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*Research Article*

# Genetic Differentiation of Wild Boar Populations in a Region Endangered by African Swine Fever

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**ABSTRACT** For areas at risk for African swine fever (ASF) introduction from neighboring regions, it is important for epidemic control to know how wild boar (*Sus scrofa*) dispersion dynamics could be used to combat the spread of ASF. In this regard, long-term information based on population genetic data makes an important contribution. We selected our study area as Rhineland-Palatinate, Germany, because it had a high density of wild boars and was threatened by ASF via infected wild boars from neighboring Belgium. On an area of around 20,000 $km^2$, we collected almost 1,200 blood samples from 22 wild boar hunting grounds. The study area included a network of potential barriers to movement, including roads and rivers. We assessed genetic differentiation based on microsatellite data. We used 2 spatial (Bayesian Analysis of Population Structure [BAPS] and TESS) and 1 non-spatial (STRUCTURE) Bayesian model-based approaches to analyze the data. Each of the algorithms detected 4 clusters with different cluster compositions in different areas and identified the highest degrees of differentiation between hunting grounds east and west of the Rhine River, between Pfalz and Eifel-Hunsrück, and to a lesser degree between Westerwald and Taunus and between Eifel and Hunsrück. Thus, genetic evidence suggests barriers of different strength that might be helpful in a setup of complex and expensive measures against the spread of animal diseases such as ASF. The described approach could also provide valuable information for other threatened regions to contain ASF. © 2021 The Authors. *The Journal of Wildlife Management* published by Wiley Periodicals LLC on behalf of The Wildlife Society.

**KEY WORDS** African swine fever, barrier, epidemic, genetic differentiation, population genetics, *Sus scrofa*, wild boar.

Changing agro-ecosystems (Hebeisen et al. 2008), structural changes in the landscape (Morelle et al. 2016), and climate change (Markov et al. 2019) have led to a significant spread and increase of wild boars (*Sus scrofa*) in Europe since the 1970s (Massei et al. 2015, Morelle et al. 2016). Their expansion was accompanied with conflicts, including crop damage (Schley et al. 2008), damage to ecosystems (Giménez-Anaya et al. 2008, Graitson et al. 2019), and threats to public health and food security. A particular threat arises from the infection of large wild boar populations in eastern Europe with the African swine fever (ASF) virus (Guinat et al. 2017). African swine fever has already been detected in Belgium (Linden et al. 2019, Pikalo et al. 2020) and some eastern states in Germany (Sauter-Louis et al. 2021), and spread to countries in central and western Europe is a distinct possibility (Sanchez-Vizcaino et al. 2015; Śmietanka et al. 2016; World Organisation for Animal Health 2017*a*, *b*). This continued expansion leads to social and administrative demands for countermeasures to prevent and limit the spread of ASF (Keuling et al. 2016, Liordos et al. 2017, Podgórski and Śmietanka 2018, Vajas et al. 2019). The management of ASF in free-roaming animals is of primary importance to reduce the risk of introduction and continued spread of ASF in a given region (Chenais et al. 2018, Saegerman 2018, Petit et al. 2020).

Hosts can contribute to disease spread through their spatial behavior by moving away from the outbreak area and transmitting pathogens to susceptible individuals, leading to disease transmission between populations (Conner and Miller 2004, Oyer et al. 2007). Thus, migration of hosts is an integral part of disease dynamics, depending on the host's mobility and dispersion dynamics. Combating the spread of the disease by preventing movement of infected animals (e.g., eliminating hunting in the center of the infected area, increasing hunting pressure at the periphery, and fencing to prevent infected animals from migrating) are the main tactics to control ASF in wild boar (European Food Safety

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Authority [EFSA] 2018). Such measures are complex and expensive. Understanding the host's dispersion dynamics can help to optimize disease control measures and allow management efforts to be focused on specific areas, thus saving costs and time (Van der Waal et al. 2013, Hirsch et al. 2016, Podgórski and Śmietanka 2018). Consequently, EFSA has recommended increasing understanding of wild boar movement behavior for non-affected areas at risk of ASF introduction (EFSA et al. 2020).

A number of methods have been used to evaluate the distribution of wild boar (Peris et al. 2020). Such studies are expensive, but provide a good overview. Results depend mainly on the characteristics of the consigned individuals, habitats, weather, and season, among others. It is unlikely to be possible in the short-term to demonstrate long-term migration or even identify barriers using home range estimators. The identification of barriers and areas that are particularly permeable in the long-term, might help to better target resources (e.g., fences) for the containment of the spread of ASF by wild boar. Genetic differentiation among natural populations using gene markers, could provide a measure of the long-term connectivity of the species in a region. Such data are often used to demonstrate the differentiating effect of gradients of landscape resistance and barriers on populations (Frantz et al. 2012, Goedbloed et al. 2013, Rutten et al. 2019). No previous studies have provided results on the use of gene marker data for the prediction of spreading tendencies in the case of a disease outbreak in wild boar.

The study area was part of a larger study that included all of western Germany, the Netherlands, and Belgium (Goedbloed et al. 2013). In the study of Goedbloed et al. (2013), the Rhine alone showed a differentiating effect and the wild boars of all regions were traced back to a single historical population. Nevertheless, we expected to be able to detect a finer differentiation for regions and thus indications of possible barriers within Rhineland-Palatinate with greater sample sizes.

Our objectives were to determine the connectivity for wild boars in a region threatened by ASF from neighboring regions based on genetic differentiation in a descriptive study. Further, we wanted to identify existing barriers to movement that could be useful for preventing the spread of the disease.

## STUDY AREA

We conducted the study in 2018 to 2019. The study area covered the entire region of the federal state of Rhineland-Palatinate, Germany, with a low mountain topography (Fig. 1; Table 1), neighboring the province of Luxembourg in Belgium, where ASF in wild boar was detected. The state has a north-south extension of 225 km, a west-east extension of 150 km and covers an area of about 19,800 $km^2$. The country has a west European-Atlantic climate with 4 seasons, characterized by mild winters ($\bar{x} = 1.4$°C), moderate summers ($\bar{x} = 16.8$°C), and high annual rainfall (20.6 cm). The elevation varies between 53 m (Rhine near Rheinbreitbach) and 817 m (Erbeskopf in the Hunsrück). The predominant biome is the temperate broadleaf forest biome (deciduous forest biome), consisting of 5 different zones: the stratum tree zone, the small tree and sapling zone, the shrub zone, the herb zone, and the ground zone. The stratum zone is made up of big trees such as oak (*Quercus* spp.), beech (*Fagus* spp.), or maple (*Acer* spp.) trees. Dominant mammalian species include wild boar, red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), red fox (*Vulpes vulpes*), and badger (*Meles meles*). Human population density in 2018 was approximately 206 people/$km^2$. The state was largely rural with about 42% forest, 20% arable land, 13% permanent grassland, 3% viticulture, 8.5% settlement area, and 6.1% transport area. Rhineland-Palatinate is crossed by 2 major rivers, the Rhine and the Moselle, and 10 freeways. Because of the relief of Rhineland-Palatinate, the freeways cross over numerous viaducts. Furthermore, the freeways were not fenced in the largest sections. The number of boars in the area was estimated between 240,000 and 400,000.

## METHODS

### Sample Collection and Laboratory Methods

We obtained wild boar blood samples ($n = 1{,}186$) from 22 hunting grounds (Fig. 1) from the Institute for Animal Diseases at the State Office of Investigation in Koblenz-Rhineland-Palatinate. The office routinely collected blood samples during the 2018–2019 hunting season to monitor the health of wild boar including the incidence of Classical Swine Fever and ASF. The number of individual boars per hunting ground ($n = 54$) provided a reliable genetic characterization of the local populations (Reiner et al. 2019). We did not sample any living animals and we did not hunt or otherwise kill animals for the study. Thus, we did not require an animal care and use approval. We did not implement detailed landscape genetics analyses because the goal was to identify major barriers to boar gene flow as a potential countermeasure for the spread of ASF and not to determine specific landscape features associated with gene flow.

