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Polymorphism of pyruvate carboxylase gene and its relation with productive and reproductive performance of dairy cows

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Abstract Genetic selection in dairy cattle can be enhanced by identifying the candidate genes and variants associated with economic traits. This study examined the productive and reproductive characteristics of Holstein cattle based on the genotypes of the pyruvate carboxylase (*PC*) gene. A total of 50 cows, aged three to five years, was included in the study, with identical feeding and maintenance protocols applied to all animals. All cows completed a lactation period of 305 days. The genotype and allele frequencies were determined through genotyping. Sequencing analysis of 816 bp amplicons detected two single-nucleotide polymorphisms (SNPs), rs42195003 and rs110476776. The association analysis of the two SNPs was not significantly associated with lactation period, dry period, milk composition, and reproductive performance ($P>0.05$). In contrast, the highest milk production (4305.63 ± 222.01 kg), was associated with the CT genotype of the *PC* (rs110476776) polymorphism. The results revealed that the SNP rs110476776 in the *PC* gene was associated with total milk production in Holstein cows. This study may provide valuable insights into the role of the analyzed gene in the functional traits of dairy cattle; however, it is essential to validate the results by researching larger populations and different breeds of cattle.

Keywords: cattle, milk traits, pyruvate carboxylase, polymorphisms, reproductive traits

Introduction

Breeding dairy cattle primarily focuses on economically enhancing productive and reproductive traits. The most significant economic traits of dairy cows include milk production characteristics, such as milk yield, fat and protein percentages, and the overall fat and protein content (Du et al., 2024). Milk quality is assessed based on its protein and fat content, as these factors directly influence the nutritional value of the milk (Du et al., 2024; Saleh et al., 2024). Numerous factors affect milk production, including genetics, nutrition, and environ-

mental conditions. With advancements in technology and genetics, livestock breeding has become increasingly cost-effective and efficient (Al-Jaryan et al., 2023; Saleh et al., 2024). When functional genes or variations associated with milk production traits are identified, they can provide invaluable molecular information for marker-assisted or genomic selection in dairy cattle (Li et al., 2020). Several candidate genes have recently been investigated for their polymorphisms, particularly those involved in milk production traits, including yield, protein content, and fat content (Chouhan et al., 2023).

Several mutations within candidate genes are associated with productivity and reproduction in cattle. An analysis of the statistics revealed that cows with the AC genotype of the oxidized low-density lipoprotein gene (*OLR*) exhibit higher milk yield, protein levels, and fat content, with the difference in fat yield being statistically significant (Kowalewska-Łuczak and Czerniawska-Piątkowska, 2018). The analysis also indicated that for the SAA2 (serum amyloid A2) c.-84 G>C polymorphism, the CC genotype was associated with the highest milk protein concentration and the lowest milk fat concentration. In comparison to the SAA2 c.114G>A polymorphism, cows with the GA genotype had the shortest calving interval ($P < 0.05$) (Kowalewska-Łuczak and Czerniawska-Piątkowska, 2021). In crossbred dairy cattle, kappa-casein (*kappa-CN*) genotypes were linked to milk production traits. The AA variant produced more milk and a higher proportion of fat, casein, protein, and solids not fat (SNF), with increases of 1.397 kg, 0.75%, 0.31%, 0.27%, and 0.68%, respectively, compared to the BB variant (Albazi et al., 2023). Variants of the GH-IGF-I somatotrophic axis were examined in a recent study involving Holstein cattle to determine their effects on milk composition and production. The GH-Alul-AG and IGF-I-SnaBI-TC genotypes significantly enhance milk yield and reproductive performance. In contrast, the IGF-I-SnaBI-CC and GH-Alul-AA genotypes exhibit the highest proportions of fats and proteins (Saleh et al., 2024).

