Ghazian et al. 2020) have found the advantages of the shelters by providing shade for the seedlings, slowing down the evaporation of moisture, protecting the seedling from sudden temperature changes and increasing the humidity inside the shelter by condensing moisture on the walls.

Height

The results of descriptive statistics of pedunculate oak seedlings heights with and without shelters, as well as with and without polymer addition, during the research period (2015–2022), i.e. after the first, second, fifth, sixth and eighth growing season are shown in Table 6. Seedlings protected by shelters had a higher average height than

Table 3. The difference in the survival of seedlings of different variants in planting after the first, second, fifth, sixth and eighth growing season.

Comparison of variants	**2015**	**2016**	**2019**	**2020**	**2022**
			(%)		
SS and S	5.99	10.42	17.10	15.43	16.56
SSP and SP	10.49	2.59	18.96	19.30	23.76

Table 4. Monthly and annual precipitation according to data from the nearest meteorological station Gradište (DHMZ) for the research period (2015–2022).

Year	**Precipitation** (mm)												
	Month												**Annual**
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	
2015	69	70	46	24	99	26	10	49	103	90	57	2	644
2016	71	71	71	58	36	44	112	51	95	66	69	2	746
2017	27	46	45	68	43	42	72	17	74	59	43	47	581
2018	2	66	77	29	53	257	88	54	60	24	42	40	852
2019	43	24	20	97	113	94	87	37	60	26	65	51	717
2020	17	40	35	17	76	113	72	77	21	82	21	86	657
2021	59	41	33	51	47	22	83	50	11	90	105	82	671
2022	15	32	9	69	52	39	12	74	119	29	89	65	605
AVERAGE	38	49	42	52	65	80	67	51	68	58	61	47	684

Table 5. The average level of precipitation during the growing season (IV-IX month) for the research period (2015–2022) (DHMZ).

Year	Precipitation (mm)						
	Month						Total
	IV	V	VI	VII	VIII	IX	
2015	24	99	26	10	49	103	310
2016	58	36	44	112	51	95	395
2017	68	43	42	72	17	74	314
2018	29	53	257	88	54	60	542
2019	97	113	94	87	37	60	488
2020	17	76	113	72	77	21	377
2021	51	47	22	83	50	11	262
2022	69	52	39	12	74	119	365
AVERAGE	52	65	80	67	51	68	382

seedlings without shelters in all five measurements during the monitoring period of eight growing seasons.

After the eighth growing season, the average height of seedlings of S variant was 129.02 cm (range 28–390 cm), and the average height of seedlings of SS variant was 176.27 cm (range 50–353 cm). The average height of seedlings of SP variant was 137.26 cm (range 23–410 cm), and the average height of seedlings of SSP variant was 202.75 cm (range 38–412 cm). Seedlings of SSP variant had the biggest height growth of 202.75 cm, while on average the smallest seedlings were of S variant, achieving the height of 129.02 cm.

In the case of seedlings without shelters (S and SP variants), the transplant shock that lasts at least two years (the first and the second growing season) is visible on the chart in Figure 5, during which the seedlings did not increase in height, while seedlings with shelters increased in height significantly already after the first growing season. Transplant stress is a condition that occurs during transplanting and is associated with the loss of a significant part of the root system when removing seedlings, as well as lack of moisture and nutrients, which often leads to higher mortality in the first growing season (Rietveld 1989, Struve

Figure 4. Detail of the experimental planting with shelters, Forest Office Županja, FMU Kragujna, subcompartment 35a (photo: Dubravac, May 2020).

and Joly 1992, Jacobs et al. 2005). Based on Figure 5, it can be concluded that the use of shelters has a favourable effect on height growth, alleviates the transplant stress and thus has a positive effect on survival (Figure 4). The above has been confirmed by previous research (Liović et al. 2019, Benko 2020), which stated that the height of oak seedlings protected by shelters is greater than that of seedlings without shelter protection.

The average heights in the period from the planting of seedlings in 2014 until 2022 are shown in Figure 6. The seedling image presents the difference in height growth from planting to the end of the second growing season,

Table 6. Number of survived seedlings (N) and descriptive statistics of height, during measurements of pedunculate oak seedlings without shelters (S and SP) and with shelters (SS and SSP) after the first, second, fifth, sixth and eighth growing season.

Year	Variant	N	Height (cm)				
			Mean	Median	Min.	Max.	SD
2015	S	694	34.36	34	5	73	9.70
2016		687	39.04	37	9	79	12.62
2019		562	73.05	70	12	250	36.17
2020		566	94.64	88	18	298	46.63
2022		537	129.02	122	28	390	62.55
2015	SP	687	34.71	35	5	69	9.41
2016		681	37.97	38	4	82	12.01
2019		561	83.82	77	17	213	43.74
2020		561	102.27	95	20	290	52.65
2022		504	137.26	129	23	410	67.14
2015	SS	258	56.64	55	10	115	17.37
2016		249	117.27	132	22	199	41.27
2019		236	146.04	150	21	251	41.02
2020		233	160.54	158	36	290	38.76
2022		224	176.27	164	50	353	49.64
2015	SSP	270	54.94	52	5	115	17.26
2016		245	128.72	138	38	187	33.42
2019		244	167.63	165	50	293	35.45
2020		245	182.43	173	64	340	46.25
2022		233	202.75	187	38	412	61.50

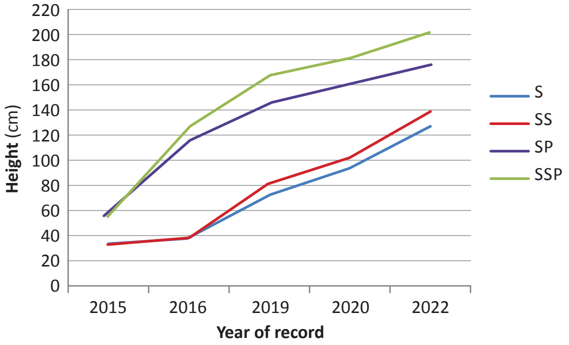

Figure 5. Height growth of seedlings, during measurements of seedlings without shelters (S and SP variants) and seedlings with shelters (SS and SSP variants) after the first, second, fifth, sixth and eighth growing season.

when seedlings with shelters (SSP and SS variants) had three times higher average height than seedlings without shelters (S and SP variants).

In the period from the end of the first to the end of second growing season seedlings of SSP variant had the highest annual height growth (73.78 cm). In the period from the third to the end of the fifth growing season (coloured green), the SSP and SS variants had slightly more than twice the average height of seedlings of the S and SP variants. Seedlings of SP variant had the highest height growth (45.85 cm). In the period from the end of the fifth to the end of the sixth growing season (coloured purple), the seedlings of SSP and SS variants also had about twice the average height of the seedlings of S and SP variants. Seedlings of S variant had the highest annual height growth (21.59 cm). In the period from the seventh to the eighth growing season (coloured blue), the seedlings of SSP and SS variants had about 1.5 times the average height of the seedlings of S and SP variants. Seedlings of SP variant had the highest height growth (34.99 cm).

By analysing the data from Table 7, we found a significant difference in the height of plants protected by shelters, which was supported by ANOVA statistical method, during which a significant difference in the average plant height (ANOVA F=48.09, p<0.001) was found within the variants.

The statistical results were processed with an additional Tukey-Kramer post-hoc test in order to determine whether there is a statistically significant difference between the variants of planting seedlings of SS variant with an average height of 176±49.64 cm and seedlings of SSP variant with an average height of 203±61.50 cm. Seedlings of SSP variant were on average 26.48 cm higher than the seedlings of SS variant, and they also had a statistically significant difference (p=0.000000574). Tukey-Kramer post-hoc test revealed statistically significant differences in the average height of the seedlings of SSP variant compared to the seedlings of SS variant, which is why it can be concluded that the addition of polymers influenced the increase of the average height of seedlings protected by shelters.

In the case of seedlings of S variant with an average height of 129±62.55 cm and seedlings of SP variant with an average height of 137±67.14 cm, the difference in average height was 8.25 cm, and the variants had no statistically significant difference (p=0.020449), so it can be concluded that in this case the effect of polymers on height growth was almost insignificant.

Table 7. ANOVA analysis of variance for height.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1120852	6	186808.7	48.09493	3,8839E-54	2.104652
Within Groups	5791291	1,491	3884.165			
Total	6912143	1,497				

SS - sum of squares, df - degrees of freedom, MS - mean sum of squares, F - *F*-statistic, F crit - *F* critical value.

Figure 6. Average height growth and seedling heights by measurement periods.

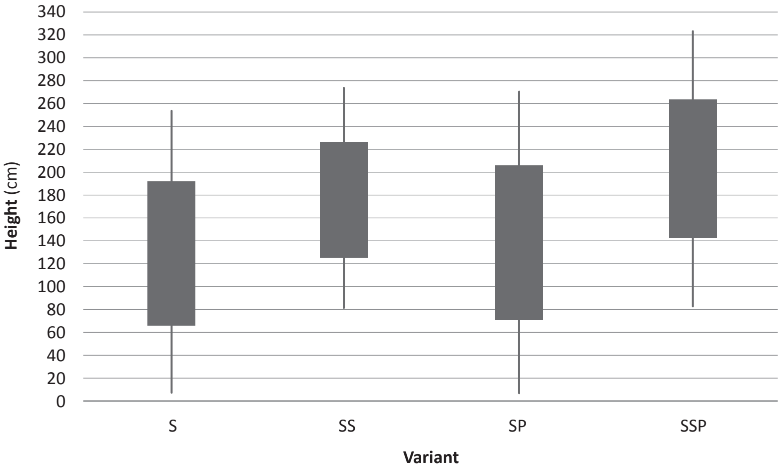

Figure 7. Average height of seedlings by measurement periods (Box-and-whisker chart). The upper and lower bounds of the rectangle represent the range within which there is a standard deviation of height. The line segment above and below the rectangle represents the circumference within which there is 1.96 standard deviations of seedling height, which includes 95% of all data).

CONCLUSIONS

During five measurements (2015, 2016, 2019, 2020 and 2022) in eight growing seasons (2015–2022), survival and seedling heights of S, SP, SS and SSP variants were recorded and measured.

Seedlings of SSP variant had the highest survival rate, amounting to 82.14%, along with the seedlings of SS variant with a rate of 80.00%. Seedlings of SP variant had the lowest survival rate of 62.63%, while the survival rate of the seedlings of S variant was slightly better, amounting to 66.75%. Seedlings of SSP variant had a 23.76% better survival rate compared to SP-variant seedlings, while SS-variant seedlings had a 16.56% better survival rate compared to S-variant seedlings.

Seedlings of SSP variant had the highest height growth, with an average height of 202.75 cm (range 38–412 cm), along with seedlings of SS variant with an average height of 176.27 cm (range 50–353 cm). The average height of SP variant seedlings was 137.26 cm (range 23–410 cm), and S-variant seedlings had the lowest average height of 129.02 cm (range 28–390 cm).

Statistically significant differences were found with regard to SS and SSP variants. The seedlings of SSP variant were on average 26.48 cm higher than the seedlings of SS variant, and it can be assumed that the addition of the polymers had a favourable effect on the increase in the average height of the seedlings protected by shelters due to the synergistic effect of the polymers and the shelters. For seedling of S variant and SP variant, the difference in average height was 8.25 cm, which indicates that these variants had no statistically significant difference (the effect of polymers on height growth is almost insignificant in this case).

Author Contributions

ŽT, BL and TD conceived and designed the research, RL, BL, TD and SŠ carried out the field measurements, RL processed the data and performed the statistical analysis, ŽT supervised the research RL, TD, BL and ŽT wrote the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Wild Boar (*Sus scrofa* L.) as the Biomonitor of Cadmium and Lead Pollution in the Republic of North Macedonia

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ABSTRACT

Until the outbreak of African swine fever in almost the entire Europe and consequently in the Republic of North Macedonia (2022), the population of wild boars was in increase. The biological characteristics of this species make it suitable to be used as a bioindicator for environmental pollution. The concentrations of cadmium and lead (Cd and Pb) were measured using atomic absorption spectrophotometry in the liver of free ranging wild boars. Samples were taken from 608 animals, harvested in a period of 7 years (2016-2022), in 11 hunting locations that cover the whole territory of the Republic of North Macedonia. The mean value of Pb in the liver in all hunting areas was 0.225 mg·kg^{-1} wet weight with range of 0.154-0.722 mg·kg^{-1}, while mean Cd was 0.366 mg·kg^{-1} and ranged from 0.177 to 0.464 mg·kg^{-1}. A statistically significant difference in Cd was found between the hunting locations, while Pb differed between hunting years. The mean Cd concentrations were significantly higher in Pelagonisko, Krivorečansko and Ohridsko-Prespansko hunting areas than in those from Pološko hunting area, while the mean Pb values in 2019 and 2020 were significantly higher than the mean value determined in 2021. For Cd, 18.2% of the total number of samples exceeds the maximum allowed limit provided in the legislation, while for Pb it was 6.7%. The maximum mean value for Pb (0.336 mg·kg^{-1}) was measured in 2020 and for Cd (0.449 mg·kg^{-1}) in 2016. The mean values for Pb and Cd obtained in this study were in line with data reported for wild boar from other European countries.

Keywords: wild boar; Pb; Cd; liver; hunting areas; pollutants

INTRODUCTION

Regardless of whether it comes from natural processes on the Earth's surface or from human activities, elements and compounds that are released into the environment and cause damage to the biosphere are treated as pollutants (Kłos et al. 2018). About 13,000 tons of cadmium (Cd) are produced annually worldwide, mainly intended for chemical stabilizers, nickel-cadmium batteries, pigments, metal coatings and alloys (Flora et al. 2008). Through the soil and the water, cadmium compounds can be easily adsorbed by plants. With the increased accumulation of toxic metals in the plants and the soil, the risk of their transfer to herbivorous wild mammals, game and livestock may increase (Bilandzić et al. 2010). Lead (Pb) is a highly toxic metal, which with its wide distribution, mainly as a result of human activities (mining, production and combustion of fossil fuels), can cause environmental contamination and health problems in many parts of the world (Jaishankar et al. 2014). The exposure to lead contamination can be manifested in lead-induced encephalopathy, gastroenteritis, and peripheral nerve degeneration (Lénárt et al. 2023). For the domestic and the wild animals and for the humans, acute or chronic exposure to Pb and Cd may have severe consequences because these heavy metals accumulate in almost all tissues, mainly in liver and kidneys as target organs (Satarug et al. 2003, Kramárová et al. 2005, Pandey and Madhuri 2014).

