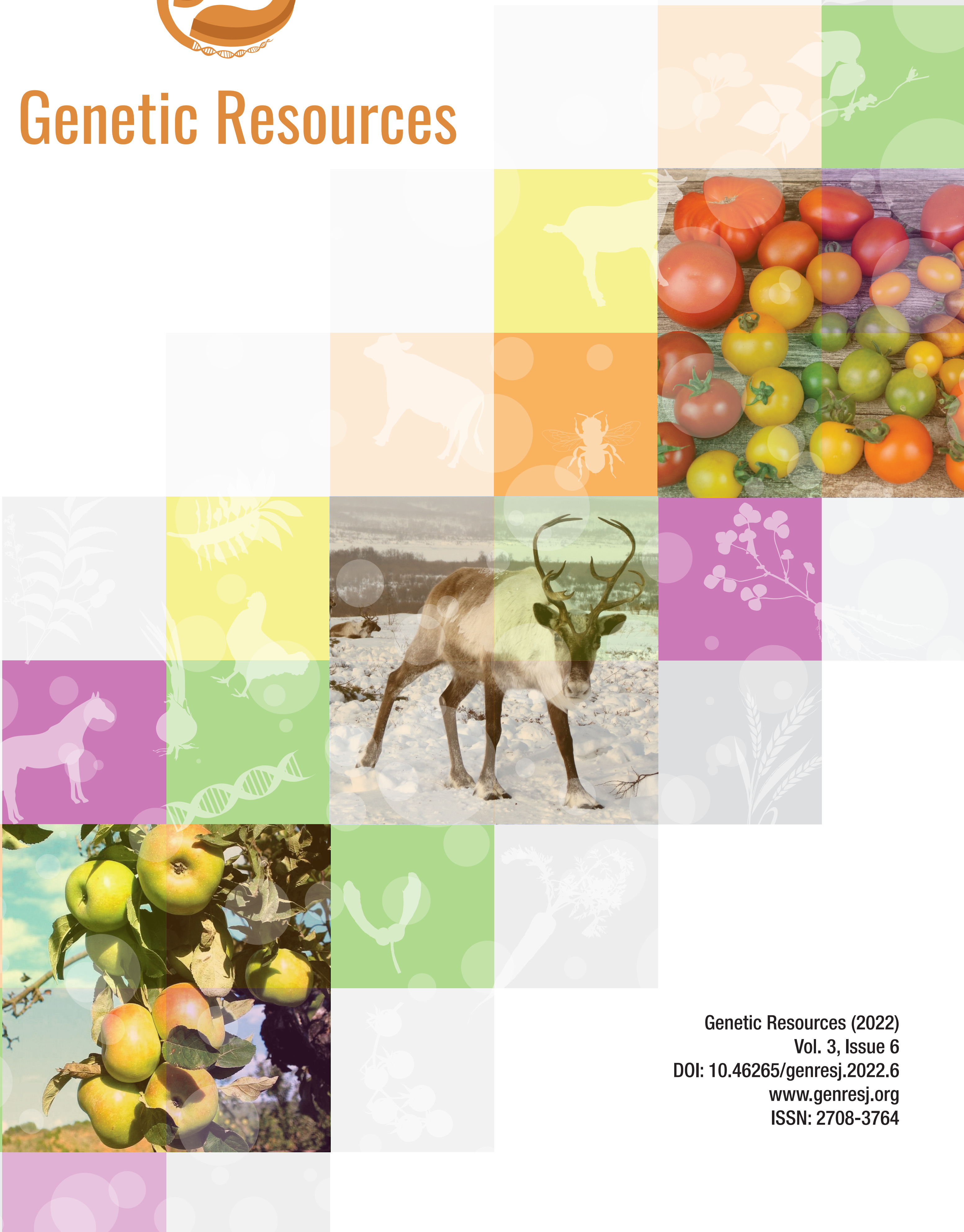
# Genetic Resources



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# Genetic differentiation between coexisting wild and domestic reindeer (*Rangifer tarandus* L. 1758) in Northern Eurasia

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**Abstract:** *Rangifer tarandus* L. 1758 is one of the few modern hoofed species in which domestic and wild forms coexist in the same territory. The genetic differentiation of domestic and wild reindeer in Northern Eurasia was examined using microsatellite data. A total of 780 animals were studied at 16 microsatellite loci. Samples of wild reindeer were taken from seven populations inhabiting different natural areas, and samples of domestic animals were selected from the Evenki, Evens, Chukchi and Nenets breeds, including two ecotypes, Tofalar and Todzha reindeer. The levels of genetic diversity and variation in wild reindeer were higher than in domestic ones. Bayesian clustering analysis allowed us to distinguish domesticated reindeer populations by the degree of taming, but failed to detect differences in genetic structure between wild reindeer populations. These differences were found using the pairwise *Fst* values. Overall, the microsatellite analysis revealed a significant genetic differentiation between domestic and wild forms and the structuring of populations within each form, which may be important for the development of strategies for animal conservation.

**Keywords:** Domestic and wild forms of reindeer, geographic population, microsatellites, phylogenetic tree, biodiversity

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## Introduction

Reindeer (*Rangifer tarandus* L. 1758) is a widespread circumpolar species on the planet. The geographic distribution of reindeer mainly covers the northern part of the Arctic region, including arctic and subarctic regions of Eurasia and North America (Williams and Heard, 1986). Although the range of reindeer is very large, the species has been classified as vulnerable by the International Union for Conservation of Nature (IUCN) because of a 40% decline over the last decades (Gunn, 2016). The decline in population size is the result of the intensification of industrial development in the Arctic and climate warming (Yannic *et al*, 2014).

For the indigenous Arctic ethnic groups living in Northern Eurasia, reindeer are of great economic, social, cultural and ecological importance, since they are a source of meat, hide and milk, as well as a means for transportation. In the historical process, the employment of reindeer has been of crucial importance in the colonization of the northernmost parts of Eurasia. Currently, reindeer continue to play a central role in the cultures of the Indigenous Nenets, Chukchi, Evenki, Sami, Evens Peoples and other Peoples of Northern Eurasia (Helskog and Indrelid, 2011; Bjørklund, 2013).

Reindeer husbandry is undoubtedly a historical branch of animal keeping in the northern regions of Russia. Unlike other Arctic countries, reindeer husbandry in Russia is much differentiated. Representatives of 18 peoples are engaged in the industry, thereby preserving their national traditions, and 16 of them are included in the

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official list of Indigenous Small-Numbered People of the North (Jernsletten and Klokov, 2002).

By now, four native (Nenets, Evens, Evenki and Chukchi) breeds of reindeer have been officially registered in Russia. The Nenets breed is widespread in the Taymyr District of the Krasnoyarsk Territory, as well as in the Yamal-Nenets, Nenets and Khanty-Mansi Autonomous Districts, the Komi Republic and the Murmansk Region. Currently, this reindeer breed is the most numerous (over 880,000 heads) and prosperous. The Chukchi breed inhabits the territory of the Chukotka and Kamchatka peninsulas and the northeast of Yakutia. The Evenki breed is bred in the taiga zone of Siberia and the Far East, from the Yenisei River region to the shores of the Sea of Okhotsk and Sakhalin Island. The Evens breed habitat is the mountain-taiga regions of Yakutia and Kamchatka, as well as the region of Magadan (by its type, this breed occupies an intermediate position between the Chukchi and Evenki breeds). All breeds differ in productivity and conformation, as well as in adaptation to specific natural and climatic conditions. Based on our own observations and FAO data (Zabrodin and Borozdin, 1989), we summarized the descriptive characteristics of the four breeds mentioned above (Table 1 ). The breed-forming processes in reindeer husbandry have much in common with the breed genesis of other species of domesticated aboriginal animals, such as horses, sheep, yaks, camels, etc. The reindeer breed specialty is based on the folk traditional breeding system and the complete lack of commercial incentives, features that later also appear in stud breeding in Europe and North America (Kharzinova *et al*, 2015). All the reindeer breeds are aboriginal and the result of folk selection by various northern people (Zabrodin and Borozdin, 1989; Kharzinova *et al*, 2015). Within the breeds, there are some ecotypes that are characterized by their own morphological features, but they are taxonomically indistinguishable (Davydov *et al*, 2007). In particular, within the Evenki breed, two independent ecotypes, Tofalar and Todzha reindeer, have been identified (Kharzinova *et al*, 2015). The Tofalar reindeer, bred by the small indigenous ethnic group, Tofalars, are the largest of the domesticated reindeer. Currently, Tofalar reindeer husbandry numbers less than 100 heads, compared with several thousand in the 1990s, and the population of the Todzha reindeer, bred by Todzha-Tuvans, is less than 1,000 animals. The Tofalar and Todzha herds, which define the southern borders of reindeer husbandry, are the most isolated from other reindeer herds bred by the Indigenous Peoples. The reindeer husbandry of the Tofalars and that of the Todzha-Tuvans can be combined into one Sayan group of reindeer husbandry, based on their common characteristics. In Russia, along with domesticated reindeer, there are many wild herds, whose ranges cover almost the entire tundra, forest-tundra and taiga zones, including mountainous areas. Depending on the habitat of wild reindeer, several ecological forms (macro-ecotypes) are distinguished, namely island (inhabiting arctic deserts), tundra (migrating to the forest-tundra for the winter), taiga and mountain-taiga (carrying out vertical migrations) (Davydov *et al*, 2007). The most significant differences in size are observed between the tundra and taiga forms of reindeer. As a rule, forest reindeer are more long-legged animals, with elongated body. However, the intraspecific taxonomy of *Rangifer tarandus* L. is still controversial and needs to be specified using molecular genetic markers.

On the territory of Eurasia, several subspecies of wild reindeer are distinguished (Danilkin, 1999). The Svalbard reindeer (*R. tarandus platyrhynchus*) is a non-migratory tundra subspecies inhabiting the high-arctic archipelago of Svalbard. The Novaya Zemlya reindeer (*R. tarandus pearsoni*) is an island subspecies living only on the northern island of the Novaya Zemlya archipelago. The European reindeer (*R. tarandus tarandus*) is a subspecies that lives in the European part of Eurasia in the tundra and northern taiga regions up to the Ural Mountains. The Siberian reindeer (*R. tarandus sibiricus*) is a subspecies that lives in tundra and partly forest-tundra zones of Siberia (from the Ural Mountains to the Lena River and Lake Baikal). Finally, the Okhotsk reindeer (*R. tarandus phylarchus*) is a subspecies inhabiting the coast of the Sea of Okhotsk, the Amur River basin, the northern part of Sikhote-Alin, the Kamchatka peninsula and Sakhalin Island (from the Lena River and Lake Baikal to the Sea of Okhotsk). These geographic subspecies differ in the body type, increasing in size with the transition of the habitat from west to east, but genetic and phenotypic variabilities and differences in subspecies have been insufficiently studied.

It is worth noting that the bulk of the wild reindeer populations (~85%) is concentrated in the Taymyr Peninsula, northern Yakutia, and the central part of Chukotka (Kolpaschikov *et al*, 2015; Kharzinova *et al*, 2018). The Taymyr herd of wild reindeer is the largest and most monitored wild reindeer population in Eurasia, inhabiting a vast area in the north of central Siberia (Petrov *et al*, 2012; Kolpaschikov *et al*, 2015). Three large, relatively isolated herds of wild reindeer are found in the continental tundra of Yakutia, namely Yana-Indigirka, Sundrun (Indigirka-Kolyma) and Lena-Olenek (Bulun) (Safronov, 1996). In addition, there are sedentary taiga reindeer populations, distributed primarily in the mountains of the south and west Yakutia. In recent years, a difficult demographic situation has developed with the populations of wild reindeer on the Kola Peninsula (Baranova *et al*, 2016). The main direct cause of the decline in these populations is poaching, while forest fires and deforestation, leading to the depletion of food resources, are considered indirect causes. Wild reindeer of the Murmansk region of the Russian Federation (the Kola Peninsula) are endangered and listed in the latest Red Data Book of Murmansk Region (Konstantinova *et al*, 2014). On the contrary, the number of wild reindeer in the Magadan region is growing thanks to constant monitoring and restrictions on poaching.

Wild and domesticated reindeer have distinct craniological parameters and differ genetically according to results obtained by various methods of biochemical genetics (Kharzinova *et al*, 2017). DNA markers are the most popular tool for studying genetic diversity of reindeer populations. The analysis of mitochondrial DNA polymorphism is widely used in phylogenetic studies (Flagstad and Røed, 2003; Davydov *et al*, 2007; Wang *et al*, 2019; Røed *et al*, 2020). Microsatellite markers are widespread for studying the genetic structure and allelic pools of individuals, as well as for their identification and differentiation (Røed and Midthjell, 1998; Jepsen *et al*, 2002; Mcloughlin *et al*, 2004; Mcdevitt *et al*, 2009; Kharzinova *et al*, 2016; Zhai *et al*, 2017; Stolpovsky *et al*, 2020). With the development of high-throughput SNP genotyping platforms, SNP markers are getting more popular in animal agriculture. Although SNP platforms are becoming available for genomic research in reindeer, they are not cost-effective for genotyping several hundred animals.

This study aimed to identify genetic diversity among poorly examined wild reindeer populations and genetic differences between wild and coexisting domesticated reindeer living in different eco-climatic zones using microsatellite data.

# Materials and methods

## Animal sample information

Initially, 790 animals were collected, which were selected from all breeds registered in Russia (Nenets, Evens, Evenki and Chukchi), including two ecotypes (Tofalar and Todzha reindeer), as well as from seven wild populations of Magadan, Amur, Taymyr, Yakutia, Tura, Murmansk and Chukotka regions (Figure 1 and Supplemental Table S1). For the study of domesticated reindeer, we selected clinically healthy animals according to phenotypic traits corresponding to their breeds. The age of the selected animals varied from 3 to 7 years. Special attention was paid to calving females, since only females with a good conformation, regularly bearing healthy calves and capable of raising strong offspring, should be selected for further reproduction. For females, the presence of a calf was determined by the condition of the udder and by interviewing reindeer herders. Males were selected in August-September, and females were selected in October-November, i.e. during periods of their maximum fatness. For the study of wild reindeer, we used the biological material from animals that were obtained from hunters. As biological material, pieces of the cartilaginous part of the horn or auricle were taken, which were placed in test tubes and fixed with ethanol (96%). A total of 13 population samples were tested. It is worth noting that the sample of Nenets reindeer breed combined three subsamples taken from different breeding regions, since no significant genetic differences have been found between these subsamples (Stolpovsky *et al*, 2020).

## DNA extraction and microsatellite genotyping

Animals were genotyped using 16 polymorphic microsatellite markers (BMS1788, BMS745, C143, C217, C276, C32, FCB193, NVHRT16, OHEQ, RT1, RT24, RT30, RT6, RT7, RT9 and T40). The description of microsatellites is given in Supplemental Table S2. DNA was extracted from velvet antlers, muscles, skin, and ear notches using the COrDIS SPRINT PCR compatible reagent kit (OOO Gordiz Moscow, Russia, https://gordiz.ru/en/products/animal-kits/cordis-rangifer/) according to the protocol of the manufacturer. PCR was performed using Applied Biosystems thermal cyclers under the conditions recommended by the manufacturer of the used reagent kit. Separation of PCR products was carried out by capillary electrophoresis using an ABI 3130 automatic genetic analyzer (Applied Biosystems). Data analysis and determination of allele variants in the specimens for each locus were performed using the GeneMapper ID-X 1.4 software (Applied Biosystems). The genotyping quality was controlled using a standard specimen supplied as part of the reagent kit.

For each animal, the genotypes of microsatellites were obtained as the allelic lengths in base pairs. The used multiplex short tandem repeat (STR) panel consisted of ten dinucleotide loci (Rt6, BMS1788, Rt30, Rt1, Rt9, Rt7, Rt24, FCB193, BMS745 and NVHRT16) and six tetranucleotide STR markers (OHEQ, C217, C32, 40, C276 and C143). Of these, ten loci have been described for caribou: NVHRT16, BMS745, FCB193, OHEQ, BMS1788, RT6, RT24, RT30, RT9, RT1 and RT7 (Yannic *et al*, 2014), and other six loci have been described for North American subspecies of red deer: C32, C143, C276, T40 and C217 (Jones *et al*, 2002) (Meredith *et al*, 2005) . This panel has recently been tested on reindeer (Stolpovsky *et al*, 2020; Dodokhov *et al*, 2021).

## Statistical analysis

The minimum number of markers needed to discriminate between animals was estimated using the *genotype curve()* function with 50,000 replicates from the poppr R package (Kamvar *et al*, 2014). In each population sample, the individuals with duplicated genotypes were removed using the *clonecorrect()* function from the same package. Linkage disequilibrium between loci was assessed using the *pair.ia()* function with 50,000 replicates from the same package. Testing the departure from Hardy–Weinberg equilibrium (HWE) was performed using the *hw.test* function from the pegas R pack-age (Paradis, 2010). Here we applied two tests: the classical $c^2$-test based on the expected genotype fre-quencies calculated from the allelic frequencies, and an exact test based on Monte Carlo permutations of alleles (with 50,000 replicates). To control the false discovery rate, the p-values of both HWE tests were

**Table 1.** Comparative characteristics of four domestic breeds of reindeer in Russia. Characteristics were summarized from our observations and FAO data (Zabrodin and Borozdin, 1989).

| Breed | Predominant pelage colour | Animal physique | Live weight of males before rut (♂) and females (♀) [kg] | Slaughter yield of live weight [%] |
|---|---|---|---|---|
| Chukchi | Dark brown | Short height, squat, wide and at the same time elongated body, short limbs, well-developed skeleton and musculature. | ♂: 125-130 ♀: 93-96 | ~51-55 |
| Nenets | Brown and dark brown | Medium height, rather long and wide body, well-developed skeleton, relatively wide head. | ♂: 130-135 ♀: 90-95 | ~50 |
| Evenki | Light brown and grey of various shades | Tall height, rather long body, deep chest, well-developed skeleton and musculature | ♂:140-175 ♀: 108-120 | ~49 |
| Evens | Light brown and grey colour of various shades | Tall height, long body, a narrow deep chest, narrow long pelvis, well-developed skeleton, narrow long head. | ♂: 130-140 ♀:91-103 | ~49-51 |

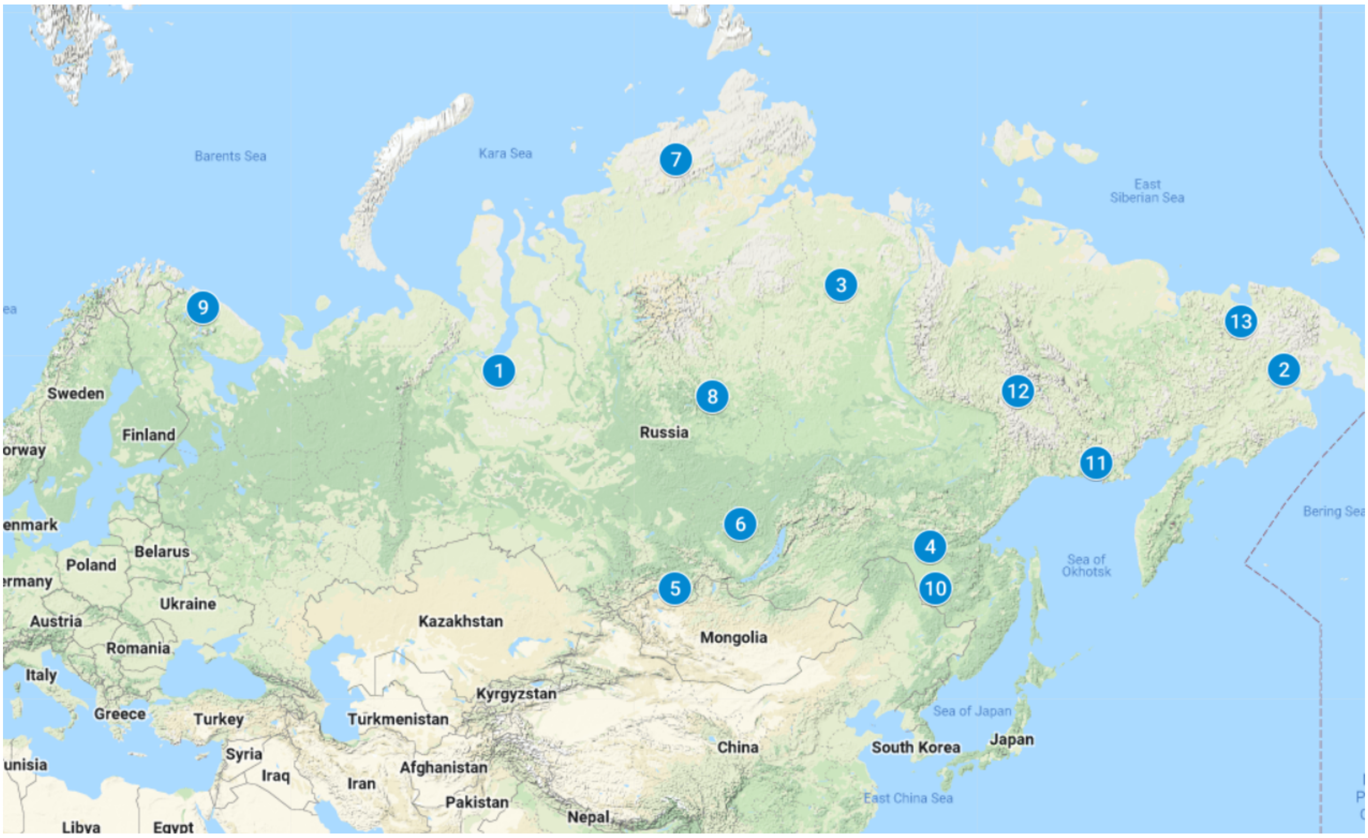

**Figure 1.** Geographical positions of the studied reindeer samples. The population samples are numbered as follows. Breeds: 1. Nenets; 2. Chukchi; 3. Evens; 4. Evenki; 5. Todzha; 6. Tofalar. Wild reindeer herds: 7. Taymyr; 8. Tura; 9. Murmansk; 10. Amur; 11. Magadan; 12. Yakutia; 13. Chukotka. The map was downloaded from https://www.google.com/maps.

adjusted using the *p.adjust* R function with the option *method='fdr'* (Benjamini and Hochberg, 1995). The main population genetic statistics for each reindeer sample were computed in the adegenet R package (Jombart, 2008). Allele frequencies, allelic richness and the number of private alleles were assessed by the PopGenReport R package. Polymorphism information content was estimated using the polysat R package (Clark and Jasieniuk, 2011). For each 'locus-population' pair, fixation index and the observed and expected heterozygosity were estimated using the *divBasic()* function from the the diveRsity R package (Keenan *et al*, 2013). Nei's pairwise *Fst* and corresponding p-values were obtained by the *pairwise.fst()* function from the hierfstat R package (Goudet, 2005) and visualized by the corrplot R package (Wei *et al*, 2017). *Fit*, *Fst* and *Fis* for each locus were calculated using the *Fst()* function from the pegas R package. The Bayesian clustering approach implemented in the STRUCTURE v.2.3.4 programme (Pritchard *et al*, 2000) was used to study population differentiation. The optimal number of clusters was determined by the method proposed by (Evanno *et al*, 2005) and implemented in the Structure Harvester and Clumpak programs (Earl, 2012; Kopelman *et al*, 2015). The dendrogram based on Nei's genetic distances was constructed by the neighbour-joining algorithm implemented in the poppr R package, with bootstrap support from 5,000 replicates. Isolation by distance was assessed by regressing pairwise genetic distance against the natural logarithm of geographical distance (km) with the Mantel test (Mantel, 1967) with 10,000 permutations using the ade4 R package (Dray and Dufour, 2007). Slatkin's linearized *Fst* was adopted as the measure of genetic distance (Rousset, 1997). The geographic centre of allelic richness among the studied populations was calculated as the weighted midpoint from a series of latitude/longitude coordinates by a method that converts longitude/latitude coordinates (in radians) to a 3D Cartesian coordinate system *(x, y, z): x = cos(lat) cos(lon)*, *y = cos(lat) sin(lon)* and *z = sin(lat)*. This method then calculates the weighted means of these coordinates $(\bar{x}, \bar{y}, \bar{z})$ and converts them back to longitude/latitude coordinates using the formulas: $lon = atan2\left(\bar{y}, \bar{x}\right)$ and $lat = atan2\left(\bar{z}, \sqrt{\bar{x}^2 + \bar{y}^2}\right)$, where atan2 is a function defined as the angle in the Euclidean plane.

# Results

## Data quality checks

Initially, 790 individuals were genotyped at 16 loci. To ensure that we had enough information to accurately identify multilocus genotypes, we estimated the minimum number of loci needed to discriminate between animals to be 15 markers and determined the presence of clones. According to the results, four individuals genotyped by less than 15 microsatellites and six individuals with duplicated genotypes were removed from further analyses. Linkage disequilibrium between loci was assessed using the standardized index of association, *rd* (Agapow and Burt, 2001), which corrects for sample size; *rd* between loci was not high and varied from -0.019 to 0.072. A significant deviation from HWE was detected by both HWE tests considered here only in the sample of wild Yakut reindeer at the C276 locus (Supplemental Tables S3–S5). Since deviation from HWE is most often associated with genotyping errors, we had to exclude C276 from the data for the wild Yakut sample. Thus, the final dataset included 780 individuals.

## Genetic variability

For the 16 microsatellites analyzed, a total of 236 alleles were found, with 3 (C143 and C217) to 30 (OHEQ) alleles per marker. The mean number of alleles per marker across all samples was 14.8. The average percentage of the total number of alleles observed per marker varied from 40.94% (domestic Tofalar reindeer) to 81.8% (wild Yakut reindeer) (Table 2). Detailed results for each marker and reindeer population studied are available in Supplemental Table S6.

In all studied samples of reindeer, we found alleles with high frequency (*AF*) ($AF > 0.7$). In particular, C217.215, T40.302, C32.330 and C143.180 had the highest frequencies. The polymorphism information content (*PIC*) values were calculated for each combination 'sample-locus' (Supplemental Table S7). The highest polymorphism levels were found for pairs 'W.Yakut–OHEQ' ($PIC = 0.920$), 'W.Taym–BMS1788' ($PIC = 0.915$) and 'W.Yakut–BMS1788' ($PIC = 0.911$). The mean *PIC* value over loci and samples appeared to be rather high, 0.71 ± 0.05.

Out of 236 alleles in 780 reindeer genotyped, 16 alleles were private alleles with allelic counts $\geq 2$ and $AF > 0.008$ (Table 3). The largest number of private alleles per marker (3) was detected for BMS1788 and OHEQ, and the largest numbers of private alleles adjusted for sample size were in the wild Chukchi herd and Todzha ecotype (24.4% and 14.4%, respectively of their total numbers). The presence of private alleles with frequencies above 0.01 in the wild herds suggests that each of these herds most likely has a unique genepool (Svishcheva *et al*, 2020). In total, private alleles were found in four wild reindeer herds and two domesticated ones (Table 3).

In each sample of animals, we evaluated genetic variability in terms of the number of alleles (*A*), allelic richness (*Ar*), observed (*Ho*) and expected (*He*) heterozygosity, and fixation index (*Fis*) (Table 2 and Supplemental Table S6). *Ar* ranged from 3.29 (Tofalar herd) to 5.39 (wild Yakut herd) with a mean of 4.60 ± 0.17. Overall, we found a significant correlation between *Ar* and *He* ($R^2$. 0.96, p-value $= 2.92 \times 10^{-9}$). The highest *Ho* value (0.77) corresponded to the wild Taymyr herd and the lowest (0.57) to the Tofalar herd. For each sample, we computed *Fis* as (*He-Ho*)/*He*. For Evens and Chukchi breeds the *Fis* values were equal to zero, while for other samples, we observed a slight

**Table 2.** Population parameters for the domesticated and wild reindeer samples studied. Size: population sample size; N: the average number of animals genotyped per marker; A: the number of alleles per sample; %: the percentage of total alleles observed across population samples per population sample per locus; Ar: the mean allelic richness across markers; Ho and He: observed and expected heterozygosity, respectively; Fis: fixation index; IC(se): mean (standard error) of inbreeding coefficients estimated for all animals. Herds of wild reindeer from Taimyr, Tura, Murmansk, Amur, Magadan, Yakutia and Chukotka are designated as 'W.Taym', 'W.Tura', 'W.Murm', 'W.Amur', 'W.Magad', 'W.Yakut' and 'W.Chuk', respectively.

| **Domestic form** | **Size** | **N** | **A** | **%** | **Ar** | **Ho** | **He** | **Fis** | **IC (se)** |
|---|---|---|---|---|---|---|---|---|---|
| Nenets | 224 | 223.88 | 132 | 60.94 | 4.31 | 0.64 | 0.66 | 3.03E-02 | 0.167(0.017) |
| Todzha | 42 | 42 | 89 | 46.66 | 3.77 | 0.64 | 0.62 | -3.23E-02 | 0.171(0.043) |
| Tofalar | 47 | 46.94 | 77 | 40.94 | 3.31 | 0.57 | 0.56 | -1.79E-02 | 0.182(0.039) |
| Evens | 44 | 43.56 | 130 | 60.98 | 4.78 | 0.73 | 0.73 | 0.00E+00 | 0.152(0.040) |
| Evenki | 50 | 50 | 113 | 52.96 | 4.27 | 0.67 | 0.68 | 1.47E-02 | 0.165(0.041) |
| Chukchi | 118 | 118 | 131 | 58.87 | 4.43 | 0.7 | 0.7 | 0.00E+00 | 0.156(0.023) |
| **Wild form** | | | | | | | | | |
| W.Taym | 21 | 21 | 142 | 65.88 | 5.34 | 0.77 | 0.76 | -1.32E-02 | 0.130(0.047) |
| W.Tura | 12 | 11.81 | 115 | 54.12 | 5.01 | 0.73 | 0.75 | 2.67E-02 | 0.138(0.048) |
| W.Murm | 29 | 28.94 | 124 | 57.44 | 4.83 | 0.7 | 0.71 | 1.41E-02 | 0.161(0.053) |
| W.Amur | 20 | 20 | 111 | 52.76 | 4.53 | 0.71 | 0.7 | -1.43E-02 | 0.154(0.058) |
| W.Magad | 6 | 6 | 89 | 43.31 | 4.44 | 0.71 | 0.68 | -4.41E-02 | 0.134(0.075) |
| W.Yakut | 126 | 118.12 | 193 | 81.8 | 5.27 | 0.74 | 0.77 | 3.90E-02 | 0.151(0.024) |
| W.Chuk | 41 | 41 | 163 | 73.89 | 5.38 | 0.74 | 0.76 | 2.63E-02 | 0.154(0.043) |

**Table 3.** Private alleles by locus and sample

| **Sample** | **Locus** | **Allele size [bp]** | **Allelic Frequency** | **Allelic count** |
|---|---|---|---|---|
| W.Yakut | RT6 | 168 | 0.036 | 9 |
| W.Yakut | OHEQ | 302 | 0.024 | 6 |
| W.Yakut | RT24 | 260 | 0.02 | 5 |
| W.Yakut | RT24 | 262 | 0.02 | 5 |
| W.Yakut | BMS1788 | 162 | 0.008 | 2 |
| W.Yakut | C32 | 314 | 0.008 | 2 |
| W.Taym | T40 | 335 | 0.045 | 2 |
| W.Murm | OHEQ | 268 | 0.052 | 3 |
| W.Chuk | RT7 | 262 | 0.122 | 10 |
| W.Chuk | FCB193 | 124 | 0.049 | 4 |
| W.Chuk | BMS1788 | 176 | 0.024 | 2 |
| W.Chuk | FCB193 | 120 | 0.024 | 2 |
| Todzha | BMS745 | 131 | 0.071 | 6 |
| Todzha | OHEQ | 322 | 0.024 | 2 |
| Chukchi | RT6 | 192 | 0.042 | 10 |
| Chukchi | BMS1788 | 166 | 0.008 | 2 |

deficiency and excess of microsatellite heterozygotes. The mean Fis value in the pooled sample was 0.0023 ± 0.0071.

## Pairwise *Fst* values

To assess the levels of genetic differentiation between the populations over all microsatellites, Nei's pairwise *Fst* values were computed (Figure 2, Supplemental Table S8). According to a 5% significance level, the wild reindeer samples, except for 'W.Yakut' and 'W.Chuk', were genetically differentiated. The wild reindeer from Chukotka differed only from the Amur wild population sample, and the wild reindeer from Yakutia were genetically similar to all sampled wild reindeer populations. When comparing only domesticated reindeer, we found stronger genetic differentiation relative to each other (all p-values $< 0.03$) than in group of wild reindeer. When comparing wild and domestic forms, we found significant differences (p-values $< 0.05$) for most (38 out of 42) pairs of populations, except for the pairs 'W.Magad'–'Nenets', 'W.Magad'–'Chukchi', 'W.Yakut'–'Evens' and 'W.Yakut'–'Evenki'. The Tofalar and Todzha samples were the most distant from all other breeds and from each other (all p-values $< 0.03$).

## Wright's F statistics for each locus

For 16 loci, the overall inbreeding coefficient (*Fit*) varied from 0.0411 to 0.2296 (Table 4) with a mean of

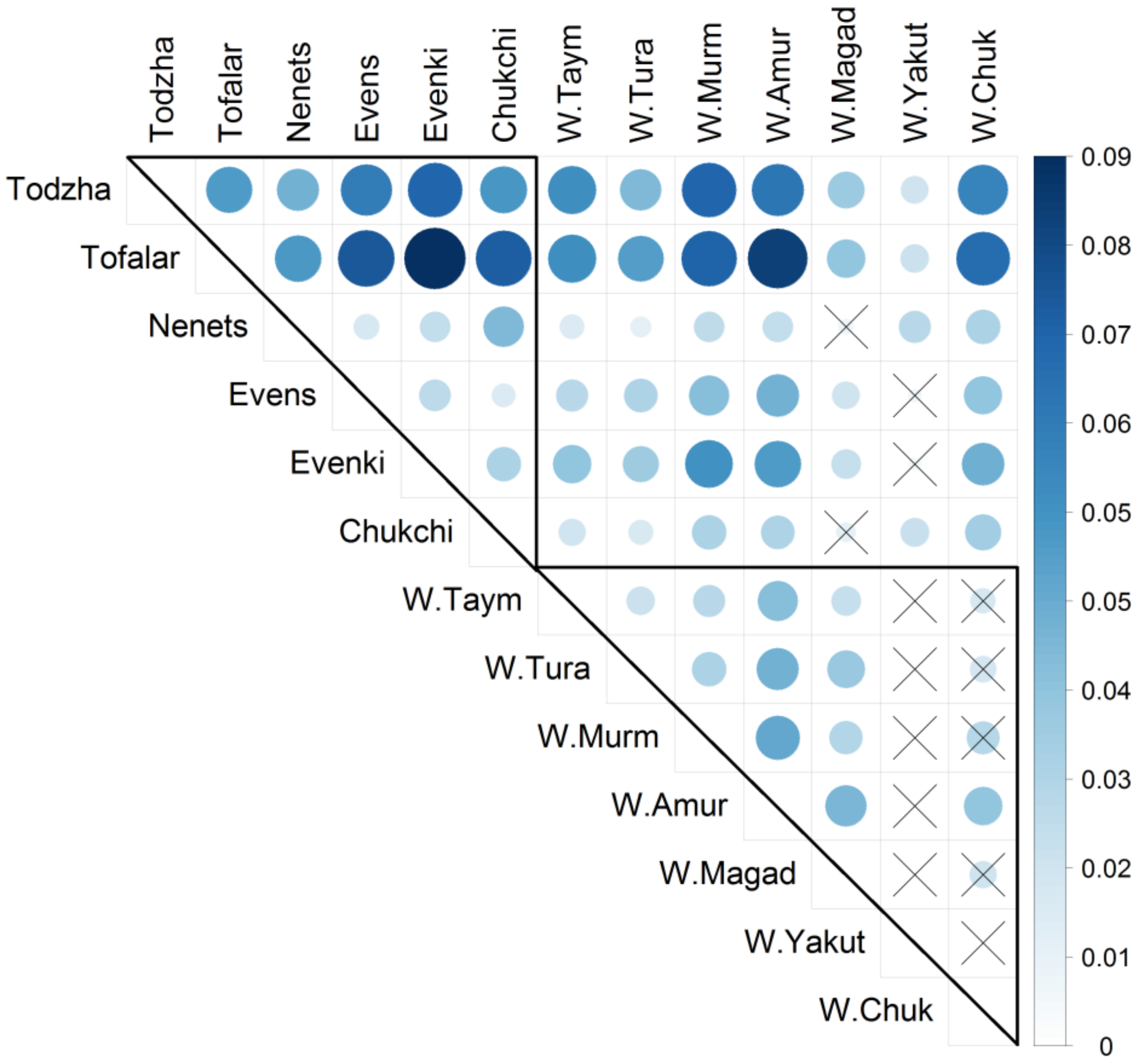

**Figure 2.** A visualization of pairwise *Fst* values. Colour intensity and size of circles are proportional to the pairwise *Fst* values between two population samples. Crosses indicate that no significant differences were found between two samples (p-value threshold = 0.05). The two black triangles separate domestic and wild reindeer forms.

0.0077. The highest *Fit* value reflecting a heterozygosity deficiency corresponded to marker C143, likely due to the small number of alleles at this locus (Cornuet and Luikart, 1996). The inbreeding coefficient, *Fis*, ranged from -0.0388 to 0.0977 with a mean of 0.0188, and the fixation index, *Fst*, ranged from 0.0486 to 0.1462 with a mean of 0.0808. Based on *Fst*, all loci (especially C143, RT30 and RT6) contributed to the differentiation between the herds.

