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Copy number variation within *CLEC4E locus* as a molecular tool for distinguishing DNA from domestic pigs and wild boars

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Wild boar, the primary wild ancestor of the domestic pig, frequently interbreeds with its domesticated counterpart. Distinguishing between the domestic pig (*Sus scrofa domestica*), wild boar (*Sus scrofa*), and their hybrids is ambitious due to their close evolutionary relationship and the various genetic markers they have in common. The aim of the study was to develop a reliable and straightforward method to distinguish pig and wild boar DNA based on CNV in the *CLEC4E* gene, preventing food fraud and ensuring product safety.
The study population consisted of domestic pigs (n=42), wild boars (n=40) and their hybrids (n=6). The domestic pig group is comprised of four breeds: Polish Large White , Duroc, Polish Landrace and Puławska. Wild boars were sampled from the South West region of Poland. The pair of primers (F/R) designed for the *CLEC4E* gene were used for analysis. A quantitative-comparative CT reaction with a melting curve (SybrGreen) was performed for the tested samples.
The obtained results indicate different CT values for pigs and wild boars. The CT values below 18.70 cycles and melting temperature equal or above 90°C indicate wild origin, while the CT below 17.67, and melting temperature lower than 89.20°C additionally supported by the shape of the melting

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curve, indicate the presence of a domestic pig or hybrid. The analysis of 85 out of 88 tested samples, supported the assumptions based on the CT value, while for the remaining 3 the result was doubtful due to inconsistent interpretation for CT and / or Tm.

KEY WORDS: CNV / *CLEC4E* gene / real-time PCR / swine

Differentiating between domestic pigs (*Sus scrofa domestica*) and wild boars (*Sus scrofa scrofa*) is crucial for identifying and preventing food fraud. This research, which provides a reliable species identification method, is essential for consumers, food and animal feed producers, and prosecuting authorities [Lorenzini *et al*. 2020]. The reliability of the method, demonstrated through the development and commercial implementation of molecular tests to detect animal components in human food and animal feed, instils confidence in its effectiveness. Techniques based on PCR (e.g., RFLP, Real-Time PCR) already enable the successful and accurate identification of cattle, horse, and poultry DNA in food and animal feed [Natonek-Wiśniewska *et al.* 2013, Safdar *et al*. 2014]. Therefore, our research using a PCR based technique aims to simplify the identification of porcine components, ensuring the quality and safety of meat products for consumers. [Koutsogiannouli *et al.* 2010, Lorenzini *et al*. 2020].

Fajardo *et al.* [2008] pioneered studies differentiating wild boars from commercial pig breeds by combining nuclear (melanocortin one receptor, MC1R) and mitochondrial DNA (D-loop) analysis. Their research found that analyzing the MC1R gene was significantly more effective than the traditional method based on mitochondrial DNA polymorphism [Fajardo *et al*. 2008], providing the users with confidence in the research findings. The NR6A1 gene, a strong candidate for a vertebrae quantitative trait locus (QTL), has also been studied. A substitution of proline to leucine at NR6A1 codon 192 (p.Pro192Leu) results in an increased number of vertebrae in commercial pig breeds [Mikawa *et al*. 2007].

Recently, the genotyping-by-sequencing method has been implemented to differentiate both subspecies [Koseniuk *et al*. 2023].

The European population of wild boars descends from the Asian population. Both lineages stayed separated due to the geographical boundaries, which are the Ural mountains. The separation of the European and Asian wild boar populations occurred around 1 million years ago [Groenen *et al*. 2012]. The European pigs were divergent from European wild boars, while the Asian pigs were from Asian wild boars. Consequently, both Asian and European domestic pigs and wild boars differ genetically. However, neither European subspecies has yet been successfully discriminated. This is due to the frequent crossbreeding incidents, which are confirmed by numerous genetic studies [Lacolina *et al*. 2018, Lorenzini *et al*. 2020, Groenen *et al*. 2012, Larson *et al*. 2013].

