



# Animal

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## Genomic characterisation and diversity assessment of eight endangered Belgian sheep breeds



R. Meyermans<sup>\*</sup>, W. Gorssen, N. Aerts, K. Hooyberghs, B. Chakkingal Bhaskaran, L. Chapard, N. Buys, S. Janssens

Center for Animal Breeding and Genetics, Department of Biosystems, KU Leuven, Kasteelpark Arenberg 30 – 2472, 3001 Leuven, Belgium

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### ABSTRACT

Assessing the genetic diversity of local breeds is essential for conserving these unique breeds, which may possess unique traits. This study provides the genomic characterisation of eight indigenous sheep breeds in Belgium based on pedigree and single nucleotide polymorphism (SNP) analysis. A total of 687 sheep were genotyped and were subjected to a rigorous quality control, resulting in a set of 45 978 autosomal SNPs. Pedigree analysis showed breed-average inbreeding estimates between 3.3% and 11.3%. The genomic analysis included an assessment of runs of homozygosity (ROH) to examine the genomic inbreeding coefficient, with breed-average inbreeding coefficients estimated between 4.1% and 8.5%. Runs of homozygosity islands were identified in six of the eight breeds studied, with some exhibiting an incidence of up to 58%. Interestingly, several ROH islands overlapped with other breeds included in this study, as well as with international sheep breeds. Pedigree-based effective population sizes were estimated below 100 for all breeds, whereas genomic-based effective population sizes were below 24, indicating that these eight local sheep breeds are endangered. Principal component analysis, admixture analyses, and  $F_{st}$  computations were used to study the population structure and genetic differences. A neighbour-joining tree using 95 international sheep breeds positioned the eight local breeds in the group of milk sheep, Texel sheep and the Scandinavian breeds. Additionally, the investigation of paternal *oY1* genotypes revealed diverse lineage origins within the Belgian sheep population. This study refines and deepens our knowledge about the local sheep breeds in Belgium, thereby improving their management and conservation. Moreover, as these breeds are linked to other international breeds, these insights are significant for the global scientific community.

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### Implications

Screening genetic diversity of local livestock breeds is of significant importance for their conservation. Currently, 85% of global sheep populations are endangered or their status is unknown. This study evaluates for the first time the genetic diversity of eight Belgian sheep breeds using both pedigree and genomic information. By assessing effective population sizes and inbreeding coefficients, we found that all eight breeds were considered endangered. Furthermore, we positioned these eight Belgian sheep breeds within the context of other international sheep breeds. These findings will serve as a valuable resource for international researchers exploring the genetic connections among diverse sheep breeds.

### Introduction

Conserving genetic diversity in local livestock breeds is crucial (Groeneveld et al., 2010). Local breeds often suffer from decreasing population sizes, which threatens their genetic variation (Groeneveld et al., 2010; Meuwissen and Oldenbroek, 2017). The diversity that is present in local breeds equips them with the ability to adapt to environmental changes and resist diseases, a capability that is increasingly crucial in the face of climate change (Hoffmann, 2010; Meuwissen and Oldenbroek, 2017). Local breeds can significantly contribute to climate mitigation efforts not only in extreme environments such as arid or alpine regions (Hoving et al., 2014; Naqvi et al., 2017) but also in temperate climates like Western Europe (Medugorac et al., 2011). Moreover, local breeds often harbour unique characteristics adapted to their local climate and can be invaluable in sustainable and resilient farming systems (FAO, 2013). If the conservation of these (genetic) characteristics is not guaranteed, they may fail to exist for future generations

<sup>\*</sup> Corresponding author.

E-mail address: [roel.meyermans@kuleuven.be](mailto:roel.meyermans@kuleuven.be) (R. Meyermans).

(Meuwissen and Oldenbroek, 2017). Moreover, local sheep breeds often play an important role in the cultural heritage and can play a pivotal role in local economies through the production of unique wool, meat, and dairy products, as well as the maintenance of landscapes and biodiversity (Meuwissen and Oldenbroek, 2017). The Food and Agriculture Organisation reports that only 15% of all known sheep breeds are “not at risk”, whereas 4% are considered vulnerable, 14% are considered endangered, 9% are considered critical, 11% are considered extinct and the status is unknown for 47% (FAO DAD-IS, 2024). These data imply that the diversity of 85% of all breeds registered by the Food and Agriculture Organisation is under threat. It underscores the necessity of not only monitoring known populations but also researching previously unstudied sheep breeds (Meuwissen and Oldenbroek, 2017). Furthermore, for effective global monitoring, it is crucial that national coordinators regularly report population statistics to the Food and Agriculture Organisation.

A comprehensive way to study genetic diversity in livestock is by using both pedigree and genomic data. Correct and deep pedigree data provide insights into the historical lineage of a breed and the (assumed) relatedness between animals within a breed. Genomic data offer direct evidence of the existing diversity levels within a breed as well as the similarities and distinctions between various breeds. Nowadays, genomic data are commonly acquired by using cost-effective single nucleotide polymorphism (SNP) genotyping arrays (Eusebi et al., 2020; Olschewsky and Hinrichs, 2021). The utilisation of SNP data enables efficient screening of individuals and populations for inbreeding, identification of cross-breeding and/or parentage, verification of population admixture and history, detection of selection signatures, and the execution of genome-wide association studies. This approach was first employed in sheep by Kijas et al. (2012), hereby initiating the world’s first SNP-based genome-wide analysis of 74 sheep breeds.

One of the common methods used to study the genomic inbreeding pattern of livestock is by investigating runs of homozygosity (ROH), first identified by Broman and Weber, 1999 in humans and first studied in livestock by Sölkner et al. (2010). Since then, ROH studies have become state of the art to study genomic inbreeding patterns (Peripolli et al., 2017). In sheep, studies such as Purfield et al. (2012), Mastrangelo et al. (2018) and Cesarani et al. (2022) used ROH. Moreover, ROH can also signify genomic regions with a high level of inbreeding, called ROH islands (Nothnagel et al., 2010). These ROH islands are often linked to signatures of selection. In sheep, several ROH islands have been reported, both with a known underlying genomic cause and with an unknown basis (Gorssen et al., 2021).

Three flockbook organisations register nine local sheep breeds in Belgium (Scherf, 2000, FAO DAD-IS, 2024). The flockbooks *Kleine Herkauwers Vlaanderen* and *Steunpunt Levend Erfgoed* are active in Flanders (northern Belgium), whereas *Association Wallonne des Éleveurs – Elevéo asbl – Service Ovin-Caprin* is active in Wallonia (southern Belgium). The nine local sheep breeds are the Belgian Milk sheep, the Flemish sheep (FLS), the Ardense Voskop (Ardennais Rouge) (AVK), the Entre-Sambre-et-Meuse (ESM), the Houtlandschaap (HLS), the Kempen Heath (KES), the Mergelland Schaap (MLS), the Vlaams Kuddeschaap (VKS) and the Lakens Kuddeschaap (LAS). The FLS played a crucial role in the Flemish cloth industry starting from the Middle Ages (Steunpunt Levend Erfgoed, 2023). The AVK, another old breed, is a red-brown sheep common in both Flanders and Wallonia. The ESM is believed to have originated from the AVK, with introgressions of several English breeds and Merino in the 19th century. The HLS likely emerged from the AVK, FLS, and ESM breeds. The closely related KES and MLS both originate from eastern Belgium. The VKS is considered a recent breed, originating from crosses of VLS, AVK, KES, and ESM breeds. Lastly, the LAS was created from a flock at the Belgian

royal palace, utilising both indigenous breeds and English bloodlines (Steunpunt Levend Erfgoed, 2023). Dumasy et al. (2012) were the first to use microsatellite markers to study Entre-Sambre-et-Meuse, Ardense Voskop, and Belgian Milk sheep whereas Meyermans et al. (2020a) used SNP genotyping in the Belgian Milk sheep, demonstrating the versatility of the methodology to describe the breed and position it amongst related breeds.