## Bayesian clustering analysis

To infer the population structure, Bayesian clustering was carried out using the Markov chain Monte Carlo methods. We performed 50 runs for each number of clusters from 1 to 13. We selected an admixture model with correlated allele frequencies. To find the optimal number of clusters, we performed simulations of 1,000,000 iterations with a burn-in period of 100,000 generations. For each animal, the genetic origin was assessed as the proportion of genetic membership to each cluster. Based on the statistic *DeltaK* with values of 216.98, 2.58, 520.04, 2.97, 0.14 and 0.72 for K = 2-6 and K13, respectively, we found the optimal number of clusters, ($K = 4$, *DeltaK* = 520.04) (Figure 3). The first cluster consists only of the Nenets breed (red bars), the second one includes Tofalar and Todzha ecotypes (green bars), the third one (purple bars) comprises the remaining three breeds (Evens, Evenki and Chukchi), while the fourth cluster combines all wild reindeer. At $K = 13$, when the maximum proportion of a sample's membership in any of these clusters (*Pm*) is above 75%, we acknowledge 'pure ancestry' (Svishcheva *et al*, 2020). Pure ancestry was thus detected at four population samples: Tofalar (92.5%), wild Amur (87%), Todzha (82%) and wild Murmansk reindeer (79%). Other samples were found to have mixed ancestry. The

**Table 4.** Wright's F statistics for each locus. *Fit*: the inbreeding coefficient of an individual relative to the entire population; *Fst*: the inbreeding coefficient of the subpopulation relative to the entire population expected under random mating; *Fis*: the inbreeding coefficient of an individual relative to its own subpopulation.

| Locus | Number of alleles | *Fit* | *Fst* | *Fis* |
|---|---|---|---|---|
| BMS1788 | 28 | 0.109 | 0.078 | 0.034 |
| BMS745 | 13 | 0.071 | 0.049 | 0.023 |
| C143 | 3 | 0.23 | 0.146 | 0.098 |
| C217 | 3 | 0.041 | 0.077 | -0.039 |
| C276 | 8 | 0.085 | 0.066 | 0.021 |
| C32 | 9 | 0.118 | 0.085 | 0.037 |
| FCB193 | 15 | 0.099 | 0.081 | 0.019 |
| NVHRT16 | 13 | 0.047 | 0.052 | -0.005 |
| OHEQ | 30 | 0.078 | 0.077 | 0.001 |
| RT1 | 17 | 0.072 | 0.074 | -0.002 |
| RT24 | 17 | 0.117 | 0.084 | 0.037 |
| RT30 | 15 | 0.163 | 0.131 | 0.037 |
| RT6 | 17 | 0.087 | 0.091 | -0.004 |
| RT7 | 14 | 0.055 | 0.074 | -0.02 |
| RT9 | 14 | 0.081 | 0.08 | 0.001 |
| T40 | 20 | 0.11 | 0.049 | 0.065 |

wild reindeer sample from Tura showed the lowest *Pm* values (< 20%).

## Phylogenetic analysis

We reconstructed an unrooted phylogenetic tree by the neighbour-joining algorithm using Nei's genetic distances (Figure 4). The domestic reindeer were grouped into two clusters with 100% bootstrap support (BS). The cluster highlighted in green contained only main breeds (BS = 100%), while the cluster highlighted in red included two ecotypes (BS = 100%). Among the wild populations, we did not identify any group (all BS < 45%).

## Isolation by distance analysis and centre of allelic richness

We performed an isolation-by-distance (IBD) analysis based on the Mantel test. The original value of the correlation between genetic distances, measured as pairwise $Fst/(1-Fst)$, and geographic (Euclid) distances is represented by the black dot (Figure 5a), and the histogram describes the permuted values in the absence of spatial structure. Since the original value is inside the reference distribution, the spatial structure is non-significant (p-value = 0.135, $R^2 = 0.03$). For the studied population samples, we theoretically determined the geographic region/centre of allelic diversity using their geographic coordinates as well as allelic richness and indices defining natural (climatic) zones as weights (see Supplemental Table S1). We estimated that this region is located in the north-western part of Yakutia (along the coast of the Laptev Sea), which is the core area of seasonal ranges and migration routes traditionally used by reindeer. We detected a significant negative correlation ($cor = -0.57$, $p = 4.1\times10^{-2}$) between the level of allelic richness in the population sample and the geographic distance from the sample to the theoretically calculated geographic centre (latitude ≈72°, longitude ≈125°). Figure 5b demonstrates the distribution density of population samples plotted using a bivariate kernel density estimate.

## Discussion

In recent years, a difficult demographic situation has developed in wild reindeer herds of some geographic regions of Eurasia due to the industrial development of the Arctic and adjacent territories, uncontrolled hunting, loss of grazing land and climatic changes (Kirpotin *et al*, 2021). A severe economic and cultural disruption has been observed in some herds of domesticated reindeer, such as Tofalar and Todzha ecotypes. These reindeer are bred by small Indigenous groups and mark the southern limits of reindeer husbandry. Although the reindeer is an extremely important species for the Indigenous Arctic ethnic groups living in the arctic and subarctic regions of Eurasia, information on the population structure of this species is limited. It is clear that genetic studies provide knowledge that can be useful in the conservation of genetic variation by identifying the intraspecific genetic structure and also in the management of wild hunting and poaching. The first step to this is to identify the population structure of this arctic species.

We studied the genetic structure of *Rangifer tarandus* L. from the Eurasian part of the range. Our study included 13 population samples of animals, covering most of the range, six of which were domesticated to varying degrees, and the remaining seven samples belong to the wild form of reindeer from tundra, taiga and mountainous nature zones. The studied samples from wild reindeer populations relate to the different subspecies according to (Danilkin, 1999). The Murmansk reindeer selected from the western part of the range, namely from the Kola Peninsula, belongs to a subspecies *R. tarandus tarandus*; the Taymyr and Tura reindeer selected from the central part of the range, namely from the Taymyr Peninsula and Central Siberian Plateau, belong to a subspecies *R. tarandus sibiricus*; the Amur, Magadan, Yakutia and Chukotka reindeer samples selected from the eastern part of the range belong to a subspecies *R. tarandus phylarchus*. All studied wild populations of reindeer differ in morphological features. Our population structure analysis used a newly developed panel of 16 polymorphic microsatellites with high polymorphism information content.

When analyzing allele content, we detected private alleles specific for four wild reindeer herds ('W.Yakut', 'W.Taym', 'W.Murm' and 'W.Chuk') and for two domesticated ones ('Todzha' and 'Chukchi'), which suggests that each of these herds probably has formed a unique genepool under different (selection, geographical or feed) conditions. Besides, for the Todzha ecotype and

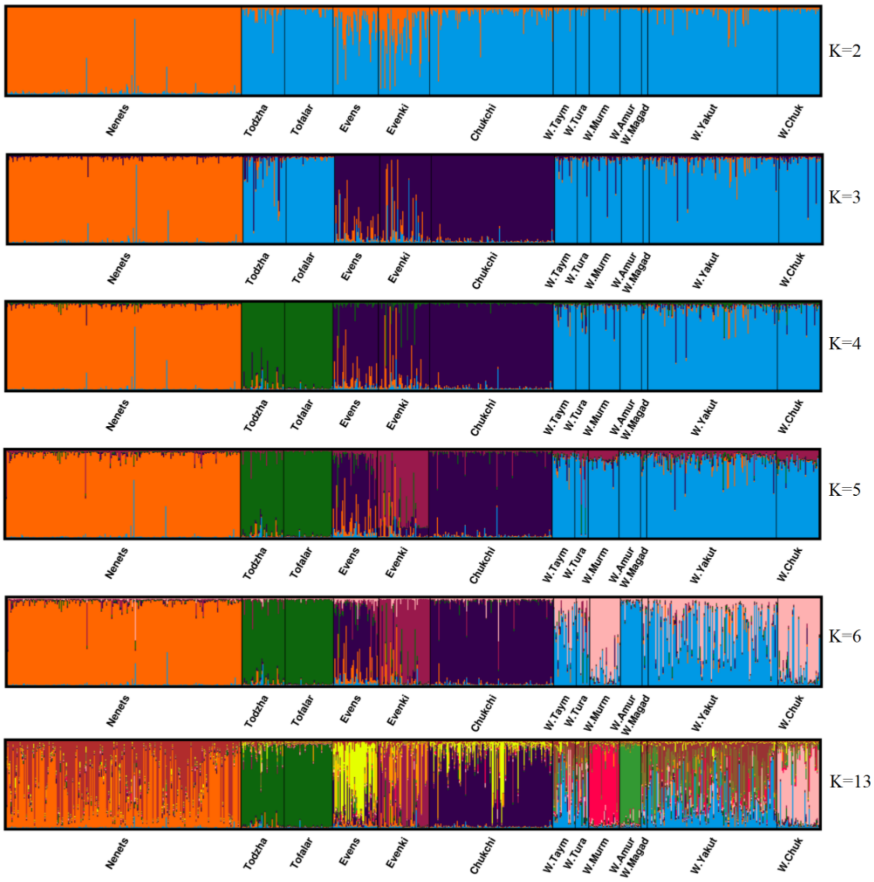

**Figure 3.** Results of STRUCTURE analysis based on microsatellite genotypes. Colour indicates the proportion of membership of each animal to *K* assumed clusters.

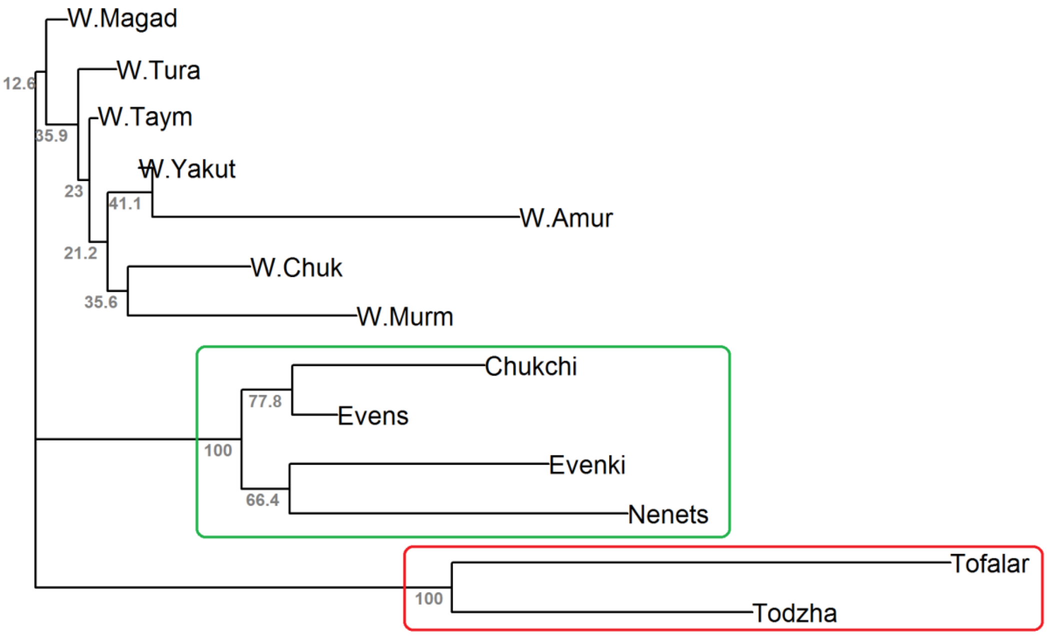

**Figure 4.** Phylogenetic tree constructed by the neighbour-joining algorithm. Numbers at the branch nodes indicate the confidence values for each branch obtained using the bootstrap procedure. The red and green rectangles show clusters with BS > 95%.

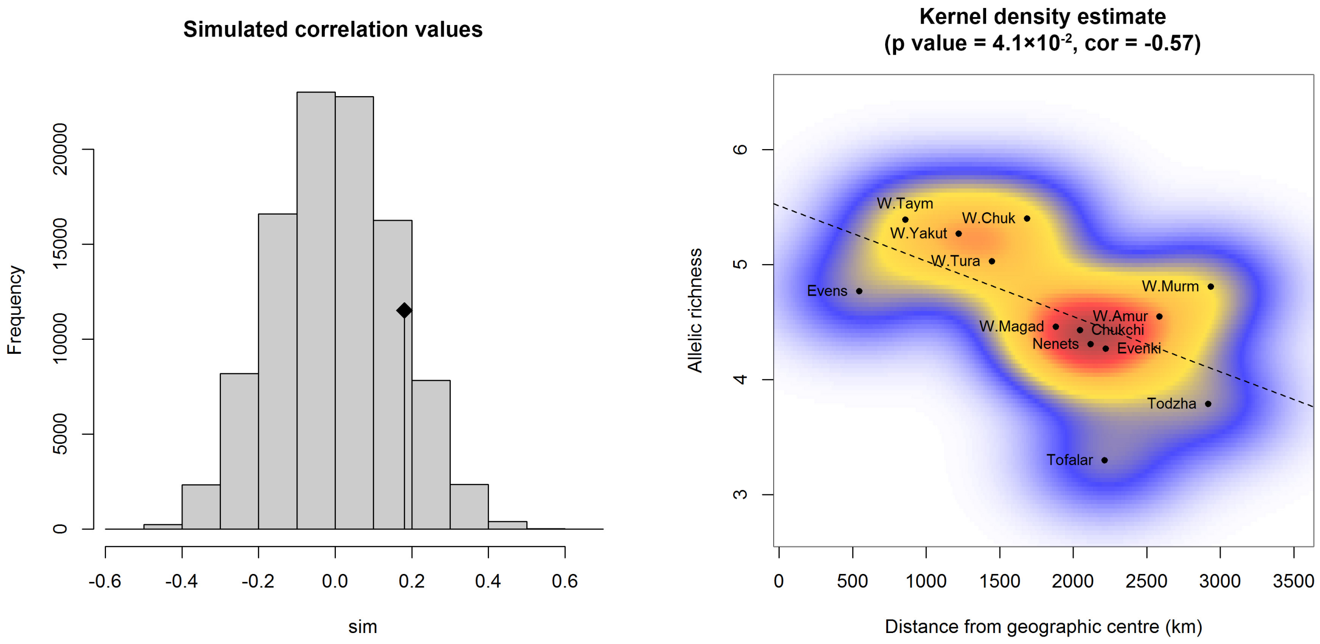

**Figure 5.** a) Histogram of simulated values. Results of Mantel test of correlations between genetic differentiation (Slatkin's linearized *Fst*) and geographic distance (spatial Euclidean). The black dot represents the original value of the correlation between genetic distances, measured as pairwise *Fst*/(1− *Fst*), and geographic (Euclid) distances. b) Distribution density of population samples plotted using a bivariate kernel density estimate. The linear regression trend is shown with a dashed line; colours represent degrees of density (blue: low; yellow: medium; red: high density).

the wild Murmansk reindeer, we found a high degree of 'pure ancestry', since the estimated maximum proportion of animal membership in these herds was 82.0% and 79%, respectively. Along with these two herds, we identified two more genetically homogeneous herds: the Tofalar ecotype (92.5%) and the wild Amur (87%) reindeer. For the Todzha and Tofalar samples, this is explained by a high degree of taming of reindeer due to taiga-specific (spatially limited) breeding and keeping conditions and, of course, indigenous traditions (Klokov, 2020). For the wild population samples from the Amur and Murmansk regions, 'pure ancestry' can be explained by their geographical remoteness from the crossings of major migration routes. By analyzing the heterozygosity and allelic richness, we suggest a comparatively low genetic variability in the Tofalar and Todzha ecotypes, whereas a high genetic variability was observed in the wild herds from the Taymyr, Chukotka and Yakut regions. This is due to the large population sizes and long-distance migration capability of the wild reindeer herds compared to domestic Tofalar and Todzha reindeer. It is interesting that according to the *Fis* index (Table 2), we observed no noticeable inbreeding in the studied populations. However, analysis of the distribution of inbreeding coefficients calculated for each animal, based on maximum likelihood method, showed comparably high inbreeding in the isolated samples of Todzha and Tofalar reindeer (Supplemental Table S9).

Results of Bayesian clustering analysis showed that all wild reindeer herds were clustered together. However, the samples of domesticated animals were distributed among the three clusters according to the degree of their domestication. As expected, the Todzha and Tofalar herds having the deepest level of taming, without seasonal migrations, formed a separate cluster, while the main breeds living in conditions of large herd formation, including long seasonal migrations (Stolpovsky *et al*, 2020), formed a cluster including only the Nenets breed and a cluster including the remaining breeds. The special genetic status of the domesticated Nenets reindeer is associated with the peculiarities of their historical formation (Dotsev *et al*, 2017) (Kharzinova *et al*, 2015). However, the phylogenetic analysis did not provide a high bootstrap support for joint clustering of wild herds and did not distinguish the Nenets breed from the rest, but confirmed the joint clustering of two ecotypes of reindeer, as well as the joint grouping of reindeer from three breeds (Evenki, Evens and Chukchi), adding to them the Nenets breed.

The analysis of paired *Fst* confirmed the population genetic differentiation between domestic and wild forms of the Eurasian reindeer, as well as the genetic structuring within each form (Figure 3). When comparing these two forms, we found significant differences only for 4 out of 42 pairs of populations. We suppose that for pairs 'W.Magad'–'Nenets', 'W.Magad'–'Chukchi' this can be attributed to the small size of the Magadan sample (n = 6) versus the large sample sizes of the Chukchi (n = 120) and Nenets breeds (n = 228). The genetic isolation of tame reindeer from wild ones living in the same geographic region is also worth mentioning. In particular, for the 'W.Amur'–'Evenki' pair from the Stanovoy Range region and the 'W.Chuk'–'Chukchi' pair from the Chukotka Mountains region we did not see the effect from an exchange of some individuals. The comparison of only the five population samples of the wild herds ('W.Taym', 'W.Tura', 'W.Murm', 'W.Amur' and 'W.Magad') showed that they are genetically differentiated from each other, but to a lesser extent than breed samples. The 'W.Yakut' sample has no genetic differences from all others, and 'W.Chuk' differs only from 'W.Murm' and 'W.Amur', which have characteristic feeding and behaviour patterns (Baskin, 1986). The wild reindeer from Yakutia and Chukotka belong to very large herds (with a high level of genetic diversity) that during seasonal migrations cover vast territories of the tundra and taiga. Probably, the exchange of genetic material between the wild reindeer herds takes place on overlapping migration routes. The population from Yakutia is especially distinguished in the group of samples of wild reindeer, which occupies the central part of the range relative to the studied populations, and has the maximum level of genetic diversity. We showed that the number of common alleles in 'W. Yakut' is about 82% of the total allelic diversity of the studied population samples, which significantly exceeds these indicators for the rest of the regional reindeer samples (Table 3). According to the results of our study, Yakutia is the centre of the allelic diversity of the genepool of the *Rangifer tarandus* L. species in the Eurasian part of the range. When comparing only the domestic herds, we found a higher level of genetic differentiation than among wild reindeer. This fact is consistent with an ecologically determined selection process that led to the emergence of divergent breeds in different geographic regions due to their different adaptability. In particular, the isolated samples of the Todzha and Tofalar herds showed the highest level of differentiation from all the breed samples ($Fst$ = 0.019-0.091, p-values $< 0.05$), while a low but significant difference was observed between the Evens and Chukchi breed samples ($Fst$ = 0.014, p-value $< 0.05$), which can be explained by the fact that the breeds have been created on the basis of the same constantly migrating natural populations of the eastern part of the reindeer range.

Although the IBD analysis did not reveal a spatial structure of the population samples, which may be associated with long-term seasonal migrations of animals, we theoretically determined the geographic region of allelic diversity for the studied group of population samples. As expected, this region is located in the north-western part of Yakutia: the coast of the Laptev Sea, where the major seasonal ranges and migration routes pass. The Yakutsk, Taymyr and Chukotka wild populations were the geographically closest to the centre of allelic diversity.

Among the factors that limit species distributions and effect on genetic structure, undoubtedly the last glacial maximum (LGM) has been the major force shaping reindeer geographic ranges in northern Eurasia. As it has been shown (Grosswald, 1999), in this part of the continent, exactly in the area of the Taymyr Peninsula, the LGM boundary has been discovered, extending to the northeast. However, the Laptev Sea region, which is part of West Beringia, has not been glaciated since at least the Tazov Glacial (Wetterich *et al*, 2011). Therefore, an eastern part of the species range has been significantly preserved. The species *Rangifer tarandus* L. has lived here both during the ice age and in the subsequent period of natural disasters caused by the melting of glaciers. The Pleistocene glaciation undoubtedly has influenced the genogeography of the species. This confirms the maximum genotypic diversity of reindeer in the eastern part of the range. From this region, resettlement has likely occurred throughout Eurasia range at the end of the LGM period. Long-term migrations have been inevitably accompanied by a loss of genetic diversity, a fact also known from studies of other species inhabiting this territory, for example, sable (Kashtanov *et al*, 2015).

## Conclusion

Overall, statistical analysis of microsatellite data revealed a significant genetic differentiation between domestic and wild reindeer and confirmed population structures within each form. Our results highlight the importance of collecting microsatellite data from wild and domesticated reindeer in providing new insights into the genetic diversity and population structure of reindeer, which can help design strategies for genetic conservation and improvement as well as support resource use.

## Author contributions

GRS: development of statistical methods, statistical analysis, interpretation of results, development of a concept, draft of the work, writing of the manuscript, final approval of the manuscript; SNK: interpretation of results, writing of the manuscript, final approval of the manuscript; OVB: microsatellite analysis, final approval of the manuscript; TPS: collecting of biological material, discussion of the results, final approval of the manuscript; MTS: DNA extracting; MVK: collecting of biological material, discussion of the results, final approval of the manuscript; YAS: development of a concept, draft of the work, collecting of biological material, interpretation of results, writing of the manuscript, final approval of the manuscript.

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## Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Ethics statement

All experiments were approved by the Local Ethics Committee of the Vavilov Institute of General Genetics of the Russian Academy of Sciences, Russia. The animal use procedures were carried out in accordance with the national veterinary guidelines.

## Data accessibility

Microsatellite genotyping data was deposited in Zenodo: 10.5281/zenodo.6376769.

## Supplemental data

Supplemental Table S1. Characteristics and geographic locations of the analyzed population samples of reindeer

Supplemental Table S2. Description of microsatellite markers

Supplemental Table S3. P-values of the Hardy–Weinberg equilibrium test with the FRD-based correction for each combination of sample and locus

Supplemental Table S4. P-values of the Hardy–Weinberg equilibrium test for each locus

Supplemental Table S5. The proportions of loci that are out of HWE for each population

Supplemental Table S6. Population parameters calculated for each locus and each population sample.

Supplemental Table S7. The polymorphism information content values for each combination of sample and locus

Supplemental Table S8. Nei's pairwise *Fst* values among samples.

Supplemental Table S9. The distribution of maximum-likelihood-based estimates of inbreeding coefficients

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# Farmers using local livestock biodiversity share more than animal genetic resources: Indications from a workshop with farmers who use local breeds

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**Abstract:** Recognizing the products from farms that use local breeds is key to *in situ* conservation of local animal biodiversity. Recognition often focuses on a small number of specific breeds or products but could be expanded to include multiple local breeds and products. This paper shows that several farmers who use local breeds can share principles among the multiple dimensions of their farming systems. We analyzed the exchanges among nine farmers who use local breeds on the different dimensions of their farming systems at a workshop held in November 2017. We present the principles they shared and discuss (i) the fact that bringing the principles to the fore requires a collective participatory approach, (ii) the fact that shared principles may also concern dimensions often neglected in livestock farming systems approaches, and (iii) how a collective participatory approach can help recognize the products and activities of farmers who use local breeds.

**Keywords:** Local breeds, livestock farming systems, value, recognition, practices

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## Introduction

As reported by the Food and Agriculture Organization of the UN (FAO, 2015), local livestock biodiversity is under threat. As livestock and livestock diversity contribute to livelihoods, food security and rural development, as well as having cultural and environmental dimensions (FAO, 2015), it is important to maintain the diversity of and add value to local breeds, through their sustainable use and development. To this end, several examples of using geographical indications to support the products of these breeds have been reported (Mathias *et al*, 2010). These labels often focus on a specific product associated with a single breed. Beyond labels focused on local breed products, it is hypothesized that, if different civil society stakeholders have a positive image of farmers who use local breeds, this will contribute to their conservation (Gandini *et al*, 2010). In this line of thought, it is important to raise public awareness of farming activities and products linked to local breeds. General recognition of products and activities of farmers who use local breeds should not necessarily focus on a single breed and product, but could, for instance, apply to a whole territory. Recognition at this scale would then concern a wide range of products and could include several local breeds of different species. It is recognized that the choice of a breed and the way the breed is managed are an integral part of the logic of a whole livestock farming system (Lauvie *et al*, 2015), and that breeds and the types of farming systems within which they are used are linked (Sturaro *et al*, 2013). However, the same breed can be used in different farming systems (Perucho *et al*, 2020). Despite this diversity, to better recognize the activities and products of farms at the territory scale, it is important to identify what they have in common. Indeed, recognition often

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depends on putting the spotlight on elements that justify recognition. These elements could for instance be included in a product specification or charter, or through communication campaigns. A prerequisite condition for recognition is thus to identify shared elements that could be highlighted. We therefore questioned if certain elements are shared by the different farming systems that use local breeds.

To answer this question, we aimed to identify the characteristics shared by several farming systems that use local breeds. We refer to these common underpinnings of a system as shared 'principles'. In this definition, shared principles can be associated with an existing diversity of practices. This paper aims to characterize the nature of common principles identified as shared by several farming systems that use local breeds.

The paper is organized in three sections: First, we present the methodology chosen to analyze the exchanges between local breed farmers on the different dimensions of their farming systems during a workshop on production systems and territory. We then identify the shared principles, and show how farmers who use local breeds may share farming system practices or values that go beyond technical or genetic aspects. Finally, we discuss (i) the fact that bringing these principles to light requires a specific participatory approach and the difficulties in applying this approach to a larger group of farmers, (ii) the fact that these principles concern dimensions often neglected in livestock farming systems approaches, and (iii) how to recognize the specificities of farming systems that use local breeds while supporting diversity.

## Materials and methods

This paper analyzes the content of the exchanges that took place during a workshop held in November 2017. The workshop was one of several organized during an action research project conducted in partnership between a research team of the French National Research Institute for Agriculture, Food and Environment, and the *Fédération des Races de Bretagne* (Federation of Local Breeds in the region of Brittany, Federation hereafter). The action research aimed to promote recognition of the products and activities linked with local breeds in Brittany.

The project was organized by the Federation, which gathers local breed associations in the region. Participation was voluntary and open to farmers who are members of the Federation. As a consequence, a variety of farmers using local breeds attended, and several different species were concerned.

In this paper, we analyze the content of a workshop on the theme: *Production systems and territory*. The workshop brought together nine farmers, four researchers (three participating in the action research and one involved in another part of the project, as an observer), and one facilitator from the Federation. Among the breeds raised by the nine farmers, those concerned by the Federation were Nantaise, Armoricaine, Bretonne Pie Noir, and Froment du Léon cattle breeds, Chèvre des fossés goats, and Landes de Bretagne sheep, all of which were included in this study. Animals of other breeds are also raised on some farms, but these breeds were not among those covered by the Federation.

Six of the farming systems produced meat, three mainly produced dairy products, and one produced both wool and meat. At the time of the workshop, three farmers mentioned they had another activity combined with raising livestock – one grew vegetables, and the other two had off-farm activities.

The four participants from the research institute came from three different fields: one from management sciences, one from sociology and two (including the observer) from livestock farming systems.

The exchanges were organized under three topics: (1) livestock feeding system and land use, (2) breeding practices, and (3) processing and sale of products and other activities such as opening the farm to visitors. The discussion on each topic was divided into two parts: (1) a roundtable to present each farmer's practices related to the topic, and (2) a collective discussion to agree on what they had in common about the topic. The exchanges were audio-recorded for internal report writing, and used for further analysis.

We analyzed the characteristics shared by the farming systems identified by the workshop participants, using the audio recordings and report to stay close to the collective expression of principles shared among the workshop participants.

We analyzed the exchanges concerning individual practices to capture their diversity in more detail. We conducted a thematic analysis of the notes reporting the exchanges. The diversity of practices described for each topic was reported with the aim of illustrating the diversity within a shared principle. We used this empirical data to better characterize the nature of the shared principles.

## Results

### Elements shared by farmers referred on different dimensions of the livestock farming systems.

Table 1 lists the results of the collective discussion aimed at reaching an agreement on what the farmers' systems had in common. The three dimensions of the farming systems reported in the table are the same as those used to organize the workshop, and correspond to the three topics.

The shared elements listed in Table 1 are phrased in a way that is as close as possible to the way they were expressed by the group of farmers. "Grazing-based systems" and "free-range (quasi integral)" refers directly to the characteristics of the livestock farming systems. "Orientation toward autonomy" refers to the shared desire to move towards livestock feed autonomy, even if the farmers underlined this is more difficult

**Table 1.** What the participant farmers considered they had in common in their farming systems

| Dimensions of farming systems | What farmers considered they had in common |
|---|---|
| Livestock feeding systems and land use | - Grazing-based systems<br>- Free-range (quasi integral)<br>- Orientation toward autonomy (principle to be modulated depending on the species)<br>- Use of hedges<br>- Purchased feed is not industrially processed<br>- Floristic diversity<br>- Match between the choice of environment and the choice of breed<br>- Attention to animal health and animal wellbeing |
| Animal breeding and genetics | - Management of a genepool: “farmers are gene passers” |
| Product processing and sales and other activities | - Farmers make their animals and activities visible<br>- Farmers establish direct links with their customers<br>- Trust is based on mutual knowledge and not on a label<br>- The taste of products is co-built and shared with consumers (and farmers consume their own products)<br>- Association between market relations and friendship<br>- Proximity can be interpersonal and/or geographical<br>- Everything is used/no waste<br>- Farmers seek balance and not expansion |

with some species than with others. Alongside their aim to achieve feed autonomy, they pointed out that if they had to buy feed, they avoided buying industrially processed feed. Some of the shared elements concerned the farm ecosystem and how it is valorized: “use of hedges”, “floristic diversity” of the pastured areas was considered important. More generally, they underlined the consistency between the breed and the farm agroecosystem: “match between the choice of environment and the choice of breed”. They also mentioned they all pay attention to “animal health and animal wellbeing”. The only principle linked with genetic management they share is that they consider themselves responsible for the “management of a genepool”. Several shared principles refer to what farm gate sales enable: “farmers can make their animals and activities visible”, there is a “direct link with the customer”, there may be an “association between market relations and friendship”, “trust [is] based on mutual knowledge and not on a label”, “the taste of products is co-build and shared with the consumers”. Another principle linked with farm gate sales is that “proximity can be interpersonal and/or geographical”, as in some cases farmers sell their products at a distance from the farm but where they know the customers. Finally, the last two principles are values shared by the farmers: “Everything is used”, is a declaration of their desire to avoid waste, and “farmers seek equilibrium balance and not expansion”.

## Shared principles refer to both practices and values

The elements the farmers consider as shared are expressed in different ways, and may be of a different nature. Some are formulated as generic practices (e.g. “when feed is purchased it is not industrially processed”). When principles are expressed as a shared generic practice, they may include different ways of expressing the practice. Some are not expressed as practices (e.g. “farmers seek balance and not expansion”) but as values, i.e. they refer to elements that are important in the farmers’ views, something they care about. When principles are expressed as values, the values can also be expressed through different practices.

## Shared principles associated with a diverse range of practices

We inventoried the diversity of practices expressed during the roundtables. Linking them to the corresponding shared principle revealed the diversity of farmers’ practices as illustrated in Table 2.

## Several shared principles refer to relational values and practices

All farmers who attended the workshop practised direct sales (farm gate sales), but when talking about what they have in common, the farmers did not mention this practice per se as a shared principle; instead, they mentioned the values associated with the practices that were not focused on economic but on relational values such as “direct link with the client” and “proximity that can be interpersonal and/or geographical”.

The shared principle “the taste of products is co-built and shared with the consumers” also refers to relational values and interactions. Indeed, the interactions with consumers lead to better mutual knowledge: farmers learn about consumers’ preferences, while the customers learn about the farming activity and the animals. For instance, one farmer mentioned that customers became accustomed to watching the animals they will eat in

**Table 2.** Examples of the range of practices associated with a shared principle

| Shared principle | Illustration of the diversity of associated practices |
|---|---|
| "Floristic diversity" | - Soil preparation practices (e.g. new flora are observed after subsoiling, or the use of limestone to treat acid soil)<br>- Plan to sow pasture (given the difficulties encountered in spontaneously obtaining floristic diversity due to local conditions)<br>- Leaving sowed multispecies grasslands to evolve in the long term (i.e. to become permanent grassland) and improve "by themselves". |
| "Farmers are gene passers" | - The future of young female animals: when referring to their conservation objective, the farmers pointed out that they do everything possible to keep young female livestock, while other farmers mention situations where it is difficult to keep a young cow (a cow whose behaviour is dangerous, for instance)<br>- Several farmers said they follow the advice on the genetic management of rare breeds, where there are differences in organization among breeds. |
| "Direct link with customer" | - All farmers used farm gate sales (some farmers were also part of a short supply chain with one intermediary)<br>- Farmers chose to sell individual cuts of meat. This requires a dedicated place and time<br>- Farmers chose to sell several different cuts of meat in boxes weighing several kilos. For these purchases, farmers use a dedicated booking organization, and define a sales period linked with the slaughtering and processing period<br>- Farmers may sell both individual cuts and mixed cuts in boxes. |

the end, grow, and two farmers mentioned people who had given up eating meat and started eating meat again. Several farmers mentioned receiving feedback about their products, and two farmers who process milk explained that when they develop a new product, they have some customers taste it, and they take the feedback they receive into account when adjusting the recipes.

The shared principle "farmers make their animals and activities visible" refers to a diversity of relational practices and to the description of individual practices illustrating how farmers interact with the public: organizing barbecues or dinners and shows on the farm with the possibility to visit the farm, opening their farm to visits on demand, receiving a helping hand from customers who have become friends, holding art exhibitions on the farm through the intermediary of an association.

## Discussion

### Identifying shared principles underlying livestock farming systems through collective approaches

The approach we present in this paper consisted of identifying shared principles that underpin livestock farming systems. We show that these principles can be expressed both as practices and values. Agroecology is a scientific field in which the principles underpinning farming systems are widely acknowledged. Wezel *et al* (2020) reviewed the different agroecological principles reported in the literature with the aim of proposing consolidated principles. These authors defined principles as actionable statements that contain both normative aspects (that assert values) and causative aspects (that explain relationships) (Wezel *et al*, 2020). Agroecological principles are generally defined by scientists and experts to provide a permanent generic scope that can be used to guide analysis, support transition and evaluate systems. The principles are generically formulated but can be applied locally through a range of practices suited to local conditions (Wezel *et al*, 2020). In this context, some agroecological studies aim to establish links between generic agroecological principles and the diversity of practices and ways of acting on agroecosystems (Toffolini *et al*, 2018). The principles we identified in this study are actionable statements that can be expressed through a diversity of practices depending on the farm. However, these principles are formulated as statements shared by a group of farmers, to support work to achieve better recognition of their activities and products. The shared practices and values identified are consequently the result of bringing together diverse views – the product of a collective process within a group, the contours of which may change. Therefore, in contrast to generic agroecological principles, these principles may change over time. As the identification of shared principles necessarily results from the views of a group, a participatory approach is required.

The need for a collective process leads to a methodological challenge. The workshop reported in this paper involved only a small number of farmers. Although this study confirms that this group of farmers who use local breeds share more than genetic resources, it is impossible to conclude what all farmers using local breeds share at the Federation scale. The Federation has many members, and it would be impossible to include all the farmers of the member associations in a face-to-face collective reflection. One possible way to proceed would be to design a collective form of governance to enable each farmer to monitor the process and give their view, even if they do not participate actively in the workshop discussions.

## The shared principles of livestock farming systems concern several dimensions.

This study showed that farmers who use local breeds not only share the genetic resources they use but also other elements of their farming systems. Those elements are expressed as values and practices, and are interrelated, as values are related to "how farmers 'make sense' of their practices" (Darnhofer *et al*, 2012). This calls for further developments in the field of genetic resources management to better understand what farmers who use local breeds do share.

We have shown that principles shared by all the farmers are reflected in practices that vary with the farm. Diversity among farming systems is indeed a key research object of livestock farming systems, and is often tackled through the use of different kinds of typologies depending on the aim of the study (Alvarez *et al*, 2018). Here, in accordance with the aim of our study, our approach was more focused on the identification of common principles than on the characterization of the diversity of possible practices that each principle covers.

Our work also shows that shared principles of livestock farming systems using local breeds are not only linked with breeding and genetics but with other dimensions, including feeding and sanitary systems. The systemic approach to livestock farming considers several dimensions and their associated practices in a given farming system. Although feeding, sanitary, reproduction and renewal practices are at the core of the livestock farming systems approach, what Landais (1994) termed the "valorization practices" of animal products (processing, sales, etc.) are also an integral part. Our study confirms the importance of the processing and sale of farm products in the overall functioning of the system. Few studies of livestock farming systems pay sufficient attention to the sale of farm products (Nozières, 2014), even though it may play an important role. Indeed, here we have shown the key role of farm gate sales or short supply chains. Although direct sales strategies are important in terms of the farm's overall economic strategy as well as work organization, our results show that the relations established with consumers are also very important and interact with other dimensions of the system. Milk and meat processing, for instance, can be influenced by direct sales, as the range of products and the recipes can evolve in response to consumer feedback about the products. The relational dimension of short supply chains is studied in social and economic sciences (Chiffoleau *et al*, 2019), and livestock farming system approaches also consider this relational dimension increasingly relevant, as underlined by Darnhofer *et al* (2012).

More generally, this work underlines the importance of the different relationships created by the farmers around their farms, not only through direct sale, but also farm visits or other activities. These different interactions could be the subject of further studies in livestock farming system approaches.

## Challenges: how to recognize specifics of farming systems that use local breeds without excluding diversity?

Gaining recognition for the products and activities of farms using local breeds is a challenge. This is fully consistent with the wider challenge of adding value to local breeds and helping ensure their continued viability (Ligda and Casabianca, 2013). Several studies focused on products made from local breeds, with or without specific labelling, showing the advantages of adding value to breeds by adding value to products, particularly in an approach focused on economic value. (Verrier *et al*, 2005; Mathias *et al*, 2010). However, other kinds of value may also be at play, and an approach focused on products and activities could broaden the perception of adding value to one or several local breeds. This would include the interrelated strategies identified by Ligda and Casabianca (2013):

> ”1. linkage of local breeds to traditional products and/or tourism/agritourism; 2. promotion of local breeds in specific farming systems, such as organic production, conservation grazing, sylvopastoral systems and small-scale low-input farms and hobby farms; and 3. general strategies focusing on the promotion of local breeds (marketing, legislation, organizational issues and raising public awareness) (Papachristoforou *et al*, 2013)."