We extracted DNA by using a commercially available kit (Instant Virus RNA Kit, Analytik Jena, Jena, Germany). We processed 150 µl of blood per sample according to the manufacturer's instructions. We eluted DNA in 60 µl of RNAse-free water. We determined DNA concentration photometrically with a Qubit Flex fluorometer (Thermo Fisher Scientific, Dreieich, Germany) and adjusted the concentration to 5 ng/µl with RNAse-free water. This DNA concentration gives the best results in capillary electrophoresis. We confirmed the presence of high molecular weight DNA by agarose gel electrophoresis. Each DNA extraction was accompanied by a blank extraction without sample material, which we used as a negative control in a polymerase chain reaction (PCR). For each PCR analysis, we used the same wild boar sample as the positive control. We used the sample subsequently as standard in capillary electrophoresis (see below). We genotyped wild boar with 14 microsatellites. We purchased the primers from Biomers (Schwalbach, Germany) and combined them in 2 multiplex PCRs (Table 2). We performed PCR in a volume of 10 µl consisting of 5 µl of 2× Multiplex Mastermix (Qiagen, Hilden, Germany), 4 µl of primermix, and 1 µl (5 ng) of extracted DNA. We amplified DNA after an initial denaturing step of 15 minutes in 26 cycles

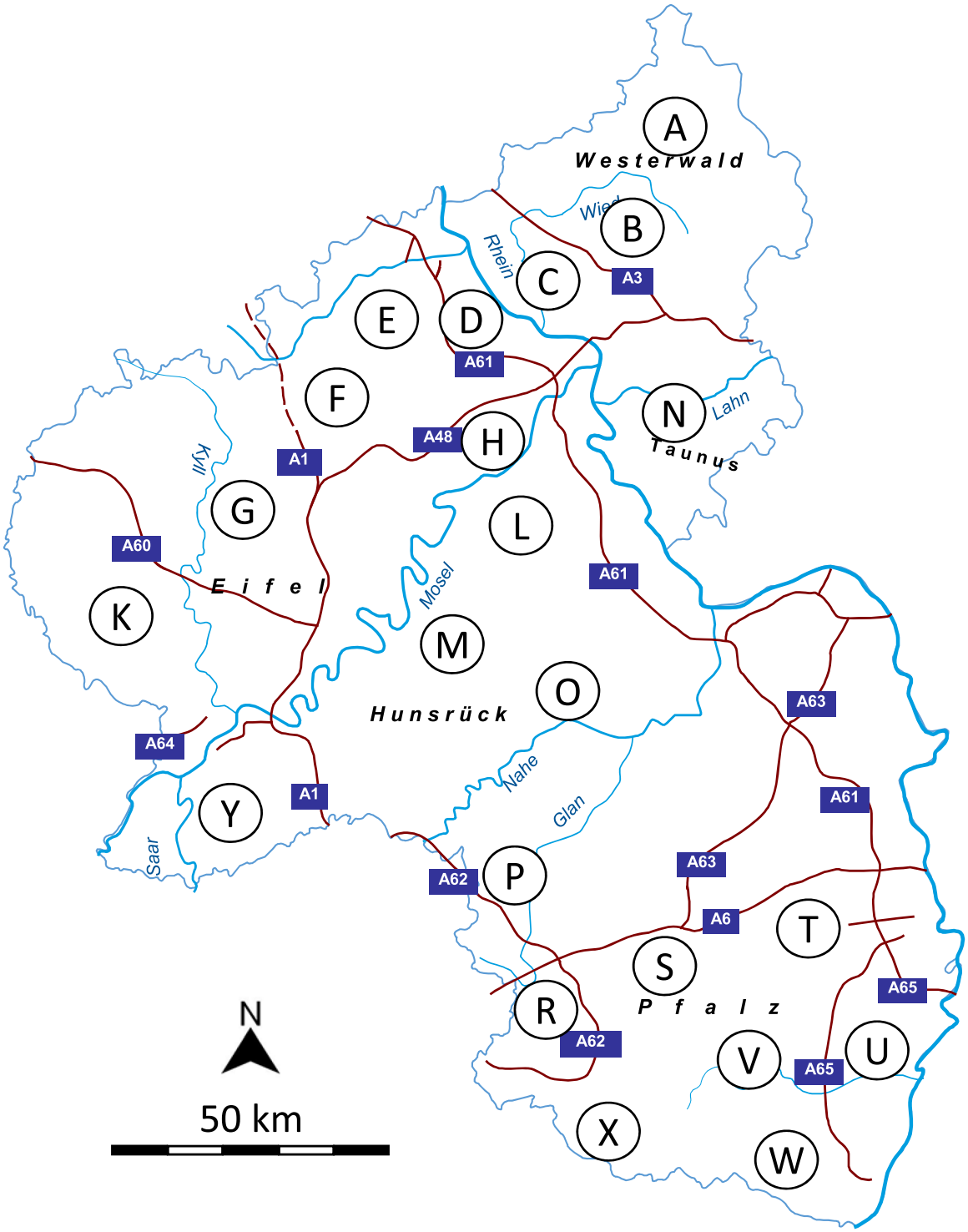


**Figure 1.** Location of the wild boar hunting grounds A through Y in Rhineland-Palatinate, Germany, 2018–2019. Blue lines indicate rivers and red lines indicate roads.

of denaturing at 94°C for 30 seconds, annealing at 57°C (multiplex PCR 2 at 60°C) for 90 seconds, and extension at 72°C for 30 seconds. After a final step at 60°C for 30 minutes, we cooled down PCR reactions to 4°C.

We added 1 µl of the fluorescently labeled PCR product and 0.375 µl DNA size standard 500 Orange (NimaGen, Nijmegen, Netherlands) to 12 µl Hi-Di Formamide (Thermo Fisher Scientific) and electrophoresed it on an ABI 310 capillary sequencer (Thermo Fisher Scientific). We routinely analyzed all homozygous samples twice. We determined allele sizes with the Peakscanner 2.0 software (Thermo Fisher Scientific). We averaged allele sizes from the positive control sample (see above) over 10 runs and used them as standard. In each run we electrophoresed

**Table 1.** Location of the wild boar hunting grounds in Rhineland-Palatinate, Germany, 2018–2019 and number of individuals sampled.

| Wild boar hunting ground | Latitude | Longitude | Region | Location[a] | *n* |
|---|---|---|---|---|---|
| A | 50°74′69″95‴N | 7°85′89″54‴E | Westerwald | NE | 40 |
| B | 50°54′22″-1‴N | 7°52′73″65‴E | Westerwald | NE | 79 |
| C | 50°52′79″93‴N | 7°41′83″43‴E | Westerwald | NE | 62 |
| D | 50°48′17″47‴N | 7°30′93″77‴E | Eifel | W | 50 |
| E | 50°42′58″90‴N | 7°38′-3″-3‴E | Eifel | W | 122 |
| F | 50°11′59″58‴N | 7°38′-3″-3‴E | Eifel | W | 54 |
| G | 50°20′63″51‴N | 6°75′53″32‴E | Eifel | W | 49 |
| H | 50°19′75″18‴N | 7°18′82″24‴E | Eifel | W | 62 |
| K | 49°96′49″6.‴N | 6°43′86″31‴E | Eifel | W | 67 |
| L | 50°17′61″84‴N | 7°11′61″16‴E | Hunsrück | C | 82 |
| M | 50°29′-2″-2‴N | 7°14′59″22‴E | Hunsrück | C | 27 |
| N | 50°27′51″36‴N | 7°82′68″53‴E | Taunus | E | 72 |
| O | 49°76′16″0.‴N | 7°52′88″61‴E | Hunsrück | C | 64 |
| P | 49°50′40″64‴N | 7°43′84″16‴E | Hunsrück | C | 24 |
| R | 49°28′54″-4‴N | 7°44′27″33‴E | Pfalz | S | 33 |
| S | 49°34′91″90‴N | 7°80′59″4.‴E | Pfalz | S | 44 |
| T | 49°46′47″-4‴N | 8°16′27″49‴E | Pfalz | S | 21 |
| U | 49°20′20″27‴N | 8°29′79″3.‴E | Pfalz | S | 53 |
| V | 49°52′95″9.‴N | 7°91′98″25‴E | Pfalz | S | 66 |
| W | 48°98′68″50‴N | 8°17′64″75‴E | Pfalz | S | 19 |
| X | 49°14′12″71‴N | 7°62′39″65‴E | Pfalz | S | 59 |
| Y | 49°73′72″13‴N | 6°73′33″99‴E | Hunsrück | C | 41 |

