ACTA UNIVERSITATIS CAROLINAE

European Journal of Environmental Sciences

VOLUME 13 / NUMBER 1
2023

CHARLES UNIVERSITY
KAROLINUM PRESS

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ISSN 1805-0174 (Print)
ISSN 2336-1964 (Online)

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EFFECTS OF THE HERBICIDE GLYPHOSATE [N-(PHOSPHONOMETHYL) GLYCINE] ON BIODIVERSITY AND ORGANISMS IN THE SOIL – A REVIEW

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ABSTRACT

Glyphosate is an organophosphate herbicide manufactured by Monsanto, which eliminates annual and perennial weeds by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) involved in the production of aromatic amino acids in plants and microorganisms. As this herbicide is used extensively, there is a lot of research on its effect on plants, animals and microbes, and human health. Glyphosate contaminates different ecosystems by spray drift, volatilization, and erosion by wind of it adsorbed on soil particles. Soil and aquatic microbiota play a signficant role in this process. This molecule is resistant to abiotic degradation. Degradation by microbes is important. The aim of this review is to provide a concise and comprehensive survey of certain relevant aspects related to its effect on the biodiversity in soil. The effect on human health is also discussed.

Keywords: biodiversity; environment; glyphosate; health; microorganisms; soil

Introduction

Glyphosate (N-phosphonomethyl glycine), was synthesised by Henri Martin of the Swiss pharmaceutical company Cliag and is the most widely used herbicide in the world. In the 1970s glyphosate was tested for its herbicidal activity (Duke and Powles 2008; Fu et al. 2017) and then sold by Monsanto in 1974 under the trade name Roundup® (Namratha et al. 2019), which consists of the active substance glyphosate (78.5%) (Çağlar and Kolankaya 2008), and a surfactant to facilitate the penetration of the active ingredient and increase its efficiency (Mesnage and Antoniou 2020).

Furthermore, glyphosate is a systemic, broad-spectrum, post-emergence and non-selective organophosphonate (Zhan et al. 2018; Namratha et al. 2019), which can be used to control annual and perennial species of weeds and grasses (Singh and Singh 2014) in agricultural, forest and aquatic systems, and is applied as a foliar spray (Villarreal-Chiu et al. 2017; Yang et al. 2019). Some of the spray may fall directly on the surface of the soil or on non-targeted plants (Gomes et al. 2014). Its mode of action is to inhibit enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is involved in aromatic amino acid synthesis (Singh et al. 2019) as well as in reducing the rates of synthesis of proteins (Solomon 2016) and secondary metabolites that interfere with the vital energy pathways of plants and microorganisms (Zhan et al. 2018).

The shikimate pathway is present in plants and microorganisms, but not in mammals, including humans, because they get amino acids from their diet (Aristilde et al. 2017). Excessive use of glyphosate and its persistence have adverse effects on human health and ecosystems (Sihtmäe et al. 2013), such as, genotoxicity, cytotoxicity, and reproductive toxicity, and can cause or triger cancer, chronic kidney disease, hypothyroidism and birth defects (Manogaran et al. 2017). This herbicide can be transformed or degraded and removed from the environment, which is generally carried out by microbes, as the very stable bonds of glycophosphate inhibit chemical degradation (Manogaran et al. 2017). Other studies have shown that bioremediation is a more promising way of removing chemical pollutants from the environment (Zhao et al. 2015). The purpose of this review is to present a summary of the scientific literature on the mode of action of glyphosate, the accumulation of its residues in humans, the air, water, and food products and to specify their effects on soil microorganisms, microbial biodiversity, plants and animals.

Proprieties and herbicidal activities of glyphosate

Glyphosate belongs to the glycine family (Ovono et al. 2019) and is non-volatile (Singh and Singh 2014). Glyphosate is an herbicide that is highly soluble in water (12 g l^{-1}) and insoluble in organic solvents due to a very stable carbon-phosphorus (C–P) bond (Hadi et al. 2013). The half-life of glyphosate in soil is 2–215 days and 2–91 days in an aquatic environment (Battaglin et al. 2014;

Badani, H., Djadouni, F., Haddad, F. Z.: Effects of the herbicide glyphosate [n-(phosphonomethyl) glycine] on biodiversity and organisms in the soil – a review
European Journal of Environmental Sciences, Vol. 13, No. 1, pp. 5–14
https://doi.org/10.14712/23361964.2023.1
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Mesnage and Antoniou 2020). Glyphosate can be adsorbed by humus and form complexes with the metal cations Fe^{2+}, Cu^{2+}, Mn^{2+} and Ni^{2+} (Singh and Singh 2014) and due to its phosphonic acid fraction it accumulates in soil (Lane et al. 2012; Zhao et al. 2015). A significant percentage of the glyphosate in soil can infiltrate the groundwater (Simonsen et al. 2008). This herbicide is effective against 100 annual species of grasses and broadleaved weeds, and more than 60 species of perennial weeds (Dill et al. 2010).

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which converts PEP (phosphoenolpyruvate) and S3P (shikimate-3-Phosphate) into 5-enolpyruvylshikimate-3-Phosphate (EPSP) (Cao et al. 2012). Current studies have shown that due to its structural similarity glyphosate competes with PEP (phosphoenolpyruvate) and then binds to the S3P-EPSPS complex to produce EPSP (Aristilde et al. 2017). This interaction is between the hydroxyl group 5 of S3P and glyphosate nitrogen (Rueda-Ruzafa et al. 2019), which is a common precursor of three aromatic amino acids, chorismate (Aristilde et al. 2017). So, glyphosate action is generated by the chelation of manganese necessary to reduce the MNF co-factor; which is a key element in the shikimate pathway (Shehata et al. 2013; Myers et al. 2016).

Then glyphosate induces the suppression of protein synthesis and secondary metabolites, e.g., flavonoids, lignin inducing cell death (Cao et al. 2012; Sviridov et al. 2015; Fu et al. 2017). The shikimate pathway is found only in microorganisms and plants, never in animals and humans (Samsel and Seneff 2013; Ovono et al. 2019), because they do not make their own aromatic amino acids (phenylalanine, tyrosine, and tryptophan), but obtain them from food (Padgette et al. 1995)

There are two classes of EPSPS: class I are naturally sensitive to glyphosate and occur in plants and many Gram-negative bacteria (e.g., *Escherichia coli* and *Salmonella typhimurium*), while class II EPSPS are involved in resistance to glyphosate and are found only in bacteria, including *Agrobacterium* sp. strain CP4 and *Pseudomonas* sp. PG2982 and some Gram-positive bacteria. Both have a similar structure but a different amino acid sequence (Fan et al. 2012; Rueda-Ruzafa et al. 2019).

Biodegradation of glyphosate

Glyphosate can either be degraded by biotic or abiotic means such as oxidation (with chlorine, permanganate, air or ozone), filtration and flocculation, adsorption, thermolysis and photodegradation, the latter of which is capable of breaking down glyphosate into non-toxic products such as carbon-dioxide, inorganic ions and water (Zhan et al. 2018).

This means of degradation is generally used in water and wastewater treatment plants, but it is expensive since it is difficult to devise a single method because of the very stable bonds (carbon-phosphorus bond) in glycophosphate (Manogaran et al. 2017).

The main pathway of glyphosate degradation in soils is biodegradation by enzymes produced by some microorganisms, such as *Pseudomonas* sp. strain LBr (Jacob et al. 1988). Numerous bacteria of the genus *Escherichia*, *Pseudomonas*, *Agrobacterium*, *Klebsiella*, *Arthrobacter*, *Bacillus* and *Rhizobium*, and basidiomycete and ascomycete fungi (Ermakova et al. 2008) can degrade glyphosate in soil and water (Zhan et al. 2018). The main metabolites of this degradation are AMPA, sarcosine and acetyl glyphosate (Zhan et al. 2018). This degradation is considered to be a co-metabolic process because it produces nutrients (Zabaloy et al. 2012), which can be used by soil microorganisms as a source of phosphorus, carbon and nitrogen (Fu et al. 2017). It is important to optimize the conditions for degradation, which include pH, temperature, glyphosate concentration, biomass, and incubation period (Namratha et al. 2019).

The biodegradation of glyphosate takes place by two alternative pathways, one involving the cleavage of the C–P bond by the enzyme C–P lyase producing sarcosine, which is then broken down to glycine, used by microorganisms for the biosynthesis of proteins (Karpouzas and Singh 2006), and formaldehyde, which is then mineralised to carbon dioxide and water (Fig. 1), this pathway is used by bacteria that use glyphosate as a phosphorus source (Guijarro et al. 2018; Mesnage and Antoniou 2020) (Table 1). Among these microorganisms are *Pseudomonas* PG 2982, *Geobacillus caldoxylosilyticus* T20 and *Pseudomonas* LBr strain, which are able to convert about 5% of the initially added glyphosate via the formation of sarcosine and glycine (Karpouzas and Singh 2006; Fu et al. 2017; Zhan et al. 2018), and fungi, including *Penicillium janthinellum*, *Penicillium simplicissimum*, *Mucor* sp. (Karpouzas and Singh 2006).

The second pathway is by the cleavage of the C–N bond of glyphosate by the enzyme glyphosate oxidoreductase releasing AMPA, which is the main metabolite of glyphosate and is then mineralized into methylamine and phosphate with a final decomposition producing CO_2, NH_3 and glyoxylate, which *Ochrobactrum anthropi* GPK 3 uses as a source energy (Shushkova et al. 2010) (Fig. 1).

This is the main natural pathway in the environment by which soil microorganisms use glyphosate as a source of nitrogen (Guijarro et al. 2018). The following microorganisms use this pathway: *S. meliloti* (Hove-Jensen et al. 2014), *Arthrobacter* sp. GLP-1 uses glycine for the biosynthesis of basic peptide proteins and amino acids (serine, threonine) (Shehata et al. 2013; Mesnage and Antoniou 2020), *Arthrobacter atrocyaneus* ATCC 13752 (Shehata et al. 2013; Fu et al. 2017). Strains 4ASW and 7b (Fu et al. 2017; Rueda-Ruzafa et al. 2019) of *Pseudomonas pseudomonalli* (Karpouzas and Singh 2006) and *Pseudomonas* sp. strain LBr (Shahata et al. 2013)

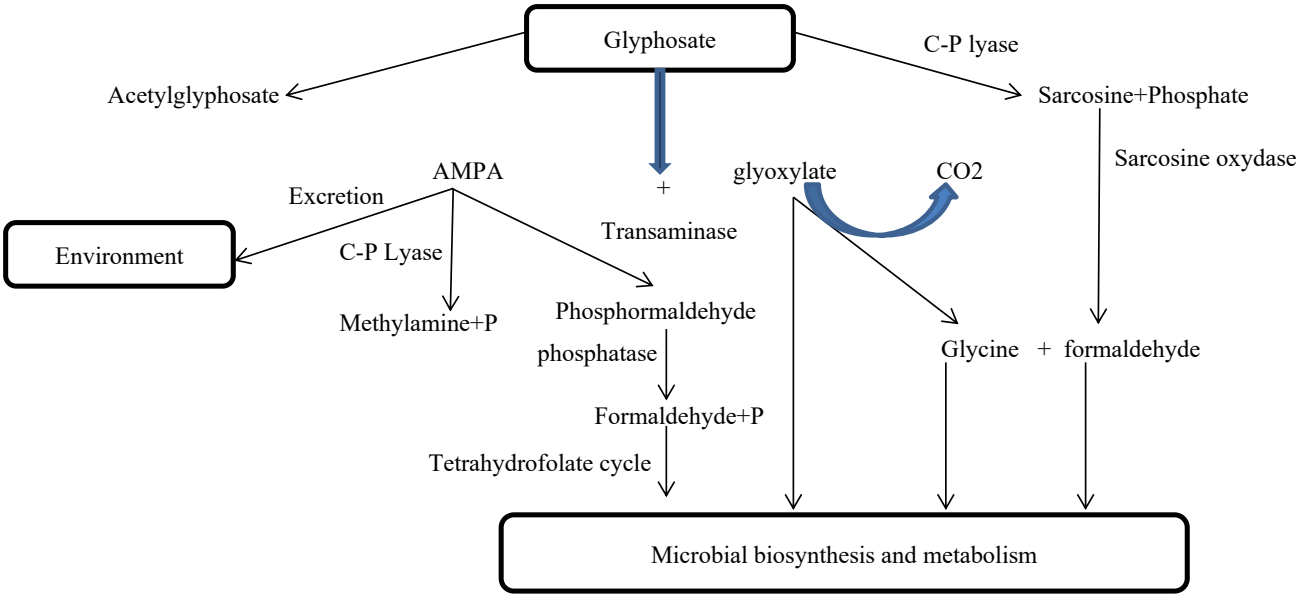

Fig 1. Pathways in the bacterial degradation of glyphosate.

Table 1 Glyphosate degrading microorganisms.

Microorganism	Origin	Degradation pathway and source	Metabolites detected	References
Bacteria				
Strains				
***Achromobacter* sp. MPS 12A**	Sites contaminated with AMPA	Sarcosine pathway as the sole source of phosphorus	Sarcosine, Glycine, Formaldehyde	Sviridov et al. (2011)
Arobacterium radiobacter	Sludge from water treatment plant	Sarcosine pathway as the sole source of phosphorus	No data	Wackett et al. (1987)
***Arthrobacter atrocyaneus* ATCC 13752**	Collection of microorganisms and cell cultures Germany	AMPA pathway as the sole source of phosphorus	AMPA CO_2	Pipke and Amrhein (1988)
***Bacillus cereus* CB4**	Glyphosate contaminated soil China	Both AMPA and sarcosine pathway as sole sources of phosphorus and carbon	AMPA Glyoxylate Sarcosine Glycine Formaldehyde	Fan et al. (2012)
***Enterobacter cloacae* K7**	Rhizoplane of various plants in Russia	Sarcosine pathway as the sole source of phosphorus	Sarcosine Glycine	Kryuchkova et al. (2014)
***Flavobacterium* sp. GD1**	Monsanto activated sludges	AMPA pathway as the sole source of phosphorus	AMPA Phosphate	Balthazor and Hallas (1986)
***Pseudomonas pseudomallei* 22**	Soil	AMPA putative pathway of the sole source of phosphorus	No data	Peñaloza-Vazquez et al. (1995)
***Pseudomonas* sp. SG-1**	Aerobic digester liquid	AMPA pathway as the sole source of phosphorus	AMPA	Talbot et al. (1984)
***Pseudomonas* sp. LBr**	Glyphosate process waste stream	Both AMPA (95%) and sarcosine (5%) pathways as sources of phosphorus	AMPA Glycine	Jacob et al. (1988)
***Rhizobiaceae meliloti* 1021**	Spontaneous mutation of a wild type strain	Sarcosine pathway as the sole source of phosphorus	Sarcosine Glycine	Liu et al. (1991)
***Streptomycete* sp. StC**	Raw sludge from a municipal sewage treatment plant	Sarcosine pathway as the sole source of phosphorus, nitrogen or nitrogen and phosphorus	Sarcosine Glycine	Obojska et al. (1999)
***Comamonas odontotermitis* P2**	Glyphosate-contaminated soil in Australia	Both AMPA and sarcosine pathways (putative) as the sole sources of carbon and phosphorus	No data	Firdous et al. (2017)

Microorganism	Origin	Degradation pathway and source	Metabolites detected	References
***Pseudomonas* sp. strains GA07, GA09, GC04**	Glyphosate contaminaited soil China	Both AMPA and Sarcosine pathways as the sole sources of carbon and phosphorus	AMPA Glyoxylate Glycine Formaldehyde	Zhao et al. (2015)
***Enterobacter* sp. strain Bisph2**	sandy soil Algeria	AMPA pathway as the sole source of phosphorus	No data	Benslama and Boulahrouf (2016)
***Ochrobactrum* sp. GDOS**	Soil	Sarcosine pathway as the sole source of phosphorus	AMPA	Hadi et al. (2013)
***Achromobacter* sp. MPK 7A**	Glyphosate contaminated soil	AMPA pathway as the sole source of phosphorus	Sarcosine	Ermakova et al. (2017)
Fungi				
***Aspergillus oryzae* A-F02**	Sludge of glyphosate manufacture	AMPA pathway	AMPA Methylamine	Fu et al. (2017)
Penicillium chrysogenum	Soil	AMPA pathway putative source of nitrogen	No data	Klimek et al. (2001)
Aspergillus niger* *Scopulariopsis* sp. *Trichoderma harzianum	Soil	AMPA pathway as the sole source of phosphorus	AMPA	Krzysko-Łupicka and Orlik (1997)

Table 2 Glyphosate doses degraded or tolerated by bacteria.

Microorganisms	Doses of glyphosate	Comments	References
***Aeromonas acetobacter* sp.**	100 mg l^{-1} 100 to 250 mg l^{-1} 7.2 mg ml^{-1}	Increase in number of bacteria sensitive to this dose of glyphosate Strong degradation	Moneke et al. (2010)
***Bacillus cereus* strain CB4**	6 g l^{-1} for 7 days 12g	94.16% degradation in 5 days Inhibition of degradation	Fan et al. (2012)
***Bacillus subtilis* Bs-15**	5000 mg l^{-1}	In treated soil 66.97% degradation was recorded 71.57% in untreated soil	Yu et al. (2015)
***Enterobacter cloacae* K7**	5 mM	40% degradation	Kryuchkova et al. (2014)
***Comamonas odontotermitis* P2**	1.5 g l^{-1}	Complete degradation of glyphosate within 104h	Firdous et al. (2017)
***Pseudomonas* sp. LBr**	0.5–0.7 mM	Glyphosate degraded at this dose	Jacob et al. (1988)
***Fusarium solani* H30** ***Fusarium solani* H50** ***Fusarium oxysporune* H80**	1 to 1.5 mM 2.0 mM	Significant growth Sensitive to glyphosate at this concentration	Krzysko-Lupicka and Sudol (2008)

use glyphosate as a source of phosphate (Hove-Jensen et al. 2014), *Penicillium chromogenum* does not use nitrate, but uses glyphosate as its sole source of nitrogen (Karpouzas and Singh 2006) and *Achromobacter* sp. kg16 converts glyphosate to acetylglyphosate (Zhan et al. 2018) (Table 2).

Some bacteria degrade glyphosate using both these mechanisms, like *Bacillus cereus* CB4, *Ochrobactrum anthropi* GPK3 1 and *Pseudomonas* sp. LBr and *Bacillus subtilis* uses another enzyme, glycine oxidase to metabolizes glyphosate (Zhan et al. 2018).

Mineralisation in soil occurs in two phases, the first is rapid and attributed to direct microbial action followed by a slow phase, which may be due to microbial metabolism after adsorption of glyphosate (Villarreal-Chiu et al. 2017) (Table 1). Adsorption of glyphosate in soil slows down its degradation by soil microorganisms, leading to accumulation over time (Van Bruggen et al. 2018). Glyphosate degrades rapidly in soil, with more than 20 to 70% of the glyphosate mineralised into CO_2 in about 5 weeks and up to 79 to 86% over a period of six months (Dill et al. 2010), depending on the type of soil. The AMPA metabolite can accumulate in soil corresponding to 10–20% of the glyphosate initially applied (Reddy et al. 2008). AMPA is toxic to bacteria and can be released into the environment (Villarreal-Chiu et al. 2017). Adsorption of glyphosate in soil slows down degradation by soil micro-organisms and causes it to accumulate over time (Van Bruggen et al. 2018). Intracellular metabolism of AMPA does not occur and it is released into the environment resulting in the contamination of several bacteria, such as, *Bacillus megaterium* 2BLW, *Pseudomonas* sp. 4 ASW, *Pseudomonas* sp. 7B and *Pseudomonas* sp. LBr (Zhan et al. 2018).

Glyphosate and AMPA residues in agricultural products

Glyphosate residues are frequently found in the food chain, as they are sprayed on cereals to accelerate ripening and more uniform drying of the grain (Mesnage and Antoniou 2020). Traces of this herbicide have been found in breast milk, honey, cereals and soybeans (Rueda-Ruzafa et al. 2019), with 95% of the levels of glyphosate in most human beverages, such as beer and wine being between 51 and 3.5 ppb (Rueda-Ruzafa et al. 2019). Concentrations of glyphosate and AMPA varies considerably in agricultural products, ranging from 0.1–100 mg kg^{-1} in legumes (including soybeans), 0.1–25 mg kg^{-1} in cereals and rice, 0.1–28 mg kg^{-1} in oil seed and 1–344 mg kg^{-1} in various types of forage (Van Bruggen et al. 2018). In Europe, MRLs are defined separately for each type of product. For barley and oats (cereals) it is 30 mg kg^{-1}, whereas the ADI is 0.5 mg/kg body weight per day (EFSA 2015).

Glyphosate and AMPA residues in soil, water, air, and humans

Glyphosate residues in humans

Humans may be exposed to glyphosate residues by consuming fruit, vegetables, and other agricultural products, as well as by drinking water (Nielsen et al. 2018). Glyphosate and AMPA residues are absorbed by animals and humans from water and plant products and then excreted in their faeces and urine (Van Bruggen et al. 2018), where they are reported in the urine of farmers and public, including children, with an incidence of 60–80% in the USA and in 44% of the public in Europe (Krüger et al. 2014; Niemann et al. 2015). The concentration is generally low but much higher in people in the United States (average of 2–3 μg l^{-1} and maximum of 233 μg l^{-1}) than in Europe (average of b 1 μg l^{-1} and maximum of 5 μg l^{-1}) (Niemann et al. 2015). Studies report high levels of glyphosate in the breast milk of women in the United States; of 10 samples sent in by mothers; 3 women had detectable levels of glyphosate, with 166 μg l^{-1} for a mother in Florida, 76 μg l^{-1} for one in Virginia and 99 μg l^{-1} for one in Oregon (Honeycutt and Rowlands 2014). The glyphosate level in 182 samples of urine from 18 European countries ranged from 0.16 μg l^{-1} in Switzerland to 1.82 μg l^{-1} in Latvia (Honeycutt and Rowlands 2014).

These levels were compared with those in breast milk, which are higher and can therefore influence the health and development of infants. In 21 samples of drinking water from the USA 13 had concentrations between 0.085 μg l^{-1} and 0.33 μg l^{-1}, which is much lower than in urine and breast milk (Honeycutt and Rowlands 2014). The study of Conrad et al. (2017) shows that concentration of glyphosate in urine collected over the course of a day were significantly higher in 2013 (1.12 μg l^{-1}) and in 2014 (0.80 μg l^{-1}) than in other years. In addition, glyphosate and AMPA concentrations are generally higher in male than female urine (Conrad et al. 2017).

Glyphosate residues in soil

Glyphosate and AMPA residues recorded in soil using GC-MS are both 0.05 mg kg^{-1}. In drinking water, groundwater, and surface water the concentration of glyphosate and AMPA is 0.03 μg l^{-1} measured using LC-MS/MS and glyphosate in air using GC-MA with LOQ is 5 μg m^{-3} (EFSA 2015).

Glyphosate residues in air

There are very few studies on the atmospheric transport of glyphosate. In one study, glyphosate concentrations in air are reported to be less than 15.7 mg m^{-3} during silvicultural spraying (Chang et al. 2016). The frequency of detection of glyphosate in air and rain samples ranges from 60% to 100% and deposition rates from 0.01 to 1.51 μg m^{-2} per day measured at 7-day intervals during the growing season at three sites in Alberta and Canada (Humphries et al. 2005).

Glyphosate residues in water

Glyphosate can reach aquatic ecosystems through uncontrolled runoff, aerial drift, accidental overexploitation or when sprayed directly on aquatic weeds. All these substances influence aquatic organisms (Cuhra 2015). In general, there are several ways in which herbicide can be degraded, such as photodegradation, oxidation (with chlorine, permanganate, air, or ozone), filtration and flocculation, adsorption and by using membranes, but these are costly and difficult to use for treating wastewater (Manogaran et al. 2017). Glyphosate has been detected in seawater at 0.1–2.5 μg l^{-1} in surface waters in Germany, Switzerland, and Hungary and 165 μg l^{-1} in Spain (Van Burggen et al. 2018).

In European surface waters, glyphosate and AMPA occur at up to 370 and 200 μg l^{-1}, respectively, while in groundwater the concentration is 0.1 μg l^{-1} (Mertens et al. 2018). Glyphosate has an aquatic half-life of 2–14 days (Howe et al. 2004).

Effects of glyphosate on microorganisms, plants, animals, and humans

Effect of glyphosate on soil microorganisms

Soil microorganisms play a central role in the degradation of herbicides and the maintenance of the functions of soil ecosystems, including nutrient cycling and bioremediation. For example, if the concentration of glyphosate is above 200 mg kg^{-1}, microbial biomass increases in less than 10 days at a pH below 5.5 and decreases in more than 100 days at a neutral pH (Liu et al. 2018).

Glyphosate can cause structural changes in local soil microbial communities by inhibiting the growth of soil microorganisms and facilitating the growth of soil fungal path-

ogens of plants that form the basis of ecosystem services such as pollutant transformation and nutrient cycling (Zabaloy et al. 2012; Zhan et al. 2018). Thus, the presence of free glyphosate in the soil profile changes the composition of microbial communities, resulting in a marked increase in the population of the phytopathogenic fungi Fusarium and Phytophthora (Kryuchkova et al. 2014).

Glyphosate application can change the balance between pathogenic *Fusarium* sp. and antagonistic microorganisms such as *Pseudomonas fluorescens* in favour of root pathogens, similarly, the human and animal pathogen *Staphylococcus aureus* is insensitive to glyphosate and can become more dominant in glyphosate-treated soil (Van Bruggen et al. 2018). This organophosphorus herbicide is the only one capable of inhibiting the mycelial growth and sexual reproduction of *Pythium* and *Fusarium* (Azouaoui-Ait Kettout et al. 2007) (Table 2).

The application of Roundup (glyphosate) (50 and 100 mg l^{-1}) results in an increase in *Aeromonas* compared to controls (Amoros et al. 2007). These microorganisms are tolerant of concentrations above 100 mg l^{-1}. The highest growth of *Acetobacter* sp. and *Pseudomonas fluorescens* was recorded in the control, which had the lowest concentration of glyphosate (7.2 mg l^{-1}).

A recent meta-analysis indicated that the effect of glyphosate on soil microbial communities is very variable and depends on many different factors, including the concentration and formulation of glyphosate, number of applications, soil pH and exposure. For example, glyphosate concentrations > 200 mg kg^{-1} induce a short-term increase (100 days) in soil microbial biomass in soils with a pH of 5.5, whereas lower concentrations of glyphosate reduce the long-term (> 100 days) increase in microbial biomass in soils with a neutral pH (Liu et al. 2018), *Bacillus subtilis* strain Bs-15 degraded 67% of 5000 mg glyphosate l^{-1} in sterile soil after 96 h, and the degradation up to 72% greater in unsterilised soil, probably due to the stimulation of endogenous microorganisms (Shehata et al. 2013; Yu et al. 2015; Villarreal-Chiu et al. 2017). Significant decrease in Xanthomonada-substituted gamma-proteotic acid and limbs (Villarreal-Chiu et al. 2017).

