


High domestic pig contribution to the local gene pool of free-living European wild boar: a case study in Poland

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Abstract Rates of hybridization between wild and domesticated animals appear to be increasing worldwide. Recent results suggest that genetic introgression from domestic swine into European wild boar is much more common in local populations than expected, based on pan-European studies. Thus, we screened the genetic purity of 265 free-living wild boars from two hunting areas in Poland by genotyping the melanocortin receptor 1 gene (*MC1R*) for polymorphism. Unexpectedly, high numbers of individuals with domestic genes (24%) were identified. This suggests that mixed ancestry may be common in Polish wild boar. Among admixed individuals, backcrosses with domestic pig and/or introgressed wild boars were detected (2%). Multiple commercial domestic pig breeds are possibly involved in the introgression observed in the study populations. In addition, the absence of significant differences in the frequency of wild-type allele among two hunting areas suggests high dispersal of individuals and gene flow among populations. We conclude that further study is needed to better understand the mechanisms and sources of introgression in wild boars in Poland.

Keywords Wild boar · Domestic swine · *Sus scrofa* · Introgression · *MC1R*

Introduction

Hybridization is the phenomenon of gene pool mixing between different taxa. If hybrid offspring survive, are fertile, and contribute their alleles to future generations by backcrossing, the process is called introgressive hybridization (introgression). Introgression is one of the primary threats to global biodiversity (Rhymer and Simberloff 1996; Allendorf et al. 2001). For example, evidence is accumulating that rates of hybridization between wild species and their domestic relatives are increasing (Randi 2008; Canu et al. 2014). This hybridization may occur intentionally or accidentally (Fulgione et al. 2016). Domestic genes may provide a fitness advantage for individuals of some species (Anderson et al. 2009; Goedbloed et al. 2013a; Fulgione et al. 2016). However, there is growing concern that introgression from domestic into wild animals may compromise the genetic integrity of the latter (Largiadèr 2008). The resulting loss of genetic diversity may correspond to a reduction in fitness and adaptive potential, as well as favor disease transmission (Allendorf et al. 2001; Todesco et al. 2016).

Wild boar (WB; *Sus scrofa*) is an important game species that is highly managed throughout its range in Eurasia and Northwest Africa. This species is the ancestor of domestic swine (DS; *Sus scrofa domestica*), and WB can successfully crossbreed with DS. This can unintentionally occur where there is open-air domestic swine farming, which is a common management system in Bulgaria, Croatia, Iberia, Corsica, and Sardinia (Scandura et al. 2008; Apollonio et al. 2010). Intentional hybridization between WB and DS also commonly occurs for the purpose of producing less aggressive animals,

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obtaining larger litter sizes, increasing piglet growth rates, achieving hybrid vigor, creating “wild boar-like” hybrids to be released for hunting, or fraudulent WB meat substitution (Booth 1995; Aravena and Skewes 2007; Randi 2008; Fontanesi et al. 2014).

Wild boar population sizes have been increasing worldwide, and WB is considered an agricultural and forestry pest in many countries (Dzięciołowski and Clarke 1989; Gipson et al. 1998; Waithman et al. 1999; Apollonio et al. 2010; García et al. 2011; Wilson 2013). Wild boar is currently one of the world’s most widespread large mammals and is the second most abundant ungulate in Europe (Herrero et al. 2008; Apollonio et al. 2010; Massei et al. 2015). More than three million WB are harvested annually in Europe (Massei et al. 2015). The trend of increasing WB populations is also evident in Poland, where the harvest of WB has increased from 118,000 in 2000 to 264,000 in 2015 (Central Statistical Office of Poland 2015).