The Republic of North Macedonia, as part of the former Yugoslavia, was an important producer of metals such as copper, steel, ferroalloys, lead, zinc, cadmium, nickel, silver and gold, with long-term mining and mining-related activities contributing greatly to the pollution of the environment (Barandovski et al. 2020). The studies conducted in North

Macedonia (Rogan et al. 2010, Stafilov et al. 2010, Stafilov 2017, Barandovski et al. 2020, Stafilov et al. 2023), where the concentration of heavy metals was measured in soils and mosses, offer a good contribution to getting to know the situation regarding the representation of heavy metals in this country. On the other hand, very little is known about the exposure of game (herbivores and omnivores) to heavy metals as a direct consumer of food from nature, which is often contaminated around critically endangered areas (industrial zones and mines). The anthropogenic pollution and the environmental areas naturally enriched in toxic heavy metals where game animals reside can increase the risk of contaminants entering the food chain and induce elevated level of them in game (Lehel et al. 2016). The diet is considered to be the most important pathway of metal transfer from soil and plants to the tissues of animals (Stankovic et al. 2014).

A well-known and commonly used bioindicator in modern research is wild boar (*Sus scrofa* L.) (Bilandzić et al. 2009, Bilandzić et al. 2010, Danieli et al. 2012, Gasparik et al. 2012, Lénárt et al. 2023). Wild boar is a potentially good bioindicator for monitoring of heavy metals in the environment because it is an opportunistic omnivore ingesting both flora and fauna (invertebrates, vertebrates, crops (Lee and Lee 2019) and mushrooms (Brzezicha-Cirocka et al. 2016)). Wild boars have a characteristic way of diet; they search for food by browsing or grazing (grasses, herbs, stems, leaves), on the surface of the ground (fruits and fungi) or below the surface of the ground by rooting (roots, invertebrates, rhizomes) (Bueno et al. 2011, Ballari and Barrios-García 2014). They have relatively long lifespan and thus long period of accumulating chemicals (Bilandzić et al. 2010). Also, they are relatively mobile and have the large effective population sizes (Frantz et al. 2012), so they can cover a large area in search for food and thus provide an early warning of adverse toxic effects in the ecosystem as a whole. Wild boars are widely distributed in the world and can be found in Eurasia, the southern part of Asia, and some of the islands od Indonesia (Lénárt et al. 2023).

The presence of wild boar in the last years in European areas has increased (Jori et al. 2021). In the Republic of North Macedonia, wild boars are represented in the entire territory of the country, while the last decade their number has increased drastically. This is confirmed by data from the State Statistical Office, where the number of the population in 2010 was estimated to be around 2,872 individuals, and in 2022 it was estimated to be around 9,400 individuals (MakStatDatabase 2021a). Since the largest number of hunters in North Macedonia are focused on wild boar hunting, in recent years the number of wild boars harvested during the hunting seasons has increased dramatically (in 2010, about 472 were shot, and in 2022, about 2,800 individuals) (MakStatDatabase 2021b), which makes biomonitoring with this species much easier. The good reflection of environmental pollution in wild boar tissues demonstrated in numerous papers in the last decade and the relevance for the consumers of boar meat (transmission to humans) are just a few of the reasons why we decided to investigate the occurrence of Pb and Cd in the liver of wild boar as the biomonitor of the pollution in the Republic of North Macedonia.

MATERIALS AND METHODS

The Study Area

The Republic of North Macedonia is a country situated in the central part of the Balkan Peninsula, bordering Serbia to the north, Bulgaria to the east, Greece to the south and Albania to the west, and it has a total area of 25,713 km^2. The country is mountainous and has deep basins and valleys with three large lakes and is bisected by the Vardar River. It has a water area of 857 km^2, while its land area is 24,856 km^2.The country has 16 mountains higher than 2,000 m, but most of the surface is between 500 and 1,000 meters above sea level. The highest point (2,764 m) in the country is the peak Golem Korab of Mount Korab and the lowest point (44 m) marks the area at the crossing point of the Vardar River (Barandovski et al. 2020).

In North Macedonia, the state establishes hunting grounds as basic spatial units for game management according to hunting law (2009). However, in the general hunting management plan for the Republic of North Macedonia (period 1997–2016), according to Trpkov and Maletic (1997), the total territory of the country is divided into 11 hunting areas as broader categories for game management. This division was made for the purpose of implementing a unique hunting policy and appropriate measures for protection, breeding and promotion of game management. In fact, the hunting area represents an ecological unit with a large area, conditioned by a complex of environmental factors and the vital characteristics of game populations, where it resides throughout the year and has the greatest impact on the environment. Given the mobility of game, especially large game, and its relatively large radius of action during all seasons to satisfy life's needs, such an organization is fully justified, especially when planning for the joint implementation of hunting policies in the hunting grounds that are part of the same hunting area, especially for the shooting. Also, when monitoring certain types of big game at the hunting ground level, wrong data on the numerical condition of the game are often obtained because the hunting ground is a small spatial unit, so there is a fear of the possibility of counting the same game in two or three hunting grounds which border.

Therefore, this study was carried out on the same 11 hunting areas that cover the territory of the entire country, namely: Pelagonisko hunting area (1) including the municipalities of Prilep, Bitola, Demir Hisar and Kruševo, with a total area of 408,279 ha; Ohridsko-Prespansko hunting area (2) including the municipalities of Resen, Ohrid, Struga and Debar, a total area of 183,743 ha; Kičevsko-Brodsko hunting area (3) including the municipalities of Kičevo and Makedonski Brod, a total area of 173,624 ha; Pološko hunting area (4) including the municipalities of Gostivar and Tetovo, a total area of 168,717 ha; Skopsko-Kumanovsko hunting area (5) including the municipalities of Skopje and Kumanovo, a total area of 276,105 ha; Sredno-Vardarsko hunting area (6) including the municipalities of Veles and Sveti Nikole, a total are of 220,525 ha; Krivorečansko hunting area (7) including the municipalities of Kratovo and Kriva Palanka, a total area of 109,524 ha; Bregalnčko hunting area (8) including the municipalities of Kočani, Vinica, Probištip and Štip, a total area of 214,435 ha; Vlainsko-Maleševsko hunting area (9) including the municipalities of Delčevo, Berovo, Pehčevo and Makedonska Kamenica, a total area of 139,233 ha; Strumičko hunting area (10) including the municipalities of Strumica and Radoviš, a total area of 168,695 ha; Dolno-Vardarsko hunting area (11) including the municipalities of Kavadarci, Negotino, Valandovo and Gevgelija, a total area of 282,772 ha.

Sampling

From 11 hunting areas (number 1 to 11) covering the whole territory of the Republic of North Macedonia, during the regular hunting season, which starts on 1 October and lasts until 31 January every year (between 2016 and 2022), a total number of 608 liver samples of wild boar (aged between 2 and 4 years) harvested by the active hunters were collected. The collected samples do not have a normal numerical arrangement in the hunting areas. From hunting area number 1, 143 samples were collected, from number 2 – 106 samples, from number 3 – 57, from number 4 – 50, from number 5 – 17, from number 6 – 15, from number 7 – 57, from number 8 – 3, from number 9 – 26, from number 10 – 53 and from number 11 – 81. After collection, the samples were individually packed in polyethylene bags and transferred to the laboratory in refrigerated bags. The tissue samples were frozen and stored at -20°C until analysis. During sampling operations, special care was taken to avoid tissues near the bullet pathway; all tissue samples were taken from >40 cm away from the areas of bullet damage (Dobrowolska and Melosik 2008, Danieli et al. 2012).

Method of Analyzing

Approximately 1 g of homogenized samples (measured with accuracy of ±0.01 g) were digested with 5 mL HNO_3 (67% v/v, purity for atomic absorption, Merck, Darmstadt), 1 mL of hydrogen peroxide (30%, analytical grade, Merck Darmstadt) with high-performance microwave oven (model Ethos Up, Milestone Srl, Sorisole, Italy) according to EN 13805:2002 (CEN 2002a). In brief, the temperature was ramped for 20 min to 220°C, after which samples were held at the same temperature for 15 min. During the digestion the potency was automatically adjusted by temperature and pressure control in each digestion vessel. Digested and cooled samples were diluted to the final volume of 25 mL with deionized water with Milli-Q quality.

Analysis of Pb and Cd was conducted by electrothermal atomic absorption spectrometer (ETAAS) with Zeeman background correction, model AAnalyst 600 (Perkin Elmer, Waltham, Massachusetts), and using 0.005 mg Pd (NO_3) as matrix modifier for each atomization of Pb and Cd (CEN 2003). The selected wavelengths for Pb and Cd measurement were 283.3 nm and 228.8 nm, respectively. Furnace programs for ETAAS determination were optimized for liver matrix (Table 1). Calibrations were prepared from commercial solutions of Pb and Cd (1,000 mg·L^{-1}) in 2% HNO_3 (Carl Roth GmbH, Karlsruhe, Germany). For recovery and precision validation, reference material offal liver FAPAS test material 07199 was used.

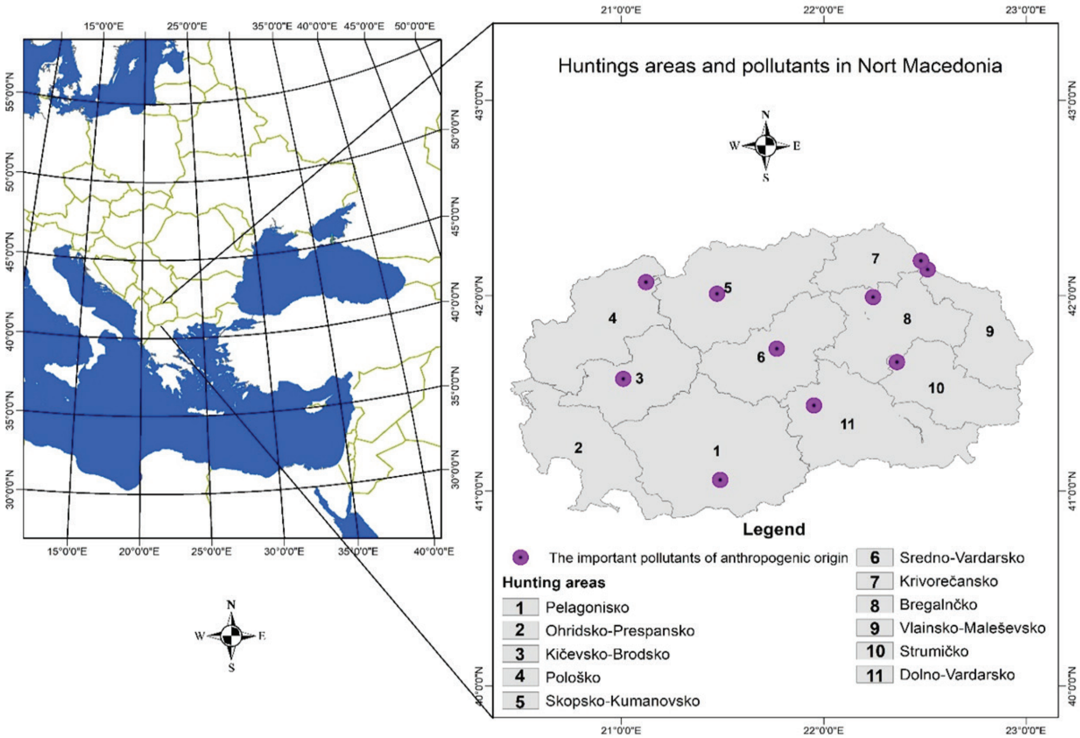

Figure 1. Hunting areas and pollutants of anthropogenic origin in North Macedonia.

Table 1. Furnace programs for lead (Pb) and cadmium (Cd) slit with 0.7 nm.

Step	Element	Temperature (°C)	Ramp time (s)	Hold Time (s)	Internal flow (mL·min^{-1})
1	Pb	110	10	30	250
	Cd	110	15	20	
2	Pb	130	20	30	250
	Cd	130	40	30	
3	Pb	950	35	35	250
	Cd	600	35	20	
4	Pb	1,850	0	5	0
	Cd	1,500	0	5	
5	Pb	2,450	1	3	250
	Cd	2,500	2	2	
6	Pb	20	15	20	250
	Cd	20	15	25	

Method Performance Characteristics

The quality assurance of the ETAAS method was performed by validation in accordance with the requirements proscribed in Commission Regulation (EC 2007). The obtained method of linearity range was 3.716-50 µg·L^{-1}, 0.319-6.0 µg·L^{-1} for Pb and Cd, respectively, with R^2>0.99, with limits of quantification of 12.39 µg·L^{-1} for Pb and 1.06 µg·L^{-1} for Cd. Method precision expressed as relative standard deviation was 6.90% and 8.56% for Pb and Cd, respectively, and the recovery was 104.25% (Pb) and 102.07% (Cd). Internal quality control was performed by running a reagent blank sample and reference material with every batch of samples. External quality control was assured by satisfactory participation in proficiency tests organized by FAPAS and the European Union Reference Laboratory for metals, EURL-MN. Matrix liver was within the ETAAS method scope which was accredited according to EN ISO/IEC 17025:2017.

Statistical Analysis

Statistical analysis was performed using the Statistica software version 14 (StatSoFT STATISTICA Software) for descriptive analysis and for analysis of variance. To examine differences between sampling areas we used the two-way analysis of variance (ANOVA) test. All statements of significance were based on the 0.05 level of probability (p≤0.05).

RESULTS AND DISCUSSION

The concentrations of lead and cadmium found in livers of wild boars, collected during the period of 7 years on 11 different hunting areas in North Macedonia, are presented in tables 2 and 3. According to Commission Regulation (EC) no.1881/2006 (EC 2006), Food and Veterinary Agency of the Republic of North Macedonia have established maximum levels of Cd and Pb in liver of bovine animals, sheep, pigs, poultry and horses, which amounts to 0.5 mg·kg^{-1} (EC 2006). In this study, no statistically significant difference in hepatic Pb was found between 11 hunting areas locations. The mean concertation for Pb, looking at all hunting areas, ranged from 0.154 to 0.722 mg·kg^{-1}, and mean concentration for all in total was 0.225 mg·kg^{-1}. Compared to other studies, somewhat lower values were found in Croatia (Bilandzić et al. 2009), where the mean values ranged from 0.061 to 0.202 mg·kg^{-1}. Quite similar mean values were obtained in a study carried out in Italy (Danieli et al. 2012), with the mean value of 0.329 mg·kg^{-1}. However, somewhat higher values for Pb were found in Poland (Durkalec et al. 2015), with the mean value of 0.083 to 0.903 mg·kg^{-1}. Significantly higher values than those in our study were obtained in Southern Spain (Santiago et al. 1998), with mean value of 0.44 to 5.05 mg·kg^{-1}. The higher values obtained in Poland and Southern Spain compared to those in our study are probably a result of the choice of locations where the research was conducted. Namely, in Poland, the research was conducted in 3 locations, 2 of which are known as industrial areas, and the third location was taken as a reference area. In Southern Spain, the situation was similar, each of the four zones that were the subject of research had its own sources of possible pollution. In this study, the 11 hunting ground locations on which the research was carried out are of such a setting that they cover the entire territory of the country, and not only the known industrial facilities where higher values can be expected.