Copy number variation (CNV) can be defined as unbalanced, structural rearrangements of the genome, which lead to significant increases or decreases in DNA content. The insertion or deletion DNA segments >50 bp are considered as CNVs [Zarreli *et al*. 2015]. This variability in the length of particular DNA segments

appears between individuals representing the same or other populations [Pos *et al*. 2023]. To date, in the porcine genome, several CNVs have been determined to be correlated with selective traits like coat colour, backfat thickness, or reproduction [Zheng *et al*. 2020, Fowler *et al*. 2013; Rubin *et al.* 2012]. Considering the above, comparing the CNV of certain potentially selective traits can help identify a recently evolved differentiating region between domestic pigs and wild boars. Jang *et al*. [2023] defined CNVs as vast samples covering 328 pigs and wild boars. They found that CVNs of gene *CLEC4E* are present in wild populations from Europe and Asia, whereas they are lost in the domestic species .

Despite the numerous research in this field, there is still a lack of useful genetic tools to enable the distinction between domestic pigs and wild boars based on DNA samples. Therefore, our study aimed to identify the variation in the copy number of *CLEC4E* and investigate the possibility of implementing it to differentiate wild boars from domestic pigs.

Material and methods

Blood samples were collected from 40 wild boars, 42 unrelated domestic pigs(*Polish Large White (WBP), n*=3; Polish landrace (PBZ), n=13; Duroc (DUR), n=15, Puławska (PUŁ)n=11), and pig-wild boar hybrids (n=5). All hybrids were the F1 generation. All samples were obtained from routine diagnostic DNA profile tests. Before the analysis, the samples were stored in the laboratory at -20 C. All tested samples are presented in Table 1. Total genomic DNA was extracted using Sherlock (A&A Biotechnology, Poland) according to the standard protocol for frozen blood.

To determine the DNA concentration and purity of the obtained DNA isolates, the absorbance was measured at 260 nm and 280 nm using a Nanodrop spectrophotometer (Thermo Scientific, USA). The DNA isolates were stored at 4°C. The obtained DNA extracts were optimized for further analysis to a 50 ng/ul concentration.

Domestic pig and wild boar discrimination test

CNVs can be detected using PCR-based tests by identifying: (1) the amplification cycles required to reach a relative threshold fluorescence intensity in real-time quantitative PCR (qPCR) assays [Higuchi *et al*. 1993, Heid *et al*. 1996], (2) the denaturation properties reflected in the melting temperatures or the shapes of the melting curves during conventional or high-resolution melting analysis [Radvansky *et al.* 2010].

We amplified a 1286-bp fragment of *CLEC4E* between 63219461 and 63220746 bp of the pig reference genome (NC_010447.5) using Sybr Green real-time PCR with the primers: F 5′-GGGCACATCATTCCAACTTT-3′ and R 5′-GACTGACGGACCAGGTGATT-3′. PCR was performed in a 10-ul volume containing 1x RT HS-PCR Mix SYBR buffer and 0.4 ul HiRox (A&A Biotechnology, Poland) and 0.10 mM of each primer. Thermal conditions were: 95°C for 3 min,

followed by 45 cycles at 95°C for 20 s, 54°C for 40 s, and 72°C for 45 s. The final extension was at 72°C for 5 min. Directly after PCR, the PCR products were melted at a constant rate and the decrease in fluorescence was monitored as the strands dissociated. The PCR conditions involved an initial denaturation of 1 min at 95°C; followed by 35 cycles (denaturation: 95°C for 15 s.; annealing and extension at 60°C). The melting cycle started from 65°C to 95°C.