In addition to autosomal SNP analysis, another prevalent method for tracing the ancient lineage of various sheep breeds is through the Y-chromosomal *oY1* polymorphism. Meadows et al. (2006) were the first to use this method to explore the roots and domestication of various sheep breeds. Their findings revealed that the ancestral A allele is prevalent in most breeds from Europe, the Mediterranean, Asia, and Africa. On the other hand, the G allele is predominantly found in sheep breeds from England and Northern Europe, including Finland (Meadows et al. 2006, Niemi et al., 2013). Meyermans et al. (2020a) showed that Belgian Milk sheep mostly carried the A allele whereas the Flemish sheep predominantly carried the G allele. For the remaining local Belgian sheep breeds, the *oY1* status remains unknown to date.

This study presents the first genomic characterisation using SNP genotyping of eight local sheep breeds in Belgium (all sampled in Flanders and registered by the flockbook). Every population was screened genomically, and results were compared to the calculated pedigree statistics. Subsequently, an inter-breed analysis was conducted to examine breed relationships and trace the history of admixture. Finally, these eight breeds were positioned among a large international dataset of breeds with publicly available SNP genotypes. On the one hand, this study is valuable for the future breed management and conservation efforts of these eight local breeds. On the other hand, comparing Belgian breeds with other international sheep breeds provides significant insights for the scientific community studying the relationships among different sheep breeds.

Material and methods

Blood samples of sheep were provided by the breeding association *Steunpunt Levend Erfgoed* and were collected during their routine sample collection. From this routine, a representative set of animals from active members across all breeds was selected for genotyping. An overview of the total number of genotyped animals per breed (total n = 687) and their number of sampled owners is given in Table 1 (total n = 82). The birth dates of the sampled animals range from 2012 to 2023, with the majority of samples coming from animals born since 2020. Pedigree data were provided by the flockbook, with records starting in the early 1990 ’s and with 2023 as the most recent complete year.

**Table 1**  
Overview of the number of genotyped sheep per breed, with their original local names, abbreviations and the number of sampled owners per breed.

Breed name	Abbreviation	Number of genotyped animals	Number of owners
Ardense voskop (Ardennais Rouge)	AVK	100	15
Entre-Sambre-et-Meuse	ESM	82	7
Houtlandschaap	HLS	75	15
Kempens schaap	KES	78	10
Lakens schaap	LAS	93	11
Mergellandschaap	MLS	100	9
Vlaams kuddeschaap	VKS	83	3
Vlaams schaap	VLS	76	12

### Pedigree analysis

Pedigree completeness was assessed following MacCluer et al. (1983). Inbreeding estimates based on pedigree records ( $F_{\text{ped}}$ ) were calculated following Falconer and Mackay (1996) and Groeneveld et al., 2009. Pedigree-based effective population size ( $N_e$ ) estimates were generated based on POPREPORT (Groeneveld et al., 2009) and its decision tree. Active breeding animals were defined as rams or ewes that have registered progeny. The number of active males and females was calculated using a custom R script for the last two complete record years (2022–2023). The same was done for the average number of litters per year. Selected progeny per sire or dam was defined as the number of offspring that was used for breeding and was calculated using POPREPORT on the full pedigree (Groeneveld et al., 2009).

### Genotyping and quality control

All sheep were genotyped on the GeneSeek® Genomic Profiler™ Ovine 50 K array (Neogen Corporation), containing 52 260 SNP markers. All samples and SNP markers were subjected to quality control following Anderson et al. (2010) and Meyermans et al. (2020b). On the individual level, samples were checked for call rate ( $> 90\%$ ), outlying heterozygosity ( $\pm 3$  SD), discordant sex information when comparing to pedigree records, and potential missampling of related individuals based on pedigree records. On SNP marker level, only autosomal SNPs with known genomic position and a call rate  $> 95\%$  were retained for analysis (45 978 remaining SNP markers). The Y-chromosomal oY1 allele was included on the GGP Ovine array, and genotypes for all males were extracted. Potential oY1 genotyping errors were checked with the oY1.1\_dup SNP.

### Genetic diversity analysis

The genetic diversity of the eight sheep populations was evaluated by estimating  $N_e$ , based on LD measurements using a custom R script following Weir and Hill (1980) and Waples (1991, 2006). Average heterozygosity per population over all autosomal SNPs was calculated using PLINK (--het) (Chang et al., 2015). Next, an ROH analysis was conducted using PLINK v1.9 (--homozyg). The minimum ROH length was set to 1 000 kb, and the minimum number of SNPs in one ROH was determined following Purfield et al. (2012). It was set at 42 for VKS, VLS HLS, ESM, KES and at 43 for AVK, LAS, MLS. Only ROHs with no heterozygote and maximally one missing SNP were allowed. The sliding window length was set equal to the minimal ROH length (in SNPs). A minimum density of 1 SNP/100 kb and a maximal gap between two consecutive SNPs was set at 1 000 kb, and the threshold value was equal to 0.05. The inbreeding coefficient based on ROH ( $F_{\text{ROH}}$ ) was estimated taking into account the genome coverage of the analysis (Meyermans et al., 2020b). Additionally,  $F_{\text{ROH} > 5 \text{ Mb}}$  and  $F_{\text{ROH} > 16 \text{ Mb}}$  were estimated for ROHs longer than 5 Mb and 16 Mb, respectively, as these metrics give an indication on the more recent inbreeding events, approximately up to ten and three generations ago (Curik et al., 2014). Genome-wide overviews of ROH incidence were visualised using the qqman R package (Turner, 2018). ROH islands were identified following Gorssen et al. (2021).

### Inter-breed analysis

Principal component analysis was conducted with PLINK (--pca), and plots were visualised using ggplot2 (Wickham, 2016). The analysis of ancestry was carried out with ADMIXTURE (v1.3.0) for  $K = 2$  to  $K = 12$  clusters (Alexander et al., 2009). The optimal level of  $K$  was determined using 5-fold cross-validation,

and admixture results were visualised using Pophelper (v2.3.1) (Francis, 2017). A neighbour-net graph was constructed based on pairwise Weir and Cockerham's  $F_{\text{st}}$  values, calculated using PLINK's --fst --family function. The visual representation was generated using SPLITSTREE5 (Huson and Bryant, 2006). To position the eight sheep breeds internationally, different datasets of genotyped sheep breeds were used: Kijas et al. (2012) with 2 819 animals from 71 breeds, Ciani et al. (2015) with 671 animals from 38 breeds; Meyermans et al. (2020a) with 116 animals from two breeds; Rochus et al. (2020) with 93 animals from five breeds. A complete list of all available breeds and the number of animals per breed can be found in Supplementary Table S1. After merging the different datasets (GGP ovine array, Illumina OvineSNP50 and the Ovine Infinium HD SNP BeadChip), 17 351 SNPs were available for analysis. Only breeds with at least 10 individuals were retained for the final dataset, which consisted of 3 393 individuals of 95 breeds. A neighbour-joining graph was constructed based on pairwise Weir and Cockerham's  $F_{\text{st}}$  values, calculated using PLINK's (--fst --family) and SPLITSTREE5.

