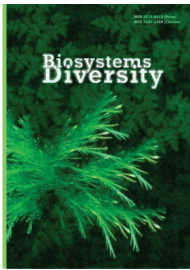

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Analysis of polymorphism and development of a molecular-genetic system for genotyping by the telomerase reverse transcriptase (*TERT*) gene

A. Saienko*, M. Peka*, **, O. Tsereniuk*, M. Babicz***, K. Kropiwiec-Domańska***, A. Onyshchenko*, P. Vashchenko****, *, V. Balatsky*, **

**Institute of Pig Breeding and Agroindustrial Production of National Academy of Agrarian Sciences of Ukraine, Poltava, Ukraine*
***V. N. Karazin Kharkiv National University, Kharkiv, Ukraine*
****University of Life Sciences, Lublin, Poland*
*****Poltava State Agrarian University, Poltava, Ukraine*

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Institute of Pig Breeding and Agroindustrial Production of National Academy of Agrarian Sciences of Ukraine, Shvedska Mohyla st., 1, Poltava, 36000, Ukraine.
Tel.: +38-066-727-24-93.
E-mail: pigbreeding@ukr.net

V. N. Karazin Kharkiv National University, Svobody sq., 4, Kharkiv, 61022, Ukraine.
Tel.: +38-057-707-55-00.
E-mail: pekapoltava@gmail.com

University of Life Sciences, Akademicka st., 13, Lublin, 20950, Poland. Tel.: +48-81-445-66-91.
E-mail: biuro.rektora@up.lublin.pl

Poltava State Agrarian University, Skovorody st., 1/3, Poltava, 36003, Ukraine.
Tel.: +38-096-944-98-12.
E-mail: pavlo.vashchenko@pdaa.edu.ua

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This article explores the genetic variability of the gene that encodes telomerase reverse transcriptase (*TERT*), which plays a key role in maintaining telomere length and, as a result, genome stability in various eukaryotic species. The study employs a comprehensive approach that combines phylogenetic and bioinformatic analysis with molecular-genetic research methods. The research involved the screening of genetic databases to investigate *TERT* gene orthologues across organisms belonging to different systematic groups. The *TERT* gene, which is prevalent in a wide range of eukaryotic biological species, exhibits polymorphisms that have the potential to influence *TERT* enzyme function and, consequently, animal phenotypes. The primary focus of this study centers on the pig *TERT* gene, selected as a model organism due to its genetic similarity to humans and its importance as a productive agricultural species. The article explores the exon-intron structure of the *TERT* gene, analyzing the size of the corresponding transcript and protein product. Furthermore, it provides data on polymorphisms in the pig *TERT* gene, including missense and synonymous variants. The chromosomal localization of these polymorphisms is characterized and correlated with the domain structure of the *TERT* enzyme. For the evaluation of the impact of polymorphisms on the structural and functional properties of the *TERT* enzyme, a molecular-genetic system based on the PCR-RFLP method has been developed. This PCR-RFLP system serves as a basis for subsequent experimental analyses of missense and synonymous variants in population and association studies, allowing for an assessment of the prevalence of these polymorphisms and their significance for animal phenotypes. Given the significance of further laboratory investigation of the pig *TERT* gene, the developed PCR-RFLP system becomes necessary for the assessment of the functional implications of the polymorphisms within this gene and the potential identification of causative ones among them. The synergy of bioinformatics and molecular-genetic methods in this study lays the groundwork for future impactful research in this field. The presented study holds promise for marker-associated selection, as it opens the way for the use of the *TERT* gene as a marker in the genetic improvement of agricultural animal species.

Keywords: telomerase reverse transcriptase; single nucleotide polymorphism; genetic marker; polymerase chain reaction; phylogenetics; bioinformatics; marker-associated selection.

Introduction

In eukaryotes, cellular aging processes and age-related changes are linked to telomere length (Seeker et al., 2018; Ilska-Warner et al., 2019; Schrumpfová & Fajkus, 2020). Telomeres, located at the ends of chromosomes, protect them from shortening during DNA replication. The functioning of telomeres is of fundamental importance for the existence of living species since processes associated with maintaining their length ensure genome stability and cell viability. Telomere shortening leads to cellular senescence and may ultimately result in apoptosis (Shay, 2016; Zhu et al., 2019; Chakravarti et al., 2021). On the other hand, telomere dysfunction can lead to abnormal cell proliferation and the neoplasm development, emphasizing the importance of finely regulating processes associated with maintaining telomere length in cells (Lu et al., 2013; Lansdorp, 2022).

Various mechanisms of telomere elongation are known, with the most studied being regulation by the telomerase complex. In addition to telomerase, there are also alternative ways of telomere elongation (Monaghan & Ozanne, 2018). The telomerase complex consists of several components, with the main ones being telomeric RNA, which acts as a template for synthesis during the elongation of the telomere, and telomerase reverse transcriptase enzyme (*TERT*), which directly carries out the synthesis (Wang et al., 2019). *TERT* belongs to RNA-dependent DNA polymerases (Schrumpfová & Fajkus, 2020) and is encoded by the gene of the same name. *TERT* is essential for extending telomeres at the molecular level, preserving genome stability, and preventing chromosome fusion (Autexier & Lue, 2006).