Among the most candidate genes, the pyruvate carboxylase (*PC*) gene which is located on cattle chromosome 29 (NCBI Reference Sequence NC_037356.1), encodes PC, a rate-limiting enzyme that is essential in various metabolic pathways, including glucose production, lipogenesis, amino acid metabolism, and neurotransmitter synthesis (Agca and Donkin, 2007; Weld et al., 2020). Seven SNPs identified in previous reports—rs109496284, rs110561408, rs137492467, rs42194999, rs42195000, rs42195007, and rs42197370—were significantly associated with milk protein percentage, milk yield, and protein yield (Lillehammer et al., 2007). In total, six SNPs in the *PC* gene—g.44965658G>A, g.44883644G>A, g.44862106C>T, g.44861428A>G, g.44861419C>T, and g.44861340T>C—were significantly associated with milk production traits in Chinese Holstein cows. The SNP g.44965658G>A may represent a crucial mutation site for milk production traits, potentially regulating the transcriptional activity of the *PC* gene by binding transcription factors (Du et al., 2024). While the *PC* gene is recognized as a major gene responsible for milk production and reproduction in dairy cattle, only a few studies have confirmed this association. Therefore, this study examined the polymorphisms of the *PC* gene (rs42195003 and rs110476776) in Holstein cows and their relationship with reproductive traits and productivity.

Materials and methods

Animals, phenotypic data, and milk collection

An investigation of dairy cattle at the Great Diwanayah Cattle Station was conducted during the productive season of 2023/2024, following the international recommendations (Federation of Animal Science Societies, 2010), under approval number Agri, No. 020,9,24. The study included fifty Holstein cows aged three to five years, all of which received the same feeding and maintenance regimen. Each cow completed a lactation period of 305 days. Data were collected on the animals' phenotypes, including age, milking, lactation, calving, and sire/dam identifications. Two sires were randomly selected to mate with approximately 25 to 30 dams within the studied herd. The fat, protein, and solid content were determined by analyzing a ten-milliliter milk sample using an industrial milk analyzer (EKOMILK).

Blood sampling, DNA extraction, and SNP genotyping

A total of 3 to 4 mL of blood was collected in anticoagulant tubes from the jugular veins of 50 cows. Genomic DNA was extracted from the whole blood using the gSYNCTM DNA extraction kit (Geneaid, New Taipei City, Taiwan), following the manufacturer's instructions. The extracted DNA was assessed using a Nanodrop (BioDrop Lite, Biodrop, UK) and analyzed by agarose gel electrophoresis at 0.8% (Al-Jumaili et al., 2023).

A PCR amplification of an 816 bp fragment containing the coding region of bovine *PC* exon 7 was conducted. The primers used for amplifying the *PC* gene fragments were reported by Du et al. (2024), with the following nucleotide sequences: forward (F) 5'-GTGAATGGAGCGAGTGAGGT-3' and reverse (R) 5'-CTCCGGTAGCTTTCCCAA-3'. The PCR was performed using a Bioneer PCR premix containing 50 mM dNTPs, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM MgCl₂, and 1 U of Top DNA polymerase. The PCR setup included denaturation at 94°C for 4 minutes, followed by 35 cycles of annealing, elongation, and extension at 94°C, 60°C, and 72°C for 45 seconds each, with a final extension at 72°C for 5 minutes (Mohammed et al., 2022). Prior to Sanger sequencing, the PCR products were electrophoresed on 2% agarose gels, and the gel images were captured using a Chemidoc Gel Imager (Bio-Rad, USA) (Ali et al., 2022). After sequencing, potential SNPs were identified using NCBI-BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) based on reference sequences (ARS-UCD1.2).

Studied traits

The traits studied in this research included milk production traits (total milk production, lactation period, and dry period), milk composition (fat %, solids-not-fat (SNF) %, protein %, glucose %, and specific gravity), and reproductive performance (services per conception, calving interval, and days open).

Statistical analysis

PopGen32, version 1.31, was utilized to calculate genotype and allele frequencies (Yeh et al., 1999). The General Linear Model procedure was used to detect the effect of pyruvate carboxylase-PC gene (rs42195003 and rs110476776) polymorphism on parameters. The statistical model was $Y_{ij} = \mu + G_i + e_{ij}$

in which:

Y_{ij} = Dependent variable.

μ = Overall mean of traits.

G_i = Effect of genotype/ pyruvate carboxylase-PC gene (TT and GG -rs42195003) and (CC and CT -rs110476776).

e_{ij} = Error term.

