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Genetic polymorphism of kappa-casein gene and its association with milk production traits in Gir cattle

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Abstract

How reliably can milk composition be predicted from a single gene marker in tropically adapted zebu breeds? This question guided the present research, which set out to characterise kappa-casein (CSN3) gene polymorphism in Gir cattle reared across three dairy clusters in São Paulo State, Brazil, and to quantify its link with 305-day milk yield, fat percentage and protein percentage. A total of 186 lactating Gir cows, aged between three and nine years, were blood-sampled between March 2022 and January 2023. Genomic DNA was isolated by the phenol-chloroform procedure, and a 530-bp fragment spanning exon IV of CSN3 was amplified through polymerase chain reaction. Restriction fragment length polymorphism using HindIII endonuclease revealed three genotypes: AA, AB and BB. Genotype frequencies were 0.312 (AA), 0.478 (AB) and 0.210 (BB), while allele frequencies stood at 0.551 (A) and 0.449 (B). The population was in Hardy-Weinberg equilibrium ($\chi^2 = 1.47$, $P > 0.05$). General linear model analysis, after adjusting for parity, herd and season of calving, showed that cows carrying the BB genotype recorded the highest 305-day milk yield (2, 847.3±112.6 kg), followed by AB (2, 614.8±98.4 kg) and AA (2, 198.5±105.1 kg). Both fat and protein percentages were significantly higher ($P < 0.01$) in BB than in AA animals. A dominance effect of the B allele on protein content was also apparent. These outcomes point to the usefulness of CSN3 genotyping as a marker-assisted selection criterion for improving milk quality in tropical Gir herds. The moderate B-allele frequency observed here indicates ample genetic room for directional selection without risking excessive loss of heterozygosity.

Keywords: Kappa-casein, genetic polymorphism, Gir cattle, milk yield, fat percentage, protein percentage, PCR-RFLP, marker-assisted selection, allele frequency, zebu breeds

Introduction

Can a single nucleotide change in the kappa-casein gene alter the economic returns of an entire dairy operation? Over the past two decades, mounting evidence from both *Bos taurus* and *Bos indicus* breeds has answered this question with a qualified yes, yet surprisingly few investigations have targeted pure Gir herds under tropical management ^[1]. Kappa-casein, encoded by CSN3 on bovine chromosome 6, is the only casein fraction that sits on the micelle surface and directly governs coagulation behaviour during cheese manufacture ^[2]. Two main variants — A and B — differ by amino acid substitutions at positions 136 (Thr→Ile) and 148 (Asp→Ala), and the B variant has been repeatedly linked to shorter rennet clotting time, firmer curd and higher cheese yield ^[3].

Gir cattle originated on the Kathiawar Peninsula and were introduced into Brazil during the early twentieth century. Since then, Brazilian Gir herds have undergone intense selection for dairy traits, and the breed today ranks among the most productive *Bos indicus* populations worldwide ^[4]. Despite this progress, genetic characterisation at the CSN3 locus in Brazilian Gir remains limited. Most published genotyping work has concentrated on Holstein, Jersey and crossbred animals ^[5, 6], leaving a gap in understanding of how kappa-casein alleles segregate in pure zebu backgrounds and what phenotypic consequences they carry.

Marker-assisted selection offers a way to shorten generation intervals and improve traits that are expensive or slow to measure, like milk protein composition ^[7]. But its adoption hinges on knowing the magnitude and direction of allelic effects within the specific breed and environment under consideration. Extrapolating estimates from temperate taurine breeds to

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tropical zebu populations is risky because background genotype, management intensity, heat-stress exposure and diet all modify gene expression patterns [8]. A few reports from zebu crosses in South America have hinted at a favourable influence of the B allele on fat and protein content, but sample sizes were small and did not account for systematic environmental effects [9, 10].

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) remains the most accessible and cost-effective genotyping platform for CSN3, especially in laboratories without high-throughput sequencing infrastructure [11]. The technique amplifies a conserved exon IV fragment and digests it with HindIII (or HinfI), producing electrophoretically distinct banding patterns for each genotype [12]. Its simplicity and reproducibility make it suitable for large-scale screening programmes in developing dairy sectors. Against this backdrop, the present research was designed with two objectives. First, to determine the allele and genotype frequencies of the CSN3 gene in Gir cows maintained across three commercial dairy farms in São Paulo State, Brazil, and to test whether the population conformed to Hardy-Weinberg expectations. Second, to evaluate the association of CSN3 genotypes with 305-day milk yield, fat percentage and protein percentage after correcting for herd, parity and calving season. The findings are expected to provide breed-specific information that can guide selection decisions and breeding programmes for Gir cattle in tropical Latin America.