The mechanism of maintaining telomere length through the functioning of the telomerase complex shares commonalities across a wide range of eukaryotic species (Schrumpfová & Fajkus, 2020). Furthermore, the possible long-standing origin of *TERT* has been previously reported, which first arose as a reverse transcriptase and then evolved in eukaryotes (Podlevsky & Chen, 2016; Dey & Chakrabarti, 2018). However, there are reports of potential differences in telomere characteristics and the maintenance of their length at both the interspecific and intraspecific levels (Dugdale & Richardson, 2018; Young, 2018). Telomere dynamics are considered one of the aspects towards which evolutionary selection is directed (Monaghan & Ozanne, 2018). Given the above, we can also assume the presence of connections between the *TERT* gene and animal phenotypes.

Mutations and polymorphisms in this gene can affect the enzyme's ability to perform its functions in maintaining telomere length, which, in turn, can impact factors such as longevity, growth rate, reproductive ability, and, for agricultural species, the duration of useful productive use.

Population studies investigating the genetic structure of the *TERT* gene and association studies seeking to establish correlations between telomere length, telomerase complex activity, and phenotype have been conducted across diverse animal species, including cattle (Brown et al., 2012; Seeker et al., 2018; Iannuzzi et al., 2022), pigs (Fradiani et al., 2004; Russo et al., 2006), sheep (Froy et al., 2021), horses (Argyle et al., 2003; Ząbek et al., 2012), dogs (McAloney et al., 2014), chickens (Badmus et al., 2021), and turkeys (Adikari et al., 2013). However, these studies are limited in number, possess a fragmented nature, and specific associations with distinct physiological and pathological processes resulting from *TERT* gene mutations have only been conclusively established in humans (Zhao et al., 2015; Tong et al., 2020; Dratwa et al., 2021). Thus, researching specific polymorphisms and mutations that may alter the structure or expression of the *TERT* gene in animals, or affect the enzyme's functionality, consequently impacting the lifespan and productive traits of animals, stands as a pressing task.

In our previous study (Peka et al., 2023), we conducted a bioinformatic analysis of the pig *TERT* gene and predicted the potential influence of several missense polymorphisms on the structural and functional properties of pig *TERT* using software resources. In the current study, our aim is to analyze the intron-exon structure of the *TERT* gene, identify regions saturated with missense and synonymous polymorphisms, and develop an appropriate PCR-RFLP genotyping technique for each of these SNPs. The results obtained hold promise for using in population and association studies to establish correlations with animal phenotypes and identify causative mutations.

Materials and methods

The experiment protocol was approved by the Scientific Council of the Institute of Pig Breeding and Agroindustrial Production of the National Academy of Agrarian Sciences of Ukraine. All animal handling procedures conformed to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasburg, 1985).

Bioinformatic and phylogenetic analysis. A search was conducted for sequences corresponding to the *TERT* gene and its homologs in various biological species in the NCBI and Ensembl databases (Martin et al., 2023). For this purpose, in particular, BLAST search was used (Altschul et al., 1990; Boratyn et al., 2013). To perform phylogenetic analysis, reference coding sequences of biological species were utilized. Multiple alignment of coding sequences was carried out according to the MUSCLE algorithm (Edgar, 2004) within the Mega11 software (Version 11 64-bit; Tamura et al., 2021). The JTT matrix-based model and the Maximum Likelihood approach were used to create the resulting phylogenetic tree similarity (Jones et al., 1992) within the Mega11 software.

Data on the reference sequence of the pig *TERT* (Ensembl ID: ENSSSCG00000017118), gene structure, and the number and localization of polymorphic variants were obtained from the Ensembl database. To develop a technique for amplifying *TERT* gene fragments, primers were designed using the Primer3Plus (Untergasser et al., 2007). These primers were verified for lack of self-complementarity and formation of dimers using the Multiple Primer Analyzer (Thermo Scientific Web Tools), a calculator that utilizes a modified nearest-neighbor method based on the approach described in Breslauer (1986).

Restriction endonucleases were designed using NEBcutter V2.0 (Vincze et al., 2003). Restriction product lengths for each endonuclease were determined using the Restriction Analyzer (Molbiotools, USA, 2023).

Sampling and DNA extraction. Blood samples (1 mL) were collected from pigs of the Large White breed at the State Enterprise "Experimental Farm Stepne," Stepne village, Poltava district, Poltava region, Ukraine. The blood samples were mixed with 0.05 M EDTA as an anticoagulant and stored for up to seven days at +4 °C before being used for DNA isolation. Genomic DNA was isolated using the Chelex 100 reagent (Walsh et al., 2013; Gautam, 2022).

Polymerase chain reaction / PCR, analysis of amplificates and restriction fragments. The PCR reaction mixture contained genomic DNA, forward and reverse primers (0.2 µM), dNTP (0.25 mM), $MgCl_2$ (2.0 mM), and 1 unit of recombinant Taq DNA polymerase (Thermo Scientific, EU), with a total volume of 25 µL. Twenty-five microliters were layered onto the reaction mixture, followed by the addition of mineral oil. The reaction was carried out in a Tertsik-2 thermal cycler (Tertsik TP4-PCR01, DNA-Technology) according to the standard protocol / procedure (Lorenz, 2012).