Sequencing of the CLEC4E fragment selected for testing

To evaluate the analysed amplicon the Sanger sequencing of the *CLEC4E* fragment was applied. A sequencing reaction was performed to ensure that the PCR products obtained were the specific DNA fragment of interest. We have chosen several domestic pigs and wild pigs samples and performed PCR and sequencing using the primer pair (5'-GTGTGGCTGGCTTACAATG-3'; 5'-TGCAATATGTCACT GGCACACA-3') flanking the sequence of *CLEC4E* gene which was also analysed using the real-time PCR. The web-based software Primer-BLAST (NCBI, National Center for Biotechnology Information) (NCBI) was used for creating primers. The reaction mixture contains 5 ul AmpliTaq Gold 360 Master Mix with Polymerase and 1.2 ul 360 GC Enhancer (Thermofisher Scientific, USA) and 0.5 pM of each primer and was conducted according to the producer's manual. The amplification products were purified with Eppic purification kit (A&A Biotechnology, Poland)

Table 1. Tested samples and interpretation of the C_T and T_m results

Sample symbol	declared breed / subspiecies	CT	Tm	shape of Tm	interpretation of results
A1	PBZ	23.43	88.73	d.pig	+
B1	PBZ	22.24	88.28	d.pig	+
C1	PBZ	21.43	88.43	d.pig	+
D1	PBZ	22.47	88.28	d.pig	+
E1	PBZ	22.07	88.28	d.pig	+
F1	PBZ	21.98	88.43	d.pig	+
G1	PBZ	23.81	88.43	d.pig	+
H1	PBZ	23.42	88.73	d.pig	+
A2	PBZ	20.81	88.43	d.pig	+
B2	PBZ	20.88	88.43	d.pig	+
C2	PBZ	21.60	88.43	d.pig	+
D2	PBZ	21.53	88.28	d.pig	+
E2	PBZ	18.38	88.13	d.pig	+
F2	PUŁ	21.72	88.58	d.pig	+
G2	PUŁ	21.89	88.28	d.pig	+
H2	PUŁ	22.77	88.28	d.pig	+
A3	PUŁ	23.36	88.72	d.pig	+
B3	PUŁ	22.73	88.57	d.pig	+
C3	PUŁ	21.25	88.27	d.pig	+
D3	PUŁ	22.44	88.12	d.pig	+
E3	PUŁ	21.56	88.12	d.pig	+
F3	PUŁ	20.17	88.12	d.pig	+
G3	PUŁ	21.09	88.57	d.pig	+
H3	PUŁ	22.44	88.42	d.pig	+
A4	DUR	19.80	88.27	d.pig	+
B4	DUR	19.16	89.17	d.pig	+
C4	DUR	19.94	88.12	d.pig	+
D4	DUR	18.70	87.83	d.pig	+
E4	DUR	18.82	88.12	d.pig	+
F4	DUR	18.97	87.83	d.pig	+
G4	DUR	19.07	88.12	d.pig	+
H4	DUR	19.11	88.87	d.pig	+
A5	DUR	19.33	88.58	d.pig	+
B5	DUR	19.31	87.83	d.pig	+
C5	DUR	18.87	87.98	d.pig	+
D5	DUR	18.95	88.43	d.pig	+
E5	DUR	18.98	88.73	d.pig	+
F5	DUR	18.72	87.68	d.pig	+
G5	DUR	18.87	87.98	d.pig	+
F6	WBP	19.27	88.13	d.pig	+
G6	WBP	20.29	89.02	d.pig	+
H6	WBP	19.68	88.28	d.pig	+
E5	hybrid	19.50	88.13	d.pig	+
A6	hybrid	18.73	88.73	d.pig	+
B6	hybrid	20.20	88.13	d.pig	+
C6	hybrid	18.52	87.98	d.pig	+
D6	hybrid	18.48	88.13	d.pig	+
E6	hybrid	18.60	89.02	d.pig	+
A7	w. boar	17.41	90.22	w. boar	+
B7	w. boar	17.30	90.37	w. boar	+
C7	w. boar	19.06	91.86	w. boar	+
D7	w. boar	12.26	91.56	w. boar	+
E7	w. boar	20.52	88.88	d.pig	ID
F7	w. boar	10.18	89.03	d.pig	+
G7	w. boar	20.24	90.22	w. boar	+
H7	w. boar	8.78	90.67	w. boar	+
A8	w. boar	10.25	90.37	w. boar	+
B8	w. boar	8.85	90.37	w. boar	+
C8	w. boar	6.62	90.37	w. boar	+
D8	w. boar	17.67	88.28	d.pig	ID
E8	w. boar	5.47	91.26	w. boar	+
F8	w. boar	5.93	90.52	w. boar	+
G8	w. boar	11.47	90.52	w. boar	+
H8	w. boar	17.23	91.89	w. boar	+
A9	w. boar	20.83	89.47	d.pig	ID
B9	w. boar	19.33	90.22	w. boar	+
C9	w. boar	11.82	89.92	d.pig	+
D9	w. boar	15.60	90.22	w. boar	+
E9	w. boar	16.75	90.36	w. boar	+
F9	w. boar	8.32	92.01	w. boar	+
G9	w. boar	7.93	91.86	w. boar	+
H9	w. boar	4.62	92.01	w. boar	+
A10	w. boar	10.76	91.86	w. boar	+
B10	w. boar	24.24	92.01	w. boar	+
C10	w. boar	10.37	92.01	w. boar	+
D10	w. boar	10.05	91.86	w. boar	+
E10	w. boar	6.66	92.01	w. boar	+
F10	w. boar	5.41	91.86	w. boar	+
G10	w. boar	6.88	92.01	w. boar	+
H10	w. boar	9.02	92.01	w. boar	+
A11	w. boar	16.84	91.70	w. boar	+
B11	w. boar	17.55	90.36	w. boar	+
C11	w. boar	9.64	91.70	w. boar	+
D11	w. boar	6.15	91.70	w. boar	+
E11	w. boar	7.01	91.70	w. boar	+
F11	w. boar	5.52	91.70	w. boar	+
G11	w. boar	7.79	90.21	w. boar	+
H11	w. boar	16.02	89.91	d.pig	+