The spread of WB may be due to several factors (Massei and Genov 2004; Vetter et al. 2015). One recent hypothesis is that introgressive hybridization between WB and DS increases WB fitness and invasiveness (García et al. 2011; Frantz et al. 2013; Goedbloed et al. 2013a). However, the degree to which WB and DS hybridization occurs is currently uncertain. One viewpoint is that intensive farming during the last two centuries has progressively reduced the risk of hybridization (Scandura et al. 2011a). This is supported by data suggesting that there are marginal DS gene contributions to the genetic make-up of free-living WB in Europe (5–10% in Scandura et al. 2008, 2011a and 11% in Canu et al. 2016). However, more extensive introgression has recently been reported in WB populations located in Luxembourg (27%) and the Emilia-Romagna region in Italy (17.1%) (Frantz et al. 2013; Fontanesi et al. 2014). Recent preliminary research using genome-wide single-nucleotide polymorphism (SNP) data documents variable levels of introgression in Europe and suggests the possible existence of a hybrid zone(s) in Europe (Iacolina et al. 2016a).

In Poland, the risk of unintentional hybridization between WB and DS is considered minimal because DS management is primarily intensive and indoors, thereby reducing the opportunities for direct contact between domestic and wild animals. Moreover, intentional hybridization is prohibited by Polish law except on registered farms. Thus, high levels of recent introgression are not expected. However, because research to date is based on only 44 individuals, data on introgression into WB in Poland is inconclusive. While Babicz et al. (2013) found three individuals with domestic alleles among 10 WBs from Lublin (eastern Poland), Canu et al. (2016) found only one introgressed individual among seven Polish WBs. Neither Gongora et al. (2003) ($n = 15$) nor Fang et al. (2009) ($n = 12$) found evidence of hybridization among Polish WBs.

Sequence diversity at the melanocortin receptor 1 (*MC1R*) locus is widely used to distinguish between meat originating from WB, DS, and their hybrids, as well as to detect introgression from DS into WB (Giuffra et al. 2000; Koutsogiannouli et al. 2010; Frantz et al. 2013; Fontanesi et al. 2014; Canu et al. 2016). In addition, mtDNA sequencing, microsatellite genotyping, and a single-nucleotide polymorphism (SNP) assay have been used to look for evidence of introgression (Giuffra et al. 2000; Scandura et al. 2008, 2011b, Frantz et al. 2012, 2013, 2015, Goedbloed et al. 2013b, a; Herrero-Medrano et al. 2013; Iacolina et al. 2016b).

Unfortunately, the resources of most wildlife geneticists are inadequate for using these markers. Thus, we used a simple diagnostic test for *MC1R* gene polymorphism to screen for the presence of domestic alleles in a large number of Polish WBs sampled in two hunting areas. We also genotyped DS breeds used for commercial crossbreeding in Poland and used these genotypes as reference material for study of WB genetic purity. Specifically, we seek to answer the following questions: (1) has contact with DS resulted in introgression of domestic genes into free-living WB populations and (2) is there a significant difference in the frequency of wild-type alleles between hunting areas in Poland?

Methods

Blood sample collection

A total of 359 blood samples were genotyped. Ten samples from each of eight DS breeds ($n = 80$) were obtained from either the National Research Institute of Animal Production (Kraków-Balice, Poland) or breeders who participate in “The National Breeding Program” and “The Programme of Genetic Resource Protection”. These samples included both commercial breeds (Pietrain, Hampshire, Duroc) and Polish native pig breeds (Polish White Landrace, Polish Large White, Żłotnicka White, Żłotnicka Spotted and Puławska). Three WB × DS (Duroc) hybrids housed at the Institute of Applied Biotechnology and Basic Sciences, University of Rzeszów, and eleven DS hybrids (Polish Large White × Duroc) were also used in this study. In addition, blood samples were taken from 265 free-living animals identified in the field as WBs. All animals were legally hunted in accordance with the national WB regulations during the 2011–2015 hunting seasons. Sample collection was performed by licensed hunters as a part of routine wildlife management and hunting club shooting programs without bias towards age, sex, or coat color. Animals were shot in two neighboring hunting areas in north-central Poland: Krajna ($n = 93$) and Kujawy ($n = 172$). These natural populations are relatively well isolated from each other by landscape barriers, including rivers (Noteć, Warta, and Wisła) and a road network (Fig. 1).