## Results

### Pedigree analysis

Pedigree completeness in 2023 for the last five generations was  $> 95\%$  for all populations except for KES (82%) and MLS (71%). Table 2 presents the outcome of the pedigree analysis of all eight local sheep breeds. The lowest number of active males in the most recent years was found in MLS and ESM sheep (16 and 19 rams, respectively), with VLS having the highest number of active males (84). For active females, MLS and ESM also had the lowest numbers (143 and 157, respectively), whereas the highest number of active females was found in KES (1 858). The average number of litters per year was lowest in MLS and ESM (83 and 110) and highest in KES (1 126). The average number of selected progeny per dam was equal in all eight sheep breeds (2); however, SD was highest in VLS, and the highest maximum of selected progeny per ewe was found in HLS. For sires, the average selected progeny varied between 3 (MLS) and 23 (KES), with KES and VKS having the highest SD (23.3 and 22.0, respectively). In VKS, one ram sired 140 selected offspring. Average  $F_{\text{ped}}$  for animals born in the most recent complete year varied between  $\pm 3.2\%$  for MLS, AVK and KES, and 11% for ESM. Pedigree-based  $N_e$  was estimated below 20 for LAK and MLS and up to 90 for AVK (Table 2).

### Genomic diversity analysis

LD-based  $N_e$  estimates are shown in Table 3 and reveal for all eight sheep breeds an  $N_e$  below 24, with the ESM as the least diverse breed (15.3). Moreover, average SNP heterozygosity per population is indicated in Table 3, showing the lowest autosomal SNP heterozygosity in MLS, although with a high SD. The ROH analysis (Table 4) reveals variation among the eight breeds. The highest average number of ROH per individual was found in MLS (36.6) and the lowest in KES (16.0). The variation in ROH numbers was highest in MLS and VLS. The average ROH length was highest in LAS (7.5 Mb), followed by VKS (7.0 Mb). For 6 animals (five registered as MLS and one registered as KES), no ROH were detected. The highest average  $F_{\text{ROH}}$  was observed in VKS (8.5%), LAS (8.2%) and MLS (8.1%). The highest variation in  $F_{\text{ROH}}$  was found in VLS (5.0%) and ESM (5.0%). Outliers with  $F_{\text{ROH} > 20\%}$  were observed in all but two populations (KES, VLS), and even spiked to 29.7% in ESM. Average  $F_{\text{ROH} > 5 \text{ Mb}}$  was highest in LAS (6.7%), followed by VKS (6.6%), whereas KES had the lowest  $F_{\text{ROH} > 5 \text{ Mb}}$  estimate (3.0%). Average  $F_{\text{ROH} > 16 \text{ Mb}}$  was highest in LAS (2.1%), followed by VLS (1.7%),

Table 2

Overview of pedigree-based census data and diversity estimates of the eight studied local sheep breeds in Belgium, sampled in Flanders. The active males and females and the mean number of litters per year were computed for the period 2022–2023. Selected progeny statistics were computed for the full available pedigree. The pedigree-based inbreeding estimate ( $F_{ped}$ ) is the breed average for animals born in 2023. Effective population size ( $N_e$ ) is estimated for animals born in 2023.

Breed	Animals in pedigree	Total number		Mean number of litters per year	Selected progeny		$F_{ped}$ (in %)	$N_e$
		Active males	Active females		Per sire (mean / SD / max)	Per dam (mean / SD / max)		
AVK	16 047	74	550	378	7 / 9.3 / 66	2 / 1.3 / 13	3.3	90
ESM	3 507	19	157	110	9 / 14.6 / 67	2 / 0.9 / 6	11.3	23
HLS	8 861	62	474	312	9 / 11.1 / 97	2 / 1.3 / 12	7.2	39
KES	17 648	59	1 858	1 126	23 / 23.3 / 131	2 / 1.0 / 10	3.3	66
LAS	3 645	28	181	125	9 / 9.0 / 37	2 / 1.2 / 8	9.4	16
MLS	2 203	16	143	83	3 / 5.7 / 51	2 / 1.0 / 8	3.1	17
VKS	13 422	31	827	551	18 / 22.0 / 140	2 / 1.0 / 8	7.6	33
VLS	12 672	84	865	509	8 / 12.6 / 99	2 / 1.5 / 10	6.7	28

Abbreviations: AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kuddeschaap; VLS=Vlaams schaap.

Table 3

Overview of the estimated LD-based effective population size ( $N_e$ ) and the observed average heterozygosity for all eight studied sheep populations.

Breed	$N_e$	Average heterozygosity (SD)
AVK	23.9	0.375 (0.020)
ESM	15.3	0.392 (0.029)
HLS	19.4	0.373 (0.020)
KES	18.3	0.394 (0.017)
LAS	18.7	0.377 (0.024)
MLS	21.0	0.353 (0.032)
VKS	21.0	0.373 (0.019)
VLS	16.5	0.379 (0.033)

Abbreviations: AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kuddeschaap; VLS=Vlaams schaap; LD=Linkage Disequilibrium.

whereas AVK had the lowest  $F_{ROH > 16 Mb}$  estimate (0.7%). Pearson correlation between  $F_{ROH}$ ,  $F_{ROH > 5 Mb}$ , and  $F_{ROH > 16 Mb}$ , averaged at 0.99 (between  $F_{ROH}$  and  $F_{ROH > 5 Mb}$ ), 0.80 (between  $F_{ROH}$  and  $F_{ROH > 16 Mb}$ ) and 0.85 (between  $F_{ROH > 5 Mb}$  and  $F_{ROH > 16 Mb}$ ) for all populations, showing consistent results between the different ROH classes. Correlations of  $F_{ped}$  with  $F_{ROH}$  and  $F_{ROH > 16 Mb}$  are given in Table 5 and were  $\pm 0.5$  for AVK, VLS and LAS, low for ESM, VKS, KES and MLS and negative ( $-0.19$ ) for HLS. Fig. 1 shows the incidence plots for all autosomal SNPs in a ROH for the eight studied breeds. In all but two breeds (KES and ESM), ROH islands were detected. These ROH islands are described in Supplementary Table S2. MLS showed the largest number of detected ROH islands (7), and the highest incidence of one ROH island was observed on chromosome OAR13 in AVK.

Table 4

Overview of the detected ROH (number of ROH, average ROH length and total ROH length per individual) and estimated inbreeding coefficient based on ROH ( $F_{ROH}$ ), including the inbreeding coefficients based on larger ROH fragments ( $F_{ROH > 5 Mb}$  and  $F_{ROH > 16 Mb}$ ) for all eight sheep breeds.