Glyphosate can disrupt freshwater microbial communities and reduce species biodiversity in aquatic communities, as *Vibrio ficheri*, a marine bacterium, is sensitive to glyphosate at a concentration of EC50 5.4 to 7.6 mg l^{-1} (Van Bruggen et al. 2018). The concentration of glyphosate required to inhibit the growth of *Escherichia coli, Bacillus subtilis*, *Bacillus jabonicum* and *Pseudomonas aeruginosa* by 50% is estimated to be 75 μM, 174 μM, 1.1 mM and 1.1 mM, respectively (Duke et al. 2012). Specifically, at 4 kg ha^{-1} of glyphosate, nitrogenase activity decreased by 22% in *Azotobacter vinelandii*, but only by 2% in *Azotobacter chroococcum*. A higher application of glyphosate (12 kg ha^{-1}) resulted in nitrogenase activity of 45% and 13%, respectively, in *Azotobacter vinelandii* and *Azotobacter chroococcum* (Aristilde et al. 2017).

Release of AMPA into the environment as a result of intracellular metabolism leads to contamination of *Bacillus megaterum* 2BLW, *Pseudomonas* sp. 4ASW, *Pseudomonas* sp. 7B, *Pseudomonas* sp. LBr that use glyphosate as a source of phosphorus (Zhan et al. 2018).

Effect of glyphosate on plants

Glyphosate is transported throughout a plant in 4 hours via the phloem (Mesnage and Antoniou 2020), is toxic to monocotyledons (such as grasses) and dicotyledons (broadleaved plants) (Gomes et al. 2014), affects photosynthesis by degrading chlorophyll and AMPA disrupts the biosynthesis of chlorophyll resulting in yellowing and necrosis of foliage (Gomes et al. 2016).

Glyphosate affects the metabolism of carbon, nutrition, and oxidative events, and disrupts interactions between plants and microorganisms (Kremer and Means 2009; Zobiole et al. 2012). This adversely effects nitrogen fixation and inhibits PSII activity and undiluted photochemical energy dissipation processes (Gomes et al. 2014). Low Mg content in leaves, results in a decrease in chlorophyll content and photosynthesis (Gomes et al. 2014).

In susceptible plants, it inhibits CO_2 uptake and depletes photosynthetic intermediates (Gomes et al. 2017). For example, glyphosate reduces the ability of bean plants to defend themselves against anthracnose (Johal and Rahe 1988). Plants treated with glyphosate do not produce secondary aromatics, including antimicrobial phytoalexins that defend them against pathogens, which can lead to changes in the endophytic microbiome and rhizosphere (Van Bruggen et al. 2018). Glyphosate and its breakdown product AMPA inhibit the activities of antioxidant enzymes and induce the accumulation of species oxygen reactants (ROS) that cause physiological dysfunction and cell damage (Gomes et al. 2016).

Effect of glyphosate on animals

Glyphosate in animal feed affects not only intestinal bacteria but also fungi, such as mucorales, which are fast-growing fungi that often form ball spores on fungal threads and are therefore sometimes called mussel pins (Van Bruggen et al. 2018). The absence of the shikimate pathway in animals is the reason why glyphosate is not toxic for animals such as mammals, amphibians and reptiles even when exposed to relatively high doses (Van Bruggen et al. 2018), but animals can ingest glyphosate and AMPA by drinking water and eating contaminated plants, which may damage or reduce the survival of many animals, including benthic insects, fish, birds and earthworms (Tsui and Chu 2003). It can also damage the DNA and chromosomes of fish (Zhan et al. 2018).

It directly affects the morphology, behaviour and reproduction of several species and adversely affects the long-term survival of arthropods in the soil (Villarreal-Chiu et al. 2017). Glyphosate concentrations above 400 μg l^{-1} are potentially toxic to certain aquatic species,

including amphibians and fish (Mesnage and Antoniou 2020). Exposure of zebrafish embryos to 50 mg l^{-1} Roundup® results in developmental defects in the forebrain, midbrain, and eye lesions (Roy et al. 2016). A concentration of Roundup® of 3.6 mg l^{-1} for 4 h causes DNA damage in the blood, gills and liver of the European eel, *Anguilla anguilla* (Van Bruggen et al. 2018). Changes in liver cells and mitochondria occur in freshwater carp (*Cyprinus carpio*) exposed to Roundup® at 205 or 410 mg l^{-1} (Van Bruggen et al. 2018).

The International Organisation for Biological Control found that exposure to freshly dried Roundup® killed more than 50% of three species of beneficial insects: a parasitoid wasp, a nymph, and a ladybird beetle (Hassan et al. 1988) and more than 80% of a predatory beetle. Glyphosate is extremely toxic to birds, but only in large quantities.

Effect of glyphosate on humans

Acute use of glyphosate is correlated with a wide variety of human diseases, including various forms of cancer, mental problems, and disorders such as ADHD, Autism, Alzheimer's and Parkinson's (Hadi et al. 2013; Fu et al. 2017; Namratha et al. 2019). Inhalation of droplets of spray is a minor route of exposure to glyphosate, whereas contact with skin is the main route of exposure (Acquavella et al. 2004). The use of soybeans (as a dietary supplement) contaminated with glyphosate may pose a risk of breast cancer due to its potential for additive estrogenicity. They hypothesize that glyphosate may behave as a xenoestrogen (Thongprakaisang et al. 2013).

Indeed, glyphosate can also kill human cells, by disrupting mitochondrial succinate dehydrogenase, 3/7 caspases and adenylate kinase, and is even responsible for oxidative damage to human epidermal cells (Clair et al. 2012). Richard et al. (2005) report that glyphosate inhibits aromatase Cyp 450, an enzyme crucial for the synthesis of the sex steroid hormone (Krüger et al. 2014).

At the genomic and cellular level, it affects the regulation of the cell cycle (Santovito et al. 2018). In 2001, Barbosa proposed that glyphosate may contribute to parkinsonism because of its chemical similarity to glycine, which is a necessary cofactor for the activation of the N-methyl-d-aspartase receptor (NMDA), which controls the excitatory actions of the central nervous system and is also involved in memory and learning, however, clinical studies have shown no evidence of NMDA activity in relation to glyphosate toxicity (Krüger et al. 2014). Thongprakaisang et al. (2013) and Cattani et al. (2014) report that it is teratogenic and cytotoxic to the human placenta by inhibiting the aromatase effect of cytochrome P450 (Shehata et al. 2013). Studies on Ecuadorians have shown that aerial spraying of glyphosate on coca crops damages the DNA of erythrocytes and induces an increase in haemolysis and metahaemoglobin, at moderate to high concentrations (85 to 1690 mg l^{-1}) and decreased DNA methylation at 42 mg l^{-1} glyphosate *in vitro* leading to DNA damage, cancer and appoptosis in human cell lines (Honeycutt and Rowlands 2014; Villarreal-Chiu et al. 2017; Van Bruggen et al. 2018). In 2015, the International Agency for Research on Cancer (IARC) classified glyphosate as a "known human carcinogen" (Group 2A) (Chang and Delzell 2016; Drašar and Poc 2017; Tarone 2018). AMPA inhibits the activities of antioxidant enzymes that induce the accumulation of ROS, causing physiological dysfunction and cell damage. It is a glutamic acid receptor in the CNS, so the activity of acetylcholine esterase in the body can be affected by exposure to 70 mg/kg/day (Van Bruggen et al. 2018).

Conclusion

This study summarizes the literature on environmental pollution due to the excessive use of glyphosate. This herbicide persists in the environment for long periods of time due to its adsorption properties, can also occur in groundwater, changes the composition of bacterial and fungal communities, which in turn adversely affects the functions of the soil ecosystem and animal and plant health. Microorganisms have a crucial role in the transformation of toxic organic compounds such as pesticides into harmless products, which allows them to be used in bioremediation. For this reason, it is suggested that the harmful effects of glyphosate could be due to the adjuvants in the GBH formulations.

Acknowledgments

The authors gratefully acknowledge the time given by expert reviewers in their critical assessment and improvement of the quality of this paper.

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HOW LAND USE AFFECTS BIODIVERSITY: AN ANALYSIS OF THE DIFFERENCES IN THE EFFECTS RECORDED ON DIFFERENT CONTINENTS

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ABSTRACT

Biodiversity provides humans with abundant natural resources, but due to human activities, land use has become one of the main factors determining the loss of biodiversity. Previous research has shown that land use has different effects on different species. To illustrate this phenomenon, this study used a wide range of sets of data to determine how land use affects species diversity worldwide, and whether this effect depends on the continent. This study mainly uses linear mixed-effects models (LMM) and generalized linear mixed-effects models (GLMM) to address the questions from two aspects: abundance and species richness. The results show that the responses of both abundance and species richness differ significantly between continents, which in Europe are significantly lower than in countries with primary vegetation. However, due to the sample size for Europe being much larger than that for Asia and Oceania, this result also indicates that the level of sampling could have biased the results.

Keywords: biodiversity; continents; land use; impact

Introduction

Biodiversity, the number of species, differs across the planet. There are three highly correlated levels of biodiversity: genetic diversity, ecosystem diversity and species diversity (Glowka et al. 1994). The number and variety of species in an ecosystem determine the biological characteristics that affect ecosystem processes, so species diversity has functional consequences. Species diversity also affects the resistance and adaptability of the ecosystem to environmental change (Chapin III et al. 2000). As a species, human beings depend on the oxygen and food provided by nature to sustain life. However, organisms not only provide humans with abundant natural resources, but also indirectly provide many other basic ecological services and economic values. They provide a variety of market-oriented products, such as wood, resin, fibre and organic chemicals; and have an aesthetic value (Alho 2008), which also provides an economic return. While benefiting mankind, it also provides a living environment for the animals, plants, and various microorganisms in forests. But since the 1970s, human influence on life on earth has increased dramatically, due to the demand created by an increase in the per capita income and population growth. Humans are rapidly changing the world landscape by cutting down forests and turning natural habitats into areas for subsistence farming (Foley et al. 2005). Therefore, there has been much research into how biodiversity responds to human threats, such as land use and agricultural intensification.

China is one of the most diverse countries in terms of biodiversity and ranks third in terms of the number of species (after Brazil and Colombia) (Anonymous 1996). But due to the increasing size and wealth of the human population, China's biodiversity is facing tremendous pressure from human activities. China's land use, as in many other countries in the region, has undergone tremendous changes in the past few decades. The area of cultivated land in northern China has increased, while the area of cultivated land in the south has decreased and the centre of reclaimed cultivated land has shifted from northeast to northwest. The urban areas surrounding cities in East China are expanding and gradually developing in central and western regions. The total area of grassland and woodland is also decreasing (Li et al. 2010; Zhao et al. 2015). Given the rapid economic development of China over recent decades, the original plan was to ask Chinese researchers who have undertaken comparable biodiversity surveys at multiple sites that differed in land use or levels of management for access their data. Each of the raw data sets of each of these authors were curated and uploaded to the PREDICTS database.

Nature is now providing more resources and products for humans than before, but at a high cost: The scope and integrity of ecosystems around the world are declining at an unprecedented rate, the uniqueness of local ecological areas, the numbers of wild species and that of local livestock have also declined dramatically (Diaz et al. 2019). Currently, land use or habitat change is one of the main factors that is reducing biodiversity in many areas (De Baan et al. 2013). Several previous syntheses have shown that in terms of changes in the composition of the atmosphere and extensive current changes in the earth's ecosystem, global land use has had a huge effect on the environment (Matson et al. 1997; Vitousek et al. 1997; Tilman et al. 2001; Wackernagel et al. 2002). But most of the case

Yidi, J., Purvis, A.: How land use affects biodiversity: an analysis of the differences in the effects recorded on different continents
European Journal of Environmental Sciences, Vol. 13, No. 1, pp. 15–22
https://doi.org/10.14712/23361964.2023.2
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studies consider changes in one place and often assume that the changes are much the same everywhere (Newbold et al. 2015), so there are still very few articles exploring the differences in the effects recorded on different continents in the world.

Past research has shown that disturbance has a greater effect on the biodiversity in tropical forests in Asia than in other regions (South America, Central America, and Africa) (Gibson et al. 2011). There are several possible reasons for this. Firstly, the types of land use and intensities vary in different regions and the sampling of different taxa varies, so the biodiversity recorded may differ (Phillips et al. 2017). In addition, there are differences in the sensitivity of species to land use and land use intensity. This sensitivity is mainly the intrinsic sensitivity of biological communities, determined by natural selection (Gibson et al. 2011; Gerstner et al. 2014; De Palma et al. 2016; Newbold et al. 2016). The reasons for this difference in sensitivity may be the difference in the size of the geographical range or difference in regional land use (Lambin et al. 2003; Schipper et al. 2008). That is, in some areas, long-term land use might have already filtered out relatively sensitive species, so the current difference in land use has less effect. This is also known as the "extinction filter" (Balmford 1996).

This study is part of the ongoing PREDICTS project. The data on abundance, species richness and the GPS coordinates of each research site come from the PREDICTS database (Hudson et al. 2014, 2017). The PREDICTS project (Projecting Responses of Ecological Diversity In Changing Terrestrial Systems; www.predicts.org.uk) is a collaboration between the Natural History Museum London, the United Nations Environment Program-World Conservation Monitoring Centre and others in the development of better models of how human activity affects biodiversity, which is endorsed by the Group on Earth Observations Biodiversity Observing Network (GEO-BON). Because of the hierarchical structure of the PREDICTS data the statistical analysis used in this study is a generalized linear mixed-effects model (GLMM) if the biodiversity values for two sites in the same study will tend to be much more similar than values for two sites chosen at random. The purpose of this study is to investigate how land use affects the level of biodiversity worldwide and to see if the effects differ on different continents. Three questions are addressed: 1. How do species richness and abundance respond to land use worldwide? 2. Are there any significant differences in the effect of land use on the level of biodiversity on different continents? 3. What are the possible reasons and mechanisms determining the results?

Methods

Data

The data came from numerous published studies on the effects of land use on abundance and species richness. Since 2012, the PREDICTS project has been collating records on the abundance and composition of species, and composition and diversity information on communities to simulate likely local changes in biodiversity attributable to human activity at a global scale (Hudson et al. 2017).

The predominant types of land use in the PREDICTS project are primary vegetation (local vegetation that is not known or inferred to have ever been completely destroyed), secondary vegetation (where the original primary vegetation was completely destroyed), forest plantations (previously cleared areas that were planted with crop trees or shrubs for commercial or subsistence harvesting, in which the trees are not harvested), cropland (land that people have planted with herbaceous crops), pasture (land where livestock is known to be grazed regularly or permanently) and urban areas (areas with human habitation and/or buildings, where the primary vegetation was removed) (full descriptions are given in Hudson et al. 2014). All the research sites in the database were classified according to the description in the source document or text provided by the author. The data are arranged into Sources (= papers), within which there are one or more Studies (= sampling methodology). That is why the data are hierarchical and mixed-effects models are needed.

Data analysis

Due to the differences in sampling standards and methods all the statistical analysis was done using R v4.0.0 with the "lme4" package (to run mixed-effects models, which could be used to analyse very heterogeneous data compilations). Therefore, when random effects are involved, generalized linear mixed models (GLMMs) provide a method of analysing non-normal data (Bolker et al. 2009). Several packages were used in the analysis. The first two packages "predictsFunctions" and "StatisticalModels" were useful for dealing with PREDICTS data and plotting PREDICTS models and testing spatial autocorrelation, respectively. Another package called "raster" was used for dealing with spatial data. Both "dplyr" and "tidyr" were used as handy functions for manipulating data. Package "car" was used to produce ANOVA tables with significance values and "DHARMa" to produce model criticism plots. Finally, "MuMIn" was used to check the explanatory power of mixed-effects models.

To select the random-effects structure, the method using the most complex fixed-effects structure, including all interactions, was used to test the second stage of the modelling, while comparing the fit of different random-effects structures (Zuur et al. 2009). When the response variable was abundance, a linear mixed-effects model (LMM) was used in this study. Abundance was also transformed into rescaled abundance (abundance divided by the maximum recorded in each study) for calculation. Source was included as a random intercept (termed Source_ID). As the differences in methods and

sampling effort in the different studies result in differences in the diversity metrics, the study identity was also included as a random intercept (termed SS). Block was often used as a random intercept (termed SSB) to reflect the spatial configuration of sites into spatial blocks within some studies. Sometimes the mixed-effects model included random slopes within a study so that the effects of the explanatory variables varied from study to study. When species richness is the response variable, a generalized linear mixed-effects model (GLMM) with a Poisson distribution of errors was adopted and due to overdispersion, a site-level random effect (SSBS) was added, together with (SS) and (SSB), which effectively turned the model into a quasipoisson model. The basic structure of the mixed-effects models looked roughly like this (taking predominant land use as the fixed effect as an example):

Species_richness ~ Predominant_land_use + (1|SS) + + (1|SSB) + (1|SSBS)

(1|SS) is the study-level random intercept, and (1|SSB) the block-level random intercept, both of which were considered to be random effects.

Another model used the interaction between land use and continents as a fixed effect to explain the relationship between it and biodiversity:

Species_richness ~ Predominant_land_use × UN_region + + (1|SS) + (1|SSBS)

In the PREDICTS database, UN_region is a geographical factor, with Asia, Americas, Europe, Africa and Oceania as the levels.

Model simplification, which produced the minimal adequate model (MAM), was done by checking the ANOVA table, deleting variables that had no significant effect and gradually deleting the next most complex and least important term and repeating the process until everything in the model was statistically significant. More specifically, if $p > 0.05$, the interaction variable was deleted first and then any single variable that did not participate in the remaining interactions when $p > 0.05$ (Zuur et al. 2009). The remaining model was the minimal adequate model. The "ANOVA" function in the "car" library was used to obtain the p value.

Results

Overall, the data contained 480 sources, 666 studies and 22678 sites. These sites are distributed in various countries in the world, across five continents (Fig. 1). As shown in Fig. 2, compared with the primary vegetation, the abundance in plantation forest, pasture, cropland and urban areas is significantly lower while that in young secondary vegetation and intermediate secondary vegetation is lower but not significantly so. In contrast, the abundance of mature secondary vegetation is a little bit higher than that of primary vegetation. The (square root rescaled) abundance of primary vegetation is 0.66 and pasture is 0.05 lower. This means that the (square root rescaled) abundance of pasture is 0.66 – 0.05 = 0.61 (Table 1). This model includes Study (SS), Block (SSB) and (Source_ID) as random intercepts, also, a random slope of Predominant_land_use to allow the effects of explanatory variables to vary among studies. It is worth mentioning that when the abundance is used as the response variable, the data is continuous and normally distributed, so the Student's t test is used to test whether the abundance has changed significantly from that of the primary vegetation, which it has when the absolute value of t is greater than 2.

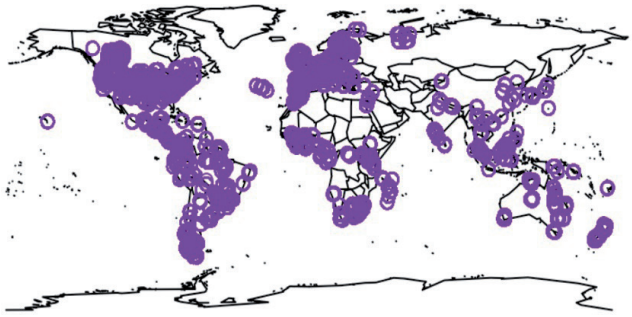

Fig. 1 Geographic distribution of the studies.

Table 1 Result of the linear mixed-effect model (LMM) with land use classes as fixed effects related to abundance, with 95% confidence intervals.

Terms	Estimate	Standard error	*t*-value
Primary vegetation	0.66	0.011	58.67
Young secondary vegetation	−0.02	0.015	−1.30
Intermediate secondary vegetation	−0.01	0.015	−0.48
Mature secondary vegetation	0.02	0.016	1.32
Plantation forest	−0.04	0.017	−2.58
Pasture	−0.05	0.017	−3.01
Cropland	−0.08	0.020	−3.78
Urban areas	−0.06	0.024	−2.59

In addition, the types of land use at all research sites were modelled with species richness as a response variable (Fig. 3). Considering overdispersion and convergence, the random effects in this model are SS and SSBS. Also, due to the overdispersion of data, this model is a quasipoisson model, so the p value is used to test the significance. The result of the GLMM shows that the species richness in young secondary vegetation, intermediate secondary vegetation, plantation forest, pasture, cropland, and urban areas is significantly lower than in primary vegetation (Table 2). Regardless of whether the response variable is abundance or species richness, the level of biodiversity in each of the four

types of land use (plantation forest, pasture, cropland and urban areas) is significantly less than recorded in primary vegetation.

Table 2 Result of the generalized linear mixed-effect model (GLMM) with land use classes as fixed effect related to species richness, with 95% confidence intervals (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Terms	Estimate	Standard error	*p* value
Primary vegetation	2.62	0.049	<2e−16 ***
Young secondary vegetation	−0.16	0.017	<2e−16 ***
Intermediate secondary vegetation	−0.16	0.016	<2e−16 ***
Mature secondary vegetation	−0.04	0.021	0.09
Plantation forest	−0.28	0.015	<2e−16 ***
Pasture	−0.20	0.014	<2e−16 ***
Cropland	−0.27	0.016	<2e−16 ***
Urban areas	−0.25	0.031	<2e−16 ***

To test whether continent matters, ANOVA (Analysis of Variance) was used to compare the model that included it with a model in which predominant land use was the only fixed effect. The results indicate that not only do these two fixed effects have significant effects on biodiversity, but the effect of their interaction is also significant (Table 3). In addition, the model of the interaction between the two fixed effects fits the data better (the lower AIC value).

Table 3 Result of the one-way and two-way ANOVA with different fixed effects related to abundance and species richness, with 95% confidence intervals (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Terms	Fixed effect	Df	*p* value
One-way ANOVA	Predominant_land_use	7	< 2e−16 ***
Two-way ANOVA	Predominant_land_use: UN_region	28	< 2e−16 ***

A model in which the combination of land use types and the world's five continents (Asia, Americas, Europe, Africa, and Oceania) were included as fixed effects was developed. This model indicates the relationship between each land use type and each continent and provides a comparison between continents. As shown in Fig. 4, the effect of land use on abundance varies significantly from region to region. In Africa, abundance in young secondary vegetation, mature secondary vegetation, pasture, and cropland is significantly lower than in primary vegetation. The abundance in Americas is less sensitive to land use, but in plantation forest and urban areas is significantly lower than in primary vegetation but is significantly higher in mature secondary vegetation. In Asia, abundance in young secondary vegetation, plantation forest and cropland are significantly lower, whereas in Oceania, abundance is significantly lower in young secondary vegetation and pasture. Moreover, abundance in Europe is significantly lower in all types of land use and is also the lowest of all the continents in intermediate secondary vegetation.

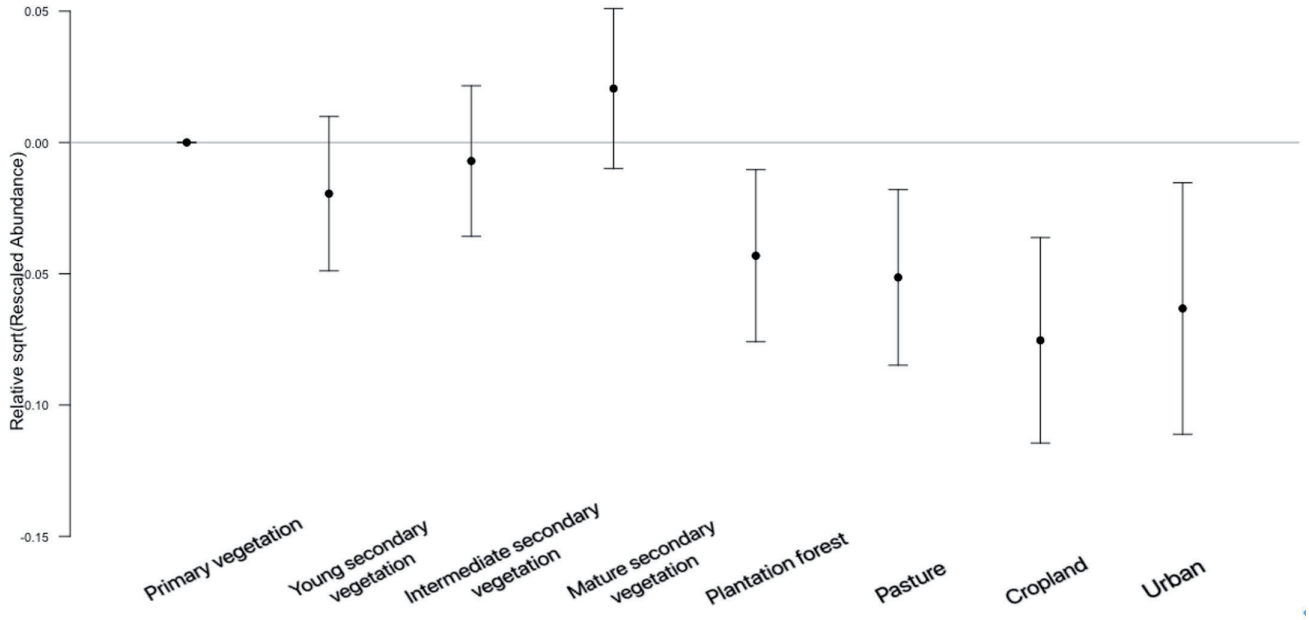

Fig. 2 Estimated average effect worldwide of different classes of land use on (square root rescaled) abundance. Error bars show 95% CIs.