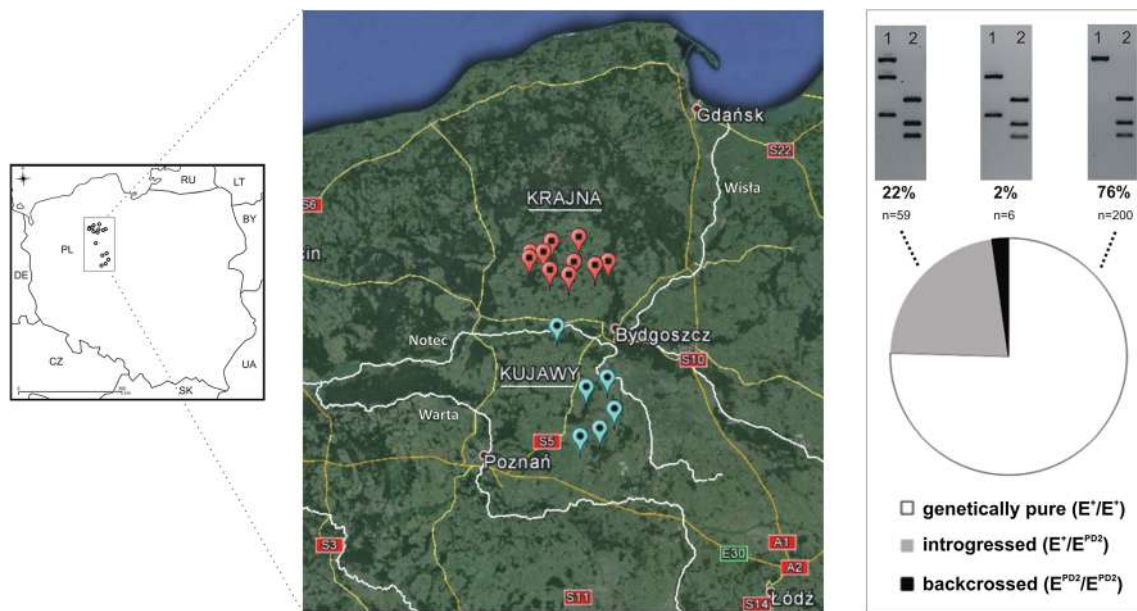


Fig. 1 Distribution of sampling sites and polymorphism of wild boar in Poland. Numbers represent digestion patterns of *MC1R* gene with BspHI and BstUI (1 and 2, respectively)

Analysis of the *MC1R* gene

Two loci, *Extension* (*E*) and *Agouti* (*A*), control much of variation in coat color and pattern in mammals (Barsh 1996). The melanocortin receptor 1 (*MC1R*) encoded by *Extension* locus is a G-protein coupled receptor determining the switch between production of black/brown eumelanin and red/yellow pheomelanin. A series of alleles of the *MC1R* gene have been found so far in pigs. The wild-type (E^+) allele has been identified in WBs, the Hungarian Mangalica DS breed and DS raised in free or semi-free conditions in Sardinia (Canu et al. 2016). Meanwhile, in other DS breeds, because of different human needs or cultural preferences, domestication and human selective pressures caused coat color variation generated by non-synonymous mutations (Fang et al. 2009; Li et al. 2010). The phenotypically defined alleles in porcine *MC1R* gene are black (E^{D1} and E^{D2}), black spotting (E^P), and red (e). Moreover, several additional allelic variants are also reported based on subsequent sequence analyses (Andersson 2003; Fang et al. 2009). The black alleles (E^{D1} and E^{D2}) are dominant over E^P and e , whereas E^P is incompletely dominant over e (Aravena and Skewes 2007; Dun et al. 2007). *MC1R* genotypes and corresponding coat color phenotypes are presented in Table 1.