Inbreeding parameter		AVK	ESM	HLS	KES	LAS	MLS	VKS	VLS
Number of ROH	Mean (SD)	24.2 (9.7)	17.7 (13.3)	31.7 (7.9)	16.0 (7.6)	27.6 (9.9)	36.6 (14.6)	31.3 (9.8)	21.5 (14.1)
	Range	8 – 61	1 – 63	13 – 47	0 – 41	10 – 51	0 – 75	15–61	0 – 47
ROH length (Mb)	Mean (SD)	5.4 (1.1)	6.8 (2.2)	6.4 (1.4)	6.3 (2.2)	7.5 (1.6)	5.4 (1.6)	7.0 (1.2)	6.2 (2.1)
	Range	3.4 – 10.0	2.0 – 15.0	3.4 – 11.8	2.0 – 14.7	4.1 – 9.0	1.8 – 9.2	4.5 – 9.6	2.6 – 12.8
Total ROH length per individual (Mb)	Mean (SD)	136 (90)	130 (132)	189 (77)	107 (7.2)	215 (117)	212 (107)	223 (89)	152 (130)
	Range	29 – 612	4 – 778	65 – 543	0 – 374	65 – 617	0 – 531	77 – 540	2.6 – 498
$F_{ROH}$ (%)	Mean (SD)	5.2 (3.4)	5.0 (5.0)	7.6 (2.9)	4.1 (2.8)	8.2 (4.3)	8.1 (4.1)	8.5 (3.4)	5.8 (5.0)
	Range	1.1 – 23.3	0.1 – 29.7	2.5 – 20.7	0 – 14.3	2.5 – 23.5	0 – 20.3	2.9 – 20.6	0 – 18.9
$F_{ROH > 5 Mb}$ (%)	Mean (SD)	3.4 (3.1)	3.9 (4.5)	5.6 (2.8)	3.0 (2.5)	6.7 (4.0)	5.4 (3.4)	6.6 (3.0)	4.4 (4.4)
	Range	0.2 – 19.6	0 – 27.8	0.3 – 18.4	0 – 12.4	1.1 – 21.4	0 – 16.3	1.4 – 17.5	0 – 18.0
$F_{ROH > 16 Mb}$ (%)	Mean (SD)	0.7 (1.8)	1.4 (2.6)	1.5 (1.8)	0.9 (1.4)	2.1 (2.6)	1.0 (1.6)	1.4 (1.7)	1.7 (2.5)
	Range	0 – 14.0	0 – 16.1	0 – 11.3	0 – 6.7	0 – 14.5	0 – 9.1	0 – 10.5	0 – 10.5

Abbreviations ROH: run of homozygosity; AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kuddeschaap; VLS=Vlaams schaap.

Table 5

Pearson correlations between the pedigree-based inbreeding estimates ( $F_{ped}$ ) and the ROH-based inbreeding estimates ( $F_{ROH}$  and  $F_{ROH > 16 Mb}$ ) for all eight investigated sheep breeds.

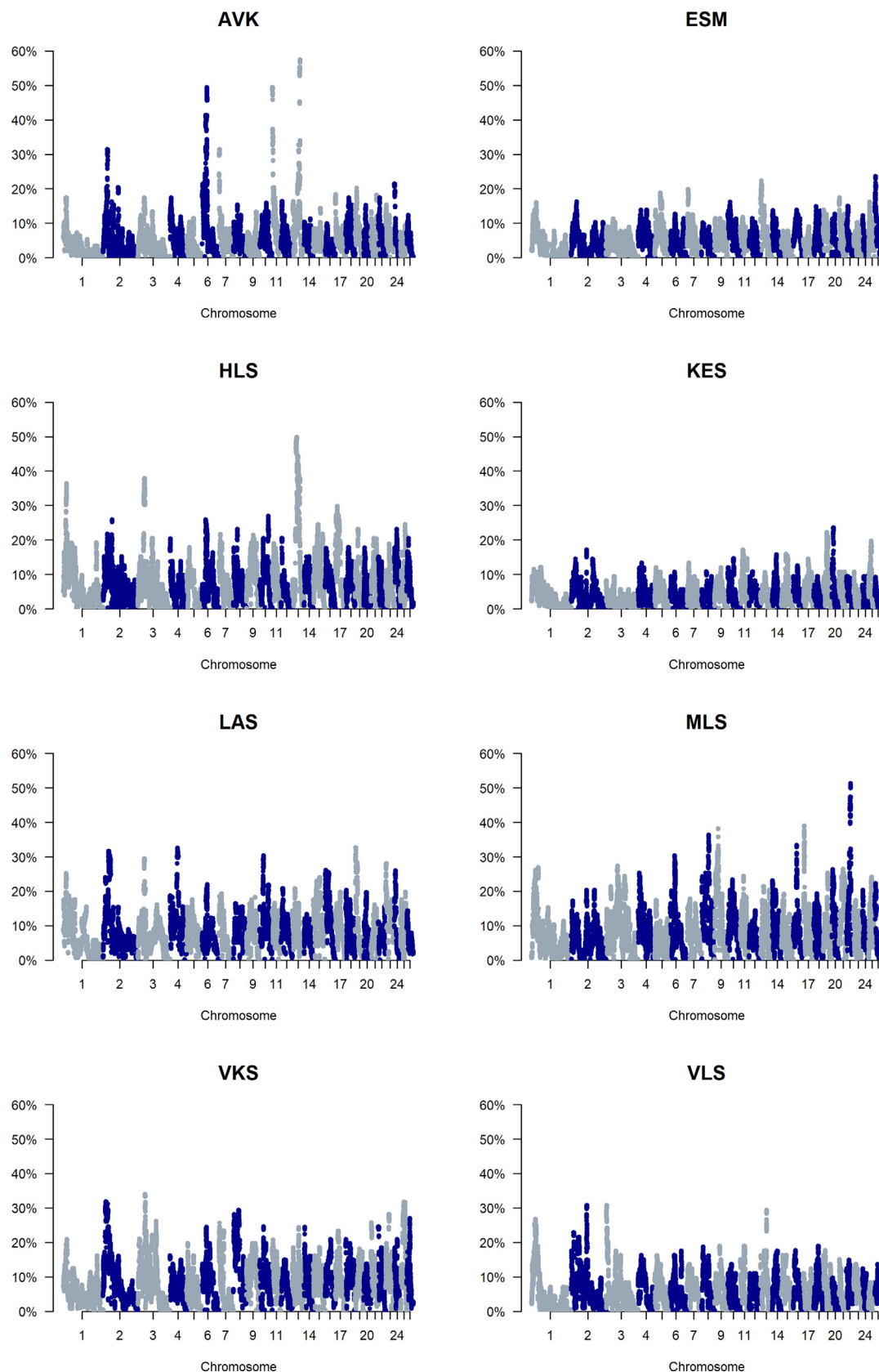
Breed	$F_{ROH}$	$F_{ROH > 16 Mb}$
AVK	0.45	0.52
ESM	0.24	0.26
VLS	0.59	0.68
VKS	0.02	0.13
KES	0.20	0.21
MLS	0.34	0.44
LAS	0.55	0.56
HLS	−0.19	−0.18

Abbreviations: ROH: run of homozygosity; AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kuddeschaap; VLS=Vlaams schaap.