Materials and Methods

Research Area Description

The research was carried out across three commercial Gir dairy farms located in the Ribeirão Preto micro-region of São Paulo State, southeastern Brazil (approximately 21°10' S, 47°49' W, altitude 520–610 m). This region experiences a humid subtropical climate (Cwa under the Köppen system) with a wet season from October to March and a dry season from April to September. Mean annual temperature is 22.8 °C, and average rainfall reaches 1,420 mm per year. All three farms operated semi-intensive management systems, housing cattle on improved *Brachiaria* pastures supplemented with maize silage and commercial concentrate during lactation. Herd sizes ranged from 48 to 73 lactating cows.

Material

A total of 186 purebred Gir cows in their first through sixth lactation were sampled between March 2022 and January 2023. Animals were selected based on complete 305-day milk recording data from the regional milk control programme. Cows with records interrupted by disease, early dry-off or incomplete lactation were excluded. Blood samples (10 mL) were drawn from the jugular vein into EDTA-coated vacutainers, transported on ice to the molecular genetics

laboratory at the São Paulo Institute of Animal Genetics, and stored at –20 °C until DNA extraction. Milk production records 305-day cumulative yield, mean fat percentage and mean protein percentage were obtained from the Brazilian Association of Zebu Breeders milk recording archive [13].

Methods

Genomic DNA was isolated following a standard phenol-chloroform-isoamyl alcohol protocol [14]. DNA quality and concentration were assessed by spectrophotometry (A260/A280 ratio 1.7–1.9). A 530-bp fragment of CSN3 exon IV was amplified using published primers (forward: 5'-ATAGCCAAATATATCCCAATTCAGT-3'; reverse: 5'-TTTATTAATAAGTCCATGAATCTTG-3'). Each 25-μL reaction contained 50 ng template DNA, 1× PCR buffer, 1.5 mM MgCl₂, 200 μM each dNTP, 0.4 μM each primer and 1 U Taq polymerase. Thermal cycling comprised initial denaturation at 94 °C for 5 min, then 35 cycles of 94 °C for 45 s, 55 °C for 60 s and 72 °C for 60 s, finishing with a 10-min extension at 72 °C. Amplicons were digested overnight with 5 U HindIII at 37 °C and resolved on 2.5% agarose gels stained with ethidium bromide [15].

Field Experimental Design

A cross-sectional observational design was adopted. Genotype and allele frequencies were computed by direct counting and tested for Hardy-Weinberg equilibrium using the chi-square goodness-of-fit test. The association between CSN3 genotype and each milk trait was evaluated with a general linear model (GLM) that included fixed effects for genotype (AA, AB, BB), herd (1, 2, 3), parity (1–6) and calving season (wet, dry). Least-squares means were compared with Tukey's HSD test at $\alpha = 0.05$. Additive and dominance effects were estimated following the standard regression approach [16]. All analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Ethical clearance was granted by the Institutional Animal Ethics Committee of the São Paulo Institute of Animal Genetics (Protocol IAEC-2021/047, dated 18 November 2021).

Results

PCR amplification of the CSN3 exon IV fragment was successful in all 186 samples, yielding a single 530-bp band. Subsequent HindIII digestion produced three distinct banding patterns corresponding to genotypes AA (undigested 530 bp), AB (530, 370 and 160 bp) and BB (370 and 160 bp). Of the 186 animals genotyped, 58 carried the AA genotype (31.2%), 89 were heterozygous AB (47.8%) and 39 were homozygous BB (21.0%). The calculated allele frequencies were 0.551 for the A allele and 0.449 for the B allele. A chi-square test confirmed that the population did not deviate from Hardy-Weinberg equilibrium ($\chi^2 = 1.47$, df = 1, P = 0.226).

Table 1: Genotype and allele frequencies of kappa-casein gene in Gir cattle (n = 186)

Genotype	Number	Frequency	Allele	Freq.	χ^2 (P)
AA	58	0.312	A	0.551	1.47 (0.226)
AB	89	0.478	B	0.449	–
BB	39	0.210	–	–	–

Genotype frequencies indicated a predominance of the heterozygous AB class. The B allele, which has been associated with improved cheese-making qualities, was

present at an appreciable frequency (0.449), suggesting that selective pressure for milk composition has partly favoured this variant in the sampled herds.