We adopted methodology based on PCR-RFLP analysis of the *MC1R* gene (Fajardo et al. 2008). We used nomenclature for porcine *MC1R* alleles described by Fang, with modifications proposed by Fontanesi et al. (Fang et al. 2009; Fontanesi et al. 2014). Since alleles E^P and E^{D2} cannot be separated from each other, they were combined as one allele E^{PD2} . Briefly, we were able to differentiate between six genotypes in WB, DS,

and their hybrids: E^{PD2}/E^{PD2} ; e/e ; E^{PD2}/e ; E^+/E^+ ; E^+/E^{PD2} ; E^+/e (for details see Fig. 2).

Amplification of the *MC1R* gene was performed using the Phusion Blood Direct PCR Kit (Thermo Scientific, Affibody AB, Sweden) according to manufacturer's directions, with minor modifications. Briefly, PCRs were carried out directly from whole blood placed onto Whatman FTA Classic Cards (Whatman, UK) that were pretreated by washing in Purification Reagent (Whatman, UK) and TE Buffer (Whatman, UK). PCRs were performed in a total volume of 30 μ l containing 1 \times Phusion Blood PCR Buffer, 1 μ M each *MC1R* primer, 0.6 μ l of Phusion Blood II DNA Polymerase, and a 2.0-mm diameter circle of FTA Classic Card.

The primers *MC1R*-FW and *MC1R*-REV failed to amplify under our PCR conditions (Fajardo et al. 2008). Consequently, we redesigned both primers based on the DNA sequences of the *MC1R* gene of *S. scrofa* deposited in GenBank (accession numbers, EU443645 and DQ191184). Finally, we analyzed the variation at the *MC1R* using forward primer: 5'-AGTG

Table 1 Genotypes and phenotypes at the *MC1R* locus in wild boar and domestic swine

<i>Extension</i> genotype	Coat color phenotype	Origin	Breed examples
E^+/E^+	Wild type (reddish/brown)	European/Asian	WB
E^{D1}/E^{D1}	Uniform black	Asian	Meishan
E^{D2}/E^{D2}	Uniform black	European	Hampshire
E^P/E^P	Black spotting on a red or white background	European	Pietrain
e/e	Uniform red	European	Duroc