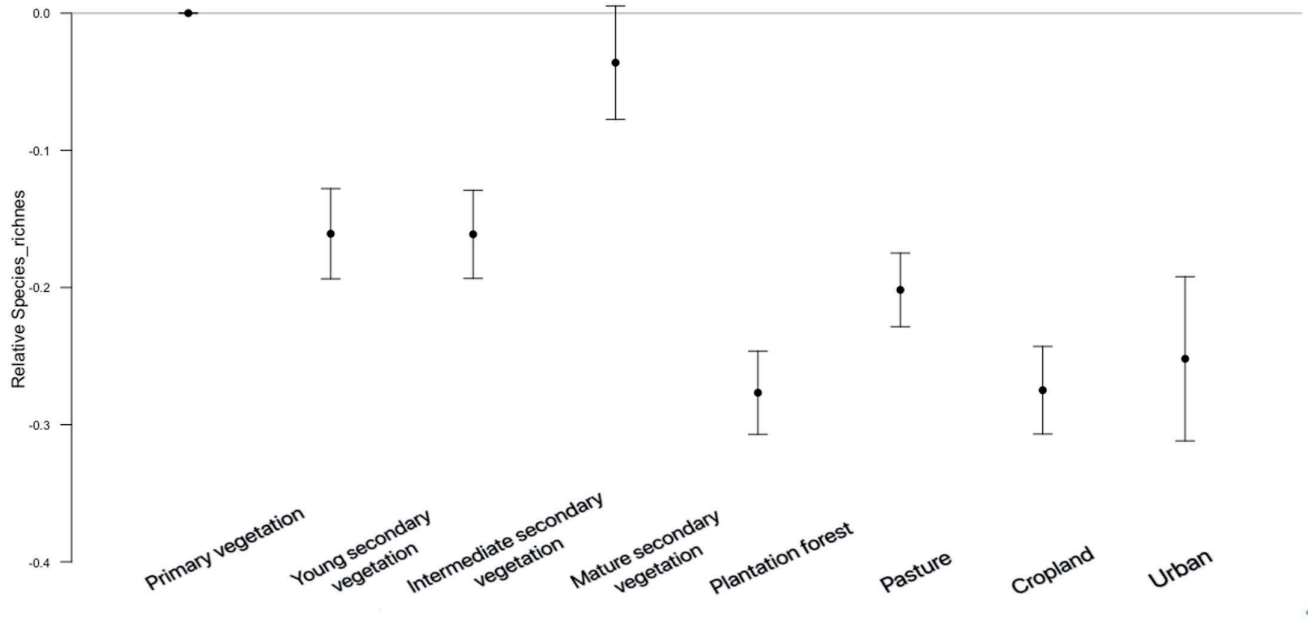

Fig. 3 Estimated average effect worldwide of different classes of land use on species richness. Error bars show 95% CIs.

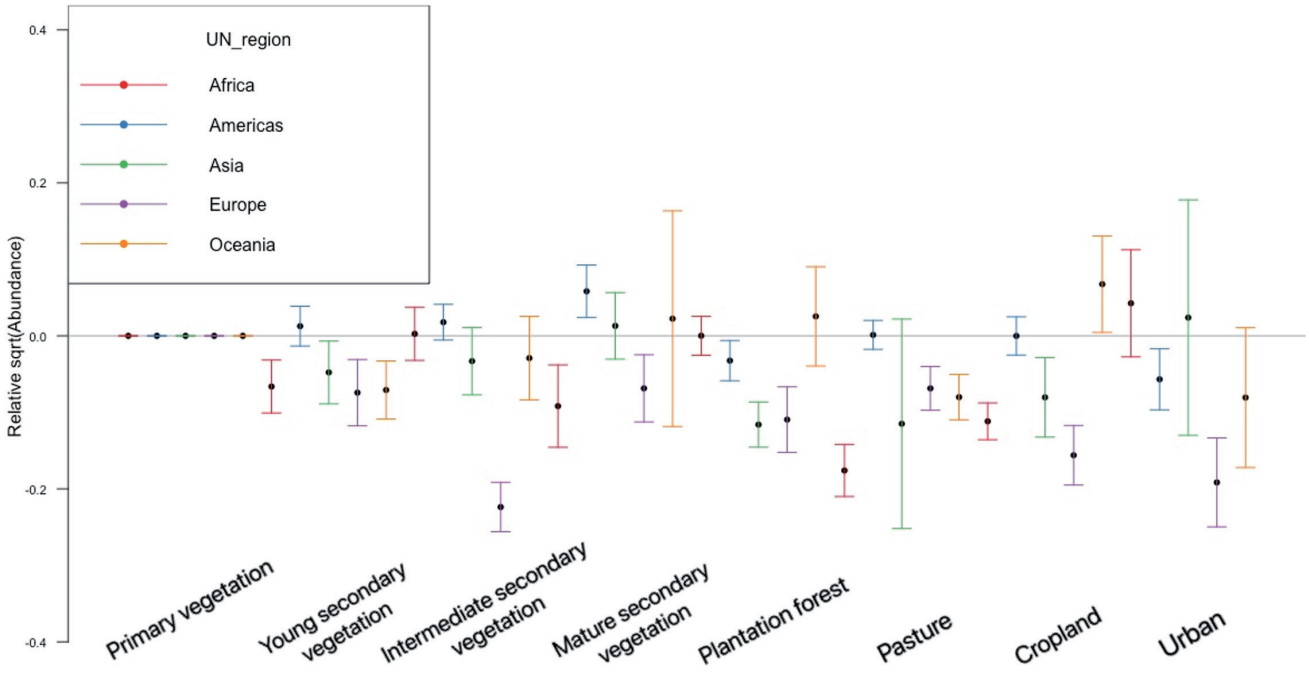

Fig. 4 Estimated average effect of different classes of land use and continents on (square root rescaled) abundance. Error bars show 95% CIs.

When species richness is the response variable, the results for Africa and Europe are similar, that is, species richness in almost all classes of land use is significantly lower than in primary vegetation (Fig. 5). Species richness in young secondary vegetation, plantation forest and pasture in America, Asia and Oceania is also significantly lower than in primary vegetation. In addition, species richness in cropland and urban areas in the Americas and intermediate secondary vegetation and cropland in Asia is significantly lower.

Discussion

The global models presented indicate that abundance and species richness recorded in plantation forest, pasture, cropland, and urban areas, are significantly lower than those in primary vegetation, with particularly low levels of diversity in cropland (Figs 2–3). Today, nearly 38% of the world's total land area is farmland (Ramankutty et al. 2008). Cropland accounts for 12% of the world's land area (about 1.53 billion hectares) and the net

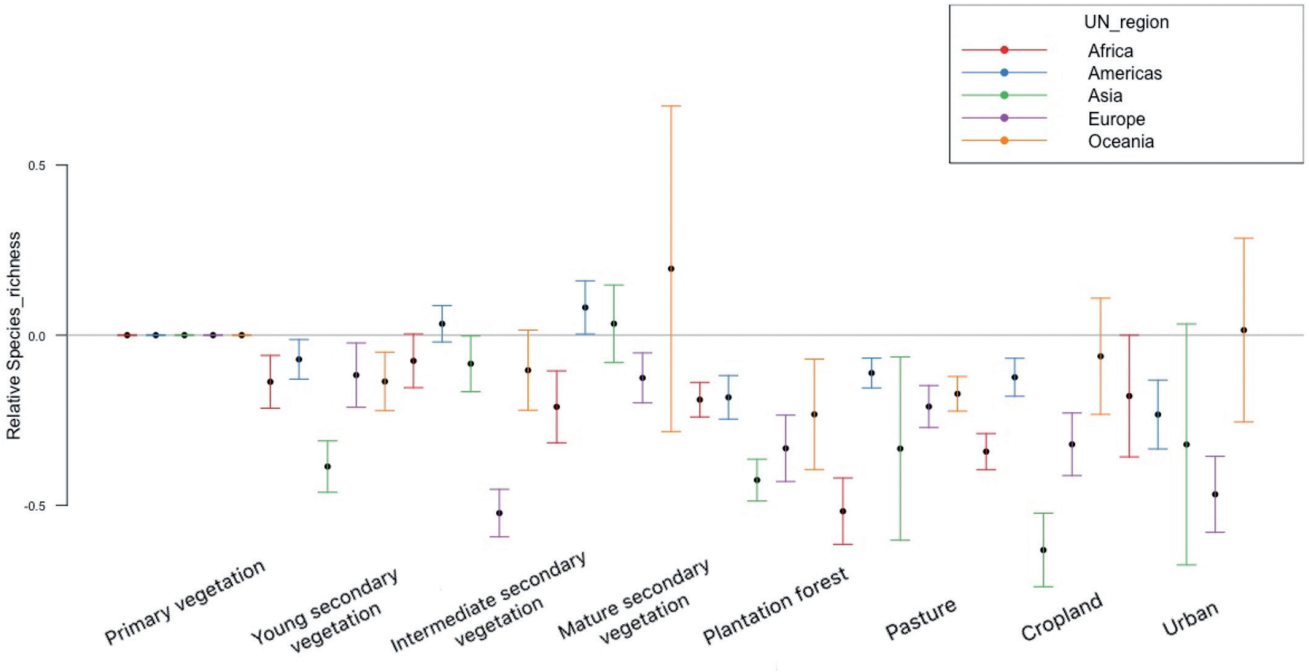

Fig. 5 Estimated average effect of different classes of land use and continents on species richness. Error bars show 95% CIs.

primary production suitable for human use is about 30% (FAOSTAT 2011; Haberl et al. 2007). It is also estimated that by 2050, the world will need to increase food production by 60–110% to feed the growing population (Tilman et al. 2011; Kastner et al. 2012). As a result, the global population growth and increase in human demand for food and energy, the expansion and intensification of cropland has become the main method of promoting agricultural production, which has resulted in a decrease in biodiversity (Garnett et al. 2013; Zabel et al. 2019).

The regional models show that the effects of land use on biodiversity differ in the five major regions (Figs 4–5). Both abundance and species richness in Europe in all types of land use are significantly lower than in primary vegetation due to the change in land use. In Europe, farmland is the most important type of land use, with 34% of its land area used for agricultural production, and grassland accounting for 14% (Reidsma et al. 2006; Verburg et al. 2006). In addition, due to the agricultural intensification that has occurred during recent decades, Europe currently also has some of the most intensively used arable lands in the world (Haberl et al. 2007; Mueller et al. 2012; Kuemmerle et al. 2016). But this result may have limitations because statistical significance depends on two things: effect size and sample size. Europe has a very large sample size, so the confidence intervals are narrow. However, perhaps its effect size is greater (more negative) than elsewhere. In addition to the models showing European biodiversity to be badly affected by changes in land use, it also faces a major effect of climate change. With global warming and significantly increasing extreme weather events, the annual average temperature in Europe has risen by over 1.1 degrees compared to the pre-industrial period, which is higher than the global average increase (Change IPCC 2007). The largest increases have occurred in southwestern and north-eastern Europe, central Europe, and alpine regions. Climate change has resulted in a high loss of species in mountainous areas, such as the mid-altitude Alps, central Spain, the Balkans, mid-altitude Pyrenees, French Cévennes and the Carpathians in Europe (Thuiller et al. 2005). In addition, in the past two decades, frequent droughts, severe fires and many destructive storms have resulted in a decline in forest productivity and the loss of biodiversity (Schelhaas et al. 2003; Ciais et al. 2005; Dobbertin and DeVries 2008).

The main limitation of this study is that data on the biodiversity in urban areas are only available for far fewer sites than for other types of land use. The PREDICTS database includes 6926 sites of primary vegetation, 3788 sites of secondary vegetation (excluding indeterminate age and undecidable types), 2345, 3275, and 3179 sites of plantation forest, pasture and cropland, respectively. But there are only 922 sites for urban areas. Therefore, the relative lack of data on types of urban land use may cause errors in the response of abundance and species richness to different types of land use. In addition, because the data in the PREDICTS database comes from articles and data collected by scholars from different regions and countries, there are biases caused by factors such as regional differences in biophysics, evolution, and socioeconomic history (Sodhi et al. 2005; Corlett et al. 2006; Gardner et al. 2009, 2010), also, different levels in taxonomic understandings, which may result in unobjective data. Considering the above limitations, future research should collect and include more data on urban

land use types, as well as data for Asia (great difficulty experienced in getting data for China) and Oceania as there are only 2719 and 2320 research sites in Asia and Oceania, respectively, whereas for the other three continents there are at least 4500. Since biodiversity in different areas is affected differently by land use, comparing the effect of land use on different continents and on different species or considering countries rather than regions may also increase the level of understanding of the interaction between continents and land use.

Data and code availability

The data and code can be obtained from https://data.nhm.ac.uk/dataset/the-2016-release-of-the-predicts-database and https://github.com/didi970428/How-Land use-Affects-Biodiversity-an-Analysis-of-Differences-in-Impacts-between-Continents.

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HOW DIFFERENT REPRODUCTION PROTOCOLS CAN AFFECT THE GERMINATION OF SEEDS: THE CASE OF THREE STENOENDEMIC SPECIES ON MT. OLYMPUS (NC GREECE)

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ABSTRACT

Mt. Olympus, the highest mountain in Greece, is a biosphere reserve and a magnet for countless visitors. In the wider area of Olympus, at least 1,700 species and subspecies of plants are recorded, 26 of which are endemic. Most of the endemics only occur in the high or subalpine zone, which is expected to be strongly affected by climate change or in specific microsites that might be affected by several other factors. Thus, the unique flora of the mountain will probably become extinct. *Ex situ* conservation can be utilized to prevent and even reverse this trend and preserve plant diversity for future generations. The aim of the present study was to develop reproduction protocols for the endemic species on Mt. Olympus, *Centaurea incompleta*, *Centaurea litochorea* and *Viola striis-notata*, to facilitate their mass production, either for *ex situ* conservation or reintroduction into their natural habitats, if necessary. Seeds of the target species were collected in summer 2021. In a sample of the collected seeds, the embryo viability was checked using sequentially 1% w/v tetrazolium solution and Evans blue solution concentration of 0.25% w/v. As for the germination tests, two treatments were used to terminate seed dormancy: (a) cold stratification at ± 2 °C, and (b) imbibition in gibberellic acid (250 ppm) for 48 hours. The results showed that more than 75% of the embryos in the fertile seeds were viable. In the seed germination tests, treatment with gibberellic acid resulted in germination percentages for *Centaurea incompleta* and *Viola striis-notata* are equal to or very close to the seed viability percentage. In contrast, no treatment was successful for *Centaurea litochorea*, as the control germination percentage was higher.

Keywords: endemics; gibberellic acid; plants; seed germination; stratification; viability test

Introduction

The conservation of biodiversity is one of the most significant global issues that scientists face. The intensity and range of human effects on habitat loss and degradation has resulted in a reduction in biodiversity at an unprecedented rate (Rogan and Lacher 2018). It is estimated that, at least, 25% of the world's plant species are threatened with extinction due to habitat loss (Holtz et al. 2022). Endemic species have the highest rates of global extinctions, as they usually have a limited geographical distribution, small population sizes and low adaptive capacity (Kraus et al. 2022). Moreover, these species are also being gradually more affected by climate change, at both species and community levels, which will eventually result in a modified plant distribution (Román-Palacios and Wiens 2020). Southern European Mountain systems are among the ecosystems most affected by climate change (Engler et al. 2011) and the endemic mountain plants there are expected to be severely stressed (Dagnino et al. 2020; Manes et al. 2021).

In the southern part of Europe, the Balkan Peninsula acted as a glacial refugium for many vascular plants and is one of the main biodiversity centres in Europe (Thompson 2005; Hewitt 2011; Nieto Feliner 2014; Rešetnik and Španiel 2022). Mount Olympus is one of the important mountains in the Balkans and the highest in Greece (2,918 m a. s. l.). It is situated in the southern-east part of the north-central (NC) floristic region in Greece and currently at least 1,700 plant species and subspecies are recorded in its wider area (c. 25% of the Greek flora). Of these, 60 are Greek endemics and 28 local endemics (Strid 1980; Strid and Tan 1986; Strid and Tan 1991; Tsiftsis and Antonopoulos 2017). To protect its unique wildlife (flora and fauna), Mount Olympus was established by the Greek Government as Greece's first national park.

Protecting plants in their natural environment (*in situ* conservation) is the main method used in their conservation. However, *in situ* conservation is not always efficient, despite the efforts and resources invested in it (Johnson et al. 2017). In such cases, conserving plants away from their natural habitat (*ex situ* conservation) could be more efficient way of protecting endangered species from external threats. Moreover, material for reintroduction, translocation, reinforcement, and habitat restoration can be produced by *ex situ* conservation. This material can be utilized to halt and even reverse the extinction trend and preserve plant diversity for future generations (Mounce et al. 2017).

Varsamis, G., Merou, T., Tseniklidou, K., Goula, K., Tsiftsis, S.: How different reproduction protocols can affect the germination of seeds: The case of three stenoendemic species on Mt. Olympus (NC Greece)
European Journal of Environmental Sciences, Vol. 13, No. 1, pp. 23–30
https://doi.org/10.14712/23361964.2023.3
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The practice of plant conservation using *ex situ* propagation has been around for several years. The reproduction of wild plants from seed is a generally effective *conservation* action. However, there are many uncertainties, concerning appropriate species-specific propagation techniques, for most plant species of conservation concern, particularly rare and/or endemic species (Cerabolini et al. 2004). Nevertheless, studying seed germination is crucial because it is an essential step in the successful reproduction of a given plant species (Baskin and Baskin 2014).

Germination tests are the most effective method for providing a protocol for *ex situ* conservation (Katsalirou et al. 2019; Margreiter et al. 2020). Knowing the viability level is crucial for developing a reproduction protocol, since it reveals the potential reproduction dynamics, because germination is usually a lengthy process. Such knowledge will allow for the calculation of the number of seeds needed to reproduce a certain number of individuals for restoration purposes.

The seeds of many species of plants often undergo periods of dormancy with innate mechanisms securing the appropriate timing of seed germination in the wild (Baskin and Baskin 2014). To break the seed dormancy of a species, it is necessary to use different pre-treatments depending on the type of dormancy (e.g., morphological, physiological) and its intensity (deep, light, or intermediate) (Baskin and Baskin 2001). Usually, the type of dormancy in a population of a plant species is strongly affected by the climate, without this being a panacea (Cotado et al. 2020). For example, it is quite common for species that grow in colder environments or on northern slopes to produce seeds with physiological dormancy. Cold stratification is suggested as a trigger to initiate germination, because this pre-treatment imitates the low winter temperatures that prevail in their natural environment. Although this pre-treatment is simple, it is quite effective, especially for species that originate from high altitudes.

The pre-treatment of seed with various hormones has replaced the time-consuming stratification that is often required to initiate the germination of deeply dormant seeds. The hormones are naturally present in the seeds and appear to be the keys to breaking dormancy and initiating germination. It is commonly thought that dormancy release is due to an increase in the levels of cytokinin (KIN) or gibberellic acid (GA3) or both, even if it is not yet completely clear how exactly they work (Shu et al. 2016). However, placing seeds in a hormone solution seems to have a positive effect on terminating dormancy in several species and at the same time, germination is more uniform (Bewley and Black 1985).

Although optimal germination protocols are available for numerous species of plants, the specific requirements of narrowly distributed species (e.g., endemics, species with narrow niche) are usually unknown. Therefore, the aim of the present study was to determine the germination requirements of three stenoendemic species on Mount Olympus (*Centaurea incompleta* Halácsy, *Centaurea litochorea* T. Georgiadis and Phitos and *Viola striis-notata* [J. Wagner] Merxm. and W. Lippert) and to evaluate the effects of cold stratification and the use of gibberellic acid (GA3) for improving germination.

Materials and methods

Species studied and seed collection

In total, the following three stenoendemic species, characterized by different ecological preferences, were studied:

(a) *Centaurea incompleta* Halácsy is one of the rarest endemic species on Mt. Olympus, known only from three microsites on limestone rocks, occurring at 400–800 m (Strid 1980).
(b) *Centaurea litochorea* T. Georgiadis and Phitos is an endemic and known only from a few microsites on the eastern and southern slopes. It prefers rocky slopes at 950–1,800 m on limestone (Strid and Tan 1991; Constantinidis 2009).
(c) *Viola striis-notata* (J. Wagner) Merxm. and W. Lippert is a rare subalpine species, which occurs at 2,400–2,900 m, where it is exclusively found growing in mobile screes (Strid 1980; Strid and Tan 1986).

Mature seeds of *Centaurea incompleta, C. litochorea* and *Viola striis-notata* were collected from Mt. Olympus during the summer of 2021, extracted from the infructescence of plants and kept at room temperature until February 2022, when viability and germination tests began.

Seed viability tests

Two random samples (i.e., repetitions) of 25 seeds each were used for the estimation of seed viability. Initially, the seeds were soaked in water for 12 hours after the testa of each seed was abraded using a dissecting needle in order make it easier to remove the embryos. Seeds were grouped into two categories during seed dissection: filled (contained an embryo) and empty (did not contain any gametophytic tissue). Empty seeds or seeds with atrophic embryos were considered non-viable (Figs 1a,b). The viability of the extracted embryos was determined by staining them sequentially with two dyes. The method used for staining was:

– Staining with a 1% w/v tetrazolium chloride solution (abbr. TTZ) (ISTA 1999), and
– Staining with 0.25% w/v Evans blue solution (Busso et al. 2005; Busso et al. 2015)

The embryos were treated with tetrazolium chloride solution, and the embryos that were not stained and therefore initially considered to be non-viable were then immersed in a solution of Evans blue dye. Thus, the percentage of non-viable embryos is the percentage of stained embryos after the Evans blue test. Consequently, the percentage of non-germinable seeds is the

sum of the percentages of empty seeds and those with a non-viable embryo. Both solutions were used due to the different properties of their active substances. Tetrazolium chloride solution only stains tissues red that are metabolically active (so-called viable tissues), whereas the Evans blue solution only stains dead tissues, which results in an accurate evaluation of embryo viability (Busso et al. 2015).

In the Evans blue staining of embryos they were left in the dye for 30 min, then examined every 30 min for 6 hours to determine the time of imbibition. There was no difference in the staining patterns of embryos after 30 minutes (Figs 1c,d) in both species of *Centaurea*, whereas even the viable embryos of *Viola striis-notata* turned blue after two hours in Evans blue dye (Figs 2a,b). Therefore, the staining pattern in *Viola striis-notata* was estimated after staining with Evans blue dye for 90 min.

An embryo with more than 50% of its surface tissue stained red in TTZ was considered viable. Unstained TTZ embryos that did not turn blue in Evans blue dye were also considered viable (Figs 2c,d). Finally, the embryos were grouped into four categories, stained and unstained in TTZ, as well as stained and unstained in Evans blue dye. Average percentages in each category were calculated based on 25 seeds in each repetition.

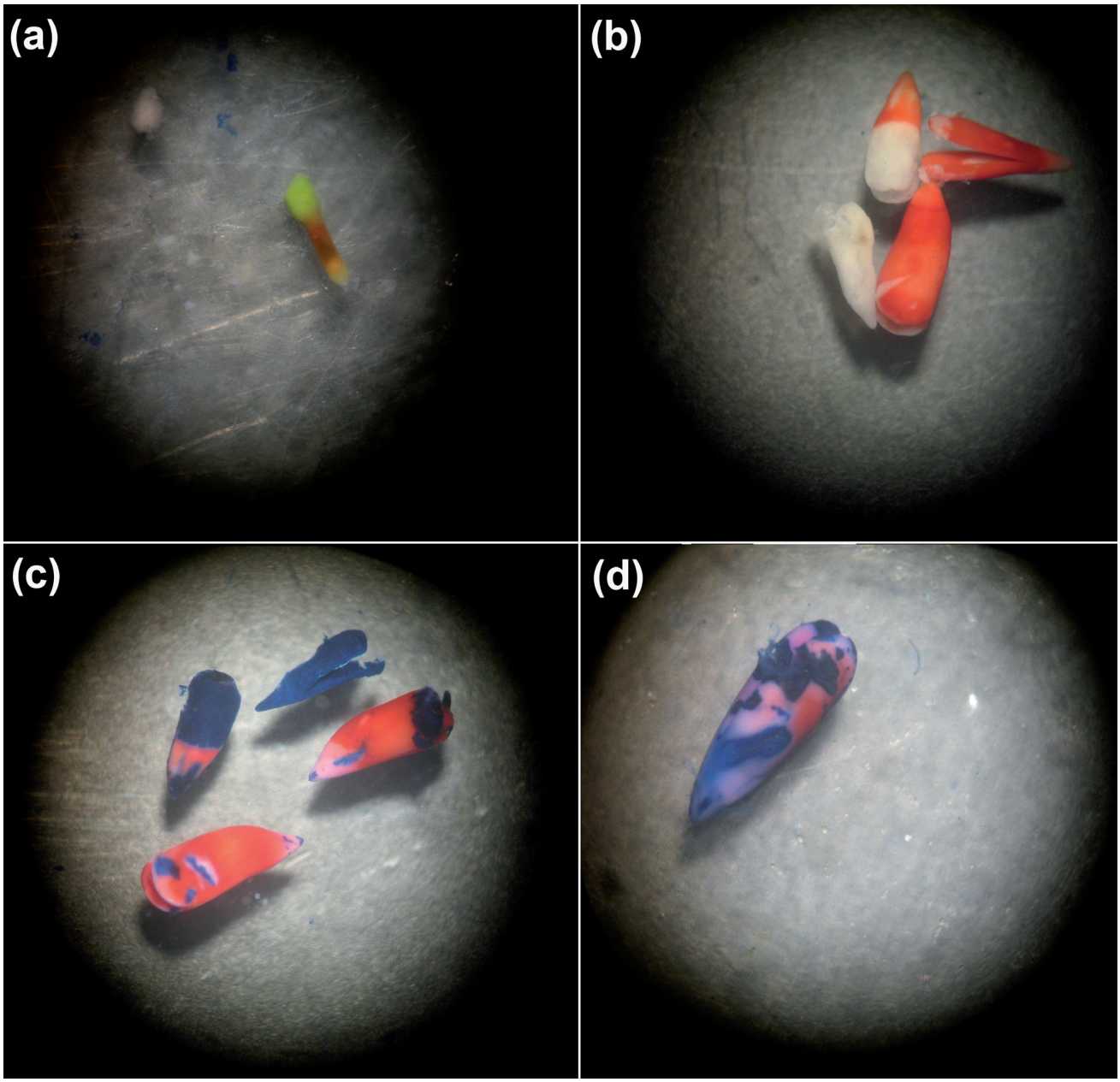

Fig. 1 (a) Atrophic (non-viable) embryo of *Viola striis-notata*; (b) Stained (viable), partially unstained (non-viable) and unstained using Tetrazolium (atrophic *Centaurea incompleta* embryos were considered to be non-viable); (c) Stained (non-viable) and unstained (viable) embryos of *Centaurea incompleta* using Evans blue; (d) Unstained (viable) embryos of *Centaurea litochorea* using Evans blue (local blue colouring in some areas is superficial due to slight damage caused by dissecting needle during extraction of the embryo).