Looking at all locations, out of the total number of samples (n=608), in 18% of samples Pb was not detected (<LODs). Seven percent of samples (41 of 608) exceeded the maximum levels for Pb in liver of domestic animals (0.5 mg·kg^{-1}). The maximum measured concentrations of Pb in individual locations ranged from 0.443 to 4.338 mg·kg^{-1}, and the maximum measured value of 4.338 mg·kg^{-1} was obtained from a sample at hunting area no. 7, which is 8.5

Table 2. Lead (Pb) and cadmium (Cd) concentrations in the livers of wild boars from 11 hunting areas of North Macedonia.

Concentration in wet weight (mg·kg^{-1})							
Pb							
Hunting areas	**N**	**N < LODs**	**Mean**	**±SD**	**Min**	**Max**	**N > Max. l.**
1	109	34 (5.6)	0.244	0.384	0.006	2.443	11 (1.8)
2	86	20 (3.3)	0.178	0.184	0.009	0.968	6 (1)
3	44	13 (2.1)	0.190	0.204	0.015	1.067	3 (0.5)
4	50	0	0.247	0.541	0.017	3.512	3 (0.5)
5	14	3 (0.5)	0.154	0.117	0.027	0.443	0
6	15	0	0.205	0.269	0.023	1.060	2 (0.3)
7	44	13 (2.1)	0.352	0.700	0.021	4.338	6 (1)
8	3	0	0.722	1.122	0.021	2.017	1 (0.2)
9	21	5 (0.8)	0.214	0.286	0.030	1.358	1 (0.2)
10	42	11 (1.8)	0.190	0.214	0.015	1.289	2 (0.3)
11	67	14 (2.3)	0.197	0.194	0.004	0.959	6 (0.9)
∑	495	113(18.5)	0.225	0.370	0.004	4.338	41 (6.7)
Cd							
Hunting areas	**N**	**N < LODs**	**Mean**	**±SD**	**Min**	**Max**	**N > Max. l.**
1	134	9 (1.5)	0.423^{a}	0.418	0.012	1.929	40 (6.5)
2	98	8 (1.3)	0.381^{a}	0.315	0.015	1.468	22 (3.6)
3	56	1 (0.2)	0.372^{ab}	0.342	0.006	1.333	12 (2)
4	49	1 (0.2)	0.177^{b}	0.153	0.003	0.732	3 (0.5)
5	14	3 (0.5)	0.305^{ab}	0.138	0.111	0.644	1 (0.2)
6	15	0	0.293^{ab}	0.140	0.026	0.495	0
7	51	6 (1)	0.464^{a}	0.403	0.039	2.531	12 (2)
8	3	0	0.177^{ab}	0.089	0.091	0.268	0
9	22	4 (0.6)	0.387^{ab}	0.242	0.119	1.27	4 (0.6)
10	45	8 (1.3)	0.320^{ab}	0.520	0.008	3.573	3 (0.5)
11	71	10 (1.6)	0.354^{ab}	0.251	0.019	1.093	14 (2.3)
∑	558	50 (8.2)	0.366	0.354	0.003	3.573	111 (18.2)

N – number of samples above the limit of detection; N < LODs – number of samples bellow the limit of detection; N > Max. l. – number of samples exceeding maximum levels of Cd/Pb in liver of bovine animals, sheep, pigs, poultry and horses, set out by law; % of samples from the total number of samples is presented in brackets.

times higher than the maximum allowed concentration for lead. This is most likely due to contamination from Toranica, which is present in this area with a production capacity of 700,000 tons of ore per year. This is confirmed also by the results of the study conducted during the moss biomonitoring of air pollutinon where high contents of Pb and Zn were discovered (average values of 60 and 75 mg·kg^{-1} of moss) in the area very close to the source of pollution (Angelovska et al. 2014).

Moreover, this hunting area is bordered by two more lead and zinc mines, , Zletovo and , Sasa, which are characterized by high deposition of Pb and Zn, especially near the source of pollution (Balabanova et al. 2014, Balabanova et al. 2017) . This has also been confirmed by one of the latest studies conducted in the Republic of North Macedonia (Stafilov et al. 2023) in which the distribution of various chemical elements was determined in soils and vegetables from gardens in the region of Probištip, North Macedonia (with the main point being the Zletovo mine). The researchers obtained the results in which the lead content in 8 of 19 soil samples studied was higher than the target value (85 mg·kg^{-1}) according to the Dutch standards, and the highest lead content measured in soil was 478 mg·kg^{-1}, which is 5.6 times higher than the target value (the target values indicate the level at which there is a sustainable soil quality). In the Republic of North Macedonia, the regulations for the maximum allowed content of pollutants in the soil and vegetables have not yet been adopted, so in the previous studies regarding the soil and vegetables, the values are compared with the Dutch regulations.

The results of the occurrence of lead in vegetables and cereals showed that a larger number of samples (18 samples out of 38) had an elevated value that exceeds the maximum allowed levels of 0.10 mg·kg^{-1} for vegetables, 0.30 mg·kg^{-1} for leafy vegetables and 0.20 mg·kg^{-1} for cereals. In the mentioned study, the highest content of Pb was found in the barley samples with the concentration 6.65 mg·kg^{-1}, exceeding the maximum allowed content by 33 times. Since the wild boar does not know natural boundaries and obstacles, and since omnivores when searching for food can cover a large area, the occasional high values that exceed the maximum permitted limit may be the result of contamination right near such industries.

The mean concertation for Cd, looking at all hunting areas together, ranged from 0.177 to 0.464 mg·kg^{-1}, and the total mean concentration was 0.366 mg·kg^{-1}. Similar values were obtained in two studies conducted in Croatia, where the mean values ranged from 0.3 to 0.49 and 0.162 to 0.308 mg·kg^{-1} (Bilandzić et al. 2009, Florijancic et al. 2015), and to one study in Spain (0.17 to 0.34 mg·kg^{-1}) (Santiago et al. 1998), while the lower mean value was obtained in Italy amounting to 0.084 mg·kg^{-1} (Danieli et al. 2012). From a total of 608 samples, cadmium was not detected in 50 samples or 8.2% of the total number of samples (Cd<LODs). This study indicates location differences, so the mean Cd concentrations were significantly higher in Pelagonisko, Krivorečko and Ohridsko-Prespansko hunting areas than in the samples collected from Pološko hunting area (p=0.004 both.). The most acceptable explanation for these differences is the availability and distance from pollutants and mining activities, although it is a fact that there is no large-scale pollutant in the Ohrid-Prespa hunting ground. However, Cd and Pb are transported over long distances by air, so the dry and wet deposition of the particles may be due to pollutants from greater distances. In the Krivoreško hunting area or near its borders, as mentioned previously, there are 3 potential sources of pollution on a huge scale. In the research conducted near the Zletovo mine (Stafilov et al. 2023), high concentrations of cadmium were found in soil samples (16 out of 19 exceeded the maximum allowed limits of 0.8 mg·kg^{-1}) and vegetables (14 out of 19 samples exceeded the maximum allowed limits of 0.05 mg·kg^{-1} for vegetables, 0.20 mg·kg^{-1} for leafy vegetables, and 0.10 mg·kg^{-1} for cereals). In the vicinity of the Sasa and Toranica mines, high concentrations of cadmium in moss were detected, especially near the source of contamination (Angelovska et al. 2014, Balabanova et al. 2017). Compared to the area where much lower concentrations of cadmium were determined, near the eastern border of the Pološko hunting area, a polluter of this scale is the previously active high-melting ferrochromium plant, Jugohrom - Tetovo, which stopped working in 2016. In the research carried out in North Macedonia, and referring to the Tetovo region, in general this area is characterized as highly contaminated with Sc, Cr, Fe, Co, and Ni, as a consequence of the former operation of the ferrochromium plant, and not with cadmium (Barandovski et al. 2008). Also, some of the hunting grounds in this area border the Mavrovo National Park, and most of them today are already part of the newly formed Shar Planina National Park. Characteristic of the Pelagonian hunting area, which applies to all other hunting areas and can be seen from the obtained results, is the fact that the largest number of samples that exceed the maximum permissible limit for cadmium (N>0.5 mg·kg^{-1}), i.e. 40 out of 111 samples or 6.5 out of 18.2%, were determined exactly in this area. The major polluter of the environment in the region is the thermo-electric power plant REK Bitola. The average value of cadmium of 0.423 mg·kg^{-1} for this locality does not exceed the maximum permissible limit, but it is still significantly higher than the average value of the Polog hunting area. Our results correspond with the recent study (Stafilov et al. 2018)indicating that the concentrations of cadmium in the soil in the Bitola region do not exceed the maximum allowed according to Dutch Standards. Furthermore, compared to the average values of European soils, they are lower in relation to them, but some samples in the area of REK Bitola have slightly increased content over the target value ranging from 1.0 to 1.3 mg·kg^{-1}. Pollution of soil with some elements present in fly ash deposited in this region influence the appearance of high content of arsenic, cadmium, lead and zinc.

Table 3 shows Pb and Cd concentration in livers of wild boars during the period of 7 years (2016-2022) in North Macedonia. The mean values for lead over the years ranged from 0.095 to 0.336 mg·kg^{-1}, or the mean for all years was 0.225 mg·kg^{-1}. The highest mean value for lead (0.336 mg·kg^{-1}) was measured in 2020. In the same year, the largest number of liver samples exceeding the maximum allowed limits (N>0.5 mg·kg^{-1}) were determined, 20 out of 41 samples or 3.4 out of 6.7%. The maximum measured lead concentration in wild boar liver was 4.333 mg·kg^{-1} and it was measured in 2019.

In our study, results show that the mean lead values in 2019 and 2020 are significantly higher than the mean value determined in 2021 (p=0.0002). It is very difficult to explain and determine the specific reason why the mean value for lead was significantly lower in 2021 compared to the previous two years. Additional research is needed, where many more factors that could influence the differences that occur over the years will be monitored to determine the specific causes.

In general, the mean values for cadmium over the years have showed no statistically significant difference. Mean values ranged from 0.324 to 0.449 mg·kg^{-1} and mean value for all years was 0.366 mg·kg^{-1}. The maximum mean value for Cd (0.449 mg·kg^{-1}) was measured in 2016. It can be indicated that cadmium is present in the environment, but its average amount over the years has remained stable and no changes in an upward or downward direction have been observed. Also, in each of the researched years, certain values exceeding the maximum allowed limits for North Macedonia were observed (N>0.5 mg·kg^{-1}), namely 7 in 2016, 18 in 2017, 18 in 2018, 19 in 2019, 19 in 2020,

Table 3. Lead (Pb) and cadmium (Cd) concentrations in the livers of wild boars for the period 2016-2022 in North Macedonia.

Concentration in wet weight
(mg·kg^{-1})

Pb

Years	N	N < LODs	Mean	±SD	Min	Max	N > Max. l.
2016	25	4 (0.6)	0.318^{ab}	0.710	0.004	3.512	2 (0.3)
2017	78	18 (3)	0.207^{ab}	0.116	0.026	0.549	1 (0.2)
2018	82	21 (3.4)	0.197^{ab}	0.392	0.012	2.443	4 (0.7)
2019	59	13 (2.1)	0.322^{a}	0.625	0.006	4.338	7 (1.1)
2020	92	20 (3.3)	0.336^{a}	0.379	0.016	2.017	21 (3.4)
2021	84	14 (2.3)	0.095^{b}	0.103	0.021	0.684	1 (0.2)
2022	75	23 (3.8)	0.174^{ab}	0.157	0.030	0.968	5 (0.8)
∑	495	113 (18.5)	0.225	0.370	0.004	4.338	41 (6.7)

Cd

Years	N	N < LODs	Mean	±SD	Min	Max	N > Max. l.
2016	29	0	0.449	0.460	0.017	1.551	7 (1.1)
2017	92	4 (0.6)	0.406	0.288	0.006	1.333	18 (3)
2018	99	4 (0.6)	0.372	0.363	0.008	1.929	18 (3)
2019	65	7 (1.2)	0.406	0.394	0.003	1.522	19 (3.1)
2020	100	12 (2)	0.324	0.423	0.005	3.573	19 (3.1)
2021	93	5 (0.8)	0.346	0.315	0.015	2.531	17 (2.8)
2022	80	18 (3)	0.328	0.266	0.012	1.316	13 (2.1)
∑	558	50 (8.2)	0.366	0.354	0.003	3.573	111 (18.2)

N – number of samples above the limit of detection; N < LODs – number of samples bellow the limit of detection; N > Max. l. – number of samples exceeding maximum levels of Cd/Pb in liver of bovine animals, sheep, pigs, poultry and horses, set by law; % of samples from the total number of samples is presented in brackets.

17 in 2021, and 13 in 2022, or 111 of total 608 samples during 7 years. This indicates that cadmium can accumulate in larger amounts in game organs, which has also been confirmed by the numerous studies conducted in the surrounding countries of the region, where different species of game such as brown hare, roe deer and red deer were used as bioindicators for the presence of heavy metals in the environment (Bilandzić et al. 2009, Durkalec et al. 2015, Beuković et al. 2022, Beuković et al. 2023).

CONCLUSION

From this study it can be concluded that wild boars, as free-migrating animals on the hunting areas of North Macedonia, are show low contamination with Cd and Pb. The mean values for Pb and Cd obtained in this study were in line with data reported for wild boars from some other European countries. The research was conducted on the entire territory of the country, and not only on the industrially polluted areas, as it is the case in most studies where game is used as a bioindicator. Even though the average values of these heavy metals did not exceed the maximum permissible limits, in each year and on each location a certain number of samples exceeded the maximum permissible limits. This indicates the need for additional research, in order to assess the risk while consuming liver, which is especially important for the population of hunters who more often incorporate wildlife in their nutrition.

Author Contributions

All authors listed under the title contributed to the research and development of the paper. KC, VM, DB and MPH conceived and designed the study, KC, VM and DB carried out the field activities in the collection of wild boar samples, EDS and VE performed the laboratory analysis, MPH and MV performed the statistical analysis, KC, DB and MPH wrote the manuscript except the part of мethod of laboratory analyzing which was written by EDS and VE.