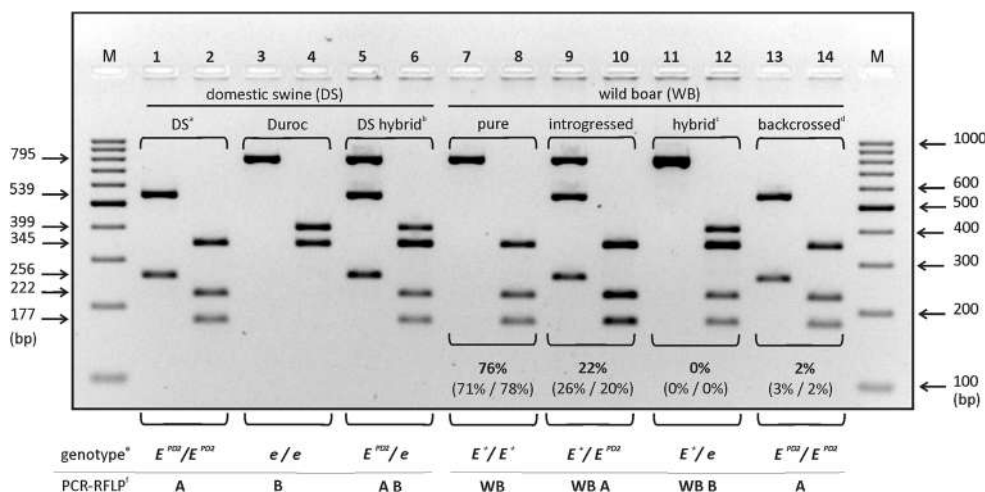


Fig. 2 PCR-RFLP analysis of *MC1R* gene in domestic swine and wild boar using BspHI (lines 1, 3, 5, 7, 9, 11, 13) and BstUI (lines 2, 4, 6, 8, 10, 12, 14) and corresponding genetic profiles (below the picture). Lengths of restriction fragments are shown on the left. Frequencies of *MC1R* genotypes are given for total population and two hunting regions in Poland—Krajna and Kujawy, respectively (in parentheses). *M* molecular weight marker (size of DNA fragments on right); ^a genetic

profile common to domestic swine breeds: Pietrain, Hampshire, Polish White Landrace, Polish Large White, Złotnicka White, Złotnicka Spotted, Puławska, ^b Polish Large White × Duroc; ^c WB × Duroc, ^d backcrosses with domestic swine and/or introgressed individuals, ^e alleles described by Fang et al. (2009) with modification proposed by Fontanesi et al. (2014), ^f PCR-RFLP profiles according to Fajardo et al. (2008)

CCTGGAGGTGTCCATTCCC-3', and reverse primer: 5'-CGTAGATGAGGGGTCACGATGGA-3' (modifications underlined).

Negative and positive PCR controls were included in each set of reactions (water and Duroc, respectively). Amplification was run in a C1000 Touch Thermal Cycler (Bio-Rad, USA) using a two-step thermal profile without an annealing step, as recommended for the Phusion Blood Direct PCR Kit. Pre-PCR denaturation occurred at 98 °C for 5 min. This was followed by 35 cycles of denaturing at 98 °C for 1 s, extension at 72 °C for 15 s, and a final extension step at 72 °C for 1 min. The PCR products were checked by electrophoresis on 1% agarose gels containing 0.5 µg/ml ethidium bromide in 1× TBE buffer. Ten microliters of each PCR products was digested with 10 U of BspHI or BstUI restriction endonuclease and 1× CutSmart Buffer (New England Biolabs, UK) in a total volume of 20 µl for 1 h. Digested DNA fragments were separated on 2% high resolution agarose (Nu Micropor, Prona, EU) in 1× TBE buffer at 5 V/cm. DNA fragments were visualized with the Gel Doc XR+ System (Bio-Rad, USA). Generuler™ 100 bp DNA Ladder (Thermo Scientific, USA) was used as a DNA molecular weight marker.

Significance of difference in the frequency of the wild-type allele between two hunting areas was tested using the chi-square test.

Results

We were able to amplify 795 bp *MC1R* fragments in all samples. Restriction analyses with BspHI and BstUI

endonucleases provided DNA fragments in expected sizes (Fig. 2). Duroc breed pigs had genotype *e/e*. All Polish native DS breeds and two commercial breeds (Pietrain and Hampshire) carried the common genotype (E^{PD2}/E^{PD2}) (Table 2).

Most WBs carried only the wild-type E^+ allele (76% in the total sample; 71 and 78% in Krajna and Kujawy, respectively). However, 24% of WB showed signs of DS contribution (E^+/E^{PD2} and E^{PD2}/E^{PD2}) (Fig. 1). Differences in the frequency of WBs carrying the domestic allele E^{PD2} among the two hunting areas (29 and 22% in Krajna and Kujawy, respectively) were not statistically significant.