The Chi-squared test was used to determine the percentage of genotype distribution/ calculator of allele frequency of the pyruvate carboxylase-PC gene according to Hardy Weinberg's equilibrium (Falconer and Mackay, 1996).

$$PA = \frac{2 * \text{No. of Homozygotes} + 1 * \text{No. of Heterozygotes}}{2 * \text{Total number of sample}}$$

$$p + q = 1$$

$$qB = 1 - PA$$

The age effect was not significant and was excluded from further analysis.

Results

Genotyping of the PC gene and genetic diversity

Fifty samples were amplified using PCR (Figure 1). Sequencing of the 816 bp amplicons revealed two single-nucleotide polymorphisms (SNPs), rs42195003 and rs110476776 (Figures 2 and 3). Genetic diversity analysis of the rs42195003 SNP indicated that the TT genotype was the most prevalent (n = 34), followed by the GG genotype at a frequency of 0.32 (n = 16). For the second SNP, the CC genotype was the most common (n = 34), with the CT genotype also at a frequency of 0.32 (n = 16). The Chi-squared test showed significant deviations from the Hardy-Weinberg equilibrium for both SNPs, rs42195003 and rs110476776 (Tables 1 and 2).

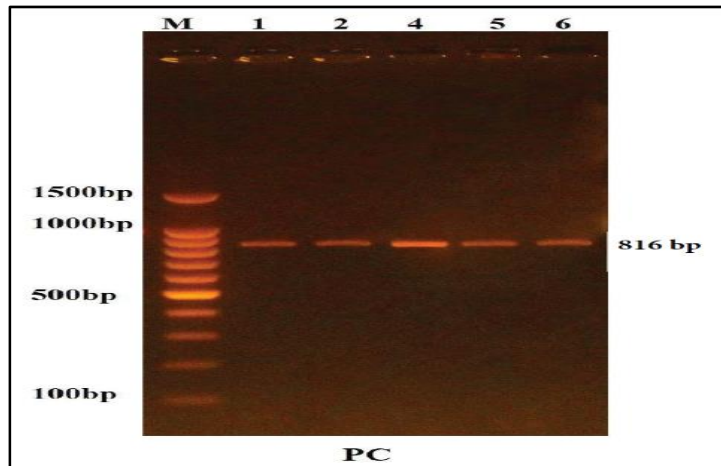


Figure 1. Agarose gel electrophoresis for PCR product amplification of the PC gene. Lane M: 100 bp DNA ladder, lanes 1–6 indicate samples of Holstein cows. Electrophoresis conditions: 2% agarose concentration, Electrophoresis time 25 min. The buffer used TBE pH 8.3

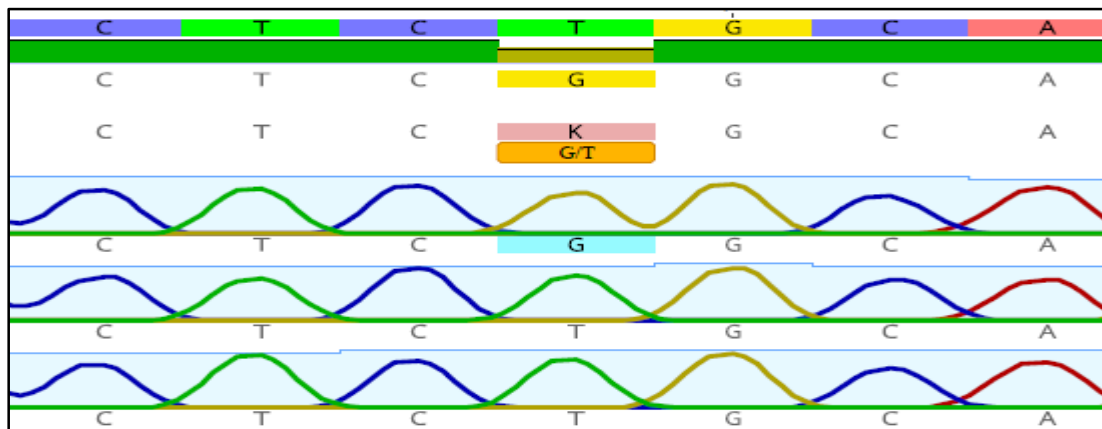


Figure 2. DNA sequencing revealed a mutation of the PC gene (rs42195003)