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REVIEW PAPER

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A Review of the Factors Influencing Variations in the Heartwood Proportion for Solid Wood

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ABSTRACT

Using 146 journals, 6 chapters, 4 reports, 2 books, and a proceeding, this research seeks to investigate the factors that influence variations in the proportion of heartwood. The data were collected through an online search in databases such as Scopus, Science Citation Index, Science Direct, Google Scholar, and ResearchGate with seven search queries, namely heartwood variability, heartwood proportion, tree age of heartwood, growth rate of heartwood, silvicultural treatment of heartwood, as well as genetic and environmental effects of heartwood. By eliminating papers that were out of topic or title, purpose and year of publication, 67 journal articles, 4 chapters, 3 reports, a book, and a proceeding were selected. The review results showed that the amount of heartwood was influenced by its position in the tree, tree variations, such as age, growth rate, and species/genera, as well as the growing conditions, including location and environmental factors. It is also affected by various silvicultural treatments, namely spacing, thinning, pruning, fertilization, and irrigation. These findings indicate that the amount of heartwood is more controlled by the diameter of the wood than the age. Furthermore, several studies reported that the growth rate of sapwood rings was significantly higher than the heartwood rings, which can reduce the quality of solid wood used for construction. Therefore, it is necessary to control the proportion of heartwood through genetics and environmental factors, especially genetics, which has more effects on the growth rate of tree diameter than environmental factors. Previous studies revealed that tree diameter has a weak or negative correlation with other wood properties, such as basic density, color, and wood stiffness, which are generally important in global markets. It is important for breeders to concentrate on using species with superior genetic variations as well, especially the highly demanded traits in further studies.

Keywords: heartwood formation; heartwood variation; silvicultural treatment; genetic effect; environmental effect

INTRODUCTION

The majority of tree species have two histologically similar, but physiologically different xylem zones, namely sapwood and heartwood. Sapwood contains living cells that are physiologically active as well as reserve materials located in the outer zone. The outer ring allows the transport of water and minerals from the roots to the cambium and leaves. Meanwhile, heartwood is located in the inner zone of the xylem, and it is physiologically inactive in terms of water conduction (Pinto et al. 2004).

Piqueras et al. (2020) revealed that the formation of heartwood is the final step in the life cycle of ray cells. Furthermore, these cells undergo metabolic changes in the transition zone between sapwood and heartwood before death, which leads to increased synthesis of secondary metabolic compounds, such as extractives. Pande (2013) also stated that the formation process is a natural growth regulation mechanism that is associated with tree development. The process also helps to maintain optimal sapwood volume (Taylor et al. 2002).

Heartwood and sapwood have different properties and proportions in the trunk, which have a significant effect on wood utilization. The presence of heartwood is often used to determine the wood value (Yang et al. 2020). In pulping, it has a negative effect because its extractives can affect the process and properties of the final product. Meanwhile, for solid wood applications, the properties of heartwood and sapwood affect the drying, durability, and aesthetic value of panels and furniture. Although there is a large color difference between the two zones of the xylem, the selection of wood components based on color also plays an important role in some applications (Pinto et al. 2004).

Meunpong et al. (2021) revealed that differences in humidity, wind exposure, climate area, elevation, and aspect are closely associated with the width of heartwood. Silvicultural practices can also affect its formation and quality, for example, proper thinning and fertilization promote stem growth, which accelerates the formation (Yang et al. 2020). The content of heartwood and sapwood varies between and within species. It is also associated with growth rates, stands, individual tree biometric features, site conditions, and genetic control (Pinto et al. 2004). A better understanding of the heartwood formation process helps to control and maintain its production (Taylor et al. 2002). Therefore, this study aims to review the factors that cause variation in heartwood proportion. The results are expected to help foresters and farmers plan or control its formation based on the end-use of wood, especially in construction.

MATERIALS AND METHODS

While conducting this review, we performed a semi-systematic or narrative review. We explored several databases such as Scopus, Science Citation Index, Science direct, Google Scholar, and ResearchGate, to identify and to download the abstracts, chapters, reports, books, proceedings, review articles and research papers related to the factors influencing variations in the heartwood proportion for solid wood. The search queries used during searching of information were: “heartwood variability”, “heartwood proportion”, “tree age of heartwood”, “growth rate of heartwood”, “silvicultural treatment of heartwood”, “genetic effect of heartwood”, and “environmental effect of heartwood”. The publication years were restricted from 1970 to 2022. Subsequently, a total of 159 documents (146 journals, 6 chapters, 4 reports, 2 books, and a proceeding) were found from the databases, but they were reduced to 67 journal articles, 4 chapters, 3 reports, a book, and a proceeding, based on the criteria of topic or title, objectiveness, and year of the publication. Both published and unpublished articles, such as journal articles, chapters, reports, books, and proceedings related to the heartwood formation and heartwood variation were grouped (Table 1). They were then analysed using qualitative analysis to identify, analyse and report patterns in the form of themes in a text.

RESULTS AND DISCUSSION

Figure 1 shows the various factors that affect the proportion of heartwood based on the review, namely 1) within tree variation (position), among tree variation, such as age, growth rate, and species/genera/family; 2) growing conditions, namely site and environment, and 3) silvicultural treatment, including spacing, thinning, pruning, irrigation, and fertilization. Furthermore, they can be divided into two groups based on their effect, namely into direct, and indirect categories. Direct factors affect the proportion of heartwood directly, while the indirect variants affect heartwood through other intermediaries. The results showed that all the aforementioned influential factors were controlled by genetic and environmental factors, as shown in Table 2.

Table 1. Reviewed publications.

Theme	Number of publications	References
Heartwood, sapwood, heartwood formation, positive and negative impacts of heartwood	6	Meunpong et al. 2021, Pande 2013, Pinto et al. 2004, Piqueras et al. 2020, Taylor et al. 2002, Yang et al. 2020
Within tree variation	24	Bamber 1976, Björklund 1999, Brix and Mitchell 1983, Climent et al. 1993, 2003, Fernandez-Solis et al. 2018, Gominho et al. 2001, Gominho and Pereira 2000, 2005, Hillis 1987, Knapic et al. 2006, Knapic and Pereira 2005, Miranda et al. 2006, Morais and Pereira 2007, Moya et al. 2014, Pinto et al. 2003, 2004, Rink and Phelps 1989, Sellin 1994, 1996, Stokes and Berthier 2000, Wilkins 1991, Wilkes 1991, Yang and Murchison 1992
Among tree variation	31	Anish et al. 2015, Anoop et al. 2014, Bamber 1976, Bamber and Fukazawa 1985, Bhat 1995, Bradbury et al. 2005, 2011, Climent et al. 2002, Fernandez-Solis et al. 2018, Gominho et al. 2001, Gominho and Pereira 2000, 2005, Hazenberg and Yang 1991, Hillis 1987, 1972, 1987, Karkkainen 1972, Miranda et al. 2006, 2014, Morais and Pereira 2007, Moya and Munoz 2010, Nicholas et al. 1994, 2007, Pillai et al. 2013, Pinto et al. 2004, Sotelo Montes et al. 2008, Wadsworth and Gonzales 2008, Wilkins 1991, Woeste 2002, Yang and Hazenberg 1991, Yang and Murchison 1992
Growing condition	7	Bhat 2000, Bhat et al. 2004, Crespo et al. 2008, Kjær et al. 1999, Kokutse et al. 2004, Perez and Kanninen 2003, Thulasidas and Bhat 2009
Silvicultural treatment (site and environmental)	14	Bergstrom et al. 2004, Gominho and Pereira 2005, Langstrom and Hellqvist 1991, Miranda et al. 2003, 2006, Morling and Vallinger 1999, Moya and Perez 2008, O'Hara 2012, Pérez and Kanninen 2005, Pimentel et al. 2008, Ramanan et al. 2018, Santos et al. 2020, Vallejos et al. 2015, Viquez and Perez 2005
Genetic x environmental	13	Bradbury et al. 2011, Bush 2011, Ericsson and Fries 1999, Freitas et al. 2019, Harrison 1975a, 1975b, Kurinobu et al. 1992, Miyamoto et al. 2016, Nakada 2007, Nicholas et al. 2007, Rodríguez-Pérez et al. 2022, Searle and Owen 2005, Zobel and Jett 1995

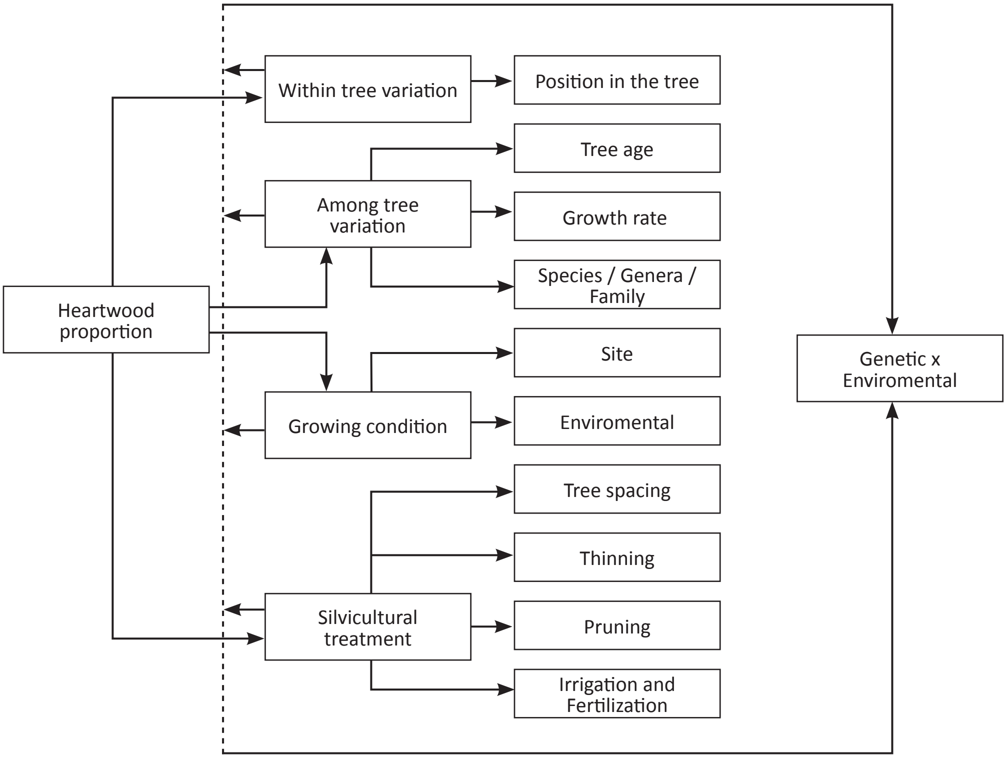

Figure 1. Factors that affect the proportion of heartwood.

The heartwood percentage has significantly decreased from base of the stem to top of the stem, with the highest value at the base part. This is a general trend of within-tree variation in heartwood proportion and has been reported for many hardwood and softwood species, such as *Picea abies* (L.) Karst (Sellin 1994, 1996), *Juglans nigra* L. (Rink and Phelps 1989), *Acacia melanoxylon* R.Br. (Knapic et al. 2006), *Pinus pinaster* Aiton (Stokes and Berthier 2000, Pinto et al. 2003, 2004, Knapic and Pereira 2005), and *Pinus canariensis* C.Sm. ex DC. (Climent et al. 1993, 2003). In addition, it was also found in *Eucalyptus globulus* Labill. wood from Chamusca, Penamacor, Odemira, Quinta do Paço, Crato, Sertã, V. F.Xira, Azambuja, and Obidos in Portugal (Gominho and Pereira 2000, 2005, Morais and Pereira 2007, Miranda et al. 2006), *Eucalyptus hybrid urograndis* (Gominho et al. 2001), *Eucalyptus grandis* W.Hill. (Wilkins 1991), and *Tectona grandis* L.f. (Fernandes-Solis et al. 2018).

Corresponding to the fact that the development of the inner heartwood of trees in the lower part correlates with tree growth, the greater part is larger in the lower trunks of large trees (Gominho and Pereira 2005). In previous studies, some authors also reported that there were positive or negative correlations with regard to the impact of growth rate and size on heartwood formation (Hillis 1987, Wilkes 1991, Wilkins 1991, Pinto et al. 2004). In addition, the distribution of heartwood at a certain height and age is not present. Gominho and Pereira (2000) observed that the proportion of *E. globulus* heartwood at the age of nine had disappeared by about 60% of the total tree height in Penamacor and by 74% in Chamusca and Odemira. Heartwood content was also absent in the relative height levels at 65% in *E. hybrid urograndis* (5.6 years old) from Brazil (Gominho et al. 2001) and 90% of the total tree height in *E. globulus* (12 to 15 years old) from Central Portugal (Morais and Pereira 2007).

Furthermore, the thickness of sapwood increased from bottom to top and at a certain height sapwood thickness is the same or stable. Moya et al. (2014) assumed that the thickness of sapwood for different tree ages of *T. grandis* is stable with height, varying from 2 to 5 cm in thickness. Miranda et al. (2006) reported that the area and width of the sapwood showed little variation due to the small thickness of the sapwood at the bottom of the trunk which contains a lot of heartwood. The opposite pattern was found at the end of the trunk, where the thickness of the sapwood was greater, which made the variation of the sapwood greater than at the base of the tree. This variation trend in the tree was also previously obtained in *E. globulus* (Gominho and Pereira 2000, 2005), *E. grandis* (Bamber 1976, Wilkins 1991), *A. melanoxylon* (Knapic et al. 2006), *Pinus contorta* Dougl. ex Loud. (Yang and Murchison 1992), *P. pinaster* (Knapic and Pereira 2005, Pinto et al. 2003, 2004), *Pinus silvestris* L. (Björklund 1999), and *Pseudotsuga menziesii* Mirb (Brix and Mitchell 1983). Fernandez-Solis et al. (2018) stated that a tree regulates its physiological processes to

Table 2. List of research related to the factors causing variation in the proportion of heartwood in several wood species.

Factors		Effect on the heartwood	Relationship	Species	References
Within tree variation	Position in the tree (bottom to top of the tree)	Direct	Negative	*Acacia melanoxylon* R.Br., *Eucalyptus globulus* Labill., *Eucalyptus grandis* W.Hill., *Eucalyptus hybrid urograndis, Juglans nigra* L., *Picea abies* (L.) Karst., *Pinus canariensis* C.Sm. ex DC., *Pinus pinaster* Aiton, *Tectona grandis* L.f.	Climent et al. 1993, 2003, Fernandes-Solis et al. 2018, Gominho and Pereira 2000, 2005, Gominho et al. 2001, Knapic and Pereira 2005, Knapic et al. 2006, Miranda et al. 2006, Morais and Pereira 2007, Pinto et al. 2003, 2004, Rink and Phelps 1989, Sellin 1994, 1996, Stokes and Berthier 2000, Wilkins 1991
Among tree variation	Tree age	Direct	Positive	*Picea abies, Populus tremuloides* Michx., *Tectona grandis*	Fernandez-Solis et al. 2018, Sellin 1994, 1996, Yang and Hazenberg 1991a, 1991b
	Growth rate	Direct	Positive	*E. globulus, E. grandis, Eucalyptus tereticornis* J.E.Sm., *J. nigra, P. canariensis, Pinus contorta* Dougl. ex Loud., *T. grandis*	Bhat 1995, Climent et al. 2002, Gominho and Pereira 2000, 2005, Miranda et al. 2006, Morais and Pereira 2007, Pillai et al. 2013, Wilkins 1991, Woeste 2002, Yang and Murchison 1992
			Negative	*Cryptomeria japonica* (L.f.) D.Don., *Picea abies, Pinus sylvestris* L.	Hillis 1987, Karkkainen 1972
	Species/ Genera/ Family	Direct	Positive	*E. globulus*	Miranda et al. 2014
Growing condition	Site	Direct	Positive	*T. grandis*	Anish et al. 2015, Kjær et al. 1999
		No effect		*T. grandis*	Bhat 2000, 2009, Bhat et al. 2004
	Environmental	Direct	Positive	*T. grandis*	Crespo et al. 2008, Kokutse et al. 2004, Perez and Kanninen 2003
Silvicultural treatment	Tree spacing	Direct	Positive	*E. globulus, T. grandis*	Gominho and Pereira 2005, Miranda et al. 2003, Pimentel et al. 2008
		No effect		*Eucalyptus grandis × E. urophylla hybrid*	Santos et al. 2020
	Thinning	Direct	Positive	*Gmelina arborea* Roxb.	Vallejos et al. 2015
		Indirect	Positive	*Acacia mangium* Willd, *T. grandis*	Pérez dan Kanninen 2005, Ramanan et al. 2018
	Pruning	Direct	Positive	*P. sylvestris, T. grandis*	Langstrom and Hellqvist 1991, Viquez and Perez 2005
		No effect		*P. sylvestris, Sequoia sempervirens* (D.Don) Endl.	Bergstrom et al. 2004, O'Hara 2012
	Fertilization and irrigation	Direct	Positive	*E. globulus*	Miranda et al. 2006
		No effect		*P. sylvestris*	Morling and Valinger 1999

keep the sapwood area, holding the formation of heartwood during the first years of growth, because the tree uses its physiological processes to achieve a greater height quickly. Miranda et al. (2006) stated that in several species, sapwood width increased with tree age in absolute measurements and declined in relative measurements.