Table 2 Individual genotypes identified at the *MC1R* locus in domestic swine and wild boar populations in Poland

Origin	No. of animals	Genotypes (no. of animals)			
		E^+/E^+	E^+/E^{PD2}	E^{PD2}/E^{PD2}	<i>e/e</i>
Domestic swine					
DS		–	–	70	–
Duroc		–	–	–	10
Wild boar					
Krajna	93	66	24	3	–
Kujawy	172	134	35	3	–
Total	265	200	59	6	–

DS domestic swine breeds (*n* = 10 each): Pietrain, Hampshire, Polish White Landrace, Polish Large White, Złotnicka White, Złotnicka Spotted, Puławska

Discussion

Our newly developed primers and direct PCR method (without DNA extraction) were easy to employ and have wide applicability. We used *MC1R* polymorphism to show that WBs from two hunting areas are not genetically purebred. We also showed that the Duroc DS breed had genotype *e/e*, while all Polish native DS breeds and two commercial breeds (Pietrain and Hampshire) carried the common genotype (E^{PD2}/E^{PD2}). The same results for Złotnicka White, Złotnicka Spotted, and Puławska were observed in Poland by Babicz et al. (2013). However, this is the first report on genetic variation in *MC1R* gene in two local pig breeds: Polish White Landrace and Polish Large White.

Mixed ancestry has been found worldwide in WB populations. The Polish WB populations we studied have some of Europe's highest levels of DS introgression. These levels are similar to those found in regions where DS are reared in semi-free conditions (up to 10–20% in Canu et al. 2016) or WB populations that have been restocked with captive-bred individuals that have been cross-bred with DS (27% in Frantz et al. 2013). Since analysis using *MC1R* alleles is likely to severely underestimate the number of WB with a domestic ancestor (Frantz et al. 2012, 2013), we cannot exclude the possibility that the real rate of introgression may be much higher than observed in this study. However, our result should be interpreted with caution and confirmed by DNA markers that would reveal the admixture level (e.g., SNPs or microsatellites).

Our results contradict Scandura et al. (2011a) and Goedbloed et al. (2013a) suggesting that the contribution of DS genes to the WB gene pool in Europe is marginal. In fact, introgression of domestic genes may reach much higher levels at very local scale (Fulgione et al. 2016), especially in regions where open-air pig farming (Canu et al. 2016) and/or hybridization in captivity (Gongora et al. 2003; Canu et al. 2014) and/or restocking with farmed WB that had been cross-bred with DS (Frantz et al. 2012, 2013; McDevitt et al. 2013), are practiced.

Crosses between WB and DS were relatively common during domestication processes but also noted throughout Europe at present (White 2011). Nowadays, illegal and unauthorized hybridization in captivity and subsequent accidental escapes or intentional releases of captive-bred individuals constitutes the major source of the spread of domestic genes into wild boar populations (Koutsogiannouli et al. 2010; Apollonio et al. 2010; Frantz et al. 2012, 2013; McDevitt et al. 2013; Canu et al. 2014; Murakami et al. 2014). We believe that high DS contribution to the local gene pool of free-living WB in Krajna and Kujawy may result from the clandestine release and/or escape of farmed WBs, that had been crossed with DS in captivity. Moreover, the potential sources of hybridization in the study area may include five registered WB × DS hybrid farms located within a radius of 60 km surrounding the study sites.

It should be noted that the intentional hybridization is prohibited by Polish law since 2008 and breeding of hybrids

is allowed only in 37 farms registered by the General Directorate for Environmental Protection. Similarly, breeding of the WB is allowed exclusively in 34 farms registered by the Polish Ministry of the Environment. Unfortunately, the numbers of farmed WBs and hybrids are unknown and the registration of WB farms does not guarantee that WBs are genetically pure. Thus, it seems that genetic controls in Polish WB farms should be enforced to identify and selectively remove admixed individuals. Moreover, it seems that the threat to the native gene pools and genetic integrity of WB in Poland is represented by both legal and illegal WB × DS hybrids farms. Although, to date, no massive escape or release of farmed individuals was recorded in Poland, we cannot confidently exclude the possibility that escapes or illegal releases from farms were performed in the study area. Alternatively, hybrid origin could result from historic crosses between WB and DS in semi-wild condition, followed by genetic drift (Frantz et al. 2013). Until the modern era, the seasonal practice of releasing DS in the forest provided plenty of opportunities of cross-breeding with WB (White 2011; Marshall et al. 2014). Although, recessive or semi-dominant *MC1R* alleles may be masked by dominant genes, the coat color is a remarkable trait which is likely to undergo strong selective pressure in the wild. Thus, the introgressed alleles driving color variation are likely to be purged within a limited number of generations. A recent study by Battocchio et al. (2017) seems to support the hypothesis that phenotypically anomalous WBs are not fully fit for life in the wild and poorly accepted by their cohort. Hence, the presented results suggest ongoing or very recent hybridization rather than historic processes. However, these hypotheses need further insights.