Restriction of amplificates was carried out according to the protocols of the manufacturers of restriction endonucleases. Amplifications and restriction fragments were electrophoresed in 1.5–2.0% agarose and 8.0% acrylamide gels in tris-borate electrophoresis buffer (TBE: 0.0879 M tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0). DNA staining in the gel was carried out in a solution of ethidium bromide (0.5 µg/mL). Visualization of DNA fragments was conducted under UV light at a wavelength of 340 nm on a transilluminator (Ota et al., 2009).

Results

Phylogenetic analysis on the TERT gene. An analysis of the Ensembl and NCBI data conducted in this study revealed the presence of the *TERT* gene in a broad array of animal species. At the time of writing, the NCBI database contained 648 entries for genes with a protein architecture similar to that of *TERT* among various eukaryotic species, of which 539 are *TERT* orthologues among vertebrates.

Phylogenetic analysis was performed to demonstrate similarities and relationships between different species based on the coding sequences of the *TERT* gene. The resulting phylogenetic tree is displayed in Figure 1.

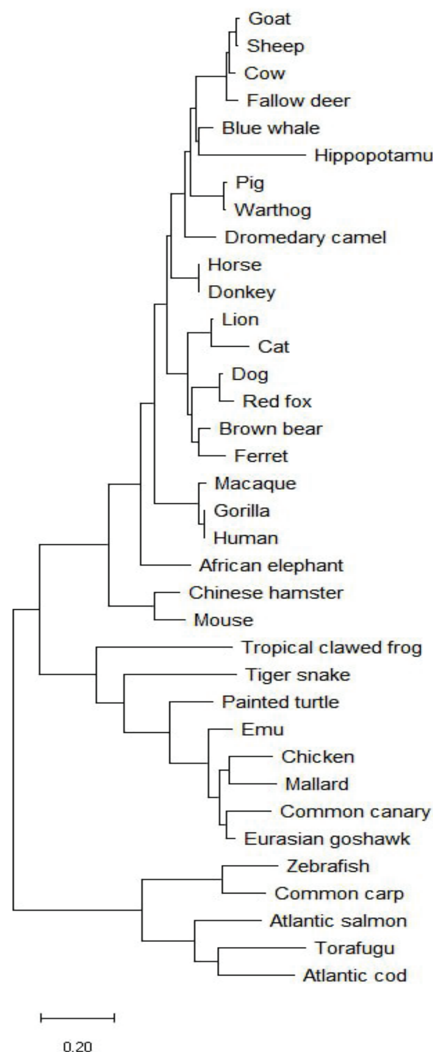

Fig. 1. Phylogenetic tree built based on the alignment of *TERT* coding sequences

One of the primary subjects of biological research is domestic pigs, as they exhibit a relatively high degree of similarity to humans, including by the *TERT* gene. Additionally, they possess sufficient prevalence and population size, and they hold significant value as productive agricultural species. Consequently, the development of approaches for molecular genetic analysis of the *TERT* gene in this study is carried out using the pig as a model organism.

The analysis of TERT gene polymorphism. Current data from Ensembl at the time of writing regarding the pig *TERT* gene and its polymorphisms were juxtaposed with findings from a prior study to evaluate updates in the Ensembl database and incorporate them into the present study. Through meticulous analysis and systematic review of literature and data archived in Ensembl several key generalizations were derived, holding significance for the development of molecular genetic analysis techniques.

The *TERT* gene in pigs is situated on chromosome 16, occupying the genomic coordinates between 79,258,591 and 79,276,421 on the forward strand, as per the reference genome assembly. The coding region encompasses 17 exons of varying lengths. The largest Exon 2 extends to 1,348 base pairs (bp), while the smallest Exon 6 spans 21 bp. Notably, these exons are flanked by the largest and smallest introns, respectively: Intron 2–3 measures 2,765 bp, and Intron 5–6 is merely 2 bp.

The cumulative length of all 16 introns excised from the primary transcript amounts to 12,334 bp. Consequently, the mature transcript comprises 5,497 bp, with 16 bp constituting the 5'-UTR, 3,393 bp comprising the coding sequence, and 2,088 bp constituting the 3'-UTR. Upon translation, this transcript is anticipated to yield a protein comprising a chain of 1,130 amino acid residues.

Previously obtained data regarding the structure of the *TERT* enzyme and the localization of functional domains (TEN, TRBD, RTD, CTE) within the protein molecule were cross-referenced with data pertaining to the exon structure of the coding region of the *TERT* gene. Consequently, the TEN domain (1-228 aa) is encoded by the Exon 1 and 2, TRBD (320-548 aa) by the Exon 2 and 3, RTD (603-933) from the Exon 4 to 12, and the CTE domain (934-1130) from the Exon 12 to 17. Moreover, there exist two linker regions in the enzyme structure: Linker 1 (229-319 aa) is entirely encoded by Exon 2, and Linker 2 (549-602 aa) by the Exon 3 and 4.

As per Ensembl data at the time of writing, there are 689 known polymorphisms in the pig *TERT* gene. Of these, 368 are localized in intronic regions, while 56 polymorphisms are situated in the exonic regions (coding part of the transcript). Among these exonic polymorphisms, 24 are missense variants, and 32 are synonymous variants. No polymorphisms were identified in the 5'-UTR region, whereas 77 polymorphisms were found in the 3'-UTR region. The remaining polymorphisms are localized in the upstream and downstream regions of the pig *TERT* gene. Information regarding known polymorphisms in the coding segment, including their rsID and the type of polymorphism (missense or synonymous), is presented in Table 1.