Among Tree Variation

Among trees, heartwood variation occurs partly because of the age of the wood and the rate of growth. The heartwood proportion increases with the increasing age of tree (Fernandez-Solis et al. 2018). Moya and Munoz (2010) reported that heartwood percentage at diameter and breast height (DBH) of a 14-year-old *Swietenia macrophylla* King. grown in Costa Rica was 41.2%. The values of heartwood percentages were lower compared to *S. macrophylla* (the total height ranged from 13 to 20 m), with a heartwood content of 89% at the bottom area from the Olavakkode Research Range, India (Anoop et al. 2014). In addition, Wadsworth and Gonzales (2008) also observed that 40-year-old *S. macrophylla* trees obtained heartwood volumes ranging from 70 to 80%. In *Eucalyptus* species, Gominho and Pereira (2000) reported that the heartwood percentage of a 9-year-old *E. globulus* was 43% of the cross-sectional area at breast height. This value was lower than the percentage of heartwood reported by Morais and Pereira (2007) of 12-15-year-old *E. globulus* trees amounting to 54%, and by Miranda et al. (2006), of 18-year-old *E. globulus* trees amounting to 77% of the cross-sectional area of the tree. Gominho et al. (2001) also reported that heartwood content is related to the tree's age. Furthermore, heartwood growth increased with the age of the tree until a maximum tree height was reached (Anish et al. 2015, Fernandez-Solis et al. 2018). This suggests the role of tree age in increasing the proportion of heartwood, as already mentioned for *E. grandis* (Bamber 1976), *P. menziesii* (Hillis 1987), *Pinus tremuloides* Michx (Yang and Hazenberg 1991), *P. pinaster* (Pinto et al. 2004), and *Abies balsamea* (L.) Mill. (Hazenberg and Yang 1991). It seems clear that heartwood develops with tree ageing, as has been shown in several reviews of heartwood formation (Hillis 1972, 1987, Bamber and Fukazawa 1985).

On the other hand, Gominho and Pereira (2005) stated that heartwood proportion increased with tree diameter. Pillai et al. (2013) also reported that growth rate affected heartwood proportion due to increased radial growth. Miranda et al. (2006) reported that the heartwood area of *E. globulus* was positively correlated with the radial growth of the stem. As reported by Gominho and Pereira (2000), a positive correlation was found between heartwood content and growth for 9-year-old *E. globulus* trees. Wilkins (1991) found that the percentage of heartwood in *E. grandis* was higher in the faster growing trees. Similar results were also obtained for other species, i.e., *T. grandis* (Bhat 1995), *P. contorta* (Yang and Murchison 1992), *J. nigra* (Woeste 2002), and *P. canariensis* (Climent et al. 2002). However, some authors have reported reverse correlations for species such as *P. silvestris, P. abies*, and *Cryptomeria japonica* (L.f.) D.Don. (Karkkainen 1972, Hillis 1987). In several previous studies, the proportion of heartwood was more influenced by tree diameter than tree age, as reported by Miranda et al. (2006) in *E. globulus* wood and by Bradbury et al. (2011), Nicholas et al. (1994), and Nicholas et al. (2007) in *A. melanoxylon*. This is very beneficial for tree breeding programs to select bigger diameter trees. In addition, tree diameter is also known to be positively correlated with the color of heartwood in *A. melanoxylon* and *Calycophyllum spruceanum* (Benth.) K.Schum. (Bradbury et al. 2005, Sotelo Montes et al. 2008).

Apart from the effect of tree age and diameter, family differences also affect the proportion of heartwood as reported by Miranda et al. (2014). They stated that family affects this relation of heartwood dimension with tree growth, as shown by the differences between genetic and phenotypic correlations. Heartwood proportion of cross-sectional area on average amounted to 41% with significant between-family variation (*P*=0.016), ranging from 27 to 53%. In addition, there were also important within-family differences with coefficients of variation of the mean between 4 and 48%.

Growing Condition (Site and Environmental)

The variation in heartwood percentage is a major parameter studied in relation to the site and environmental conditions. Thulasidas and Bhat (2009), who examined teak trees from wet and dry areas in India, revealed that there was no significant difference in the proportion recorded at the sites. This finding is consistent with Bhat (2000) and Bhat et al. (2004), where similar results were also obtained. However, large differences were found in relation to the tree and heartwood diameter, where larger values were recorded in the wet sites. Crespo et al. (2008) and Perez and Kanninen (2003) reported that a larger proportion was obtained in the dry areas of Ecuador and Costa Rica compared to the wet areas.

In the wet regions, tree growth occurred almost continuously for 8-12 periods, while growth was more intense in dry areas containing inert material, and heartwood was produced in a shorter growing season. An increase in diameter often occurs in wet locations, but the development of the xylem tissue was slower compared to the dry region where tree growth stopped for months and led to annual heartwood formation. Kjær et al. (1999) revealed that the proportion obtained in 5 countries, namely in Puerto Rico, India, Mexico, Indonesia, and Ghana was significantly different. Furthermore, the values recorded in Indonesia and Ghana were lower than those of India and Mexico. The amount of rainfall can also affect the proportion of heartwood. Kokutse et al. (2004) revealed that higher amounts of annual rainfall, such as 1,200-1,500 mm per year, were more favorable for tree growth and the wood content of teak growing in Togo.

Silvicultural Treatment

Several studies reported that silvicultural activities, such as widening tree spacing, thinning, and pruning have effect on the proportion of heartwood. This is in line with Gominho and Pereira (2005), who found that the content obtained in *Eucalyptus globulus* was affected by an increase in spacing. Furthermore, heartwood percentages of 45%, 52%, and 69% were obtained for 2x1 m, 3x2 m, and 3x3 m of distancing, respectively. Similar results were also obtained in previous studies for the *E. globulus* tree (Miranda et al. 2003). They reported that the use of 4x4 m, and 4x5 m spacing can reduce pulp quality and paper due to the high extractive content and low degree of delignification. This condition is associated with an increase in heartwood proportion along with diameter. Pimentel et al. (2008) used different patterns for teak wood in Brazil, namely 3x1.5 m, 3x2 m, and 3x2.5 m. The results showed that the 3x2 m, and 3x2.5 m spacing resulted in the largest proportion. Moya and Perez (2008) also revealed that the heartwood percentage in 14-year-old teak trees was related to the stand density as well as diameter. However, Santos et al. (2020) stated that plant spacing affects growth rate, but has no effect on the xylem tissue content of eucalyptus clonal plantation (*E. grandis* × *E. urophylla* hybrid).

Several studies also reported that thinning has a significant effect on the percentage of heartwood. Vallejos et al. (2015) investigated the effect of thinning intensities of 60%, 70%, and 80% on the percentage and diameter of the tissue in a 8-year-old *Gmelina arborea* Roxb. tree. The results showed that the highest proportion was obtained with the 80% treatment, while there was no significant difference between the 60% and 70% intensities. However, thinning does not always increase the proportion, because it only has a temporary effect. Ramanan et al. (2018) stated it does not have a direct association with the increase in heartwood content, but the mechanism functions through an increase in tree diameter caused by the treatment. The result is based on thinning experiments carried out on 7-year-old *Acacia mangium* Willd. stands from Kerala, India. These findings are consistent with those of Pérez and Kanninen (2005), who used different treatments for teak plantations in Costa Rica. The results showed the impact of thinning on wood properties, such as heartwood percentage and density, tended to be temporary in nature, and the effects were nullified after a long period. Furthermore, the content obtained in young teaks of 4 and 6 years with light thinning was slightly higher compared to the moderate and heavy categories. The moderate treatment involves the removal of 25% of the original trees in the 4th year and another 25% in the 5th year or only 40% in the 4th year, while 60% was removed in the 4th year for the heavy treatment.

Previous studies revealed that pruning can also increase the proportion of heartwood. Langstrom and Hellqvist (1991) conducted 4 different pruning treatments on a 25-year-old pine stand in Jadraas, Central Sweden, namely: (1) unpruned control (UC); (2) unilateral pruning (UP), where all live branches were cut on one side of the crown; (3) pruning from below (PB), where all live circles were removed below the 1981, leaving the top four, and the expanding current circle was kept intact; and (4) pruning from above (PA), including all branches from 1984-1982 as well as the tops of others (segments 1985-1982 and its side branches) in the live circles below 1982, as shown in Figure 2. The result showed that UP reduced growth along the stem, while PB caused greater reductions in the lower stem. Furthermore, PA increased radial growth above and decreased development below the pruned crown fraction. The branch bottom area (needle biomass) of the developing new whorls was increased in the trees were pruned from above, and height growth was only slightly affected by the treatments. The treated groups had a total growth volume loss of 24-33% during the study period, compared to the controls. The pruning caused a decline in the conducting sapwood area as well as an increase in the non-conducting heartwood at breast level. The results also showed that the homeostatic adjustment was still incomplete after four growing seasons, except for the upper whorls, which developed after the process.

Viquez and Perez (2005) carried out different treatments on four experimental plants with a density of 3.5x3 m and an age of 2.2 years, namely the control group, as well as pruning at a height of 3 m, 4 m, and 5 m. The second treatment was carried out at the age of 5.2 years to a height of 7 m in all groups, except the control. The results showed that larger volumes of heartwood were found at 3 m, but they were not statistically different from the control group. The treatment also produced 142% more volume than 4 and 5 m, as well as 35% more than the control group.

Other studies stated that pruning has no effect on the heartwood proportion of trees. Bergstrom et al. (2004) reported that there were no changes in the proportion or the number of growth rings for five growing seasons. The study was carried out on 50 years old *P. sylvestris* trees from a natural stand, north-west of Umeå, belonging to the Swedish University of Agricultural Sciences. However, increases can still occur over a longer period. This indicates that pruning is not a practicable silvicultural method for regulating heartwood formation in *P. sylvestris*. These findings are consistent with O'Hara (2012), stating that it had no effect on the proportion. The results revealed that the effect of the treatment on heartwood was minimized six years after pruning. It was observed that the pruned trees have reestablished their crown foliage, and required the same amount of sapwood for water transport as the unpruned variants.

Apart from tree spacing, thinning and pruning, fertilization, and irrigation, several factors were also

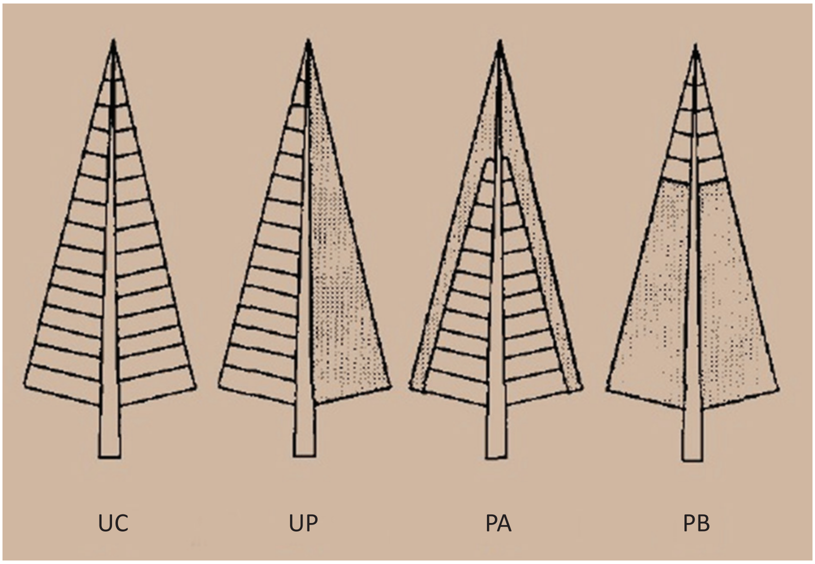

Figure 2. Schematic drawing of the treatment showing the appearance of the tree after pruning in June 1985 (Langstrom and Hellqvist 1991).

reported to affect the proportion of heartwood. Miranda et al. (2006) revealed that an 18-year-old *E. globulus* wood treated with fertilization and irrigation (FI) in the first 6 years of growth showed a larger volume than the control (C), namely 65.6% and 55.6%, respectively. Furthermore, in the FI and C trees, the tissue's distribution decreased by 77.7% and 67.6% at the bottom, as well as by 7% and 4.8% at a height of 29.3 m, respectively. Morling and Vallinger (1999) reported different results in *P. sylvestris*, where the increase in heartwood area after fertilization and thinning was not statistically significant, while sapwood thickness increased significantly. The number of growth rings in the heartwood at breast height was not influenced by treatment.

Relationship Between Genetic and Environmental Factors on Heartwood Proportion

Tree growth is influenced by genetic factors as well as environmental conditions, and this interaction affects the nature of the wood formed (Freitas et al. 2019). The heartwood, bark, and pith tissue in tree formation are often influenced by the environment, but are also under strong genetic control (Zobel and Jett 1995). Several studies have explored their effect on wood properties (Bradbury et al. 2011, Li et al. 2018, Rodríguez-Pérez et al. 2022). However, the factor that controls the proportion of heartwood in trees is still discussed the most. Freitas et al. (2019) stated that the environment did not affect the xylem content of *Eucalyptus grandis* × *Eucalyptus urophylla* clones, but it was influenced by clonal effects. Similar results were also obtained in *Eucalyptus cladocalyx* F.Muell (Bush 2011) and *P. sylvestris* (Ericsson and Fries 1999), where the proportion of heartwood was affected by genetics. Miyamoto et al. (2016) reported that its color was mainly controlled by genetics rather than the environment based on the spatial autocorrelation analysis. Previous studies also revealed that the color was influenced genetically, and there were clear differences between the clones (Kurinobu et al. 1992, Nakada 2007). However, other studies showed that *C. japonica* was affected by environmental factors, such as water and soil type (Kansai Regional Office of Breeding, Forest Tree Breeding Center 1977), and a similar effect was observed in *A. melanoxylon* (Harrison 1975a, Harrison 1975b, Nicholas et al. 2007).