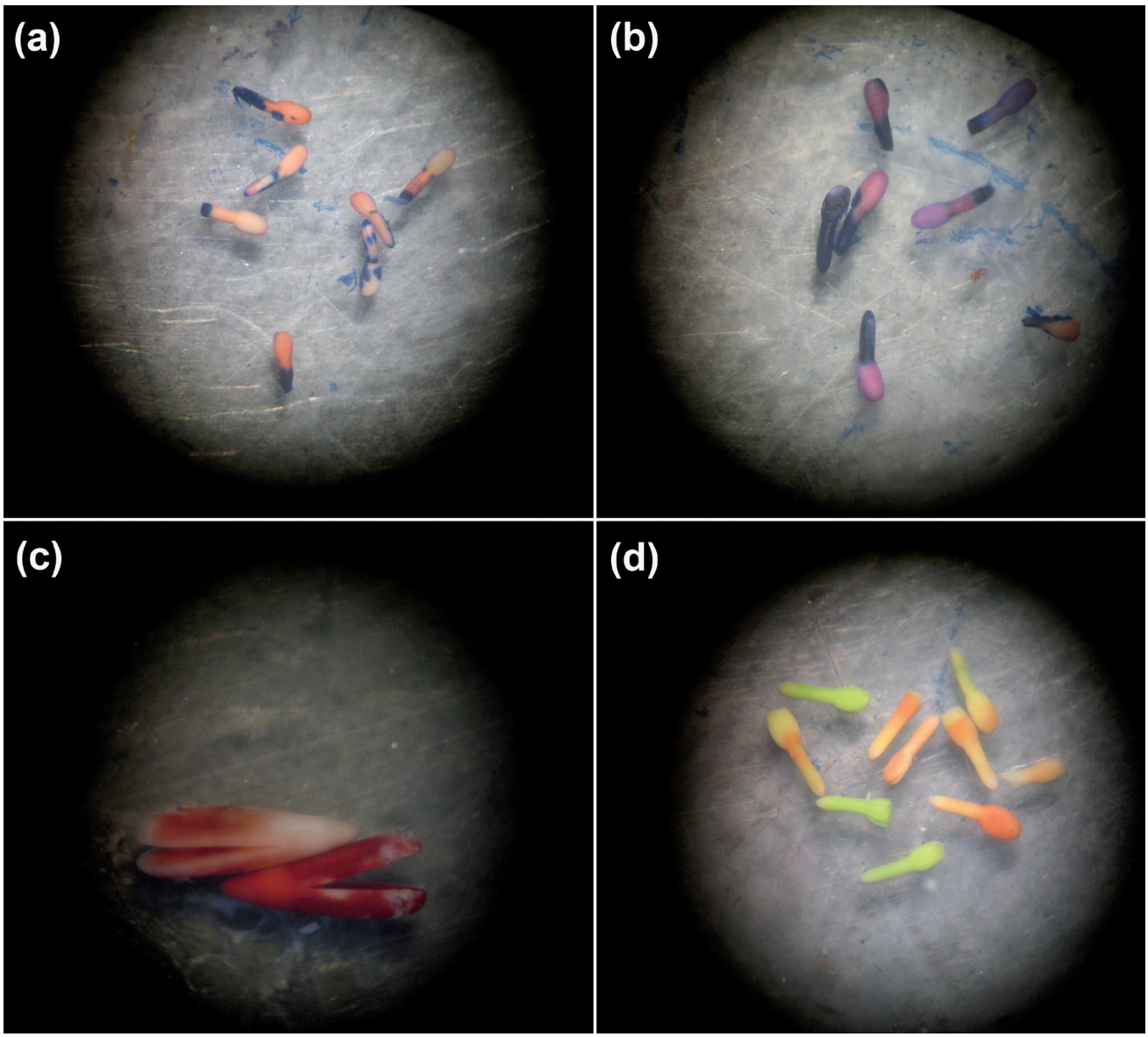

Fig. 2 (a) Stained (non-viable) and unstained (viable) embryos of *Viola striis-notata* using Evans blue after 90 min; (b) *Viola striis-notata* embryos using Evans blue after 2 hours; (c) Stained (viable) and partially unstained (non-viable) embryos of *Centaurea litochorea* using Tetrazolium; (d) Stained (viable) and unstained embryos of *Viola striis-notata* using Tetrazolium.

Germination test

Germination test was based on 4 repetitions of 50 seeds for *C. incompleta* and *C. litochorea* and 4 repetitions of 25 seeds for *Viola striis-notata*. Seeds in each repetition were visually inspected under a ZEISS STEMI 2000-C stereoscope and empty seeds were discarded and replaced. It should be noted here that, especially in *Centaurea* species, it is easy to distinguish empty from filled seeds when magnified.

Seeds were subjected to one of two treatments, cold stratification or GA3hormone. For the hormone treatment, the seeds were soaked in a solution of 250 ppm gibberellic acid for 48 hours at room temperature in the dark between two moistened filter papers. After 48 hours, the seeds were placed in Petri dishes on top of filter paper and wet sterile sand. As for the stratification, the seeds were placed in Petri dishes on top of filter paper and wet sterile sand and were put in a refrigerator at 2 °C for two months. Upon completion of treatments, seeds were placed for germination in a growth chamber under alternating conditions of temperature (25/15 °C) and photoperiod (8/16 h, light/dark) for 12 weeks. To evaluate the treatment effectiveness, control Petri dishes were also put in the growth chamber, containing 4 × 50 untreated seeds of *C. incompleta* and *C. litochorea* and 4 × 25 untreated seeds of *Viola striis-notata*. Germination was recorded every seven days for 12 weeks. When the germination test ended, the non-germinated seeds were dissected, and any empty seed left removed. Germination percentages were corrected based on the total number of full seeds per repetition.

Data analysis

Germination data were checked for normality and homogeneity using Shapiro-Wilk's and Levene's tests, respectively, and found to meet both assumptions. Differences in mean values were checked using one-way ANOVA followed by Tukey's test at 5% level of significance.

Results

A significantly higher percentage of empty seeds (without embryo or any gametophytic tissue) was recorded during the viability test for both species of *Centaurea* (*C. incompleta*: 40%; *C. litochorea*: 54%; Table 1). However, the two-dye treatment revealed that the embryos in full seed were viable. In contrast, no empty seeds were recorded in the case of *Viola striis-notata* and the viability test also revealed a high percentage of potentially germinable seeds (80%). After the two-staining test, most of the full seeds was recorded as viable (*C. incompleta*: 93.3%; *C. litochorea*: 100%; *V. striis-notata*: 80%).

The highest percentage germination of *C. incompleta* (57.73%) was recorded after the seed was treated with GA3 and was significantly higher ($P < 0.001$) than the germination percentage after cold stratification (49.74%), which in turn was significantly higher ($P < 0.001$) than the control (14.60%) (Fig. 3). It should also be noted that the maximum germination was recorded in the control and the cold stratification by the 4th week, whereas for the GA3 treatment it was by the 6th week.

Unlike in *C. incompleta*, the control rather than the treatment with GA3 or cold stratification of *C. littochorea* resulted in the highest germination (68.00%, 53.45% and 44.68% respectively) (Fig. 4). Statistically significant differences were only recorded in the comparison between the control and the cold stratification treatment ($P < 0.01$). The maximum germination in the cold stratification and control treatments was recorded by the 3rd and 4th week, respectively, whereas the percentage in the GA3 treatment increased rapidly until the 6th week, and then slightly up to the 10th week.

For *Viola striis-notata*, treatment of seed with GA3 hormone resulted in the highest germination (71.21%), whereas significantly lower germination percentages ($P < 0.001$) were recorded for seed either cold stratified or subjected to no treatment at all (control seeds) (Fig. 5). Although the percentage germination when cold stratification was used was higher than in the control, the differences were not statistically significant according to the one-way ANOVA and Tukey's test. The maximum percentage germination was recorded by the 4th (control) and 5th week (cold stratification and GA3).

Table 1 Results of the viability tests on the three species studied.

Species	Seed category		Tetrazolium staining		Evans blue staining		Total number of seeds with viable embryos	Total number of seeds that did not germinate	Total
	Empty seeds	Full seeds	Stained embryos (viable)	Unstained embryos (non-viable)	Unstained embryos (viable)	Stained embryos (non-viable)			
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8) = (4) + (6)	(9) = (2) + (7)	(10)
Centaurea incompleta	40%	60%	40%	20%	16%	4%	56% (93.33%*)	44%	100%
Centaurea litochorea	54%	46%	36%	10%	10%	0%	46% (100%*)	54%	100%
Viola striis-notata	0%	100%	60%	40%	20%	20%	80% (80%*)	20%	100%

* Percentages are for full seeds.

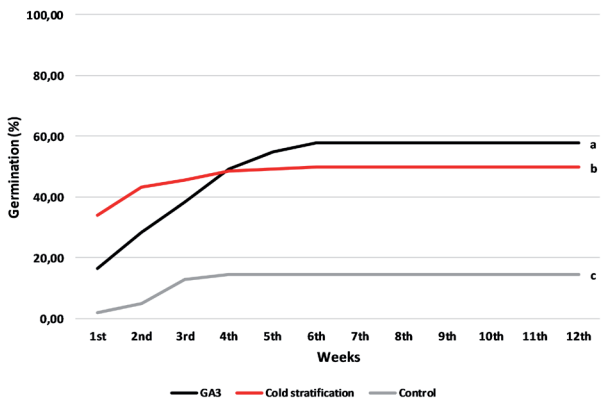

Fig. 3 Cumulative germination recorded for seed of *Centaurea incompleta* in the different treatments.

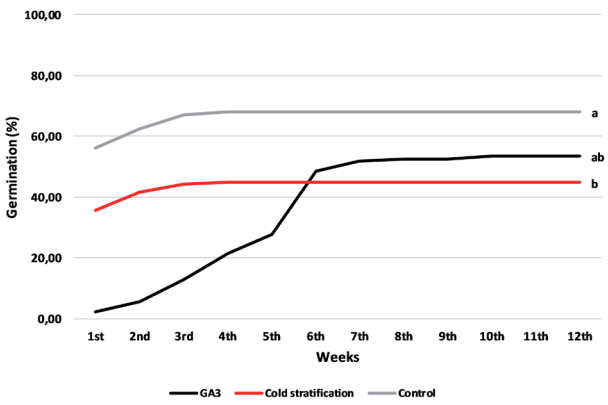

Fig. 4 Cumulative germination recorded for seed of *Centaurea litochorea* in the different treatments.

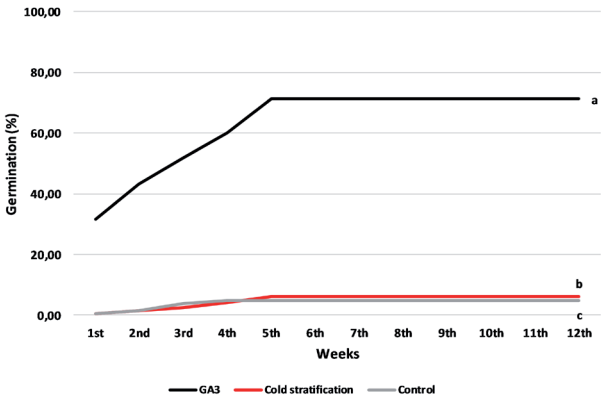

Fig. 5 Cumulative germination recorded for the seed of *Viola striis-notata* in the different treatments.

Discussion

Mount Olympus has a rich and diverse flora, which is characterized by a significant number of local endemic species (Strid 1980; Strid and Tan 1986; Strid and Tan 1991). Some of them occur widely above 2,000 m a. s. l. (e.g., *Potentilla deorum, Achillea ambrosiaca*), whereas others, despite their broad altitudinal range, occur at a limited number of microsites (e.g., *Centaurea litochorea*). Endemic species, and especially those that are range-restricted, are expected to become vulnerable and strongly affected by climate change or stochastic events (Trigas et al. 2012). The increasing threat to these species requires urgent action for their conservation. With this in mind, it is highly advisable to develop successful reproduction protocols with which species' genetic diversity will be preserved and their *ex situ* conservation, with a view to their reintroduction, will be guaranteed.

Here, the specific germination requirements of three stenoendemic species on Mount Olympus (*Centaurea incompleta, Centaurea litochorea* and *Viola striis-notata*) were determined to find the optimal treatment for maximizing their percentage germination. The two species of *Centaurea* studied (*C. incompleta* and *C. litochorea*) produced a high number of empty seeds (40% and 54%, respectively). This can be attributed to various reasons, such as pollination failure due to random environmental constraints (i.e., low spring temperature), genetic drift or correlated paternity if we consider that both species occur in isolated, small populations (Hardy et al. 2004; Bossuyt 2007). In addition, the reproductive (i.e., mating) system can affect successful pollination and later embryo formation (Zheljazkov et al. 2022). Even though there is no information in the literature on the mating system of the species of *Centaurea* studied, it is generally known that other species of *Centaurea* species are self-incompatible (e.g., Sun and Ritland 1998; Bellanger et al. 2015; Abrahamczyk et al. 2021).Thus, it is possible that self-incompatibility of these narrow endemic species in combination with their small effective population size may lead to pollination failure, low embryo formation and consequently to an increased proportion of empty (non-viable) seeds.

Unlike the two species of *Centaurea*, the percentage of empty seeds recorded for *Viola striis-notata* was nil. This may be because unfertilized ovules are aborted (Miyajima 2006), which in turn might be related to abiotic stresses (Sun et al. 2004). Moreover, the genus *Viola* includes both cleistogamous and non-cleistogamous taxa (Marcussen et al. 2015). In the Northern Hemisphere, most species of *Viola* can produce cleistogamous flowers (Culley and Klooster 2007). Although cleistogamy is not reported for *V. striis-notata*, it is an adaptation ensuring seed production in harsh environments in which pollinators are rare or absent.

The tetrazolium chloride viability test is a standard procedure for estimating embryo viability and potential germination since it stains seed tissues that are metabolically active and has long been used in studies on different plant taxa (França-Neto and Krzyzanowski 2022). *Viola striis-notata* and *Centaurea incompleta* produced seeds with embryos that were not stained by tetrazolium dye. A percentage of them, also not stained by Evans blue, were considered viable. Thus, it is possible that some of the seeds are in deep dormancy, which would indicate intrapopulation variability in the expression of seed dormancy (Kildisheva et al. 2020). In contrast, all the seeds of *C. litochorea* with embryos that were not stained by the tetrazolium dye were unstained by Evans blue and were all considered to be viable. The three species studied differed in the percentages of full seeds and seeds with viable embryos. Although *C. litochorea* had the lowest percentage of full seed, they were all viable. In contrast to *C. litochorea*, all seeds of *V. striis-notata* were full, but only 80% of them were viable. This cannot be accounted for and should be the subject of future research.

The period of dormancy of the seeds of many species of plants varies depending on innate mechanisms that result in them germinating at the appropriate time in the wild. Species-specific research on the treatments required to overcome seed dormancy are required, but this can sometimes be extremely difficult and time consuming (Baskin and Baskin 2014). The existence of deep dormancy was verified in *C. incompleta* since GA3 and cold stratification resulted in higher percentages of germination than recorded in the control. Dormancy was also recorded in *Viola striis-notata*, in which seeds treated with GA3 germinated better than control seeds. However, this was not recorded in *C. litochorea* were both treatments resulted in lower percentages of germination than recorded in the control indicating a weaker dormancy, which was easily terminated, whereas the two treatments (cold stratification and GA3) delayed germination.

Species-specific reproduction protocols enabling propagation from seeds are very important for plant conservation and the ecological restoration of disturbed

habitats (Kildisheva et al. 2020). Although the treatments used did not increase the percentage germination above that of the control for one (*C. litochorea*) of the species studied, they were successful for two species (*C. incompleta* and *V. striis-notata*), as they terminated seed dormancy and initiated germination. However, in addition to the studies on germination and the conditions required for terminating dormancy, the successful transplantation and establishment of young seedlings in pots are also crucial for *ex situ* plant conservation.

Acknowledgements

This research was funded by the Natural Environment & Climate Change Agency (N.E.C.C.A.) through the Management Unit of Olympus National Park under the call "Providing Services for the Creation of Germination Protocols for Endemic Plants".

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THE ANALYSIS OF THE INFLUENCE OF GRAZING INTENSITY ON THE DIVERSITY AND ABUNDANCE OF PLANTS AND SPIDERS (ARACHNIDA: ARANEAE)

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ABSTRACT

In restoration projects, low stock grazing has become a popular means of management. However, an accurate understanding of the effects of grazing on plants and spiders is often lacking. Although spiders and plants are not closely related evolutionarily or genetically, the relationship between them can be complex and diverse. Many species of spider build their webs or nests on plants, using the leaves, stems, or flowers as anchoring points. This provides them with protection from predators, access to prey and a stable habitat. On the other hand, spiders can provide a number of benefits to grassland ecosystems, such as helping to control populations of insects and other arthropods that can damage or consume grassland plants.This study addresses the effects of different grazing regimes on plant and spider diversity in siliceous grasslands. Plant and spider diversity was studied for four months in the Sharri Mountains (Kosovo) in order to determine the biodiversity in ungrazed, moderately grazed and overgrazed siliceuous grasslands. The responses of plant height, plant biomass, plant species diversity and spider species diversity to three grazing intensities at 12 sites were recorded. Vegetation structure (plant height and plant biomass) was significantly higher in ungrazed grasslands compared to grazed and overgrazed grasslands. This was not the case, however, for spider species richness and diversity, as these were higher in moderately grazed than ungrazed grassland. On overgrazed grasslands, spider diversity was extremely low, as only one species of spider (*Pardosa saltuaria*) was recorded. Plant and spider diversity increased in the following order: overgrazed grasslands < ungrazed grasslands < moderately grazed grasslands, in all the habitats studied. Different grazing intensities significantly affected the abundance of particular plants on siliceous grasslands, for example, *Deschampsia cespitosa*, one of the most dominant plants on siliceous grasslands had an abundance of 4.77% in ungrazed grasslands., but only 4.94% in moderately grazed grasslands and was absent in overgrazed grasslands. There were other species of plants that were most abundant in intensively grazed silicate grasslands. One of them was *Nardus stricta*, whose percentage in ungrazed, moderately grazed and overgrazed grasslands was characterized by a multiple exponential increase in % (s1 – ungrazed grasslands = 0.99%, s2 – moderately grazed grasslands = 1.25% and s3 – overgrazed grasslands = 10.50%). It is concluded that the intensity of grazing of natural grasslands directly affects biodiversity and that this information may be valuable for long-term management and conservation programs in similar habitats in SE Europe and beyond.

Keywords: biodiversity; plant ecology; siliceous grasslands; spider species composition

Introduction

Due to human activities that directly threaten biodiversity, the need for conservation measures and actions is becoming increasingly urgent (Galli et al. 2014; Hoban et al. 2021). This is particularly evident in natural habitats and within national parks, where the negative effects of human activities are already visible (Reimann et al. 2011). One of the natural habitats under such negative influence and known for their high plant diversity are silicate grasslands, which are classified as habitats of priority interest due to their high biodiversity (Anonymous 1992). Particularly important in this context are the so-called "species-rich *Nardus* grasslands", which are widely distributed on siliceous substrates in the alpine and mountainous habitats in Europe (Galvánek and Janák 2008; Wilson et al. 2012; Pittarello et al. 2017). Due to the high level of negative effects on a continental scale, these habitats were classified as vulnerable (VU) in Europe in 2017 (Janssen et al. 2017). It is known that extensive and continuous grazing of these grasslands can result in large-scale destruction and gradual conversion of them into semi-natural habitats (Tscharntke et al. 2002; Steffan-Dewenter and Leschke 2003). Indeed, most European grasslands are now considered to be semi-natural due to prolonged grazing, burning and other detrimental factors (EEA 2016). Given these negative factors, which are permanent and of varying intensity, efforts to conserve biodiversity in semi-natural grasslands in Europe remain a real challenge (Mills et al. 2007; Dumont et al. 2009). One of the most common practices used to enhance the biodiversity of grazed grasslands is to reduce the stocking rates of such grasslands. In general, there are few studies on how grazing intensity simultaneously affects insect and plant communities (Scohier and Dumont 2012; Zhu et al. 2015; Ravetto Enri et al. 2017). However, there are many more that separately address the effects of grazing on plant diversity (Porensky et al. 2017; Zhang et al. 2018; González-Hernández et al. 2020) or insect diversity (Williams et al. 2012; Davidson et al. 2020).

As for the effects of grazing on spider diversity, they can be divided into short- and long-term effects. In the short term, the effects of grazing are associated with the oversimplification of general plant architecture, which directly affects the ability of spiders to forage for insects and reduces the diversity of primary sources in the com-

Berisha, N., Geci, D.: The analysis of the influence of grazing intensity on the diversity and abundance of plants and spiders (Arachnida: Araneae)
European Journal of Environmental Sciences, Vol. 13, No. 1, pp. 31–38
https://doi.org/10.14712/23361964.2023.4
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plex food chain, thereby reducing the diversity of insects (Purvis and Curry 1981). A more direct long-term effect is caused by the obvious changes in the composition of plant communities and structure of the vegetation (Kruess and Tscharntke 2002). In these semi-natural grasslands, one of the most traditional management practices is low intensity grazing or mowing once a year, which in turn is often associated with a generally higher biodiversity (Barbaro et al. 2001; Plantureux et al. 2005; Dostálek and Frantík 2008; Török et al. 2018).

The aim of this study, which was carried out at Brezovicë, in the Sharri National Park, was to assess whether different grazing intensities will result in different levels of biodiversity in terms of plants and spiders. In the area studied, grazing occurs in three seasons (spring, summer and autumn), with sheep being the predominant grazing animal. The prediction was that less grazed grasslands will have a higher biodiversity than ungrazed and overgrazed grasslands. However, such an expectation is particularly difficult to predict when comparing plants and spiders (Dennis et al. 2015; Torma et al. 2023). There is an abundance of data on the diversity of spiders (Geci and Naumova 2021a, 2021b; Grapci-Kotori et al. 2022) in the area, which provides a good basis for further studies on this aspect. Based on this hypothesis, three grassland plots with different levels of grazing: ungrazed, moderately grazed and overgrazed, were selected for this study.

Material and Methods

Area studied

The area studied was in Sharri National Park, at Brezovicë, Shtërpce municipality, 54 km SW of Prishtina. The landscape structure was predominantly characterized by grasslands, gorges with springs and scattered forests consisting mainly of Balkan pine (*Pinus peuce* Griseb.) and dwarf juniper (*Juniperus communis* subsp. *nana* Syme). The average annual temperature is 7.6 °C, with August being the warmest month and January the coldest (Çavolli 1997; Ivanović et al. 2016). The annual precipitation ranges from 900 to 1100 mm, with the maximum in November and the minimum in August (Çavolli 1997). This study was carried out between May and August 2021 and included three types of grassland: *i.* ungrazed, *ii.* moderately grazed and *iii.* overgrazed. Grazing classification was based only on that year. In each of the three types of grassland four plots were sampled (= 12 plots sampled). Since the area is part of the Sharr National Park, it was agreed with the shepherds employed by the central management that the plots in the grasslands would not be grazed. In addition, a 4,000 m^2 mountain meadow was grazed at a low intensity (only one short period of grazing per month by 10 sheep). This was done under supervision and only during the 4-month survey period. The grasslands in the overgrazed group were constantly grazed and were on that part of the hillside that was somewhat flatter and more accessible for the sheep.

Vegetation characteristics, survey design and sampling protocol

Vascular plant taxa were recorded throughout the four-month season, with the aim of obtaining the total number of plant taxa per site. All three types of grassland studied belonged to the same type of vegetation, had the same geological base and similar ecological conditions. This enabled a comparative analysis of silicate grasslands exposed to different grazing pressures. For each plant taxon, the percentage total cover per sampling unit was recorded in the field. Vegetation characteristics of the same subalpine to alpine silicicolous grasslands (*Poion violaceae* Horvat et al. 1937) differed in their general composition of plant taxa and abundance depending on the intensity of grazing.

To minimize other effects in the selection of the plots, care was taken to ensure that each plot in each grazing group (s1 – ungrazed, s2 – moderately grazed and s3 – overgrazed grasslands) was similar in its general ecology (including moisture and soil conditions). The distance between plots was 500 meters. In this way, erroneous comparisons and discrepancies in data were avoided. A total of 12 plots were analysed.

In addition to the composition of plant taxa and the percentage cover of each plant taxa, plant height and biomass were measured in each sampling unit. Plant height and biomass were measured in 25 m^2 quadrants, along transects set 10 meters apart. Plant height was measured as the height of the plant cover at five different points within the transect, four near the corners (1.2 m) of the square plot and one exactly in the middle of the plot. The protocol for height measurements was repeated in the same manner in all plots. Vegetation biomass was determined using a direct destruction technique. Samples of plants (all aboveground parts of grasses were cut off) were collected at the same sampling points, including the dry biomass and litter on the ground, where plant height was measured at five points within plots. The quadrants used were 50 × 50 cm. Samples were placed in marked bags and then oven dried (24 hours at 80 °C) and their weight recorded.

Spiders (*Araneae*) were collected using an aspirator and sweeping an entomological net (42 cm diameter) 22 times over vegetation while traversing random transects at each site sampled. In addition, spiders were collected using pitfall traps. Two pitfalls per plot were set 10 m apart from each other. All spiders collected were preserved in 75% ethanol, sorted and identified to species level using the identification key Araneae-Spiders of Europe (Nentwig et al. 2022).

Statistical analysis

To better understand the effects of grazing on grasslands of the same composition (subalpine grasslands on acidic soils), the abundance (number of individuals per plot) and number of species (number of species per plot) record in the three types of grasslands: not grazed,

moderately grazed, and overgrazed by sheep, were compared. Data collected from May to August included vascular plants and spiders. Because the number of spiders recorded at each site was particularly low, the plant and spider diversity data were combined for further analysis. The combined data collected from May to August were analysed using linear mixed-effects models calibrated with a Poisson distribution using the R package Ime4 (Bates et al. 2015). For carrying out a range of numerical analyses and operations, PAST statistical software was used (Hammer et al. 2001). To measure the similarity of the different types of habitats, Czekanowski coefficient (Czekanowski 1909) was used.

Results

Vegetation and plant species diversiy

Mean plant species richness (per 25 m^2 plots) differed slightly (0.75) between the ungrazed and moderately grazed grasslands, whereas the differences between the moderately grazed and over-grazed grasslands (0.27) and between ungrazed and over-grazed grasslands (0.31) were much greater according to the Czekanowski Coefficient of Similarity (Table 1). The total number of plant taxa recorded per site (as a sum of all plots) was also noticeably different between the two first sites (s1 = 95 plant taxa, s2 = 99 plant taxa) and the 3rd one (over-grazed site), where only 55 plant taxa were recorded (Table 1). A list of all taxa in terms of presence/absence is presented in the supplementary material (Annex Table 1).

Table 1 Similarity in terms of % composition of the three types of grasslands (s1 – ungrazed, s2 – moderately grazed and s3 – overgrazed grasslands) based on the intensity of grazing, according to the Czekanowski Coefficient of Similarity and the the total number of plant taxa recorded per grassland site.