An analysis of the data presented in Table 1 reveals that missense polymorphisms are exclusively identified in Exons 2 to 5 and Exons 14 to 17. Remarkably, no polymorphisms, including both missense and synonymous variants, have been currently identified in Exons 1, 6, and 10. Notably, Exon 2 harbors the highest number of polymorphisms, with 12 missense variants, constituting half of the presently known missense variants in *TERT* gene. This observation can be attributed to the considerably greater length of Exon 2 in comparison to other exons.

Primer design, restriction enzymes search, and PCR amplification conditions for genotyping TERT gene regions. A frequently used technology for determining allelic variants of specific polymorphisms in a genotype is PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism). Its laboratory use is preceded by the design of primers and search of restriction endonucleases through *in silico* analysis. In the present study, the design of primers targeting *TERT* gene regions encompassing polymorphisms within Exon 2 and Exon 4 was conducted. The respective localization of *TERT* gene polymorphic regions and their corresponding primers are shown in Figures 2 and 3.

The selection of specific segments within Exons 2 and 4 is based on practical feasibility of their study. Amplification Fragment 1 encompasses 4 missense SNPs (among which the most important are rs789641834 and rs706045634), alongside 1 synonymous SNP. Concurrently, Fragment 2 encompasses 6 missense (including rs325294961) and 4 synonymous SNPs. These first two amplification fragments pertain to regions within Exon 2. Amplification fragment 3 covers Exon 4 polymorphisms, encompassing 3 missense (including rs705602819) and 2 synonymous SNPs.

Table 1
Polymorphisms in the coding region of pig *TERT* gene

# of Exon	Type of PMs	Number of PMs	Variant IDs
Exon 1			—
Exon 2	m	13	rs789641834 (C/A), rs698799571 (A/T), rs706045634 (G/C), rs696805316 (G/A), rs331656839 (C/G), rs338419951 (G/A), rs318799866 (A/G), rs705219838 (C/T), rs330770291 (C/T), rs325294961 (C/G), rs333763227 (G/A), rs789588487 (G/A), rs324158660 (T/C)
	s	11	rs338788293 (G/A), rs320317081 (A/T), rs331656839 (C/A), rs791103453 (C/T), rs691116673 (C/G), rs341711614 (G/C), rs325294961 (C/A), rs1108662349 (G/A), rs340247149 (G/T), rs792811218 (G/C), rs790128312 (G/A)
Exon 3	m	1	rs329843407 (C/G)
	s	2	rs711015510 (G/A), rs336951316 (G/C)
Exon 4	m	3	rs698738374 (G/T), rs705602819 (C/T), rs332450148 (C/G)
	s	2	rs1113181409 (C/T), rs324499091 (C/T)
Exon 5	m	2	rs339601952 (G/A), rs332245175 (G/A)
	s	3	rs787762715 (C/T), rs324498699 (G/A), rs340294883 (T/C)
Exon 6			—
Exon 7	s	2	rs1109618740 (G/A), rs327589845 (A/C)
Exon 8	s	1	rs318961175 (C/T)
Exon 9	s	1	rs702373657 (C/T)
Exon 10			—
Exon 11	s	1	rs329893408 (C/T)
Exon 12	s	4	rs792761413 (C/T), rs793278375 (C/T), rs336114613 (T/C), rs341929237 (A/G)
Exon 13	s	1	rs344396736 (C/T)
Exon 14	m	1	rs787080565 (G/A)
Exon 15	m	1	rs791792095 (C/T)
	s	4	rs338329906 (T/C), rs321561105 (A/G), rs325803871 (C/G), rs792308146 (A/G)
Exon 16	m	1	rs335037856 (G/A)
Exon 17	m	2	rs336411058 (G/A), rs320614499 (A/G)

Note: m – missense polymorphism, s – synonymous polymorphism, dash indicates that no polymorphism is known in exon to date.

Fig. 2. Location of primers and amplification fragments within Exon 2 of the pig *TERT* Gene: amplification Fragments 1 and 2 are depicted, respectively, in green and yellow (Fig. 1), while amplification Fragment 3 is highlighted in cyan (Fig. 2); missense and synonymous SNPs are denoted in red and pink, respectively; primers are in bold and underlined; sequences corresponding to adjacent intronic sections to the investigated exons are crossed out; arrows indicate the positions of SNPs, primer, as well as exon and intron regions on the *TERT* gene; Exon 2 in Figure 1 is presented partially on its 3'-end

F3→
TCTGCCGGGA CCCAGGTGTC CGGCCCCGCC AGGGCGCTGG GCGTGGGCTC CGCCAGCCTC
←intron//
CCTAGCTCTG GTGTCCCCCA GGCTCACCTG CCTGCCTTTC CACGGGCCCG TCTGTTTCAG
Exon 4→ rs698738374(G/T)↘
GCAACACTTA GATCGTGTGC GGCTTCGAGA ACTGTCGGAA GCAGAGATCA GGCGACGCCG
rs1113181409(C/T)↘
GGAGGCCAGG CCCGCTGTAC TGACCTCCAA GCTCCGCTTC GTCCCCAAAC CCGACGGGCT
↙rs705602819(C/T) rs332450148(C/G)↘
GCGGCCCATC GTGAACATGG CGAACGTCGT GCGAGCCAGG ACAGGCCCCG GAGACAAGAA
//intron→ ↖rs324499091(C/T) ←R3
GGTCACTGCC GGTGTCGTTT TTAGGCGAAG TGCATTTCAG CCCCAGGCCT GGGTTTCTGT

Fig. 3. Location of primers and amplification fragments within Exon 4 of the pig *TERT* Gene: see Figure 1

Table 2 provides details on the primer structure delineating the selected gene regions with their respective polymorphisms and outlines the amplification conditions. Figure 3 illustrates the amplification fragments corresponding to the designated gene regions following their electrophoretic separation in an agarose gel.