PBZ - Polish Landrace; PUŁ – Puławska; DUR – Duroc; WBP- *Polish Large White*, d. pig – domestic pig; w. boar - wild boar; CT - number of cycles in which an increase in the detection signal can be observed/ cycle of crossing with the threshold line; Tm – melt temperature; shape of Tm - shape of Tm typical for subspecies; interpretiation of results - interpretation of the sample subspecies based on the obtained CT, melt temperature and shape of melt curve; results + means that the interpreted subspecies is consistent with reality; ID – indistinguishable - means impossible to identify.

according to the manufacturer's protocol. The sequencing PCR was conducted using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and with the same primers as for PCR used. The sequencing products were finally purified with BigDye XTerminator_ Purification Kit (Applied Biosystems, USA) and capillary electrophoresis was performed on the SeqStudio Genetic Analyzer 3500 XL (Applied Biosystems, USA). The sequences were analyzed using BioEdit Sequence Alignment Editor [Tom Hall; https://bioedit.software.informer.com], FinchTV v1.4.0 [Geospiza Inc. https://digitalworldbiology.com/FinchTV] and BLAST (Basic Local Alignment Search Tool).

Results and discussion

The *CLEC4E* gene codes for the C-type lectin domain family 4 member E protein, also called Mincle (macrophage-inducible C-type lectin). The *CLEC4E* protein is an immune receptor upregulated during a mycobacterial infection [Behler *et al.* 2012]. Regarding Suidae, the most common mycobacterium-induced infection is tuberculosis, and wild boars are the significant reservoir of this pathogen [Behler *et al.* 2012]. Therefore, it is hypothesized that in wild boars, increased copy numbers of the *CLEC4E* gene compared to domestic pigs, might impact the expression of proinflammatory cytokine release against tuberculosis. The average copy number for the European and Asian domestic pig is 0.91 and 0.94, respectively. The same parameter for the European and Asian wild boar is 1.82 and 1.55 [Jang *et al*. 2023].

Based on different copy numbers of the *CLEC4E* gene, we provide an in-lab time-saving and cost-effective test for discriminating between domestic pigs and wild boars.