Czekanowski Coefficient			Plant taxa richness per site		
s1 – s2	**s2 – s3**	**s1 – s3**	**s1**	**s2**	**s3**
0.75	0.27	0.31	95 *taxa*	99 *taxa*	55 *taxa*

A direct comparison of plant diversity revealed that there are three species of plants unique to the ungrazed grasslands: *Primula minima* L., *Scleranthus perennis* subsp. *marginatus* (Guss.) Nyman and *Cirsium heterophyllum* (L.) Hill. Moderately grazed grasslands, on the other hand, are characterized by a greater number of species only occurring there, with a total of seven plant species: *Carduus acanthoides* L., *Veratrum album* L., *Potentilla aurea* L., *Pedicularis verticillata* L., *Knautia midzorensis* Formánek, *Crepis aurea* (L.) Cass. and *Antennaria dioica* (L.) Gaertn. A total of ninety-two species were recorded in these two types of grassland. It is interesting to note that of the fifty-five species of plants in the overgrazed grassland sites, not one is characteristic and only occurring there.

The average plant biomass differed in the three types of grassland; in ungrazed grasslands the average biomass was 234.2 g, in moderately grazed grasslands 54.5 g and in overgrazed grasslands it was 33.6 g (Fig. 1), although these differences are not statistically significant, with the probability of difference between the first and the second being $p = 0.08$, first to the third $p = 0.06$ and second to the third ($p = 0.26$). The average height of the vegetation was significantly lower in overgrazed grasslands (23.6 cm), 47.7 cm in the moderately grazed grasslands, while in the ungrazed grasslands the average height was 144 cm (Fig. 1). On the other hand, total vegetation cover ranged from 91% to 100% at the sites sampled in the three types of grassland and were significantly different.

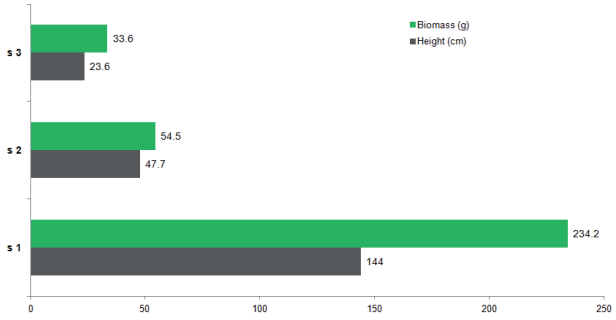

Fig. 1 The biomass (g) and the average height (cm) of plants in the three types of grasslands: s1 – ungrazed , s2 – moderately grazed and s3 – overgrazed grasslands.

The syntaxonomic affiliation of the grasslands studied based on the presence of dominant plant taxa, general ecological characteristics and indicator species, was that they all belong to the alliance: *Poion violaceae* Horvat et al. 1937 [Order: *Seslerietalia comosae* Simon 1958 and Class: *Juncetea trifidi* Hadač in Klika et Hadač 1944]. This vegetation alliance includes alpine and subalpine siliceous grasslands on deep acid soils in wind-protected habitats in the Balkan Peninsula. At all 12 sites sampled, vegetation was dominated by the following ten plant taxa: *Deschampsia caespitosa* (L.) P. Beauv., *Bellardiochloa variegata* (Lam.) Kerguélen, *Calamagrostis arundinacea* (L.) Roth., *Phleum phleoides* (L.) H. Karst., *Galium anisophyllon* Vill., *Festuca adamovicii* (St.-Yves) Markgr.-Dann., *Vaccinium myrtillus* L., *Pimpinella saxifraga* L., *Nardus stricta* L. and *Jasione orbiculata* Velen.

It is worth noting that grazing intensity significantly affected the abundance of individual plant taxa at many of the sites sampled. Of the plant taxa mentioned above: *D. caespitosa* (s1 = 4.77%, s2 = 4.94% and s3 = 0.00%), *Ph. phleoides* (s1 = 3.69%, s2 = 1.62% and s3 = 0.00%), *C. arundinacea* (s1 = 2.97%, s2 = 0.81%, and s3 = 0.00%), *P. saxifraga* (s1 = 2.16%, s2 = 2.43%, and s3 = 0.00%) and *G. anisophyllon* (s1 = 3.24%, s2 = 2.43%, and s3 = 0.84%),

which occurred mainly in ungrazed (s1) and moderately grazed (s2) grasslands, but were almost completely absent in overgrazed grasslands (s3). This clearly indicates that these plants, among others at somewhat lower percentages, occur at a lower abundance in intensively grazed grasslands. *J. orbiculata* had particularly inconsistent responses to grazing intensity (s1 = 1.17%, s2 = 0.96%, and s3 = 4.20%). On the other hand, *N. stricta* (s1 = 0.99%, s2 = 1.25% and s3 = 10.50%), *Geum montanum* (s1 = 0.27%, s2 = 0.52% and s3 = 4.62%) and *F. adamovicii* (s1 = 2.97%, s2 = 2.58% and s3 = 7.14%), belong to the second group of plants that benefited from intensive grazing. Their percentage cover was > 4.5% in the overgrazed grasslands. In addition, there was a group of plants that were consistently present regardless of grazing intensity, such as *V. myrtillus* (s1 = 3.33%, s2 = 3.10%, s3 = 3.78%).

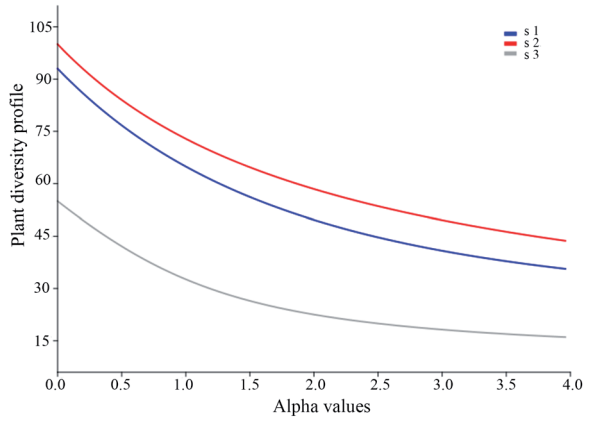

Fig. 2 The overall plant diversity profiles for the three types of grassland, based on grazing intensity. s1 – ungrazed, s2 – moderately grazed and s3 – overgrazed grasslands.

As this study was of an ecologically uniform grassland ecosystem that was subjected to different degrees of grazing, the total number of plant taxa at each site sampled was analysed. When analyzing the α-diversity of plant taxa at the sampling sites (s1, s2, and s3), it was found that the highest α-diversity was recorded in s2 – moderately grazed, followed by s1 – ungrazed and the lowest in s3 – overgrazed grasslands (Fig. 2).

Spiders

A total of 10 species of spiders (Annex – Table 2) were collected by sweepnetting and using an apirator, from May to August. The most abundant genus was *Araneus* with three species (*A. diadematus*, *A. quadratus* and *A. opisthographa*). *Pardosa saltuaria* was the most abundant in terms of the number of individuals, with a total of 20 individuals (18 individuals, or 100% of spider taxa in s3, and 2 individuals in s2 or 14.2%, consisting of 4 males and 16 females). A total of six individuals was recorded for *Tibellus oblongus,* of which five were recorded in s1 (or 35.7%) and one in s2 or 7.14%), consisting of 2 males and 4 females. For *Aculepeira ceropegia* it was six individuals, three in s1 (21.4%) and three in s2 (21.4%), 1 male and 5 females. For *Araneus diadematus* it was six individuals, four in s1 (28.5%) and two in s2 (14.2%), 2 males and 4 females. For *Araneus quadratus* it was three individuals, 1 in s1 (7.1%) and two in s2 (14.2%), all female. In addition in s1 one female of the species *Microlinyphia pusilla* was recorded and in s2 four species (each by one individual): *Xysticus audax* (female), *Platnickina tincta* (female), *Araniella opisthographa* (male) and *Linyphia triangularis* (female).

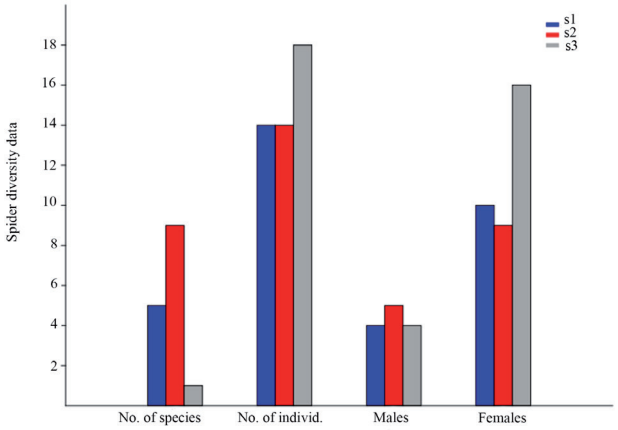

Fig. 3 The total number of species and numbers of spiders, and males and females recorded in s1 – ungrazed , s2 – moderately grazed and s3 – overgrazed grasslands.

The diversity of spiders in all three type of grassland types was very low. The largest number of species (Fig. 3) was recorded at site 2 (s2 grasslands) with a total of 9 species, followed by site 1 (s1 – ungrazed grasslands) with a total of 5 spider species, while at site 3 (s3 – overgrazed grasslands) only one species of spider (*Pardosa saltuaria*) was recorded. At site 3 (overgrazed grasslands) the highest number of spiders was recorded, but they were individuals of the same species (*Pardosa saltuaria*). In terms of the sex ratio, as can be seen in Fig. 3, females dominate with 72.9% being female and 27.1% male.

Analyzing all of the data recorded in this study (Fig. 4) the ungrazed grasslands (s1) were characterized by a greater plant biomass and, consequently, by higher values for the height of the of vegetation (in cm) and the moderately grazed grasslands (s2) by a higher biodiversity than the other two. This is especially evident in the greater number of species of plants and spiders present. Thus, it is clear that the biodiversity recorded in overgrazed grasslands (s3) was very low, so intense grazing by sheep greatly negatively affected their overall diversity.

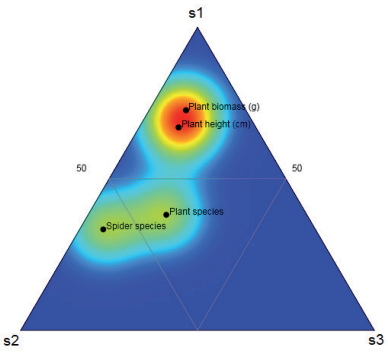

Fig. 4 A density map showing the distributions of the number of plant species, number of spider species, plant biomass (g), and plant height (cm) in three triangles corresponding to grassland types (s1, s2, and s3).

Discussion

The results indicate that increasing or decreasing grazing intensity in natural grasslands can have a direct effect on plant and spider diversity. It is a well-known ecological hypothesis that reduced grazing can directly affect grasslands and may result in the development of mosaic landscapes of heavily and lightly grazed grasslands (WallisDeVries and Raemakers 2001), which may be the basis for heterogeneous vegetation (Kruess and Tscharntke 2002). This hypothesis is supported by the diversity indices recorded in this study. Many species of plants responded to grazing intensity and differed significantly in abundance in the three types of grassland, such as: *Deschampsia caespitosa* (L.) P. Beauv., *Phleum phleoides* (L.) H. Karst., *Calamagrostis arundinacea* (L.) Roth, *Pimpinella saxifraga* L. and *Galium anisophyllon* Vill. In all these species, as well as in many others at a lower incidence, a reduction in abundance was recorded with increase in grazing intensity. There were other plant species like: *Geum montanum* L., *Nardus stricta* L., *Sesleria comosa* Velen. and *Festuca adamovicii* (St.-Yves) Markgr.-Dann. that were either highly tolerant of grazing or even favoured by intense grazing (Cole 1995). Most likely due to the low incidence of grazing and thus damage to plants, the ungrazed grasslands (s1) had the highest level of plant biomass and percentage cover of vegetation. In terms of the total number of species of plants, slightly higher numbers were recorded in the moderately grazed grasslands (s2), but did not differ significantly from that recorded in ungrazed grasslands (s1). The difference between s1 and s2 was more pronounced in the diversity of the few species of spiders recorded. This indicates that habitat composition structure either directly or indirectly determines spider diversity (Greenstone 1984; Dennis et al. 1998; Buchholz 2010; Ávila et al. 2017). That the moderately grazed grasslands (s2) had the highest spider populations reveals something about the natural habitat conditions that spiders prefer. Overgrazed grasslands (s3), on the other hand, are not only unfavourable for the development of plants, plant diversity and vegetation, but also for spiders.

The effects of grazing on plant and spider diversity became apparent when grasslands with three different grazing regimes were compared. A general trend towards higher diversity in moderately grazed and ungrazed, compared to overgrazed grasslands was evident. Although these results are based on a short-term analysis of grassland grazing intensity, the apparent differences in species diversity in the three types of grassland may prove valuable for habitat specialists, conservation efforts and heterogeneity analyses of the natural habitats in Sharri National Park. In ungrazed and moderately grazed grasslands, more spiders that rely on tall plants to hunt, such as building webs (Araneidae and Linyphiidae) or waiting for prey (Philodromidae and Thomisidae) were recorded, whereas in overgrazed grasslands where were nearly exclusively only active hunting spiders (Lycosidae).

It is well known that ungrazed grasslands are likely to be more heterogeneous than grazed (and especially overgrazed) grasslands. This is mainly due to the height of the vegetation in the former, which has a more complex architecture (Southwood et al. 1979; Southwood 1988; Pittarello et al. 2017).

The natural habitats of acid grassland belong to a group of habitats whose conservation is a priority not only at regional and national levels, but also at the European level. The European Habitats Directive (Anonymous 1992) defines *Nardus stricta* communities on siliceous substrates as "species-rich" because they host a higher diversity of vascular plants compared to other siliceous habitats. Based on the results presented, as well as those of other studies (Millaku et al. 2013; Berisha et al. 2020), conservation efforts should focus primarily on siliceous grassland habitats with *Nardus stricta*, as they contain many important plant taxa and belong to a group of very high conservation importance.

Conclusions

In conclusion, in terms of maintaining habitats with high natural values and their proper management, efforts to reduce grazing intensity remain of crucial importance. This would have a direct effect on increasing plant and animal biodiversity, as revealed by this study of plants and spiders. There was correlation between plant and spider diversity in three types of differently grazed grasslands. The results indicate the importance of small-scale, moderate grazing of grasslands, as well as the conservation of longstanding, ungrazed grasslands. This would result in the conservation and restoration of plant and spider diversity. Because natural grasslands are one of the most important habitats in terms of biodiversity, proper management, extent and control of grazing, mowing, or general use of grasslands may be the key to the long-term conservation of these natural habitats.

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Annex – Table 1 Plant taxa presence/absence at each site.

No.	Plant taxa	s1	s2	s3	No.	Plant taxa	s1	s2	s3
1.	*Achillea multifida*	1	1	0	**2.**	*Patzkea paniculata*	1	1	1
3.	*Agrostis capillaris*	1	1	1	**4.**	*Luzula luzuloides*	1	1	0
5.	*Anthyllis vulneraria*	1	1	0	**6.**	*Geum montanum*	1	1	1
7.	*Festuca rubra*	1	1	1	**8.**	*Juncus trifidus*	1	1	0
9.	*Deschampsia cespitosa*	1	1	0	**10.**	*Vaccinium myrtillus*	1	1	1
11.	*Campanula rotundifolia*	1	1	1	**12.**	*Myosotis alpestris*	1	1	0
13.	*Filipendula vulgaris*	1	1	0	**14.**	*Jasione orbiculata.*	1	1	1
15.	*Geranium subcaulescens*	1	1	0	**16.**	*Campanula spatulata* subsp. *spatulata*	1	1	0
17.	*Poa alpina*	1	1	1	**18.**	*Pedicularis verticillata*	0	1	0
19.	*Plantago atrata*	1	1	1	**20.**	*Calamagrostis arundinacea*	1	1	0
21.	*Bellardiochloa variegata*	1	1	1	**22.**	*Knautia midzorensis*	0	1	0
23.	*Ochlopoa annua*	1	1	0	**24.**	*Bupleurum falcatum*	1	1	1
25.	*Stachys alopecuros*	1	1	0	**26.**	*Silene sendtneri*	1	1	1
27.	*Rumex acetosa*	1	1	1	**28.**	*Homogyne alpina*	1	1	0
29.	*Carex curvula*	1	1	1	**30.**	*Luzula spicata*	1	1	1
31.	*Arabis hirsuta*	1	1	0	**32.**	*Lotus corniculatus*	1	1	1
33.	*Hypericum richeri* subsp. *grisebachii*	1	1	0	**34.**	*Avenella flexuosa*	1	1	0
35.	*Dianthus deltoides*	1	1	0	**36.**	*Anthoxanthum odoratum*	1	1	1
37.	*Viola gracilis*	1	1	1	**38.**	*Poa media*	1	1	0
39.	*Silene vulgaris*	1	1	0	**40.**	*Thymus praecox* subsp. *zygiformis*	1	1	1
41.	*Primula veris*	1	1	1	**42.**	*Cerastium alpinum*	1	1	1
43.	*Carduus acanthoides*	0	1	1	**44.**	*Achillea lingulata*	1	1	1
45.	*Cirsium heterophyllum*	1	0	0	**46.**	*Viola elegantula*	1	1	1
47.	*Senecio squalidus* subsp. *rupestris*	1	1	0	**48.**	*Muscari botryoides*	1	1	1
49.	*Trifolium medium subsp. balcanicum*	1	1	1	**50.**	*Gentiana utriculosa*	1	1	1

No.	Plant taxa	s1	s2	s3	No.	Plant taxa	s1	s2	s3
51.	*Alchemilla hybrida*	1	1	1	**52.**	*Pilosella hoppeana*	1	1	1
53.	*Pilosella officinarum*	1	1	1	**54.**	*Crepis aurea*	0	1	1
55.	*Verbascum longifolium subsp. pannosum*	1	1	1	**56.**	*Trifolium pratense*	1	1	1
57.	*Euphrasia rostkoviana* subsp. *montana*	1	1	0	**58.**	*Vaccinium uliginosum*	1	1	0
59.	*Ranunculus montanus*	1	1	1	**60.**	*Campanula scheuchzeri*	1	1	1
61.	*Leucanthemum vulgare*	1	1	0	**62.**	*Ligusticum mutellina*	1	1	1
63.	*Scabiosa columbaria*	1	1	0	**64.**	*Armeria canescens*	1	1	0
65.	*Veronica chamaedrys*	1	1	1	**66.**	*Centaurea nervosa*	1	1	0
67.	*Agrostis stolonifera*	1	1	1	**68.**	*Gentianella bulgarica*	1	1	0
69.	*Dianthus carthusianorum*	1	1	0	**70.**	*Gymnadenia conopsea*	1	1	0
71.	*Phleum phleoides*	1	1	0	**72.**	*Minuartia recurva*	1	1	0
73.	*Luzula sylvatica*	1	1	0	**74.**	*Scleranthus perennis* subsp. *marginatus*	1	0	0
75.	*Genista depressa*	1	1	0	**76.**	*Vaccinium vitis-idaea*	1	1	1
77.	*Galium anisophyllon*	1	1	1	**78.**	*Festuca adamovicii*	1	1	1
79.	*Veratrum album*	0	1	1	**80.**	*Sesleria comosa*	1	1	1
81.	*Silene viscaria*	1	1	0	**82.**	*Crocus veluchensis*	1	1	1
83.	*Asperula cynanchica*	1	1	0	**84.**	*Primula minima*	1	0	0
85.	*Bistorta vivipara*	1	1	0	**86.**	*Botrychium lunaria*	1	1	0
87.	*Nardus stricta*	1	1	1	**88.**	*Clinopodium alpinum*	1	1	0
89.	*Phyteuma pseudorbiculare*	1	1	1	**90.**	*Thesium alpinum*	1	1	1
91.	*Hieracium villosum*	1	1	1	**92.**	*Juniperus communis* subsp. *nana*	1	1	0
93.	*Poa badensis*	1	1	1	**94.**	*Antennaria dioica*	0	1	1
95.	*Pimpinella saxifraga*	1	1	0	**96.**	*Hieracium naegelianum*	1	1	1
97.	*Potentilla aurea*	0	1	1	**98.**	*Festuca halleri* subsp. *scardica*	1	1	0
99.	*Anemonastrum narcissiflorum*	1	1	1	**100.**	*Hypericum maculatum*	1	1	1
101.	*Linum capitatum*	1	1	1	**102.**	*Bruckenthalia spiculifolia*	1	1	0

Annex – Table 2 Spider species numbers and presence at each site.

No.	Spider taxa	s1	s2	s3
1.	*Tibellus oblongus* (Walckenaer, 1802)	**5**	**1**	0
2.	*Pardosa saltuaria* (L. Koch, 1870)	0	**2**	**18**
3.	*Xysticus audax* (Schrank, 1803)	0	**1**	0
4.	*Aculepeira ceropegia* (Walckenaer, 1802)	**3**	**3**	0
5.	*Platnickina tincta* (Walckenaer, 1802)	0	**1**	0
6.	*Araneus diadematus* Clerck, 1757	**4**	**2**	0
7.	*Araneus quadratus* Clerck, 1757	**1**	**2**	0
8.	*Araniella opisthographa* (Kulczyński, 1905)	0	**1**	0
9.	*Microlinyphia pusilla* (Sundevall, 1830)	**1**	0	0
10.	*Linyphia triangularis* (Clerck, 1757)	0	**1**	0

SYSTEMATIC REVIEW AND ANALYSIS OF THE TAXONOMY OF MUSK DEER IN KASHMIR

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ABSTRACT

Elucidating the taxonomy of species is important for conservation purposes, but unfortunately musk deer in Kashmir have not been studied extensively. Examination of the museum specimens of musk deer from the Kashmir region indicated that in Kashmir there are possibly two species. Field studies in Jammu and Kashmir revealed that the musk deer occurring in the Wardwan – Kishtwar belt are apparently different from those that occur in the main part of Kashmir. This study is based on a systematic review of the literature on the taxonomy of musk deer in Kashmir along with some personal field observations. The species most predominantly occurring in Kashmir is the Kashmir musk deer (*Moschus cupreus*). The other possible species is Himalayan musk deer (*Moschus leucogaster*) occurring mostly in the Wardwan – Kishtwar belt of Jammu and Kashmir. The affinities of musk deer in Kashmir with other species of musk deer are also studied.

Keywords: Himalayan musk deer; Jammu Kashmir; Kashmir musk deer; *Moschus cupreus*; *Moschus leucogaster*; taxonomy

Introduction

Taxonomy is the foundation of biodiversity conservation and validating the genetic distinctness of extant sub-species and isolated populations remains an important goal, with implications for conservation (Grubb and Gardner 1998). The basic premises of taxonomy, the science of biological classification, is still undergoing a wide-ranging rethink (Groves 2003).

Musk deer are evolutionarily primitive (Fig. 1) and not included in the Cervidae (Groves and Grubb 1987). The taxonomy of *Moschus* is unrefined (Flerov 1952; Groves 1975; Groves 1980; Groves and Grubb 1987; Groves et al. 1995; Grubb and Gardner 1998; Groves 2003). Further, the taxonomy of musk deer in India and some of its adjoining areas is further confused by giving too much emphasis to the species *Moschus chrysogaster* (Green 1979, 1985, 1986, 1987, 1989, 2002, etc.).

In the absence of detailed scientific and biological studies, is it possible to establish how many species of musk deer there are in Kashmir? To address this question, the present study compiled the morphological and other observations on the musk deer found in Kashmir. The details presented below may initially appear fuzzy, but succeeding elaboration will throw more light on the taxonomy of musk deer in Kashmir and reveal its similarities and differences from other species of musk deer.

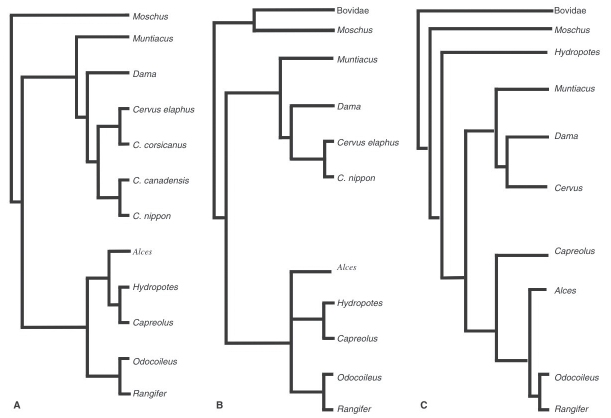

Fig. 1 Cladograms (maximum parsimony) of the phylogenetic relationships of Cervids based on: (A) Cytochrome b mtDNA gene data (Randi et al. 1998; Pitra et al. 2004); (B) Cytochrome b and CO_2 mtDNA and nuclear fragments: exon 2 of alpha-lactalbumin and intron 1 of the gene encoding protein kinase C iota data (Gilbert et al. 2006); (C) morphological data (Groves and Grubb 1987) (Source: Cap et al. 2008).

Methods

Literature on musk deer was analysed with particular emphasis on those occurring in Jammu Kashmir. Some of the personal field observations made during various studies on musk deer relevant to their taxonomic status are also presented.

Results and Discussion

In cladistic terms, *Moschus moschiferus* is probably the sister-taxon of all other species (Groves et al. 1995). Kao's (1963) description of *Moschus moschiferus*, which perhaps is a modification of that of Flerov's (1952), according to Groves (1975), is as follows: "a large species; dark brown, usually spotted; two white stripes on lower part of neck, extending to shoulder. Ear-backs dark. Individual hairs are grey white for two-thirds of their length, then brown-grey, with a darker brown tip; commonly there are whitish rings near the tips, which, when clumped, give the overall spotted effect. The fur is soft

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compared to that of the other two species, 45–60 mm long on the withers, 65–75 mm on the rump (in the Siberian race; but in Korean skins these lengths are only 34–37 mm and 45–62 mm respectively)". Only the Siberian musk-deer (provisionally called *Moschus moschiferus*) has white rings on its hair; in both other species the rings tend to be yellowish to some degree (Groves 1975).

On the basis of museum samples, Groves (1975) states that "there exist two colour types from the Indian region: a dark type with dark ears, indistinguishable from the Chinese *berezovskii*, and a light type with yellow-rimmed ears, recalling *sifanicus,* but not identical with it (ears only rimmed with yellow, not broadly tipped; colour perhaps greyer and less yellow); and as such there are certainly two species represented". Two of the museum samples at the British Museum (Natural History) pertaining to the Kashmir region have been described by Groves (1975) as:

"London, BM (NH): Skull only";

"91.8.7.221. Kashmir. Length 150 mm; lacrimal somewhat longer than broad; midpoint in orbit; arches not much elevated."

"91.8.7.222. Kashmir. Incomplete; lacrimal longer than broad; arches somewhat elevated."

Codes 91.8.7.221 and 91.8.7.222 mentioned above refer to the museum cataloguing of specimens and numbers for locating the samples within the museum.