[a] NE = Northeast; W = West; C = Center; E = East; S = South.

the positive control sample along with the other samples. We monitored run-to-run allele size variations by comparing allele sizes of the positive control sample with those of the standard. We used deviations between both to correct allele sizes of the other samples.

## Analysis of Population Genetic Parameters

We performed most of the population genetic analyses within the statistical software R (R Core Team 2017). We calculated frequencies of null alleles with the function null.all implemented in the R package PopGenReport

**Table 2.** Composition of the multiplex polymerase chain reaction for amplifying microsatellites of wild boar from Rhineland-Palatinate, Germany, 2018–2019.

| | | | | | Allele size (bp) | |
|---|---|---|---|---|---|---|
| Multiplex | Marker | Chromosome | 5′-label | Primer sequence (5′-3′) | Min. | Max. |
| 1 | SW936F | 15 | FAM | tctggagctagcataagtgcc | 89 | 113 |
| | SW936R | | | gtgcaagtacacatgcaggg | | |
| | S0155F | 1 | FAM | tgttctctgtttctcctctgtttg | 150 | 166 |
| | S0155R | | | aaagtggaaagagtcaatggctat | | |
| | S0226F | 2 | FAM | aaagcacttttaactttcatgatactcc | 180 | 208 |
| | S0226R | | | ggttaaacttttncccccaataca | | |
| | S0227F | 4 | FAM | gatccatttataattttagcacaaag | 226 | 252 |
| | S0227R | | | atggtgtgatgctatgtcaagc | | |
| | S0026F | 16 | HEX | aaccttcccttcccaatcac | 100 | 114 |
| | S0026R | | | catatattcacagactgctttttactcc | | |
| | S0225F | 8 | HEX | gctaatgccagagaaatgcag | 146 | 204 |
| | S0225R | | | caggtggaaagaatggaatga | | |
| | SW240F | 2 | Atto550 | agaaattagtgcctcaaattgg | 95 | 133 |
| | SW240R | | | aaaccattaagtccctagcaaa | | |
| 2 | SW951F | 10 | FAM | tttcacaactctggcaccag | 111 | 135 |
| | SW951R | | | gatcgtgcccaaatggac | | |
| | SW911F | 9 | FAM | ctcagttctttgggactgaacc | 155 | 173 |
| | SW911R | | | catctgtggaaaaaaaaagcc | | |
| | S0101F | 7 | FAM | gaatgcaaagagttcagtgtagg | 197 | 213 |
| | S0101R | | | tccctcacacttaccgcag | | |
| | SW72F | 3 | HEX | atcagaacagtgcgccgt | 98 | 124 |
| | SW72R | | | ctttgaaaatggggtgtttcc | | |
| | SW24F | 17 | Atto550 | ctttgggtggagtgtgtgc | 101 | 123 |
| | SW24R | | | gatccaaatgctgcaagc | | |
| | SW632F | 7 | Atto550 | tgggttgaaagatttcccaa | 153 | 183 |
| | SW632R | | | ggagtcagtactttggcttga | | |
| | Swr1941F | 13 | Atto550 | agaaagcaatttgatttgcataatc | 209 | 261 |
| | Swr1941R | | | acaaggacctactgtatagcacagg | | |

(Gruber and Adamack 2019). Because the frequency of missing data was <5%, we estimated null allele frequencies with the method described by Brookfield (1996). We used 1,000 bootstraps to compute the 95% confidence interval. If the 95% confidence interval included zero, the null allele frequencies did not significantly differ from zero.

We tested deviations from the Hardy-Weinberg equilibrium (HWE) with the function hw.test implemented in the R package pegas (version 0.12; Paradis 2010). We performed the test as an exact test based on Monte Carlo permutations ($n = 1{,}000$) of alleles (Guo and Thompson 1992). We determined private alleles and evenness of allele distribution with functions implemented in the R package poppr (version 2.8.3; Kamvar et al. 2015).

We calculated population genetic parameters (number of alleles/hunting ground, percentage of alleles/locus/hunting ground, mean number of alleles, allelic richness, effective number of alleles, observed heterozygosity, expected heterozygosity, inbreeding coefficient [Fis]) with the function divBasic implemented in the R package diveRsity (Keenan 2017). We presented Fis values with their 95% confidence intervals obtained after 1,000 bootstrap iterations. We used the same R package (diveRsity version 1.9.90) to determine pairwise population differentiation using Fst (Weir and Cockerham 1984) as population statistics.

We evaluated population structure with a non-spatial (STRUCTURE 2.3.4; Pritchard et al. 2000) and 2 spatial (TESS 2.3, Francois et al. 2006, Chen et al. 2007; BAPS 6.0, Corander and Marttinen 2006) methods. The 3 methods applied individual-based Bayesian clustering algorithms to detect genetic discontinuities. We performed hierarchical STRUCTURE analysis to detect underlying genetic structures on a finer resolution. For this purpose, we used clusters inferred from the first round as input to a further STRUCTURE analysis. We repeated this procedure until there was no further clustering. We assumed population admixture and correlated allele frequencies. We ran simulations with 200,000 Markov chain Monte Carlo iterations after a burn-in of 100,000. We varied the number of clusters (K) from 1 to 10 with 10 independent runs per K. We determined the optimal K with STRUCTURE HARVESTER 0.6.94 (Earl and vonHoldt 2012). To determine the population assignment probability of each individual across all simulations and to visualize population structure, we used the R package pophelper (Francis 2017).

We performed TESS with relaxed parameters (1,200 sweeps with a burn-in of 200 sweeps, maximum number of clusters Kmax fixed to 10, 10 runs for each K) in a first run to determine the optimal number of clusters from the lowest Deviance Information Criterion value. After that, we performed 100 independent runs at the optimal K with 50,000 sweeps after a burn-in of 10,000 sweeps. We performed all runs under the assumption of admixture. We ran BAPS with the spatial clustering of individuals option.

We used individual assignment probabilities from STRUCTURE in a generalized linear model (IBM-SPSS version 27, IBM, Munich, Germany) to quantify the genetic connectivity between neighboring hunting grounds and to investigate the relevance of the differentiating regions between them. We applied the model to test whether the distribution of the assignment probabilities to 2 clusters determined with STRUCTURE ($K = 2$), differed significantly between the hunting grounds. We made pairwise comparisons and used a Bonferroni correction. We considered $P$-values $\leq 0.05$ statistically significant.