The desire to better recognize the products and activities of farms that use local breeds also reflects a general need to raise awareness of local animal biodiversity. In a previous study on the motives for buying products, conducted with short supply chain consumers who buy products from five French rare local breeds, it appeared that the breed was not spontaneously mentioned as a reason for the purchase. This was considered interesting from a global perspective for consumers to better understand the origin of the food they eat (Couzy *et al*, 2017). Although the conclusion of this study cannot be extrapolated, it illustrates the dual need to improve awareness of local biodiversity and provide information about the breeds raised, along with additional information on the production process.

Identifying the principles shared by farming systems based on the use of local breeds is a first step towards better recognition of the activities and products of farms using local breeds. Achieving recognition could be inspired by existing strategies (e.g. labels on products, labels on farms, labels on sales outlets, logos, charters, specifications) or designed ad hoc. In a study of participatory guarantee systems for organic agriculture, Lemeilleur and Allaire (2018) provide insights into the different dimensions of recognition setups. They distinguished three types of shared resources in such setups: ideas (shared principles, shared knowledge about practices etc.), equipment (frames of reference, e.g. specifications or charters, guarantee mechanisms, e.g.participatory guarantees or third party certification, etc.) and artefacts (logos, denominations, etc.) (Lemeilleur and Allaire, 2018). The results of the present study offer more possible ideas for such a setup. However, as we explained earlier, it is a challenge to develop a collective governance that would allow each farmer, who cannot be directly involved in a discussion held to define common principles, to nonetheless be able to follow the process and give their views.

Diversity is a key notion for farmers who use local breeds. As shown in a previous study (Lauvie *et al*, 2014), diversity is considered not only in terms of genetic resources but also in terms of the farmers involved, the different farming systems, farm products, etc. Identifying the activities and products of farmers who use local breeds to make them easier to recognize without reducing the existing diversity is a challenge. Finding a balance between diversity inclusion and differentiation and/or protection, depending on the final aim of such a setup, questions the degree to which products and activities should be specified.

## Conclusion

This article presents the results of an action research project aimed at identifying the principles shared by farming systems that use local livestock breeds. We observed that farmers who use local breeds share more than just a breed: they also share principles concerning livestock feeding systems and the sale of products, for instance. We also observed that the shared principles are expressed both as practices and values. We identified the methodological challenges associated with identifying common principles shared by livestock farming systems using local breeds. We finally discussed what can be done to increase recognition of the activities and products produced by farmers using local breeds – a process that can both maintain and develop the use of local breeds and, consequently, favour local livestock biodiversity.

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## Authors contributions

Anne Lauvie: study conception and design, data collection, analysis and interpretation of results, draft manuscript preparation and manuscript revision. Nathalie Couix and Jean Michel Sorba: Study conception and design, data collection, analysis and interpretation of results, manuscript revision.

## Conflict of interest statement

The authors declare no conflict of interest.

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# Morphological characterization of 23 *Malus domestica* Borkh cultivars from central Spain

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**Abstract:** The purpose of this work was to morphologically characterize an apple tree collection composed of 67 individuals from 41 accessions belonging to 23 old Spanish apple cultivars (*Malus domestica* Borkh) alongside 9 reference cultivars. The studied germplasm was collected previously in rural areas of central Spain (Sierra Norte de Madrid and Tagus river basin) and it was analyzed through 67 descriptors mainly from IBPGR and UPOV. We found a very high morphological diversity in the studied old apple cultivars, as 48% of the descriptors (most of them devoted to fruit traits) were significantly different between types of cultivars. In addition, the sample cultivars resulted clearly distinct from reference cultivars in multivariate analysis. In general, no particular structure was found in old cultivars, but a strong differentiation of 'Agridulce' and 'Hojancas' is reported due to their bigger fruits. Our results support the molecular analysis and call for further analysis of the local apple germplasm and long-term conservation actions.

**Keywords:** Apple genetic resources, old apple cultivars, phenotype, multivariate analysis

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## Introduction

The apple tree (*Malus domestica* Borkh.) is the most important temperate fruit tree crop, with more than 126 million tonnes harvested worldwide in 2020 (FAO, 2022). Such production is in line with the Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture (FAO, 2010), which reported *Malus* L. genetic resources to be among the largest *ex situ* collections. Morphological characterization of *Malus* has been essential for an adequate description of germplasm collections, for breeding programmes (Božović *et al*, 2015) and taxonomic studies (Höfer *et al*, 2014; Wagner *et al*, 2014). Currently, although the information provided by genetic markers (such as microsatellites) is preferred against phenotyping due to their stability and economy (Reddy *et al*, 2002; Ban *et al*, 2014), the study of agricultural germplasm by morphological traits is still relevant and useful in diversity analysis (Božović *et al*, 2015; Király *et al*, 2015; Kumar *et al*, 2018).

As a result, apple morphological descriptions were conducted in Bosnia and Herzegovina (Gaši *et al*, 2011), Canada (Watts *et al*, 2021), Hungary (Király *et al*, 2015), India (Dolker *et al*, 2021), Iran (Farrokhi *et al*, 2013), Italy (Martinelli *et al*, 2008), the Kashmir Valley (Dar *et al*, 2015), Macedonia (Kiprijanovski *et al*, 2020), Montenegro (Božović *et al*, 2015), Serbia (Mratinić *et al*, 2012) and Turkey (Karatas, 2022), reporting high morphological diversity. The most common morphological descriptors used in those works belong to international guidelines such as IBPGR (1982) and UPOV (2005) and they focus on fruit characteristics because sensorial characteristics and consumer demand focus on fruits (Pereira-Lorenzo *et al*, 2018).

In Spain, several studies also reported great phenotypic apple diversity (Royo and Itoiz, 2004; Ramos-Cabrer *et al*, 2007; Santesteban *et al*, 2009; Pérez-Romero *et al*, 2015), but they did not include old cultivars from some central regions. This lack of informa-

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tion should be filled, as some of these undescribed old apple cultivars are valued for their sensorial qualities, others are suspected to be exclusive to central Spain, and the abandonment of rural landscapes threatens their survival (Aceituno-Mata, 2010; Comunidad de Madrid, 2018; Arnal *et al*, 2020).

This study aims to quantify the morphological diversity of old apple cultivars growing in rural areas of central Spain, compare it with a previous genetic study with simple sequence repeats (Arnal *et al*, 2020) and see whether the old traditional cultivars were distinguishable by morphological descriptors. This work also provides the foundations for further agronomic and sensorial studies to complete the Spanish apple morphological description and provide valuable information that will increase the knowledge of apple genetic resources.

## Material and Methods

### Plant material

A collection of 67 individuals from 41 accessions belonging to 23 old Spanish apple cultivars was evaluated to assess their morphological diversity (Table 1). The collection is located in Arganda del Rey (Madrid) and belongs to the Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario IMIDRA (Figure 1). For each accession, in 2009 two scions were grafted onto seedlings of *M. domestica* in a frame of 5m, being the aisles oriented in the SW-NE direction. Two individuals of nine main reference varieties and sports (grafted in rootstocks from the same nursery) curated in the same orchard and environmental conditions were included as controls: 'Fuji Aztec', 'Fuji Kiku 8', 'Gala Buckeye', 'Gala Schniga', 'Golden Delicious', 'Golden Reinders', 'Granny Smith', 'Reineta Blanca' and 'Verde Doncella' (Table 2). Those references were selected because they are widespread in Spain (Iglesias *et al*, 2009).

The annual maintenance of the collection was conducted as follows: goblet pruning at the end of autumn, soil amendment at the beginning of winter and a preventive application of pesticides against aphids at the end of spring. Trees were irrigated every two weeks from May to September to reduce water stress during summer.

### Morphological descriptors

A set of 67 morphological descriptors (25 quantitative and 42 qualitative, of which 3 were discrete, 23 nominal and 16 ordinal) were assessed on for 4 organs: 8 descriptors on winter 1-year-old wooden branches (or shoots), 15 on leaves, 16 on flowers and 28 on fruits (Table 3). The descriptors were obtained from IBPGR (1982), UPOV (2005) and Urbina and Dalmases (2014) and new descriptors and further categories in some qualitative traits were also considered, such as watercore (Arnal, 2021). Ten (10) to 20 fruits, 20 leaves, 10 flowers and 20 shoots were collected from different orientations of the tree crown in two years (except flowers) from summer 2016 to autumn 2019 and stored at 4–7°C until processing. In particular, leaves and shoots were collected in 2016 and 2017, and flowers and fruits through the four years of the study.

Continuous descriptors of shoots, flowers and fruits were measured manually using a JP Selecta model 5900601 digital caliper with a precision of 0.01mm. Leaf quantitative descriptors were captured with ImageJ (Schneider *et al*, 2012), so leaves were previously scanned attached to a 2D-scale. Finally, apple fruit weight (ten per individual) was registered with a Sartorius CP 2202 S digital scale with a precision of 0.01g.

### Data analysis

*Phenotypic diversity.* Arithmetic means for 25 quantitative, medians for 16 discrete and ordinal, and modes for 26 nominal descriptors were calculated to obtain the central values by accession and cultivar. All qualitative descriptors were translated into a numerical value to meet computing requirements. Student's t- and Cohen's d-tests were conducted to identify ifferences between types of cultivar descriptors (references and old apple cultivars). Lastly, Tukey's HSD test was performed to detect different groups among old apple cultivars. The significance level ($\alpha$) was set at 0.05.

*Correlations*. A correlation matrix was calculated to explore significant correlations between descriptors in old traditional cultivars. Correlations between continuous descriptors were done with Pearson, whereas the rest were computed with Spearman. No correlations between nominal descriptors were performed. Descriptors with no variance were removed at this stage.

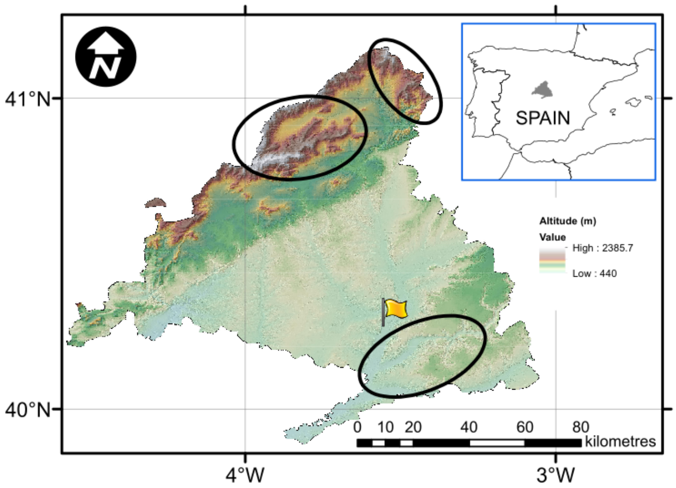

**Figure 1.** Collection sites of the old apple cultivars from central Spain. The two upper ellipses indicate accessions from Sierra Norte de Madrid and the lower one, from the Tagus River basin. The yellow flag indicates the location of the IMIDRA collection. MDT25 2015 and BDLJE 2018, CC-BY 4.0 ign.es.

**Table 1.** Cultivar name, individual, accession andcollection site of the 23 old apple cultivars sampled in rural areas of central Spain.

| Old cultivar name | Individual/s | Accession | Collection site |
|---|---|---|---|
| Amarillo de El Paular | APRA1/2 | APRA | Rascafría |
| Camuesa | CABU1/2 | CABU | Bustarviejo |
| | CAPR1/2 | CAPR | Prádena del Rincón |
| Camueso tardío | CTAP1/2 | CTAP | Puebla de la Sierra |
| Camueso temprano | CTEP1 | CTEP | Puebla de la Sierra |
| de Chapa | CHCA1/2 | CHCA | Canencia |
| | CHVA1 | CHVA | Valdemanco |
| Esperiega | ESPU1/2 | ESPU | Puebla de la Sierra |
| Agridulce | MAMO1/2 | MAMO | Montejo de la Sierra |
| Hojancas | MHHO1/2 | MHHO | Horcajuelo de la Sierra |
| | MHPR1/2 | MHPR | Prádena del Rincón |
| del Ortel | ORCA1/2 | ORCA | Canencia |
| | ORMO3 | ORMO | Morata de Tajuña |
| Pero de Aragón | PAHO1/2 | PAHO | Horcajuelo de la Sierra |
| | PAPR1/2 | PAPR | Prádena del Rincón |
| | PAPU1/2 | PAPU | Puebla de la Sierra |
| Pepita de melón | PECA1/2 | PECA | Canencia |
| | PEHI1/2 | PEHI | La Hiruela |
| | PEVA2 | PEVA | Valdemanco |
| Pero gordo | PGHI2 | PGHI | La Hiruela |
| Pero pardo | PPHI1 | PPHI | La Hiruela |
| | PPMO1/2 | PPMO | Montejo de la Sierra |
| | PPPU1/2 | PPPU | Puebla de la Sierra |
| Pero real | PRBU1/2 | PRBU | Bustarviejo |
| | PRHI1/2 | PRHI | La Hiruela |
| Rabudas | RAHI1/2 | RAHI | La Hiruela |
| Reineta | REHO11 | REHO1 | Horcajuelo de la Sierra |
| | REHO21 | REHO2 | |
| | REMO1 | REMO | Montejo de la Sierra |
| | REPR1/2 | REPR | Prádena del Rincón |
| Rojillo | RJHO1/2 | RJHO | Horcajuelo de la Sierra |
| Rojo | RJPR1/2 | RJPR | Prádena del Rincón |
| | RJPU1/2 | RJPU | Puebla de la Sierra |
| de Rosa | RORA1/2 | RORA | Rascafría |
| Rojillo temprano | RTEV1 | RTEV | Valdemanco |
| San Felipe | SFCA1 | SFCA | Carabaña |
| Temprano | TEPI1 | TEPI | Pinilla del Valle |
| Verde Doncella | VDCA1/2 | VDCA | Canencia |
| | VDHO1/2 | VDHO | Horcajuelo de la Sierra |
| | VDTI1 | VDTI | Tielmes |

**Table 2.** Reference cultivars curated in the IMIDRA collection.

| Reference cultivar name | Accession number | Origin |
|---|---|---|
| Fuji Aztec | 4 | Worldwide cultivar |
| | 7 | |
| Fuji Kiku 8 | 11 | Worldwide cultivar |
| | 14 | |
| Gala Buckeye | 115 | Worldwide cultivar |
| | 117 | |
| Gala Schniga | 109 | Worldwide cultivar |
| | 113 | |
| Golden Delicious | 104 | Worldwide cultivar |
| | 108 | |
| Golden Reinders | 101 | Worldwide cultivar |
| | 203 | |
| Granny Smith | 206 | Worldwide cultivar |
| | 210 | |
| Reineta Blanca | 211 | Worldwide cultivar |
| | 215 | |
| Verde Doncella | 307 | National cultivar |
| | 311 | |

*Multivariate analysis.* A principal coordinate analysis (PCoA) was performed to visualize the possible groups of cultivars and detect the descriptors that better describe the differences among individuals. In the cluster analysis, a distance matrix between accessions was calculated with Nei's distance (Nei, 1973) and the dendrogram was plotted using the unweighted pair group method with arithmetic mean (UPGMA) hierarchical agglomerative method (Sokal and Michener, 1958).

*Analysis computing.* Statistics were performed in R Studio v.3.4.1 (R Core Team, 2017) and a set of packages: ‘effsize’ for Cohen's d-test Torchiano (2018), ‘corrplot’ (Wei and Simko, 2017) for the correlation matrix and ‘agricolae’ (De Mendiburu, 2019) for Tukey HSD. Multivariate analysis was computed with an adapted version of the ‘MorphoTools’ script (Koutecký, 2015).

# Results

## Phenotypic diversity

Thirty-two morphological descriptors (48% of the total; 19 continuous and 13 non-continuous), showed significant differences between references and old apple cultivars (Table 4). In general, old apple cultivars registered lower quantitative values than references, but level frequencies in many qualitative descriptors were less skewed.

Shoots, leaves and flowers from both types of cultivars were similar, as there were only 14 significant descriptors out of 39 (36%). Some significantly different descriptors found in those organs were the width of the apical bud (ShW; P = $6.63 \times 10^{-14}$), the petal length (FlPetL; P = 0.0012) and the petal width (FlPetW; P = 0.023).

In fruit, 18 out of 28 descriptors were significantly different (64%). The calyx opening diameter (FrCCD; P = $4.26 \times 10^{-24}$), fruit peduncle length (FrPedL; P = $5.57 \times 10^{-37}$), and peduncle width (FrWP; P = $7.37 \times 10^{-18}$) stood out by their significance and effect size (Cohen's d). In fact, it was observed that the peduncles from old apple cultivars were around 1cm shorter than those from references (Figure 2a). Other important quantitative descriptors such as the peduncular cavity width (FrSCW; P = 0.0163), the calyx cavity width (FrCCW; P = 0.0163), fruit length (FrL; P = 0.0026) and fruit weight (FrM; P = 0.0275) were significant, but the effect size (Cohen's d) was not large. Alternatively, no significant differences existed in fruit width (FrW; P = 0.2081). In the fruit qualitative descriptors, the depth of the calyx cavity (FrCCDep) was ’intermediate’ in old apple cultivars and ’strong’ in references, with significant differences (P = 0.006), describing more diversity in old apple cultivars, as it was relatively easy to find apples with a ’weak’, ’intermediate’, ’strong’, or ’very strong’ calyx. Regarding over colour (FrUpCol), it was found that old apple cultivars had significantly less over colour than references (P = $2.69 \times 10^{-06}$). Nevertheless, among apples with cheeks, ’red’ and ’yellow’ were the most abundant colours.

Means, medians and modes were also computed by cultivar. As a result, means of quantitative descriptors from references were contained in the Tukey's HSD groups of old apple cultivars. The average fruit length (FrL) of the apples was in the 43–63mm range and the fruit width (FrW) was between 50 and 81.5mm. Regarding fruit weight (FrM), apples weighed 125g on average. Their shape (FrShp) was mostly conical (sum of ’conical globose’, ’conical oblong’ and ’conical truncated’), with a minority of ellipsoidal and flat globose shapes.

The cultivar ‘Agridulce’ showed significant larger sizes than the other old apple cultivars and even references, as its measurements belonged to the ’a’ group of Tukey's HSD in 17 out of the 25 quantitative descriptors (almost 50%), such as fruit length (FrL), fruit weight (FrM) and fruit width (FrW). ‘Hojancas’ and ‘Pero gordo’ also tended to have larger organs. The rest of the cultivars presented intermediate size organs, except ‘Esperiega’, and ‘San Felipe’, which showed small organs.

## Correlations

There was significant correlation in 44 out of 67 descriptors studied, as the total average significant correlation was 0.43 (Figure 3). Shoot colour (ShCol) was removed as no variance was detected. By organ, the means of correlation was 0.37 in the shoot, 0.54 in the leaf, 0.42 in the flower, and 0.44 in the fruit. Correlations within each organ were mainly positive, such as leaf area

**Table 3.** Morphological descriptors evaluated in 23 old apple cultivars curated at the IMIDRA collection. Type of descriptor (C, Continuous;D, Discrete; N, Nominal; O, Ordinal). The hash (#) indicates that the descriptor has been altered (by adding or removing some levels of the published descriptors). Sources:1, IBPGR (1982); 2, UPOV (2005); 3, Urbina and Dalmases (2014); 4, this paper.

| Organ | Descriptor name | Code | Type | Levels | Source |
|---|---|---|---|---|---|
| Shoot | Pubescence on the apical bud | ShBPub | O | 0, glabrous; 1, intermediate; 2, tomentose | 3 |
| | Shoot colour | ShCol | | 1, brown; 2, reddish brown, 3; green; 4, grey; 5, purple; 6, red; 7, brown reddish; 8, light brown | 2 |
| | Shoot diameter (mm) | ShDia | C | – | 2 |
| | Length of the apical bud (mm) | ShL | C | – | 3 |
| | Lenticels# | ShLent | O | 1, very few; 3, few; 5 frequent; 7, densely populated | 2, 3 |
| | Apical shoot shape# | ShShp | | 1, semispherical; 2, ovoid; 3 intermediate; 4, conical | 3 |
| | Pubescence on shoot | ShSPub | O | 0, glabrous; right, 9, tomentose | 2 |
| | Width of the apical bud (mm) | ShW | C | – | 4 |
| Leaf | Leaf area (cm2) | LeArea | C | – | 4 |
| | Asymmetry of the leaf blade | LeAsim | | 0, symmetric; 1, asymmetric | 4 |
| | Shape of the base of the leaf blade | LeBas | | 1, cuneate; 2, rounded cuneate; 3, rounded; 4, asymmetric; 5, cordate; 7, truncated | 3 |
| | Petiole colour | LeCol | | 1, purple; 2, green and purple; 3, green | 2 |
| | Leaf edge shape | LeEdg | | 1, crenate; 2, bicrenate; 3, serrate-1; 4, serrate-2; 5, biserrate-2; 6, biserrate-1; 7, triserrate | 2, 3 |
| | Foliar blade folding | LeFold | | 1, folded; 2, turned; 3, convex; 4, ondulate; 5, flat | 3 |
| | Leaf blade length (cm) | LeL | C | – | 2, 3 |
| | Maximum width of the leaf blade (cm) | LeMWL | C | – | 4 |
| | Petiole length (cm) | LePetL | C | – | 3 |
| | Pubescence on the reverse | LePub | O | 0, not pubescent; 1, pubescent at the base of the midrib; 3, little pubescent; 5, pubescent; 7, very pubescent; 9, tomentose | 2, 3 |
| | Leaf blade shape | LeShp | O | 1, ovate; 2, elliptical; 3, obovate | 4 |
| | Leaf petiole stipules | LeSti | | 1, rudimentary; 3, short filiform; 5, long filiform; 7, narrow foliar; 9, wide foliar | 3 |
| | Leaf apex length (mm) | LeTip | C | – | 3 |
| | Shape of the leaf apex | LeTipShp | | 2, rounded; 3, acute; 5, mucronate; 7, acuminated; 9, cuspidate | 4 |
| | Leaf blade width (cm) | LeW | C | – | 2, 3 |

*Continued on next page*

*Table 3 continued*

| | | | | | |
|---|---|---|---|---|---|
| Flower | Androecium length (mm) | FlAnd | C | – | 4 |
| | Dominance of the gynoecium over androecium | FlDom | O | 1, dominated; 2, balanced; 3, dominant | 2 |
| | Gynoecium length (mm) | FlGin | C | – | 3 |
| | Pedicel colour | FlPedCol | O | 1; purple; 2, purple and green; 3, green | 4 |
| | Flower pedicel length (mm) | FlPedL | C | – | 4 |
| | Petal colour | FlPetCol | | 0, white; 1, pink white; 2, purple white; 3, purple | 3 |
| | Petal length (mm) | FlPetL | C | – | 2, 3 |
| | Number of petals | FlPetN | D | Integer counting | 3 |
| | Petal width (mm) | FlPetW | C | – | 2, 3 |
| | Pubescence on pedicel | FlPub | O | 1, glabrous; 2, slightly pubescent; 3, tomentose | 4 |
| | Relative position of the petals | FlRPP | O | 0, free; 1, tangent; 2, overlapped | 2, 3 |
| | Sepal length (mm) | FlSepL | C | – | 4 |
| | Flower shape | FlShp | | 1, flat turned; 2, turned cupuliform; 3, cupuliform; 4, slightly cupuliform; 5, flat | 3 |
| | Flower pedicel stipules | FlSti | | 0, no present; 1, present | 4 |
| | Type of petal | FlTyp | | 0, flat; 1, wavy; 2, concave; 3, convex | 4 |
| | Welding point of the stamens | FlWeld | O | 1, welded at the base; 2 welded in pairs up to a certain height; 3 completely welded | 3 |
| Fruit | Calyx opening diameter (mm) | FrCCD | C | – | 2, 3 |
| | Depth of the calyx cavity[#] | FrCCDep | O | 0, external; 1, very weak; 2, weak 3, intermediate; 4, strong; 5, very strong | 2, 3 |
| | Length of the calyx cavity (mm) | FrCCL | C | – | 2, 3 |
| | Shape of the opening of the calyx cavity | FrCCShp | | 0, without sepals; 1, convergent; 2, partially extended or extended; 3, erect | 2, 3 |
| | Calyx cavity width (mm) | FrCCW | C | – | 2, 3 |
| | Over colour distribution[#] | FrDisCol | O | 0, uniform (no cheeks); 1, blurred; 2, blurred and stripped; 3, stripped | 1, 3 |
| | Opening of the calyx cavity[#] | FrEye | | 0, closed; 1 open | 3 |
| | Flattening[#] | FrFlat | O | 1, dominated; 2, balanced; 3 dominant | 2, 3 |
| | Surface colour | FrGroCol | | 1, green; 2, light green; 3, yellowish green; 4, light yellow and 5, yellow | 1, 2, 3 |
| | Opening of the locules | FrHea | O | 0, closed; 1, semi-open; 2 open | 2, 3 |
| | Fruit length (mm) | FrL | C | – | 2 |
| | Number of loculi | FrLoc | D | Integer counting | 1 |

*Continued on next page*

*Table 3 continued*

| | | | | | |
|---|---|---|---|---|---|
| | Fruit weight (g) | FrM | C | – | 1, 3 |
| | Pulp colour | FrMCol | | 0, white; 1, greenish white; 2, green; 3, yellowish green; 4, white-yellowish; 5, Greenish yellow; 6, yellow | 2, 3 |
| | Fruit peduncle length (mm) | FrPedL | C | – | 2, 3 |
| | Russeting in the calyx cavity# | FrRCC | | 0, no russeting; 1, russeting | 2, 3 |
| | Russeting on fruit faces# | FrRF | | 0, no russeting; 1, russeting | 2, 3 |
| | Ribs | FrRib | O | from 1, absent; to 5, very prominent | 2, 3 |
| | Russeting in the peduncular cavity# | FrRS | | 0, no russeting; 1, russeting | 2, 3 |
| | Peduncular cavity length (mm) | FrSCL | C | – | 2, 3 |
| | Peduncular cavity width (mm) | FrSCW | C | – | 2, 3 |
| | Number of seeds | FrSeed | D | Integer counting | 4 |
| | Fruit shape | FrShp | | 1, Globose 2, conical globose; 3, wide conical globose; 4, flat; 5, flat globose; 6, conical; 7, narrow conical; 8, conical truncate; 9 ellipsoidal; 10, conical ellipsoidal; 11, oblong; 12, conical oblong; 13, asymmetric | 1, 2, 3 |
| | Over colour | FrUpCol | | 0, without over colour (no cheeks); 1, white; 2, yellowish; 3, yellow; 4, orange; 5, reddish-pink; 6, red; 7, purple; 9, brown | 1, 2, 3 |
| | Vitrification (or watercore) | FrVitr | | 0, absent; 1, present | 4 |
| | Fruit width (mm) | FrW | C | – | 2 |
| | Bloom of skin | FrWax | O | 1; weak; 2, moderate; 3, intense | 2, 3 |
| | Peduncle width (mm) | FrWP | C | – | 2, 3 |

(LeArea) with leaf width (LeW; 0.91), petal length (FlPetL) with petal width (FlPetW; 0.81) and fruit weight (FrM) with fruit width (FrW; 0.92) and fruit length (FrL; 0.71).

Some significant correlations were negative, such as the one found between the fruit peduncle length (FrPedL) and peduncle width (FrWP; -0.68, Figure 2b), and the length of the apical bud (ShL) with peduncle width (FrWP; -0.63). Correlations between two different organs were also positive, for example between the fruit peduncle length (FrPedL) and flower pedicel length (FlPedL; 0.86) and with length of the apical bud (ShL; 0.72). We found some negative significant correlations, such as the ones that involved the flower pedicel length (FlPedL) with peduncle width (FrWP; -0.63) and with the length of the apical bud (ShL; -0.55).

## Multivariate analysis

The two multivariate analyses showed that the morphological diversity of apple references was lower than the one from old cultivars. Results from PCoA (Table 5) showed that the first three PCos explained 31% of the variability assessed, with seven coordinates necessary to reach 50% variability. By coordinates, PCo 1 represented 14% of the variance, PCo 2 10%, and PCo 3 7%. The most important correlations in PCo 1 were negative: outstanding fruit weight (FrM; -0.76), fruit length (FrL; -0.75), leaf area (LeArea; -0.75), peduncular cavity width (FrSCW;-0.74) and leaf width (LeW; -0.71). In PCo 2, the balance between negative and positive correlations was similar, with important correlations for peduncle width (FrWP; -0.77), peduncular cavity width (FrSCW; -0.740), leaf width (LeW; -0.71), length of the apical bud (ShL; 0.70), flower pedicel length (FlPedL; 0.67) and lenticels (ShLent; 0.62). In PCo 3, the strongest positive correlations involved the maximum width of the leaf blade (LeMWL; 0.65), leaf length (LeL; 0.54), and apical shoot shape (ShShp; 0.49). Among the negative correlations in PCo 3, most noticeable were the width of the apical bud (ShW; -0.51) and over colour (FrUpCol; -0.48). In the plot that represents apple individuals in PCo 1 and PCo 2 (24% of variance), we detected that clonal replicates of references were closely grouped while old apple cultivars appeared separated from apple references, but no further structure was detected in the traditional pool (Figure 4). Despite their low structure in the plot, 'Agridulce' (MAMO1 and MAMO2) and 'Hojancas' (MHHO1) were clearly distinct from the rest of the cultivars thanks to quantitative descriptors such as leaf area (LeArea), fruit weight (FrM) and fruit width (FrW). Finally, 'Verde Doncella' with VDCA, VDHO and VDTI clustered together.

In the cluster analysis, the two 'Agridulce' individuals (MAMO1 and MAMO2) split off very early from the rest of the individuals (Figure 5). They were followed by a 'Rojillo temprano' (RTEV1), two 'Pero de Aragón' (PAPR2 and PAPU1), a 'Camueso tardío' (CTEP1), a 'Pero gordo' (PGHI2), and a 'Temprano' (TEPI1). The rest of the dendrogram was structured in three clusters. The first cluster was composed of two 'peros' (PPPU2 and PRBU2), two 'reineta' ('Reineta Blanca' and REPR2), 'Hojancas' (MHHO1), and one 'Camueso' (CAPR1). The second cluster contained 28 individuals, including the two reference 'Verde Doncella' (VDHO2). Here also appeared a pool consisting of the cultivar 'Esperiega' (ESPU1, ESPU2), 'de Chapa' (CHVA1, CHCA1, and CHCA2) and 'Pepita de melón' (PECA2, PEVA2), as well as a group that nested the cultivars 'Rojo' and 'Rojillo' (RJHO1, RJPU1, RJPU2, and RJPR2). This second cluster also included a 'Camuesa' (CABU2) and the three remaining individuals of 'Pepita de melón' (PEHI1, PEHI2, and PECA1). The third cluster was composed by references ('Fuji', 'Gala', 'Golden', and 'Granny Smith'), as well as one 'Reineta' (REPR1) and two 'Pero de Aragón' (PAPR1 and PAPU2).

# Discussion

## Phenotypic diversity

The results of the present morphological analysis of shoots, leaves and flowers were similar to other morphological studies (Božović *et al*, 2015; Hassan *et al*, 2017). Fruit size measures were highly variable, especially fruit length (FrL), fruit weight (FrM) and fruit width (FrW). Although averages of these three descriptors were similar to results reported by other works, the registered range was larger than those described in studies by Mratinić *et al* (2011), Özrenk *et al* (2011), Király *et al* (2012), Božović *et al* (2015), Pérez-Romero *et al* (2015) and Posadas-Herrera *et al* (2018).

According to Pereira-Lorenzo *et al* (2003), Gaši *et al* (2011), Božović *et al* (2015) and Pérez-Romero *et al* (2015), quantitative descriptors related to apple cavities (FrSCL, FrSCW, FrCCL, FrCCW) are informative because they are genetically controlled. Those four descriptors detected statistically significant differences among studied cultivars, but such significance may be due to their correlation to fruit length (FrL) and fruit width (FrW). In fact, the depth of the calyx cavity (FrCCDep), a qualitative descriptor that relativizes the calyx cavity width and the calyx cavity length with the global size of the fruit, showed an intermediate diversity, since the depth of this cavity was found to be 'intermediate' in many of our studied old apple cultivars, similarly to Božović *et al* (2015). Furthermore, no 'external' cavity was found neither in our collections nor in Božović *et al* (2015), being 'Sisa' the unique old apple cultivar reported with such characteristic (Zovko *et al*, 2010).

Related to fruit shape, our results agree with Božović *et al* (2015), since their predominant shapes were 'conical' and 'obloid', with some presence of 'ellipsoidal' and 'globose'. Nevertheless, not all studies reported conical shapes as dominant, as Pırlak *et al* (2003) found that the 'flat', 'conical' and 'spherical' shapes were all abundant. Similarly, Hassan *et al* (2017) reported predominantly the shapes 'globose', 'obloid'

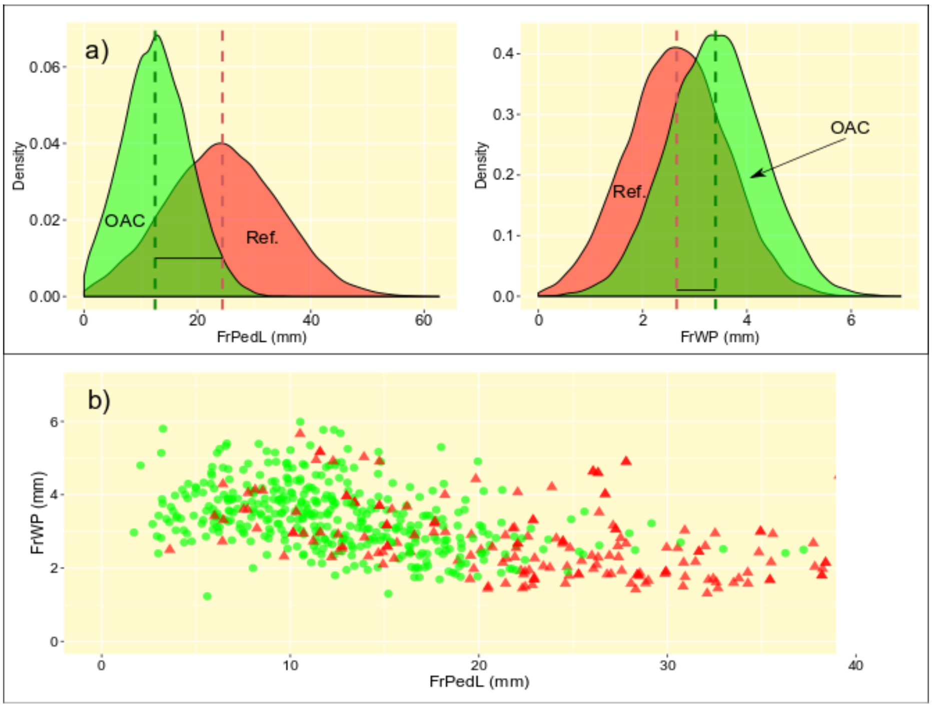

**Figure 2.** Diversity in the morphological descriptors of fruit peduncle length (FrPedL) and peduncle width (FrWP): a) density model of fruit peduncle length (FrPedL; left) and peduncle width (FrWP; right) from each mean and variance. Curves were computed with the rnorm(20000, $\bar{x}$, $s^2$) code from R. Red, reference cultivars (Ref.); Green, old apple cultivars (OAC); b) significant negative correlation between both continuous descriptors. Red triangles, reference cultivars; green circles, old apple cultivars.

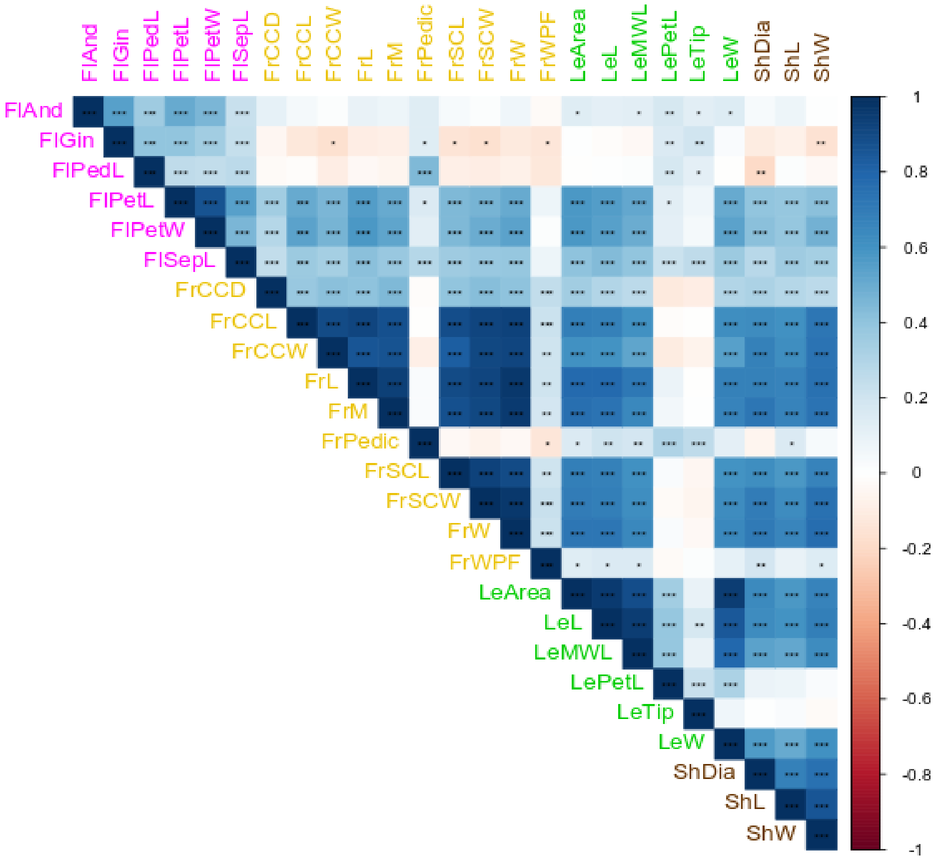

**Figure 3.** Correlation matrix plot for continuous and ordinal apple descriptors assessed on old traditional apple cultivars. Shoot colour (ShCol) could not be computed due to a lack of variation at a tree level. Descriptors are coded as in Table 3. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; no asterisk, non-significant correlation.