The additional specimens examined by Groves (1980) are described as:

"1. Moschus sifanicus

Two skins, one skull and a head skin in the Powell-Cotton Museum, Birchington, Kent, England. T.31.2 is skin and skull; the skin is light tobacco brown, fading to off-white on head, shoulders and again on rump. The ears are yellow-rimmed. Skull broken, but its length is approximately 160 mm, lacrimal 21×14; mid-point of skull probably in orbit. Locality is Baltal, Kashmir. The other complete skin (no number) and head skin (M.46.99) have no locality beyond 'Kashmir' but are clearly of this species."

"2. Moschus chrysogaster chrysogaster

The Powell-Cotton Museum possesses an incomplete skull that is probably of this form, number T.31.3, from Srinagar, which (if it is the actual locality rather than a base camp) is in the forest zone. The length would have been about 145 mm; lacrimal 23×20; mid-point would have been approximately at the front edge of the orbit."

Since both specimens are from Kashmir, it is possible that there are two species of musk deer in Kashmir. During the present study it was apparent that the musk deer occurring in the Wardwan – Kishtwar belt differ from those that occur in the Kashmir division. The musk fragrance and the form of the musk pods (and musk grains) differ in the musk deer in these two areas. According to Ali (2016), "The musk pods of the musk deer in Wardwan and Marwah are larger and differ in appearance from those of the deer in Kashmir. The outer skin covering the musk glands of those in the Wardwan-Marwah range is more delicate than that covering the musk pods of those in Kashmir. The musk from Wardwan and Marwah deer is highly granular and reddish brown in colour, similar to the colour of dried dates, and musk grains are wood-like in consistency. By comparison, the musk from Kashmir is powdery in appearance with a predominantly brownish tinge. Kashmir musk is not so fragrant; the fragrance of the musk from the Wardwan-Marwah range is well developed, breath taking and pleasing to the mind."

The earliest name for musk-deer from the Indian region is Hodgson's *Moschus chrysogaster* (1839). According to Groves (1980), two species of musk deer are found in India and Nepal: *M. sifanicus* has light brown fur, the backs of the ears are rimed with pure yellow, the skull length averages about 160 mm, and the lacrimal bone is long and low; *M. chrysogaster* has dark brown fur, the earbacks are wholly dark, the skull length is about 150 mm, and the lacrimal is relatively short and high. The former, which lives above the tree-line, is represented in China by a race in which the whole tip of the ear, not just the rim, is yellow, but which is otherwise poorly distinguished and is in any case unnamed; the latter species, which lives in forest and is represented in India and Nepal by its nominate form, is smaller and short-faced, whereas in China there are probably two subspecies.

Further, Grubb (1982, cited in Groves and Grubb 1987) describe *Moschus chrysogaster* as, "*M. chrysogaster* [= *M. sifanicus*] that occurs in the Alpine zone of the eastern and southern edge of the Tibetan Plateau, extending onto the southern side of the Himalayas. It is the largest of the musk deer, with a characteristic long-snouted skull. The pelage is a pale speckled yellow brown in colour, with yellow-tipped ears and a broad whitish band down the throat. These last two features are sometimes absent in the north Indian population, which apparently bridge the gap between nominate *chrysogaster* and a newly described subspecies, *cupreus*, from Kashmir."

For the musk deer of Himalaya, the Kashmir form is described by Groves et al. (1995) as: "A Kashmir form, described as *Moschus chrysogaster cupreus* by Grubb (1982); apart from the specimens listed in the type description, Colin P. Groves has seen two in the Calcutta collection, from Gilgit (ZSI 19942) and from Peshawar (ZSI 9968). It is grey-brown, often vaguely spotted, distinguished by having a coppery-brown unspeckled dorsal saddle; it has a very dark, grizzled grey rump, light grey underparts, white throat, whitish lower limb segments, and dark brown ears white at the base, with frosted rims. The hairs have long white bases; their length is 33–38 mm. on the withers, 37–58 mm. on the rump."

There are four, perhaps five distinct taxa in the Himalayan region; two of these (*Moschus cupreus* and the *Kulu form*) are allopatric to the others, whereas the other four occur close to each other in the Nepal / Sikkim / S.E. Tibet region. As three of them (*M. fuscus, M. leucogaster* and the true *M. chrysogaster*) are all said to occur in Sikkim, while *M. fuscus* and the enigmatic Zhangmu form

are both recorded from the Nepal side of Mt. Everest, it would appear that there are at least three species (Groves et al. 1995).

An analysis of skull morphology using craniometric characteristics in a multivariate analysis (Groves et al., 1995) indicates, as shown in Figs 2 and 3, the affinities of musk deer in Kashmir with other species of musk deer.

"The polygon for *M. moschiferus* overlaps those for *M. berezovskii* and *M. cupreus*; those for *M. berezovskii* and *M. fuscus* overlap somewhat and those for *M. cupreus*, *sifanicus* and the *Zhangmu/ Khumjung* sample are separate from all the others. When the *M. moschiferus* and *M. cupreus* polygons are omitted, *M. chrysogaster* is close to the *sifanicus* sample, which contains the reputed type of *M. saturates*; the *anhuiensis* specimen is close to the *berezovskii* sample; and the *M. fuscus* and *M. berezovskii* samples no longer overlap", according to Groves et al. (1995). *Moschus chrysogaster* almost certainly includes *sifanicus* as a subspecies; there is no evidence that it occurs south of the Himalayas, but seems to be restricted to the alpine zone in the southeastern and eastern margins of the Tibetan plateau (Groves et al. 1995). Groves et al. (1995) conclude by proposing a taxonomy for *Moschus* in which *Moschus cupreus* is recorded as:

" *? M. cupreus* Grubb, 1982 (or as *Moschus leucogaster cupreus*)

Kashmir. Alt. over 3000 m.

Dorsal reddish; rump very dark, with grizzled grey bottom patch; limbs lighter. Rump usually dark or grizzled grey."

The multivariate analysis of the craniometric data (Groves et al. 1995) indicates that there is no overlap of the polygons for *Moschus leucogaster* and *Moschus cupreus*; therefore, it is plausible to record the Kashmir musk deer as "*Moschus cupreus* Grubb, 1982".

Groves (2003) raised six taxa, commonly regarded as subspecies, to specific rank, two of which are *Moschus cupreus* and *Cervus hanglu*. These species were earlier considered as subspecies and were named *Moschus leucogaster cupreus* and *Cervus elaphus hanglu,* respectively.

On the basis of Groves (2003) it can be inferred that *Moschus chrysogaster* does not occur in India and its distribution extends from eastern Nepal to Sikkim and Bhutan, entirely in the plateau zone.

Groves (2003) describes the Kashmir musk deer as:

"*Moschus cupreus* Grubb, 1982. Kashmir musk deer

From Kashmir, at over 3,000 m above m s. l.

Colour grey-brown, often vaguely spotted, with a copper-reddish dorsal saddle; rump dark, grizzled grey; underside light grey; throat white; lower segments of limbs whitish. Ears dark brown, white at base, with frosted rims. Large species."

This description can be compared with the photographs of the Kashmir musk deer in Fig. 4. Further images of the deer are presented in Figs 5–8. The colour details of the individual hairs of musk deer (measuring 48 mm and 58 mm in length) collected from Dachigam National Park (Kash-

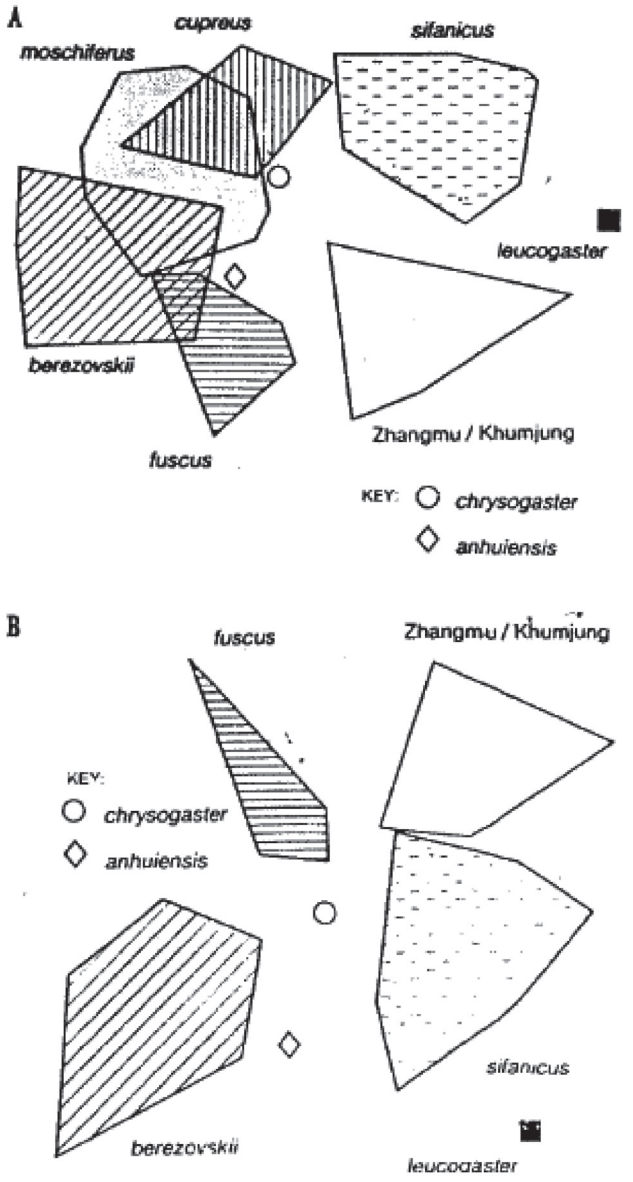

Fig. 2 Discriminant function plots; full variables (the *M. moschiferus* and *M. cupreus* dispersions are omitted); A – horizontal: Functions 1, vertical: Functions 2; B – horizontal: Functions 1, vertical: Functions 3 (Groves et al. 1995).

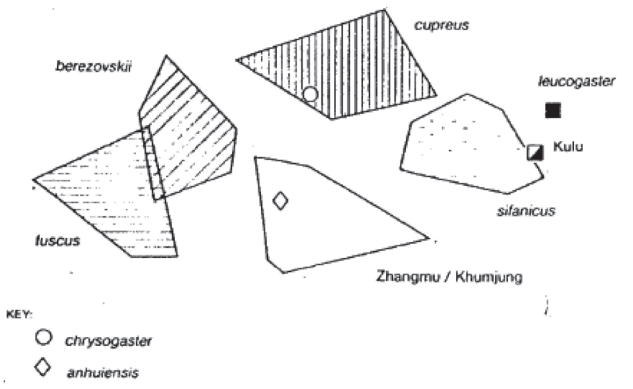

Fig. 3. Discriminant function plot of a reduced list of craniometric characteristics of *Moschus* (to enter the Kulu skull). Horizontal: Functions 1, Vertical: Functions 2 (Groves et al. 1995).

mir) during the present study are illustrated in Fig. 9 for comparison with the descriptions provided above. In Siberian musk deer, the hairs covering the body are reported to be 65 to 95 mm in length (Vaisman and Fomenko 2004) and the length of those of the Alpine musk deer (*Moschus chrysogaster*) in China is 38 mm (Sheng et al. 1993).

Morphometric and morphological traits are frequently used by taxonomists for differentiating between species. The photographs in figs 4 to 8 provide first-hand information on the musk deer in Kashmir that can be compared with the descriptions of the specimens of musk deer in Kashmir in various museums and described above.

Fig 4. A sub-adult female musk deer photographed in Kashmir, 2005 (Ali 2009).

Fig. 5 An approximately five-month-old musk deer fawn injured by stray dogs in a residential locality in Firdousabad (Batamaloo) in southern part of Srinagar City (Kashmir), which was rescued by locals and wildlife department officials.

Fig. 6 Photograph of a male musk deer that was probably driven by a predator from the Dachigam National Park or its adjoining hills, which was caught by people in Merakshah Colony Habak (Srinagar, Kashmir) and handed over to Nigeen Police, 2007.

Fig. 7 Photographs of a female musk deer rescued by locals from a stream in Pethpora Nallah Bramsar locality of Chatergul (Kangan, Kashmir) on July 25, 2019. It had a bruised neck and was suffering from skin disease and infested with small wingless bloodsucking insects; and died after a few days in a rescue hut in the Dachigam National Park.

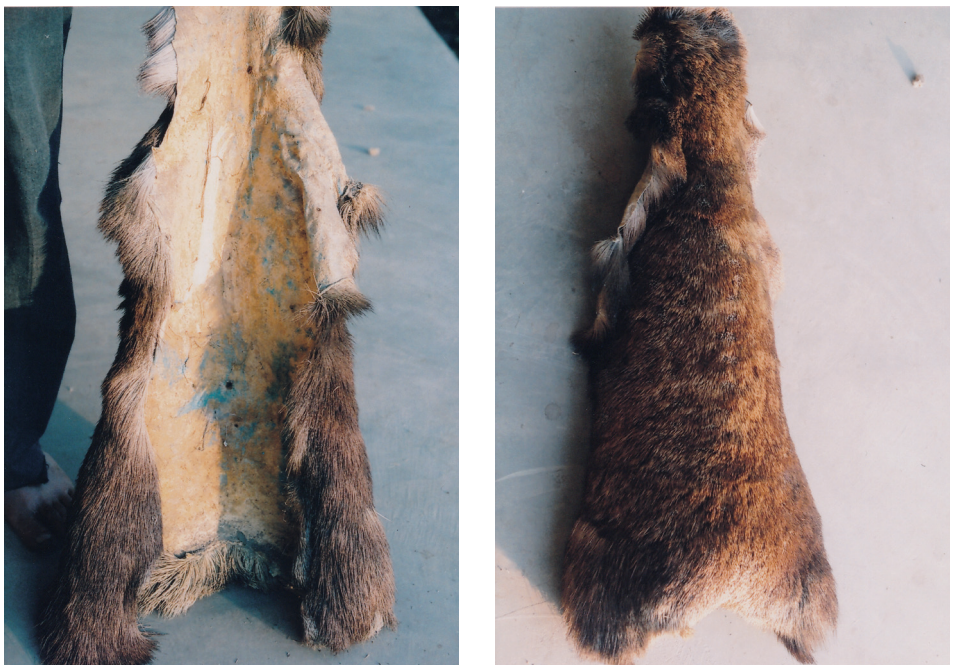

Fig. 8. An old skin of musk deer photographed in 2005 (type locality for the above skin is Yamhur Nai (~≥3000 m), northeast of Dachigam) (Ali 2016).

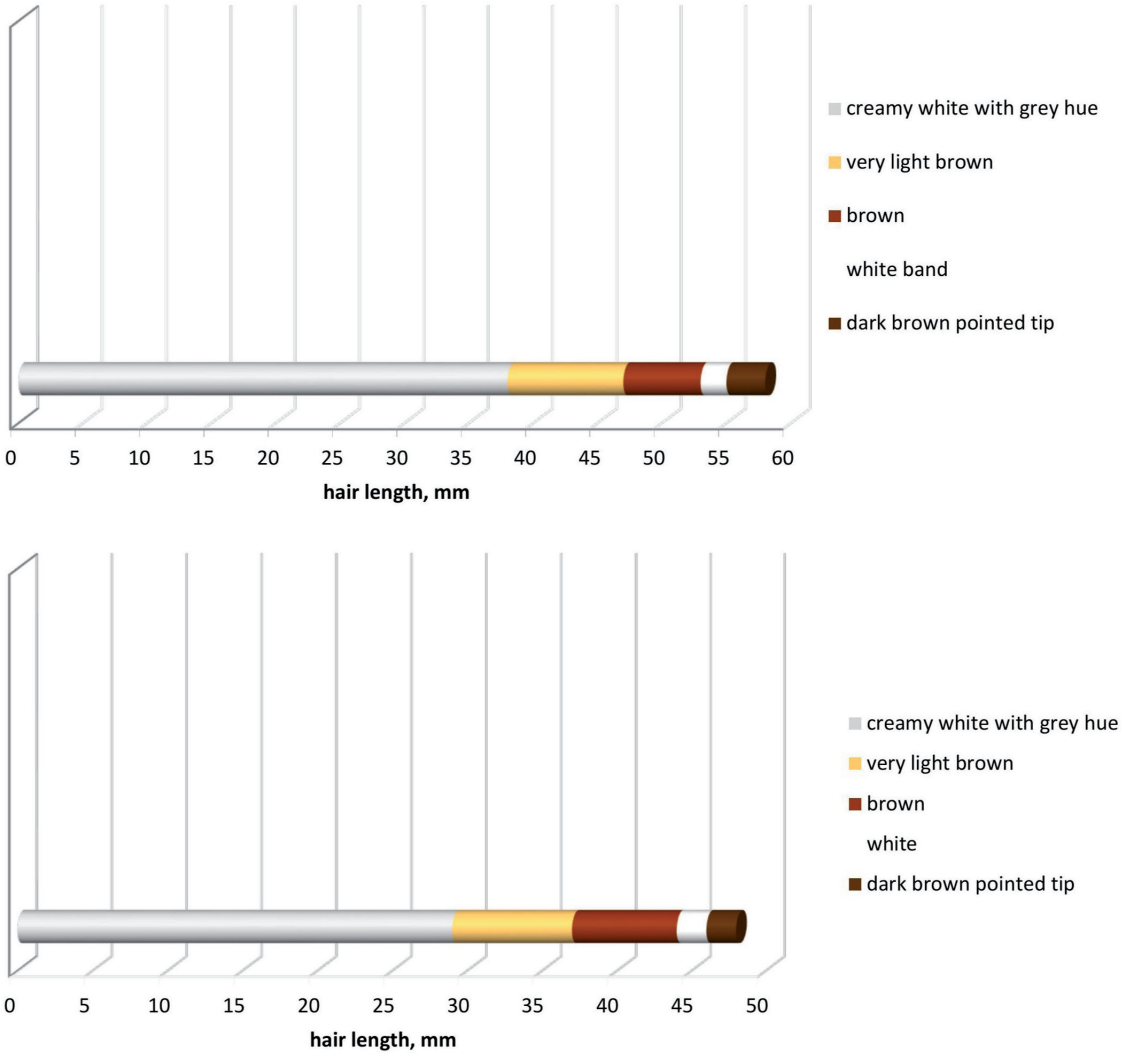

Fig. 9 Colouration of two hairs of different lengths collected from Kashmir musk deer in Dachigam National Park, Kashmir.

Gergan (1962) reports the height of musk deer at the shoulders is about 50 cm and at the rump about 55 cm. Colonel A. Ward, a well-known authority on Kashmir and mountain sports reports that musk deer are 22 inches (56 cm) in height at the croup and weigh 20 to 25 pounds (9 to 12 kg) (Lawrence 1895). The young are spotted on the back and sides (Lawrence 1895).

The updated IUCN Red List of threatened species (2008) recognizes the specific rank of the Kashmir musk deer and lists it as endangered. The status and global distribution of musk deer is shown in Figs 10 and 11. The most recent study on the habitat of Kashmir musk deer in Kashmir is that of Ali (2014).

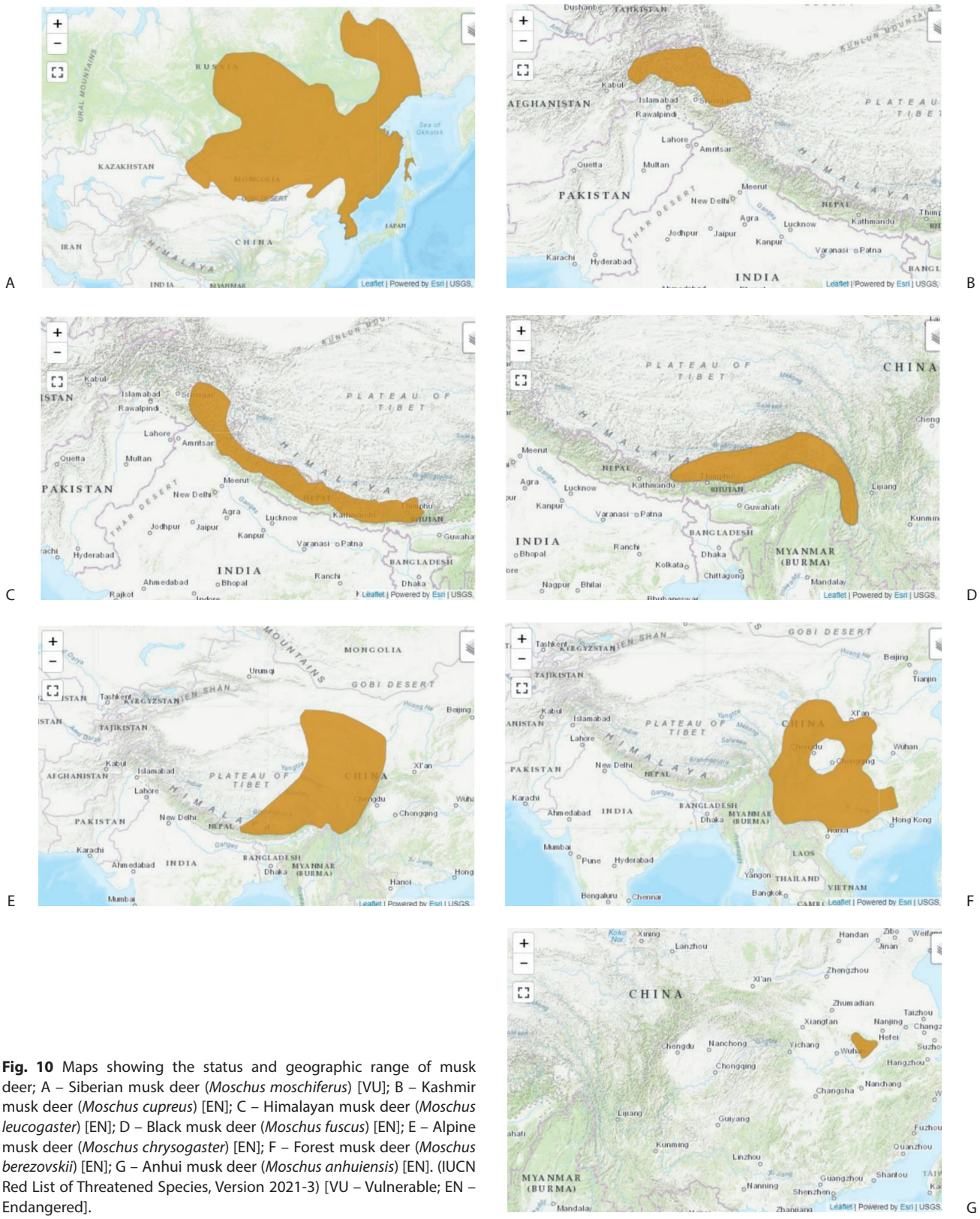

Fig. 10 Maps showing the status and geographic range of musk deer; A – Siberian musk deer (*Moschus moschiferus*) [VU]; B – Kashmir musk deer (*Moschus cupreus*) [EN]; C – Himalayan musk deer (*Moschus leucogaster*) [EN]; D – Black musk deer (*Moschus fuscus*) [EN]; E – Alpine musk deer (*Moschus chrysogaster*) [EN]; F – Forest musk deer (*Moschus berezovskii*) [EN]; G – Anhui musk deer (*Moschus anhuiensis*) [EN]. (IUCN Red List of Threatened Species, Version 2021-3) [VU – Vulnerable; EN – Endangered].

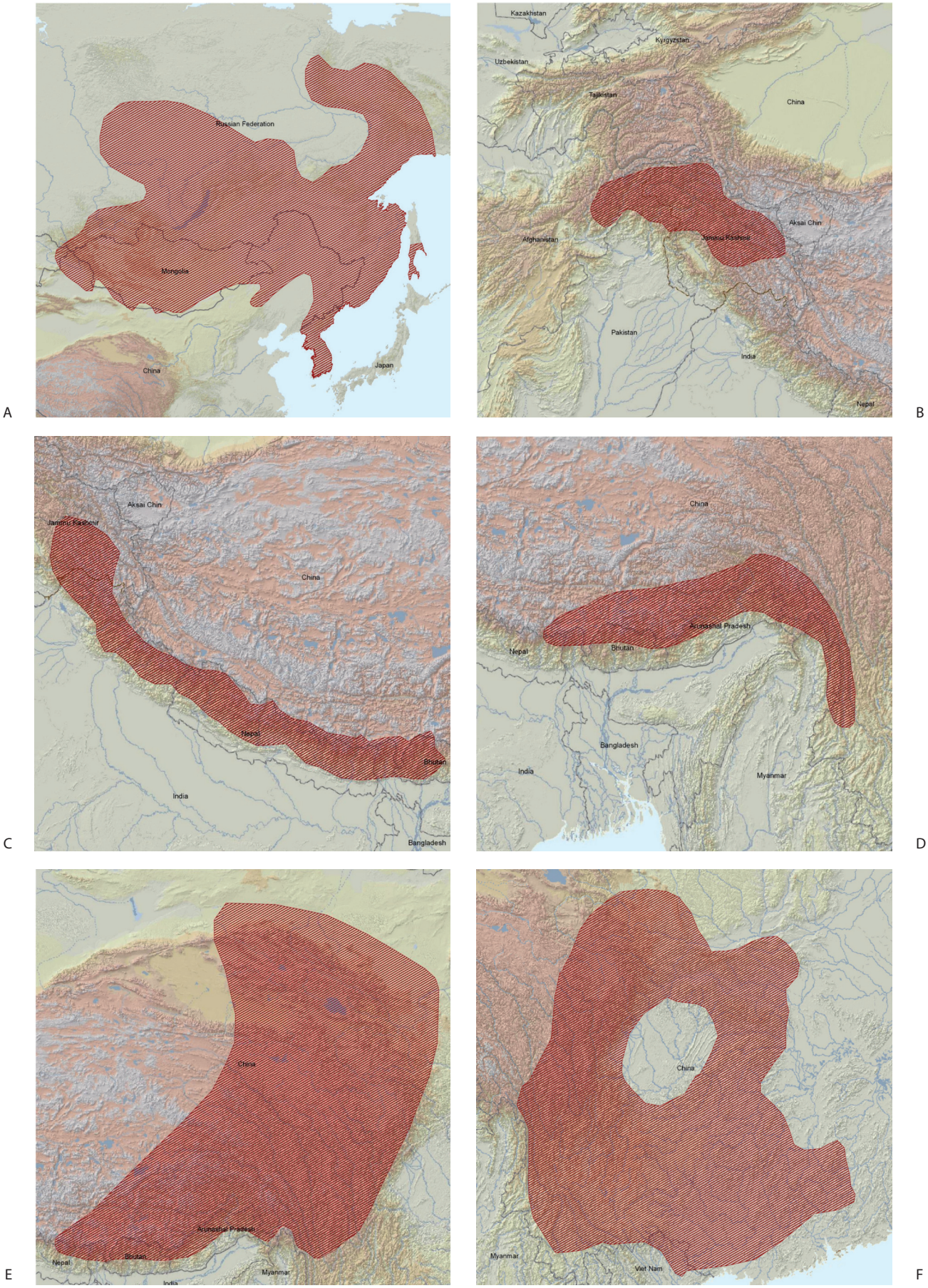

Fig. 11 Maps showing in cross hatched red the geographical distribution of musk deer; A – Siberian musk deer (*Moschus moschiferus*); B – Kashmir musk deer (*Moschus cupreus*); C – Himalayan musk deer (*Moschus leucogaster*); D – Black musk deer (*Moschus fuscus*); E – Alpine musk deer (*Moschus chrysogaster*); F– Forest musk deer (*Moschus berezovskii*). (IUCN Red List of Threatened Species, 2008/2022).