To use all available information on gene structure from the Bayesian algorithms to quantify genetic differentiation between hunting grounds, we analyzed the individual assignment probabilities of all gene clusters by binary logistic regression. The result was the degree of differentiation between neighboring hunting grounds. The measure for the differentiation was the coefficient of determination according to Nagelkerke ($R^2$; range = 0–1), expressed as a percentage ($R^2\% = 0$–$100\%$). A complete differentiation ($R^2\% = 100\%$) means that 100% of the genetic differentiation within and between 2 hunting grounds is based on the separation of both hunting grounds, whereas the Bayesian method does not detect any genetic differentiation within the individual hunting grounds. This means that the individuals of both hunting grounds are assigned to completely different gene clusters. If the genetic differentiation is within the hunting grounds (i.e., the individuals of the hunting grounds represent exactly the same gene clusters), then $R^2\%$ is 0% and the hunting grounds are not separated. We performed this binary regression for all neighboring hunting grounds based on the results of all 3 Bayesian methods (STRUCTURE, BAPS, TESS). We presented the degree of differentiation ($R^2\%$) as a number between each of 2 neighboring hunting grounds in steps of 0–15%, >15–30%, >30–45%, >45–60%, >60–75%, and >75%.

Isolation by distance can significantly influence the results of Bayesian clustering methods (Perez et al. 2018). Therefore, we evaluated isolation by distance using a Mantel test. We used Slatkin's linearized Fst values (Fst/[1−Fst]) as population genetic metrics. We analyzed the existence of spatial patterns of the overall Mantel correlative relationship within geographic distance classes using Mantel correlogram analysis. We considered a positive spatial autocorrelation of genetic distance to indicate that individuals are more genetically similar than if randomly sampled over the whole area, which we designated as a genetic patch.

## RESULTS

Null allele frequencies of markers significantly different from zero were detected in all wild boar hunting grounds (A–Y) except F, K, M, R, and W. The most prominent markers prone to null alleles were Sw0155 and S0026 with null allele frequencies ranging from 7.9% to 28.7% ($16.8 \pm 5.7$ [SD]) and 7.1% to 44.0% ($19.3 \pm 13.0$), respectively. Therefore, we removed these 2 markers from the dataset. All other null allele frequencies significantly different from zero were distributed across different markers and different wild boar hunting grounds and ranged from 4.6% to 23.4% ($12.3 \pm 5.5$).

**Table 3.** Population genetic parameters for the wild boar hunting grounds in Rhineland-Palatinate, Germany, 2018–2019.

| | Population genetic parameters[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Wild boar hunting ground** | ***n*** | **A** | **Na** | **%** | **Ar** | **Ho** | **He** | **Fis** | **Fis low** | **Fis high** |
| A | 40 | 58 | 4.8 | 45.9 | 3.98 | 0.43 | 0.51 | 0.162 | 0.092 | 0.234 |
| B | 79 | 68 | 5.7 | 53.6 | 4.08 | 0.43 | 0.51 | 0.154 | 0.103 | 0.201 |
| C | 61 | 67 | 5.6 | 52.1 | 4.15 | 0.42 | 0.49 | 0.139 | 0.071 | 0.196 |
| D | 50 | 82 | 6.8 | 64.8 | 4.87 | 0.48 | 0.55 | 0.127 | 0.073 | 0.179 |
| E | 122 | 82 | 6.8 | 65.0 | 4.64 | 0.48 | 0.52 | 0.079 | 0.036 | 0.126 |
| F | 54 | 55 | 4.6 | 42.5 | 3.80 | 0.49 | 0.51 | 0.032 | −0.027 | 0.092 |
| G | 49 | 60 | 5.0 | 46.6 | 4.26 | 0.48 | 0.52 | 0.066 | 0.006 | 0.121 |
| H | 62 | 65 | 5.4 | 50.0 | 4.24 | 0.51 | 0.54 | 0.059 | 0.009 | 0.110 |
| K | 67 | 64 | 5.3 | 49.4 | 4.24 | 0.50 | 0.51 | 0.018 | −0.041 | 0.078 |
| L | 81 | 62 | 5.2 | 47.5 | 3.91 | 0.48 | 0.51 | 0.058 | 0.013 | 0.104 |
| M | 27 | 48 | 4.0 | 37.7 | 3.58 | 0.46 | 0.47 | 0.018 | −0.073 | 0.107 |
| N | 72 | 54 | 4.5 | 42.5 | 3.57 | 0.46 | 0.49 | 0.068 | 0.009 | 0.124 |
| O | 64 | 72 | 6.0 | 56.4 | 4.69 | 0.52 | 0.56 | 0.072 | 0.009 | 0.138 |
| P | 24 | 54 | 4.5 | 41.0 | 3.89 | 0.47 | 0.52 | 0.105 | 0.001 | 0.205 |
| R | 33 | 57 | 4.8 | 44.4 | 3.99 | 0.57 | 0.56 | −0.013 | −0.113 | 0.091 |
| S | 44 | 65 | 5.4 | 51.1 | 4.31 | 0.50 | 0.56 | 0.119 | 0.061 | 0.182 |
| T | 20 | 51 | 4.3 | 38.5 | 3.81 | 0.42 | 0.51 | 0.161 | 0.081 | 0.231 |
| U | 52 | 64 | 5.3 | 50.1 | 4.00 | 0.47 | 0.50 | 0.061 | −0.003 | 0.129 |
| V | 66 | 58 | 4.8 | 45.2 | 3.87 | 0.47 | 0.50 | 0.071 | 0.014 | 0.127 |
| W | 19 | 51 | 4.3 | 39.5 | 3.73 | 0.40 | 0.46 | 0.122 | −0.036 | 0.264 |
| X | 59 | 62 | 5.2 | 48.3 | 4.14 | 0.51 | 0.55 | 0.067 | 0.011 | 0.124 |
| Y | 41 | 52 | 4.3 | 40.0 | 3.64 | 0.47 | 0.49 | 0.048 | −0.029 | 0.124 |
| Mean | 53.9 | 61.4 | 5.1 | 47.8 | 4.06 | 0.47 | 0.52 | 0.082 | 0.012 | 0.149 |
| SD | 23.7 | 8.9 | 0.7 | 7.5 | 0.35 | 0.04 | 0.03 | 0.049 | 0.054 | 0.053 |

[a] A = total number of alleles in the population; Na = mean number of alleles/population; % = percentage of alleles/locus/population; Ar = allelic richness; Ho = observed heterozygosity; He = expected heterozygosity; Fis = fixation index; Fis low = lower (2.5%) confidence interval for fixation index; Fis high = upper (97.5%) confidence interval for fixation index.

Marker Sw240 had the highest ($n = 17$) and marker Sw951 the lowest number of alleles ($n = 6$). This is also reflected in the informativeness (In) of the markers (Rosenberg et al. 2003), which was highest for SW240 (In = 0.85) and lowest for Sw951 (In = 0.054).

We detected 44 private alleles, most of which were for markers Swr1941 ($n = 6$), Sw240 ($n = 5$), Sw72 ($n = 5$), S0226 ($n = 5$), and S0227 ($n = 19$). Private alleles were predominantly spread over wild boar hunting grounds D ($n = 12$), E ($n = 8$), and S ($n = 6$). We did not detect private alleles in hunting ground A, F, G, L, M, P, T, U, W, and X.

Alleles of marker S0101 ($n = 9$) were most evenly distributed (evenness = 0.83) over all hunting grounds, whereas allele frequencies of marker SW951 with 6 alleles varied substantially (evenness = 0.34). Although observed heterozygosity (Ho) of markers was consistently lower than expected heterozygosity (He), differences were statistically not significant ($P = 0.8$). None of the markers showed a consistent deviation from HWE. Markers SW240 and SW911 deviated in 9 and 8 hunting grounds, respectively, from HWE. Only marker SW632 was in HWE for all wild boar hunting grounds.

Based on an average of 54 animals/wild boar hunting ground (19–122), the mean number of alleles was 5.1 (4.0–6.8), amounting to 48% of the total alleles/locus. The highest mean number of alleles (Na) was in the hunting grounds D and E (Na = 6.8 for both sites, 65% of the alleles/locus). Hunting ground M had the lowest mean number of alleles (Na = 4.8), which is 37% of the alleles/locus (Table 3).