**Table 4.** Central statistics of the 32 significant descriptors analyzed by type of cultivar (references and old apple cultivars). Descriptor codes are as used in Table 3. C, continuous; D, discrete; N, nominal; O, ordinal; Ref., reference cultivar; OAC, old apple cultivar; Sig., significance; ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05.

| Organ | Descriptor | Type | Mean | | Median | | Mode | | P-value | Sig. | Cohen's d |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ref. | OAC | Ref. | OAC | Ref. | OAC | | | |
| Shoot | ShCol | N | | | | | Brown | | $2.69 \times 10^{-5}$ | *** | - |
| | ShDia | C | 4.95±0.06 | 6.81±0.07 | | | | | $8.74 \times 10^{-94}$ | *** | Large |
| | ShL | C | 6.75±0.08 | 5.41±0.05 | | | | | $1.74 \times 10^{-41}$ | *** | Large |
| | ShW | C | 4.54±0.05 | 4.13±0.04 | | | | | $6.63 \times 10^{-14}$ | *** | Medium |
| Leaf | LeFold | N | | | | | Folded | | 0.003 | ** | - |
| | LeMWL | C | 4.48±0.17 | 4.72±0.07 | | | | | 0.013 | * | Small |
| | LeTip | C | 0.62±0.03 | 0.68±0.01 | | | | | 0.006 | ** | Small |
| Flower | FlAnd | C | 10.46±0.53 | 9.96±0.24 | | | | | 0.001 | ** | Small |
| | FlPedL | C | 17.43±0.93 | 8.66±0.25 | | | | | $2.20 \times 10^{-16}$ | *** | Large |
| | FlPetL | C | 23.28±1.18 | 21.85±0.52 | | | | | 0.001 | ** | Small |
| | FlPetN | D | | | Five | | | | 0 | *** | - |
| | FlPetW | C | 16.27±0.82 | 15.58±0.38 | | | | | 0.023 | * | Small |
| | FlSepL | C | 7.54±0.39 | 7.01±0.17 | | | | | 0.014 | * | Small |
| | FlShp | N | | | | | Flat | | 0.034 | * | - |
| Fruit | FrCCD | C | 5.88±0.22 | 3.81±0.13 | | | | | $4.26 \times 10^{-24}$ | *** | Large |
| | FrCCDep | O | | | Strong | Intermediate | | | 0.006 | ** | - |
| | FrCCL | C | 11.93±0.38 | 10.44±0.26 | | | | | $3.47 \times 10^{-10}$ | *** | Medium |
| | FrCCW | C | 24.82±0.76 | 24±0.57 | | | | | 0.016 | * | Negligible |
| | FrDisCol | O | | | Blurred | Uniform | | | 0.012 | * | - |
| | FrGroCol | N | | | | | Yellow | | $2.46 \times 10^{-10}$ | *** | - |
| | FrL | C | 57.35±1.71 | 55.57±1.29 | | | | | 0.003 | ** | Small |
| | FrLoc | D | | | Five | | | | $2.20 \times 10^{-16}$ | *** | - |
| | FrM | C | 131.87±4.47 | 124.37±3.27 | | | | | 0.028 | * | Negligible |
| | FrMCol | N | | | | | Greenish-yellow | | $6.12 \times 10^{-05}$ | *** | - |
| | FrPedL | C | 24.36±1.01 | 12.54±0.38 | | | | | $5.57 \times 10^{-37}$ | *** | Large |
| | FrRS | N | | | | | Presence | | 0.017 | * | - |
| | FrSCL | C | 2.42±0.47 | 3.64±0.29 | | | | | $7.67 \times 10^{-20}$ | *** | Medium |
| | FrSCW | C | 29.71±0.91 | 27.94±0.67 | | | | | $2.64 \times 10^{-05}$ | *** | Small |
| | FrUpCol | N | | | | | Absent | | $2.69 \times 10^{-06}$ | *** | - |
| | FrVitr | N | | | | | Absent | | - | *** | - |
| | FrWax | O | | | Very intense | | | | 0.011 | * | - |
| | FrWP | C | 2.65±0.10 | 3.42±0.09 | | | | | $7.37 \times 10^{-18}$ | *** | Large |

and ’ellipsoid’, finding only one conical old apple cultivar. Although a great morphological diversity in apple shapes is generally reported, probably some of these differences could be attributed to the high subjectivity of this descriptor (Currie *et al*, 2000).

Discrepancies in shape should have affected other descriptors such as flattening (FrFlat), but our results were similar to those reported in other collections (Božović *et al*, 2015; Salkić *et al*, 2017), leading us to consider that old apple cultivars are, in general, wider than longer. We also support this conclusion, as although fruit length (FrL) was larger in references, no significant differences between types of cultivars were found in fruit width (FrW).

Apple skin colour is supposed to be a distinctive trait in apple cultivars. The fruit ground colour (FrGroCol) ranged in our collection from ’greenish white’, ’green’, ’greenish yellow’ to ’yellow’, similarly to Božović *et al* (2015), Mišić (2002) and Zovko *et al* (2010). Regarding over colour (FrUpCol), apples herein described were mainly cheekless, as the most common level for this descriptor was ’absent’. Therefore, the studied old apple cultivars have a more uniform colour than those reported in the collection of Božović *et al* (2015) and Šebek (2013). Concerning our cheeked apples, the most common colour was ’red’, as in Mratinić *et al* (2012) and Božović *et al* (2015).

## Correlations

Many of the significant correlations computed were logical, supporting the botanical description of the apple tree (Terpó, 1981; Aedo *et al*, 1998) and agree

**Table 5.** Correlation coefficients between the first three principal coordinates (PCo) and the morphological descriptors, abbreviated as in Table 3. Eigenvalues below -0.5 and above 0.5 are highlighted in bold.

| Descriptor | PCo 1 | PCo 2 | PCo 3 | Descriptor | PCo 1 | PCo 2 | PCo 3 |
|---|---|---|---|---|---|---|---|
| FlAnd | **-0.59** | 0.08 | -0.04 | FrRib | 0.12 | -0.09 | -0.15 |
| FlDom | -0.26 | -0.45 | -0.06 | FrRS | 0.03 | -0.21 | -0.21 |
| FlGin | **-0.66** | -0.34 | -0.03 | FrSCL | **-0.68** | 0.45 | 0.07 |
| FlPedCol | -0.13 | 0.39 | -0.22 | FrSCW | **-0.74** | -0.26 | -0.08 |
| FlPedL | **-0.61** | **0.51** | -0.30 | FrSeed | 0.10 | **0.55** | -0.14 |
| FlPetCol | -0.13 | 0.23 | 0.14 | FrShp | -0.26 | **0.53** | 0.25 |
| FlPetL | **-0.60** | -0.01 | 0.06 | FrUpCol | 0.08 | 0.43 | -0.48 |
| FlPetN | 0.24 | -0.15 | 0.12 | FrVitr | 0.22 | -0.12 | 0.19 |
| FlPetW | **-0.69** | -0.22 | -0.05 | FrW | **-0.70** | -0.41 | -0.10 |
| FlPub | -0.01 | 0.07 | 0.21 | FrWax | -0.14 | -0.12 | 0.22 |
| FlRPP | -0.12 | -0.34 | -0.07 | FrWP | 0.11 | **-0.77** | -0.03 |
| FlSepL | **-0.68** | 0.04 | 0.14 | LeArea | **-0.75** | -0.19 | 0.29 |
| FlShp | -0.13 | -0.05 | 0.38 | LeAsim | -0.03 | 0.10 | 0.24 |
| FlSti | -0.30 | -0.03 | -0.30 | LeBas | -0.18 | -0.44 | -0.29 |
| FlTyp | -0.16 | -0.14 | -0.23 | LeCol | -0.22 | -0.26 | 0.15 |
| FlWeld | 0.04 | 0.07 | 0.29 | LeEdg | -0.40 | -0.09 | 0.24 |
| FrCCD | **-0.55** | 0.07 | -0.44 | LeFold | -0.09 | -0.06 | -0.004 |
| FrCCDep | -0.15 | 0.18 | 0.20 | LeL | **-0.54** | 0.09 | **0.54** |
| FrCCL | **-0.63** | 0.12 | -0.05 | LeMWL | -0.43 | 0.15 | **0.65** |
| FrCCShp | -0.18 | -0.02 | 0.10 | LePetL | -0.04 | 0.24 | 0.38 |
| FrCCW | **-0.52** | -0.31 | -0.15 | LePub | 0.19 | -0.39 | -0.16 |
| FrDisCol | 0.10 | 0.37 | -0.37 | LeShp | -0.03 | 0.21 | 0.44 |
| FrEye | 0.08 | -0.04 | -0.29 | LeSti | -0.49 | -0.08 | -0.47 |
| FrFlat | 0.12 | **-0.64** | -0.36 | LeTip | -0.13 | -0.22 | 0.17 |
| FrGroCol | 0.02 | 0.18 | 0.40 | LeTipShp | -0.17 | -0.21 | -0.20 |
| FrHea | -0.26 | -0.25 | -0.13 | LeW | **-0.71** | -0.31 | 0.09 |
| FrL | **-0.75** | 0.06 | 0.17 | ShCol | 0.29 | -0.05 | 0.18 |
| FrLoc | -0.07 | -0.07 | 0.16 | ShDia | 0.10 | **-0.59** | 0.36 |
| FrM | **-0.76** | -0.33 | -0.03 | ShL | -0.29 | **0.70** | -0.30 |
| FrMCol | -0.20 | 0.46 | 0.09 | ShLent | -0.23 | **0.62** | -0.19 |
| FrPedL | **-0.57** | **0.67** | -0.14 | ShShp | 0.03 | -0.13 | 0.49 |
| FrRCC | -0.21 | -0.35 | -0.24 | ShSPub | 0.16 | 0.19 | 0.01 |
| FrRF | 0.03 | 0.07 | -0.14 | ShW | -0.12 | -0.002 | **-0.51** |

with Ganopoulos *et al* (2018) and Farrokhi *et al* (2013), as the strongest correlations occurred among quantitative descriptors and in the same organ. Also, we obtained a strong correlation between leaf area and descriptors related to fruit size, as mentioned by Migicovsky *et al* (2018).

A significant correlation, important in breeding, was detected between the fruit peduncle length (FrPedL) and peduncle width (FrWP). Salkić *et al* (2017) consider that short peduncles are not desirables. We agree with these authors, as probably short and wide peduncles may suffer from lack of growth space, causing some injuries to the fruits and decreasing their commercial quality (Figure 6). Although this behaviour was not deeply studied in this morphological characterization, our observations suggest that correlation analyses help breeders select descriptors that have a lever effect on genetic improvement (Chen and Lübberstedt, 2010; Ganopoulos *et al*, 2018).

## Multivariate analysis

The PCoA decomposed the variance of the morphological descriptors analyzed. The sedimentation rate along the PCo is almost identical to the Spanish study of Pereira-Lorenzo *et al* (2003), but it was slower compared to other collections (Gaši *et al*, 2011; Božović *et al*, 2015; Ganopoulos *et al*, 2018). For example, our PCo 1 only gathers 14% of the variance, whereas PCo 1 from Gaši *et al* (2011) gathered almost 30% with 18 descriptors. A slow sedimentation rate does not necessarily indicate that our collection is more diverse than others. Probably, the greater the number of descriptors and accessions analyzed, the slower the sedimentation process tends to be. In fact, our study analyzed the largest number of descriptors, followed by Pereira-

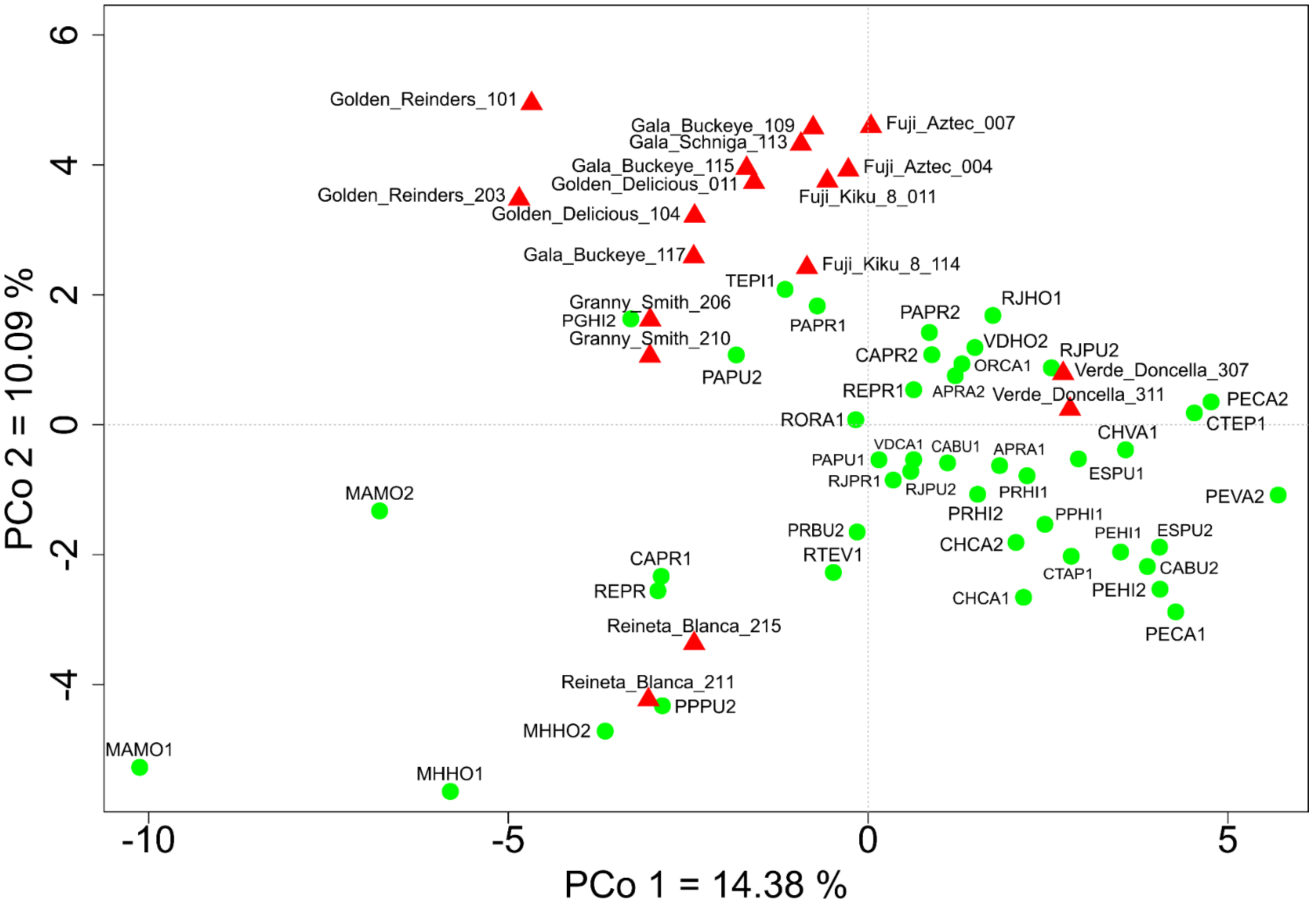

**Figure 4.** Plot between PCo 1 and PCo 2 for all 67 old apple cultivar individuals and 18 reference individuals from 'Fuji', 'Gala', 'Golden', Granny Smith', 'Reineta Blanca' and 'Verde Doncella' based on morphological descriptors. Red triangles, reference cultivars; green circles, old apple cultivars.

Lorenzo *et al* (2003), who used 49 descriptors in 350 trees.

Regarding PCo eigenvalues, fruit descriptors were usually predominant, especially fruit weight and size. Our results were very similar to other studies, such as Božović *et al* (2015), Gaši *et al* (2011), Farrokhi *et al* (2013) and Pereira-Lorenzo *et al* (2003). Some results reported from other collections do not totally agree with ours. This is the case of Ganopoulos *et al* (2018) where they highlighted other types of fruit characteristics, such as the number of loculi (FrLoc), pulp colour (FrMCol), russeting on fruit faces (FrRF) and calyx opening diameter (FrCCD). The importance of fruit descriptors in the total variance can be explained because it is the organ where selection is performed (Šebek, 2013; Božović *et al*, 2015; Dar *et al*, 2015; Pérez-Romero *et al*, 2015; Salkić *et al*, 2017; Posadas-Herrera *et al*, 2018).

Results from PCoA are consistent with the cluster analysis, as both detect a high morphological diversity in the old apple cultivars. Differences may be due to the lower number of references analyzed, as 'Gala', 'Golden', 'Fuji', and 'Granny Smith' are few, but they are the most widespread varieties in Spain and represent almost the whole apple production (Iglesias *et al*, 2009). In addition, we could discriminate references from old apple cultivars and we found an early separation of 'Agridulce', the presence of two clusters composed of 'de Chapa', 'Esperiega', 'Pepita', and 'Camuesa' and 'Pepita', respectively, and the inclusion of 'Verde Doncella' within old apple cultivars. The closeness of 'Verde Doncella' to other traditional apples is congruent with its breeding history, as this cultivar is autochthonous to Spain (Iglesias *et al*, 2009; Urrestarazu *et al*, 2012; Pina *et al*, 2014).

We did not find further classification in old apple cultivars, as for instance we could not separate 'camuesas' from 'peros'. A clear separation of references from old traditional cultivars without a strong structure has been reported before (Božović *et al*, 2015; Ganopoulos *et al*, 2018), indicating that old apple germplasm is different from references, in contrast with Posadas-Herrera *et al* (2018) and Király *et al* (2015), who could not differentiate between both type of cultivars. Regarding descriptors, no single descriptor can distinguish among cultivars, but some of them may be informative and should be considered in cultivar classification, such as the flower pedicel length (FlPedL), depth of the calyx cavity (FrCCDep), fruit peduncle length (FrPedL) and peduncle width (FrWP).

Difficulties in old apple cultivar classification are probably due to boundaries among old apple cultivars being more diffuse than in modern cultivars, whose

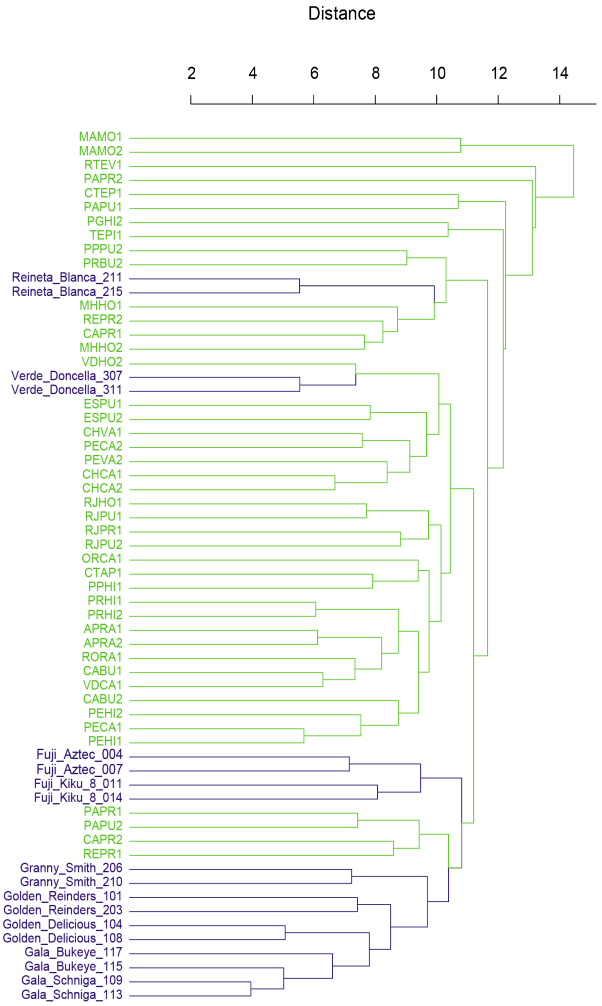

**Figure 5.** Dendrogram constructed based on unweighted pair group method with arithmetic mean (UPGMA) cluster analysis calculated with the Euclidean distance for all 67 old apple individuals and 18 reference individuals from 'Fuji', 'Gala', 'Golden', Granny Smith', 'Reineta Blanca' and 'Verde Doncella' based on 67 morphological descriptors. Purple, references; green, old apple cultivars. Accession and individuals are numbered as in Table 1 and Table 2.

genealogy is always known (Noiton and Alspach, 1996; Laurens, 1999). For example, 'Crisp Pink' (Pink Lady) derives genetically from 'Golden' and 'Lady Williams' (Iglesias *et al*, 2009). The lack of information on the origin of old apple cultivars is also accompanied by homonymies. Probably, any morphological or sensory trait may be enough to link two cultivars not necessarily parented. For example, flattened apples may evoke a 'reineta', as Martinelli *et al* (2008) found that 'Reineta grigia' was not really a 'reineta'. Something similar was reported by Mratinić and Fotirić (2012), who informed that some accessions named 'Šerbetka' (which means 'too sweet') were later clustered separately.

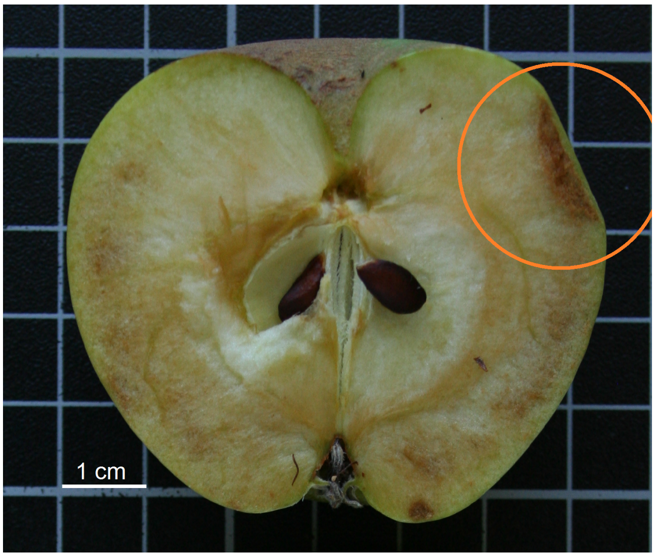

**Figure 6.** Apple damaged due to lack of growth space, possibly triggered by the presence of a short or wide peduncle.

## Support of SSR molecular data in old apple cultivars identities

Morphological and DNA characterization are two complementary techniques, although conclusions about diversity and parental analysis are more robust with molecular analysis (Király *et al*, 2012). Therefore, we recently published a molecular analysis of the same accessions in this study based on 13 microsatellites (Arnal *et al*, 2020) in which we reported germplasm with breeding potential that should be further considered. Interestingly, PCoA and clustering analysis between the two studies are very comparable, as both clearly differentiate references from old apple cultivars and in general no further groups could be defined.

Our two studies pointed out two singular old apple cultivars ('Agridulce' and 'Hojancas'), which may derive from 'reineta'. In the morphological study, these two old cultivars (especially 'Agridulce') showed traits that differentiated them better than microsatellites, as their differential morphology allowed us to segregate them even earlier than in the molecular study. In consequence, they could be considered for *ex situ* conservation and further studies. Moreover, both methodologies closely related 'Camuesa', 'de Chapa' and 'Pepita'. Also, 'Rojillo' and 'Rojo', which seemed synonyms in our molecular analysis, showed similar morphological profiles, as they appeared together in the multivariate analysis. In contrast, the present morphological study does not gather all triploids in a cluster, nor detect the two groups of 'peros' found with microsatellites, as PGHI2, PAPU1, and PAPU2 fell each one in different clusters and one of them (PAPU2) was closely related to PAPR1.

In conclusion: 1) a great morphological diversity of old apple cultivars was detected in rural areas of central Spain; 2) the presented results confirm our previous analysis with microsatellites; 3) both approaches will help to better understand Spanish and global apple genetic resources; 4) the described collection contains two old apple cultivars ('Agridulce' and 'Hojancas') with a very distinct morphology, which may deserve further studies (such as flowering and ripening times, productivity, resistance to pests, etc.); 5) two old apple cultivars ('de Chapa' and 'Pepita') may be a variation of 'Camuesa', and 6) the cultivars 'Rojillo' and 'Rojo' are likely synonyms.

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## Author contributions

A. Arnal, J. Tardío and A. Lázaro designed the research. A. Arnal sampled the individuals with the help of J. Tardío and A. Lázaro. A. Arnal adapted the R Code and analyzed the data. A. Arnal, J. Tardío and A. Lázaro wrote the manuscript. A. Arnal, J. Tardío and A. Lázaro revised the manuscript.

## Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Nutritional and phenotypic variations among newly selected African eggplant (*Solanum aethiopicum* L.)

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**Abstract:** African eggplant (*Solanum aethiopicum* L.) is an important but underutilized leafy and fruit vegetable. Systematic characterization of available eggplant accessions for morphological and nutritional traits is paramount to their genetic improvement. This study characterized the diversity among selected *S. aethiopicum* accessions from Nigeria to identify promising genotypes for future eggplant breeding activities in the region. Twenty new purified African eggplant accessions collected from farmers' fields were characterized using morphological and nutritional descriptors. The accessions varied significantly in qualitative, quantitative and nutritional parameters. Top performers for selected yield-contributing traits and nutritional parameters were NHEPA54, NHEPA39-1, NHEAP10, NHEPA10, NHEPA1, NHEPA56, NHEPA23 for vitamin C, iron, calcium, days to flowering, number of branches, plant height at maturity and number of fruits per plant respectively. The first four principal components accounted for 72.42% of total variability. The first principal component with the largest variation (28.77%) was loaded with number of branches, plant height at maturity, number of fruits per cluster, number of fruits per plant, and fruit width. A significant positive association was exhibited between iron and yield-increasing traits such as number of fruits per plant (r = 0.532) and number of fruits per cluster (r = 0.551). Plant height at maturity positively correlated with vitamin C (r = 0.492) indicating predictable success in selecting top-performing eggplant genotypes combining high-yield potential and nutritional content. Top-performing eggplant genotypes identified in this study could be deployed as donors for a hybridization programme to develop new eggplant varieties with higher yield potential and improved nutritional quality.

**Keywords:** Diversity, accessions, breeding, principal component, variability, correlation

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## Introduction

The African eggplant (*Solanum aethiopicum* L.) is one of the important indigenous fruit vegetables widely grown and consumed across most regions of tropical Africa. It is the third most consumed fruit vegetable after tomato, pepper and onion both in quantity and value in the region (Osei *et al*, 2010). Mature fruits of African eggplant are eaten fresh, with fried groundnuts or used to prepare special delicacies called 'African salad' in southern Nigeria (Igwe *et al*, 2003). A significant increase has been observed in its production across sub-Saharan Africa from 606,672 tonnes in 1994 to 2,079,920 tonnes in 2018 (FAO, IFAD, UNICEF, WFP and WHO, 2018).

Eggplant is considered amongst the healthiest fruit vegetables for its low calories and high concentration of various macro and micro minerals essential for maintaining good health (Docimo *et al*, 2016). They are rich sources of fibres, vitamins (A, B1, B2, B6, B12, C, D), magnesium, calcium and iron even though potassium is the most abundant mineral ranging from 200 to 600mg/100g of fresh matter (Kowalski *et al*, 2003; Nyadanu and Lowor, 2015; Nimenibo and Omotayo, 2019). The crop has been reported to play an essential role in meeting the nutritional needs of the Igbo-speaking tribe in southern Nigeria

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where consumption of fresh fruits might be of great benefit to glaucoma patients and to prevent heart disease (Igwe *et al*, 2003; Denkyirah, 2013). *S. aethiopicum* is used in the management and treatment of diarrhoea and hypertension (Adeniji and Aloyce, 2012). The high yield and nutritive value of the leaves and fruits complemented with resistance to pests and diseases endear the crop to consumers, farmers and researchers (Bonsu *et al*, 1998; Toppino *et al*, 2008; Taher *et al*, 2019).

Eggplants belong to the Solanaceae family, which encompasses three closely related cultivated species endemic to Afro-Eurasia. Two sections exist at the subgenus level, namely *Melongena* and *Oliganthes* sections. The section *Melongena* comprises two species (*S. melongena* and *S. macrocarpon*) while the *Oliganthes* group has only one species (*S. aethiopicum*). *S. aethiopicum* has been grouped into four different ecotypes or cultivars including Aculetum, Gilo, Kumba and Shum groups as revealed by similarities in genotypic characterization through varied phenotypes (Sharmin *et al*, 2011). Aculetum is mostly used as ornamental, Gilo is used for its fruits, Kumba is for both fruits and leaves while Shum is used for its leaves (Lester and Daunay, 2003).

Consumer preferences for an African eggplant cultivar are based on a number of traits including fruit size, form, fruit colour and taste (sweet or bitter). Morphological characterization using conventional descriptors has proved useful for describing and establishing relationships among cultivar groups and accessions in scarlet eggplants (Adeniji *et al*, 2013). The enormous morphological variability present in the eggplant family, despite being characterized by a narrow genetic base, might be attributed to new segregants emanating from natural hybridization and backcrossing (Meyer *et al*, 2012).

Despite their socioeconomic significance and their role in meeting the nutritional needs of the ever-increasing population across sub-Saharan Africa, these heirloom and indigenous adapted cultivars are becoming less popular, and the efforts to improve them for traits of interest to farmers and end-users are scarce (Bationo-Kando *et al*, 2015).

Continuous planting and selection of many diverse cultivars of *S. aethiopicum* by small-scale farmers as well as the existence of germplasm collections have helped to conserve the majority of desired traits within families over the years. The long period of selection by these poorly resourced farmers has resulted in a number of landraces exhibiting different variants with unique traits such as earliness, colour, size and taste. In essence, the African eggplant has long been neglected by formal crop improvement programmes except in breeding programmes where it is used as a source of specific traits. Furthermore, they are considered neglected and underutilized crops since their nutritional and economic potentials are mostly underexploited (Padulosi *et al*, 2019).

Systematic characterization of African eggplant accessions using morphological and nutritional traits is an important prerequisite toward their conservation and use in further studies and genetic improvement in the region (AVRDC, 2003). Unfortunately, minimal efforts have been directed to identify and select promising genotypes with a good combination of desired agronomic and nutritional qualities that could be used as parental materials for hybridization or released as new open-pollinated varieties. The process of germplasm characterization and trait screening to identify and select desired gene combinations can be challenging.

This study characterized newly selected and purified eggplant accessions collected in Nigeria to ascertain their mineral composition and to identify promising elite lines with the best combination of desired agronomic traits that could further be deployed for eggplant genetic improvement programmes.

## Materials and methods

### Experimental materials

Twenty new eggplant accessions (Table 1) were selected from the 2019 characterization of germplasm collections from farmers' fields across the south-west and north-central regions of Nigeria based on observable traits under field conditions. These materials have gone through two cycles of selection and selfing.

### Experimental design and conditions

The experiment was conducted at the experimental field of the National Horticultural Research Institute (NIHORT), Ibadan, Oyo State, Nigeria. NIHORT is located in the humid forest-savannah transition zone (210m above sea level, 7° 30′ N, 3° 54′ E) with a bimodal annual rainfall pattern of about 120–128 rainy days amounting to 1,200–1,400mm. Pan evaporation is between 1,550–1,600mm. The wet season is from March through October and the dry season from November through February with an annual maximum temperature ranging between 27°C and 34°C and an annual minimum temperature of 20–23°C (Ogungbenro and Morakinyo, 2014).

The eggplant accessions were first raised in a nursery and transplanted to the field after 35 days using a randomized complete block design with three replications. The plot size was 2 x 1m with a spacing of 0.5 x 0.6m between and within rows having 10 plants per plot. Manual weeding was carried out to reduce the competitiveness of soil nutrients. Fertilizer was not applied while insecticides (Cypermethrin) were used at the rate of 200ml/20l of water when needed to reduce damage caused by insects.

### Phenotypic characterization

Phenotypic data collection was carried out on 5 uniform tagged plants out of 10 plants from each plot for the 20 accessions using 12 quantitative (number of branches, number of days to flowering, number of days to 50%

flowering, plant height at maturity, number of fruits per cluster, number of harvested fruits, weight of harvested fruits, petiole length, fruit length, fruit width, stem girth, pedicel length) and 10 qualitative traits (fruit colour, stem colour, petiole colour, leaf hairs, sepal colour, fruit colour, fruit shape, fruit position, fruit-end shape, presence/ absence of stripes). Physiochemical variables were iron, vitamin C and calcium, using the descriptor list for eggplant by the International Board for Plant Genetic Resources (IBPGR, 1990).

**Table 1.** Status, collection source and states of *Solanum aethiopicum* L. accessions collected in Nigeria

| | Accessions | Status | Source | States |
|---|---|---|---|---|
| 1 | NHEPA01 | Farmers cultivar | Local Market | Ogun |
| 2 | NHEPA03 | Farmers cultivar | Local Market | Ogun |
| 3 | NHEPA10 | Farmers cultivar | Local Market | Ogun |
| 4 | NHEPA12 | Farmers cultivar | Farmers | Kogi |
| 5 | NHEPA17 | Farmers cultivar | Farmers | Kogi |
| 6 | NHEPA19 | Farmers cultivar | Farmers | Kogi |
| 7 | NHEPA23 | Farmers cultivar | Farmers | Kogi |
| 8 | NHEPA35 | Farmers cultivar | Farmers | Kogi |
| 9 | NHEPA36 | Farmers cultivar | Farmers | Kogi |
| 10 | NHEPA38 | Farmers cultivar | Farmers | Kogi |
| 11 | NHEPA39-1 | Farmers cultivar | Farmers | Kogi |
| 12 | NHEPA39-2 | Farmers cultivar | Farmers | Kogi |
| 13 | NHEPA39-3 | Farmers cultivar | Farmers | Kogi |
| 14 | NHEPA51 | Farmers cultivar | Farmers | Kaduna |
| 15 | NHEPA52 | Farmers cultivar | Farmers | Kaduna |
| 16 | NHEPA53 | Farmers cultivar | Farmers | Kaduna |
| 17 | NHEPA54 | Farmers cultivar | Farmers | Kaduna |
| 18 | NHEPA55 | Farmers cultivar | Farmers | Kaduna |
| 19 | NHEPA56 | Farmers cultivar | Farmers | Kaduna |
| 20 | YALO | Farmers cultivar | Green seed company | Oyo |

## Calcium, iron and vitamin C determination

Fruit samples were dried in an oven at 600°C for 4 hours. Ashes and crucibles were previously decontaminated with a solution of 10% nitric acid at rest for a night and rinsed. Then, 10ml of 5% nitric acid was added to the sample, and this mixture was heated until complete dissolution of the ash which was then filtered. After the sample had reached room temperature, the solution was put into a 25ml volumetric flask and the volume supplemented with deionized water.

The determination of calcium and iron contents was performed according to AOAC METHOD 2005 using an atomic absorption spectrophotometer flame (BULKS SCIENTIFIC® model AA 240). Calibration curves for each element were plotted using standard mineral diluted with deionized water. All analyses were performed in triplicate; the results were expressed in milligrams per 100g (mg/100g) of sample on a dry basis.

The amount of vitamin C in analyzed samples was determined by titration using the method described by Mondal et al., (1995). About 0.5g of sample were soaked for 10 minutes in 40ml metaphosphoric acid-acetic acid (2%, w/v). The mixture was centrifuged at 3,000rpm for 20 minutes and the supernatant obtained was diluted and adjusted with 50ml of bi-distilled water. Ten (10)ml of this mixture was titrated to the endpoint with dichlorophenol-indophenol (DCPIP) 0.5g/l (AOAC. 1990).

## Statistical analysis

Analysis of variances (ANOVA) was calculated using Plant Breeding Tools (ver.1.1.0, http://bbi.irri.org/product) to determine significant variations in quantitative characters among the eggplant genotypes. The estimate of co-efficient of variation (CV) was calculated using the standard formulae (Burton, 1952) and expressed in percentage. Inter-species diversity pattern was analyzed through Ward's minimum variance while correlation, dendrogram clustering and principal components analysis (PCA) were carried out using STAR software.

## Results

The frequency distribution of qualitative traits observed in all 20 accessions is presented in Table 2. All the genotypes (100%) exhibited green stems, petioles and sepals. At the reproductive stage, 48.48% of the fruits had a white colour, 45.45% expressed lemon green while 3.03% were light green and 3.03% exhibited deep green fruit colour. Three prominent fruit shapes were observed: oval (51.51%), long (30.30%) and round (18.18%). All accessions exhibited perpendicular fruit position with 69.69% and 30.30% of the populations having pointed and flat ends respectively. The fruits of selected eggplant accessions are presented in Figure 1.

Table 3 lists the descriptive statistics measures of spread: mean, range, standard deviation and coefficient of variation (CV). The partitioning of the means revealed high significant variations for all traits at $P \leq 0.01$. For most traits, higher variations in terms of range and CV were observed in the nutritional data compared to the phenotypic data. The highest CV was recorded for calcium (43.91%) followed by average

**Table 2.** Qualitative traits of 20 *Solanum aethiopicum* accessions

| | Traits | Modality | Frequency (%) |
|---|---|---|---|
| 1 | Stem colour | Green | 100 |
| 2 | Petiole colour | Green | 100 |
| 3 | Leaf hairs | Very few | 100 |
| 4 | Sepal colour | Green | 100 |
| 5 | Fruit colour | White | 48.48 |
| | | Lemon green | 45.45 |
| | | Light green | 3.03 |
| | | Deep green | 3.03 |
| 6 | Fruit shape | Oval | 51.51 |
| | | Long | 30.3 |
| | | Round | 18.18 |
| 7 | Fruit position | Direct | 100 |
| 8 | Fruit end | Pointed | 69.69 |
| | | Flat | 30.3 |
| 9 | Stripe presence | Present | 100 |

**Figure 1.** Sample of fruits for selected eggplant accessions, where V04, V10, V12, V17, V19, V24, V29 and V35 represent NHEPA4, NHEPA10, NHEPA12, NHEPA17, NHEPA19, NHEPA24, NHEPA29 and NHEPA35 respectively.

number of fruits per plant (37.13%) and average yield per plant (32.21%). The top performers for selected yield-contributing traits and nutritional parameters are NHEPA54, NHEPA39-1, NHEPA10, NHEPA10, NHEPA1, NHEPA56, NHEPA23, for vitamin C, iron, calcium, days to flowering, number of branches, plant height at maturity and number of fruits per plant, respectively, while NHEPA54 was outstanding for high yield potential and vitamin C content. Accession NHEPA10 was the top performer for calcium and days to flowering while average number of fruits per cluster had NHEPA17, NHEPA19, NHEPA23 as the top performers.