Table 2
PCR primers specification

Fragment #	Exon	Primer	Primer sequence	T_m, °C	$MgCl_2$, mM	Fragment lenght, bp	Variant IDs (SNPs)
Frag. 1	2	F1	5'-ACGACGTGCTCACCCACCT-3'	60	2.0	270	rs789641834, rs698799571, rs320317081 rs706045634, rs696805316
		R1	5'-GCCTCTTGGCTGGAAGTGG-3'				
Frag. 2	2	F2	5'-TGAACAGGGTCCCGAAGG-3'	62	2.0	444	rs331656839 rs338419951, rs318799866, rs705219838, rs791103453 rs330770291, rs691116673 rs341711614 rs325294961 rs1108662349
		R2	5'-TTAAGCAGCTCCCGGAACA-3'				
Frag. 3	4	F3	5'-AGCCTCCCTAGCTCTGGTGT-3'	60	2.0	279	rs698738374, rs1113181409 rs705602819, rs324499091 rs332450148
		R3	5'-GCACTTCGCCTAAAAACGAC-3'				

The design of endonucleases necessitated identifying enzymes that, upon processing the PCR amplificates, would produce restriction fragments detectable through electrophoretic separation in either polyacrylamide or agarose gels. The summarized information is presented in Tables 3–5. For each SNP, multiple endonuclease variants are provided where feasible, ensuring a maximum of one endonuclease from a specific group of isoschizomers.

Table 3
Restriction enzymes specification for Fragment 1

Variant ID (SNP)	Alleles	Enzyme	Sequence	Restriction products
rs789641834	C/A	HhaI	GCG/C	C: 103+58+46+28+26 A: 103+58+46+30+26
		HinP1I	G/CGC	C: 103+60+46+28+24 A: 103+60+46+30+24
		Cac8I	GCN/NGC	C: 192+45+26+7 A: 192+52+26
		BtgZI	GCGATG(10/14)	C: 270 A: 222+48
		CdiI	CATCG(-1/-1)	C: 270 A: 236+34
rs698799571	A/T	CviQI	G/TAC	A: 232+38 T: 270
		RsaI	GT/AC	A: 231+39 T: 270
		BspMI	ACCTGC (4/8)	A: 221+49 T: 270
		SetI	ASST/	A:121+40+30+24+24+12+12+7 T: 121+54+40+24+12+12+7
rs320317081	A/T	MnlI	CCTC (7/6)	A: 104+57+45+37+15+11 T: 94+57+45+37+15+11+10
rs706045634	G/C	HhaI	GCG/C	G: 103+58+46+28+26 C: 149+58+28+26
		HinP1I	G/CGC	G: 103+60+46+28+24 C: 149+60+28+24
		MwoI	GCNNNNN/NNGC	G: 70+61+61+37+30+9 C: 98+70+61+30+9
		BstUI	CG/CG	C: 131+106+26+7 C: 237+26+7
rs696805316	G/A	FauI	CCCGC(4/6)	G: 164+69+37 A: 201+69
		MnlI	CCTC (7/6)	G: 104+57+45+37+15+11 A: 104+57+37+23+22+15+11