Based on the results, genetic factors have more influence on the proportion of heartwood than the environment. Bradbury et al. (2011) reported that the absolute amount of heartwood in blackwood was mainly affected by the growth rate, but the relative amount was related to variations in the width of the sapwood, which has a strong genetic basis. This finding indicates that increasing the growth rate as well as the heartwood content through genetic selection can be carried out independently. Several studies also reported that its percentage was influenced by genetic control in other *Acacia* species (Searle and Owen 2005). Tree diameter is more significant in the initiation of heartwood production in *A. melanoxylon* compared to age (Bradbury et al. 2011). Increasing the growth rate helps to increase the proportion, although it has a minimal effect, and can also have a negative impact on other properties, such as color, basic density, and stiffness. Breeding efforts also need to concentrate on increasing provenance collections and native families, which increase their best genetic base.

CONCLUSIONS

A high proportion of heartwood will increase the quality of timber for construction, as it is more durable and more aesthetic. In this study, heartwood proportion varied greatly in each species and this affected the end use of the wood. Furthermore, the influential factors include within tree variation and among tree variation, such as age, growth rate, and species/genera as well as growth conditions, including location and environment. The percentage of heartwood was also affected by various silvicultural treatments, namely spacing, thinning, pruning, fertilization, and irrigation. The amount of heartwood in a tree can be controlled by the growth diameter, because it has more influence than age. The faster the growth rate of a plant, the greater the effect on the proportion. Previous studies reported that its increase also led to the formation of larger sapwood rings. This indicates that the growth rate caused a decrease in the percentage of heartwood. Efforts through genetic and environmental control are expected to produce a tree with faster heartwood growth than sapwood thickness, especially genetic factors, which have more influence than the environment. Therefore, further studies need to investigate the genetic factors that influence or play a role in controlling these properties. Apart from the proportion of heartwood, which is often considered in global markets, other wood properties, such as basic density, color, and stiffness are also important. Therefore, it is necessary for tree breeders to also concentrate on other traits that are of interest for further studies.

Author Contributions

RA conceptualized and designed the study, derivated models, analyzed data and wrote the original draft of the manuscript, RA and GL collected and processed the data, SNM, JPGS and GL checked and edited the manuscript, GL performed methodological discussions and gave final approval of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Wild Animal Attacks on Humans in Croatia

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ABSTRACT

Contacts between humans and wild animals are common and frequent, but very rarely end in attacks by wild animals on humans. The aim of this paper is to identify the circumstances of wildlife attacks on humans in Croatia, while the idea behind the paper is that a better understanding of interactions between humans and wildlife should subsequently lead to fewer attacks. As there is no database on attacks by wild animals on humans in Croatia, the data for this analysis was collected from scientific publications, media, and relevant reports. The variables analysed refer to the type of attacks and the victims of the attacks. The results showed that there were 33 attacks by wild animals on humans in Croatia in the period from 2005 to 2023; the most frequent were attacks by wild boars, brown bears, and grey wolves. The characteristics of the attacks differ depending on the animal that perpetrated the attacks, but there are some common characteristics: the attacks mainly took place in rural areas; the victims were mostly male and over 40 years old; in almost all attacks they suffered minor injuries; and during the attacks the victims were in most cases hunting, walking dogs or herding livestock. The data obtained from this analysis can be used to develop preventive measures and define appropriate behaviour in areas with wildlife.

Keywords: wild boar; brown bear; grey wolf; injuries; safety

INTRODUCTION

In the recent decades, the number of attacks by large carnivores (e.g., wolves, bears) on humans has increased, both in industrialised and developing countries (Penteriani et al. 2016, Bombieri et al. 2023). This increase has been caused by several factors: intentional feeding of wildlife (Penteriani et al. 2016), unintentional feeding by leaving food in litter in places accessible to wildlife (Nowak et al. 2021), and the increase of people participating in outdoor activities (Bombieri et al. 2019). Most attacks by large carnivores occur between late spring and early autumn, which coincides with the time when people are most engaged in outdoor activities (Penteriani et al. 2016). In addition to environmental factors, attacks are also influenced by socio-economic factors: large global studies have shown that victims of wildlife attacks in developed or urban societies are usually engaged in recreational activities such as hiking or visiting national parks. In developing countries, i.e., in rural societies, victims mainly perform their daily tasks during attacks, such as working in the fields, herding livestock or picking forest fruits (Löe and Röskaft 2004, Bombieri et al. 2023).

Although, in general, the number of wild animals and the number of attacks by wild animals on humans is constantly increasing, the probability of such an attack is very low (Penteriani et al. 2016, Bombieri et al. 2019). However, if such an attack does occur, the consequences are unpleasant, can be long-lasting and affect both the victim and the surrounding community (Löe and Röskaft 2004). Overestimating the risk of attacks by wildlife can lead to anxiety and an unjustified fear of encountering wildlife (Moscardo et al. 2006, Penteriani et al. 2016), which can subsequently lead to biophobia, an irrational fear of spending time in nature due to the possibility of being attacked by wildlife (Goulding and Roper 2002). Furthermore, excessive fear of attack by wildlife can have a negative impact on conservation efforts by creating negative public attitudes towards these endeavours (Löe and Röskaft 2004).

Since only a few people, such as hunters, come into frequent or direct contact with wildlife or have much experience with it, the attitudes and opinions of the general population towards wildlife are mainly shaped by the media (Bombieri et al. 2019). Attacks by wild animals on humans are of great interest to the media (Löe and Röskaft 2004), although in Europe, for example, the likelihood of such an event actually occurring is low (Arbieu et al. 2021). Unfortunately, the media often approach this topic in an unobjective way, which includes speculations and exaggeration (Ambarli 2019). Instead of educating the public on how to behave when encountering wild animals, they are often unnecessarily frightened by attacks that are actually very rare (Bombieri et al. 2019), because "media coverage of such attacks generally includes sensational texts and dreadful pictures appealing more to the public's emotions than their logic" (Penteriani et al. 2016). As an example of biased reporting about wild animal's attacks on humans, Arbieu et al. (2021) note the death of a British female tourist in Greece in 2017, who deceased after being attacked by an animal, at first unclear whether it was a dog or a wolf. This tragic event attracted a lot of media attention (Iliopoulos et al. 2022), and most Greek media blamed the wolf for the death of the tourist, believing that an attack by a wolf on a human would attract more public attention than an attack by a dog (Arbieu et al. 2021).

To avoid reactions that are "based on myth, ignorance, exaggeration, and sensationalism" (Floyd 1999), Arbieu et al. (2021) argue that when reporting about wildlife attacks to public, a greater role should be given to scientists and experts who approach the topic objectively and utilise interdisciplinary knowledge to objectively present both positive and negative facts about these events to the public. Löe and Röskaft (2004) state that the circumstances of attacks by wild animals on humans have only recently begun to be researched and that the role of scientists and experts is crucial, considering that aggression by wild animals towards humans is a complex problem due to various environmental and social factors (Nowak et al. 2021). These factors include the behaviour of the animals prior to the attack, the demographic characteristics of the victims, the seasonality of the attacks and the activities that the victim was engaged in prior to the attack. A comprehensive understanding of the circumstances or patterns of attacks that have already occurred can help prevent future attacks (Behdarvand and Kaboli 2015).

The literature review shows that attacks by wild animals on humans have rarely been studied in the context of tourism or recreational activities (Moscardo et al. 2006); notable exceptions are Durrheim and Leggat (1999), Thompson et al. (2003), Toovey et al. (2004), Moscardo et al. (2006), Penteriani et al. (2017) and Ikeda et al. (2019). In Croatia, most of the studies on attacks by wild animals on humans were conducted by medical professionals (see Lojkić et al. 2009, Šprem et al. 2013, 2014, Vodopija et al. 2016, 2021, Sarajlić et al. 2022, Tunjić Pejak et al. 2022), so, as expected, they mainly analysed the medical aspects of the attacks. More comprehensive, interdisciplinary studies analysing different aspects of the attacks are much rarer and have been conducted as part of larger studies involving experts from several countries who examined one species (see Bombieri et al. 2019) or a guild (see Penteriani et al. 2016, Bombieri et al. 2023).

This paper aims to determine the circumstances of wild animal attacks on humans in Croatia: which animals most frequently attack humans, the spatial and temporal circumstances of the attacks, the activities of the victims during the attacks and the consequences of the attacks. In addition, several wild animal species are analysed in this paper. In this way, a broader and more detailed picture of wildlife attacks will be obtained.

MATERIALS AND METHODS

Löe and Röskaft (2004) argue that sources of data on wolf attacks on humans are "few, often fragmentary and difficult to find". In general, this statement could also be applied to data on other wild animals' attacks on humans. In Croatia, there is no database on attacks by wild animals on humans. There is also no standardised system for collecting data on such incidents in other countries (Moscardo et al. 2006). There are several systems in the USA, Canada, Sweden and India, but they are not linked or comparable as they do not collect identical or standardised information (Löe and Röskaft 2004).

Therefore, the data for this paper was collected on the internet, using common search engines such as Google and the literature database Google Scholar. Word combinations in Croatian and English were entered into the search engines, e.g., "attack", "human" and specific wildlife species. This method of data collection has been used in literature that studied attacks by wild animals on humans, such as Penteriani et al. (2016, 2017), Ambarli (2019) and Bombieri et al. (2019, 2023).

The variables analysed relate to the attacks and the victims of the attacks. The following information was collected about the attacks: (1) wildlife species involved; (2) location; (3) general location category; (4) date of attack; (5) time of attack; and (6) the number of animals involved in the attack. The following data were collected on the victims of the attack: (1) gender; (2) age; (3) group composition at the time of the attack (alone or in a group); (4) the activity of the victim at the time of the attack; (5) the circumstances of the attack; (6) the type of injuries; and (7) the nature of injuries (possible outcomes were fatal and nonfatal). The information collected was defined using variables from published papers such as by Tough and Butt (1993), Durrheim and Leggat (1999), Herrero and Higgins (2003), Mayer (2013) and Bombieri et al. (2019, 2023).

In the data processing, three groups were formed depending on the location of the attack (Group 1 = urban area, Group 2 = rural area, and Group 3 = suburban area). For the variable relating to the time of the attack, two groups were formed (Group 1 = morning, and Group 2 = afternoon). Two groups were also formed according to the season in which the attack took place (Group 1 = spring-summer period, and Group 2 = autumn-winter period). In terms of the number of endangered individuals, two groups were formed (Group 1 = individual, and Group 2 = group of individuals). For the activity of the affected individuals, three groups were formed (Group 1 = recreation/hiking, Group 2 = hunting, and Group 3 = daily work activities).

Data on 33 attacks was collected, for the period from 2005 to 2023. In cases where one animal attacked more than one person on the same day, these attacks were recorded as one attack. The primary processed data was entered and systematised in the Microsoft Excel® software package, and further data analysis was carried out using statistical software Statistica® (TIBCO Software Inc., version 14, Palo Alto, CA, USA) and SPSS® (IBM-SPSS Inc., version 28, Armonk, NY, USA). The nonparametric Chi-Square Test of Independence was used to determine whether there is an association between categorical variables by comparing the type of different species with the above-mentioned categorical variables such as location of the attack, time of the attack, etc.

RESULTS

The following section presents the attacks of wild animals on humans in Croatia. It analyses the conditions of the attacks and their consequences. The number of attacks by certain wild animal species per year is shown in Figure 1. The geographical distribution of wildlife attacks is shown in Figure 2.

As the p-value is greater than the significance level we chose (p=0.05), no correlation was found between the wildlife analysed and the location of the attack (χ^2=11.375, p=0.077).

Wild Boar (*Sus scrofa* L.)

Between 2007 and 2023, there were 14 attacks on humans by wild boar, which is the highest number of attacks by any wild animal included in this study. Details of these attacks can be found in Table A1 in the Appendix. The wild boar attacks took place throughout the whole territory of the Republic of Croatia. Nine attacks occurred in the continental part and five in the coastal part; 64.29% of the attacks took place in rural areas, 28.57% in suburban areas and 7.14% in urban areas. There were one to two attacks per year in the observed period, and in some years, such as 2008, 2013 and 2020, as well as in the period from the end of 2014 to the end of 2019, there were no attacks.

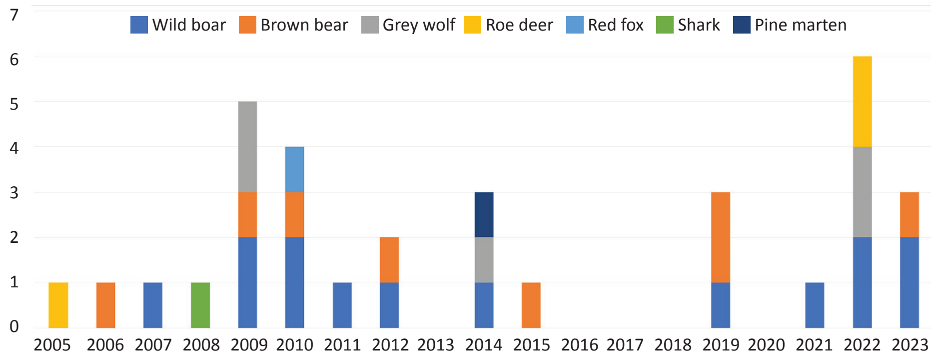

Figure 1. Wild animal attacks on humans (2005-2023).

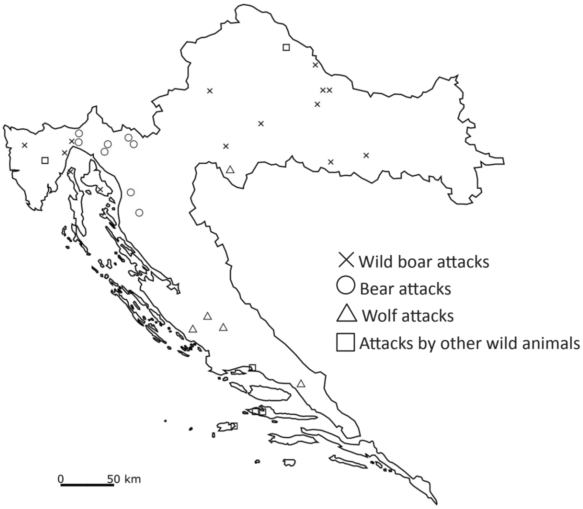

Figure 2. Geographical distribution of wild animal attacks.