Principal component analysis (PCA), which is a statistical technique used to emphasize variation and bring out strong patterns in data sets, was performed to show the traits that best contributed to the observed genetic variation. The eigenvalues and proportion of accounted variance for each variable are shown in Table 4. PC1 had an eigenvalue of 4.027 while PC2, PC3 and PC4 had eigenvalues of 2.889, 1.861 and 1.363, respectively. The first four principal component axes (PCA) accounted for 28.77%, 20.64%, 13.29%, 9.73% of the total variation individually and, cumulatively, 72.42% of the total variability while the first two PCs contributed 49.41% (Figure 2). The first PC axis, which accounted for the highest proportion (28.77%) of the variability, was dominated by traits with relatively high factor scores ($> 2.60$) corresponding to number of branches, plant height at maturity, number of fruits per cluster, number of fruits per plant, and fruit width. The second PC axis was dominated by days to flowering, fruit length, stem diameter, petiole length and pedicel length. Also, the third PC axis was dominated by average yield per plant, fruit length, stem diameter, petiole length and pedicel length while the fourth PC axis was dominated by days to flowering, number of branches, fruit length, fruit width and petiole length.

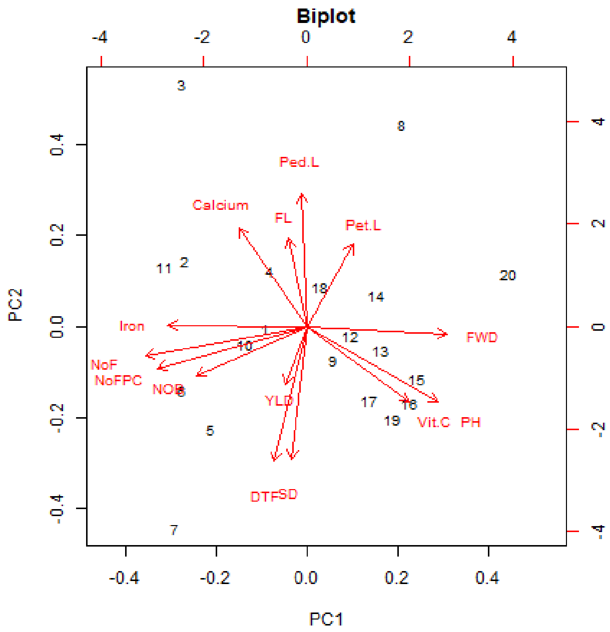

**Figure 2.** Distribution of 20 eggplant accessions for the first two principal components based on 14 quantitative traits. DTF, days to flowering; NoB, number of branches; PH, plant height at maturity; NoFPC, average number of fruits per cluster; NoF, average number of fruits per plant; YLD, average yield per plant; FL, fruit length; FWD, fruit width; SD, stem diameter; Pet.L, petiole length; Ped.L, pedicel length. Numbers 1-20 represent genotypes: 1, NHEPA01; 2, NHEPA03; 3, NHEPA10; 4, NHEPA12; 5, NHEPA17; 6, NHEPA19; 7, NHEPA23; 8, NHEPA35; 9, NHEPA36; 10, NHEPA38; 11, NHEPA39-1; 12, NHEPA39-2; 13, NHEPA39-3; 14, NHEPA51; 15, NHEPA52; 16, NHEPA53; 17, NHEPA54; 18, NHEPA55; 19, NHEPA56; 20, Yalo.

Correlations between pairs of quantitative variables are recorded in Table 5. There was no significant association between the nutritional parameters except for a negative moderate significant association between vitamin C and iron ($r = -0.50$, $P < 0.05$). Iron content correlated positively with number of branches ($r =$

**Table 3.** Nutritional and phenotypic data for relevant yield-contributing traits on 20 African eggplant accessions, including descriptive statistics measures of spread. Means vary significantly for all traits at P = 0.05. DTF, days to flowering; NoB, number of branches; PH, plant height at maturity; NoFPC, average number of fruits per cluster; NoF, average number of fruits per plant; YLD, average yield per plant; FL, fruit length; FWD, fruit width; StemD, stem diameter; Pet.L, petiole length; Ped.L, pedicel length; CV, coefficient of variation; SD, standard deviation.

| Nutritional data (mg/100g) | | | | Phenotypic data | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accessions** | **Vit.C** | **Iron** | **Calcium** | **DTF** | **NoB** | **PH (cm)** | **NoFPC** | **NoF** | **YLD (g)** | **FL (mm)** | **FWD (mm)** | **StemD (mm)** | **Pet.L (mm)** | **Ped.L (mm)** |
| NHEPA01 | 3.11 | 0.43 | 7.73 | 109.12 | 7.13 | 57.66 | 2.70 | 58.55 | 1370.10 | 65.90 | 37.37 | 19.50 | 23.08 | 20.01 |
| NHEPA03 | 2.02 | 0.47 | 4.47 | 104.00 | 5.79 | 48.58 | 4.98 | 74.61 | 1699.17 | 67.31 | 32.45 | 11.84 | 18.57 | 15.17 |
| NHEPA10 | 2.90 | 0.50 | 14.24 | 97.91 | 4.39 | 46.31 | 4.49 | 81.03 | 1320.72 | 58.54 | 22.73 | 10.44 | 19.96 | 19.06 |
| NHEPA12 | 2.90 | 0.25 | 8.88 | 103.04 | 5.01 | 54.58 | 4.49 | 81.68 | 1673.41 | 50.49 | 36.32 | 12.57 | 20.24 | 15.06 |
| NHEPA17 | 3.15 | 0.43 | 4.86 | 109.44 | 4.70 | 50.69 | 5.79 | 65.29 | 1726.48 | 45.37 | 32.66 | 17.58 | 14.72 | 14.86 |
| NHEPA19 | 3.99 | 0.50 | 10.69 | 106.56 | 5.48 | 52.47 | 5.79 | 76.38 | 1522.60 | 44.91 | 33.05 | 13.76 | 12.93 | 11.78 |
| NHEPA23 | 4.81 | 0.46 | 4.42 | 107.52 | 6.42 | 58.80 | 5.79 | 111.71 | 1731.05 | 45.62 | 26.84 | 19.12 | 13.96 | 12.58 |
| NHEPA35 | 3.78 | 0.25 | 7.53 | 100.79 | 3.92 | 60.09 | 3.34 | 36.87 | 1666.91 | 78.18 | 41.88 | 13.07 | 26.63 | 22.54 |
| NHEPA36 | 4.24 | 0.18 | 5.62 | 104.96 | 5.48 | 60.42 | 3.51 | 71.56 | 1747.09 | 57.16 | 31.49 | 15.55 | 18.09 | 15.84 |
| NHEPA38 | 3.15 | 0.42 | 4.10 | 104.00 | 6.26 | 57.34 | 4.16 | 78.14 | 1682.95 | 54.81 | 32.11 | 14.56 | 19.42 | 15.18 |
| NHEPA39-1 | 3.53 | 0.51 | 12.77 | 102.08 | 6.16 | 52.80 | 4.65 | 77.66 | 2019.68 | 76.44 | 30.42 | 16.02 | 11.51 | 17.77 |
| NHEPA39-2 | 3.92 | 0.43 | 6.85 | 102.72 | 5.84 | 62.52 | 4.11 | 45.54 | 1309.34 | 51.03 | 64.21 | 12.77 | 15.50 | 14.43 |
| NHEPA39-3 | 5.80 | 0.33 | 11.27 | 103.36 | 5.32 | 61.12 | 4.21 | 34.94 | 989.17 | 53.52 | 60.30 | 14.91 | 13.60 | 14.12 |
| NHEPA51 | 4.28 | 0.36 | 7.53 | 99.19 | 5.01 | 60.69 | 2.48 | 36.22 | 2035.71 | 47.56 | 38.84 | 12.26 | 15.06 | 14.04 |
| NHEPA52 | 4.84 | 0.21 | 5.95 | 105.28 | 4.80 | 60.80 | 3.13 | 35.26 | 1666.91 | 56.52 | 45.62 | 16.11 | 21.60 | 12.10 |
| NHEPA53 | 4.95 | 0.37 | 5.45 | 103.68 | 3.66 | 62.52 | 2.59 | 47.15 | 2420.55 | 54.42 | 51.03 | 18.11 | 16.01 | 14.26 |
| NHEPA54 | 6.01 | 0.25 | 3.31 | 104.00 | 4.70 | 58.53 | 3.56 | 53.89 | 2805.39 | 75.11 | 36.08 | 15.37 | 15.68 | 13.21 |
| NHEPA55 | 2.58 | 0.45 | 7.04 | 102.08 | 5.43 | 59.06 | 3.24 | 64.81 | 1522.60 | 56.94 | 59.61 | 14.07 | 21.02 | 13.78 |
| NHEPA56 | 3.50 | 0.36 | 4.29 | 104.96 | 4.70 | 66.96 | 3.56 | 44.26 | 2335.03 | 37.03 | 51.94 | 16.58 | 20.99 | 14.34 |
| YALO | 5.05 | 0.21 | 4.11 | 104.00 | 3.61 | 57.01 | 1.88 | 21.45 | 219.29 | 47.08 | 70.86 | 11.87 | 16.18 | 16.30 |
| Mean | 3.93 | 0.37 | 7.06 | 103.93 | 5.19 | 57.45 | 3.92 | 59.85 | 1673.21 | 56.2 | 41.79 | 14.8 | 17.74 | 15.32 |
| Min | 2.02 | 0.18 | 3.31 | 97.91 | 3.61 | 46.31 | 1.88 | 21.45 | 219.29 | 37.03 | 22.73 | 10.44 | 11.51 | 11.78 |
| Max | 6.01 | 0.51 | 14.24 | 109.44 | 7.13 | 66.96 | 5.79 | 111.71 | 2805.39 | 78.18 | 70.86 | 19.50 | 26.63 | 22.54 |
| CV | 27.23 | 29.73 | 43.91 | 2.78 | 17.92 | 8.95 | 28.83 | 37.13 | 32.21 | 20.12 | 32.30 | 17.23 | 21.76 | 17.62 |
| SD | 1.07 | 0.11 | 3.10 | 2.89 | 0.93 | 5.14 | 1.13 | 22.22 | 538.91 | 11.31 | 13.5 | 2.55 | 3.86 | 2.70 |

**Table 4.** Eigenvalues and the proportion of accounted variance for each trait across 20 accessions of eggplant for the first four principal components (PC). DTF, days to flowering; NoB, number of branches; PH, plant height at maturity; NoFPC, average number of fruits per cluster; NoF, average number of fruits per plant; YLD, average yield per plant; FL, fruit length; FWD, fruit width; StemD, stem diameter; Pet.L, petiole length; Ped.L, pedicel length.

| Variables | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| Vitamin C | 0.2777 | -0.2423 | 0.1295 | -0.3933 |
| Iron | -0.3759 | 0.0049 | 0.1498 | 0.0992 |
| Calcium | -0.1837 | 0.3163 | 0.2492 | -0.1366 |
| DTF | -0.0896 | -0.4322 | -0.1783 | 0.3571 |
| NoB | -0.2992 | -0.1570 | -0.1479 | 0.2603 |
| PH | 0.3553 | -0.2406 | -0.1429 | 0.0086 |
| NoFPC | -0.4063 | -0.1331 | 0.1653 | -0.0500 |
| NoF | -0.4371 | -0.0926 | -0.0639 | -0.0384 |
| YLD | -0.0591 | -0.1850 | -0.3516 | -0.5664 |
| FL | -0.0488 | 0.2823 | -0.4034 | -0.3045 |
| FWD | 0.3804 | -0.0242 | 0.2446 | 0.2843 |
| StemD | -0.0412 | -0.4246 | -0.3557 | 0.0549 |
| Pet.L | 0.1243 | 0.2639 | -0.4563 | 0.3129 |
| Ped.L | -0.0135 | 0.4255 | -0.3351 | 0.1488 |
| Proportion of Variance | 0.2877 | 0.2064 | 0.1329 | 0.0973 |
| Cumulative Proportion | 0.2877 | 0.494 | 0.6269 | 0.7242 |
| EigenValues | 4.0274 | 2.8891 | 1.8605 | 1.3625 |

0.487, $P < 0.05$), average number of fruits per plant ($r = 0.532$, $P < 0.05$) and number of fruits per cluster ($r = 0.551$, $P < 0.05$). Plant height had a negative but moderate significant association with iron ($r = -0.461$, $P < 0.05$) and calcium ($r = -0.407$, $P < 0.05$) but was positively correlated with vitamin C ($r = 0.492$, $P < 0.05$). The strongest and most persistent correlation was recorded for association between fruit width and plant height ($r = 0.574$, $P < 0.01$) and number of fruits per plant ($r = -0.737$, $P < 0.01$); stem diameter with days to flowering ($r = 0.734$, $P < 0.01$). A positive significant correlation was observed between pedicel length and fruit length ($r = 0.561$, $P < 0.05$), and between pedicel length and petiole length ($r = 0.528$, $P < 0.05$).

Based on variation in the phenotypic parameters the 20 eggplant accessions were clustered into four unique groups (Figure 3). Clusters I and II contained seven and three accessions, respectively, while clusters III and IV both had five accessions. Means of variables, ranges and standard deviation for each cluster are presented in Table 6. Cluster III was unique in having accessions with high vitamin C content and high yield potential while clusters II was characterized by accessions with high iron content, an increased number of fruits per plant and a higher number of fruits per cluster. Clusters I and IV were characterized by early maturing accessions dominated by top-performing accessions in fruit-related traits (fruit length and fruit width respectively).

## Discussion

The success of genetic improvement programmes in enhancing desired traits of interest to farmers and breeders depends on the magnitude of genetic variability available in the germplasm and the extent to which the desirable traits are heritable. The high significant variation observed for most qualitative and quantitative traits considered in this study establishes the feasibility of imposing selections towards the improvement of desired traits of interest in African eggplant. Frequency distribution among the qualitative traits with a preponderance of fruits characterized by white to cream colours and lemon green suggests that the majority of the accessions belong to the *S. aetihiopicum* group. This supports earlier reports by Osei *et al* (2010) that eggplant accessions belonging to *S. aethiopicum* had mixtures involving cream white to light yellow fruits; thus, fruit colour combined with fruit shape might be considered a strong phenotypic marker in characterizing eggplant taxa in Africa.

The high CVs and range for some of the quantitative characters could be attributed to genetic variations from natural crossings and ecogeographical factors. The maximum and minimum mean values could present a rough estimate of the variation in magnitude of variability present among genotypes. Traits such as average number of fruits per plant and fruit width that exhibited a high range of variation had more scope for improvement in the eggplant population.

The principal component analysis identified traits that contributed the most to observed variations within a group of entries (Sneath and Sokal, 1973; Grittins, 1975). The first four principal component axes in the current study accounted for 72.42% of the total variability measured. The first principal component analysis had the highest discriminating ability (contributing 28.77% out of 79.47% of variability from the first four axes) and was dominated by traits with relatively high factor scores (> 2.60) corresponding to number of branches, plant height at maturity, number of fruits per cluster, number of fruits per plant, and fruit width. This is in agreement with Clifford and Stephen (1975) who reported that the first principal component axis was the most important in reflecting the variation patterns among accessions and that the characters highly associated with these should be used in differentiating the accessions. Furthermore, this is in line with the findings of Iezzoni and Pritts (1991) and Chikaleke (2018) who reported that the implication of principal components can be accessed from the contribution of the different variables to each principal component (PC).

Correlation analysis is used to identify the relationship between variables (Anshori *et al*, 2018) and to facilitate the identification of elite traits to rely on in selection exercises of a breeding programme. The positive significant association between iron content, number of branches, number of fruits per cluster and number of fruit per plant; number of fruit per plant with number

**Table 5.** Correlation coefficients for nutritional and phenotypic parameters of the20 eggplant accessions evaluated. *, significant at $P \leq 0.05$, **, significant at $P \leq 0.01$, DTF, days to flowering; NoB, number of branches; PH, plant height at maturity; NoFPC, average number of fruits per cluster; NoF, average number of fruits per plant; YLD, average yield per plant; FL, fruit length; FWD, fruit width; StemD, stem diameter; Pet.L, petiole length; Ped.L, pedicel length.

| Traits | Vit.C | Iron | Calcium | DTF | NoB | PH | NoFPC | NoF | YLD | FL | FWD | StemD | Pet.L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Iron | -0.500* | | | | | | | | | | | | |
| Calcium | -0.162 | 0.377 | | | | | | | | | | | |
| DTF | 0.064 | 0.028 | -0.446 | | | | | | | | | | |
| NoB | -0.308 | 0.487* | 0.091 | 0.370 | | | | | | | | | |
| PH | 0.492* | -0.461* | -0.407* | 0.051 | -0.100 | | | | | | | | |
| NoFPC | -0.279 | 0.551* | 0.225 | 0.293 | 0.370 | -0.537* | | | | | | | |
| NoF | -0.418 | 0.532* | 0.151 | 0.200 | 0.545* | -0.510* | 0.730** | | | | | | |
| YLD | 0.063 | 0.040 | -0.233 | -0.001 | -0.007 | 0.213 | 0.086 | 0.193 | | | | | |
| FL | -0.045 | -0.050 | 0.181 | -0.260 | 0.097 | -0.229 | -0.127 | 0.005 | 0.217 | | | | |
| FWD | 0.319 | -0.316 | -0.200 | -0.085 | -0.339 | 0.574** | -0.554* | -0.737** | -0.410 | -0.255 | | | |
| StemD | 0.239 | 0.065 | -0.303 | 0.734** | 0.336 | 0.350 | 0.068 | 0.174 | 0.363 | -0.058 | -0.125 | | |
| Pet.L | -0.394 | -0.315 | -0.142 | -0.123 | -0.133 | 0.154 | -0.387 | -0.186 | -0.032 | 0.256 | 0.029 | -0.099 | |
| Ped.L | -0.340 | -0.013 | 0.313 | -0.275 | -0.061 | -0.212 | -0.236 | -0.095 | -0.212 | 0.561* | -0.147 | -0.147 | 0.528* |

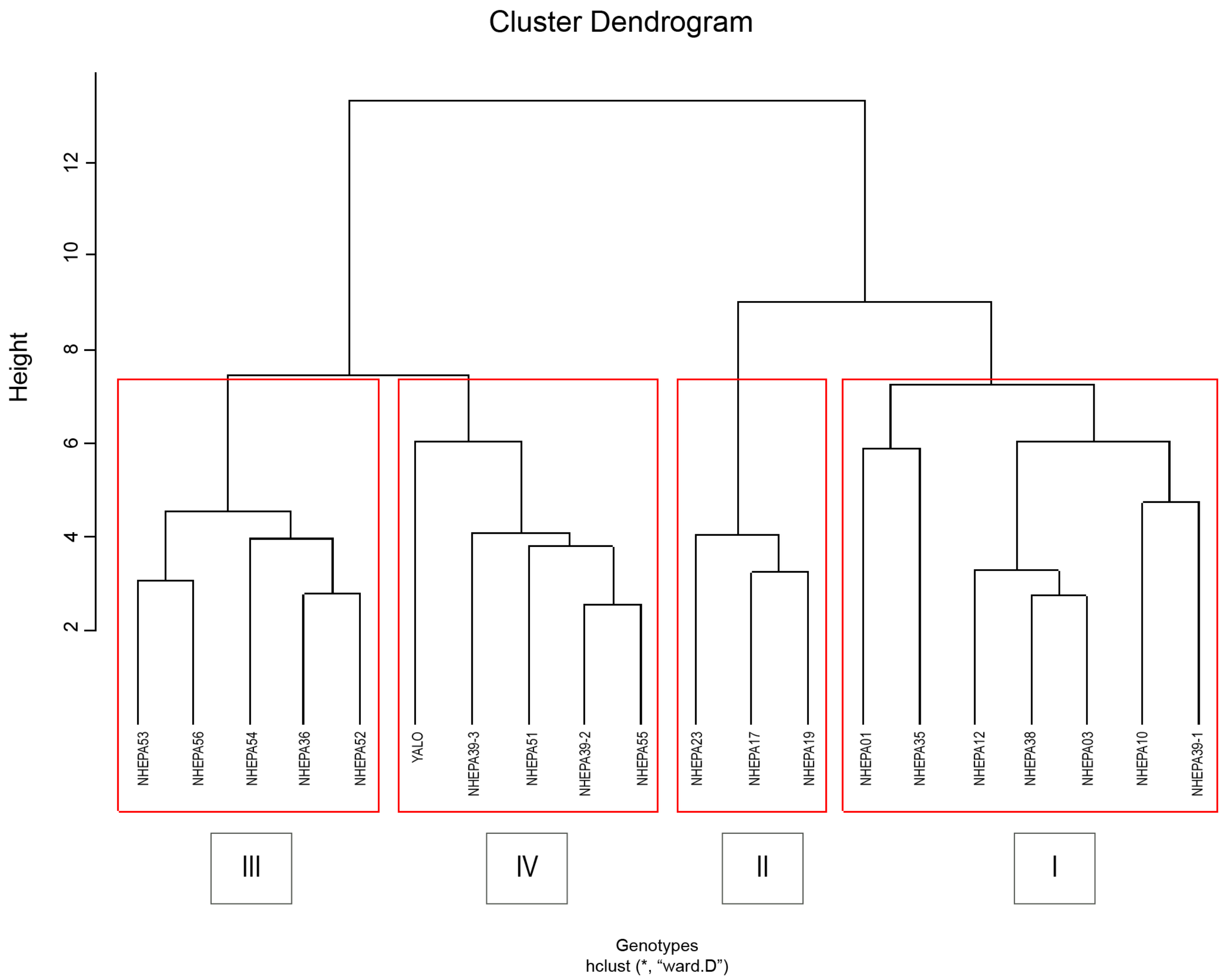

**Figure 3.** Cluster dendrogram showing the relationships among the 20 eggplant accessions with cluster tree cut value at 7.3.

of branches and number of fruits per cluster; fruit width and plant height at maturity; stem diameter and days to flowering; pedicel length with fruit length and with petiole length will facilitate selection of eggplant accessions with a good combination of these traits. The significant positive correlation displayed by these traits is in agreement with Dhaka and Soni (2013) who reported a positive significant association between yield and yield-related traits. However, where the traits had significant negative correlation coefficients (vitamin C and iron content, plant height at maturity and iron content, plant height at maturity and calcium content, number of fruits per cluster and plant height at maturity, number of fruits per plant and plant height at maturity, fruit width with number of fruits per cluster and with number of fruits per plant) indicates that an increase in one trait might lead to a decrease in the other trait or vice versa. This is in agreement with Mazer *et al* (1999) and Nyadanu and Lowor (2015) who reported that traits with significant inverse relationships could be improved independently among eggplant accessions in Ghana. However, selecting tall plants in this eggplant population might result in an indirect selection for low calcium content, while favouring increased iron content will lead to selecting genotypes with low vitamin C. Selection pressure can be deployed for an increased number of fruits per plant to simultaneously increase iron content and number of branches. Similar observations were reported by Arivalagan *et al* (2013) and Nyadanu and Lowor (2015) in their earlier works on mineral composition and morphological characterization of eggplant.

The cluster analysis emphasized further the relative contribution of various quantitative parameters to the total variability. The grouping of accessions in each cluster based on quantitative descriptors could be attributed to the fact that these accessions share some similarities. The high-yielding accessions in cluster III (NHEPA54) expressing high vitamin C and iron content could be deployed as progenitors to combine with eggplant genotypes from cluster I and create a new gene combination with improved calcium content and yield potential. Creating new eggplant varieties with high-yield potential and increased vitamins and minerals (iron, calcium and vitamin C) will not only increase farmers' income but will also help to reduce health challenges associated with hidden hunger among the

**Table 6.** Means and standard deviations for various traits in different clusters. The clusters with the highest values for each trait are highlighted in bold font. DTF, days to flowering; NoB, number of branches; PH, plant height at maturity; NoFPC, average number of fruits per cluster; NoF, average number of fruits per plant; YLD, average yield per plant; FL, fruit length; FWD, fruit width; StemD, stem diameter; Pet.L, petiole length; Ped.L, pedicel length.

| Trait | Cluster | Min | Max | Mean | StdDev | Trait | Cluster | Min | Max | Mean | StdDev |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Vit.C | I | 2.02 | 3.78 | 3.06 | 0.56 | NoF | I | 36.87 | 81.68 | 69.79 | 16.50 |
| Vit.C | II | 3.15 | 4.81 | 3.98 | 0.83 | **NoF** | **II** | **65.29** | **111.71** | **84.46** | **24.24** |
| **Vit.C** | **III** | **3.50** | **6.01** | **4.71** | **0.93** | NoF | III | 35.26 | 71.56 | 50.42 | 13.58 |
| Vit.C | IV | 2.58 | 5.80 | 4.33 | 1.22 | NoF | IV | 21.45 | 64.81 | 40.59 | 16.03 |
| Iron | I | 0.25 | 0.51 | 0.40 | 0.11 | YLD | I | 1,320.72 | 2,019.68 | 1,633.28 | 233.03 |
| **Iron** | **II** | **0.43** | **0.50** | 0.46 | 0.04 | YLD | II | 1,522.60 | 1,731.05 | 1,660.04 | 119.05 |
| Iron | III | 0.18 | 0.37 | 0.27 | 0.09 | **YLD** | **III** | **1,666.91** | **2,805.39** | **2,194.99** | **480.25** |
| Iron | IV | 0.21 | 0.45 | 0.36 | 0.10 | YLD | IV | 219.29 | 2,035.71 | 1,215.22 | 674.45 |
| **Calcium** | **I** | **4.10** | **14.24** | **8.53** | **3.84** | **FL** | **I** | **50.49** | **78.18** | **64.52** | **10.53** |
| Calcium | II | 4.42 | 10.69 | 6.66 | 3.50 | FL | II | 44.91 | 45.62 | 45.30 | 0.36 |
| Calcium | III | 3.31 | 5.95 | 4.92 | 1.10 | FL | III | 37.03 | 75.11 | 56.05 | 13.50 |
| Calcium | IV | 4.11 | 11.27 | 7.36 | 2.56 | FL | IV | 47.08 | 56.94 | 51.23 | 4.14 |
| DTF | I | 97.91 | 109.12 | 102.99 | 3.44 | FWD | I | 22.73 | 41.88 | 33.33 | 6.08 |
| DTF | II | 106.56 | 109.44 | 107.84 | 1.47 | FWD | II | 26.84 | 33.05 | 30.85 | 3.48 |
| DTF | III | 103.68 | 105.28 | 104.58 | 0.69 | FWD | III | 31.49 | 51.94 | 43.23 | 9.10 |
| **DTF** | **IV** | **99.19** | **104.00** | **102.27** | **1.86** | **FWD** | **IV** | **38.84** | **70.86** | **58.76** | **12.00** |
| NoB | I | 3.92 | 7.13 | 5.52 | 1.13 | StemD | I | 10.44 | 19.50 | 14.00 | 3.02 |
| NoB | II | 4.70 | 6.42 | 5.53 | 0.86 | StemD | II | 13.76 | 19.12 | 16.82 | 2.76 |
| NoB | III | 3.66 | 5.48 | 4.67 | 0.65 | StemD | III | 15.37 | 18.11 | 16.34 | 1.10 |
| NoB | IV | 3.61 | 5.84 | 5.04 | 0.85 | StemD | IV | 11.87 | 14.91 | 13.18 | 1.28 |
| PH | I | 46.31 | 60.09 | 53.91 | 5.03 | **Pet.L** | **I** | **11.51** | **26.63** | **19.92** | **4.62** |
| PH | II | 50.69 | 58.80 | 53.99 | 4.26 | Pet.L | II | 12.93 | 14.72 | 13.87 | 0.90 |
| PH | III | 58.53 | 66.96 | 61.85 | 3.19 | Pet.L | III | 15.68 | 21.60 | 18.47 | 2.74 |
| PH | IV | 57.01 | 62.52 | 60.08 | 2.11 | Pet.L | IV | 13.60 | 21.02 | 16.27 | 2.82 |
| NoFPC | I | 2.70 | 4.98 | 4.12 | 0.81 | **Ped.L** | **I** | **15.06** | **22.54** | **17.83** | **2.89** |
| **NoFPC** | **II** | **5.79** | **5.79** | **5.79** | **0** | Ped.L | II | 11.78 | 14.86 | 13.07 | 1.60 |
| NoFPC | III | 2.59 | 3.56 | 3.27 | 0.42 | Ped.L | III | 12.10 | 15.84 | 13.95 | 1.40 |
| NoFPC | IV | 1.88 | 4.21 | 3.18 | 1.01 | Ped.L | IV | 13.78 | 16.30 | 14.53 | 1.01 |

rural and urban populace in the region. Selection and hybridization of genotypes from clusters I and IV such as NHEPA35 and Yalo will produce new segregants characterized by bigger fruits with increased iron concentration.

## Conclusion

This study successfully characterized 20 eggplant accessions for phenotypic and nutritional traits of interest and identified top-performing new eggplant accessions with unique traits that could be deployed in crosses to facilitate the step-wise creation of new eggplant varieties with the best combination of desired traits. Furthermore, selection in favour of yield-increasing traits such as number of fruits per plant and number of fruits per cluster that showed a significant positive correlation with iron, will lead to selecting genotypes with increased iron content and higher yield potential simultaneously. Top-performing accessions for iron (NHEPA39-1), calcium (NHEPA39-1) and vitamin C content (NHEPA54) identified in this study should be deployed for hybridization to create new eggplant varieties with improved nutritional content.

## Author contributions

Olawale Olsesan Oguntolu: study design, execution, drafting.
Christian Okechukw Anyaoha: study design, data analysis and interpretation, drafting, revision.
Victor Anosie Chikaleke: drafting and revision.
Olofintoye Temidayo Joseph A, study design and execution.

## Conflict of interest statement

The authors declare no conflict of interest. All authors approved the final manuscript.

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Genetic Resources

REVIEW

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# The state of conservation of animal genetic resources in Slovakia

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**Abstract:** To effectively conserve animal genetic resources, countries need to periodically review their conservation efforts and reflect on actual problems and challenges. This study provides a review of animal genetic resources conservation activities, as well as the related existing legislative measures, strategies and funding in Slovakia. We present the development of endangered and supported breeds, discuss the impact of subsidies and the importance of awareness raising, and provide a SWOT analysis of the current animal genetic resources conservation framework in the country. In Slovakia, conservation is primarily based on animal breeding in natural conditions (*in situ*) without any limitations to breed improvement, and cryoconservation of animal genetic resources is in its initial phase. Most of the funding for conservation measures is provided by the Rural Development Programme. In general, the animal genetic resources system in Slovakia is open in terms of including new breeds eligible for support and this paper shows that the provided subsidies helped to stabilize most of the supported populations. Promoting the presence, characteristics and advantages of local breeds in times of intensive import of exotic breeds into the country is crucial to motivate breeders to prefer local livestock breeds. While the future challenge for the government is to improve conservation and facilitate related activities, research should address not only diversity, pedigree studies and cryoconservation, but also focus on the characterization of animal genetic resources for food security and climate change.

**Keywords:** biodiversity, conservation, animal genetic resources, endangered breeds, genebank

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## Introduction

Biodiversity for food and agriculture is indispensable to food security, sustainable development and the supply of many vital ecosystem services (FAO, 2019). Animal genetic resources (AnGR) are also sources of social and cultural benefits. Their contribution to a country's economy is not just in the form of animal products but also of employment opportunities for people in rural regions. AnGR are also an important part of landscape management and the agrotourism sector.

Despite all their roles and characteristics, according to the Second Report on the State of the World's Animal Genetic Resources for Food and Agriculture of the Food and Agriculture Organization of the UN (FAO), 17% of all breeds are classified as being "at risk of extinction" (FAO, 2015). In the case of local breeds, the main reason for rapid erosion is the lack of economic profitability (Gandini and Oldenbroek, 1999). This is closely related to the import of specialized and highly productive breeds, and their cross-breeding with local breeds.

Recently, local breeds are getting more attention thanks to their adaptability to local environmental conditions, their suitability for extensive agriculture and their expected roles in climate change adaptation. The increasing interest of consumers in animal production brings challenges but also opportunities to breeders. Advances in biotechnologies lead to more intensive research on the genetic level, increasing demands for establishing genebanks and providing AnGR material from already existing genebanks (Groeneveld *et al*, 2016).

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Countries adopt different policies and implement different approaches to address the conservation and use of AnGR, including local and endangered breeds, and to raise awareness of the need to conserve them. The main guidance is provided by the Global Plan of Action (GPA) for Animal Genetic Resources (FAO, 2007) and implementation progress in the four priority areas is monitored. The latest results show that globally, the greatest progress in the last years was achieved in the field of policies, institutions and capacity-building, while the lowest progress was achieved in conservation (Cao *et al*, 2021).

This review aims to provide a summary of the activities related to AnGR conservation in the Slovak Republic, with examples of the development and conservation of several breeds in the country. The information provided here should serve as a starting point in the preparation of the national strategy for the conservation of AnGR in the Slovak Republic.

## History of AnGR in the Slovak Republic

Slovakia is located in the heart of Europe and its relatively small area has made it difficult to maintain its local livestock breeds. In the past, intensive imports and crossbreeding contributed to forming the actual livestock diversity in the country. The main drivers for change in livestock diversity were improving performance and adaptedness of local breeds, often directed by landowners or the government. In the second half of the 20*th* century, the intensification of animal production and planned agriculture led to the creation of a list of recognized and allowed breeds (Act No. 110/1972 Coll. on livestock breeding, Edict 108/1974 on Act No. 110/1972), which could be kept by cooperatives and farmers, and contributed to a narrowing of livestock diversity. The massive planned use of Holstein bulls for crossing with Slovak Spotted and Slovak Pinzgau cows led to a decrease in both populations (Kadlečík *et al*, 2013). Unifying existing breeds contributed to the loss of within-breed diversity. For example, in the case of the Valachian sheep, which was considered undeveloped in the starting period of intensification, the planned unification and improvement of the population led to the loss of diversity of exterior animal characteristics (colour varieties, horn shapes). Similar to other countries, mechanization in agriculture and decline of their use in the army during the second half of the 20*th* century had a significant effect on horse populations. Their number decreased dramatically and has not recovered to this day. In those times, there was no national interest in the conservation of local breeds and some of them became completely extinct in the country (e.g., Red Carpathian and Gray Carpathian cattle). To improve production and adaptedness, new specialized breeds were created. In poultry, the Oravka breed was developed for adaptation to the colder regions of northern Slovakia following a request from the government. The breed became a favourite among small breeders, spreading around the whole country, and its current population is estimated to be up to 9,000 animals. On the other hand, some of the new breeds created were outperformed by other specialized and well-established breeds or their breeding process failed in later phases and these breeds became extinct (e.g. Slovak White and Slovak meaty pig). In horses, the creation of three breeds started in this period, including Noric of Murany, Slovak Sport Pony and Slovak Warmblood.

After the political changes in the 1990s in Central Europe, agriculture and animal production started to transform and the overall number of livestock decreased rapidly in these countries (Oravcová *et al*, 2004). For example, at the breed level, in 30 years the number of Slovak Pinzgau cattle decreased from more than 90,000 at the end of the 1980s to just 11,000 at present. The Improved Valachain sheep population decreased from almost 200,000 in the mid-1990s to 100,000 animals, currently. Some breeds became endangered according to effective population size (Table 1). Breeders started to import exotic specialized breeds to improve production and be competitive at the international level. These changes put more pressure on the breeds with a long tradition in the country. While most of the breeders were improving production, some turned their attention to endangered breeds and also breeds that were already extinct in the country. Thanks to common history (Czechoslovakia, Austro-Hungarian monarchy), animals from these breeds could still be found in neighbouring countries and the exchange of these animals allowed the recovery of their populations in Slovakia. In the case of the almost extinct Valachian sheep, in the 1990s, breeders started to select phenotypically acceptable animals from the Improved Valachian population and have continued to import animals from breeders in the Czech Republic, who have maintained the rest of the original Valachian population. Occasional imports of Valachian sheep from Germany, where a part of the Valachian sheep population was exported in the 1990s, have also occurred. Since the exchange of breeding animals between Slovakia and neighbouring countries continues, most of the breeds can be considered as being transboundary in terms of FAO definitions (FAO, 2005). Due to common history and changes in animal production, including intensive crossbreeding, it is quite difficult to strictly distinguish between native and non-native breeds. There is no legal definition of an autochthonous/native/traditional breed in the country and no legal criteria are set for the recognition of an endangered breed. However, references to generally accepted autochthonous or traditional breeds can be found across the literature (Oravcová *et al*, 2006; Weis and Hrnčár, 2009; Šidlová *et al*, 2015; Kadlečík *et al*, 2017). While a common understanding of autochthonous breeds was also reported in Poland, Austria has a more precise definition, which includes breeds having a herdbook in Austria before 1938, or ample evidence that they have always been present and bred in a part of Austria, or stem from the Austro-

Hungarian monarchy and have had relations to regions of Austria (Kompan, 2014).

In poultry, big producers use hybrids, while continuous breeding activities of small farmers have led to the creation of new colour and dwarf types. For example, in the case of Oravka chicken, three new colour types and dwarf types have been created since the original type's official recognition in 1990. The number of registered breeders, which can be considered a reliable source of breeding animals, is small and thus the number of breeding animals is also small compared to the population size (Table 2). Similarly in rabbits, big producers use hybrids. Breeding activities of small farmers are considered a hobby and are focused on the stabilization of existing and newly created breeds' characteristics as well as the creation of new breeds. The estimated size of rabbit populations and the number of breeding animals reflect the length of a breed's existence (Table 2). While populations of breeds recognized before 1990 are estimated to be more than 200 animals strong (Blue of Holic, Nitra, Slovak Greyblue rex, Zemplin), populations of breeds recognized after this year are smaller.