Sequencing of the CLEC4E fragment

The sequencing results showed that the obtained fragment of 1391 bp was 100% homologous to the reference genome of *Sus scrofa* NC_010447.5 in the range of 63219386-63220776 bp, which contains the DNA fragment of *CLEC4E*. The obtained fragment covers the part of the *CLEC4E* gene analyzed with real-time PCR.

The real-time PCR discrimination test for domestic pig and wild boars

In samples of domestic pigs and wild boars, we amplified a 1286 bp fragment of *CLEC4E* sequence. The cycle threshold (CT) values for all samples studied ranged between 4.62 and 23.81, and the melting temperature (Tm) between 87.68 and 89.17 °C (Tab. 1).

The lowest CT value was detected in samples from wild boars. Moreover, in the case of wild boars, the CT range was extensive, up to 17.67. In contrast, for domestic pigs, the starting amplification was detected from the 18.38th cycle. The obtained mean CT values indicate different values for each subspecies, at 12 cycles for wild boars, 19 for hybrids and about 21 for pigs. Moreover, the relative standard deviations

for CT, 44.92% for wild boars, 3.66% for hybrids and 7.88% for domestic pigs, respectively, confirm the wide range for the wild subspecies.

Consequently, the CT results have some potential for discriminating breeds. The starting amplification cycle is notably different for Duroc and begins from 18.7 cycle. Similarly, the WBP breed's CT values were similar but slightly higher. In turn, the PBZ and Puławska breed amplification occurring a little later was observed, and the CT range for both species was wider. The CTvalues for wild boar and domestic pig hybrids were consistent with the results observed for Duroc and WBP, i.e., within the low CT range observed for pigs (CT=18.481-20.198). The obtained results are illustrated in Figure 1 (a and b).

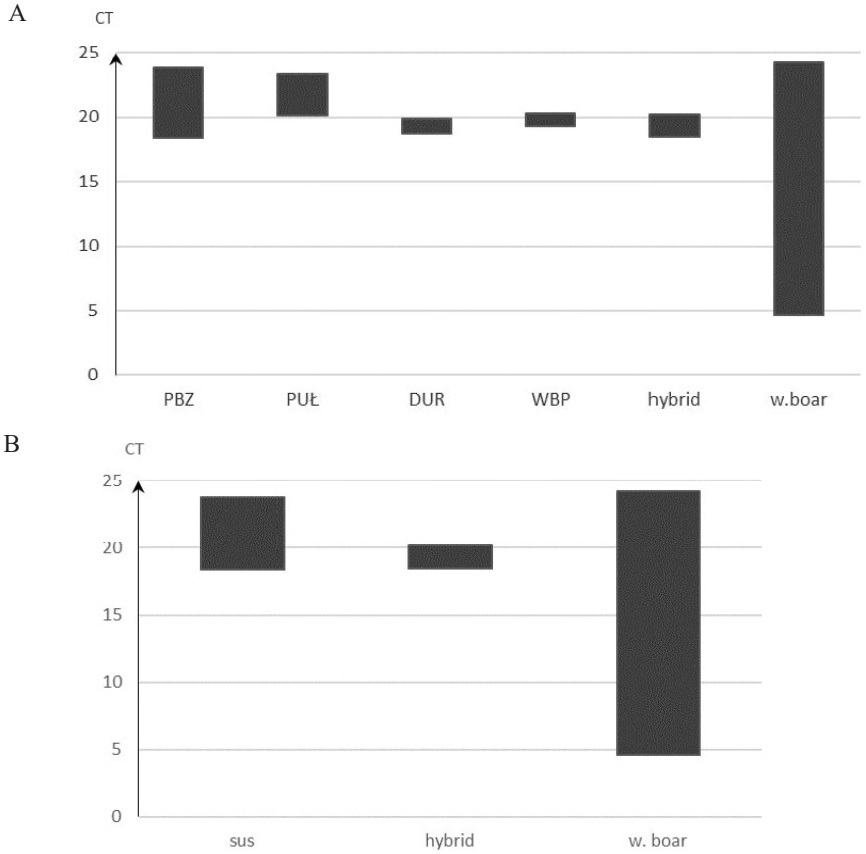

Fig. 1. Range of cycles of the minimum detection value depending on the subspecies and breed (A) and subspecies (B). CT - number of cycles in which an increase in the detection signal can be observed/ cycle of crossing with the threshold line; PBZ - Polish Landrace; PUŁ – Puławska; DUR – Duroc; WBP- Polish Large White; d. pig – domestic pig; w. boar - wild boar.