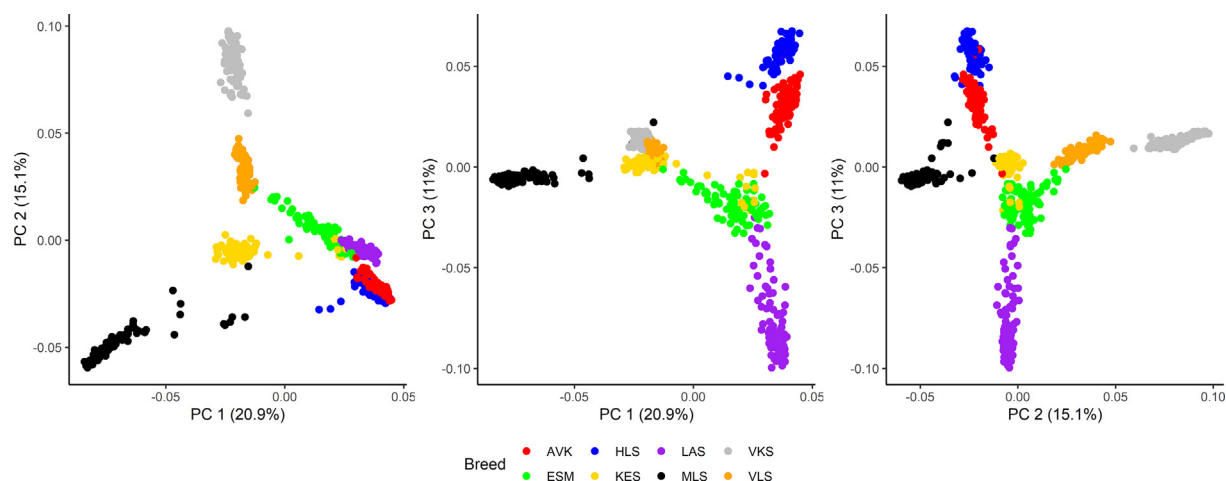
Inter-breed analysis

Principal component analysis results are shown in Fig. 2. The first three principal components explained 47.0% of the total variance present in the autosomal genome. The first principal component clearly distinguishes the AVK, HLS, ESM and LAS cluster from the MLS and the KES, VLS and VKS cluster, whereas the second principal component splits the VLS and VKS sheep. The third principal component splits off the LAS from the other populations. Next, the  $F_{st}$ -based neighbour-net graph is shown in Fig. 3 and pairwise estimates are given in Supplementary Table S3. Here, it is shown that the HLS, AVK, LAS and ESM separate from the KES, and the MLS, VLS and VKS breeds. Consequently, the admixture

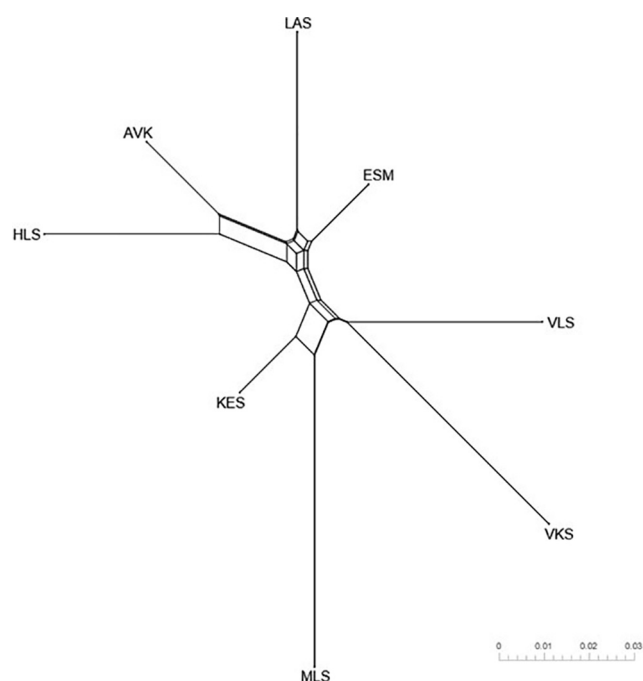




**Fig. 1.** Genome-wide incidence plots of SNPs in runs of homozygosity (ROH) for the eight studied local sheep breeds in Belgium. Abbreviations: SNP: single nucleotide polymorphism; AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kuddeschaap; VLS=Vlaams schaap.



**Fig. 2.** Principal component analysis of the eight studied local sheep with (left) principal component (PC) 1 plotted versus PC2, (center) PC1 versus PC3 and (right) PC2 versus PC3. The proportion of variance explained by each principal component is given between brackets. Abbreviations: AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kudde-schaap; VLS=Vlaams schaap.



**Fig. 3.** Neighbour net graph of the studied sheep breeds. The legend is shown in the bottom right. Pairwise estimates are shown in Supplementary Table S3. Abbreviations: AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kudde-schaap; VLS=Vlaams schaap.

analysis results for  $K = 2$  to  $K = 8$  are shown in Fig. 4. The optimal number of clusters was equal to eight, based on the  $K$ -value using 5-fold cross-validation (results not shown). Again, MLS splits off as first group, followed by the KES, VKS and VLS. Most of the eight sheep breeds are identified as separate clusters, apart from ESM, which shares genomic structures that are also present mainly in AVK, HLS and LAS. Also in the LAS, KES and VLS populations, smaller subgroups are detected that differentiate from the main breed cluster. The neighbour-joining tree containing 95 international breeds is added as Supplementary Fig. S1 and a zoomed-in version highlighting the eight Belgian breeds within a set of 49 breeds is shown in Fig. 5. Here, the VLS breed clusters are close to the Texel cluster, VKS close to Anglo-Saxon breeds and the other six more or

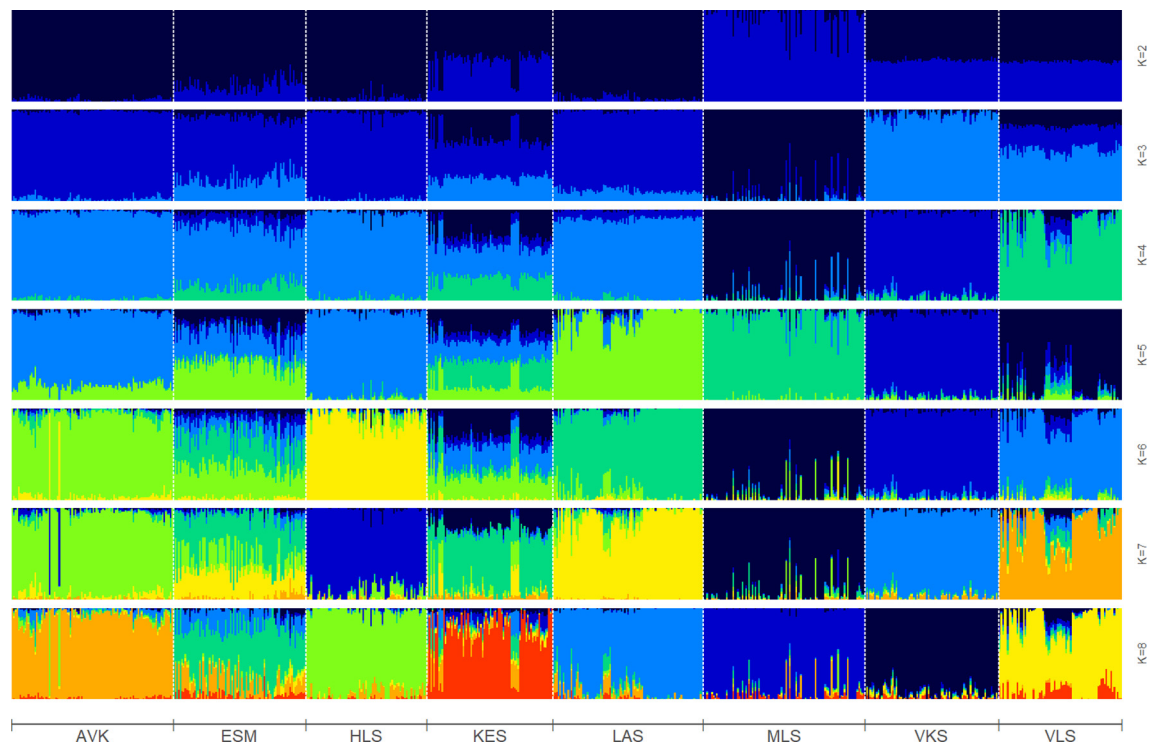
less as a distinct group (between the milksheep cluster (Belgian Milksheep and (East-)Friesian sheep) and the Scandinavian cluster). Apart from autosomal analyses, the Y-chromosomal *oY1* allele was studied. Table 6 presents the distribution of paternal A-*oY1* and G-*oY1* in the screened populations. AVK, ESM, HLS, KES and LAS are (mostly) A-allele bearing, whereas MLS, VKS and VLS are (mostly) carrying G-alleles.