## Legislation and policies

The Slovak Republic signed the Convention on Biological Diversity (CBD, 1992) in 1993 and became a Party to the CBD after approval in the following year. Issues related to biodiversity and its protection fall under the Ministry of Environment of the Slovak Republic. After affirmation of its commitments in 2007 (Interlaken Declaration on Animal Genetic Resources (FAO, 2007)), the Slovak Republic started to implement the GPA for Animal Genetic Resources. According to Strategic Priority 20 of the GPA for Animal Genetic Resources (FAO, 2007), countries should periodically review their national policies in order to investigate their direct and indirect effects on the use, development and conservation of AnGR. At present, the main responsibility for AnGR conservation in the country lies on the Ministry of Agriculture and Rural Development of the Slovak Republic and NPPC – Research Institute for Animal Production Nitra (National Focal Point for Animal Genetic Resources in Slovakia). In the Slovak Republic, the most important legislative measure is Act No. 194 of 1998 Coll. (last amended in 2020) on livestock breeding, which, in general, specifies the rights and duties of authorized breeders' associations to carry out their professional activities connected with animal breeding. It also addresses endangered breeds to a limited extent. Similar to the EU regulation 2016/1012 (EU, 2016), the national Act No. 194/1998 under Paragraph 3 Section 1e) lists the protection of endangered populations and provision of livestock genetic reserves as an important part of breeding development. Although recently not strictly followed, under Paragraph 2 Section 7 of Act No. 194/1998, protected farms are defined as farms keeping animals of endangered breeds that are used for the conservation of the breed's genepool and maintenance of its historical value. According to Paragraph 13 Section 2 of Act No. 194/1998, the transfer of breeding stock and genetic reserves originating from such farms should be controlled and permitted only after approval of the state. Contrary to this general approach, there are legislative acts of neighbouring countries, where the legislative basis for AnGR conservation, especially the setting of a national conservation programme, is part of the breeding acts (e.g. Czech Republic – Paragraph 14 of Act No. 154/2000 Coll. (last amended in 2021) (breeding act), or Poland – Art. 34 of Coll. of Laws 2021, item 36, act on the organization of breeding and reproduction of farm animals). Based on the Slovak National Act, there are authorized breeders' associations responsible for breeding the main livestock species including cattle, sheep, goats, horses, pigs, rabbits and poultry. These associations maintain breeding books, studbooks and breeding registers and are providing information to the National Focal Point for Animal Genetic Resources in Slovakia as part of the regular AnGR monitoring. Authorized breeders' associations differ in organization and number of breeds managed. For instance, the Slovak Pinzgau cattle are represented by an association solely responsible for this breed, thus making it easier to promote and take actions aimed at this particular breed. On the other hand, Valachian sheep are represented by an association responsible for several breeds of sheep and goats. All the activities are undertaken by the association. A group of breeders has been created within this association to improve collaboration and the breeding process of Valachian sheep. The same applies to the breeders of poultry (different groups according to species and breeds) and rabbits. In horses, National Stud is responsible for managing the studbook of Lipitsa, Hutsul, Arab and Shagya Arab. Studbooks of other breeds, except for English Thoroughbred, are managed by one horse breeders' association.

Regarding AnGR cryoconservation, the national legislation addresses only insemination centres with no considerations or exceptions for endangered breeds conservation and genebank activities. A description of the technical requirements for establishing genebanks is lacking, and so far, only scientific experience and rules for insemination centres are followed. The same applies to requirements for the acquisition and use of AnGR samples stored in genebanks. The actual status of the national veterinary legislation (Act No. 39/2007 Coll. (last amended in 2021) on veterinary care) therefore does not address and reflect the actual needs of AnGR conservation in the country and further steps are needed to fill this gap.

The Slovak Republic became a Party to the Nagoya Protocol (CBD, 2011) in 2016. The responsibility for the Nagoya Protocol implementation in the Slovak Republic lies with the Ministry of Environment. In order to facilitate access to its AnGR and taking into account that almost all livestock breeds in Slovakia are considered transboundary, Slovakia decided not to control access to its AnGR. Following the EU regulation 511/2014 EU (2014b), the Slovak national law (Act. No. 263/2015

**Table 1.** Numbers of purebred registered breeding males and females and endangerment class of supported breeds of livestock in the Slovak Republic according to 2020 monitoring data. Endangerment class was set according to effective population size calculated according to the formula $N_e = (4 \times M \times F)/(M + F)$ (Falconer and Mackay, 1996) and assuming mass selection $N_e(\text{sel}) = 0.7 \times N_e$ (Santiago and Caballero, 1995). The degree of endangerment was assessed according to the following limits: critically endangered ($N_e \leq 50$), endangered ($50 < N_e \leq 200$), monitored ($200 < N_e \leq 1{,}000$) and not endangered ($N_e > 1{,}000$)

| Species | Breed | Males (M) | Females (F) | Endangerment class |
|---|---|---|---|---|
| Cattle | Slovak Pinzgau | 31 | 2,025 | Endangered |
| Sheep | Valachian | 45 | 907 | Endangered |
| | Tsigai | 360 | 5,613 | Monitored |
| | Improved Valachian | 616 | 8,175 | Not endangered |
| | Askanian Merino | 6 | 68 | Critically endangered |
| Goat | White shorthaired | 50 | 708 | Endangered |
| | Brown shorthaired | 9 | 81 | Critically endangered |
| Horse | Lipitsa | 6 | 165 | Critically endangered |
| | Shagya arab | 16 | 154 | Critically endangered |
| | Hutsul | 7 | 133 | Critically endangered |
| | Furioso | 17 | 161 | Critically endangered |
| | Nonius | 7 | 28 | Critically endangered |
| | Noric of Murany | 18 | 137 | Critically endangered |
| | Slovak Sport Pony | 7 | 84 | Critically endangered |
| | Slovak Warmblood | 16 | 857 | Critically endangered |

**Table 2.** Estimated population size, purebred breeding males and females of registered breeders and endangerment class of poultry and rabbit breeds in the Slovak Republic according to 2020 monitoring data. Endangerment class was set according to effective population size calculated according to the formula $N_e = (4 \times M \times F)/(M + F)$ (Falconer and Mackay, 1996) and assuming mass selection $N_e(\text{sel}) = 0.7 \times N_e$ (Santiago and Caballero, 1995). The degree of endangerment was assessed according to the following limits: critically endangered ($N_e \leq 50$), endangered ($50 < N_e \leq 200$), monitored ($200 < N_e \leq 1{,}000$) and not endangered ($N_e > 1{,}000$)

| Species | Breed | Population | Males (M) | Females (F) | Endangerment class |
|---|---|---|---|---|---|
| Chicken | Oravka | 9,000 | 59 | 465 | Endangered |
| Goose | Suchovy | 250 | 19 | 33 | Critically endangered |
| | Slovak White | 270 | 22 | 40 | Critically endangered |
| Rabbit | Blue of Holic | 420 | 30 | 80 | Endangered |
| | Liptovsky Lysko | 160 | 20 | 42 | Critically endangered |
| | Nitra | 1,250 | 38 | 200 | Endangered |
| | Slovak Pastel rex | 105 | 15 | 40 | Critically endangered |
| | Slovak Greyblue rex | 600 | 28 | 92 | Endangered |
| | Zemplin | 280 | 15 | 55 | Critically endangered |
| | Zobor | 60 | 8 | 18 | Critically endangered |
| | Strbsky gepardi rex | 100 | 15 | 35 | Critically endangered |
| | Chrabrany | 120 | 19 | 38 | Critically endangered |

Coll. on competences in the area of access to genetic resources and sharing of benefits arising from their utilization) sets only rules for the users of genetic resources falling under the Nagoya Protocol.

The national legislation pays limited attention to endangered breeds and their conservation and lacks direct strategies for AnGR conservation. These are partly compensated for by a few cross-sectorial strategies that refer to AnGR. As part of biodiversity, some actions related to AnGR conservation were included in the Updated National Strategy For Biodiversity Protection 2020 (MZP SR, 2013). These were broadly defined, and included monitoring of AnGR, support for *in situ* conservation of traditional breeds, genetic analysis of diversity, support for *ex situ* conservation, including the development of a genebank, and identification of ecosystem services provided by AnGR. To a certain extent, the inclusion of these activities into the strategy may be considered a formality since they have been carried out even before the strategy was adopted, and the funds for most of them were allocated regardless of the strategy. Additional issues related to AnGR have been also addressed in the updated version of the Adaptation Strategy of the Slovak Republic to Climate Change (MZP SR, 2018). The disadvantage of including AnGR conservation in multiple strategies is that some activities may be duplicated, for example, the monitoring of livestock species. Moreover, the number of AnGR conservation activities has increased over time and new conservation approaches are being applied. Therefore, cross-sectoral policies are no longer sufficient to address specific activities related to AnGR conservation, as demonstrated in the case of poultry breeders. While cross-sectoral policies have been grouping the main responsible AnGR stakeholders from different biodiversity sectors, the involvement of small stakeholders that are important from an AnGR perspective has been very limited. While the overall AnGR monitoring was included in the strategy, no attention was paid to gaps and the limited registration system of local poultry breeds and farmers in the country. This led to further problems in providing financial support to these farmers. The limited implementation of the actions listed in the cross-sectoral policies resulted also from the lack of funding since not all actions have financial cover granted. In contrast to this approach, functional conservation programmes have been implemented across European countries, which have been closely tied with funds provided by the EU (Ligda and Zjalic, 2011).

## Monitoring

The livestock monitoring system in the Slovak Republic is based on the use of already existing data. Population data for monitoring purposes come from two sources. The first is the Breeding Services of the Slovak Republic, a state enterprise which provides actual sizes of cattle, horse, goat and sheep populations at the breed level. These data represent the number of obligatory registered livestock animals according to the breed. The reliability of these data is very high since this obligation applies to all farmers of the four mentioned species and the portion of non-registered animals is assumed to be negligible from a population size perspective. Horse breeding has some specificities and discrepancies exist in their registration, therefore the final number of animals is estimated based on data from the register and numbers from studbooks. The second source of data is the authorized breeders' associations which maintain the herdbooks/studbooks and breeding registers, and provide relevant information on registered breeding animals. Although it does not cover the whole population, this information is of high interest, because these animals represent the part of the populations that can actively participate in the breeding process and serve as a basis to create future generations. It provides the numbers of purebred and crossbred animals and a picture of crossbreeding in the populations. To compare between years and exclude fluctuations during the year, actual numbers relevant for the last day of the previous year are used in the monitoring. The reliability of information on cattle, horses, goats, sheep and pigs is high since it comes from reliable existing registration sources. On the other hand, information on poultry and rabbits is less reliable since the registration of animals of these species is limited and some information is based on estimates. This arises from the fact that while registration is obligatory for every animal from big livestock species, only farmers with more than 350 poultry animals are obliged to register them. Furthermore, due to the already mentioned fact that purebred animals of local breeds are mostly kept by small farmers and in small numbers as a hobby activity, limited information on the population size is available. A similar situation can be observed in the registration of purebred animals of these species, as only a few small farmers keep track of their animals' pedigree and performance. Similar obstacles were reported in the Czech Republic (MZe CR, 2017). In this situation, since the identification of eligible farmers/animals is very complicated, the breeding process becomes difficult and financial support from the state is limited.

Although population data are available for most breeds, active monitoring is performed only for those breeds that are considered established in the country. This is due to the high fluctuation of exotic breeds in the Slovak Republic and to the limited information about small populations of those breeds in the country. The examples of Tarantaise cattle or Lincoln sheep show the farmers' enthusiasm for exotic breeds. However, they were unable to keep breeding them for different reasons, and currently, there are less than ten animals of these breeds in the country. In 2020, more detailed monitoring included 15 cattle, 11 horse, 27 sheep, 5 goat, 6 pig, 49 rabbit, 21 chicken, 3 duck and 3 goose breeds. Among those breeds, 34 were considered autochthonous or traditionally kept in the country, 6 new and 100 exotic. Results of monitoring were used to evaluate the status of endangered breeds Tomka *et al* (2016); Tomka and Huba (2019) and to update population data in FAO's Domestic Animal Diversity Information System.

In addition to population monitoring, communicating with breeders of endangered breeds to understand their needs and expectations is important. This was already shown by Wanner *et al* (2021) who interviewed farmers of German-speaking parts of the Alps and found that the opinions and expectations of farmers differ from studies and opinions of other stakeholders. A short survey among the registered breeders of Valachian sheep (11 out of 14) and Askanian Merino sheep (1 out of 1), Slovak White goose(5 out of 8) and Suchovy goose (9 out of 11), was conducted by NPPC in 2017 and 2018. The survey aimed to investigate the breeders' motivation for the choice of breed, their breeding purposes and future plans for endangered breeds in Slovakia. The results of the survey can help predict trends in these populations since it provides a glimpse of the breeders' attitudes towards monitored endangered breeds. Some outputs of this survey are presented and discussed in this review to show the breeders' motivation and attitudes.

## Conservation

The AnGR conservation activities in the Slovak Republic are primarily oriented toward measures supporting *in situ* breeding of live animals by private bodies (cooperatives, farmers). This way, endangered breeds can be further developed and continually adapted, e.g., to changing climate conditions (FAO, 2013). There are no restrictions on breeding goals compared to other European countries, where breeders have to commit to maintaining the traditional characteristics of the animals. For instance, for the Slovak Pinzgau cattle, part of the population has already been transformed from dual-purpose to beef production (cow–calf system) (Pavlík *et al*, 2013). The reason for changing to a cow–calf system may be explained by the fact that the Slovak Pinzgau cattle is still predominantly kept in big herds owned by cooperatives and expected to make a profit primarily from milk production. Therefore, cooperatives have to either improve the milk performance of animals (through e.g. crossbreeding, choice of a different breed) or change the orientation of production. A positive change trend toward beef production is shown by the numbers of living cows registered in different sections of the herdbook (dual-purpose vs. beef). While 1,030 purebred dual-purpose cows and 667 beef cows were reported in 2012 in the population of Slovak Pinzgau cattle, the opposite ratio was reported in 2020, i.e. 841 purebred dual-purpose cows and 1,183 beef cows. At the moment, animals of both groups are eligible for financial support. Although this free approach allows breeds to change their breeding purposes and become more competitive, it can also bring tensions within breeding organizations (Lauvie *et al*, 2011). At the moment, there are no disagreements on the two different types of Slovak Pinzgau in the association. However, this may change in the future if beef animals divert significantly from the dual-purpose type. A different approach to *in situ* conservation of AnGR with defined requirements is applied in the Czech Republic, where conditions for animal breeding must be as similar as possible to those in which the breed was developed, and modern breeding technologies should be used to a limited extent. Most importantly, the selection of animals is not aimed at improving their performance, but at stabilizing their characteristics and/or maintaining their original characteristics (MZe CR, 2017). An approach based on maintaining the animals' original characteristics can be found in the Valachian sheep population in Slovakia. At the moment, the selection of animals is based primarily on exterior characteristics, since the breed is known for its different colours of wool and different types of horns. Less pressure on selection for productivity traits in this breed may be explained by their ownership. These animals are mostly kept by small farmers as a hobby and for agrotourism, or by cooperatives along with other more productive sheep breeds. The results of the short survey conducted by NPPC showed that resilience, low requirements for feeding and adaptation to the local environment were the most mentioned advantages of these animals. A similar situation can be observed in poultry and rabbit populations. In Oravka chicken, selection is based primarily on exterior characteristics. This comes as no surprise since the breeding of these animals is considered a hobby activity or is done to ensure small-farmers self-sufficiency.

One of the disadvantages of *in situ* conservation, especially in small populations, is the risk of increasing inbreeding. The responsibility for following breeding programmes and maintaining low inbreeding levels lies with breeders' associations. Therefore, cooperation between breeders' associations and research and academic institutions is crucial for the effective development and implementation of breeding programmes. Recently, several national research projects have been carried out to study the actual status of local breeds populations, and published papers showed different levels of inbreeding in several cattle and horse populations. While pedigree-based inbreeding under the acceptable level of 1% was estimated in Slovak Spotted and Slovak Pinzgau populations of cattle (Kukučková *et al*, 2017; Kasarda *et al*, 2019a), genomic analyses showed higher levels of inbreeding and thus increasing trends of inbreeding are expected in both populations. A low level of pedigree-based inbreeding (0.23%) and genomic inbreeding (0.11%) was observed in the relatively small population of Noric of Murany (Kasarda *et al*, 2019b). Sufficient levels of variability were observed in the populations of Lipitsa, Furioso and Nonius (Kasarda *et al*, 2018). In older studies (Pjontek *et al*, 2012), which investigated populations of horses, higher levels of relatedness and expected higher levels of inbreeding based on pedigree information were reported in the population of Hutsul (6.26%) and Slovak Sport Pony (2.67%). A preliminary assessment of inbreeding trends in Valachian sheep (Oravcová and Margetín, 2011) showed a lack of pedigree information in animals resulting in unclear coefficients of inbreeding, while a more recent analysis (Pavlík *et al*, 2017) showed a low average coefficient of inbreeding (0.85%). A low level of pedigree-based inbreeding (0.69%) was also calculated in the population of White Shorthaired goats (Oravcová, 2013).

Other conservation activities include *in situ* and *ex situ* conservation of live animals by state organizations. While horse breeds (Noric of Murany, Lipitsa, Hutsul, Shagya Arab) are maintained *in situ*, live sheep (Valachian) and chicken (Oravka) are conserved *ex situ*. Conservation activities of the state organizations are aimed mostly at stabilizing and maintaining the original characteristics of these breeds. Additionally, two of these organizations also receive national funds for the long-term storage of AnGR samples. While National Stud provides long-term storage of horse breeds samples (samples are stored as a reserve and are not distributed to breeders), the Genebank of the National Agricultural and Food Centre (GB NPPC-VUZV) stores samples of other livestock species and breeds. This is similar to other European countries, where the long-term storage of AnGR is mostly carried out by public research institutes (Passemard *et al*, 2018).

Samples stored in GB NPPC-VUZV are owned by the state and are expected to be available for cooperation with breeders and research purposes. In the past, private and public insemination centres used to cooperate on the storage of samples for conservation purposes. This is the case of the Slovak Pinzgau cattle, whose old bull lines were kept by a private insemination centre for commercial and long-term storage purposes. Due to costs associated with cryoconservation, the Slovak Pinzgau samples were later transferred to GB NPPC-VUZV. There was no involvement of Slovak Pinzgau breeders in the process of animal and sample selection. Currently, there is no information available on the number of long-term samples stored by these private insemination centres. The participation of the private sector in long-term conservation activities, however, should be restored because it can reduce collection development costs (Pizzi *et al*, 2016). Moreover, it is assumed that the private sector storing samples of transboundary commercial breeds can allow the public sector to focus more on the country's local and endangered breeds. In December 2021, GB NPPC-VUZV reported 3,058 samples, from which the majority is represented by semen samples from 12 breeds and 4 species (cattle, sheep, chicken, rabbit). These represent mainly endangered local breeds including Slovak Pinzgau cattle, Valachian sheep, Oravka chicken, Blue of Holic, Nitra, Slovak Greyblue rex and Zobor rabbit. In the case of Valachian sheep and Oravka chicken, samples are primarily collected from animals kept *ex situ in vivo* by NPPC-VUZV. This means, that there is no involvement of breeders in the process of sample selection and only limited participation of breeders in providing samples to the genebank. The predominant storing of semen is understandable here and across other countries (Leroy *et al*, 2019) and may be explained by the long and routine use of artificial insemination in some breeds. The collection of samples for long-term storage in GB NPPC-VUZV is rather random, not following any conservation plan or breed-specific attributes (e.g. endangerment, economic return). Such an opportunistic approach was already presented by Blackburn (2009), who described the initial phase of germplasm collection development in the USA. In 2021, GB NPPC-VUZV has become a member of the European Genebank Network for Animal Genetic Resources (EUGENA). This step should help to increase the genebank visibility at the national level and improve cooperation at the international level. From the transboundary breeds perspective, joining the network in combination with strategic collection and storage planning can lead to saving cryoconservation costs in the future (Silva *et al*, 2019). On the other hand, joining the network does not imply that overlapping of collected AnGR samples should be strictly avoided (Danchin-Burge *et al*, 2011).

## Funding and valorization

In order to identify endangered breeds requiring support, breed definitions by FAO (2005) have been followed and endangerment of breeds has been evaluated by the National Focal Point for AnGR in agreement with the rules laid down by the current European legislation. As a result of this approach, different breeds have been supported during the last decades based on their needs. The open approach of the Slovak Republic is demonstrated, for instance, by the Askanian Merino sheep breed. Although not originating in Slovakia, this breed has become eligible for subsidies because of its long breeding tradition in the country, its unique wool performance and its status of critically endangered breed not just at country level but also in the EU. With such an approach, immediate actions can be taken to support critically endangered breeds.

In the mid-1990s, state incentives were provided for live purebred females, but the support varied in the amount per head and number of breeds, because public funds were limited (Oravcová *et al*, 2004). In 2003 these incentives supported two cattle breeds (Slovak Spotted, Slovak Pinzgau), seven horse breeds (Hutsul, Lipitsa, Furioso, Nonius, Shagya-Arab, Noric of Murany, Slovak Sport Pony), three sheep breeds (Valachian, Improved Valachian, Tsigai) and three poultry breeds (Oravka hen, Slovak White goose), including Japanese quail.

After joining the European Union in 2004, a new funding scheme for *in situ* conservation of endangered breeds was applied. Support was provided through the Rural Development Plan (MP SR, 2003) as in many other European countries (Ligda and Zjalic, 2011). In the period 2004–2006 this support was provided only to one sheep (Valachian), one goat (White Shorthaired), eight horse (Slovak Warmblood, Hutsul, Furioso, Nonius, Slovak Sport Pony, Lipitsa, Shagya Arab, Noric of Murany) and nine poultry breeds (Oravka hen, Plymouth Rock hen, Rhode Island Red hen, New Hampshire hen, Vlaska hen, Sussex hen, Slovak White goose, Suchovy goose and Bronze turkey). Due to significant changes in the list of supported breeds, one can argue there have been inconsistencies in the approach and big pressure from breeders to include other breeds on the list of supported ones. The main changes in the list of supported breeds were the consequence of the transition from very limited national funds to European funds. Also, new conditions for support were set after joining the EU. During this period, the number of registered purebred females in herdbooks decreased in Slovak Pinzgau cattle (Table 3). Unfortunately, no data were available to present the trend of registered females of Valachian sheep and White Shorthaired goat, which were supported in this period. However, it can be assumed that this number increased in Valachian sheep and decreased in White Shorthaired goats (Table 3). In horses, the number of registered females increased in Lipitsa, Shagya Arab, Hutsul, Furioso and Slovak Sport Pony, decreased in Noric of Murany and stayed at low levels in Nonius.

**Table 3.** Development of purebred breeding females registered in herdbooks. *, Year 2003, source: (Oravcová *et al*, 2004); **, data for 2007; n.a. – data not available.

| Species | Breed | Registered purebred females (monitoring data) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2003* | 2006 | 2010 | 2012 | 2014 | 2016 | 2018 | 2020 |
| Cattle | Slovak Pinzgau | 2,500 | 1,600 | 1,969 | 1,697 | 1,491 | n.a. | 1,479 | 2,024 |
| Sheep | Valachian | 50 | n.a. | 65 | 225 | 349 | 553 | 820 | 907 |
| | Tsigai | 13,000 | 13,000** | 11,406 | 12,734 | 7,277 | 5,571 | 5,433 | 5,613 |
| | Improved Valachian | 24,000 | 21,000** | 15,724 | 14,682 | 9,412 | 7,641 | 6,971 | 8,175 |
| | Askanian Merino | n.a. | n.a. | n.a. | 34 | 27 | 39 | 52 | 68 |
| Goat | White Shorthaired | 1,000 | n.a. | 864 | 832 | 1,031 | 634 | 752 | 708 |
| | Brown Shorthaired | 3 | n.a. | 29 | 102 | 67 | 116 | 152 | 81 |
| Horse | Lipitsa | 60 | 105 | 145 | 186 | 352 | 165 | 181 | 165 |
| | Shagya Arab | 85 | 102 | 165 | 197 | 359 | 128 | 151 | 154 |
| | Hutsul | 50 | 115 | 120 | 110 | 280 | 106 | 141 | 133 |
| | Furioso | 40 | 80 | 211 | 162 | 150 | 175 | 158 | 161 |
| | Nonius | 40 | 35 | 39 | 26 | 32 | 28 | 26 | 28 |
| | Noric of Murany | 115 | 70 | 219 | 104 | 106 | 114 | 119 | 137 |
| | Slovak Sport Pony | 42 | 60 | 145 | 92 | 70 | 109 | 98 | 84 |
| | Slovak Warmblood | 320 | n.a. | 1,794 | 868 | 925 | 863 | 836 | 857 |

EU regulation 1698/2005 (EU, 2005) allowed to provide support for *in situ* conservation (under Article 39 (2,4)) and *ex situ* conservation (under Article 39(5)) of genetic resources in agriculture. Financial support for *in situ* conservation was defined as compensation of additional costs and income foregone resulting from the commitment made. EU regulation 1974/2006 (EU, 2006) and, in its Annex IV, defined the thresholds for endangered local breeds eligible for *in situ* conservation support. These criteria included a number of purebred breeding females of local breeds in all EU Member countries registered in the herdbook kept by an appropriate breeding organization. Within the period 2007–2013, support for endangered breeds from the Rural Development Programme (MP SR, 2007) was provided to one cattle breed (Slovak Pinzgau), one sheep breed (Valachian), one goat breed (White Shorthaired), eight horse breeds (Slovak Warmblood, Hutsul, Furioso, Nonius, Slovak Sport Pony, Lipitsa, Shagya Arab, Noric of Murany) and eight poultry breeds (Oravka hen, Plymouth Rock hen, Rhode Island Red hen, New Hampshire hen, Vlaska hen, Sussex hen, Slovak White goose, Suchovy goose). Contrary to other European countries, subsidies were paid solely for livestock unit, without any differences made between sexes or level of endangerment or any further required goals (Ligda and Zjalic, 2011).

During the next period (2014–2020), similar support for endangered breeds from Rural Development Programme (MP SR, 2015) continued based on EU regulation 1305/2013 (EU, 2013). Some changes were introduced in Slovakia regarding the list of eligible breeds based on the experience from the previous period, while the same conditions for support were applied. Since almost no support was provided to poultry breeds during the previous period, these breeds were excluded from the support scheme. On the other hand, three more sheep breeds (Askanian Merino, Improved Valachian, Tsigai) and one goat breed (Brown Shorthaired) were included in the support scheme. EU regulation 807/2014 (EU, 2014a), introduced transitional provisions and the role of a relevant scientific body to identify endangered breeds. In Slovakia, the assessment of endangerment was based partly on the criteria used in the previous period (number of registered purebred breeding females), as well as on effective population size calculated following Falconer and Mackay (1996).

One of the main goals of AnGR conservation is to stop the loss of biological diversity of livestock. Thanks to funding from rural development programmes, the negative trends of population sizes of endangered breeds from the 1990s were more or less stabilized in the 2000s (Oravcová *et al*, 2010), and these trends were repeatedly reported by Tomka *et al* (2016) and Tomka and Huba (2019).

The number of registered purebred Slovak Pinzgau cows decreased in the period 2003–2006 (Table 3), but it recovered to 2,000 females by 2010. The stabilized trend with small fluctuations may be observed both in population size (Table 4) and the number of registered purebred females in the recent period 2010–2020 (Table 3). It should be noted again that, while the number of registered females has been more or less stabilized at around 2,000 heads, the transfer of cows from milk production to cow–calf system is ongoing. This means breeders are trying to increase the competitiveness and profit generated from these cows. At the moment, it is unclear whether the number of registered purebred females would decrease if the subsidies were lowered for more competitive cows in cow–calf systems.

The continuous decrease of Improved Valachian and Tsigai populations and increase of the Valachian population are significant (Table 4). The number of registered purebred Improved Valachian and Tsigai females decreased in the period without subsidies (2003–2016). This number levelled off in 2016 (Table 3). This can be attributed to the start of subsidies provided to the breeders of these breeds. The decreasing trend of population size and stabilized number of registered purebred females after the implementation of subsidies suggest that further decrease could occur if the financial support is terminated. It may be assumed that in such a case, these populations will not become extinct, but their possible reduced sizes will lead to problems due to the availability of a smaller number of purebred breeding animals. On the other hand, the case of the original type of Valachian sheep shows positive trends in the population size and number of registered purebred females. While only 50 females were registered in 2003 (Table 3), recently, 900 females were registered in the herdbook. Although these trends may be attributed to subsidies provided for this breed, breeders did not state that subsidies were the motivation for choosing and breeding this breed in the short survey conducted by NPPC. The stable trend of Askanian Merino may be explained by the fact that there has been only one registered breeder of this breed in Slovakia.

Two breeds of goats are eligible for financial support in Slovakia. However, the trends in their population sizes are different (Table 4). While the larger population of White Shorthaired goats can be considered stable, the smaller population of Brown Shorthaired slightly increased during the period 2010–2020. Different patterns may be observed in the number of registered females. The number of registered White Shorthaired goats has been slightly decreasing since 2003, despite subsidies. On the other hand, a small increase of registered Brown Shorthaired goats can be observed even before support was provided (2003–2012) and the number stabilized in the following period 2012–2020. These findings show that providing financial support in Slovakia does not automatically lead to an increase in population size or in the number of registered females. These findings also support the fact that the motivation of farmers plays an important role.

In horses, the estimated population sizes are stable (Table 4). The number of registered purebred females increased compared to the year 2003 (Table 3) and can be considered stable in the last years (2016–2020). However, some of them (Nonius, Slovak Sport Pony) have been staying at very low levels since then and there is a big risk of losing these populations. While the Nonius horse is a transboundary breed and cooperation with neighbouring countries can help to prevent the loss of the breed in the country, the population of Slovak Sport Pony is extremely vulnerable due to its local distribution and the presence of only a few breeders.

A positive effect of subsidies on the demographic development of endangered breeds in Europe was presented by Gicquel *et al* (2020) who argue that correctly setting the conditions and level of subsidies are crucial elements of a subsidiary system. Based on previously published data (Kompan, 2014), it can be speculated that while some population sizes increased over time thanks to a sufficient level of subsidies (e.g. in Hungary and Poland) and/or also thanks to the support only to active breeding animals in some countries (e.g. Austria), subsidies had a stabilizing or even no effect in other countries including Slovakia. On the other hand, an insufficient level of subsidies was identified as a possible reason for the continual decrease of supported animals, especially in horses (e.g. in Austria). This may be explained by limited economic income from horse breeding, which requires higher compensation for income loss. Stabilized trends of registered animals and the number of subsidy applicants in Slovakia suggest that the support for *in situ* breeding of endangered breeds is set correctly to prevent population sizes, and most importantly the number of registered animals, from decreasing in most breeds. This means that the value of a subsidy is not so high to attract speculative breeders, who would primarily keep animals of endangered breeds only to profit from the subsidies and not for breeding and developing endangered breeds. This can be illustrated in the case of Askanian Merino. One could expect that farmers of traditional Merino sheep would switch to breeding Askanian Merino since they are phylogenetically closely related breeds. However, after the introduction of financial support for the Askanian breed, no dramatic change in the number of farmers and animals has been observed. The number of existing breeders of supported endangered breeds is not decreasing, so it can be assumed that, at the moment, subsidies provide sufficient support for these breeders to keep endangered breeds. Such an approach is in line with Strategic Priority 8 of GPA, i.e. in the long-term perspective, the emphasis should be put on the sustainable use of local breeds without the need for support from public funds or extra funding. However, it is questionable whether the support would be efficient with lower subsidies and reflect the level of endangerment of breeds. It is also questionable whether the number of animals would increase if some requirements were implemented, e.g. only active breeding animals eligible for subsidies.

Strong tools to improve the competitiveness of local breeds are the products related to the breed (Verrier *et al*, 2005). Unfortunately, no breed-related animal products exist in Slovakia. Therefore, new ways of promoting products from endangered breeds have to be exploited. As an example of efforts, activities of the sheep and goat breeders' association resulted in the official certifications of animal products that originate exclusively from Slovak sheep and goat milk and meat. The aim of the 'Golden sheep' and 'Golden goat' certificates is to ensure the quality of animal products originating from sheep and goats, and fair price for producers. Such efforts, however, are not aimed at the breed level, but rather at species. At the moment, Slovakia has no animal products registered as protected designation of origin. However, there

**Table 4.** Development of Slovak breed populations in the period 2010–2020.

| **Species** | **Breed** | **Population size (monitoring data)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **2010** | **2012** | **2014** | **2016** | **2018** | **2020** |
| Cattle | Slovak Pinzgau | 9,883 | 11,384 | 10,166 | 9,895 | 12,004 | 10,982 |
| Sheep | Valachian | 2,391 | 2,372 | 2,231 | 2,437 | 2,834 | 2,906 |
| | Tsigai | 122,253 | 142,944 | 127,071 | 123,660 | 113,135 | 91,239 |
| | Improved Valachian | 130,207 | 143,757 | 134,403 | 131,573 | 121,807 | 100,235 |
| | Askanian Merino | 350 | 541 | 341 | 345 | 418 | 440 |
| Goat | White Shorthaired | 8,389 | 6,798 | 7,685 | 8,036 | 8,166 | 7,576 |
| | Brown Shorthaired | 1,485 | 812 | 1,137 | 1,627 | 1,912 | 1,901 |
| Horse | Lipitsa | 750 | 500 | 575 | 775 | 900 | 900 |
| | Shagya Arab | 600 | 500 | 500 | 650 | 700 | 700 |
| | Hutsul | 500 | 450 | 400 | 550 | 500 | 500 |
| | Furioso | 450 | 450 | 375 | 475 | 475 | 475 |
| | Nonius | 110 | 110 | 93 | 105 | 105 | 125 |
| | Noric of Murany | 400 | 400 | 350 | 475 | 475 | 450 |
| | Slovak Sport Pony | 200 | 200 | 225 | 250 | 250 | 250 |
| | Slovak Warmblood | 2,500 | 2,500 | 2,150 | 3,250 | 2,500 | 2,500 |

are several cheese products registered as a protected geographical indication, four of which are related to certain regions and the rest is related to the whole country. The connection of local breeds to these regions may bring some interest in consumers. The promotion of animal products coming from local breeds raised in protected areas may have a similar impact. This approach, however, needs the involvement of the environmental sector and the facilitation of protected areas used for sustainable grazing. This is in line with recent European strategies (The European Green Deal, Biodiversity Strategy for 2030, Farm to Fork Strategy), which are calling for environment-friendly agriculture and high-quality animal products while referring also to local AnGR in this regard.

More straightforward use of non-productive services of livestock may be found in some cases. While national legislation sets strict rules on farming in protected areas, the environment sector is already searching for livestock species and breeds that are adapted to specific natural conditions in order to use them for maintaining valuable ecosystems in the country. These measures may be presented by including extensive pasture of different livestock species as a management tool in the projects 'Restoration and management of Danube flood plain habitats (LIFE14 NAT/SK/001306)' and 'Restoration of Natura 2000 sites in cross-border Bratislava capital region (LIFE10 NAT/SK/000080)'. Livestock animals in these projects are primarily used to maintain the traditional character of meadows and pastures while preventing the spread of invasive plant species and securing the wide biodiversity related to these ecosystems. In some cases, the production roles of livestock are neglected in favour of these non-productive ecosystem maintenance services. In the long-term perspective, such an approach may lead to the loss of the breeds' productive characteristics. Some authors have pointed out that this non-productive approach may lead to the use of livestock solely for environmental reasons without any production benefits, and may be vulnerable to lack of external payments, making the sustainability of such measures questionable (Wilson, 1996; Evans and Yarwood, 2000). Recently, however, studies suggested that conservation should aim beyond genetic and production goals since the distribution of breeds is changing in favour of more productive environments, where the diversity of breeds is lower and areas with higher breed diversity are being abandoned (Velado-Alonso *et al*, 2020).

## Awareness raising

AnGR conservation has its specific features compared to wild biodiversity conservation. The most important one is the ownership of AnGR. While the government is committed to conserving AnGR under its jurisdiction, the animals are owned by private bodies. Since the decision to keep animals lies on the private person, awareness raising of breeders about the characteristics, roles and benefits arising from breeding endangered livestock breeds is crucial. This is because in many cases, breeders of endangered breeds are hardly aware of the value of their animals as genetic resources (Herold *et al*, 2012). Economic aspects and modern technologies, which increase availability and enable the exchange of AnGR across Europe and the world, make it very difficult to motivate breeders to prefer raising endangered and less productive breeds. In some cases, breeders follow 'fashion trends' and decide to prefer an exotic breed because of its unique appearance or assumption of high profits from selling its progeny and products. They are often not aware of whether the breed is fit for their environment, and this may lead to low production. Even in cases when only non-productive livestock services are expected, exotic breeds are preferred. Of

**Table 5.** SWOT analysis of AnGR conservation activities in Slovakia

| **Strengths** | **Weaknesses** |
|---|---|
| • Breeding activities of each breed in Slovakia are covered by breeders' associations.<br>• Data from herdbooks/studbooks and data from animal registration system are available.<br>• AnGR sector has been a part of cross-sectoral policies.<br>• Open system of conservation allows to include new breeds in the list of supported breeds. | • Lack of national legislation and policies that specifically address conservation of AnGR.<br>• Lack of registration in poultry and rabbits.<br>• Limited inclusion of stakeholders and farmers in planning conservation activities and cryoconservation of AnGR.<br>• Limited transfer and implementation of results from research. |
| **Opportunities** | **Threats** |
| • Cooperation between breeders' associations of neighbouring countries.<br>• New technologies, establishment of genebanks and public funding to improve cryoconservation of AnGR.<br>• New European strategies<br>• Introduction of new ways of valorization of AnGR products and services. | • Small number of breeders of local breeds.<br>• Conservation measures may be at risk if the funding is limited.<br>• Lack of awareness and personal motivation of breeders and their successors.<br>• Trends of preferring extensive systems and decreasing livestock numbers. |

course, local breeds are not the only ones able to provide services related to landscape and ecosystem maintenance (Leroy *et al*, 2018), but some studies showed their specific abilities in harsh environments. In this case, awareness raising can help to promote local and endangered breeds, which can perform better in more diverse conditions. The already described short survey conducted by NPPC showed that one of the main reasons to raise local breeds in the country is patriotism. This finding suggested that choosing a local breed depended on breeders' enthusiasm and thus small populations may be vulnerable to changes in their motivation. Results of the survey also suggested that breeding these animals is strongly connected to the breeder and low interest from the breeder's successors can cause a further decrease in the local breed population. Similar findings were already presented by Yarwood and Evans (1998) in the UK, who suggest that the motivation of new breeders is important. This can be primarily achieved by financial support, but in many cases improving and acknowledging the status of breeders of local breeds can increase the attractiveness of local breeds.