Allelic richness (Ar) was highest for hunting ground D (Ar = 4.87) and lowest for N (Ar = 3.57). Observed heterozygosity varied between 0.40 and 0.57. The Fis values ranged from −0.013 (site R) to 0.162 (site A). We detected Fis values significantly different from zero for all wild boar hunting grounds except F, K, M, R, U, W, and Y (Table 3;

**Table 4.** Population genetic parameters of wild boar of the 4 geographical regions in Rhineland-Palatinate, Germany, 2018–2019.

| | Population genetic parameters[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Region** | ***n*** | **A** | **Na** | **%** | **Ar** | **Ho** | **He** | **Fis** | **Fis low** | **Fis high** |
| Westerwald | 180 | 84 | 7.0 | 65.78 | 5.56 | 0.43 | 0.51 | 0.168 | 0.135 | 0.200 |
| Eifel-Hunsrück | 641 | 110 | 9.2 | 88.03 | 6.15 | 0.49 | 0.54 | 0.094 | 0.077 | 0.112 |
| Taunus | 72 | 54 | 4.5 | 42.52 | 4.18 | 0.46 | 0.49 | 0.068 | 0.014 | 0.129 |
| Pfalz | 293 | 88 | 7.3 | 68.92 | 5.62 | 0.49 | 0.55 | 0.118 | 0.091 | 0.146 |

[a] A = total number of alleles in the population; Na = mean number of alleles/population; % = percentage of alleles/locus/population; Ar = Allelic richness; Ho = observed heterozygosity; He = expected heterozygosity; Fis = fixation index; Fis low = lower (2.5%) confidence interval for fixation index; Fis high = upper (97.5%) confidence interval for fixation index.

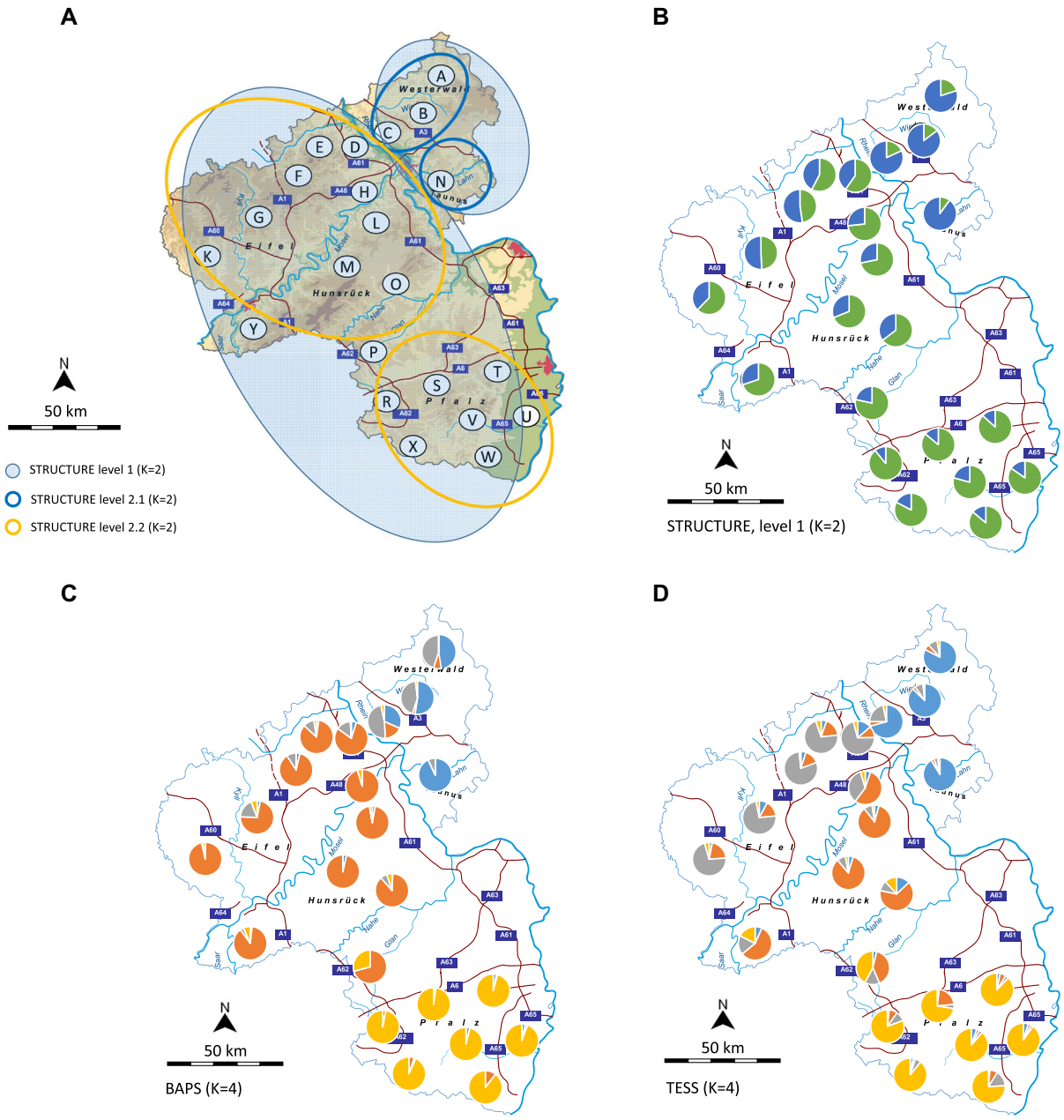


**Figure 2.** Distribution of genetic clusters in wild boar hunting grounds in Rhineland-Palatinate, Germany, 2018–2019, showing population structuring in hunting grounds A through Y. Blue lines indicate rivers and red lines indicate roads. A) Results of hierarchical STRUCTURE analysis: 2 clusters (K) at level 1 (light blue transparent areas east and west of the Rhine River) and 2 subclusters on the east side differentiating Westerwald and Taunus (blue line ovals) and on the west side differentiating Pfalz and Eifel-Hunsrück (orange line ovals). In frames B–D, we used individual assignment probabilities as a result of the Bayesian clustering approaches to visualize cluster membership of individuals from hunting grounds. For this purpose, we averaged cluster assignment probabilities of individuals from each hunting ground and expressed them as a percentage. Different colors in the pie charts represent the clusters and their size is the percentage of individuals of this hunting ground classified into the respective cluster. B) The distribution of the 2 clusters at level 1 of the STRUCTURE analysis. C) The distribution of the 4 wild boar clusters after BAPS analysis (K = 4). D) The distribution of the 4 clusters after TESS analysis (K = 4).

Fst values are presented in Table S1, available online in Supporting Information). Wild boar of the Taunus range had the lowest and wild boar of the Eifel-Hunsrück region the highest variability according to population genetic parameters (Table 4).

The Bayesian clustering approaches with non-spatial (STRUCTURE) or spatial algorithms (BAPS and TESS) agreed in that they classified individuals into 4 clusters, although with different cluster compositions (Fig. 2). All 3 methods led to a clear differentiation between hunting grounds west and east of the Rhine River. All 3 methods led to a further clear differentiation of the hunting grounds west of the Rhine River, assigning individuals of the Pfalz and Eifel-Hunsrück into different clusters.

The 4 clusters identified by STRUCTURE were determined in 2 levels, each with K = 2. The 2 clusters at level 1 were located east (cluster 1) and west (cluster 2) of the Rhine River (Fig. 2A, shown as transparent blue

areas). Distributions of the clusters in the different areas showed different patterns (Fig. 2B). The assignment probabilities of individuals belonging to cluster 1 or cluster 2 as estimated by STRUCTURE were significantly different in the regions east (Westerwald, Taunus) and west of the Rhine River (Eifel, Hunsrück, Pfalz; Table S2, available online in Supporting Information).