## Discussion

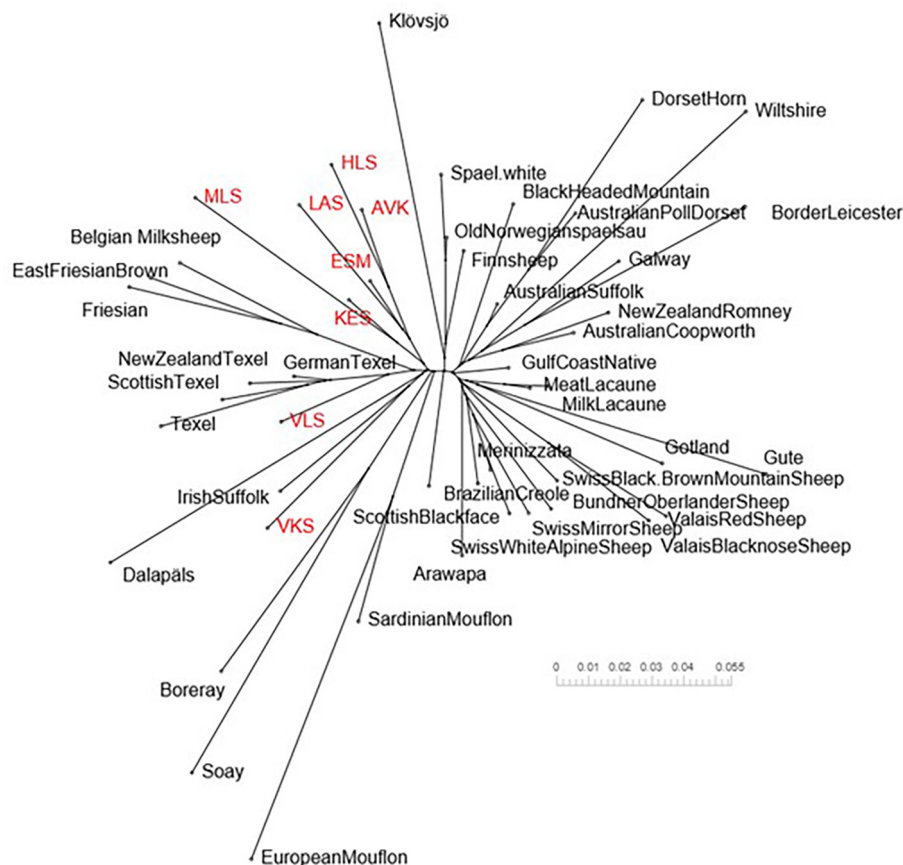
In this study, we conducted a genomic characterisation of eight local sheep breeds registered in Belgium. It offers the first thorough inter- and intra-breed analysis, as previous studies relied on microsatellite markers or focused solely on a single breed. By using genome-wide SNPs, we achieved a more precise evaluation of the genetic variation, the identification of selection signatures, and a more accurate measurement of inbreeding levels. Examining all local Belgian sheep breeds simultaneously enabled us to assess the genetic relationships among these breeds. All eight breeds were found to be endangered based on their effective population sizes. The genotyped sheep were collected from Flemish herds in northern Belgium. Some of these breeds can be considered transboundary, extending into Wallonia (southern Belgium) and the Netherlands. The KES sheep can be considered as the most transboundary breed, with about 150 animals imported and nearly 60 animals exported in recent years. The second largest imported breed is the MLS with almost 100 animals imported since 2017. For the other breeds, the number of imports to Flanders ranges from 4 (VKS) to 16 (VLS), with no exports recorded in recent years. Further research could provide more detailed verification of the transboundary nature of these breeds.

## Genetic diversity analysis

Pedigree and SNP marker information was used to investigate the genetic diversity, the population statistics and inbreeding status of each breed. Pedigree analysis revealed for all breeds that the number of active breeding males and females is rather low ( $< 100$  active males and  $< 1\,000$  active females (except for KES) (Table 2). Especially for ESM, LAS and MLS the number of active females is very low ( $< 200$  animals), which increases the risk of genetic erosion and bottlenecks impairing conservation. Danchin-Burge et al. (2013) reported that in France, seven local breeds had a range



**Fig. 4.** Admixture clustering of all eight investigated sheep breeds from K=2 to K=8, where K=8 was the optimal number of clusters. Abbreviations: AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kuddeschaap; VLS=Vlaams schaap.



**Fig. 5.** Neighbour-joining tree based on  $F_{ST}$ -distances on 49 international sheep breeds, with the eight Belgian breeds sampled in this study highlighted in red. The legend is shown in the bottom right. Abbreviations: AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kuddeschaap; VLS=Vlaams schaap.

**Table 6**

Genotypes of the paternal *oY1* SNP (A-*oY1* or G-*oY1*) for the eight local sheep breeds as a percentage of the screened population with n the number of genotyped rams.

Breed	A- <i>oY1</i> (%)	G- <i>oY1</i> (%)	N
AVK	1.00	0.00	43
ESM	0.92	0.08	24
HLS	1.00	0.00	25
KES	0.80	0.20	40
LAS	1.00	0.00	29
MLS	0.27	0.73	15
VKS	0.00	1.00	11
VLS	0.06	0.94	17

Abbreviations: SNP: single nucleotide polymorphism; AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kuddeschaap; VLS=Vlaams schaap.

of 118–3 771 active ewes, indicating similar or even larger populations. It is important to note that there often is a lack of publicly available pedigree analyses of local sheep breeds. Additionally, the information provided on Food and Agriculture Organisation's DAD-IS portal is often outdated (FAO DAD-IS, 2024), which results in less reliable population size estimates.

$F_{ped}$  was estimated as lowest for KES, AVK and MLS ( $\pm 0.03$ ), and highest for ESM (0.11). However, for KES and MLS, the average pedigree completeness was relatively low, which could lead to an underestimation of  $F_{ped}$ . For KES, VKS and VLS, there has been an increase in the number of active females (approximately 60, 83, and 330% respectively since 2014). This increase could be attributed to a growing number of small-scale grazing projects in Flanders. These grazing projects, which often focus on grassland or landscape management, are typically commissioned by local governments and nature management organisations that prefer using more robust local breeds. This shows that establishing a new (economic) objective for these local sheep breeds and their breeders could ensure a stable (genetic) future for these breeds. Interestingly, the ratio of active males to active females for VKS and KES is significantly lower (approximately 3%) compared to the average of 13% observed in six other breeds. This aligns with the fact that these two breeds are the most commonly used for grazing projects, where most flocks consist of ewes. Given the high number of litters per year, this lower number of males could potentially lead to an increase in the additive genetic relationship in future generations, thereby increasing the overall rate of inbreeding in these breeds (Meuwissen and Oldenbroek, 2017). As a result, it is strongly recommended to increase the number of (active) breeding males as a strategy for sustainable breed management.

The effective population size estimate is one of the key parameters in genetic diversity monitoring. Low  $N_e$  were estimated, using both pedigree- and LD-based computation methods. Based on pedigree data,  $N_e$  estimates were between 17 for MLS and 90 for AVK (Table 2) and based on genomic LD-based estimates  $N_e$  were below 24 for all breeds. Similar estimates were also previously reported for Belgian Milkshopeep ( $N_e = 22$ ) (Meyermans et al., 2020a). Previous research has already pointed out that genomic based measures are often more reliable, as pedigree data may contain errors and assumes that ancestors are unrelated (Curik et al., 2014). As the Food and Agriculture Organisation reports a minimal lower boundary for  $N_e$  at 100 for genetically sustainable populations (FAO, 1998), we can report that all eight screened Belgian sheep breeds are classified as endangered.