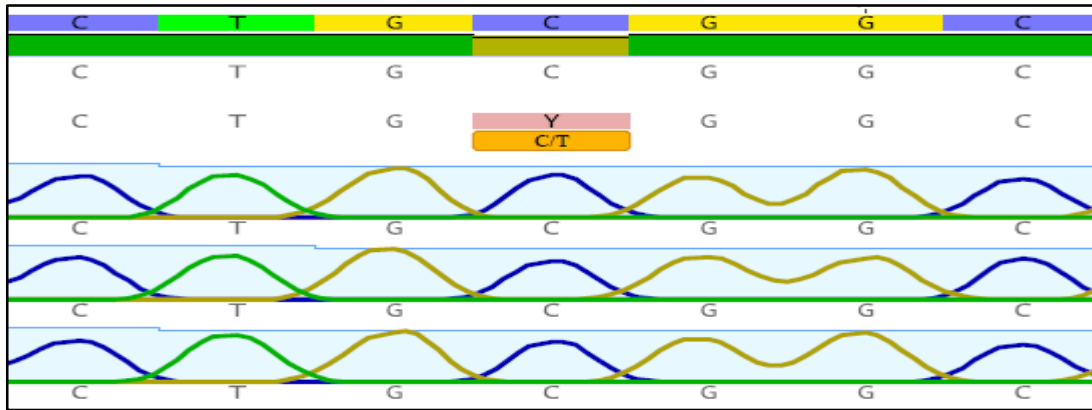


Figure 3. DNA sequencing revealed a mutation of the *PC* gene (rs110476776)

Association analysis of the *PC* gene

The association analysis of two SNPs in the *PC* gene and milk-productive traits in Holstein cows is shown in Tables 3 and 4. The rs42195003 SNP was not significantly associated with total milk production, lactation period, and dry period ($P>0.05$). The SNP rs110476776 was only associated with total milk production ($P<0.05$). The highest milk production (4305.63 ± 222.01 kg) was associated with the CT genotype for the *PC* gene (rs110476776) polymorphism. The genotypic patterns of the *PC* gene showed a non-significant effect on milk composition (Tables 5 and 6) and reproductive performance (Tables 7 and 8).

Table 1. Genotype distribution and allele frequency of pyruvate carboxylase-*PC* gene (rs42195003) in Holstein cows

Genotype	Number	Percentage (%)
TT	34	68.00
TG	0	0.00
GG	16	32.00
Total	50	100%
Chi-Squared (χ^2)	---	6.480 **
Allele	Frequency	
T		0.68
G		0.32

** ($P<0.01$)

Table 2. Genotype distribution and allele frequency of pyruvate carboxylase-*PC* gene (rs110476776) in Holstein cows

Genotype	Number	Percentage (%)
CC	34	68.00
CT	16	32.00
TT	0	0.00
Total	50	100%
Chi-Squared (χ^2)	---	6.480 **
Allele	Frequency	
C		0.84
T		0.16

** ($P<0.01$)

Discussion

Genetic variations in the *PC* gene have been observed in livestock in a few studies. Specifically, seven SNPs were identified in the *PC* gene of the Norwegian Red dairy breed (Lillehammer et al., 2007). More recently, six SNPs in the *PC* gene were detected in Chinese Holstein cows (Du et al., 2024). However, only a few studies have confirmed the variation of the *PC* gene in dairy cattle. This study provided genotypic data and identified a novel association that may be useful for marker-assisted selection in dairy cattle, thereby facilitating more effective measurement of the functional traits in future breeding programs.

Table 3. Relationship between pyruvate carboxylase-*PC* gene (rs42195003) polymorphism with total milk production, lactation period, and dry period in Holstein cows (Least squared means \pm SEM)

Genotype/ rs42195003	Total milk production (kg)	Lactation period (day)	Dry period (day)
TT	4112.88 \pm 296.92	269.29 \pm 11.29	111.88 \pm 10.43
GG	3733.75 \pm 321.62	272.06 \pm 12.87	109.62 \pm 8.63
<i>P</i> -value	0.09	0.24	0.52

Table 4. Relationship between pyruvate carboxylase-*PC* gene (rs110476776) polymorphism with total milk production, lactation period, and dry period in Holstein cows (Least squared means \pm SEM)