At level 2 of the progressive STRUCTURE analysis (Fig. 2A), cluster 1 (east of the Rhine River) split up further into 2 clusters for hunting grounds A, B, C, and N in the north-east (blue ovals), again with statistically significant distributional differences between the hunting grounds in the north (A, B, C, predominantly cluster 1.1) and the hunting ground in the east (N, predominantly cluster 1.2; Table S3, available online in Supporting Information). We also observed this differentiation with BAPS (Fig. 2C) and TESS (Fig. 2D) analysis but with different proportions of individuals within the clusters. The differences observed using BAPS and TESS, although statistically significant, were less pronounced than those obtained from STRUCTURE.

Cluster 2 of the progressive STRUCTURE analysis also split up into 2 further clusters for the hunting grounds west of the Rhine River (Fig. 2, orange ovals). Distributions of the clusters were significantly different among individuals in the Pfalz (predominantly cluster 2.1) and the Eifel-Hunsrück region (predominantly cluster 2.2; Table S4, available online in Supporting Information). Individuals on hunting grounds P and Y showed intermediate behavior and could not be clearly assigned to one cluster or the other.

Using TESS, we detected a further differentiation between hunting grounds of the Eifel and those of the Hunsrück. Differences in distribution of individuals between both areas were also implied by STRUCTURE and BAPS analysis but were statistically not significant (data not shown).

We quantified the visually recognizable differences between the neighboring hunting grounds using coefficients of determination in percent ($R^2$%), obtained from binary logistic regression. The outcome of STRUCTURE at level 1 and at level 2 quantify the differentiation between hunting grounds east and west of the Rhine River (Fig. 3A), between Westerwald and Taunus and between Pfalz and Eifel-Hunsrück (Fig. 3B). We obtained the most conservative classification by solely considering BAPS clusters (Fig. 3C). This confirmed the differentiation between the hunting grounds west and east of the Rhine River and between the Pfalz and the Eifel-Hunsrück region. A differentiation between N and the hunting grounds A to C in the north-east was also visible. The Pfalz and the entire Eifel and Hunsrück were fairly uniform.

In contrast, differences between neighboring hunting grounds were particularly evident under TESS (Fig. 3D). In addition to the differentiation between hunting grounds west and east of the Rhine River and between the hunting grounds of Pfalz and Eifel-Hunsrück, a differentiation between Eifel and Hunsrück was also clearly visible. A band along the northern Eifel (D, E, F, G, and K), the hunting grounds in the northeast and the central region of the Pfalz showed the least differentiation and thus the highest genetic connectivity.

Results of individual-based Bayesian clustering may be biased by an isolation-by-distance pattern of the data. A Mantel test (Fig. 4A) indicated that this pattern of genetic divergence was significantly predicted by distance (Mantel $r = 0.632$, $P \leq 0.001$), so that about 41% of the genetic divergence was explained by geographic distance. To evaluate the relationship between genetic and geographic distances across space, we performed a Mantel correlogram analysis. The Mantel correlogram (Fig. 4B) contains 9 distance classes showing an almost linear decrease of Mantel $r$ with increasing geographic distance. There was a positive spatial autocorrelation in genetic distance among localities ≤100 km, which may be regarded as the size of the genetic patch where individuals exhibit a greater genetic similarity than those separated by >100 km (negative spatial autocorrelation).

## DISCUSSION

The detection of genetic boundaries between or within landscapes might help to improve understanding of population connectivity for the control of introduction and spread of diseases (Conner and Miller 2004, Oyer et al. 2007). This aspect is of growing importance against the background of the real threat of ASF. Clustering algorithms can be interpreted in a spatial context (Safner et al. 2011) to investigate genetic boundaries. Because of different efficiency and reliability among methods and markers in detecting genetic boundaries in different populations and landscapes (Safner et al. 2011, Basto et al. 2016), we used 3 distinct Bayesian algorithms implemented in the non-spatial STRUCTURE and the spatial BAPS and TESS analysis programs. The wild boars of Rhineland-Palatinate broke down into 4 genetically differentiated clusters. All methods differentiated well between hunting grounds west and east of the Rhine River and between the Pfalz in the south and the Eifel-Hunsrück to the northwest. A further differentiation between the hunting grounds of the Eifel and Hunsrück, which was only slightly hinted at under STRUCTURE and BAPS, became evident with TESS. A differentiation between Westerwald and Taunus in the northeast could also be traced with all 3 algorithms, whereby the differences were smallest with TESS. The differentiation of the hunting grounds west and east of the Rhine River agrees well with data from Goedbloed et al. (2013), although in their study the Taunus and Westerwald regions had few samples and represented the southern appendages of a region that included the whole of northwest Germany (east of the Rhine River). A region from the Pfalz to Belgium is listed as the West Rhine, with no differentiation between the Eife-Hunsrück and Pfalz in the large-scale study of Goedbloed et al. (2013). According to Goedbloed et al. (2013), wild boar living west and east of the Rhine River most likely historically represent the same continuous biological population.