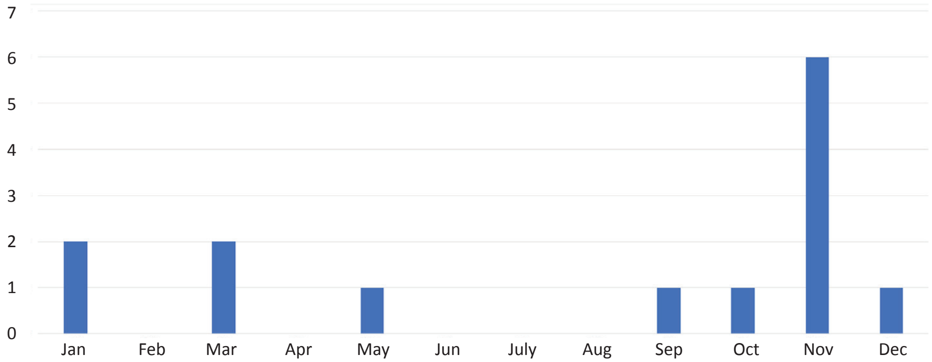

Figure 3. Wild boar attacks per month.

If we look at the distribution of attacks by month, we see that all attacks took place between September and early May, with most attacks (6 out of 14) occurring in November. Most of the attacks took place in the morning and before noon, with three attacks taking place in the afternoon. For all species in this analysis, no association was found between wildlife and time of the attack (χ^2=1.850, p=0.604), but a significant association was found in relation to time of the year (χ^2=13.645, p=0.003). It should be noted that brown bears and wild boars have a higher frequency of attacks in autumn and winter.

In all attacks, people were attacked by only one animal. Even in the attack where a female with juveniles was present, only one animal, the female, committed the attack. All attack victims were male; the age of the victims varied from 24 to 60 years, and the mean age was 47 years (M=47.45, SD=11.13). Most victims were not alone at the time of the attack but were in a group; this is to be expected as 57 % wild boar attacks occurred during hunting. Attacks where the victim was alone during the attack occurred in other situations, such as when cycling, herding livestock or gamekeeping. Regarding the circumstances of the attacks, it was found that in most cases (43% of attacks) the victims were attacked by a wounded animal, mainly while hunting or gamekeeping. The second most common attack circumstance is an attack due to a sudden encounter with an animal, and in one case the attack was initiated by a female with juveniles (Mrkić Modrić and Kućel Ilić 2022). The victims were mostly bitten or stabbed, and the injuries were mostly located in the lower part of the body, i.e., on the legs and sometimes on the hands. None of these injuries were fatal and the victims recovered after receiving medical treatment.

Brown Bear (*Ursus arctos* L.)

The next wild animal in terms of frequency of attacks on humans is the brown bear. According to available data, there were eight bear attacks on humans between 2006 and 2023.

All the attacks took place in two counties located in mountain (Dinaric Alps) region: Primorje-Gorski Kotar County, where most of the attacks took place, and Lika-Senj County; 87.5% of the attacks took place in rural areas and 12.5% in suburban areas. The attacks occurred once a year, except in 2019, when there were two attacks. Unexpectedly, the attacks took place between May and early January. Two attacks occurred in winter, one in December and one in January. The December attack occurred when the bear sanctuary staff entered the area where the bears live. The bear was irritated by the unusually high temperatures and attacked first the sanctuary volunteer and then the guard who tried to defend her (Nova TV 2006). When looking at the time of day of the attacks, no pattern can be found as the attacks occurred throughout the day. In five of the eight attacks, the reason for the attack was a sudden encounter with a female with juveniles. Similar to wild boars, the female committed the attack, and the juveniles were not involved.

All the victims were male, with the exception of the above-mentioned volunteer from the bear sanctuary. She is also the youngest victim of a bear attack, and the oldest was 82 years old at the time of the attack, while the mean age of the victims is 50 years (M=50.44, SD=21.06). Most victims were in a group at the time of the attack, and the activities they were most frequently engaged in during the attack were hunting (in 4 out of 8 cases) and dog walking. A marginally significant difference was found between the analysed wildlife attacks and the types of activities of the attacked individuals (χ^2=16.575, p=0.051). Furthermore, hunting activities were mainly associated with attacks by brown bears and wild boars, while everyday activities are more associated with attacks by wolves and other wild animals.

The victims suffered puncture and bite wounds to various parts of the body. In none of these attacks were the wounds fatal to the victims.

Grey Wolf (*Canis lupus* L.)

Besides wild boar and brown bear, the grey wolf is the third wild animal that most frequently attacked humans. In the period from 2009 to 2022, there were five wolf attacks on humans.

Of the five attacks, four occurred in the southern part of Croatia and one in continental Croatia, in Sisak-Moslavina County. The attacks took place at different times of the year, from February to September, and also at different times of the day, so it is not possible to find a temporal pattern of the attacks. In four attacks, humans were attacked by one animal, and in one attack, several animals were involved, although it is not possible to determine their exact number based on the available data and the victims' statements (Kuzmić 2009). A significant correlation was found between wild animals and the number of endangered individuals (χ^2=21.563, p=0.001). This specific result is influenced by the fact that attacks by wild boars and brown bears mainly affected groups of people, while attacks by wolves mainly affected individuals. Three attacks occurred while herding livestock, one while working in the yard and one while walking the dog. The circumstances of the attack were as follows: twice wolves attacked humans while attacking livestock; in one case the attack was perpetrated by a female with juveniles and in one by a rabid animal. In three attacks the wounds were minor and consisted mostly of bites and scratches on arms and legs. In the attack by a rabid wolf, the victim suffered severe injuries and it was necessary to amputate the left hand, nose, upper lip, and part of the upper jaw. The victim was also severely bitten on the right arm and leg (Lojkic et al. 2009).

Other Wild Animals

Except by wild boars, brown bears and grey wolves, people in Croatia have also been attacked by roe deer (*Capreolus capreolus*), red fox (*Vulpes vulpes*), pine marten (*Martes foina*), and shark. Details of these attacks can be found in Table A4 in the Appendix.

As shown in Table A4, two women were attacked by roe deer on Hvar in August 2022. The animal involved in the attacks was not hunted, so it is assumed that both attacks were perpetrated by the same animal. One victim suffered a minor stab wound and scratches to the legs and arms (Crnčević 2022b), the other a severe rib injury (Crnčević 2022a). The third deer victim was injured while hunting. The hunter was hit on the leg by a roe deer, resulting in a twisted knee and a tear of the anterior cruciate ligament (Šprem et al. 2013). The next animal that attacked humans was the red fox. The victim was attacked because he wanted to defend his dog from a fox and was bitten on the left leg (Regional Express 2010). One of the most serious animal attacks on humans was an attack by a shark on a diver. The causes of this attack are not known, but it is known that the victim was an experienced diver who was employed as a diving instructor by the Slovenian army. In this attack, the victim suffered serious injuries: a bite to the left leg, a torn calf muscle, a severely injured artery and nerves that move the foot, and disability after this attack (Express 2018). The least dangerous attacks were by pine martens. In Split in 2014, the same pine marten attacked and bit three people. Two victims were bitten when they tried to catch the animal by hand, and the third victim was attacked while riding a bicycle (Eterović 2014).

The most frequent attacks by wild animals on humans were snakebites. Between 1998 and 2019, 632 people were hospitalised due to snakebites. The highest number of hospitalisations was in 2001, when 58 people were hospitalised due to snakebites; in 2000, 57 people were hospitalised and in 2018, 40 people were hospitalised (Tunjić Pejak et al. 2022). There were three death cases due to snakebites in 2006, 2007 and 2013. Two of the victims were 69 and 80 years old, and the third victim was a child who was bitten by a viper during a picnic (Sarajlić et al. 2022).

DISCUSSION

The characteristics of the attacks differed between the attacks of different animals, but some common features were found: the attacks mainly took place in rural areas, the victims were mostly male and over 40 years old, in almost all attacks, the victims suffered minor injuries, and during the attacks, they were mainly hunting, walking dogs, or herding livestock. As mentioned in the introduction, attacks by wild animals in industrialised countries mainly occur during leisure time, whereas in developing countries they occur during work (Bombieri et al. 2023). In our analysis, 27% of attacks occurred during work and the rest during leisure activities.

Wild Boar (*Sus scrofa* L.)

Wild boars have been attacking humans since ancient times (Mayer 2013), probably because wild boars are one of the most common and widespread large mammals. It is estimated that there are more than 10,000 wild boars in Croatia today (estimate across all age and sex categories) (Gavran et al. 2019). In our analysis, wild boar attacks were the most numerous of all wildlife attacks, i.e., 14 out of 33 attacks were perpetrated by wild boars. Hunting can encourage wild boar attacks, as Šprem et al. (2013) showed in their analysis of wild animal attacks on hunters: of the seven attacks in their analysis, five were perpetrated by wild boars. In contrast to grey wolves and brown bears, which are perceived by the public and the media as dangerous animals that should be avoided, wild boars are sometimes portrayed as cute and relatively harmless. In Poland, for example, a herd of wild boars ran across a beach while tourists were resting and sunbathing (VauMijau 2023). Several wild boar attacks that ended fatally show that wild boars are not harmless animals (see Manipady et al. 2006, Shetty et al. 2008, Tumram et al. 2015).

Despite these lethal attacks, it should be noted that wild boars are shy animals (Mayer 2013) that avoid contact with humans as they run away from them (Gunduz et al. 2007), so conflicts between humans and wild boars are indeed rare (Tumram et al. 2015). However, in situations where they are provoked, wild boars may violently attack a person or animal that they perceive as a threat (Manipady et al. 2006). The number of contacts between wild boars and humans has recently increased, as wild boars enter human properties or settlements in search of food (Nagasawa et al. 2017), which they find in waste (Mayer 2013) or on agricultural land (Ikeda et al. 2019). In our analysis, wild boar attacks occurred in different areas: mostly in the countryside, but also in suburban areas, and one attack took place in an urban environment. Mayer (2013) came to similar conclusions: in a comprehensive analysis of 412 attacks that took place worldwide, he found that 73% of attacks occurred in rural areas, 22% in suburban areas and 5% in urban areas.

Although usually shy, wild boars can become aggressive during the mating season. Our analysis showed that attacks occurred from late September to early May, with most attacks occurring in November. Nagasawa et al. (2017) found similar results in Japan, where attacks occurred between October and April. Other studies also suggest that wild boar attacks tend to occur in winter: Tumram et al. (2015) describe one attack in December, Gunduz et al. (2007) describe three attacks in January, and Kose et al. (2011) describe one attack in February. It is possible that the increased occurrence of wild boar attacks in autumn and winter is also influenced by hunting activities, since most battues take place in late autumn and winter (Vujnović 2016). Several authors (Manipady et al. 2006, Gunduz et al. 2007, Mayer 2013, Tumram et al. 2015) indicate that wild boars are nocturnal or predominantly nocturnal animals; however, in our analysis, the attacks occurred between 8 AM and 5 PM. Wild boars were probably disturbed by humans, which caused their activity during the day.

The demographic characteristics of the victims are consistent with the findings of a comprehensive study conducted by Mayer (2013): the victims were predominantly male in their forties and fifties who were traveling on foot at the time of the attack. Mayer (2013) also notes that most victims were alone at the time of the attack, which differs from our analysis. This difference likely results from the fact that eight of the fourteen attacks in our analysis occurred during a hunt in which the victims were accompanied. In the attacks, the victims suffered puncture and laceration wounds mainly in the lower part of the body, which is consistent with other studies showing that the wounds in wild boar attacks occur mainly in the area from the waist down (Mayer 2013), such as the lower part of the abdomen, legs (Manipady et al. 2006, Gunduz et al. 2007) and thighs (Kose et al. 2011, Tumram et al. 2015). None of the victims in our analysis suffered fatal injuries, which is consistent with other studies showing that wild boar attacks are rarely fatal (Tumram et al. 2015).

Brown Bear (*Ursus arctos* L.)

Bear attacks on humans are “rare phenomena of significant interest to the public and wildlife managers” (Herrero and Higgins 2003). It is difficult to make assumptions about bear behaviour because the behaviour of individuals is highly variable and varies considerably from one bear to another (Penteriani et al. 2017). In North America, bears are responsible for about a quarter of all wild animal attacks on humans (Penteriani et al. 2016), and in Europe, between 2000 and 2015, most bear attacks on humans occurred in Romania, Slovakia, Sweden, and Finland (Bombieri et al. 2019). In general, the number of bear attacks on humans has been increasing over time (Cardall and Rosen 2003), as the number of people participating in outdoor recreational activities has also increased, thus raising the possibility of contact between humans and bears (Penteriani et al. 2016). Despite this increasing number of contacts, injuries and deaths due to bear encounters are “extremely uncommon” (Floyd 1999), especially considering that millions of people visit national parks or participate in other activities in areas where they might encounter bears (Floyd 1999).

Bear attacks on humans can be divided into defensive and offensive attacks. Defensive attacks occur when the bear feels threatened, e.g., during a sudden encounter with humans. Offensive attacks occur when the bear wants something, e.g., food or space, or in the most extreme cases when it sees humans as prey (Löe and Röskaft 2004). Almost all the attacks in our analysis were defensive, as in six out of eight attacks, the humans were attacked by females with juveniles who believed that by attacking the humans they would be defending the juveniles (Tough and Butt 1993). The only attack that can be described as offensive was the bear attack on sanctuary keepers who entered the area where the bears live, even though the sanctuary rules prohibit entry to bears older than 1.5 years. In the case of this attack, an additional problematic circumstance was that the bears were still awake in mid-December; they were restless because they were not hibernating due to weather disturbances (Popović 2006). Probably due to the unusually warm winters for this geographical region, the attacks in our study took place during most of the year, i.e., from May to early January. In other studies, such as by Tough and Butt (1993), Herrero and Higgins (2003) and Bombieri et al. (2019), most attacks occurred in summer and autumn.

Regarding the socio-demographic characteristics of victims, several studies (Cardall and Rosen 2003, Herrero and Higgins 2003, Bombieri et al. 2019) have found that most victims are male, in their forties and were hunting or hiking at the time of the attack. Our study found similar results: all but one of the victims were male, in their 50s on average and hunting or walking their dogs at the time of the attack. Six out of eight attacks involved a sudden encounter with a female with juveniles, which is consistent with research by Floyd (1999), who found that in 70-80% of brown bear attacks, the attack was initiated by a female with juveniles. In three attacks, dogs were present and led by the victims. It can be assumed that females with juveniles were not only irritated by the presence of humans, but also by dogs in their vicinity. Bombieri et al. (2019) argue that the presence of dogs can provoke a bear attack and that it is necessary to keep the dog on a leash during outdoor activities to reduce the possibility of such attacks.