A lack of significant difference in the frequency of wild-type allele among two WB populations in Poland may be due to opportunities for gene flow that arise during occasional migration of individuals to neighboring populations. Since WB can migrate over distances of up to 150–500 km (Andrzejewski and Jezierski 1978; Jerina et al. 2014), the isolation by roads and waterways may be not fully effective between Krajna and Kujawy.

The sources of Polish WB population introgression are unclear, but multiple DS breeds may be involved. Our results clearly indicate that only the DS allele E^{PD2} was introgressed into WB populations in the study area. This is not surprising because the vast majority of DS breeds in Poland are fixed for this allele (Fig. 2). However, the absence of introgressed WB individuals carrying the domestic allele *e* (E^+/e) suggests that the Duroc breed did not contribute to the introgression. Although, lack of genetic introgression from Duroc into WB has also been suggested in the Netherlands, Luxembourg, and Germany based on SNP analysis (Goedbloed et al. 2013a), four hybrids (E^+/e) were reported in Italy (Fontanesi et al. 2014). The reasons why allele *e* has been not introgressed into WB in Poland may be that the Duroc breed has been brought to Poland recently. Since the DS breeding system in Poland is based on crossing Polish White Landrace

or Polish Large White dams with Pietrain, Duroc, or Hampshire sires, the participation of Duroc breed in commercial crossing is rather low (4%). Unfortunately, since the allele E^{PD2} was detected in all DS breeds except Duroc (Fig. 2), the methods used in this study cannot be used to detect the origin of introgression in greater detail.

While the origins of introgression are uncertain, there are several possibilities for why individuals with the WB phenotype would carry the DS genotype (E^{PD2}/E^{PD2}) found in this study (2–3%, Fig. 1). One potential explanation is that there have been backcrosses between DS (E^{PD2}/E^{PD2}) and introgressed WBs (E^+/E^{PD2}). Another possibility is that there have been crosses between introgressed WBs (E^+/E^{PD2}). A third scenario would be crosses between backcrossed WBs (E^{PD2}/E^{PD2}). A low number of second generation or later hybrids and past introgression were observed in other European populations using the *MC1R* locus or a genome-wide single-nucleotide polymorphism (Koutsogiannouli et al. 2010; Goedbloed et al. 2013a; Fontanesi et al. 2014). However, we cannot confidently exclude the possibility that the allele E^{PD2} occurs naturally at very low frequency in other populations of free-living WBs in Poland.

Polish WB populations are reduced by approximately 80% annually, due to high harvest rates. This may unintentionally promote the spread of “domestic genes” by reducing the size of local populations to the extent that DS alleles have a greater chance of being fixed. This is most likely to occur at the local scale where there is open-air pig farming, hybridization in captivity, or restocking of farms with WB cross-bred with DS (Gongora et al. 2003; Frantz et al. 2012, 2013; McDevitt et al. 2013; Canu et al. 2014; Fulgione et al. 2016). Disruption of WB population structure could also favor migration of individuals with DS alleles. This would be consistent with the lack of significant difference in the frequency of wild-type allele among two Polish WB populations we studied. Migration and local-scale concerns raise uncertainty as to the appropriate sampling scale for identifying introgression of DS genes into WB populations. Further investigation is needed to better understand the genetic make-up of contemporary Polish WB populations, as well as the mechanisms and sources of DS allele contributions.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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