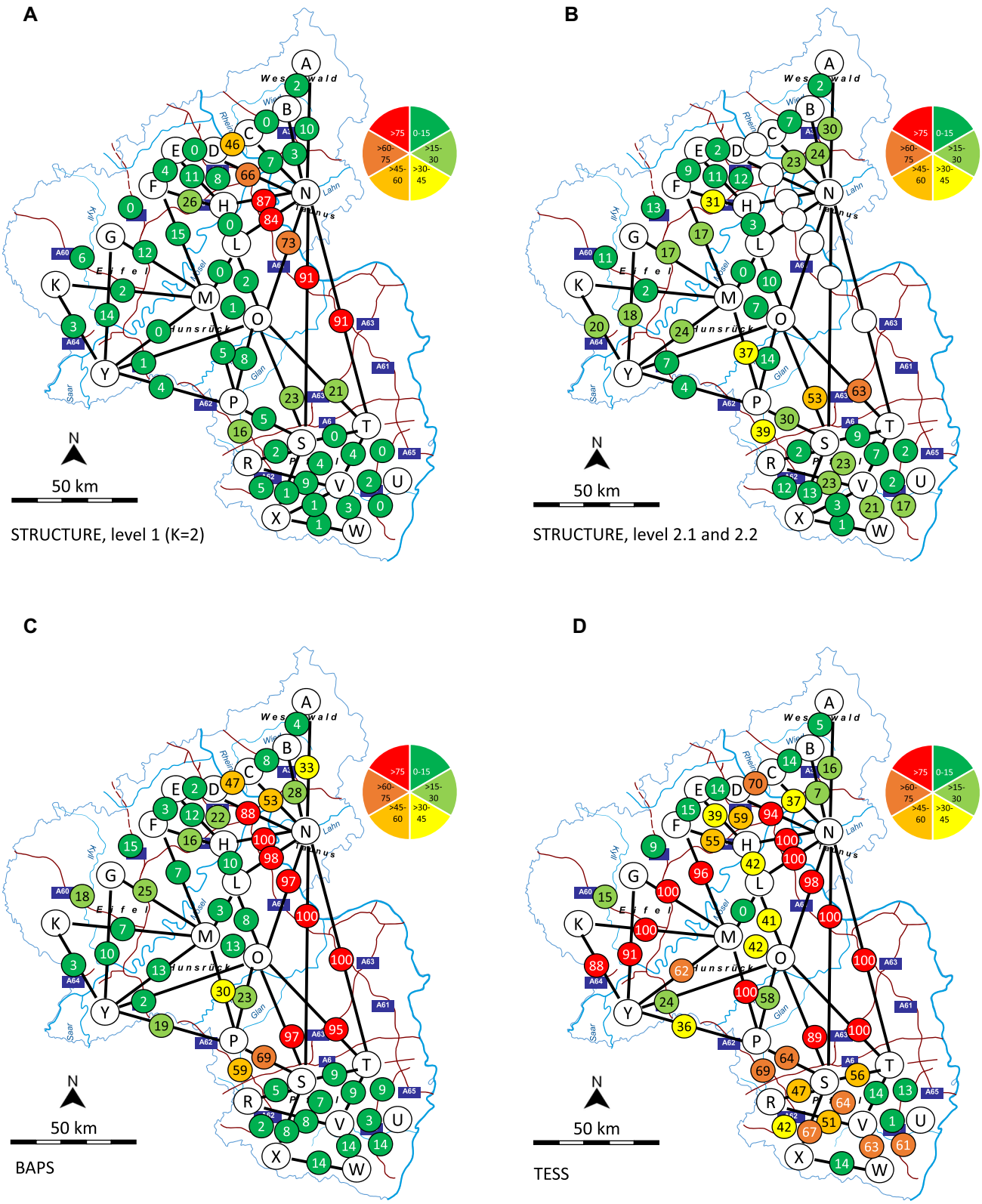


**Figure 3.** Pairwise quantification of genetic differentiation of neighboring wild boar hunting grounds in Rhineland-Palatinate, Germany, 2018–2019, in hunting grounds A through Y. Blue lines indicate rivers and red lines indicate roads. Numbers express Nagelkerke's coefficient of determination, expressed in percent ($R^2$%) calculated from a binary logistic regression; these metrics are color-coded in size bins as indicated. An $R^2$% of 100 means complete differentiation between hunting grounds; 100% of the genetic differentiation (as detected by Bayesian analysis) within and between 2 neighboring hunting grounds is based on the separation between the hunting grounds. Individuals from different hunting grounds belong to completely different clusters (K). An $R^2$% of zero can be found, if cluster distribution of individuals from neighboring hunting grounds are identical and there is no separation between the hunting grounds. A) Results based on STRUCTURE (level 1, K = 2). B) Results based on STRUCTURE level 2; presentation separated into areas west (K = 2) and east (K = 2) of the Rhine River. C) Results based on BAPS (K = 4). D) Results based on TESS (K = 4).

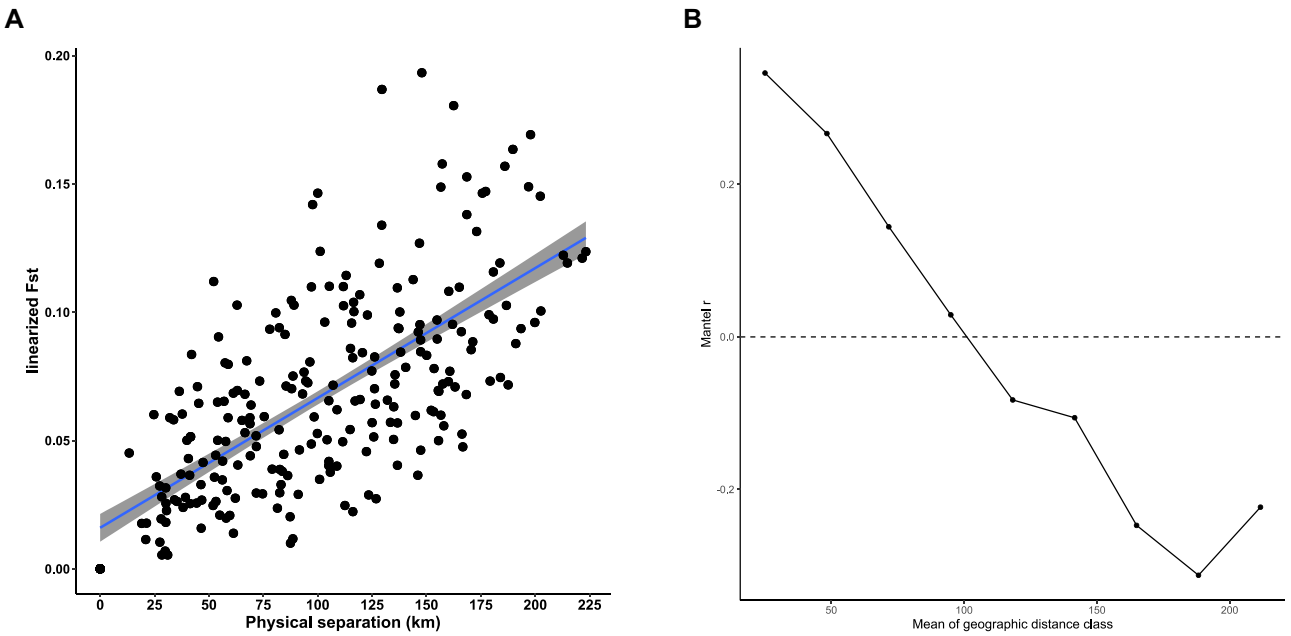


**Figure 4.** A) Variation in genetic distance (linearized Fst) between wild boar hunting grounds in Rhineland-Palatinate, Germany, 2018–2019, depending on geographical distance according to a Mantel test ($r = 0.632$; $R^2 = 40.6\%$; $P \leq 0.001$). Blue lines and shaded area describe the 95% confidence interval. B) Mantel correlogram showing positive spatial autocorrelation up to a distance of about 100 km.

The results of our study could lead to a hierarchical gradation in the relevance of the differentiating clusters, with the Rhine River being the most important. For hunting grounds separated by the Rhine River and directly adjacent to it, there was almost 100% differentiation according to $R^2$%. This result was largely independent of the Bayesian algorithm used to calculate the clusters.

Differences between the Bayesian algorithms were to be expected. Sub-structuring of a continuous natural population can result from real barriers and from gradients of landscape resistance and most importantly from isolation by distance (Cushman et al. 2006). Bayesian clustering methods tend to overestimate the number of clusters in the presence of isolation by distance (Frantz et al. 2009, Safner et al. 2011). The highly significant Mantel test including all samples suggests that isolation by distance processes structure genetic diversity at the largest scale. Mantel tests within distance classes showed a positive spatial autocorrelation up to about 100 km, indicating that evolutionary processes are dominated by gene flow up to this distance. Therefore, considering the low spatial expansion of the study area (north-south extension = 225 km; west-east extension = 150 km), strong isolation-by-distance patterns cannot be expected and thus results from the Bayesian clustering methods should be valid.

The low differentiation of wild boar within the 4 clusters is due to the dominance of forest cover and small-scale agriculture in these areas, facilitating dispersal and genetic homogenization of wild boar. This has also been observed by Rutten et al. (2019) by means of the low genetic distance in wild boar living in these landscapes.

The regions with a differentiating effect contain different landscape elements with a potential barrier function, especially rivers, freeways, and settlement areas. The comparison of the Eifel with the Hunsrück, with particular emphasis on the hunting grounds H and L, argues against an overly pronounced differentiating effect of the Moselle River, so that the differentiation between the Eifel and Hunsrück regions, identified in particular with TESS, is more likely to be on the freeways A48 and the A1 and their accompanying structures (although it is not possible to make a precise allocation).

The Moselle River, which separates the Eifel from the Hunsrück is about 40 m wide with an average discharge of 313 $m^3$/second. Obviously, enough boar are capable of crossing the river to limit detectable genetic differentiation. This would presumably also be enough movement to transmit ASF. In contrast, the Rhine River is quite different. In the north of the country, it separates the Westerwald and Taunus (east of the Rhine River) from the Eifel-Hunsrück regions. The Rhine River has an average discharge of about 2,000 $m^3$/second. Its width is 150–250 m. The Rhine River causes the greatest genetic differentiation for wild boars in the present study, independent from the algorithm applied. The A61 freeway runs parallel to the Rhine River. The population genetic comparison of wild boar in hunting grounds C (east of Rhine River and A61), D (between Rhine River and A61), and E (west of A61 and Rhine River) shows a significant differentiating effect for the Rhine River but not for the A61.