In Slovakia, breeders' associations also have limited awareness of cryoconservation activities; the recent initiatives for cryoconservation are coming from state organizations, differently from neighbouring countries (Czech Republic, Poland), where the management of preserved breeds' sample acquisition and conservation involves farmers and their associations. Limited involvement of breeders' associations in decisions on AnGR sample selection is not considered a big problem in the initial phase of sample collection, but it may result in limited harmonization of cryoconservation and breeding programmes of endangered breeds in the future. Therefore, AnGR cryoconservation should be promoted widely among breeders as a complement to *in situ* conservation. New schemes of cryoconservation funding should be investigated to improve the active involvement of breeders and the overall state of long-term conservation of AnGR.

The predominant presence of big cooperatives and the separation of farmers from their land until the 1990s resulted in a weak connection between farmers and consumers at the national and local levels. After the 1990s, people started to return to their land. However, the establishment of direct channels between farmers and consumers is still underway. In this situation, raising public awareness about AnGR, their roles and products is very important for generating demand. General promotion at the national level brings attention to AnGR. At the local level, it is very important to also acknowledge the breeders who keep endangered breeds to facilitate the creation of local niche markets.

## SWOT analysis

A SWOT analysis of AnGR conservation activities in Slovakia was conducted in order to facilitate the preparation of a national conservation strategy (Table 5). The absence of a legal basis for AnGR conservation makes it difficult to adopt any long-term conservation programme, clearly define breed categories and criteria to assess endangerment and provide related financial support, and complicates the operation of genebanks. There are also problems with records in poultry and rabbits, which hamper the identification and support of eligible farmers and animals. The adoption of a specific national strategy could facilitate this support. Cross-sectoral policies allow closer cooperation with the environment and wild biodiversity sector and this increases awareness of agricultural biodiversity as part of overall biodiversity. They can also help in achieving common goals, including ecosystem maintenance and adaptation to climate change in the future.

Breeding activities are well organized in Slovakia. This gives a good baseline for appropriate management of populations and population structure monitoring. Breeders should not only act as keepers of AnGR and providers of their samples but should be involved in the decision-making of national cryoconservation goals and related activities. Moreover, participation of private insemination and cryoconservation centres could bring new opportunities, e.g. long-term storage of samples from commercial breeds.

The majority of livestock breeds in Slovakia are considered transboundary as defined by FAO. This creates an opportunity for breeds at risk to be recovered from resources coming from neighbouring countries. In this light, data from DAD-IS as a tool to provide information on transboundary breeds in different countries are very important. In some cases, even a sign of the presence of a breed in a country presented in DAD-IS may serve as a starting point to search for animals and farmers. On the other hand, a very small number of breeders of local breeds, like Slovak Sport Pony, are considered a risk. Big efforts should be made to attract new breeders to such breeds to spread the population among more breeders and lower the risk of losing the whole population when old breeders quit breeding or decide to change breed.

Early identification of and financial support to endangered breeds can serve well as a preventive tool. On the other hand, the number of animals of some endangered breeds may decrease despite financial support. This applies in particular to horse breeds, where economic income from breeding is limited. Awareness raising and personal motivation of breeders and their successors are in many cases the key to keeping endangered breeds. Modern trends of acquiring popular exotic breeds and the decreasing interest of breeders' successors in animal breeding are a big challenge for the conservation of AnGR. Connection of products to the environment, acknowledgement of breeders and monetary expression of non-productive services related to landscape maintenance may improve the valorization of AnGR products.

Changing attitudes of the public toward animal breeding and production, and corresponding European strategies create opportunities for more research activities in the field of non-productive roles of AnGR in extensive livestock systems with a positive impact on the environment and studies of unique traits that are linked to adaptability (and resilience) of local breeds. On the other hand, these European strategies may lead to favour extensive systems and decreasing livestock numbers to reduce emissions. This may also have a harmful effect on AnGR in the country. For example, local breeds, which are at present kept in bigger herds by traditional cooperatives, may be abandoned without or with limited replacement if smaller farmers decide not to keep them or to keep them in smaller herds in more extensive systems.

## Author contributions

Ján Tomka contributed to the concept and design of the manuscript. All authors contributed to drafting and reviewing the manuscript.

## Conflict of interest statement

The authors declare that no conflict of interest exists.

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# The role of tomato wild relatives in breeding disease-free varieties

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**Abstract:** Cultivated tomato (*Solanum lycopersicum*) is one of the most economically important and widely grown vegetable crops worldwide. However, tomato plants are often affected by biotic and abiotic stresses that reduce yield and affect fruit quality. Phenotypic diversity is evident in cultivated tomatoes, particularly for horticultural traits, but genetic diversity is rather narrow. Major disease resistance genes for different pathogens such as viruses, fungi, bacteria and nematodes are mainly derived from wild tomato species and introgressed into cultivated tomatoes. Here, we list the major disease and insect-pest resistance genes identified in *S. pimpinellifolium*, *S. habrochaites*, *S. peruvianum*, *S. chilense*, *S. pennellii*, *S. galapagense*, *S. arcanum* and *S. neorickii* with perspective on the gap between current knowledge on tomato wild relatives and the knowledge that is needed.

**Keywords:** Tomato, crop wild relatives, disease resistance, genomics, plant genetic resources, plant breeding

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## Tomato wild relatives

Crop wild relatives (CWRs) are potential sources of allelic variation useful to overcome biotic and abiotic stresses as they often grow and reproduce in marginal habitats (Ortiz, 2015; Bohra *et al*, 2021). Tomato wild relatives are native to South America, distributed from the coastal region of Ecuador to northern Chile, including the Galápagos Islands (Darwin *et al*, 2003). These regions have extremely varied environments within short distances due to differences in altitude (Andean geography) and diverse ecological habitats, which led to local adaptation and generation of large genetic diversity among (Figure 1) and within wild tomato species. *Solanum section Lycopersicon* (Mill.) Wettst. consists of cultivated tomato (*S. lycopersicum*) and 12 wild relatives (Ramírez-Ojeda *et al*, 2021). The Tomato Genetics Resource Center (C.M. Rick TGRC, University of California-Davis, USA, https://tgrc.ucdavis.edu/) hosts the largest genetic stocks of wild tomato collections, with over 900 accessions. The largest collection belongs to accessions of *S. pimpinellifolium* (~300) followed by *S. habrochaites* (~120), *S. peruvianum* (~100), *S. chilense* (~100), and *S. pennellii* (~50), respectively (Table 1). The World Vegetable Center (WorldVeg, Taiwan https://genebank.worldveg.org/#/) and the United States Department of Agriculture, Agricultural Research Service (USDA-ARS, https://www.ars-grin.gov/) genebanks also maintain the second and third largest wild tomato collections, respectively (Table 1). However, the majority of their wild tomato collection was originally obtained from the TGRC collection. Furthermore, these genebanks harbour an extensive collection of introgression lines derived from different tomato wild species (Ebert and Schafleitner, 2015).

## Utilization of tomato wild relatives for biotic stress breeding

Domestication has increased the phenotypic diversity of cultivated tomatoes but may have narrowed their resistance to biotic and abiotic constraints as selection ensued (Vu *et al*, 2020). Tomato wild relatives germplasm harbour natural resistance to various diseases and insect pests. Sources of genetic resistance to many of the biotic stresses faced by cultivated toma-

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**Figure 1.** Diversity in fruit colour and leaf shape of some tomato wild relatives. From left to right: *S. pimpinellifolium* (LA1269), *S. peruvianum* (L00887-3), *S. chilense* (LA2737B) and *S. galapagense* (VI063177). The size of the marker is 14cm × 1cm. Photo credit: Hamid Khazaei

**Table 1.** Genetic resources of tomato wild relatives maintained by the Tomato Genetics Resource Center (C.M. Rick TGRC), World Vegetable Center (WorldVeg), and the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) genebanks (data accessed on June 23, 2022).

| **Species** | **No. of accessions per genebank** | | |
|---|---|---|---|
| | **TGRC** | **WorldVeg** | **USDA-ARS** |
| *S. pimpinellifolium* | 290 | 342 | 338 |
| *S. habrochaites* | 120 | 139 | 49 |
| *S. peruvianum* | 69 | 116 | 11 |
| *S. chilense* | 115 | 46 | 1 |
| *S. pennellii* | 47 | 59 | 5 |
| *S. corneliomulleri* | 53 | 10 | 12 |
| *S. neorickii* | 47 | 12 | 8 |
| *S. galapagense* | 28 | 27 | 4 |
| *S. arcanum* | 45 | 4 | 3 |
| *S. cheesmaniae* | 12 | 17 | 5 |
| *S. chmielewskii* | 16 | 11 | 0 |
| *S. lycopersicoides* | 23 | 5 | 0 |
| *S. sitiens* | 13 | 5 | 2 |
| *S. huaylasense* | 16 | 0 | 0 |
| *S. ochranthum* | 7 | 0 | 2 |
| *S. juglandifolium* | 5 | 1 | 0 |

toes have been identified in some accessions of wild tomato species that have been historically used to introduce resistance (R) genes into cultivated tomato varieties. It should be noticed that there is genetic diversity for biotic resistance response within tomato wild species, and only a few accessions within each species have been identified as sources of resistance (Ebert and Schafleitner (2015) and references). We list major R genes found in and introgressed from wild tomato species into cultivated tomatoes in Table 2, along with information about their causal pathogens as footnotes. For example, the R genes/alleles conferring resistance to the begomoviruses that cause tomato yellow leaf curl diseases (called *Ty* genes), were found in wild tomato species. Most tomato advanced lines grown in tropical and subtropical regions of the world carry at least one source of *Ty* resistance genes (*Ty-1*/*Ty-3*) or multiple *Ty* genes (*Ty-2*, *ty-5*, *Ty-6*). The pyramiding of *Ty* genes into one line leads to broad and probably more durable resistance to begomoviruses. Likewise, R genes for late blight (caused by *Phytophthora infestans*, *Ph* genes) and fusarium wilt (caused by *Fusarium oxysporum*, *I* genes) also originated from wild tomatoes (Table 2). These genes are also frequently present in the modern tomato lines worldwide. Some important major QTLs (quantitative trait locus) such as *Bwr* and *EB*, conferring bacterial wilt (caused by *Ralstonia* spp.) and early blight (caused by *Alternaria linariae*) resistance, were first introgressed to adapted cultivated tomato cultivars from *S. pimpinellifolium* and later uncovered and genetically mapped. Some R genes (*Rx-1*, *-2* and *-3*) for bacterial spot resistance (caused by several species of gram-negative bacteria in the genus *Xanthomonas*) were also derived from *S. pimpinellifolium* via cultivated tomato. Several R genes against important pathogens in tomato have mainly been introgressed, from the wild species *S. pimpinellifolium*, *S. habrochaites*, *S. peruvianum*, *S. chilense* and *S. pennellii*, into modern tomato varieties (Table 2). However, most tomato breeding programmes focus on *S. pimpinellifolium* due to its red fruit colour (Figure 1) and its close relationship to cultivated tomato, which allows breeders to easily obtain interspecific crosses. That is probably why most of the R genes have already been identified in this species.

**Table 2.** List of major biotic resistance genes incorporated into cultivated tomatoes (*Solanum lycopersicum*) from wild relatives of tomato and *S. lycopersicum* var. *cerasiforme*. Causative agents for the listed diseases are included in footnotes.

| Species | R-genes' contribution | Disease/Insect pest | References |
|---|---|---|---|
| *S. pimpinellifolium* L. | *I* and *I-2* | Fusarium wilt[1] | Stall and Walter (1965); reviewed in Chitwood-Brown *et al* (2021) |
| | *Ph-1*, *Ph-2*, *Ph-3* and *Ph-5* | Late blight[2] | Bonde and Murphy (1952); Gallegly and Marvel (1955); AVRDC (1994); Foolad *et al* (2006) |
| | *EB-5* and *EB-9* | Early blight[3*] | Anderson *et al* (2021) |
| | *Bwr-6* and *Bwr-12* | Bacterial wilt[4*] | Wang *et al* (2013) |
| | *Rx-1*, *Rx-2* and *Rx-3* | Bacterial spot[5*] | Yu *et al* (1995); reviewed in Adhikari *et al* (2020) |
| | *Rx-4* | Bacterial spot | Robbins *et al* (2009) |
| | *Sm* | Gray leaf spot[6] | Parlevliet (2002) |
| | *Cf* genes (except *Cf-4* and *Cf-5*) | Leaf mold[7] | Bailey (1950); reviewed in Scott and Gardner (2007) |
| | *Sw-1*(*a* and *b*), *Sw-2*, *Sw-3* and *Sw-4* | TSWV[8] | Finlay (1953); Roselló *et al* (1998); Zhu *et al* (2017); reviewed in Qi *et al* (2021) |
| | *Pto* | Bacterial speck[9] | Pitblado and Kerr (1980) |
| | *Cmm* genes | Bacterial canker[10] | Forster and Echandi (1972); Sotirova *et al* (1994); Sen *et al* (2021) |
| *S. habrochaites* S. Knapp and D. M. Spooner | *Ph-4* | Late blight | Lough (2003) |
| | *Cf-4* | Leaf mold | Stevens and Rick (1986) |
| | *Ty-2* | TYLCV[11] | Hanson *et al* (2006) |
| | *Tm-1* | ToMV[12] | Pelham (1966) |
| | *Ol-1*/*Ol-3*, and *Ol-5* | Powdery mildew[13] | van der Beek *et al* (1994); Huang *et al* (2000); Bai *et al* (2005) |
| | *Cmm* genes | Bacterial canker | Forster and Echandi (1972); Francis *et al* (2001); Coaker and Francis (2004); Sotirova *et al* (1994) |
| | *Rbcq* genes | Gray mould[14] | ten Have *et al* (2007); Finkers *et al* (2007a,b) |
| *S. peruvianum* L. | *ty-5* | TYLCV | Hutton *et al* (2012) |
| | *Sw-5* and *Sw-6* | TSWV | Giordano *et al* (2000); Rosello *et al* (2001) |
| | *Mi* genes | Root-knot nematodes[15] | Smith (1944); reviewed in El-Sappah *et al* (2019) |
| | *Tm-2*, *Tm-2*$^{2}$ and *Tm-2a* | ToMV | Soost (1963); Ganal and Tanksley (1996) and Tanksley and Nelson (1996) |
| | *Ve* | Verticillium wilt[16] | Diwan *et al* (1999) |
| | *Frl* | Fusarium crown[17] | Vakalounakis *et al* (1997) |
| *S. chilense* (Dunal) Reiche | *Ty-1*/*Ty-3a*, *Ty-4*, and *Ty-6* | TYLCV | Zamir *et al* (1994); Ji *et al* (2007); Ji *et al* (2009); Gill *et al* (2019) |
| | *Sw-7* | TSWV | Stevens *et al* (1994) |
| | *Cmm* genes | Bacterial canker | Sotirova *et al* (1994) |
| | *Lv* | Powdery mildew | Yordanov *et al* (1975); Chunwongse *et al* (1997) |
| | - | Gray mould | ten Have *et al* (2007) |

*Continued on next page*

*Table 2 continued*

| **Species** | **R-genes' contribution** | **Disease/Insect pest** | **References** |
|---|---|---|---|
| *S. pennellii* Correll | *I-3* and *I-7* | Fusarium wilt | Catanzariti *et al* (2015); Gonzalez-Cendales *et al* (2016) |
| | *Asc* | Alternaria stem canker[18] | Scott and Gardner (2007) |
| | *Xv-4* | Bacterial spot | Astua-Monge *et al* (2000) |
| | Acylsugar-related genes | A wide range of insects | Leckie *et al* (2012, 2016); Schilmiller *et al* (2012) |
| *S. galapagense* S. C. Darwin and Peralta | *Wf-1* and *Wf-2* | Whiteflies[19] | Firdaus *et al* (2013); Santegoets *et al* (2021) |
| *S. arcanum* Peralta | *Ol-4* | Powdery mildew | Bai *et al* (2005) |
| | *Cmm* genes | Bacterial canker | Crinò *et al* (1995); Sotirova *et al* (1994); Sen *et al* (2013) |
| *S. neorickii* D. M. Spooner, G. J. Anderson and R. K. Jansen | *V2* | Verticillium wilt | Kanagawa Agricultural Technology Center (1999) |
| | - | Gray mould | ten Have *et al* (2007); Finkers *et al* (2008) |
| *S. l.* var. *cerasiforme* | *Cf-5* | Leaf mold | Dickinson *et al* (1993); Dixon *et al* (1998) |
| | *ol-2* | Powdery mildew | Ciccarese *et al* (1998) |

[1] Fusarium wilt caused by fungal pathogen *Fusarium oxysporum* (Schlecht. emend. Snyder & Hansen).

[2] Late blight resistance caused by the oomycete *Phytophthora infestans* (Mont.) de Bary.

[3] Early blight is caused by fungal pathogen *Alternaria* spp.

[4] Bacterial wilt caused by the group of soilborne bacteria in the *Ralstonia solanacearum* species complex.

[5] Bacterial spot caused by several species belonging to the genus *Xanthomonas*. It can be caused by *Xanthomonas euvesicatoria* ex Doidge, *X. vesicatoria* ex Doidge, *X. perforans*, and *X. gardneri* Šutic.

[*] Early blight, bacterial wilt, and bacterial spot (*Rx-1*, *Rx-2*, and *Rx-3*) resistance genes are most likely derived from *S. pimpinellifolium* via cultivated tomato.

[6] Gray leaf spot caused by fungal pathogen *Stemphylium lycopersici* (*S. lycopersici*).

[7] Leaf mold caused by fungal pathogen *Cladosporium fulvum* (syn. *Passalora fulva*).

[8] TSWV, tomato spotted wilt orthotospovirus (order *Bunyavirales*, family *Tospoviridae*, genus *Orthotospovirus*) is transmitted by *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae).

[9] Bacterial speck disease caused by *Pseudomonas syringae* pv. tomato (Pst).

[10] Bacterial canker caused by *Clavibacter michiganensis* subsp. *Michiganesis.*

[11] TYLCV, tomato yellow leaf curl virus disease. TYLCV is caused by whitefly transmitted *Geminiviruses* (begomoviruses). *Ty-1* and *Ty-3* are allelic (Verlaan *et al*, 2013).

[12] ToMV, tomato mosaic virus. ToMV is a member of the family *tobamoviridae* and belongs to the genus *tobamovirus*.

[13] Powdery mildew can be caused by three species of biotrophic fungal pathogens; *Oidium lycopersici*, *Oidium neolycopersici* (syn. *Pseoudoidium neolycopersici*), and *Leveillula taurica*. *Ol-1* and *Ol-3* are allelic (Huang *et al*, 2000).

[14] Gray mould caused by fungal pathogen *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*).

[15] Root-knot nematodes can be caused by *Meloidogyne incognita*, *M. javanica,* and *M. arenaria.*

[16] Verticillium wilt caused by the biotrophic fungus *Verticillium dahliae.*

[17] Fusarium crown rot caused by *F. oxysporum* f. sp. *radicis-lycopersici*.

[18] Alternaria stem canker caused by fungal pathogen *Alternaria alternate.*

[19] Whitefly (*Bemisia tabaci*) resistance.

## Challenges

The utilization of wild tomatoes in breeding programmes is not without a cost. CWRs generally show poor adaptation beyond their natural distribution range (Bohra *et al*, 2021). Furthermore, the introgressed gene from a wild relative into advanced lines may disrupt long-accumulated horticultural traits due to linkage drag (Tanksley and Nelson, 1996). For example, *S. galapagense* has been identified as a source of insect-pest resistance (Rakha *et al*, 2017; Vendemiatti *et al*, 2021). When it is crossed with cultivated tomatoes, the fruit size and setting reduce significantly, which are undesirable traits. Linkage drag can be removed by conducting backcrosses to the recurrent parents (cultivated tomato). Applications of DNA molecular markers (MAB, marker-assisted backcrossing) allow for the monitoring of the genome around the gene/locus of interest and the genetic background, speeding up the return to the recurrent parent genome (Tourrette *et al*, 2021). Genome editing can also be used to remove the undesirable gene without having extensive backcrossing. For example, CRISPR-Cas9-based gene editing was used to overcome a linkage drag in tomato by editing the *jointless-2* gene introgressed from *S. cheesmaniae* (Roldan *et al*, 2017). The World Vegetable Center is currently testing CRISPR-Cas9 to edit genes implicated with fruit size regulation in interspecific crosses between *S. galapagense* and cultivated tomato (Schafleitner *et al*, 2022).

## Genomics-assisted breeding tools

Tomato wild relatives have more to offer. Bai *et al* (2018) stated that about 20 pathogens could be genetically controlled by resistance genes derived from a few wild species. The genome of some tomato wild species, including *S. pennellii* (Bolger *et al*, 2014; Schmidt *et al*, 2017), *S. chilense* (Stam *et al*, 2019), *S. pimpinellifolium* (Razali *et al*, 2018; Wang *et al*, 2020; Gramazio *et al*, 2020) and *S. lycopersicoides* (Powell *et al*, 2022) along with the pan-genome (Gao *et al*, 2019) have been assembled. These efforts have bolstered our knowledge and understanding of tomato wild species along with the genetics of resistance genes. Recent improvements in genomic resources have enabled us to track and genetically map the wild tomato genes in commercially adapted varieties (Anderson *et al*, 2021). The tomato community largely benefits from advanced, rich genomic resources (https://solgenomics.net/) and phenotyping tools; however, rapid generation technology (speed breeding) has not yet been developed. Robust DNA markers for major genes derived from wild relatives have been developed and widely applied in private and public breeding programmes worldwide (Foolad and Panthee, 2012; Hanson *et al*, 2016).

In the past, sources of important disease resistance genes in wild tomato relatives have been intensively investigated. However, the majority of R genes were discovered only in a few species (listed in Table 1). The other wild species that either are not easy to cross with cultivated tomatoes or are self-incompatible or allogamous have not contributed much to this journey (e.g. *S. chmielewskii*, *S. corneliomulleri*, *S. huaylalloasense*, *S. juglandifolium*, *S. ochranthum*, *S. lycopersicoides* and *S. sitiens*). Regarding these species, the literature only represents a few accessions of *S. lycopersicoides* being resistant to grey mould (caused by *Botrytis cinerea*) (Davis *et al*, 2009) or a few accessions of *S. corneliomulleri* being resistant to the TYLCV (Yan *et al*, 2018), but major genes/alleles from these species are yet to be reported. For some of these species, only a few accessions have been collected or are available in genebanks (Table 1). Among these species, some genomic studies were performed on *S. sitiens* (Chetelat *et al*, 2019) and *S. lycopersicoides* (Powell *et al*, 2022), which are potential sources of genes for adaptation to abiotic stresses (i.e. drought and heat stresses). Introgression lines were also developed from *S. chmielewskii* to study the accumulation of secondary metabolites in tomato fruit (Ballester *et al*, 2016).

## Conclusions

Now that the scientific community has access to advanced tissue culture techniques, double haploidy protocols, modern phenotyping facilities, and genomic and bioinformatic tools, tomato wild relatives could be explored even more. This may provide new sources of genetic resources and R genes that could be used to pyramid new genes into one variety leading to broad and probably more durable resistance. Furthermore, ongoing advances in sequencing technology can be used to develop reference genome sequences for undiscovered tomato wild relatives, and the development of tomato pan-genomes will be a valuable strategy in harnessing the genetic diversity of these species. Additionally, genome editing enables *de novo* domestication strategies for the targeted use of tomato relatives (Zsögön *et al*, 2018). Thus, exploring the variation in tomato wild species could be an interesting topic for future studies.

## Conflict of interest statement

The authors declare that they have no conflict of interest.

## Author contributions

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# Where access and benefit-sharing comes from: A historical overview

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**Abstract:** The international legal system of access and benefit-sharing of genetic resources (or ABS) under the Convention on Biological Diversity (CBD) is an ever-evolving field as its material, temporal and activity scope is still under discussion to meet the needs of the advancement of research and development activities as well as the questions of fairness and equity that evolve with them. Activities, such as research and development with digital sequence information (DSI), currently take considerable space in the negotiations and the lack of consensus between the Global North and the Global South continues. This paper gets its raison d'être from this lack of consensus and aims to provide a better understanding of the debate around the fair and equitable sharing of benefits arising from genetic resources as well as the sovereignty of states over their natural resources. As such, the paper provides an analysis of all relevant documents at the international level, starting from the UN Charter to the final text of the CBD with the hope of reminding the ongoing negotiations over the CBD why we have ABS in the first place and what the international community historically aimed for when regulating genetic resources at the international level. Looking back at why we had the first legally binding ABS instrument in the first place, and why we thought this instrument would achieve fairness and equity in dealing with genetic resources, will serve the interests of all Parties to the CBD and will hopefully enable them to interpret the provisions based on their overarching aim and reasoning.

**Keywords:** CBD, ABS, access and benefitsharing, Convention on Biological Diversity, benefitsharing, global multilateral benefitsharing mechanism, Nagoya Protocol, Plant Treaty negotiations, ITPGRFA, genetic resources, plant genetic resources

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## Introduction

International law provides for mechanisms for biodiversity conservation and restoration to compensate for the utilization of nature by humans. One of the major mechanisms is the fair and equitable sharing of benefits arising from the use of genetic resources (UNEP, 1992), which is a bilateral legal instrument based on state sovereignty over natural resources. The system of access and benefit-sharing (ABS) aims to fairly distribute benefits between the providers of genetic resources (such as biodiversity-rich countries) and users of genetic resources (such as biotechnology or pharmaceutical companies, universities, collections such as botanical gardens or genebanks) deriving from scientific research and development on genetic resources (GR). The ABS system prescribes the Parties to the Convention on Biological Diversity (CBD) and to the Nagoya Protocol (Secretariat of the Convention on Biodiversity, 2011) to implement national legislation on providing fair access to GR users while receiving fair and equitable benefits. States are then encouraged to channel benefits into biodiversity conservation and sustainable use.

Next to the ABS system established under the CBD, specialized ABS instruments exist on specific types of GR and their specific types of use. One of them is the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) dealing with the conservation and sustainable use of all plant genetic resources for food and agriculture (FAO, 2004). The ITPGRFA facilitates access to the genetic materials of 64 crops in the Multilateral System for research, breeding and training for food and agriculture.

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Another specialized ABS instrument is the World Health Organization Pandemic Influenza Preparedness Framework (or WHO PIP Framework). Its purpose is the sharing of H5N1 and other influenza viruses with human pandemic potential, access to vaccines and sharing of other benefits (WHO, 2021). Both the ITPGRFA and the PIP Framework function on a multilateral basis, meaning that these two specialized ABS instruments serve as pools of GR and operate under standardized agreements each Party uses. Lastly, there currently is an ongoing discussion at the international level regarding the provisions related to the bioprospecting activities taking place in areas beyond national jurisdiction under the United Nations Convention on the Law of the Sea. The negotiations are yet to be finalized, however, the issue of benefit-sharing deriving from the utilization of marine genetic resources in areas beyond national jurisdiction will be a part of this new international legal instrument (United Nations General Assembly A/RES/72/249, 2017).

ABS under the CBD, as a bilateral instrument for creating incentives for conserving biodiversity within national jurisdiction, has been an active legal concept subject to national implementation since 1992. Its success is being measured by the Global Biodiversity Outlook (Secretariat of the Convention on Biological Diversity, 2020). Additionally, ABS has found its place in several targets within the United Nations Sustainable Development Goals, such as Goal 10 Target 10a, Goal 15 Target 6, and Goal 17 Target 6. It is also highly likely that targets related to ABS will be an indispensable part of the Post-2020 Global Biodiversity Framework under the CBD (CBD/POST2020/WS/2019/8/3, 2019). Therefore, ABS provides a tangible contribution to the achievement of international targets related to biodiversity conservation.

The report published by the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) in May 2019 states that our nature and ecosystems are in rapid and constant decline (IPBES, 2019). With the current trajectories, we cannot meet global targets such as the Sustainable Development Goals. Likewise, the fifth Global Biodiversity Outlook, conducted under the auspices of the CBD to assess whether international conservation goals (such as the Aichi Global Biodiversity Targets) have been met, warns us that none of our targets has been met due to the lack of effective restoration and conservation initiatives (Secretariat of the Convention on Biological Diversity, 2020).

With the success of all global environmental targets jeopardized, it emerges the need to review international legal obligations that aim to halt biodiversity loss and thereby contribute to achieving these targets, including how ABS – being one of the major instruments in financing and incentivizing biodiversity conservation – was negotiated. This requires looking back at the original reasons and principles behind the implementation of an international access and benefit-sharing system. Additionally, the current ongoing discussions on the scope and mechanisms of the international ABS system, such as whether GR include digital sequence information (DSI), make such a retrospect even more necessary.

This paper provides a historical review of the developments at international fora that led to the negotiation and adoption of ABS provisions under the CBD, to serve as a guide for why the international community needed a bilateral ABS system in the first place. Additionally, the paper can serve for further evaluations on why ABS may or may not have achieved its anticipated objectives regarding establishing the norms of fairness and equity in dealing with GR while ensuring sustainable use and biodiversity conservation. The scope of the paper is limited to the historical events and negotiations up until the adoption of the CBD. This choice is motivated by two reasons. Firstly, the paper aims to elaborate on the discussions deriving from the concept of state sovereignty over natural resources and how sovereignty affected the use and provision of GR. Because of this, the paper solely focuses on the bilateral ABS framework under the CBD, and only refers to the ABS regime under the Food and Agriculture Organization of the United Nations (FAO) as a reference point in the historical events that led to the adoption of the ABS mechanisms under the CBD. Secondly, the paper analyzes the negotiations that led to the adoption of the CBD and not the Nagoya Protocol. This is because the paper does not aim to provide a fully comprehensive analysis of all concepts within the ABS regime generated under the CBD. It rather aims to provide a glimpse into the history of the dynamics and needs that led to the generation of the international ABS framework under the CBD starting from its establishment by the United Nations and the development of the concept of the sovereignty of states over their natural resources. These were fuelled by the aftereffects of colonization, which palpably affected the dynamics between the Global North and the Global South. Therefore, this paper does not include the analysis of the negotiations that led to the adoption of the Nagoya Protocol, nor does it include the negotiation processes and adoption of other specialized ABS instruments. Nonetheless, I wholeheartedly agree with the importance of also reviewing the post-CBD negotiations which aimed at clarifying the concept of ABS, genetic resources as well as the activities of access and benefit-sharing, their legal provenance, and their purpose. For this reason, I have previously conducted research specifically on the post-CBD negotiations that led to the adoption of the Nagoya Protocol (Sirakaya, 2022). However, the scope of the present paper is not related to the clarification of the concepts generated by the CBD, but it is related to the historical reasons why we needed these concepts in the first place.

## Sovereign rights over natural resources vs common heritage of mankind

### United Nations Biosphere Conference

Until the 1950s there existed no discussion on the provenance of GR and their utilization under international law. The first time the international community took on the subject was in 1950 during the United Nations Scientific Conference on the Conservation and Utilization of Resources. At that time, delegates acknowledged that states varied considerably in their political, economic and social institutions. Additionally, it was pointed out that world resources were not distributed proportionally to states' populations or national boundaries. Therefore, the free and full exchange of resources was seen as key for each nation to specialize in those products derived from such resources for which it enjoys the greatest comparative advantage or least comparative disadvantage. Consequently, tariffs, duties, cartels, quotas, monetary manipulations and various other political and economic devices were generally recognized as constituting the major obstacles to improved utilization of global resources. The conference had a demonstrable preference towards unrestricted access to the world's resources (as the term GR was not yet pronounced at the international level), thus neither the limits to utilization nor the subject of conservation of these resources made it on the agenda of this conference.

The introduction of the concept of states' rights over their natural resources dates to the Charter of the United Nations. The permanent sovereignty of states over their natural resources has been under discussion within the United Nations General Assembly starting from 1952. Ten years later, the United National General Assembly Resolution 1803 (XVII) (1962) was adopted, which articulated that states and international organizations shall strictly and conscientiously respect the sovereignty of peoples and nations over their natural wealth and resources in accordance with the Charter of the United Nations and the principles contained in the resolution (United Nations General Assembly A/RES/3171, 1973). These principles were set out in eight articles concerning, inter alia, the exploration, development and disposition of natural resources. The Resolution further detailed that in cases where authorization is granted for the exploration, development or deposition of these resources, the profits derived must be shared in the proportions freely agreed upon, between the investors and the recipient state. The Resolution, therefore, was the first international legal document that initiated the conversation on the states' sharing in the benefits derived from the exploration or exploitation of natural resources within their national jurisdiction.

The concerns over the conservation of the Earth's resources started building up during the 1960s when technological advances enabled humankind to develop a more enhanced understanding of the finiteness of the biosphere. The Apollo 8 mission of 1968 demonstrated to humankind the vulnerability of our planet by displaying the first photograph of the Earth ever taken from space suggesting the Earth had no other place like it anywhere close and thus was the only place life existed. This realization provided a wake-up call and moved environmentalism to mainstream international discussions (Attenborough and Hughes, 2020; Meadows and Randers, 2013)

As a response to this mainstream awakening, the United Nations Educational, Scientific and Cultural Organization (UNESCO) convened the International Biosphere Conference (IBC) in 1968. Next to being one of the first international high-level conferences that emphasized the importance of nature conservation, it was also the first international conference that introduced the concept of 'genetic resources' to policymakers. More specifically, in its recommendations related to GR utilization, the IBC suggested that special efforts had to be taken urgently to preserve the rich GR that evolved over millions of years and were being irretrievably lost as a result of human actions. Recommendations included the preservation of samples of all significant ecosystems, the establishment of special protected areas and living collections for both remnant and endangered species as well as long-domesticated species such as cereals and cattle. The IBC recommended the Member States of UNESCO, as well as FAO, to take vigorous efforts in implementing these recommended measures to avoid the loss of GR which could never be recovered.

### Stockholm Conference and Declaration 1972

During the same year as the Biosphere Conference in 1968, the Club of Rome, an informal organization consisting of scientists, members of academia, economists and civil servants started conducting a study on the factors that limited global growth. The outcome document, *Limits to Growth*, produced future projections arguing that the economic system had to be significantly altered to address the ecological capacity of the Earth (Meadows *et al*, 1972). Following these efforts, combined with the growing public awareness of global environmental problems, the United Nations General Assembly, in its meeting in 1968, agreed to organize the first international high-level conference to bring together all the United Nations bodies and Member States to generate the first global agenda for the environment. The General Assembly, in its Resolution 2398, stipulated that it was "desirable to provide a framework for comprehensive consideration within the United Nations of the problems of the human environment in order to focus the attention of Governments and public opinion on the importance and urgency of this question and also to identify those aspects of it that can only or best be solved through international cooperation and agreement." (United Nations General Assembly Resolution 2398 (XXIII), 1968) With this consensus, policymakers proceeded with the preparations for the first global conference on the environment, also known as the conference that led to the establishment of the United Nations Environment Programme (UNEP).

During the preparation of the Stockholm Conference on the Human Environment, the divide between developed and developing nations regarding their perception towards environmental problems and their willingness to take part in limiting growth became apparent to the global forum. This demonstrated that the developing world suffered from environmental problems due to poverty, and the developed world's environmental problems were related to the increased use of natural resources. The developing world initially approached the first global conference on the environment with suspicion, as they were afraid that environmental measures would result in reduced development aid and increased tariffs for products from developed countries. Nonetheless, it was apparent that the developing world also suffered from environmental problems, especially related to urbanization, and unlike the developed world, they did not have the means to deal with them. Therefore, the Stockholm Conference was the first time both parties acknowledged the interdependence of their economic development and environmental resilience (Johnson, 2012). On top of demonstrating the interdependence of the two worlds, the Stockholm Conference produced the first document introducing sovereign rights related to natural resources to the international legal arena.

Indira Ghandi's plenary speech during the conference served as a mirror to this interdependence between developing and developed nations. The speech stressed the delicate balance between environmental protection and restriction of industrial activities proposed by the developed world versus the need for economic and industrial development emanating from the developing world. Ghandi argued that, let alone conservation, the developing nations had no means of providing incentives to limit harm to nature:

> ”On the one hand the rich look askance at our continuing poverty – on the other they warn us against their own methods. We do not wish to impoverish the environment any further and yet we cannot for a moment forget the grim poverty of large numbers of people. Are not poverty and need the greatest polluters? For example, unless we are in a position to provide employment and purchasing power for the tribal people and those who live in or around our jungles, we cannot prevent them from combing the forest for food and livelihood; from poaching and from despoiling the vegetation. When they themselves feel deprived, how can we urge the preservation of animals? How can we speak to those who live in villages and in slums about keeping the oceans, the rivers and the air clean when their own lives are contaminated at the source? The environment cannot be improved in conditions of poverty. Nor can poverty be eradicated without the use of science and technology.”
>
> *Waldheim* et al *(1972)*

This speech perfectly described the need to ascertain a balance between both the needs of developing and developed countries as well as the cruciality of sharing the benefits of science and technology to eradicate poverty while conserving nature and its resources at a global scale.

Within this atmosphere, the sovereign rights of states over their natural resources became an integral part of the Stockholm Declaration. Principle 21 of the Stockholm Declaration notes that states have the sovereign right to exploit their own resources pursuant to their own environmental policies, and the responsibility to ensure that activities within their jurisdiction or control do not cause damage to the environment of other states or of areas beyond the limits of national jurisdiction (United Nations Doc. A/CONF. 48/14, 1972).