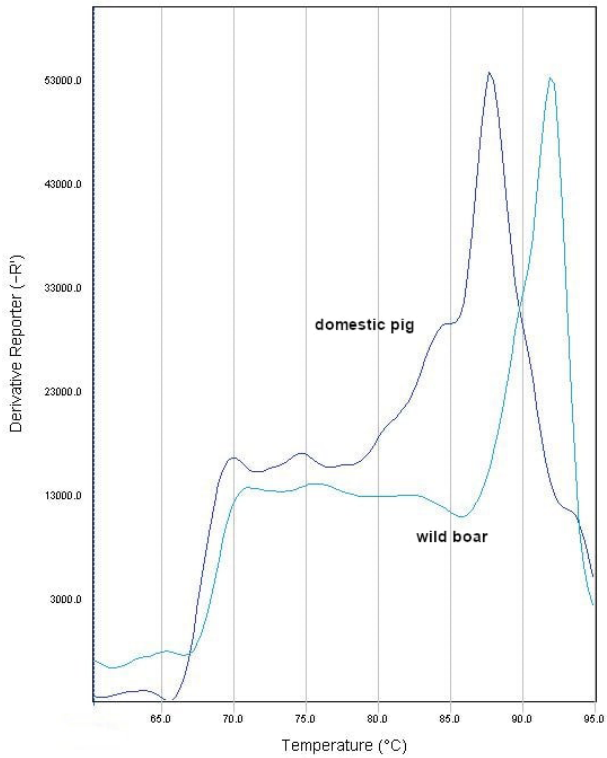

Fig. 2. Shape of melting temperature curve for wild boar and domestic pig.

In addition to the number of cycles a different melting temperature (Tm) and the shape ware correlated with both subspecies and their hybrits (Fig. 2). In the case of wild boars, Tm ranged between 88.28°C-92.01°C. Interestingly, the Tm above 89.47°C, reaching the highest value of 92.01°C, was solely detected for the wild boar samples. However, Tm for the remaining two wild boar samples was detected in the range for domestic pigs, i.e., 87.68-89.17°C. All hybrids' Tm was typical for domestic pigs range which is 87.68-89.17°C (Fig. 3a). Moreover, unlike the analysis based on the number of cycles, which was largely breed-specific, the melting point range for all pig breeds tested was very similar to each other (Fig. 3b). It is worth noting that the shape of the melting curves shown in Figure 2 was reproducible for individuals belonging to a given subspecies.

Applying the above criteria allowed us to determine how many obtained results were consistent with reality and how many gave false results. Using the number of amplification cycles for analysis enabled the correct classification of all 48 tested domestic pigs. For wild boars, 34 out of 40 samples were accurately classified. The same number of correct results was observed based on the shape of the melting