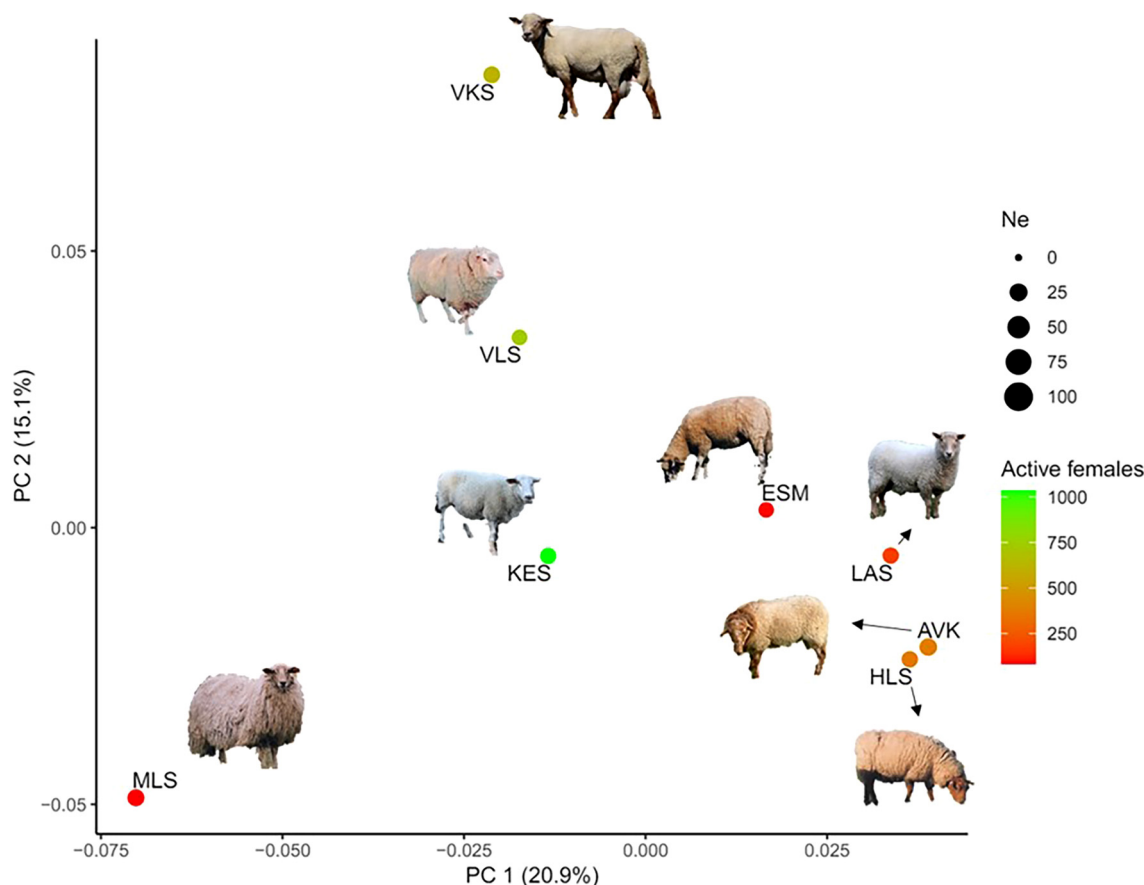
ROH-based inbreeding ( $F_{ROH}$ ) estimates were between 4.1% for KES and 8.5% for VKS, and were lower compared to the previously reported  $F_{ROH}$  in Belgian Milkshopeep (14.5%) and Friesian Milkshopeep (10.2%) in the Netherlands (Meyermans et al., 2020a). The previous  $F_{ROH}$  estimate of 12.4% for a small set of VLS in Meyermans et al. (2020a) was higher and reported a higher number of highly inbred

animals (highest  $F_{ROH>16Mb}$  was estimated at 18.4%, reported SD on  $F_{ROH>16Mb}$  was more than twice as large). Several individuals showed large  $F_{ROH} > 20\%$  in all populations, except for KES and VLS, and even as high as 29.7% in ESM which are signs of highly inbred animals. For six animals, mainly MLS, no ROH were identified. These animals also clustered separately from the main breed cluster in the principal component analysis (Fig. 2) and in the admixture analysis (Fig. 4). Even though they are registered and their physical traits align with breed standards, the lack of ROH and diverging results from principal component analysis and admixture analyses indicate a possible crossbreed origin. For sustainable, long-term breed management and to qualify an animal for flockbook registration, it could be recommended to employ ROH-based analyses for assessing breed-specificity and detecting extreme inbreeding, in addition to standard pedigree and phenotypic evaluations.

Pearson correlations between individual  $F_{ped}$  and  $F_{ROH}$  are moderate for AVK, VLS, LAS, MLS and ESM (Table 5), which can be expected and has been reported before in sheep (e.g. Mastrangelo et al., 2018). However, for VKS, this correlation is equal to 0.02, and for HLS this correlation is even negative. Although  $F_{ped}$  and  $F_{ROH}$  estimates are similar for both breeds (7.6 and 8.5 for VKS, and 7.2 and 7.6 for HLS), which suggests that the average inbreeding level is correctly assessed, pedigree correctness and (proper) animal identification might be problematic. As both breeds are frequently used for extensive grazing, mating and lambing are less well supervised, explaining this discrepancy. Correlations between  $F_{ROH > 16 Mb}$  and  $F_{ped}$  were higher, indicating that these longer ROH are more effective at capturing recent inbreeding, which is presumed to be accounted for in the pedigree.

In total, 24 different ROH islands were detected in six of the local Belgian breeds. For ESM and KES, none of the signals reached the threshold of being present in at least 30% of the population (Fig. 1, Supplementary Table S2). AVK, LAS and VKS share an ROH island on chromosome OAR2, whereas AVK and HLS share an ROH island on OAR6, and AVK and HLS on OAR13. However, additional analyses are required to study the potential different origins of these ancestral haplotypes, or underlying genes in these (potential) selection signatures. For VLS, a ROH island was identified on OAR2 at  $\pm 118$  Mb. This region is well-known for its presence of myostatin (*MTN*), a gene where mutations cause increased muscularity. Although the Flemish sheep was historically not selected for meat production, this might suggest a (indirect) selection for increased muscularity or introgression from Texel sheep. This ROH island was previously also observed in the Texel sheep, but also in Soay sheep (Gorssen et al., 2021). Further analysis on the specific haplotypes that create these ROHs could shed light on the origin of these ROH islands and whether these haplotypes could be linked to increased muscularity. Two breeds (AVK, HLS) showed an ROH island on chromosome OAR6 (27–39 Mb), a signal that has previously been found in other sheep breeds such as Merino, Scottish Blackface, Soay, Spael, Lacaune, Horned Dorset and Île de France (Gorssen et al., 2021). This region has previously been linked to milk production, growth and bone-related traits (Matika et al., 2016; Ruiz-Larriaga et al., 2018; Rodríguez-Ramilo et al., 2021). The ROH island on OAR13 (27–57 Mb) that was detected in AVK, HLS and VLS was also previously seen in Texel, Chios, Border Leicester, Boreray and Ronderib Afrikaner (Gorssen et al., 2021). The island on chromosome OAR25 (26–36 Mb), seen in VKS, was also observed in East-Friesian Brown (Gorssen et al., 2021). The island on OAR4 (56–62 Mb) that was detected in LAS was previously detected in Chios and the Cyprus fat-tailed sheep (Gorssen et al., 2021). When discarding the individuals that show as crossbreeds based on principal component analysis results (10 MLS and 3 HLS, Fig. 2), ROH islands detection did not differ ( $\pm 2\%$ ) for MLS and HLS.