Table 4
Restriction enzymes specification for Fragment 2

Variant ID (SNP)	Alleles	Enzyme	Sequence	Restriction products
rs331656839	C/A/G	BsmFI	GGGAC(10/14)	C: 434+10 A: 444 G: 444
		StyD4I	/CCNGG	C: 195+103+58+38+26+15+7 A: 195+130+58+38+15+7 G: 195+104+58+38+26+15+7
		XmaI	C/CCGGG	C: 344+73+27 A: 371+73 G: 371+73
		AvaI	C/YCGRG	C: 151+106+87+73+27 A: 151+114+106+73 G: 151+114+106+73
		HpaII	C/CGG	C: 195+104+58+38+38+14+7 A: 195+132+58+38+14+7 G: 195+105+58+38+27+14+7
		NciI	CC/SGG	C: 195+103+58+37+27+13+7 A: 195+132+58+38+13+7 G: 195+104+58+38+28+13+7
		BstNI	CC/WGG	C: 139+114+63+58+38+19+13 A: 139+114+63+58+28+19+13+10 G: 139+114+63+58+38+19+13
		ScrFI	CC/NGG	C: 195+103+58+38+28+13+7 A: 195+132+58+38+13+7 G: 195+104+58+38+28+13+7
		SmaI	CCC/GGG	C: 344+71+29 A: 373+71 G: 373+71
		EcoO109I	RG/GNCCY	C: 322+90+24+8 A: 322+90+24+8 G: 300+90+24+22+8
		NlaIV	GGN/NCC	C: 156+97+90+32+22+22+9+7+7 A: 156+119+90+32+22+9+7+7 G: 156+97+90+32+22+21+9+7+7
rs338419951	G/A	—	—	—
rs318799866	A/G	BccI	CCATC (4/5)	A: 251+193 G: 251+120+73
		BslI	CCNNNNN/NNGG	A: 271+114+24+20+14 G: 271+79+35+20+20+14
		BanI	G/GYRCC	A: 271+127+32+14 G: 271+82+45+32+14
		NlaIV	GGN/NCC	A: 156+97+90+32+22+22+9+7+7 G:156+90+52+45+32+22+22+9+7+7
rs705219838	C/T	—	—	—
rs791103453	C/T	BbvI	GCAGC (8/12)	C: 123+118+85+47+35+21+15 T: 123+118+82+47+35+21+15
		TseI	G/CWGC	C: 98+98+93+60+47+40+8 T: 98+95+93+60+47+40+8
		AluI	AG/CT	C: 436+8 T: 341+95+8
		AciI	CCGC(-3/-1)	C:120+96+66+65+32+25+13+12+8+7 T: 162+120+65+32+25+13+12+8+7
		MspA1I	CMG/CKG	C: 346+98 T: 444
rs330770291	C/T	PflMI	CCANNNNNTGG	C: 444 T: 330+114
		AvaI	CYCGRG	C: 151+106+87+73+27 T: 238+106+73+27
		Hpy188III	TCNNGA	C: 328+105+11 T: 433+11
		BsmAI	GTCTC	C: 208+119+117

Variant ID (SNP)	Alleles	Enzyme	Sequence	Restriction products
				T: 236+208
rs691116673	C/G	AciI	CCGC(-3/-1)	C:120+96+66+65+32+25+13+12+8+7 G:96+70+66+65+50+32+25+13+12+8+7
rs341711614	G/C	AvaI	C/YCGRG	G: 151+106+87+73+27 C: 257+87+73+27
		ScrFI	CC/NGG	G: 195+103+58+38+28+13+7 C: 129+103+66+58+38+28+13+7
		StyD4I	/CCNGG	G: 195+103+58+38+26+15+7 C: 129+103+66+58+38+26+15+7
		NciI	CC/SGG	G: 195+103+58+38+28+13+7 C: 129+103+66+58+38+28+13+7
		HpaII	C/CGG	G: 195+104+58+38+28+14+7 C: 128+104+67+58+38+28+14+7
rs325294961	C/G	EcoO109I	RG/GNCCY	C: 322+90+24+8 G: 412+24+8
		PspOMI	G/GGCCC	C: 300+114+30 G: 414+30
		BaeGI	GKGCM/C	C: 300+65+45+34 G: 365+45+34
		ApaI	GGGCC/C	C: 300+110+34 G: 410+34
		Bsp1286I	GDGCH/C	C: 300+65+45+34 G: 365+45+34
		BanII	GRGCY/C	C: 300+110+34 G: 410+34
rs1108662349	G/A	HinP1I	G/CGC	G: 100+81+67+56+47+39+36+16 A: 158+81+67+47+39+36+16
		HhaI	GCG/C	G: 100+81+67+54+49+39+36+16 A: 156+81+67+49+39+36+16
		BstUI	CG/CG	G: 219+169+51 A: 224+169+51
		BaeGI	GKGCM/C	G: 300+65+45+34 A: 300+54+45+34+11
		Bsp1286I	GDGCH/C	G: 300+65+45+34 A: 300+54+45+34+11
		BssHII	G/CGCGC	G: 386+58 A: 444
		AscI	GG/CGCGCC	G: 386+58 A: 444

Table 5
Restriction enzymes specification for Fragment 3

Variant ID (SNP)	Alleles	Enzyme	Sequence	Restriction products
rs698738374	G/T	CviKI-1	RG/CY	G:48+43+36+31+27+24+21+17+10+9 T:48+36+31+30+27+24+21+17+13+10+9
		BsaHI	GR/CGYC	G: 158+121 T: 279
		Hpy99I	CGWCG/	G: 123+64+59+33 T: 182+64+33
rs1113181409	C/T	—	—	—
rs705602819	C/T	TseI	G/CWGC	C: 184+95 T: 279
		AciI	CCGC (-3/-1)	C: 92+85+53+27+22 T: 119+85+53+22
		Fnu4HI	GC/NGC	C: 98+91+86 T: 193+86
rs324499091	C/T	Hpy188III	TC/NNGA	C: 104+92+83 T: 187+92
		BccI	CCATC (4/5)	C: 200+79 T: 279
rs332450148	C/G	Cac8I	GCN/NGC	C: 100+81+60+38 G: 141+100+38

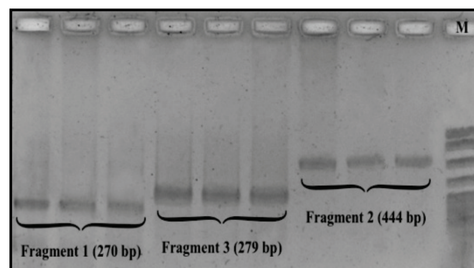

Fig. 4. Electrophoresis of PCR amplification fragments in a 1.8% agarose gel: M represents the molecular weight marker (pBR322/MspI)

Figure 5 provides a representative illustration of pig genotyping for the SNPs rs698799571 and rs706045634 within the *TERT* gene.