Conclusion and Recommendations

On the basis of an analysis of data and field observations it is tentatively concluded that in Jammu and Kashmir there are two different species of musk deer: Kashmir musk deer (*Moschus cupreus*) and Himalayan musk deer (*Moschus leucogaster*). Thus, it is recommended that a DNA study should be used to elucidate the taxonomic diversity of musk deer in this region.

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SPRUCE- AND BEECH-DOMINATED PRIMARY FORESTS IN THE WESTERN CARPATHIANS DIFFER IN TERMS OF FOREST STRUCTURE AND BIRD ASSEMBLAGES, INDEPENDENTLY OF DISTURBANCE REGIMES

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ABSTRACT

Mountain spruce- and beech-dominated forests (SDPF and BDPF) are of major importance in temperate Europe. However, information on the differences between their historical disturbance regimes, structures, and biodiversity is still incomplete. To address this knowledge gap, we established 118 circular research plots across 18 primary forest stands. We analysed the disturbance history of the last 250 years by dendrochronological methods and calculated disturbance frequency, severity, and timing. We also measured forest structure (DBH, tree density, volume of deadwood, and other parameters). Breeding bird populations were examined by point count method during the spring seasons 2017–2018 (SDPF) and 2019–2020 (BDPF). Using direct ordination analysis, we compared the disturbance history, structure and bird assemblage in both forest types. While no differences were found regarding disturbance regimes between forest types, forest structure and bird assemblages were significantly different. SDPF had a significantly higher density of cavities and higher canopy openness, while higher tree species richness and more intense regeneration was found in BDPF. Bird assemblage showed higher species richness in BDPF, but lower total abundance. Most bird species which occurred in both forest types were more numerous in spruce-dominated forests, but more species occurred exclusively in BDPF. Further, some SDPF- preferring species were found in naturally disturbed patches in BDPF. We conclude that although natural disturbances are important drivers of primary forest structures, differences in the bird assemblages in the explored primary forest types were largely independent of disturbance regimes.

Keywords: beech; birds; Carpathian Mountains; disturbance history; forest structure; mountain temperate forests; spruce

Introduction

The Central European mountain landscape has been naturally covered mostly by forest since the last Ice Age (Vera 2000; Szabó et al. 2016). The species composition of these forests changes along an altitudinal gradient. In medium elevations (500–1,200 m a. s. l.), the forest was originally a mixture of many species, but mostly dominated by beech (*Fagus sylvatica*). At the highest altitudes, near the upper treeline (1,200–1,600 m a. s. l.), forests are naturally dominated by spruce (Mirek 2013; Čada et al. 2020). However, due to the long history of human settlement, most of the Central European forests have been subjected to more or less intensive use (Mikoláš et al. 2019). Therefore, only fragments of original forest remain in the most inaccessible and remote parts of the Western Carpathian mountains, which account for less than ~10,600 ha (0.5%) of Slovakian forests (Jasík and Polák 2011; Mikoláš et al. 2019).

In comparison with managed forests, primary forests are shaped exclusively by natural processes, mainly natural disturbances (Pickett and White 1985; FAO 2020; Vandekerkhove et al. 2022). In the Central European mountain primary forests, the main disturbance agents are windstorms, bark beetles (most importantly *Ips typographus* and to a smaller extent other insect species), amongst other factors including avalanches, ice storms and large herbivores (Nagel et al. 2013; Kulakowski et al. 2017; Synek et al. 2020). Disturbances predominantly affect forest ecosystems by creating patches of dead trees varying in spatial extent and severity (Pickett and White 1985; Čada et al. 2020). In contrast with managed forests, dead trees and their components remain in unmanaged forest as disturbance legacies (Seidl et al. 2014), contribute to the total carbon pool (Commarmot et al. 2005; Glatthorn et al. 2018), help facilitate regeneration after disturbance (Zielonka 2006; Michalová et al. 2017), whilst also providing important structural elements for biodiversity (Stokland et al. 2012; Thorn et al. 2017; Kozák et al. 2020).

The recent development of dendrochronological methods has allowed our scientific understanding of the long-term dynamics of Central European mountain primary forests to increase rapidly (e.g. Svoboda et al. 2014;

Kameniar, O., Baláž, M., Svitok, M., Mikoláš, M., Ferenčík, M., Frankovič, M., Ralhan, D., Gloor, R., Svoboda, M.: Spruce- and beech-dominated primary forests in the western Carpathians differ in terms of forest structure and bird assemblages, independently of disturbance regimes
European Journal of Environmental Sciences, Vol. 13, No. 1, pp. 47–59
https://doi.org/10.14712/23361964.2023.6
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Trotsiuk et al. 2014; Janda et al. 2017; Schurman et al. 2018; Čada et al. 2020; Frankovič et al. 2021). However, large knowledge gaps remain. Although BDPF and SDPF naturally occur next to each other and their disturbance regimes can both be described as mixed-severity/mixed-scale, the regimes differ to some extent (Nagel et al. 2013). Both forest types are shaped by wind, but in BDPF wind disturbances are mostly unsynchronised over large landscapes (Frankovič et al. 2021). This typically leads to structurally rich forests with patches of all developmental stages represented in small areas (Korpeľ 1989; Orman and Dobrowolska 2017). Conversely, SDPF are mainly shaped by medium-scale and medium-severity events (Čada et al. 2020). Synchronised severe disturbances, which are typically initiated by wind and secondarily enhanced by bark beetles, also occur regularly (Wermelinger 2004; Seidl et al. 2016). However, there is emerging evidence that medium- to high-severity and scale disturbances were also historically a part of BDPF disturbance regimes, although to a much lower extent than in SDPF (Frankovič et al. 2021). The diversity of disturbance regimes has differing effects on forest structure, which thereby has divergent effects on habitat availability for different taxonomic groups of species, thereby altering biological assemblages (Kozák et al. 2020; Langbehn et al. 2021; Ferenčík et al. 2022). Therefore, disentangling the impacts of disturbances across different forest types is crucial in these times of rapid biodiversity decline.

Birds (Aves) are an ecologically important taxonomic group (Sekercioglu et al. 2004; Whelan et al. 2015), which have various demands on forest structure for nesting, foraging and other activities (Brawn et al. 2001; Hanzelka and Reif 2016). They are also important from the nature conservation perspective as umbrella species (Mikoláš et al. 2017), flagship species (Kortmann et al. 2018) and indicator species (Braunisch et al. 2019). Bird assemblages of BDPF and SDPF differ to some extent, but only a minor number of species are strictly tied to one of them (Korňan 2004; Wesolowski et al. 2018; Kameniar et al. 2021). Generalist species such as chaffinch and European robin reach comparable abundances in both forest types (Saniga and Saniga 2004; Saniga 2009), but most species typically show a stronger or weaker preference to one of them (Wesołowski et al. 2003; Tomiałojć and Wesołowski 2004). Numerous studies on bird assemblages have been conducted in European mountain temperate beech- and spruce-dominated forests, but they largely focused on forests with a human-altered disturbance regime, structure and biodiversity (Moning and Müller 2008; Topercer et al. 2009; Baláž and Kocian 2015; Birčák and Reif 2015), or they explored only one or several primary forest fragments (Korňan 2004; Saniga and Saniga 2004; Saniga 2009). Moreover, most studies which focused on primary forests did not examine disturbance history and forest structure in detail. Although the study by Kameniar et al. (2021) explored the disturbance-structure-bird assemblage relationship in SDPF in the Western Carpathians, studies investigating BDPF remain absent.

Primary forest structure is directly created or influenced exclusively by natural disturbances (Rodrigo et al. 2022). Several structural features have been identified as important for bird assemblage diversity and abundance, including the amount of coarse woody debris (Rosenvald et al. 2011) and its subtypes, especially standing dead trees (a key habitat for woodpeckers (Pechacek and d'Oleire-Oltmanns 2004)), and uprooted trees, which are used by several species for nesting (Wojton and Pitucha 2020). Other important structural characteristics for forest birds have also been identified, such as large habitat trees (Kebrle et al. 2021), age of forest stand (Poulsen 2002), richness of vertical canopy structure (Goetz et al. 2007), canopy openness (Lewandowski et al. 2021), overall stand-level heterogeneity (Kebrle et al. 2022) and the presence of various microhabitats, especially cavities (Piechnik et al. 2022). These structural features change across several time and space scales, and their actual values depend on the given disturbance agent (or their combinations), disturbance severity, spatial extent, and timing (Mikoláš et al. 2017; Kameniar et al. 2021). However, it is still unclear how they differ in BDPF and SDPF in Central Europe.

In this study, our specific aims are: 1. to compare important structural variables for birds in BDPF and SDPF; 2. to compare bird assemblages between both forest types.

Material and Methods

Study area, stand selection and study plots establishment

Our study was conducted in the Western Carpathian Mountains (Slovakia), between 48.632749° and 49.523229° N and between 19.010233° and 20.118049° E, elevation of our research plots was between 769 and 1,534 m. Research plots were located inside primary forest remnants recognised by the national inventory of primary forests in Slovakia (Jasík and Polák 2011; Mikoláš et al. 2019). During inventory, all potential primary forest areas were visually surveyed for structural elements, typical for primary forests. Localities with signs of human alteration were excluded. Selected stands of potential primary forests were also checked on historical maps and aerial imagery, whether the selected area was covered with forest during that period. For details, see Mikoláš et al. (2019).

Eighteen study stands were distributed in seven mountain ranges with the largest areas of BDPF and SDPF – the Tatra Mts. (four spruce stands), the Low Tatra Mts. (two spruce stands), the Great Fatra Mts. (two spruce and four beech stands), Low Fatra Mts. (two beech stands), the Poľana Mts. (single spruce and single beech stand), Vepor Mts. (a single beech stand) and the Orava Beskids (a single spruce stand). Most of the SDPF stands are located on intrusive and metamorphic, acidic bedrock, and beech-dominated stands were very heterogeneous. Location of stands is displayed in Fig. 1.

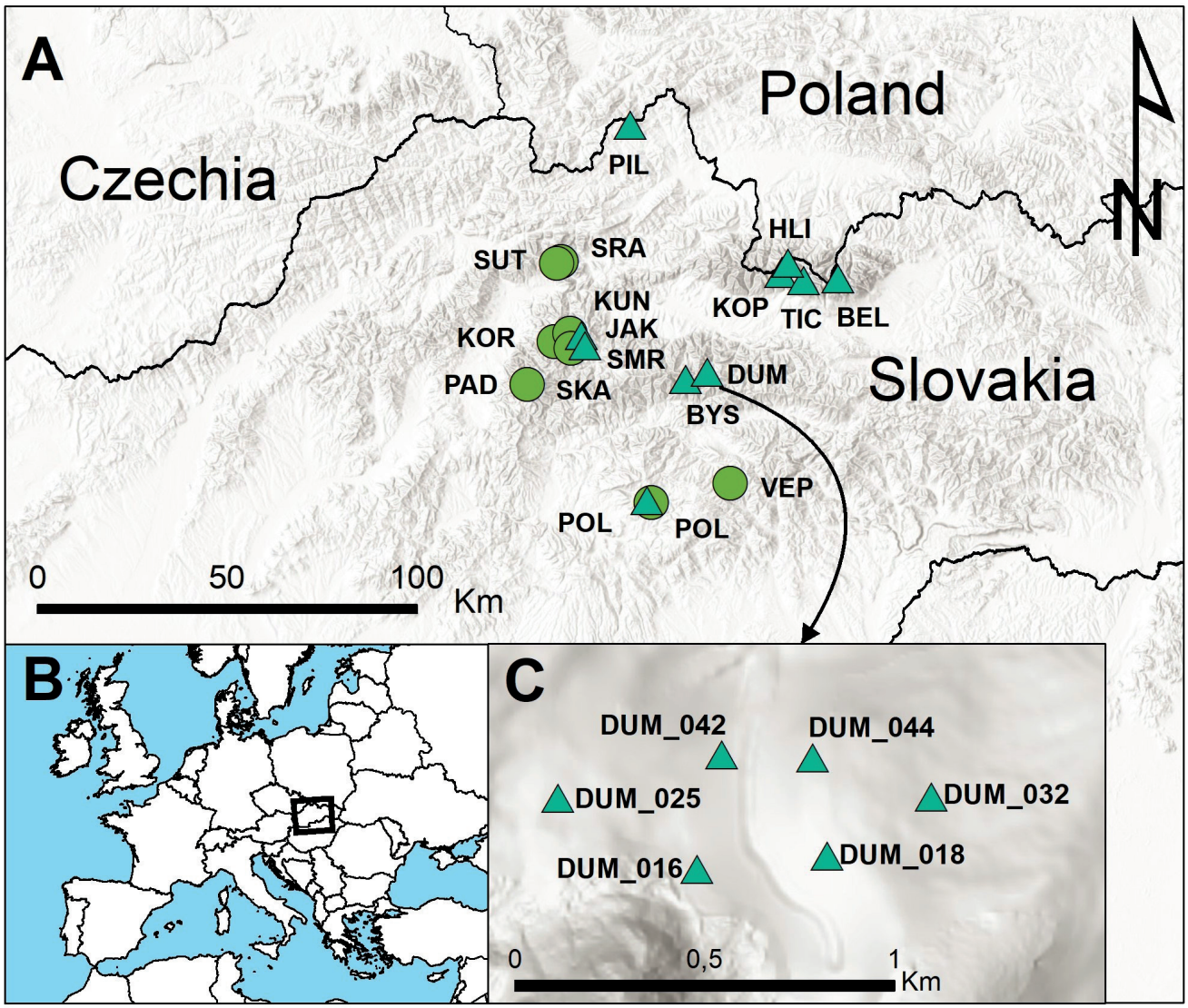

Fig. 1 a) research stands location in Western Carpathians – triangles represent spruce-dominated stands and circles beech-dominated research stands b) location of Western Carpathians in Europe, c) example of research stand with study plots. Spruce-dominated primary forest stands: BEL (Bielovodská valley, High Tatra Mts.), TIC (Tichá valley, High Tatra Mts.), HLI (Hlina, High Tatra Mts.), KOP (Kôprová valley, High Tatra Mts.), PIL (Piľsko, Orava Beskydy), JAK (Jánošíkova kolkáreň, Great Fatra Mts.), SMR (Smrekovica, Great Fatra Mts.), DUM (Ďumbier, Low Tatra Mts.), BYS (Bystrá valley, Low Tatra Mts.), POL (Mt. Poľana). Beech-dominated primary forest stands: POL (Mt. Poľana), VEP (Vepor, Vepor Mts.), SKA (Skalná alpa, Great Fatra Mts.), KUN (Kundráčka, Large Fatra Mts.), KOR (Kornietová, Great Fatra Mts.), PAD (Padva, Great Fatra Mts.), SUT (Šútovská valley, Low Fatra Mts.), SRA (Šrámková, Low Fatra Mts.).

Size of the sites studied (primary forest fragments) varied from 41 to 494 ha. In the case of the smallest fragments, several were treated as one stand. They were surrounded mostly by forests of differing naturalness: natural forests with or without recent management or intensively managed, less natural forests. Some parts are bordering with unnatural spruce plantations, salvage-logged areas, and alpine habitats. However, these environmental variables were not quantified in this study.

Tree species composition in the SDPF was strongly dominated by Norway spruce (over 90%). Other species, such as rowan (*Sorbus aucuparia* L.), fir (*Abies alba* Mill.), beech (*Fagus sylvatica* L.), maple (*Acer pseudoplatanus* L.), larch (*Larix decidua* Mill.), pine (*Pinus* spp.) and birch (*Betula* spp.), were present only as an admixture (Janda et al. 2017). Except of beech, BDPF stands contained highly variable proportion of other tree species, mainly fir, spruce and maple, but also Norway maple (*Acer platanoides*), ash (*Fraxinus excelsior* L.), wych elm (*Ulmus glabra* Huds.), European hornbeam (*Carpinus betulus* L.), Scots pine (*Pinus sylvestris* L.) and other species. Annual mean temperatures range from 1.6 to 3.4 °C in SDPF stands and from 5 to 5.5 in BDPF stands, annual precipitation varies from 1,205 to 1,365 mm in SDPF (Kozák et al. 2020) and around 1,067 mm in BDPF stands (Harris et al. 2020).

In the above mentioned 18 stands, 242 plots (97 in BDPF and 145 in SDPF) were established as part of an international primary forest research project (www.remoteforests.org). To position plot centres, a square grid

was created using the ArcView 9.3 Environment (ESRI ArcGIS 2011) for each stand, and plot centres were placed using a stratified-random design (Svoboda et al. 2014; Frankovič et al. 2021). Within the inner part of each cell, three random points were generated. If the first point was unsuitable (e.g., rocks, water, steepness), then a second (or rarely a third) randomly generated point was used. In BDPF stands, a pair of circular plots (radius of 17.84 m) was positioned along the contour, one on each side of the identified random point. Paired plot centres were 40 m from the random point and 80 m from each other. Study plots in SDPF (radius of 12.62 or 17.84 m, depending on the stand density) were established directly on randomly generated points.

For bird assemblage and forest structure sampling, 58 plots were selected in SDPF stands (six plots per stand, except for one stand in the Tatra Mts. containing only four plots) and 60 plots in BDPF stands. In each stand, study plots were selected to cover the whole gradient of disturbance severities over the last 250 years. For this purpose, we split plots according to disturbance event timing into three equally large classes. We then selected two plots within each class on every stand, with differing severity if available. At the same time, we avoided locating any additional plots within 150 m around a given plot to minimise multiple counts of individual birds at different plots.

Forest structure data

Forest structural parameters were measured in 2017 in all spruce plots and in 2020–2021 in beech plots. For each plot, the GPS position was recorded. All live and dead trees with a diameter at breast height (DBH) ≥ 6 cm were numbered and DBH was measured using a measuring tape. The trees were also precisely mapped using laser rangefinders and customised software (Field-Map; Monitoring and Mapping Solutions, Jílové u Prahy, Czech Republic). Canopy position of each tree was assessed (suppressed: trees with crowns below the general canopy layer and receiving mostly diffuse light and released: trees with crowns forming part of the canopy layer and receiving at least 50% of full light). The diameter of horizontal crown projection was measured with an ultrasound device for a sample of trees to establish statistical relationships between crown area and DBH, which was later used to estimate the percentage disturbance of the canopy.

Species of trees and growth layer (upper, lower) were also recorded. Lying deadwood with a thickness greater than 10 cm was measured using above mentioned Field-Map technology. Both ends were mapped with a laser and the diameter measured using a sliding scale. Average stage of decay (1–5) and species was also recorded for every piece (Stokland et al. 2012). Height of standing deadwood with DBH over 6 cm was estimated as either 0–10 m, 10–20 m or 20–30 m. Subsequently, the volume of deadwood (standing and lying) was calculated. Mean canopy openness was calculated using hemispherical photographs taken at six locations in each plot. They were processed and analysed using image processing software (WinSCANOPY; Regent Instruments, Ste-Foy, Quebec, Canada). Individual pixels were classified as either sky- or leaf-dominated classes based on their spectral properties. Pixel classification results were aggregated to determine the overall mean fraction made up of sky. Number of regenerating trees was counted at the plot-level in three height categories: 0.5–1.3 m; 1.3–2.5 m and > 2.5 m, (at the same time, with DBH < 6 cm.

Age structure and disturbance history

For reconstructing the history of disturbance and estimating the age of the trees, increment cores were extracted from living trees at 1 m height from the base, perpendicular to the direction of the slope. In spruce plots, 15 or 25 (depending on the radius of the plot, 12.62 or 17.84 m) randomly selected trees with DBH ≥ 10 cm and canopy status classified as currently released were sampled. If there were not enough trees on a plot, the closest trees outside the plot were selected, and rotten trees were replaced by a nearby tree with a similar DBH in order to obtain the required sample size. An additional five randomly selected suppressed trees were cored to establish a growth-rate threshold for open canopy recruitment. In BDPF plots, a subplot with a radius of 7.99 m was established at the centre, where all trees (released and suppressed) with DBH ≥ 10 cm were sampled. In mixed beech-dominated plots, a subplot with a radius of 7.99 m was established at the centre, where all trees (released and suppressed) with DBH ≥ 10 cm were sampled. On the remaining part of the plot all released trees with DBH ≥ 10 cm and all suppressed trees with DBH ≥ 15 cm were cored, in addition to three randomly selected suppressed trees with DBHs between 10 and 15 cm. Further, 12 regularly distributed points were established outside the plot within a radius of 25.23 m from the centre of the plot and at each point the closest released tree with DBH ≥ 10 cm was sampled. The study plots were established as a part of the REMOTE Primary Forests network and the differences in sampling are due to the evolving needs of this long-term project.

Cores were processed using standard dendrochronological techniques and ring-width series were measured using a stereomicroscope and a LINTAB sliding table and TsapWin software (RINNTECH, Heidelberg, Germany, http://www.rinntech.com). Cross dating was done using the marker years approach (Yamaguchi 1991) and verified with PAST4 (www.sciem.com), CDendro (Holmes 1983; Larsson 2003), and COFECHA (Holmes 1983) software. For core samples that missed the pith, the number of missing rings was estimated using the method of Duncan (1989). The total number of cores processed was 5,740 (2,284 from BDPF, 3,456 from SDPF); cores that could not be properly cross dated (rotten, damaged) were not included in the analysis, resulting in 5,092 valid samples (1,803 from BDPF and 3,289 from SDPF).

In the next step, radial growth patterns were analysed in order to identify two types of tree canopy accession events: (1) release – abrupt, sustained increase in tree growth, indicating the death of a former canopy tree, and (2) open canopy recruitment – rapid juvenile growth indicating recruitment in a former canopy gap (Lorimer and Frelich 1989). Releases from suppression were identified using the absolute increase method (Fraver and White 2005) as pulses in which the difference between average growth rates of adjacent 10-year running intervals (absolute increase) was greater than or equal to 1.25 standard deviations of all the calculated absolute increase values. To avoid false detection, when mean growth rates are largely influenced by several extreme years, increases had to be sustained for at least seven years to be considered a release event (Fraver et al. 2009). Variables characterising the age structure and disturbance history covering the last 250 years of individual plots were used to describe the disturbance histories. The reconstructed disturbance chronologies were limited to 250 years (1750–2000) to avoid potential bias due to the small number of trees sampled that were older than 250 years. Estimates of disturbance recorded after the year 2000 were not included, as the sample size was too small, which resulted in the exclusion of more recent tree recruitment.

Bird assemblages

Data on species composition of breeding bird assemblages were collected for plots from the end of April until the end of June, i.e., during the peak breeding season. Each plot was visited three times per season on average, SDPF plots in 2017 and 2018 and BDPF plots in 2019 and 2020. Some plots were visited less often due to bad weather. Point counts were used as a field technique with a census point located in the centre of each plot (Verner 1985). During each visit to the plots, all birds within an estimated distance of 60 m from the observer were counted and recorded for 10 minutes. All birds were recorded regardless of age and sex, but most records were based on bird song, particularly that of males defending their territory. After arrival at a given plot, one minute was spent silently before counting started to minimise the observer's influence on bird activity (Sutherland 2006). Counts were done early in the morning (5:00 – 10:00 AM), and only during optimal weather conditions without heavy rain and strong wind (Moning and Müller 2008). Birds recorded during all counts were summarised per plot and then standardised to account for unequal number of counts (Table 3). In the analysis we used species presence/absence data. Species numbers in BDPF and SDPF were not corrected for different sampling intensity as it was very high and almost identical (324 plot counts in BDPF vs. 329 plot counts in SDPF). At the same time, the number of species (53) was relatively low compared to the number of counts and recorded bird individuals (4,745).

Statistical analysis

An ordination analysis was used to target the aims. Redundancy analysis, RDA (Rao 1964), of the correlation matrix of structural characteristics was used to compare structural variables important for birds and disturbance characteristics in BDPF and SDPF (Fig. 2). Finally, distance-based redundancy analysis, db-RDA (Legendre and Anderson 1999), was used to test for differences in the composition of bird assemblages in the two types of forest (Fig. 3). Rarely observed species of birds (frequency of occurrence < 3 plots) were excluded from the datasets to improve the signal-to-noise ratio. Species presence/absence data were converted to Sørensen dissimilarities (1-Sørensen similarity), which disregards double absences and gives higher weight to shared occurrences (Sørensen 1948). The dissimilarity matrix was submitted to db-RDA and the differences between SDPF and BDPF were tested using randomization tests. Since the data were collected in a hierarchical design (plots nested within stands), we performed a spatially restricted randomization scheme (Anderson and ter Braak 2003) where no randomization was performed at the plot level, but the whole stands were freely reshuffled 10,000 times. The ordination analyses were performed in R v. 4.1.2 (R Core Team 2021) and the library vegan (Oksanen et al. 2019).

Table 1 All analysed structural variables with their description.

structural variable	description	units
missing_bark	number of trees with bare wood patches with bark loss and wood in a decay stage of less than 2	number
n_trees_dead_500	density of the large dead trees (DBH ≥ 500 mm, height > 1.3 m) per hectare	number of stems per hectare
volume_dead_total	amount of lying and standing deadwood	m^3/ha
openness_mean	mean openness calculated from the 6 hemispherical photos evaluated in WinSCANOPY	% of canopy area
volume_dead_lying	volume of lying deadwood with thickness on thinner end ≥ 100 mm	m^3/ha
n_trees_live_500	density of the large living trees (DBH ≥ 500 mm) per hectare	number of stems per hectare
n_trees_ha	density of the living trees (DBH ≥ 60 mm) per hectare	number of stems per hectare
dbh_mean_live_60	mean diameter of the living trees (DBH ≥ 60 mm)	mm
age_5oldest	age of 5 oldest living trees (DBH ≥ 60 mm)	years

structural variable	description	units
age_median	median age of living trees (DBH ≥ 60 mm)	years
age_mean	mean age of living trees (DBH ≥ 60 mm)	years
regeneration_250_100	density of the regeneration (height > 250 cm, DBH < 100 mm) per hectare based on the data of the plot	number of stems per hectare
regeneration_130_250	density of the regeneration (130–250 cm height) per hectare based on the data of the plot	number of stems per hectare
regeneration_50_130	density of the regeneration (50–130 cm height) per hectare based on the data of the plot	number of stems per hectare

Results

Structure in beech- and spruce-dominated primary forests

The redundancy analysis revealed that the structure of BDPF is significantly different from that of SDPF (*pseudo-F* = 15.1, $p < 0.0001$, Fig. 2). SDPF have a significantly higher density of cavities and higher canopy openness, whereas in BDPF there is a higher tree species richness and more regeneration (Fig. 2). Tree density and age characteristics were comparable in the two types of forest. The research plots were selected to cover the whole disturbance gradient to filter out the differences in disturbance regimes and redundancy analysis showed that there are no significant differences in disturbance characteristics between our plot selection in BDPF and SDPF (*pseudo-F* = 1.8, $p = 0.127$, Fig. 2).