Genotype/ rs110476776	Total milk production (kg)	Lactation period (day)	Dry period (day)
CC	3843.76 \pm 295.65	275.41 \pm 11.96	118.85 \pm 10.27
CT	4305.63 \pm 222.01	288.81 \pm 7.65	99.81 \pm 7.99
<i>P</i> -value	0.03	0.33	0.09

The association analysis of two SNPs in the *PC* gene was not significantly associated ($P>0.05$) with lactation period, dry period, milk composition, and reproductive performance in Holstein cows. The lack of effect

observed for rs42195003 may be attributed to several factors, including low allele frequency, limited statistical power, and physiological redundancy. Additionally, the small sample size further constrains these findings. In

contrast, current study identified a significant association between total milk production and the SNP rs110476776 in the *PC* gene ($P < 0.05$). The highest milk production (4305.63 ± 222.01 kg) was associated with the CT genotype compare to CC genotype (3843.76 ± 295.65 kg). The efficiency of milk production can be enhanced by increasing the synthesis of fat, protein, and lactose (Osorio et al., 2016). This can be accomplished through genetic enhancement and effective management practices, which include improving the nutritional availability of compounds utilized by the mammary gland in milk production. The discovery of DNA and the advancement of molecular biology have led to the understanding that manipulating DNA transcription could influence phenotypic traits (Osorio et al., 2016; Du et al., 2024). Throughout the lactation cycle—comprising the dry period, early lactation, and peak lactation—various physiological states, substance metabolism, and gene expression patterns are observed (Xu et al., 2019). In Rathi cattle, Chouhan et al. (2023) studied polymorphism of *LEP* and *STAT5A* genes and their association with milk production traits. The *LEP* exon 3

(454 bp), *STAT5A* intron 9-10 (224 bp), and *STAT5A* intron 15–exon 16 (379 bp) segments revealed the presence of two genotypes (AA and AB). The *STAT5A* exon 7 (215 bp) region exhibited three genotypes (AA, AB, and BB). In these genes, the AA genotype was more common than the others. The AB genotype of *LEP* exon 3 and the AA genotype of introns 9-10 and intron 15–exon 16 of the *STAT5A* gene significantly affected total milk production. Additionally, the BB genotype of the *STAT5A* exon7 gene was significantly correlated with peak milk yield. In Romanian Brown cattle, Ilie et al. (2023) also identified polymorphism of the *PRL* gene (rs211032652 SNP in exon 4) and its correlation with milk production and chemical composition. Genotyping analysis revealed three genotypes (AA, AG, and GG), with the AG heterozygous genotype occurring at a higher frequency than the homozygous AA and GG genotypes. The AA genotype was associated with a higher milk fat percentage (4.76 ± 0.28) compared to the GG genotype (4.04 ± 0.22 , $p = 0.048$), as well as a higher protein percentage ($3.96 \pm 0.32\%$ vs. $3.43 \pm 0.15\%$, $p = 0.027$) in Romanian Brown cattle.

Table 5. Relationship between pyruvate carboxylase-*PC* gene /rs42195003 polymorphism with milk composition in Holstein cows (Least squared means \pm SEM)

Genotype/ rs42195003	Fat (%)	SNF (%)	Protein (%)	Glucose (%)	Specific gravity
TT	4.01 \pm 0.22	7.73 \pm 0.25	2.86 \pm 0.11	4.27 \pm 0.14	1.02 \pm 0.003
GG	4.28 \pm 0.21	7.53 \pm 0.12	2.66 \pm 0.03	3.98 \pm 0.04	1.03 \pm 0.003
<i>P</i> -value	0.23	0.35	0.43	0.16	0.33

SNF: solids-not-fat

Table 6. Relationship between pyruvate carboxylase-*PC* gene /rs110476776 polymorphism with milk composition in Holstein cows (Least squared means \pm SEM)

Genotype/ rs110476776	Fat (%)	SNF (%)	Protein (%)	Glucose (%)	Specific gravity
CC	4.02 \pm 0.18	7.85 \pm 0.23	2.89 \pm 0.10	4.32 \pm 0.13	1.03 \pm 0.003
CT	4.25 \pm 0.34	7.25 \pm 0.22	2.59 \pm 0.07	3.88 \pm 0.11	1.02 \pm 0.004
<i>P</i> -value	0.65	0.41	0.52	0.33	0.43