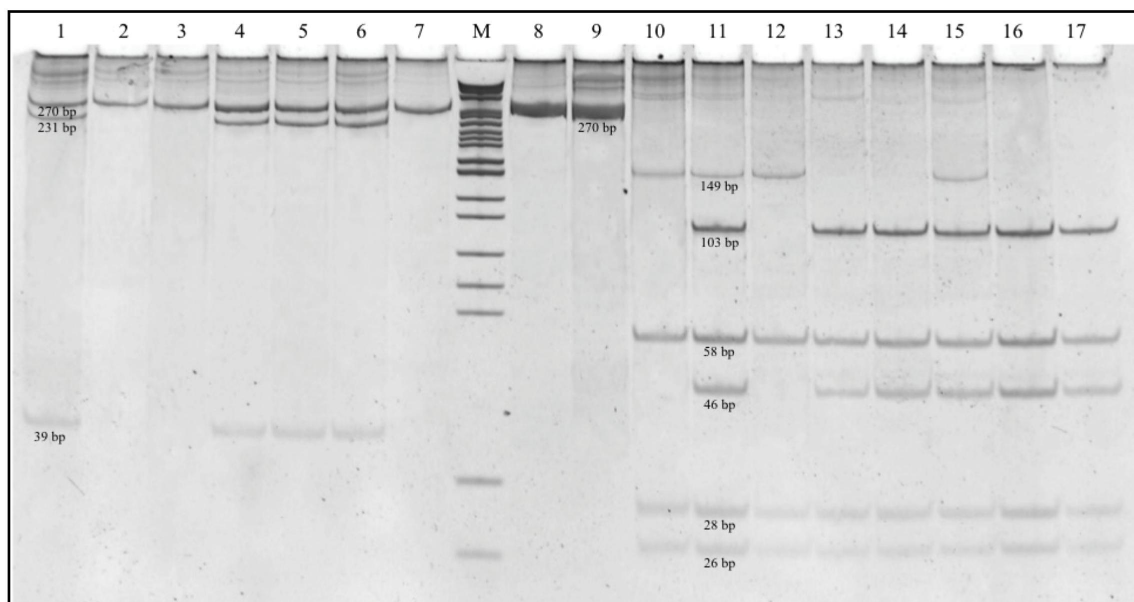

Fig. 5. Electrophoregram of the products of restriction endonucleases RsaI and HhaI for SNPs rs698799571 and rs706045634, respectively, in an 8% polyacrylamide gel of Fragment 1 of the pig *TERT* gene: M represents the pBR322/MspI molecular weight marker; numbers 8 and 9 represent Fragment 1 amplified in PCR; numbers 1, and 4–6 represent animals with AT genotype, 2, 3, and 7 denoting animals with AA genotype as per SNP rs698799571; additionally, 10 and 12 represent animals with CC genotype, 11 and 15 denote animals with GC genotype, and 13, 14, 16, and 17 represent animals with GG genotype as per SNP rs706045634

Discussion

The problem of age-related changes in the human body and other biological objects is a global problem, the relevance of which is obvious and occupies a position alongside such problems as environment preservation (Zos-Kior et al., 2021), minimization of the negative impact of agricultural production (Zos-Kior et al., 2020; Brockova et al., 2021) and antibiotic resistance (Vashchenko et al., 2022).

The *TERT* gene performs important biological functions in organisms of various species. Analysis of the NCBI database shows that orthologs of

this gene are present in a wide range of vertebrates. It is likely that this gene is present in vertebrates in general, given the mechanisms common to this taxonomic group to maintain genome stability by lengthening telomeres. However, data on the nucleotide sequence of *TERT* are so far limited to only those species for which whole-genome sequencing has been performed. Moreover, analysis of the NCBI database and BLAST search showed that *TERT* homologues (often referred to as *TERT*-like proteins) are known in representatives of priapulids, arthropods, flatworms, mollusks, brachiopods, nematodes, echinoderms, cnidarians, sponges, as well as fungi and plants.

In a previous study (Peka et al., 2023), we demonstrated that the pig *TERT* gene shares a high percentage of similarity with other animal species. For instance, the identity of the coding part is 77.7% for humans, 66.1% for mice, 45.6% for frogs, and 49.3% for zebrafish. Additionally, the identity of the genetic sequence with closely related species, such as cows, sheep, goats, and horses, is generally over 80%.

A phylogenetic tree is based on the coding sequences of *TERT* genes from various biological animal species. It is worth noting that this tree accurately reflects the real taxonomic relationships between animals: closely related animals have a smaller distance on this tree than those species belonging to different taxonomic groups. Thus, the results of the phylogenetic analysis based on the data on *TERT* of biological species can, to some extent, be used to assess the evolutionary relationships between species, as they partially reflect the general similarity of the genomes of organisms and are consistent with the data from other studies. For example, in another study, genetic similarities were evaluated, and phylogenetic analysis of biological species was carried out based on mitogenomic nucleotide sequences (Abdoli et al., 2018), which is a recognized method in phylogenetic studies. It generally agrees with the results presented here, and the phylogenetic tree built on the basis of mitogenomic sequences is very similar to the *TERT* sequences-based one.