A major freeway, the A6, runs between the area of differentiation between Pfalz and Eifel-Hunsrück. Again, it remains to be evaluated how much of this effect is due to the freeway itself, which is largely fenced in but still leads over viaducts or to parallel structures (settlement, agriculture). The areas surrounding the freeway might have a higher

resistance to movements and be the real barrier rather than the freeway itself (Frantz et al. 2012). To answer this question, a much finer and more comprehensive sampling would be necessary. Overall, however, the area of the A6 freeway together with its parallel structures has a highly significant differentiating effect. The differentiation between Westerwald and Taunus is probably due to the dense settlement in this area of Rhineland-Palatinate, close to Koblenz.

These differentiating effects of the Rhine and further differentiation between the Pfalz and the Eifel-Hunsrück, the Westerwald and the Taunus and, to a lesser extent, between the Eifel and the Hunsrück indicate areas with lower wild boar connectivity than in most parts of Rhineland-Palatinate. In these areas it should therefore be easier to prevent movement of infected animals as a major strategy to control ASF in wild boar (EFSA 2018). Infected animals can transmit the virus for 3 to 10 days, with the possibility of viral shedding persisting for up to 100 days in wild boar (Blome et al. 2020). Transmission by vectors (i.e., soft ticks [*Ornithodoros*]) is important in Africa but not in the current European outbreak (Costard et al. 2013) and the role of other arthropods seems rather limited for disease spread across areas (Blome et al. 2020). Therefore, controlling the migration of infected animals is at the heart of the European Union's prevention and control of ASF. Three measures in particular serve to implement this objective in already infected areas: eliminate hunting in the centers of their ranges, increase hunting pressure at their peripheries, and fencing to prevent infected animals from migrating. The consistent implementation of these measures led to the complete eradication of the disease from the wild boar population of the Czech Republic (Dixon et al. 2020). Even for areas at risk that are not yet infected, there is a requirement from the European Union to work on wild boar connectivity and to reduce wild boar contacts to achieve better control of the spread in the event of an outbreak (EFSA 2018). Wild boar contacts are particularly high within groups but also exist between groups because their home ranges often overlap. Lack of food and cover, supplementary feeding, and hunting pressure increase the home ranges and the chance of contacts (Johann et al. 2020). Young animals 0.5–2 years of age are disproportionately involved in contacts between groups, primarily for the biological purpose of reproduction (Podgórski et al. 2018). Thus, the targeted removal of yearlings is especially important to reduce the risk of infection.

Natural barriers to the spread of ASF in northeastern Europe (EFSA 2018) were not detected under the then given conditions of massive infection pressure and the concurrent spread of the virus via wild boar and anthropogenic sources. These results are preliminary and should be interpreted with caution (EFSA 2018). Our results show that barriers might exist in Rhineland-Palatinate. Of course, no absolute conclusion can be drawn on the basis of the available results with regard to an actual spread of ASF in case of an introduction, but the pronounced, statistically validated differences in the genetic connectivity of the wild boar could certainly be used to concentrate efforts to reduce the spread of the disease by placing barriers to movement.

Such an effect would be conceivable at least at lower infection pressure, possibly also in the case of endemic infection. There are indications that because of the high virulence of the current ASF strains (Pikalo et al. 2020), most wild boars initially die from the disease. Some animals, however, recover and are able to spread virus for an unknown period of time (Eblé et al. 2019, Ståhl et al. 2019). In such times it would be desirable if in manageable regions the introduction and spread of the disease to neighboring areas could at least be slowed down. The available data provide first indications for such possibilities. Further investigations are required for more details.

The low differentiation within large areas of the country shows that wild boar are less affected by barriers than many other mammals (e.g., red deer; Vassant et al. 1993, Dobias and Gleich 2010, Tottewitz et al. 2010, Frantz et al. 2012). Wild boars regularly cross fenced freeways (Vassant et al. 1993). Furthermore, the size of current wild boar populations is likely to counteract genetic drift due to larger effective population sizes, making differentiation difficult (Frantz et al. 2012). The potential of main rivers to restrict dispersal of the wild boar, although not completely, has also been described in other studies (Ferreira et al. 2009, Tadano et al. 2016). In contrast to the situation in Belgium (Frantz et al. 2012), Tadano et al. (2016) described significant genetic differentiation in some wild boar populations in Japan by freeways. It is therefore not possible to make a general statement about freeways, but the specific situation of the freeway and its surroundings needs consideration. Further studies are required to define them exactly for the present study area.

Against the background of the high density of wild boar and the varied landscape, wild boars in Rhineland-Palatinate show unexpectedly low heterozygosity and low allele frequencies compared to other studies. Veličković et al. (2016) reported that wild boars of the Alps, Italy, Poland, the Balkans, Spain, and central Europe show an allelic richness around 7.2–7.9 and an observed and expected heterozygosity of 0.69–0.74 and 0.74–0.79, respectively (Nikolov et al. 2009, Tajchman et al. 2018).

Comparable values to the current study were ascertained in isolated populations in Slovenia, Italy, Sardinia, and Portugal. Similar values were also described for samples from wild boars in Germany by Nikolov et al. (2009). The Fis values of the present study show a pronounced variation but are essentially in agreement with the Fis values reported in the overview by Veličković et al. (2016). Why the genetic diversity and heterozygosity is comparatively low cannot be answered conclusively. A possible cause could be the microsatellite markers used, although 11 of the 12 markers come from a panel recommended by the International Society for Animal Genetics (https://www.isag.us, accessed 23 Dec 2020). The microsatellite markers used are not directly comparable with those of the other studies. Pérez-González et al. (2017) describe a decrease in heterozygosity

in wild boar in Spain, Portugal, and Romania and propose outbreeding avoidance as the most likely biological mechanism. According to their opinion, wild boars are being selected by processes such as sequential mating, multiple paternity, male-biased dispersal, or male heterozygous advantage, which all contribute to increased genetic diversity. Thus, wild boar seem to be relatively tolerant of inbreeding (Kokko and Ots 2006, Poteaux et al. 2009, Pérez-González et al. 2014, Podgórski et al. 2014). Perhaps the relatively small-scale hunting, which allows wild boar to spread continuously through their matriarchal societies, plays a further role. Maselli et al. (2016) explain the high genetic variability of wild boar in Italy by the recurrent shifts in populations during the last ice ages and by a long tradition of anthropogenic animal displacements since historical times. In Germany, on the other hand, the wild boar population had largely collapsed by 1949 and has since then, mainly by its own efforts, increased in size and distribution. The dramatic influence of the last ice ages on the genetic diversity of wild boar and the associated markedly low genetic variability in Germany is confirmed by Vilaca et al. (2014).

## MANAGEMENT IMPLICATIONS

At present, Rhineland-Palatinate represents a non-affected area at risk of ASF introduction from Belgium and the eastern federal states of Germany. Combating the spread of diseases like ASF by spatial fixation is among the major aspects of today's epidemic control. An important requirement for this is to understand the host's dispersion dynamics. The results of the present study are based on the genetic differentiation between wild boar hunting grounds and thus, consider a longer-term scenario of exchange between regions. Genetic evidence suggests that for the entire Eifel-Hunsrück region and for the Pfalz, apart from the geographical distance, few natural or anthropogenic obstacles restrict the spread of wild boar, whereas the Rhine River caused the strongest differentiation. We therefore recommend fencing and intensive hunting before an outbreak particularly on the identified barriers to manage spread of ASF. In addition, all measures should be taken to minimize the spread of wild boar beyond their normal home range. This includes in particular the targeted removal of yearlings.

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