There were higher amounts of deadwood in SDPF (293.8 m^3 ha^{-1} on average, stand level averages 144.8–628.3 m^3 ha^{-1}), plot-level values varied between 71–978 m^3 ha^{-1}. In BDPF it was 169.3 m^3 ha^{-1} on average (stand level averages 92.2–254.4 m^3 ha^{-1}, plot-level volumes between 12–628 m^3 ha^{-1}). Average stand-level canopy openness was 4.4% in BDPF (stand averages between 2.4–6.2%, plot level values between 1.0–24.9%) and 14.4% in SDPF (stand level averages 9.6–21.0%, plot level values between 2.9–50.5%). Number of trees per hectare was higher in BDPF, with an average at stand level of 480, compared to 385 in SDPF (for details, see Table 2).

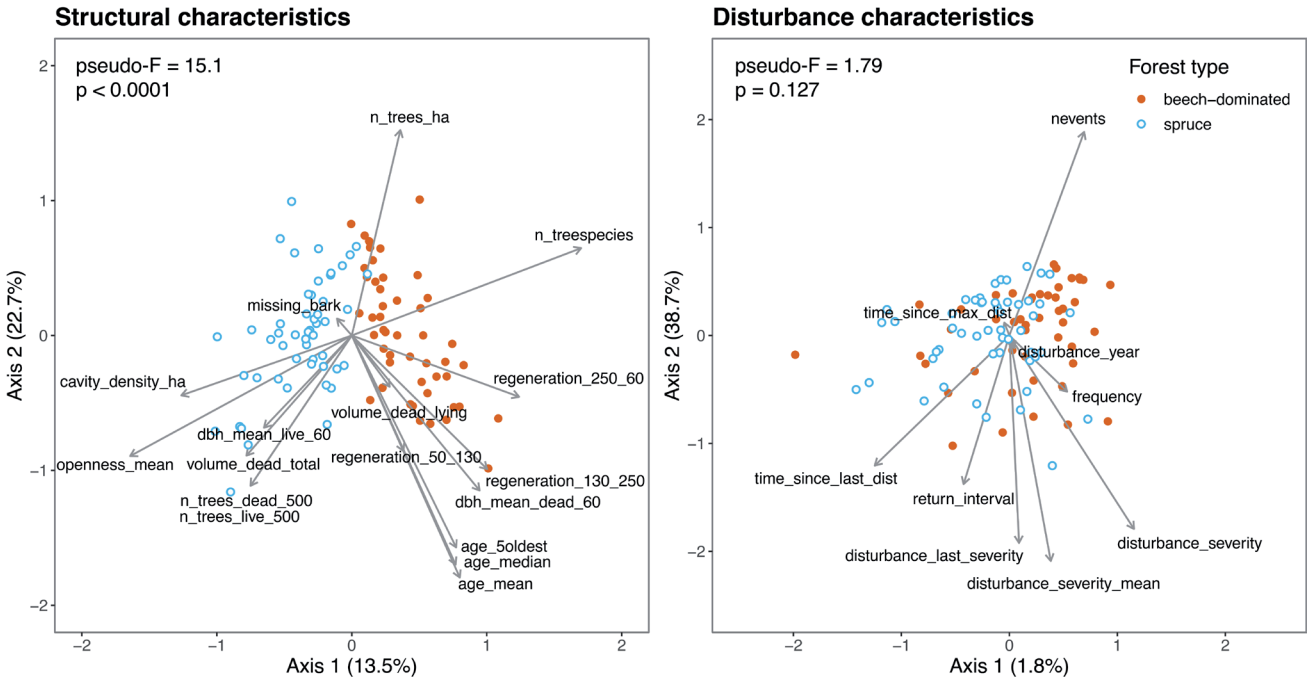

Fig. 2 Results of RDAs testing for differences between BDPF and SDPF in structural and disturbance characteristics. Ordination diagrams show scores of sampling plots (empty circles – spruce plots, full circles – beech plots) and vectors of environmental variables (arrows). The proportion of variance explained by the ordination axes is given in parentheses. The ordination plots are scaled symmetrically. Description of variables is in Table 1.

Table 2 Selected structural parameters averaged at stand level.

Stand	Forest type	Elevation (m a.s.l.)	Age mean (years)	Mean canopy openness (% of canopy cover)	Total volume of deadwood [m^3/ha]	Number of dead trees with DBH over 500 mm per ha	Number of trees per ha	Number of tree species
BEL	spruce	1361	162.0	17.6	628.3	63.3	293	1.5
BYS	spruce	1416	168.3	21.0	326.5	30.0	315	1.8
DUM	spruce	1497	158.3	11.7	144.8	13.3	383	1.8
HLI	spruce	1421	129.5	13.8	285.0	40.0	460	1.3
JAK	spruce	1307	128.4	15.9	150.8	4.0	312	1.6
KOP	spruce	1409	107.2	10.9	404.0	23.3	938	2.7
KOR	beech	1117	192.9	3.6	177.3	11.6	524	3.7
KUN	beech	1091	231.0	5.7	207.0	12.4	295	3.9
PAD	beech	1161	178.2	6.2	138.3	4.5	430	3.7
PIL	spruce	1330	186.2	12.5	200.3	15.0	263	1.0
POL	beech	1144	139.8	2.4	206.6	9.4	559	4.2
POL	spruce	1377	127.5	9.6	260.3	15.0	333	2.5
SKA	beech	1165	191.9	4.7	254.4	18.4	388	2.6
SMR	spruce	1383	135.0	14.0	233.5	20.0	210	1.8
SRA	beech	1050	104.7	5.6	161.9	7.7	751	3.7
SUT	beech	1054	153.7	3.8	92.2	10.0	565	3.0
TIC	spruce	1420	112.0	17.2	304.0	38.3	338	1.5
VEP	beech	1197	149.4	3.5	116.9	7.7	323	4.1

Bird assemblage in beech- and spruce-dominated forests

In total, 4,745 birds belonging to 53 species, were recorded, 45 species in BDPF (beech-) and 37 in SDPF (spruce-dominated primary forests). When accounting for differences in sampling effort, 17.3% fewer individuals were recorded in BDPF. 29 (53.7% of all species) occurred in both types of forest, but 17 of them were more numerous in SDPF. 24 species were recorded only in one of the two types of forest, with 16 in BDPF and 8 in SDPF. Species with dominance over 5% accounted for 60% of the total number of individuals in BDPF (6 species) and 74% in SDPF (8 species).

The composition of the bird assemblages in BDPF was significantly different from that in SDPF (*pseudo-F* = 17.6, $p < 0.0001$). Crested tit (*Lophophanes cristatus* (Linnaeus, 1758)), three-toed woodpecker (*Picoides tridactylus* (Linnaeus, 1758)), dunnock *Prunella modularis* (Linnaeus, 1758)), Eurasian bullfinch (*Pyrrhula pyrrhula* (Linnaeus, 1758)), ring ouzel (*Turdus torquatus* (Linnaeus, 1758)) and Eurasian siskin (*Carduelis spinus* (Linnaeus, 1758)) were indicative for SDPF. Collared flycatcher (*Ficedula albicollis* (Temminck, 1815)), white-backed woodpecker (*Dendrocopos leucotos* (Bechstein 1802)), wood warbler (*Phylloscopus sibilatrix* (Bechstein, 1793)) and great tit (*Parus major* (Linnaeus, 1758)) and mistle thrush (*Turdus viscivorus* (Linnaeus, 1758)) were typical for BDPF (Fig. 3). The chaffinch (*Fringilla coelebs* Linnaeus, 1758) and European robin (*Erithacus rubecula* (Linnaeus, 1758)) were the most abundant species in both types of forest, other abundant common species were coal tit (*Periparus ater* (Linnaeus, 1758)), Eurasian blackcap (*Sylvia atricapilla* (Linnaeus, 1758)) and Eurasian wren (*Troglodytes troglodytes* (Linnaeus, 1758). For a complete list of the species recorded in BDPF and SDPF with dominances see Table 3.

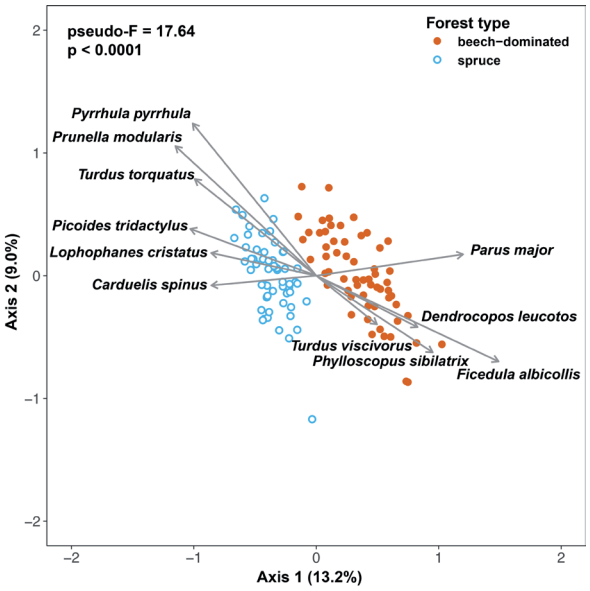

Fig. 3 Results of db-RDAs testing for differences in the composition of species of birds in BDPF and SDPF. Ordination diagrams show scores for the plots sampled (dots) and species vectors (arrows). Only species with a good fit to the ordination ($|r| > 0.4$) are displayed. The percentage of variance explained by the ordination axes is given in parentheses. The ordination plots are scaled symmetrically.

Several less numerous birds were recorded, which are of conservation concern in the Carpathians. In SPDF it was the three-toed woodpecker, capercaillie (*Tetrao urogallus* Linnaeus, 1758), Eurasian pygmy owl (*Glaucidium passerinum* (Linnaeus 1758)), boreal owl (*Aegolius funereus* (Linnaeus 1758)), golden eagle (*Aquila chrysaetos* (Linnaeus 1758)) and black woodpecker (*Dryocopus martius* (Linnaeus 1758)). In BDPF it was the Ural owl (*Strix uralensis*), peregrine falcon (*Falco peregrinus* Tunstall, 1771) and red-breasted flycatcher (*Ficedula parva* (Bechstein, 1792)).

Table 3 Differences in recorded bird assemblage species. Number of individuals in SDPF and BDPF were adjusted to account for different sampling efforts.

	Total abundance		Dominance	
species	beech	spruce	beech	spruce
Accipiter nisus	1	0	0.0	0.0
Aegithalos caudatus	2	0	0.1	0.0
Aegolius funereus	0	2	0.0	0.1
Anthus trivialis	2	6	0.1	0.2
Aquila chrysaetos	0	1	0.0	0.0
Bonasa bonasia	3	14	0.1	0.5
Buteo buteo	0	4	0.0	0.2
Carduelis spinus	1	27	0.0	1.0
Certhia familiaris	96	108	4.6	4.1
Coccothraustes coccothraustes	13	1	0.6	0.0
Columba oenas	14	0	0.7	0.0
Columba palumbus	23	15	1.1	0.6
Corvus corax	1	1	0.0	0.0
Cuculus canorus	2	18	0.1	0.7
Cyanistes caeruleus	3	0	0.1	0.0
Dendrocopos leucotos	26	0	1.2	0.0
Dendrocopos major	18	1	0.9	0.0
Dryocopus martius	4	3	0.2	0.1
Erithacus rubecula	215	257	10.3	9.8
Falco peregrinus	2	0	0.1	0.0
Ficedula albicollis	84	0	4.0	0.0
Ficedula parva	1	0	0.0	0.0
Fringilla coelebs	539	670	25.7	25.6
Garrulus glandarius	9	4	0.4	0.2
Glaucidium passerinum	0	5	0.0	0.2
Lophophanes cristatus	2	32	0.1	1.2
Loxia curvirostra	1	52	0.0	2.0
Muscicapa striata	12	0	0.6	0.0
Nucifraga caryocatactes	0	29	0.0	1.1
Parus major	38	0	1.8	0.0
Periparus ater	131	232	6.2	8.9
Phoenicurus phoenicurus	0	1	0.0	0.0
Phylloscopus collybita	172	155	8.2	5.9
Phylloscopus sibilatrix	42	0	2.0	0.0
Phylloscopus trochilus	43	31	2.1	1.2
Picoides tridactylus	2	43	0.1	1.7
Picus canus	2	0	0.1	0.0
Poecile palustris	3	0	0.1	0.0
Prunella modularis	54	180	2.6	6.9
Pyrrhula pyrrhula	23	71	1.1	2.7
Regulus ignicapilla	46	43	2.2	1.7
Regulus regulus	83	123	4.0	4.7
Scolopax rusticola	0	6	0.0	0.2
Sitta europaea	42	13	2.0	0.5
Strix aluco	1	0	0.0	0.0
Strix uralensis	1	0	0.0	0.0
Sylvia atricapilla	127	116	6.1	4.4
Tetrao urogallus	0	6	0.0	0.2
Troglodytes troglodytes	102	158	4.9	6.0
Turdus merula	68	32	3.2	1.2
Turdus philomelos	65	53	3.1	2.0
Turdus torquatus	8	65	0.4	2.5
Turdus viscivorus	13	0	0.6	0.0

Discussion

In our study, we made the first attempt to compare bird assemblages, forest structure and disturbance regimes across the largest beech- and spruce-dominated primary forest (BDPF and SDPF) remnants in the Western Carpathians in Slovakia. We showed that forest structure and bird assemblages differ significantly, despite similar disturbance regimes.

Forest structure in beech- and spruce-dominated primary forests

Natural disturbances are the main drivers of Carpathian primary forest structure (Mitchell 2013; Kameniar et al. 2021; Rodrigo et al. 2022, Kameniar et al. 2023). Their impact on forest is shaped by climatic conditions, which varies along altitudinal gradients, and by tree species composition. With increasing elevation, exposure to windstorms generally increases (Senf and Seidl 2017), whilst the risk of drought is less probable (Marchand et al. 2023). On the other hand, changes in tree species composition affects the abiotic factors and largely shapes the response to biotic factors. In lower altitudes, forests are generally more resilient to disturbance because they are more diverse in terms of species of trees and forest structures (Walker et al. 2004; Pardos et al. 2021).

Our results indicate that the important bird habitat structures of BDPF and SDPF differ significantly (Fig. 2), even though the design of the study aimed to equally represent the plot level disturbance history categories (see Methods: Study area, stand selection and study plots es-

tablishment and Fig. 2). Level of canopy openness is the main structural variable differentiating between BDPF and SDPF (Fig. 2), together with the number of tree species, which shows an opposing trend. Average stand-level canopy openness varied between 9.6–21.0% in SDPF and 2.4–6.2% in BDPF stands. Other studies also report low gap proportions, a variable more frequently used to represent canopy openness in BDPF; 1.2% at a Slovenian locality (Bončina 2000), 2.7 and 4.2% at two primary forest localities in Poland (Orman and Dobrowolska 2017) and 7–8% (or 15–16%, depending on gap characterisation) at two localities in the Slovakian part of the Eastern Carpathians (Drössler and von Lüpke 2005). We are not aware that comparable numbers have been published from SDPF. However, the study by Čada et al. (2020), which analysed the historical disturbance regimes in central European spruce primary forests, indicates that the proportion of stand disturbed varied between 25% and 75% in 69% of the researched area. Janda et al. (2017) found that 89.1% of the studied stands in the Western Carpathians SDPF experienced disturbance (35.6% loss of canopy) between 1840s–1860s. These results imply that canopy openness in this forest type is, on average, considerably higher than in beech forests. Spruce forests generally have a lower tree species diversity than mixed forests, which plays a role in canopy openness, as lower species diversity reduces productivity (Pretzsch et al. 2012).

The age variables did not differ considerably between forest types; BDPF stands were only slightly older (Fig. 2). In general, beech has been proven to be the tree with the longest lifespan among four most common tree species in temperate forests. Fir and maple also reach higher lifespans than spruce (Pavlin et al. 2021).

We found higher amounts of deadwood in SDPF (293.8 m^3 ha^{-1} on average, stand level averages 144.8–628.3 m^3 ha^{-1}) than in BDPF (average 169.3 m^3 ha^{-1}, stand level averages 92.2–254.4 m^3 ha^{-1}). This difference probably results of a higher incidence of disturbance events in SDPF (Synek et al. 2020; Frankovič et al. 2021). Other factors which likely play a role is the significantly longer decomposition time of spruce deadwood in comparison with beech, and colder climate in higher altitudes, which also slows wood decomposition (Weedon et al. 2009). In the primary forests of the Făgăraș Mts. (Southern Carpathians, Romania) the differences in the amounts of deadwood in BDPF and SDPF were smaller; on average it was 145.2 m^3 ha^{-1} (stand-level averages 83–245) in BDPF, and 151 m^3 ha^{-1} (stand-level averages 87–224 m^3 ha^{-1}) in SDPF (Kameniar et al. 2023). The lower total amounts of deadwood recorded in this study can be partly explained by the different methods used to measure lying deadwood. In our study it was measured with greater precision, which yields higher total volumes (see Methods: Forest structure data). The different ratios between BDPF and SDPF in both studies are also probably caused by higher recent mortality of trees in SDPF in the Western Carpathians (Synek et al. 2020).

The results indicate that the incidence of regeneration in BDPF is higher than in SDPF. This is attributed to the different regeneration strategies of the dominant tree species; specifically, spruce regenerate predominantly on downed deadwood (Korpeľ 1989). For example, a study in the Western Carpathians (Zielonka 2006) report that large pieces of deadwood covered only 4% of the forest floor, but it was a substrate for 43% of all seedlings and there is a 20 times higher density of seedlings on deadwood than the mineral soil. In contrast, beech and fir regenerate predominantly on mineral soil, which allow them to use more space. It is also a possible explanation for the slightly higher number of trees per hectare in BDPF. Our results also indicate a significant difference in the density of tree cavities in the two types of forest, with higher densities in spruce than beech forests. The higher cavity density in SDPF can be attributed to the higher number of large dead trees (Fig. 2), which are more likely to have cavities in addition to other microsites (Kozák et al. 2023). The population density of woodpeckers (another cause of tree cavities) is unlikely to play a significant role, as their numbers were similar in both types of forest (48 in SDPF and 50 in BDPF, for details see Table 3).

Bird assemblages in beech and spruce-dominated primary forests

In total, 53 bird species were recorded (Table 3). In SDPF we recorded 37 species, whilst 45 were identified in BDPF stands. These results are comparable to those found in other studies which also explored beech- (Korňan 2004; Saniga and Saniga 2004) and spruce-dominated mountain forests (Ślizowski 1991; Kocian et al. 2005; Saniga 2009; Baláž and Kocian 2015) in the Western Carpathians. Our work adds further evidence that naturally shaped unmanaged spruce forest supports more diverse assemblages than spruce monocultures (Kocian et al. 2005; Bashta 2007; Baláž and Kocian 2015), including rare and threatened species (see Results: Bird assemblage in beech- and spruce-dominated forests).

As the disturbance histories of BDPF and SDPF plots were not significantly different (Fig. 2) whilst the forest structure and bird assemblages' composition differed (Fig. 2 and Fig. 3), it is obvious that other factors than disturbance history are responsible for these differences. In our previous study from SDPF, where we used part of the data presented here (Kameniar et al. 2021) we also found that bird assemblage abundance, species richness and Shannon diversity remained unchanged under variable disturbance histories. However, in a study relating disturbance histories with the data on occurrence of one species, Capercaillie (*Tetrao urogallus*), a significant relationship was found (Mikoláš et al. 2017). The relationships between disturbance history variables and organism assemblages were found in other taxonomic groups such as fungi (Ferenčík et al. 2022), lichens (Langbehn et al. 2021) and saproxylic beetles (Kozák et al. 2020). In our case this relationship was probably distorted by

the high mobility of birds and by the impact of recent disturbances which occurred in approximately the last 20 years, which are not detectable by our methods. Recent disturbances (including single tree mortality) are most likely the decisive processes shaping forest structure and therefore indirectly also bird assemblages' composition (Kameniar et al. 2021).

Our results showed that bird assemblages differ in BDPF and SDPF in terms of assemblage composition and diversity; species which constituted the most significant parts of the bird assemblage occurred predominantly in BDPF or SDPF. This difference in bird assemblages between forest types can likely be attributed mainly to the differences in tree species composition: higher tree diversity in broadleaved/mixed forests offer more niches, because of various food sources, nesting and mating opportunities (Willson and Comet 1996; Reif et al. 2008). Part of the difference is also caused by more harsh environmental conditions which are tied to higher elevations – especially lower temperatures, which influence all components and processes of the local ecosystem (Micu et al. 2015).

We also found that in SDPF, although there is higher diversity of birds in lower elevations, their absolute abundance is higher. In addition, a larger part of the species shared between both forest types were more abundant in SDPF. We attribute this pattern to the fact that these species are at least to some extent specialised to spruce and therefore, they reach highest abundances in almost pure spruce forest. It partly matches with the results of Baláž and Balážová (2012). In our case, also additional species were more abundant in spruce-dominated primary forest.

Regarding BDPF and SDPF specialists and their strict avoidance of the second forest type, we also found a difference in assemblage composition between forest types. Specifically, in BDPF, species that shaped the ordination most were collared flycatcher, white-backed woodpecker, wood warbler, mistle thrush and great tit (Fig. 3), which were not recorded in SDPF. This might indicate that structural parameters other than the species composition of the trees, coincide with their habitat requirements. Other studies on SDPF or natural spruce forests also report these species as very rare or absent in this type of forest (Ślizowski 1991; Baláž and Kocian 2015). On the other hand, species typical of SDPF were not specific to this type of forest, as a few individuals also occurred in BDPF. These species are also considered as spruce or coniferous specialists in other studies: the crested tit, dunnock, ring ouzel, Eurasian bullfinch, Eurasian siskin, and three-toed woodpecker (Fuller 1995; Pechacek and d'Oleire-Oltmanns 2004; Braunisch et al. 2014).

This difference in the degree of avoidance of SDPF and BDPF by specialists can be explained by the fact that whereas beech is generally rare in SDPF (Čada et al. 2020; Synek et al. 2020), an admixture of spruce is common in BDPF (Orman and Dobrowolska 2017; Parobeková et al. 2018; Frankovič et al. 2021). In some of the beech plots spruce made up a significant part of the canopy cover (several tens of percent). Such mixed forest is suitable for spruce specialists. For example, the only two individuals of the three-toed woodpecker recorded were in two research plots in the stand Skalná Alpa, Great Fatra Mts., which are located close to a 2.5 ha patch of forest with a large proportion of recently dead large spruce trees. A high density of standing dead spruce trees, which are used by three-toed woodpeckers for foraging and nesting, is mentioned in the literature as a crucial structural element for this species (Pechacek and d'Oleire-Oltmanns 2004). The presence of spruce specialists in BDPF is also documented in other studies (Korňan 2004; Saniga and Saniga 2004; Korňan and Adamík 2014).

Along with the BDPF and SDPF specialists, several other species were recorded that occurred in both types of forest, but not at the same density. In the case of several of these species, presence or absence is probably influenced by forest structure, independently of the species composition of the trees. For example, dunnock is recorded as a species characteristic of SDPF in the ordination analysis (Fig. 3). It is considered to be a species that mainly occurs in spruce-dominated forests (Tuomenpuro 1989). However, they were also recorded frequently in BDPF. They were typically present in recently disturbed plots with low canopy cover, large amounts of deadwood and dense regeneration, as is reported in other studies (e.g., Moning and Müller 2008). This kind of structure is more common in SDPF, which likely causes this forest type to be preferred by the dunnocks and several other species (i.e., Eurasian wren). Naturally disturbed patches in BDPF are used by these predominantly SDPF species because they found suitable forest structure there, which is otherwise lacking in closed canopy BDPF.

The described patterns of bird species occurrence in BDPF and SDPF are likely to change soon due to climate change. Even currently, we are witnessing the retreat of spruce in BDPF localities in Slovakia (Parobeková et al. 2018) and in other European countries (Diaci et al. 2011; Janík et al. 2014; Jaloviar et al. 2017; Keren et al. 2017). Spruce mortality will probably temporarily create suitable habitats for spruce-related bird species (especially the three-toed woodpecker and other open-forest species), but in the long term, they are likely decrease in abundance. Thus, SDPF species will become more restricted to SDPF, which could negatively affect their populations (Braunisch et al. 2014). At the same time, the abundance of beech is reported to be increasing at high altitudes and thus transforming the species composition of trees in SDPF (Saltré et al. 2015). As a result, it is likely that the specialist birds of BDPF are likely to colonize SDPF.

Conclusions

In our study, we presented the analysis of an exceptional dataset which describes forest structure and bird assemblages in two forest types of major importance in Central

Europe in their primary state. Our results from best preserved temperate primary forests can serve as an important benchmark reference for forest management and conservation strategies focused on biodiversity conservation. We showed that bird assemblages and forest structure differ in beech- and spruce-dominated forests, independently of the disturbance regime. Both forest types with their high tree age, high standing and downed deadwood volumes and multiple tree related microhabitats provide important habitat opportunities for numerous rare bird species, which highlights the important role of primary forests in the conservation of biodiversity. Thus, protecting existing primary forests, allowing managed forests to attain older ages, and increasing the heterogeneity and availability of primary forest structures in the landscapes will maintain diverse beech and spruce forest assemblages in times of accelerating environmental change.

Acknowledgements

We thank IGA (Internal Grant Agency of the Faculty of Forestry and Wood Sciences at Czech University of Life Sciences in Prague) for supporting this research with projects IGA A 14_17, IGA A_11_18 and IGA A_19_18. Project no. 78/2021 was provided by a project financed from the OP RDE project Improvement in Quality of the Internal Grant Scheme at CZU, reg.no. CZ.02.2.69/0.0/0.0/19_073/0016944.

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