SNF: solids-not-fat

Table 7. Relationship between pyruvate carboxylase-*PC* gene /rs42195003 polymorphism with reproductive performance in Holstein cows (Least squared means \pm SEM)

Genotype/ rs42195003	Service per conception- SPC	Calving interval-CI (day)	Days open-DO (day)
TT	1.71 \pm 0.13	381.29 \pm 3.60	100.17 \pm 3.64
GG	1.87 \pm 0.22	381.62 \pm 6.43	101.31 \pm 6.38
<i>P</i> -value	0.40	0.26	0.68

Table 8. Relationship between pyruvate carboxylase-*PC* gene /rs110476776 polymorphism with reproductive performance in Holstein cows (Least squared means \pm SEM)

Genotype/ rs110476776	Service per conception- SPC	Calving interval-CI (day)	Days open-DO (day)
CC	1.73 \pm 0.14	380.29 \pm 3.90	99.08 \pm 3.94
CT	1.81 \pm 0.19	383.75 \pm 5.47	103.63 \pm 5.41
<i>P</i> -value	0.23	0.11	0.54

The *PC* gene is reported to be closely associated with quantitative trait loci (QTL: 2593, 2612, 255006), which influence milk yield, protein yield, and percentage. In this study, the rs110476776 SNP, located in the coding region, significantly affected milk production traits. A possible explanation for this finding is that the conserved coding region of DNA plays a vital role in biological

processes (Alkhammas and Al-Thuwaini, 2023). The expression of *PC* plays a crucial role in metabolic processes during milk synthesis, and its inhibition may adversely affect milk production traits (Walker et al., 2016; Pedrosa et al., 2021). Previous studies demonstrated that the *PC* gene was regulated by the concentrations and profiles of nonesterified fatty acids

(NEFAs). Correlations between PC expression and FA concentration and profile were examined to investigate whether individual fatty acids (FAs) differentially relate to PC expression at one day postpartum (Weld et al., 2020). Furthermore, PC is responsible for converting pyruvate into oxaloacetate in the mitochondria, influencing gluconeogenesis in ruminants (Lin et al., 2022; Pang et al., 2023). Gluconeogenesis in the liver is essential for maintaining whole-body glucose homeostasis and glucose production in the mammary glands, which is necessary for lactose synthesis. During the transition from pregnancy to lactation, glucose requirements increase three- to fourfold (Walker et al., 2016). As the body experiences nearly a fourfold increase in energy, protein, and mineral requirements, several organs coordinate the metabolic changes. These changes are crucial for the success of the upcoming lactation (García-Roche et al., 2021). High-producing dairy cows are more likely to require glucose than can be supplied by gluconeogenesis alone during early lactation. In late pregnancy and early lactation, dry matter intake increases by 30% to 50%. However, the mammary glucose requirement during early lactation is three times greater than the uterine glucose requirement during late pregnancy (Moran et al., 2016). However, literature on PC gene variation in ruminants is limited. Only one study on Chinese Holstein cows has demonstrated that the G allele of the 29:G.44965658G>A polymorphism in the PC gene affects milk production traits (Du et al., 2024). Although this study is the first to report a relationship between the SNP rs110476776 in the PC gene and total milk production in Holstein cows, it has some limitations. Many factors may influence the results, including environmental stressors, feed quality, and genetic variation within the sample population. Additionally, the small sample size limits the findings. Further research with a larger population of Holstein cows is needed to better understand how the rs110476776 variant in the PC gene affects total milk production.

Conclusion

The SNP rs110476776 in the PC gene has been associated with total milk production in Holstein cows. The SNPs identified in this study can serve as candidate markers for molecular breeding in dairy cows, facilitating the validation of the role of PC in milk synthesis. Although this conclusion is based on a limited sample size (n= 50), the absence of heterozygotes for the rs42195003 SNP, potential population stratification, and a lack of functional validation are important limitations. Future studies are necessary to validate rs110476776 in an independent cohort of more than 200 cows using allele-specific expression assays or to perform luciferase reporter assays to test promoter activity.

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Conflict of interest

There is no conflict of interest associated with this publication.

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