**Fig. 6.** Global representation of the genetic diversity of all eight studied sheep breeds in Belgium (sampled in Flanders, northern Belgium), representing the genetic distance between populations via principal component (PC) analysis, the active number of females registered in the pedigree, and the effective population size ( $N_e$ ) estimated based on linkage disequilibrium. Source pictures: Steunpunt Levend Erfgoed vzw. Abbreviations: AVK=Ardense voskop; ESM=Entre-Sambre-et-Meuse; HLS=Houtlandschaap; KES=Kempens schaap; LAS=Lakens schaap; MLS=Mergellandschaap; VKS=Vlaams kudde-schaap; VLS=Vlaams schaap.

### Inter-breed analysis

Based on both principal component analysis and admixture analysis, MLS was identified as most distant from the other Flemish sheep breeds. The majority of the MLS breed cluster is clearly separated from the rest, whereas a small set ( $\pm 10$  animals) of the genotyped population showed signs of crossbreeding, although they were phenotypically characterised as purebred MLS sheep. All analyses also showed VLS and VKS sheep in two genetically distinct clusters. KES was also identified as a separate cluster, however several animals showed clustering with other breeds. This could suggest crossbreeding as some KES-owners also have other local breeds. Although this potential crossbreeding cannot be excluded, the LD-based  $N_e$  estimate is still very low (18.3).

Based on the principal component analysis (Fig. 2), AVK and HLS are the two breeds within this set that are most challenging to distinguish, as the first three PC's already explain 47.2% of the total observed variance. However, the admixture analysis identifies unique genomic segments that differentiate both breeds, even when the analysis is conducted with  $K = 6$  clusters. The close link between HLS and AVK aligns with the known history of the HLS breed, which is believed to share its origins with the AVK breed from the Belgian Ardennes (Steunpunt Levend Erfgoed, 2023). This connection between AVK and HLS can also be found in the ROH island study as both breeds share two ROH islands (OAR6 and OAR13). The ESM breed is also assumed to originate from the Belgian Ardennes region, but it has a history of crossbreeding with Merino and is therefore less linked to the AVK, however, based

on PC1 and PC2 both breeds cluster together. This cluster is also visible in the  $F_{st}$  analysis (Fig. 3). Dumasy et al. (2012) found that in ESM a subgroup could be identified, that originates from an ancient splitting of the breed. However, based on the admixture analysis (Fig. 4), no clear substructure was observed, although little unique genomic segments were attributed to this breed. Fig. 6 shows a general representation of the genetic diversity of all eight studied sheep breeds, based on principal component analyses, LD-based  $N_e$  and the active number of females. In this format, we offer a comprehensive overview that encompasses multiple critical facets of genetic diversity simultaneously.

Fig. 5 and Supplementary Fig. S1 illustrate the relationships among the eight Belgian sheep breeds in comparison to a diverse collection of internationally available genotypes. LAS, HLS, and AVK exhibit genetic affinities towards the Scandinavian group, which includes KLO, Spael sheep, and Finnsheep. VLS is shown closely related to the Texel cluster, which would be in line with the detected ROH island around the *MSTN* gene, also found in Texel. VKS shows a close relationship with Irish Suffolk sheep, suggesting a potential origin of British admixture. Unfortunately, only a few Anglo-Saxon/British breeds were available in this study (Supplementary Table S1). One limitation to this analysis is the relatively low number of SNPs (17 351) that were available to conduct these  $F_{st}$  analyses ( $\pm 33\%$  of the GGP Ovine 50 K and the Illumina OvineSNP50 arrays). Despite this limitation, the obtained results align with a previous study by Meyermans et al. (2020a), where Belgian Milkshew were also grouped in the cluster of (East-)Friesian milkshew.

Previous research already indicated that the *oY1* genotype on the Y-chromosome can be used as an important marker to track domestication and links between several sheep populations world wide (Meadows et al., 2006). Table 6 shows that AVK, HLS and LAS harbour uniquely the A-*oY1* allele. For ESM and KES, the majority ( $\geq 80\%$ ) of the analysed rams also showed the A-*oY1* allele. This A-*oY1* has been frequently observed in most Southern- and Eastern Europe, Asian and African sheep breeds (Meadows et al. 2006, Niemi et al., 2013). For VKS, VLS and MLS, the predominant allele is the G-*oY1* allele, suggesting that they have been influenced by English sheep breeds. Only KES and MLS are two breeds where both alleles seem to be segregating. Moreover, it is interesting to see that this subdivision is equally present in the  $F_{st}$  analysis (Fig. 3), also here MLS, VKS and VLS seem to cluster separately from AVK, HLS, LAS and ESM that seem to cluster together. The origin of the LAS remains largely unknown, and the suggested Anglo-Saxon/British breed origin is contradicted according to the *oY1* analysis.

## Conclusion

This study presents the detailed genetic characterisation of eight local sheep breeds in Belgium. For this, we analysed pedigree data and 687 genotyped sheep using whole genome SNP arrays, with an average of 86 animals per breed. With these analyses, we revealed that all eight breeds are endangered and at risk of genetic erosion. Inter-breed analyses and paternal *oY1* allele analysis showed the relationships between these eight breeds and the group of milksheep, Texel sheep and the Scandinavian breeds. These data will contribute to public knowledge and will be made available for future (international) sheep diversity analyses. For each of these breeds, the results will aid/support future management and conservation decisions.

## Supplementary material

Supplementary material to this article can be found online at <https://doi.org/10.1016/j.animal.2024.101315>.

## Ethics approval

Not applicable. The blood samples were provided by Steunpunt Levend Erfgoed vzw and were collected during their routine farm visits.

## Data and model availability statement

The data generated in this project are publicly available and accessible from the KU Leuven Research Data Repository (RDR) via <https://doi.org/10.48804/YSLOBO>.

## Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Microsoft Copilot (GPT-4) for language editing of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

## Author ORCIDs

RM: <https://orcid.org/0000-0002-5079-5097>.  
WG: <https://orcid.org/0000-0002-7224-0746>.  
NA: <https://orcid.org/0009-0004-4373-8555>.

KH: <https://orcid.org/0000-0001-7002-8699>.  
BCB: <https://orcid.org/0000-0002-1839-3637>.  
LC: <https://orcid.org/0000-0002-9498-9977>.  
NB: <https://orcid.org/0000-0002-7313-3088>.  
SJ: <https://orcid.org/0000-0002-5588-3889>.

## CRediT authorship contribution statement

**R. Meyermans:** Writing – review & editing, Writing – original draft, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **W. Gorssen:** Writing – review & editing, Methodology, Formal analysis. **N. Aerts:** Writing – review & editing, Resources, Formal analysis. **K. Hooyberghs:** Writing – review & editing, Resources, Formal analysis. **B. Chakkingal Bhaskaran:** Writing – review & editing, Resources. **L. Chapard:** Writing – review & editing, Methodology, Formal analysis. **N. Buys:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **S. Janssens:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

## Declaration of interest

None.

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