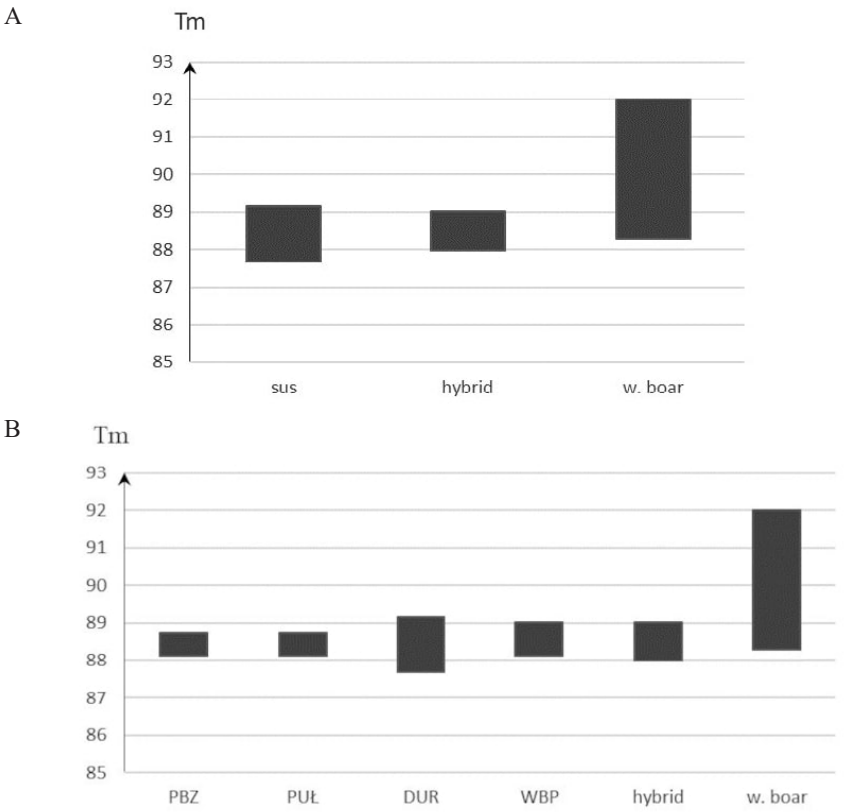

Fig. 3. Range of melt temperature value depending on the subspecies (A) subspecies and breed (B). Tm – melt temperature; PBZ - Polish Landrace; PUŁ – Puławska; DUR – Duroc; WBP- Polish Large White; d. pig – domestic pig; w. boar - wild boar.

temperature curve, although the incorrect determination of the subspecies (domestic pig instead of wild boar) was observed for other individuals than in the case of the assessment based on CT. In turn, the assessment using the melting temperature allowed for the correct determination of 46 out of 48 tested domestic pigs and 38 out of 40 tested wild boars.

CNV has been identified in many species [Perry *et al.* 2007, Axelsson *et al*. 2013, Janiak 2016, Abduriyim *et al.* 2019], starting from humans [Perry *et al.* 2007], through dogs [Axelsson *et al*. 2013] or chickens [Gorla *et al.* 2017], and even polar bears [Rinker *et al.* 2019]. CNV can be correlated with a very wide range of organism traits such as susceptibility or resistance to diseases [Chung *et al*. 2015, Patin *et al.* 2017], meat quality [Chung *et al.* 2015], fertility [Sahadevan *et al.* 2015, Samborski *et al.* 2013, Park *et al*. 2022]. CNVhas also been observed in genetically closely related species such as dogs and wolves or domestic pigs and wild boars [Jang *et al*. 2023].

Successfully used to distinguish dogs and dogs CNV in the alpha amylase (AMY2) gene [Axelsson *et al.* 2013] unfortunately has not been confirmed in domestic pigs and their wild relatives [Yoshidomi *et al*. 2021]. A promising study of CNV in AMY indicated an identical copy number in both subspecies. The method presented by us allows for species differentiation of the most of the analyzed samples.

Analyzing the obtained results, it can be seen that there are limit values of each tested parameter, which allow the tested sample to be assigned to a given subspecies with 100% certainty. For example, Tm above 90°C always indicates the origin of the sample from a wild boar, and below 88.2°C confirms the origin of a domestic pig. In turn, CT below 17.67 and above 18.70 indicate the origin of a wild boar and a pig, respectively. It is worth noting that in the case of samples giving intermediate results, the simultaneous application of all three criteria allows for the correct interpretation of almost every sample. Only in three cases was the result doubtful and these samples could not be unequivocally assigned to any of the subspecies (Tab. 1, E7, D8, A9).

Conclusion

The parameters of the developed method indicate high efficiency of the proposed strategy for distinguishing the species of domestic pig from wild boar. 85 out of 88 tested samples were correctly identified, in the remaining cases the result was doubtful. In all the tested cases there were no samples identified incorrectly.

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