Simultaneously, the following arguments can confirm the thesis about the importance of researching polymorphisms in the pig *TERT* gene. Despite the prevalence of this gene among vertebrates and the high degree of similarity between genetic sequences in different species, this gene is extremely polymorphic. For instance, according to the Ensembl database, humans have 7 transcripts of this gene, of which 4 are protein coding and give different TERT isoforms, and cats have a total of 10 *TERT* transcripts, each of which yields its own isoform of the enzyme of varying lengths. One isoform of *TERT* is known for pigs, but further research into polymorphisms in the *TERT* gene, particularly those that can influence the mechanisms of regulation of gene expression, for example, cause alternative splicing or the use of alternative promoters, can form the basis for the discovery of new *TERT* isoforms that may function differently. This, in turn, could be associated with the phenotype of animals and used in marker-associated selection.

In this study, focus was given to the examination of the SNPs in the pig *TERT* gene with known chromosomal locations, enabling their unambiguous identification through data available in the Ensembl database. Each type of polymorphism is likely to exert a distinct effect on gene expression processes and the functionality of the protein molecule it encodes. Consequently, they are anticipated to impact the animal's phenotype differently. Polymorphisms located in the upstream and downstream regions, as well as variants in the 5'- and 3'-UTR, are evidently expected to influence gene expression, its regulation by transcription control factors, pre-mRNA processing, and mature mRNA stability (Albert, 2011; Steri et al., 2018). Even though synonymous polymorphisms do not directly alter the amino acid sequence in the translation product, they indirectly influence the efficacy of this process due to the varying availability of tRNAs corresponding to specific amino acids for post-translational protein processing (Elf et al., 2003; Sauna et al., 2011). Conversely, missense polymorphisms are likely to have the most conspicuous impact, given their direct influence on the amino acid sequence in the protein molecule and its functional activity. The latter can determine the effects on specific molecular processes occurring within cells where this product of gene expression is involved, subsequently leading to phenotypic alterations. It is important to note that the effect of distinct missense polymorphisms on *TERT* enzyme functionality may not be uniform, as it also hinges on the conservativeness or radicality of each amino acid substitution caused by a particular missense polymorphism. Consequently, the substitution of amino acid residues with those possessing substantially different physicochemical properties can significantly affect the processes of folding and higher-level organization of the *TERT* molecule, as well as the enzyme's ability to interact with other components of the telomerase complex and perform its molecular function.

In a prior study (Peka et al., 2023), it was projected that certain polymorphisms, namely rs789641834 (TEN domain, substitution L158M), rs706045634 (TEN domain, R201P), rs325294961 (TRBD domain, R354G), and rs705602819 (RTD domain, R629W), could potentially have a substantial impact on the structural and functional properties of *TERT*. The first three of these polymorphisms are situated in the Exon 2, while the last is in the Exon 4. Given that these exons also encompass a notable number of other polymorphisms, their study by molecular genetic methods and an assessment of their association with phenotypic traits holds promise for the development of new genetic markers of animal productive traits.

As shown in Tables 3–5, restriction endonucleases are available for most SNPs. Consequently, these polymorphisms can be analyzed in population and association studies utilizing the PCR-RFLP method. The selection of a particular restriction endonuclease, especially in cases where multiple endonucleases from different isoschisome groups are available, may be influenced by specific experimental requirements and the economic viability associated with using a particular endonuclease.

Thus, the presented PCR-RFLP system comprehensively covers 20 polymorphisms located in two exons of the pig *TERT* gene, for the majority of which there are corresponding restriction endonucleases. This system facilitates population and association studies to investigate the distribution of polymorphic alleles within pig populations of various breeds and their associations with the animals' phenotypes. Notably, the PCR-RFLP system enables analysis of the four most critical missense polymorphisms (rs789641834, rs706045634, rs325294961, and rs705602819), whose potential influence on the structural and functional properties of *TERT* has been previously predicted. Additionally, other polymorphisms, particularly synonymous variants, hold significance for analysis as they are in proximity to missense polymorphisms and likely exhibit linkage disequilibrium, serving as markers for the latter.

Further research will allow hypotheses to be tested concerning the potential influence of *TERT* gene polymorphisms on the enzyme's functions and, consequently, on the animal phenotype. The utilization of the developed PCR-RFLP system thus lays the foundation for subsequent experimental validation of *TERT* as a marker gene in marker-associated selection of pigs and other types of farm animals.

Conclusions

The *TERT* gene is considered a promising candidate for marker-associated selection, with SNPs within it being of special interest due to their potential impact on the enzyme encoded by the *TERT* gene, crucial for telomere length maintenance and genome stability. This study thoroughly analyzed the exon-intron structure of the *TERT* gene and highlighted the significance of investigating SNPs located in Exon 2 and Exon 4. The development of the PCR-RFLP system for these SNPs involved primer and restriction enzyme design, along with optimization of PCR amplificate synthesis conditions. The subsequent step involves analyzing the effect of the studied SNPs on the phenotype and evaluating potential correlations between specific allelic variants in the animals' genotypes and their productive traits. This, in turn, lays the groundwork for utilizing *TERT* gene polymorphisms as novel markers in animal breeding and their integration into marker-associated selection practices.

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