In addition, Recommendation 39 of the Stockholm Declaration (United Nations Doc. A/CONF. 48/14, 1972) requested governments, in cooperation with FAO, to agree to an international programme on preserving the world's GR by establishing an international network to survey international conservation efforts through *in situ* and *ex situ* methods. FAO took this mandate to further expand its seed collections and later to initiate the first access mechanism to these collections. The next section provides an overview of the historical development of access to GR within FAO, predating the CBD.

## CGIAR Centres and FAO Conference of 1981

The rapid global population increase after the Second World War revealed a novel need for a smarter way of ensuring food security. Many countries started suffering from food shortages and some even famine. The Indian subcontinent had undergone severe famines during the 1940s which emphasized the need for countries to be self-sufficient in food production. This resulted in increased efforts in research on major cereal crops such as maize, wheat and rice that enabled the establishment of the Consultative Group on International Agricultural Research (CGIAR). Additionally, the CGIAR Centres' objective was to take on the task FAO was mandated regarding establishing an international network to survey international conservation efforts through *in situ* and *ex situ* methods, as prescribed by Recommendation 39 of the Stockholm Declaration. In other words, CGIAR Centres were established as centres that conserve GR and ensure the genetic diversity of crops. In addition, the CGIAR Centres started research on developing new varieties, improving the yield of cereal crops as well as irrigation techniques, pesticides and fertilizers. This clustered research effort led to the 'Green Revolution', a movement started by the plant breeder Norman Borlaug, a Nobel Prize laureate who developed dwarf maize varieties that could be adapted to various climates (Mooney, 1983).

This success in plant breeding did however generate its drawbacks. The shift from traditional to industrial agriculture, based on the use of a limited number of high-yielding varieties, generated what experts called 'genetic erosion'. As a response, CGIAR Centres started establishing their own genebanks and collections to ensure the conservation of varieties for research (Moore and Tymowski, 2005).

As the CGIAR Centres genebanks and collections kept expanding in the early 1980s, questions and concerns regarding access to and ownership of the conserved varieties were increasingly raised (Rose, 2004; Mooney, 1983). Even though CGIAR Centres claimed that they were freely accessible, there existed no legal basis at the international level that ensured this. In 1981, the FAO Conference stated that there was a need to regulate access to plant genetic resources for food and agriculture (PGRFA) at the international level. This movement resulted in the adoption of the International Undertaking of PGRFA (IUPGRFA) by the FAO Conference in 1983. With this, the Commission on Genetic Resources for Food and Agriculture (CGRFA) was created to manage the operations related to plant genetic resources (PGR) (FAO, 1983).

The International Undertaking of PGRFA was the first international – yet voluntary – instrument aiming to conserve and sustainably use agricultural crops, which would then be made available for scientific research and plant breeding purposes. The International Undertaking stated that GR are a heritage of mankind but did not use the full legal terminology 'common heritage of mankind'. It emphasized, however, that PGRFA should be made available without restriction. More specifically, the Undertaking stated that PGRFA, which include those kept within the premises of CGIAR Centres, were subject to the "universally accepted principle that plant genetic resources are a heritage of mankind and consequently should be available without restriction." (Sullivan, 2004)

The reference made within IUPGRFA to 'heritage of mankind' as opposed to 'common heritage of mankind' received criticism, mainly from the Global South, as to whether the International Undertaking followed the international law principle fully and whether PGRFA were meant to be managed as public goods (Helfer, 2003). This confusion was addressed by Resolution 3/91 of the FAO Conference, stating that the heritage of mankind principle established under the International Undertaking does not contradict the states' sovereign rights over their GR, clarifying that the states are not giving up on their sovereign rights by granting unrestricted access to their PGRFA under the International Undertaking. In its original wording, it recognized that "the concept of mankind's heritage, as applied in the International Undertaking on Plant Genetic Resources, is subject to the sovereignty of the states over their plant genetic resources." (FAO Resolution 3/91, 1991)

The IUPGRFA was also the first instrument which stipulated that governments or institutions holding PGR were expected to adopt measures that would allow access to them and permit their export for the purposes of scientific research, plant breeding or conservation, adding that the samples should be made available based on mutually agreed terms.

The International Undertaking presented measures both related to *in situ* and *ex situ* conservation of PGRFA, while also emphasizing the need to establish an international cooperation structure that enabled all countries to make use of these PGRFA for the benefit of their agricultural development. The aim of making PGRFA available for further research and breeding led to the creation of an international network of genebanks and a need to clarify their legal status as well as those of CGIAR Centres. This goal would also lead to the negotiations for the adoption of the multilateral system within the ITPGRFA, which came into force in 2004.

It is worthwhile noting that PGRFA as well as the negotiations that led to the creation of the IUPGRFA and later ITPGRFA, require a further in-depth study of the dynamics that connect PGR with farmers' rights, food security, securing genetic diversity, as well as the intellectual property regime surrounding the IUPGRFA. The establishment of CGIAR Centres and adoption of IUPGRFA have been included in this article, to the extent that clarifies the mandate provided to FAO by the Stockholm Declaration. Furthermore, even though the dynamics of the FAO multilateral system and the CBD bilateral system differ to a great extent, a historical overview of the development of the ABS system under the CBD would have contained a gap without the mention of the IUPGRFA, the first international instrument dealing with facilitated access to and benefit-sharing of PGRFA.

## Convention on Biological Diversity negotiations

Following the introduction of the North-South debate at the international environmental forum during the Stockholm Conference, developing countries expanded their request for social and economic development. They perceived the necessity of emphasizing sovereignty over natural resources within their national jurisdiction, for they aimed to free themselves from the after-effects of the economic order during colonization. Not long after the Stockholm Conference, the Group of 77, a block of developing countries established during the negotiations of the United Nations Conference on Trade and Development in 1964, put forth their Declaration on the Establishment of the New International Economic Order during the UN General Assembly of 1974 (United Nations General Assembly A/RES/S-

6/3201, 1974). While acknowledging that the "interests of the developed countries and those of the developing countries can no longer be isolated from each other, that there is a close interrelationship between the prosperity of the developed countries and the growth and development of the developing countries, and that the prosperity of the international community as a whole depends upon the prosperity of its constituent parts," the Declaration requested the following to be crystallized at the international level:

> "Full permanent sovereignty of every State over its natural resources and all economic activities. In order to safeguard these resources, each State is entitled to exercise effective control over them and their exploitation with means suitable to its own situation, including the right to nationalization or transfer of ownership to its nationals, this right being an expression of the full permanent sovereignty of the State. No State may be subjected to economic, political or any other type of coercion to prevent the free and full exercise of this inalienable right".
>
> *United Nations General Assembly A/RES/S-6/3201 (1974)*

The Declaration proved impactful as the General Assembly adopted Resolution 3281 (XXIX) containing the Charter of Economic Rights and Duties of States, reinstating the sovereignty of states over their natural resources (United Nations General Assembly Resolution 3281 (XXIX), 1974).

The repeated affirmations over the sovereign rights of states, as well as bringing the provenance of GR within CGIAR centres to a legal basis via the IUPGRFA, seemed to have provided temporary confidence to the developing world regarding how fair the global system on GR was (Shackelford, 2008). The industrialization of agriculture in developed countries resulted in the privatization of the sector, which began relying on the sales of seeds and other agricultural products for profit. As a result, the importance of intellectual property (IP) rights in the agricultural sector gained increasing importance. The International Union for the Protection of New Varieties of Plants (UPOV) Convention, adopted in 1961 (UPOV, 1961), followed this trend, aiming at encouraging plant breeding by means of breeder's rights, a sui generis form of an IP right specifically designed for plant breeders. In the case of a variety protected by a breeder's right, the breeder's authorization is required to propagate the variety for commercial purposes except for when the variety is utilized for further breeding (also known as breeder's exemption), for experimental purposes and private and non-commercial purposes as specified in Article 15(1) as compulsory exceptions. States are also invited to consider allowing for an optional exception for farmers saving seeds as specified in Article 15(2), also known as farmer's privilege (Lawson, 2015). The revisions to UPOV in 1972 and 1978 were argued to strengthen breeders' rights and diminish farmers' rights to sell, exchange or harvest seeds from protected varieties, which further raised concerns within the Global South (Tripp *et al*, 2007).

The International Undertaking was implemented specifically to curb these concerns. While serving to do so, the IUPGRFA instead raised concerns in the developed world as the seed industry expressed its worry about the definition of PGRFA. According to the seed industry, the broad definition of PGRFA would result in the necessity of making privately owned plant varieties and special genetic stocks available without restrictions. Following these concerns, a group of developed countries including Canada, France, Germany, Japan, New Zealand, Switzerland, the United Kingdom and the United States of America made reservations about the IUPGRFA arguing for the recognition of intellectual property therein (Ten-Kate and Diaz, 1997). At the same time, developing countries, under the auspices of FAO, exclaimed that the IUPGRFA did not recognize nor reward the contributions of developing countries and their farmers to the conservation and availability of PGR (Moore and Tymowski, 2005). In an attempt to calm the waters and satisfy both sides, FAO adopted a resolution with an amendment to the IUPGRFA. The resolution stated that "plant genetic resources are a common heritage of mankind to be preserved and to be freely available for use, for the benefit of present and future generations" (FAO Resolution 4/89, 1989). It also clarified that this would not extend to the protection of plant breeders' rights within UPOV, allowing the industry to exclude their varieties from the common heritage system of the IUPGRFA. This caused the need for an additional resolution from FAO (FAO Resolution 5/89, 1989) to accentuate that farmers in all countries should be able to "participate fully in the benefits derived, at present and in the future, from the improved use of plant genetic resources, through plant breeding and other scientific methods". The CBD negotiations, therefore, began in a tense atmosphere escalating both in the Global North and the Global South. On the one hand, the North aimed at conserving biodiversity via CGIAR centres, as well as conserving the rights of their rapidly evolving biotechnology sector. On the other hand, the Global South expressed its concerns regarding the IP rights over GR gaining power while establishing the initial global understanding of the need to create a mechanism to share in the benefits of development achieved through the use of GR. The Global South believed that the common heritage of mankind over GR allowed the Global North to rely on the resources of the Global South to maintain their economic prosperity.

In 1988, the Ad Hoc Working Group of Experts on Biological Diversity (AHWG), mandated by UNEP, convened for the first time to discuss the desirability and feasibility of an international framework agreement on the conservation and sustainable use of biological diversity. The Working Group agreed that the question of access, including the question of free access, to GR should be studied, yet they did not reach a consensus on the notion of biological diversity as a common resource of mankind (UNEP/Bio.Div.1/3, 1989). Additionally, the Working Group agreed that the question of placing an economic value on biological resources should be examined in detail.

The second meeting of the AHWG, convened in 1990, was opened by Dr M. K. Tolba, the Executive Director of UNEP. In his speech, he paid due attention to the preferential treatment for those having jurisdiction and control over GR with respect to genebanks containing them and to essential newly developed varieties obtained through breeding. He also emphasized the international transfer of and favourable access to biotechnology that could be usefully applied or adapted to developing countries needs (UNEP/Bio.Div.2/3, 1990). It is visible from his speech that access to GR and access to technology were regarded as two separate subjects, yet interdependent, to be dealt with rather than access to technology as a result of or deriving from access to GR (UNEP/Bio.Div.2/3, 1990). In fact, at this point, access did not only relate to access to GR but also to technology. The AHWG emphasized that “accessibility to biological diversity, including new varieties, and to related technologies, including conservation technologies, are two sides of one and the same coin and must be an integral part of the planned legal instrument.” Subsequently, it became clear to the Working Group that the issue of IP rights relating to the ownership of biotechnology and both the provision of access to GR from biodiversity-rich countries and the provision of access to technology from technology-rich countries needed to be reviewed.

Dr Tolba stipulated that “any new international agreement should not infringe upon the sovereignty of nation States over their natural resources. It must protect the interests of the States in which the resources are located and provide incentives for conservation of biological diversity without inhibiting growth or sustainable development.” (UNEP/Bio.Div.2/3, 1990)

The AHWG further discussed the common heritage principle over GR and agreed that this principle did not mean the establishment of collective international rights to resources within national jurisdictions, nor did it infringe upon the permanent sovereignty of states over natural resources. The Group underlined that free access did not mean access free of charge and accessibility should be based on mutual agreement and full respect for the permanent sovereignty of states over their natural resources. Additionally, the AHWG agreed that those having jurisdiction and control over GR should receive preferential treatment for access to their germplasm and varieties developed from these resources.

The second meeting of the AHWG discussed the two types of access and the compensation mechanisms for the provision of access and technology. The experts stated that biotechnology could assist in the conservation of GR which could be funded by enterprises that profit from the use of biotechnology. The AHWG suggested that this could be in the form of a tax to support conservation as well as biotechnology research in developing countries. Additionally, the Working Group discussed that developers of biotechnology would require compensation for the provision of access to their technology.

Regarding the relationship of access to GR with to-be established financial mechanisms for the conservation of biological diversity, the report of the AHWG expressed that there was a consensus that “those who enjoy most the economic benefits of biological diversity should contribute equitably to its conservation and sustainable management.” Some of the delegates argued for the potential of biotechnology to foster species conservation by means of adhering an immediate economic value to them and “if developing countries are given the capacity to develop and share the benefits of their biological diversity this will be a good incentive for natural resources conservation.” (UNEP/Bio.Div.2/3, 1990)

The third meeting of the AHWG discussed the draft text of the CBD prepared by the International Union for Nature Conservation (IUCN) (UNEP/Bio.Div.3/12, 1990). The Working Group commissioned a study on the relationship between IP rights and access to GR. The report did not discuss what type of access measures could be implemented by provider countries, as it was presupposed that free access would remain the norm since the experts leaned on discouraging any measures including the amendment to the UPOV Convention that would hinder free access to GR (UNEP/Bio.Div.3/6, 1990). Under paragraph 5 of the report, the experts strongly emphasized the ‘undeniable’ importance of the principle of free access and argued that the IUCN draft should not be allowed to result in a closing up of the system, for that would be against everyone’s interests.

During the time of the second meeting of the Ad Hoc Working Group, discussions were ongoing on the revision of the UPOV Convention. The draft revision of the UPOV Convention introduced the concept of dependence, which meant that a variety ‘essentially derived’ from another variety protected by plant breeder’s rights cannot be used commercially without the permission of the breeder of the protected variety. At the same time, it introduced patent coverage over living matter under the agreements of the General Agreement on Tariffs and Trade (GATT).

During the meeting, the AHWG debated that the perception of the value of biological diversity was altering as the development of biotechnology enabled humanity to potentially create technological advancements out of any organism. Therefore, the zones of biological diversity which were perceived to have no economic value, were presently considered to contain value that reflected the potential of GR contained in them (UNEP/Bio.Div.3/3, 1990).

The initial thought about sharing benefits had to do with technology transfer to improve the research capacity of developing countries. The study commissioned by AHWG on the relationship between IP rights and GR explicitly disapproved of monetary compensation for the costs of conservation (UNEP/Bio.Div.3/Inf.4, 1990). It rather supported what was described as compensation mechanisms along the lines of technology-for-nature swaps. The study also argued that developments within UPOV on restricting access to varieties were "disturbing because free access (which she [the expert consultant] stressed did not mean free of charge) had been one of the essential factors in advances in genetics." (UNEP/Bio.Div.3/12, 1990) It was recommended that the UPOV Convention would harness IP rights with the aim of ensuring the more efficient use of biodiversity and also recommended a system of 'paid open access' yet warned that such a system could be endangered by the extension of patent law.

Another study on biotechnology commissioned during the third meeting argued that the AHWG should be wary of the fact that genetic material in private collections and information thereof will not be as easily available as the information on material in public sector genebanks and that the GR collected by seed companies were not likely to be freely exchanged and might be considered as trade secrets (UNEP/Bio.Div.3/7, 1990).

The AHWG lastly commissioned a study on possible financial mechanisms for the conservation of biological diversity (UNEP/Bio.Div.3/5, 1990). As the study underlined that "the market prices of the genetic resources and functions do not reflect their real scarcity value or the ecological costs incurred by their use," it also suggested the establishment of an international multilateral fund to enable their conservation and discourage their excessive use. From the wording of this report, it could be understood that GRs were rather seen as physical, biological material whose excess use would result in negative ecological consequences. The Working Group suggested that contributions to the funding mechanism might be provided by the Parties on an assessed basis. More specifically, "the scale of assessment could be related to a United Nations scale, industrial and commercial exploitation of or trade in genetic resources or on some other equitable basis."

Following the third meeting, a Sub-working Group on Biotechnology (SWG) gathered in November 1990. Under Annex 1 regarding Possible Additional Elements for a Biotechnology Component in a Global Framework Legal Instrument on Biological Diversity, the SWG considered the inclusion of the equitable sharing of the economic benefits derived from biotechnology with the country of origin of the biomaterials used (UNEP/Bio.Div/SWGB.1/5/Rev.1, 1990). Hence, the discussions introduced monetary benefit-sharing arising from the utilization of GR for the first time, despite the previous recommendations discouraging them.

The SWG furthermore added that access to biological diversity should be based on agreements conforming with the sovereign right of states over their natural resources within their national jurisdiction. The SWG stated that access to GR as well as access to technology would not be free of charge and should be based on mutual agreement (UNEP/Bio.Div/SWGB.1/5/Rev.1, 1990). In order to enhance the contribution of biotechnology to the conservation of biodiversity, it was urgently recommended to "increase the numbers of botanical gardens, seed banks and other *ex-situ* conservation facilities in various areas throughout the world, particularly in tropical areas, and to broaden the coverage of existing ones." Consequently, the SWG has foreseen conservation via biotechnology through research and inventory on biodiversity and its conservation. It was not mentioned how shared economic benefits arising from GR utilization could enhance or how it would incentivize conservation.

Shortly after the meeting of the SWG on Biotechnology, the AHWG of Legal and Technical Experts on Biological Diversity gathered for its first meeting, in November 1990 (UNEP/Bio.Div/WG.2/2/5, 1991). This meeting discussed the first CBD draft text, which was rather contested and heavily bracketed (Lawson, 2015). The preamble of the text included the obligation of states to share in any increased knowledge as well as other benefits of the potential of biological diversity amongst bracketed suggestions for various wording such as "equitable sharing of benefits and conservation costs of biological diversity" or "the benefits derived from utilization and the cost of conservation of biological diversity should be shared" next to bracketed clarifications that free access does not mean free of charge. Title VI of the draft text covered access to biological diversity under the same title as access to technology and information thereon. The commentary to this title discussed the details on which types of technology should be subject to access, and regarding the availability of both biological diversity and technology. The text also brought up the role of IP rights regarding these two types of access.

Title VII drafted the heavily bracketed obligation directed at developed countries to transfer technology – that supported biological diversity conservation and sustainable use – to developing countries on a non-commercial and preferential basis. The text also included options for research cooperation between developing and developed countries on scientific research and training, and joint ventures, taking into account the investments made by the private sector to develop these technologies as well as the possibility of establishing a mechanism to "ensure the acquisition of technology from the technology-rich states to the gene-rich developing countries by providing funds to facilitate the necessary access to patents".

At his opening speech for the second meeting of the AHWG of Legal and Technical Experts on Biological Diversity between 25 February and 6 March 1991, Dr Tolba, the Executive Director of UNEP, stated that access to biodiversity and the availability of biotechnology and other technology relevant to the rational use of biological resources were complementary and inseparable (UNEP/Bio.Div/WG.2/2/5, 1991). He continued that states should receive fair compensation for the provision of access and at the same time, the private sector should receive fair compensation for participating in technology transfer arrangements.

Another SWG on Biotechnology gathered during the second meeting to discuss issues related to access to GR. The SWG discussed the possibility of introducing a prior informed consent mechanism to ensure that access to biological diversity would not endanger viable populations as well as to reflect the sovereignty of states over their GR. The SWG added that access should not be regulated in a manner that resulted in blanket prevention of access. While there was a general consensus on the importance of access to technology for sustainable GR utilization, some delegates in the SWG further requested the inclusion of GR utilization for other purposes such as pharmaceuticals in relation to transfer of technology to developing countries within the framework of the Convention. Some delegations requested further assessment of the transfer of both 'hard' (e.g. computers) and 'soft' (e.g. training) technologies and that technology transfer should not be specifically confined to biotechnology. Additionally, some delegates argued that countries of origin of genetic material shall have equitable and/or preferential access to the benefits and profits arising from commercial exploitation thereof. Regarding the question of how to financially incentivize all these activities, the SWG could not come to an agreement. Some delegates suggested the idea of a multilateral mechanism with a multitude of funding sources whereas some suggested bilateral settings. The SWG, as later clarified in July 1991, defined access as "the right and/or means of acquiring biological resource or technology that can exploit the resource as well as relevant information and know-how, for scientific, commercial or other purposes on conditions agreed upon multilaterally or bilaterally." (UNEP/Bio.Div./WG.2/3/6, 1991) Access to biological diversity was defined to include both physical access to the genetic material and access to information about the genetic material. Access to technology, on the other hand, was defined as access to know-how relevant to the conservation and sustainable use of biological diversity.

During the same meeting, a Multilateral Trust Fund was proposed to undertake the following activities:

"(a) to make money grants to habitat countries to enable these countries to undertake *in situ* or *ex situ* conservation of ecosystems and species;

(b) to provide fair compensation to habitat countries for the use of their genetic resources;

(c) to provide financial assistance to habitat countries to enable them to reach a technological, educational and training level that will facilitate national programmes for the conservation of biological diversity;

(d) to provide financial assistance to habitat countries to enable them to conduct ecological surveys and to monitor technical assistance and strengthen relevant legal instruments for the conservation of biological diversity." (UNEP/Bio.Div/WG.2/3/8, 1991)

In between the second and third CBD negotiating sessions, the first bioprospecting agreement was signed between a provider country and an industrial user. In September 1991, Costa Rica's National Biodiversity Institute (INBio), a private non-governmental entity, and Merck & Co., Ltd, a pharmaceutical company based in the United States announced the freshly concluded bioprospecting agreement. According to the contract, INBio would provide Merck with chemical extracts from wild plants, insects and microorganisms from Costa Rica's conserved wildlands to be used for Merck's drug-screening programme in return for a two-year research and sampling budget of US$1,135,000 and royalties on any commercial products resulting from the use of the samples. INBio agreed to contribute 10% of the budget and 50% of any royalties to the government's National Park Fund for the conservation of national parks in Costa Rica, and Merck agreed to provide technical assistance and training to help establish drug research capacity in Costa Rica (Aldhous, 1991).

This was the first agreement serving the discussions on the economic value of biodiversity as well as its ability to demonstrate how companies can agree to return a portion of the benefits of commercial development to the developing country where GR were accessed (Reid *et al*, 1993).

Following Dr Tolba's recommendation on merging negotiations related to biodiversity and biotechnology, the AHWG was mandated to negotiate both matters and was renamed the Intergovernmental Negotiating Committee (INC). After the Third Negotiating Session/First Meeting of the INC for a CBD, which lacked sufficient progress, the INC met for its fourth session between 23 September and 2 October 1991 (UNEP/Bio.Div/N4-INC.2/5, 1991). The opening speech contained considerations on the negotiations related to the General Agreement on Tariffs and Trade (GATT). This was followed up by CGIAR Centres arguing they should be allowed to freely sell their genetic material to the private sector without having to share profits with GR providers. Likewise, it was contended by some members of the private sector that, if biotechnologies were transferred to developing countries, these developing countries would only be allowed to market their products locally which would constitute a disincentive to developing countries to acquire biotechnology. This was followed up by a speech by Dr Tolba, who shared the estimates of the Global Environmental Fund (GEF) on the financial cost of biodiversity conservation ranging from $500 million to $50 billion per year. He added that the countries with the richest biodiversity were also the ones least able to afford conservation measures and followed up by stating that the proposed economic system of access to resources increasingly depended on the activity of access to biological diversity, yet the means of assessing the value of biodiversity were lacking. Secondly, regarding access to technology, he contended that "... progress was measured in terms of development and use of sophisticated technologies, yet the way in which new technologies were regulated hindered their dissemination where they were most urgently needed. One hundred or perhaps more species were being made extinct every day as a result of human action. Intensified scientific monitoring and assessment would help to fill in the gaps in knowledge, but it would take years if not decades." (UNEP/Bio.Div/N4-INC.2/5, 1991)

The INC adopted Article 14bis on 'traditional indigenous and local knowledge' in addition to access to GR. The bracketed sentence requested Contracting Parties to acknowledge the contribution of this knowledge to biodiversity conservation and sustainable use and that they should endeavour to reflect the intrinsic economic value of this knowledge within national policies and legislative decisions. Additionally, a less-bracketed version of Article 15 on access to technology was adopted, which was complemented by Article 16 on technology transfer and Article 17 on scientific cooperation, both of which were still heavily bracketed (UNEP/Bio.Div/N4/INC.2, 1991). Article 14 on access to GR was not further discussed in this session.

At the fifth session of negotiations, which took place between 25 November and 4 December 1991, Dr Tolba informed the INC of a positive development, reading the statement recently made by the Netherlands on behalf of the European Community during the United Nations General Assembly. The representative indicated that "the industrialized countries, recognizing their responsibility towards the environment, should commit themselves to reducing the burden they imposed upon it, to the extent of their legitimate share." (UNEP/Bio.Div/N5-INC.3/4, 1991)

This session did not further discuss the above-mentioned draft articles related to access to biological diversity and access to technology. The INC, however, released a document regarding the interpretation of the words fair and favourable, fair and most favourable, equitable, preferential and non-commercial, preferential, non-commercial at the relevant international fora (UNEP/Bio.Div/N5-INC3/3, 1991).

During the sixth negotiating session, gathered from 6 to 15 February 1992, the INC prioritized the discussions related to financial resources, new and additional ones, mechanisms to review and manage those financial resources, access to genetic resources, fair distribution of benefits arising from the use of those resources, fair and favourable conditions for access to technology by developing countries, the question of biotechnology, the question of commitments by developed and developing countries, as well as national regulations and policies in dealing with biological resources at the national level. While the brackets from Article 14 were largely removed and Article 14bis was reformulated as Article 7(j), Article 15 on access to technology got merged with Article 16 on technology transfer, which resulted largely in the removal of considerations related to IP rights restricting access to technology. At this point, Article 16 did not contain as strong provisions on access to technology compared to the previous draft as it became less clear what access to technology or transfer of technology stood for. Furthermore, the article read more as a mere recommendation than an obligation (UNEP/Bio.Div/N6-INC4/4, 1992). The seventh and final negotiating session held by the INC between 11 and 19 May 1992, renamed Article 14 as Article 16 and Article 15 as Article 17 (UNEP/Bio.Div/N7-INC5/2, 1992).

After these drafts, the official documentation does not provide information on how these Articles were renegotiated and what the reason behind removing the brackets and deleting certain sentences was. Regarding the final negotiating environment during the United Nations Conference on Environment and Development (also known as the Rio Conference) in 1992, Parson, Haas and Levy state the following:

> "The negotiations were plagued by the conflict over the financial mechanism, the sharing of benefits, and biotechnology regulation. France originally threatened not to sign the treaty because it did not include a list of global biodiversity-rich regions; Japan threatened not to sign because it feared biotechnology regulation. At the last moment, both relented, and only the United States refused to sign the treaty because officials felt that the financial mechanism represented an open-ended commitment with insufficient oversight and control; that the benefit-sharing provisions were incompatible with existing international regimes for intellectual property rights; and that the requirement to regulate the biotechnology industry would needlessly stifle innovation."
>
> *Parson* et al *(1992)*

The Rio Conference adopted several international environmental treaties, including the CBD. In addition, the Conference also adopted the first non-binding action plan of the United Nations with regard to sustainable development, also known as Agenda 21 (United Nations Conference on Environment and Development, 1992), which contained the following paragraph on sovereign rights of states over their GR:

> "Governments should [...] develop measures and arrangements to implement the rights of countries of origin of genetic resources or countries providing genetic resources, as defined in the CBD, particularly developing coun-

> tries, to benefit from the biotechnological development and the commercial utilization of products derived from such resources."

## Convention on Biological Diversity

The provisions of the CBD (UNEP, 1992) originate from its three overarching objectives, which are:

- Conservation of biological diversity
- Sustainable use of the components of biodiversity
- Fair and equitable sharing of benefits arising from GR

Article 15 of the CBD reaffirms the states' sovereign rights over their GR. This means that states have the right to regulate access to their GR, which includes the right to determine the conditions of such access and the fair and equitable benefit-sharing resulting from the utilization of GR (Kamau and Winter, 2013). Article 15 paragraphs 3, 4 and 5 of the CBD stipulate that the access granted by a provider country shall be subject to prior informed consent (PIC) and mutually agreed terms (MAT) unless otherwise determined by the provider country. The CBD defined the key principles of a bilateral ABS system between users and provider countries.

Article 15 of the CBD reaffirms the states' sovereign rights over their GR. This means that states have the right to regulate access to their GR, which includes the right to determine the conditions of such access and the fair and equitable benefit-sharing resulting from the utilization of GR (Kamau and Winter, 2013). Article 15 paragraphs 3, 4 and 5 of the CBD stipulate that the access granted by a provider country shall be subject to prior informed consent (PIC) and mutually agreed terms (MAT) unless otherwise determined by the provider country. The CBD defined the key principles of a bilateral ABS system between users and provider countries.

Article 2 of the CBD defines GR as "genetic material of actual or potential value." According to the same Article, genetic material is defined as "any material of plant, animal, microbial or other origin containing functional units of heredity." The right to determine the conditions of access and benefit-sharing of GR is given to the country of origin as well as the country providing GR. Article 2 of the CBD defines the former as "the country which possesses those genetic resources in *in-situ* conditions" and the latter as "the country supplying genetic resources collected from *in-situ* sources, including populations of both wild and domesticated species, or taken from *ex-situ* sources, which may or may not have originated in that country". In relation to the latter definition, the CBD Article 2 defines domesticated or cultivated species as "species in which the evolutionary process has been influenced by humans to meet their needs." This definition is important due to the fact that the CBD also considers countries as providers of those GR that "have existed for some time away from their *in-situ* conditions and have become part of new natural and cultured ecosystems." This article, therefore, refers to two situations under the definition of provider countries. Kamau and Winter (2013) consider this type of GR provider as first-level providers. The latter definition, however, also includes providers of GR from *ex situ* sources, meaning that these resources are kept and conserved outside of their natural habitat. Kamau and Winter consider this type of GR provider as second-level providers. The second-level providers become such by either rightfully obtaining GR from the country of origin (e.g. by entering into PIC or MAT when these are required by law) or by having obtained these resources before 29 December 1993 when the CBD came into force.

Article 8(j) of the CBD states that subject to their national legislation, states should promote the equitable sharing of benefits arising from the utilization of innovations and practices of indigenous and local communities. However, the CBD does not contain a definition and further description of traditional knowledge.

Article 15 and 8(j) are the two main provisions of the CBD relating to ABS. Several other articles of the CBD complement these provisions such as Article 16 on access to and transfer of technology, Article 17 on exchange of information, Article 18 on technical and scientific cooperation, Article 19/1 and 19/2 on biotechnology and distribution of its benefits, Article 20 on financial resources and Article 21 on a financial mechanism.

Article 16 is an important element in understanding the North-South debate that led to the insertion of the third objective of the CBD on fair and equitable sharing of benefits. As explained under the CBD Negotiations section of this article access, in the early drafts of the CBD, was defined as access to biodiversity and technology. This definition did not find its place in the final text that got adopted. Access to technology as an obligation was drafted as a separate article and perceived as crucial in establishing fairness and equity as access to GR would. According to this, governments would have to establish legislative measures to encourage the private sector to provide access to technology based on mutually agreed terms, and, in accordance with international obligations, refrain from imposing restrictions (such as IP rights).

Article 16 obliges Contracting Parties to provide and/or facilitate technologies relevant to the conservation of biological diversity and sustainable use of its components or technologies that make use of GR. As neither the act to "provide" nor to "facilitate" are defined, Contracting Parties have sizeable flexibility in implementing this obligation. According to Glowka *et al* (1994), this could mean the provision of technologies within the public domain. Regarding technology transfer, Article 16(3) states that the Contracting Parties (be it developing or developed states) are obliged to create a framework permitting the transfer of technologies making use of GR. Consequently, the obligation is not transferring technology yet merely creating the conditions enabling the transfer of technology, making what was an equal return for accessing GR in the beginning, a voluntary scheme in the end.

## Discussion and conclusion

The history behind the CBD negotiations demonstrates that the need to reinstate sovereign rights of states over their natural resources emanates from the Global North vs Global South debate on inequality resulting from the aftereffects of colonialism. The Global North, or the developed countries, have been historically able to develop products, processes and technologies potentially beneficial to humanity as a whole, by utilizing the genetic resources the Global South, or the developing countries had. In other words, the Global South provided the resources, and the Global North provided the technology for the development of the global society. Nevertheless, the North traded the products developed with the GR of the Global South, yet the Global South had not participated in the benefits of these products. This resulted in the perception of inequality which paved the way for the first decision on state sovereignty on GR under the Stockholm Declaration. The mandate deriving from the Stockholm Declaration initiated the attempts of FAO to establish CGIAR Centres and the first global ABS instrument (though voluntary) under the IUPGRFA. Additionally, the North during the Stockholm Conference emphasized the need to conserve the environment, whereas the Global South underlined that the poverty they were suffering would not enable them to make funds available for conservation as they had overarching priorities related to basic human needs. Therefore, the need for a financing mechanism to allow the Global South to conserve its resources became visible. Moreover, biotechnology was seen as a key to overcoming food crises and poverty, and a solution to the global decline in biodiversity.

The relief the IUPGRFA provided as a multilateral benefit-sharing system operating under the common heritage principle soon lost its power due to the mistrust elevated by IP discussions under the UPOV Convention regarding GR. The Global South believed IP rights and privatization of GR through the storage thereof in private collections would deem the Undertaking obsolete and undermine its free access principle. The Global North, on the other hand, sustained its claims for the amendment of the UPOV Convention for IP rights strengthening as their agricultural sector depended on the sales of seeds and other value-added products developed with biotechnology. With this tension, the negotiations for an international instrument on biodiversity conservation began. Separate from the attempts under the CBD, FAO further developed the multilateral benefit-sharing system of PGRFA firstly by its amendments and later by adopting the ITPGRFA to alleviate the concerns of the Global South, especially risen after the amendment of the UPOV Convention.

During the negotiations on the CBD, the AHWG initially agreed that access should not be restricted, benefit-sharing should be based on technology-for-nature swaps and that money as such would not make up for a benefit that would be mutually beneficial. The AHWG received pressure from the South on basing the CBD on the sovereign rights of states over their GR. The first CBD drafts reflected this demand and also the opinion of the AHWG on the importance of access to technology as well as to GR. Therefore, access in the context of ABS, at that stage, meant access to GR and access to technology. The North had concerns that this would overrule their IP rights on such technologies. The AHWG gathered experts to look into financing mechanisms that would serve as incentives for both access to GR and access to technology. The experts suggested the option of a multilateral benefit-sharing mechanism by which both the biodiversity-rich countries and owners of technology would be compensated for the provision of their assets. Additionally, it was suggested that the fund would provide compensation for access to provider countries by means of funding conservation projects in those countries. On the contrary, some delegations supported a bilateral negotiation mechanism by means of acquiring PIC and negotiating MAT. Both these options made it to the final text, however, the PIC and MAT appeared in the very article on access to GR, whereas the financing mechanism through a multilateral system was indirectly made an option through Article 21.

As for the definition and conceptualization of GR, it is visible from the early international documents that GR were perceived as a tangible, physical source that can potentially be subject to overexploitation. During the CBD negotiations, it was further stated that access to biodiversity meant access to GR and the information related to them. Nonetheless, neither a definition of access, nor a definition of benefit-sharing have been included in the CBD final text.

The overarching aim of the CBD is the conservation of biological diversity. However, the Global South, starting from the preparations for the Stockholm Conference, called out the lack of ability to conserve biodiversity within their territories due to the continuing gap between development and technology as well as economic advancement. During the negotiations, next to restoring justice within access to resources vs technology equilibrium, several discussions took place on how sharing of benefits would allow incentivizing biodiversity conservation. The SWG on biotechnology assumed that applications of biodiversity on GR such as research and creating inventories of GR would result in the conservation of biodiversity. It was however not clarified how sharing any other types of benefits would create incentives for biodiversity conservation. A very important note here, which surely has passed the test of time since the CBD negotiations, is that many countries of the Global South still lack the ability, economic means and technology to be able to utilize and conserve GR. The CBD originally started as an equal exchange between GR and technology, yet the negotiations as well as concerns of the Global North over the provenance of IP rights resulted in a final text unclear in its motivations, especially regarding the connection between Articles 15 on access to genetic resources and Articles 16 to 21 on transfer of technology and capacity building. Arguably, the emphasis on Article 15 in the ABS realm resulted in an international bilateral ABS framework under the CBD that is developed heavily around the concept of access and weaker around the concept of benefit-sharing. To this day, this reflects on the current discussions as the persisting lack of trust in the ABS system since the benefits of the ABS system are still blurry to many.

In other words, the negotiations to the CBD aimed at introducing fairness and equity into innovation with biotechnology, as well as incentivizing conservation. However, it is doubtful whether the PIC and MAT mechanisms enabled provider countries to acquire the technology and know-how to become users of GR themselves, nor is it clear whether provider countries have been sufficiently financially incentivized with the ABS mechanism the CBD introduced to the international legal realm. By analyzing the historical developments and negotiation documents that led to the CBD, this paper displays the narratives and needs of the Global North and the Global South with the hope of serving as guidance to the negotiations of further clarifications to the ABS system.

Although many concepts have evolved throughout the two decades since the adoption of the ABS system under the CBD, there are many lessons to be recalled regarding the tensions between the Global North and the Global South which persist today. For example, the visible demonstration of this tension often takes over the DSI debate. One of the more topical and lasting conclusions of this paper is that the current debates need to acknowledge the fact that the technological and economic prosperity gap between the Global North and the Global South, most likely on another level than in the late 1980s and early 1990s, continues to impact the prominence of the ABS system and any novel concept which evolved with the current technological advancements, relevant to bioprospecting. Without an effective solution addressing this underlying tension, the Global North and the Global South will continue to disagree on how to address the global biodiversity crisis and environmental justice, which requires all Parties to act evermore sooner than later.

## Conflict of interest statement

The author declares that there exists no conflict of interest between the author's role as a postdoctoral researcher/legal advisor and the topics covered under this article.

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