During the attack, the victims are injured in various parts of the body. When victims fall to the ground, they may suffer head and abdominal injuries (Tough and Butt 1993, Cardall and Rosen 2003). In our study, the victims mainly suffered injuries to the extremities and abdomen, and none of the victims suffered fatal injuries.

Grey Wolf (*Canis lupus* L.)

The third species that attacked humans was the grey wolf. Wolf attacks were less frequent than wild boar or bear attacks. Between 2009 and 2022 there were five wolf attacks on humans. Ambarli (2019) states that "wolves have a long history of conflict with humans". Although the wolf is generally considered the most dangerous predator in Europe (Nowak et al. 2021), the number of wolf attacks on humans worldwide has steadily decreased since 1985, in contrast to other large carnivores such as bears or coyotes, whose number of attacks has increased (Penteriani et al. 2016). Despite this actual decline in the number of attacks, conflicts between humans and wolves are portrayed negatively in the media (Ambarli 2019).

Linnell et al. (2002) divided wolf attacks on humans into three categories: (1) attacks by rabid wolves, (2) predatory attacks, and (3) defensive attacks. In a large global study covering the period from 2002 to 2020, Linnell et al. (2021) found that most attacks were perpetrated by rabid wolves, followed by predatory and defensive attacks. Of the five attacks that occurred in Croatia, only one was an attack by a rabid wolf. Rabid wolves are rare in Croatia; Lojkić et al. (2009) state that four rabid wolves were recorded in 10 years. Two cases from our study could be characterised as predatory attacks. These two attacks occurred while herding livestock, i.e., in a situation where wolves attacked the livestock, but also the herders who were trying to defend the livestock. These two attacks occurred in rural areas where sheep and goats are traditionally kept. Ambarli (2019) states that in Turkey, most predatory attacks on humans occur in areas where sheep and goats are kept and graze freely in the open. These two attacks were perpetrated by several wolves, most of which attacked livestock, while one attacked a human. This is consistent with Behdarvand and Kaboli (2015), who found that two to five wolves were involved in predatory attacks in Iran. The other two attacks were defensive. One also occurred while herding livestock, but it was not an attack on livestock but an attack by a female with juveniles. The last attack occurred while walking the dog. Similar to bear attacks (see Bombieri et al. 2019), the presence of a dog can also provoke attacks by wolves on both dogs and humans. Behdarvand and Kaboli (2015) state that their research conducted in Iran found that 19% of wolf attacks on humans were related to pets, and the study conducted by Ambarli (2019) in Turkey found that 17.2% of wolf attacks on humans were related to domestic animals.

In our study, the victims of wolf attacks mostly suffered bites to the arms and legs, with the exception of the victim who was brutally attacked by a rabid wolf. Linnell et al. (2002) found that in the past, but also in the present, shepherds are usually wounded in the arms and legs when trying to protect their livestock or dogs from wolves.

Other Wild Animals

Attacks on humans by other large carnivores were rare. For example, nine people have died from shark attacks in the last 150 years, and the last shark attack occurred in 2008 (Express 2018). The most numerous attacks by wild animals were snakebites; in the period from 1998 to 2019, 632 people were hospitalised due to snakebites (Tunjić Pejak et al. 2022), and three people died (Sarajlić et al. 2022). It is interesting to note that in Croatia, attacks by large carnivores were not fatal to humans, unlike attacks by domestic animals such as bulls (Karakaš 2023) and rams (Škavić et al. 2015). Penteriani et al. (2016) argue that bees, mosquitoes, snakes, spiders and domestic dogs are responsible for more human deaths than large carnivores such as bears or wolves.

Limitations

Certain limitations should be considered when interpreting the results of this study. Firstly, only a small number of attacks were included in the study, as no other attacks by wild animals were recorded that could be included in the analysis. Due to the small number of attacks analysed, it is difficult to draw general conclusions. The results obtained are local and can hardly be extrapolated to other countries. Secondly, as the data on attacks come from the electronic media and academic literature, it is possible that some attacks were not reported in the media and academic literature and that the actual number of attacks is higher than the number we determined. Nevertheless, this analysis provides a good insight into the attacks that occurred and enables a better understanding of this topic, which has hardly been studied in Croatia.

CONCLUSIONS

Although the exact number of wild animal attacks on humans is not known (Löe and Röskaft 2004), it is undeniable that thousands of different encounters and interactions between wild animals and humans do not end in an attack, injury, or death of the human (Penteriani et al. 2016). Šprem et al. (2014) state that Croatia is relatively safe in terms of wild animal attacks on humans, which was also confirmed by our study. In the period from 2005 to 2023 there were 33 attacks, which corresponds to an average of two attacks per year. None of these attacks were fatal; some attacks even resulted in no injuries. Most of those attacked suffered minor injuries, while a small number of attacks were more serious, as the victims were more severely injured, and the consequences of the attack were limb amputations (Lojkic et al. 2009) or physical disability (Express 2018). Of all attacks by wild animals, only snake bites were fatal.

The fact that only a few attacks can be described as predatory attacks proves that Croatia is a country safe from attacks by wild animals. In most cases, the attacks were a defensive reaction of the animals provoked by humans. To further reduce these attacks on humans, which are in fact quite rare, it is necessary to take preventive measures and define appropriate behaviour when entering an area where wild animals live (Bombieri et al. 2023). Löe and Röskaft (2004) argue that there are two general solutions to reduce wild animal attacks on humans. The first is to reduce the possibility of encounters between humans and wildlife. This can be achieved by designating an area where the animals live undisturbed by humans and human activities. The second solution is that when people encounter wild animals, they should behave in a way that does not provoke an attack. Therefore, people visiting areas where wildlife is present should be educated on how to behave when encountering wildlife (Šprem et al. 2014), especially those who live in urban areas and therefore do not frequently come into contact with wildlife. It has been shown that people contribute to attacks through their behaviour. Some of the activities that can lead to an attack include chasing a wounded animal while hunting, walking a dog that is not a leash, and approaching a female with juveniles (Penteriani et al. 2016). A key role in this education should be given to scientists and experts who use their knowledge of wildlife to inform the public objectively and without bias (Herrero and Higgins 2003).

Appendix A

Table A1. Wild boar attacks on humans.

Case	Location	Location category	Date of attack	Time of attack	Number of animals in attack	Victim gender	Victim age	Group composition	Activity of the victim	Attack circumstances	Injury type	Nature of injuries
1	Punat	Urban	3.5.2023	Morning	One	Male	Unknown	In a group	A wild boar entered the store	The victim was attacked while trying to tie up a wild boar	None	Nonfatal
2	Ivanje, Cres	Suburban	7.3.2023	8:00 AM	One	Male	60	Alone	Herding livestock	Wounded animal	None	Nonfatal
3	Ferdinandovac	Rural	22.1.2022	3:00 PM	One	Male	24	In a group	Hunting	Aggressive reaction after a sudden encounter	Torn lower leg, and open wound	Nonfatal
4	Dobreć, Lovran	Suburban	19.1.2022	5:00 PM	Four, one was involved in attack	Male	43	Alone	Dog walking	Female with juveniles	Blows to the head and thigh	Nonfatal
5	Zagreb	Suburban	9.11.2021	Morning	One	Male	Unknown	Alone	Biking	Aggressive reaction after a sudden encounter	Superficial head injuries after falling from a bicycle	Nonfatal
6	Vižinada	Suburban	6.12.2019	2:00 PM	One	Male	62	Alone	Activity near the house/in the backyard	Aggressive reaction after a sudden encounter	On the lower part of the legs, the flesh was torn in five places	Nonfatal
7	Nova Gradiška	Rural	7.10.2014	Unknown	One	Male	Unknown	In a group	Hunting	Wounded animal	Severe injuries to the leg (calf) and cuts on the arm	Nonfatal
8	Glina	Rural	25.11.2012	10:30 AM	One	Male	27	In a group	Hunting	Wounded animal	A bite wound – 4 cm long laceration of the skin and underlying soft tissue (without muscular tissue injury) of the left knee	Nonfatal
9	Pleternica	Rural	1.3.2011	11:15 AM	One	Male	46	Alone	Gamekeeping	Wounded animal	Large superficial bite wound with skin tearing left side of back	Nonfatal
10	Jurdani	Rural	15.11.2010	8:30 AM	One	Male	62	In a group	Hunting	Unknown	Bite wound left lower leg with muscle tearing	Nonfatal
11	Grubišno polje	Rural	14.11.2010	9:30 AM	One	Male	51	In a group	Hunting	Unknown	Bite wound right thigh and left lower leg with muscle tearing	Nonfatal
12	Virovitica	Rural	1.11.2009	10:00 AM	One	Male	63	In a group	Hunting	Wounded animal	Bite wound left thigh with muscle tearing	Nonfatal
13	Donja Jelenska, Popovača	Rural	27.09.2009.	Unknown	One	Male	44	In a group	Hunting	Wounded animal	Side bite under the ribs	Nonfatal
14	Virovitica	Rural	3.11.2007	10:00 AM	One	Male	40	In a group	Hunting	Unknown	Bite wound left thigh, right thigh scratches	Nonfatal

Source: Grgurinović and Putar (2009), Šprem et al. (2013, 2014), Topić (2014), Kocijančić (2019), Gavranović (2021), Konfic (2022), Mrkić Modrić and Kućel Ilić (2022), Bitići (2023), Salković (2023).

Table A2. Brown bear attacks on humans.

Case	Location	Location category	Date of attack	Time of attack	Number of animals in attack	Victim gender	Victim age	Group composition	Activity of the victim	Attack circumstances	Injury type	Nature of injuries
1	Lokve	Suburban	8.5.2023	5 PM	One	Male	26	Alone	Dog walking	Possibly female with juveniles	Several bites on the upper leg	Nonfatal
2	Vratnik, Senj	Rural	9.8.2019	Afternoon	One	Male	68	Alone	Dog walking	Unknown	The left thumb was bitten off, the left lower leg was severely injured	Nonfatal
3	Vrbovsko	Rural	12.1.2019	Unknown	Two, one was involved in attack	Male	60	In a group	Hunting	Female with juveniles	Paw kick and bite	Nonfatal
4	Moravice	Rural	25.10.2015	Morning	Three, one was involved in attack	Male	42	In a group	Hunting	Female with juveniles	Severe injuries to the left lower leg, left upper arm and head	Nonfatal
5	Klana	Rural	28.8.2012	10 AM	Two, one was involved in attack	Male	28	Alone	Dog walking	Female with juveniles	Multiple bite wounds on chest, abdomen and extremities	Nonfatal
6	Kastav	Rural	13.11.2010	7 AM	Two, one was involved in attack	Male	82	In a group	Hunting	Female with juveniles	A bite on the front of the left thigh, a small wound on the right elbow and small lacerations on the chest, head and fingers.	Nonfatal
7	Fužine	Rural	28.9.2009	7:15 PM	Three, one was involved in attack	Male	69	In a group	Hunting	Female with juveniles	Bite wound left upper arm, scratches of left hand and both legs	Nonfatal
8	Kuterevo	Rural	16.12.2006	Noon	One	Female	19	In a group	Attack on guards at the bear shelter	The guards entered the area where the bears live	Minor injuries	Nonfatal
						Male	60				Bitten hands and right thigh	Nonfatal

Source: Nova TV (2006). Net.hr (2010). Čuliat (2012). Šprem et al. (2013). Kleva (2015). Balen (2019). Radio Mrežnica (2019). RTL Danas (2023).

Table A3. Grey wolf attacks on humans.

Case	Location	Location category	Date of attack	Time of attack	Number of animals in attack	Victim gender	Victim age	Group composition	Activity of the victim	Attack circumstances	Injury type	Nature of injuries
1	Zagvozd	Rural	14.7.2022	10 AM	One	Male	64	Alone	Herding livestock	Female with juveniles	None	Nonfatal
2	Kistanje	Rural	13.6.2022	9 AM	Four animals, one was included in attack	Male	Unknown	Alone	Herding livestock	The victim was attacked during a livestock raid.	Hand injury	Nonfatal
3	Drniš	Suburban	26.9.2014	Evening	One	Female	33	Alone	Dog walking	Unknown	A bite on the left hand.	Nonfatal
4	Donji Žirovac	Rural	22.3.2009	2 PM	One	Male	67	Alone	Activity near the house/in the backyard	Encounter with a rabid animal	Amputation of the left hand, nose, upper lip and part of the upper jaw. Severe bites in the right arm and leg.	Nonfatal
5	Cista Velika	Rural	21.2.2009	11 PM	Few animals, the exact number is unknown	Male	25	Alone	Herding livestock	The victim was attacked during a livestock raid.	Scratches on the lower leg	Nonfatal

Source: Kuzmić (2009), Lojkić et al. (2009), Pavić and Tkalčević (2014), Nejašmić (2022), Pilić (2022).

Table A4. Attacks on humans by other wild animals.

Case	Species involved	Location	Location category	Date of attack	Time of attack	Number of animals in attack	Victim gender	Victim age	Group composition	Activity of the victim	Attack circumstances	Injury type	Nature of injuries
1	Roe deer	Hvar	Suburban	15.8.2022	Unknown	One	Female	Unknown	Alone	Walking	Unknown	Slight puncture wound, further scratches on legs and arms.	Nonfatal
2	Roe deer	Hvar	Suburban	12.8.2022	6:00 AM	One	Female	65	Alone	Walking	Unknown	Severe bodily injury to the rib	Nonfatal
3	Roe deer	Koprivnica	Rural	13.11.2005	11:30 AM	One	Male	26	In a group	Hunting	Rammed by a running roe deer buck	Ramming into the right leg with knee distortion and ACL rupture	Nonfatal
4	Fox	Gračišće	Rural	25.3.2010	11:20 PM	One	Male	81	Alone	Activity near the house/in the backyard	The victim tried to protect his dog from the attack of a fox	A bite to the left lower leg	Nonfatal
5	Shark	Vis	Rural	6.10.2008	Unknown	One	Male	43	In a group	Diving	Unknown	A bite to the left leg, a torn calf muscle, a badly injured artery and nerves moving the foot	Nonfatal
6	Pine marten	Split	Urban	27.5.2014	Morning	One	Male	38	Unknown	Walking	The victim wanted to catch the animal with his hand	A bite on the hand	Nonfatal
	Pine marten	Split	Urban	27.5.2014	Morning	One	Male	54	Unknown	Walking	The victim wanted to catch the animal with his hand	A bite on the leg	Nonfatal
	Pine marten	Split	Urban	27.5.2014	Morning	One	Male	20	Unknown	Biking	Unknown	A bite on the leg	Nonfatal

Source: Regional Express (2010), Šprem et al. (2013), Eterović (2014), Express (2018), Crnčević (2022a, 2022b).

Author Contributions

HM conceived and designed the research, also performed data collecting. KB and ML processed the data and performed the statistical analysis. HM supervised the research, HM, KB and ML wrote the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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