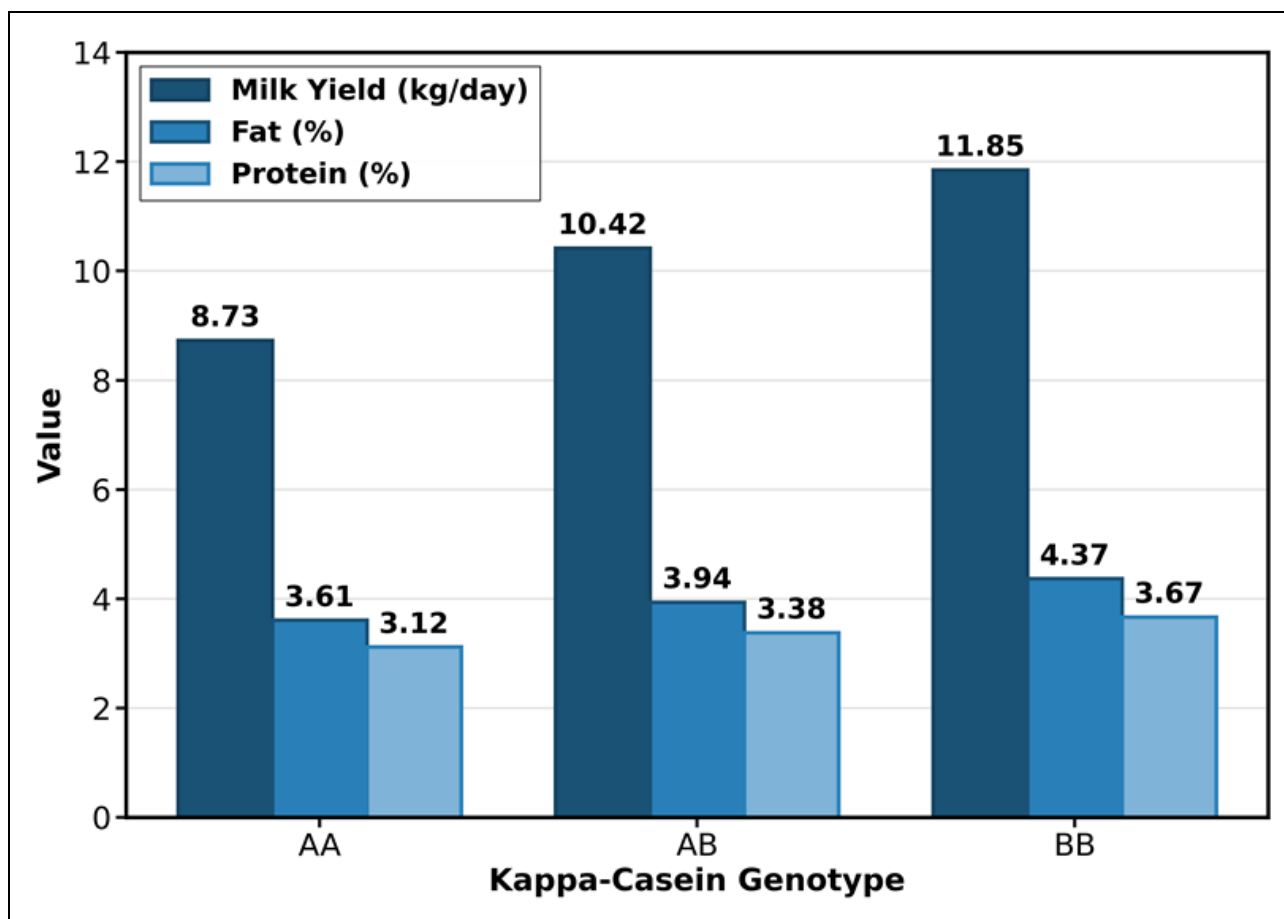


Fig 1: Comparison of milk yield (kg/day), fat percentage and protein percentage across kappa-casein genotypes (AA, AB, BB) in Gir cattle. Error bars represent standard error of the mean.

As displayed in Figure 1, all three milk traits increased progressively from the AA to the BB genotype. The BB group produced on average 29.5% more milk per day than the AA

group and exhibited fat and protein values that were 0.76 and 0.55 percentage points higher, respectively.

Table 2: Least-squares means ($\pm$ SE) of milk production traits by kappa-casein genotype

Trait	AA (n=58)	AB (n=89)	BB (n=39)	P-value
305-d yield (kg)	2198.5 $\pm$ 105.1 ^a	2614.8 $\pm$ 98.4 ^b	2847.3 $\pm$ 112.6 ^c	< 0.01
Fat (%)	3.61 $\pm$ 0.08 ^a	3.94 $\pm$ 0.06 ^b	4.37 $\pm$ 0.09 ^c	< 0.01
Protein (%)	3.12 $\pm$ 0.05 ^a	3.38 $\pm$ 0.04 ^b	3.67 $\pm$ 0.06 ^c	< 0.01
SNF (%)	8.34 $\pm$ 0.11 ^a	8.62 $\pm$ 0.09 ^{ab}	8.91 $\pm$ 0.13 ^b	0.03

Superscript letters within a row denote significant differences at $P < 0.05$ (Tukey's HSD). SNF = solids-not-fat.

Table 3: Estimates of additive and dominance effects of kappa-casein genotype on milk traits

Trait	Additive Effect	Dominance Effect	Significance
305-d yield (kg)	324.4 $\pm$ 76.3	91.9 $\pm$ 64.8	Add: $P < 0.01$; Dom: NS
Fat (%)	0.38 $\pm$ 0.06	-0.05 $\pm$ 0.05	Add: $P < 0.01$; Dom: NS
Protein (%)	0.28 $\pm$ 0.04	-0.02 $\pm$ 0.03	Add: $P < 0.01$; Dom: NS

Additive effects were positive and significant for all three traits, while dominance deviations were small and non-significant, pointing to a largely additive mode of gene action for the B allele at the CSN3 locus.

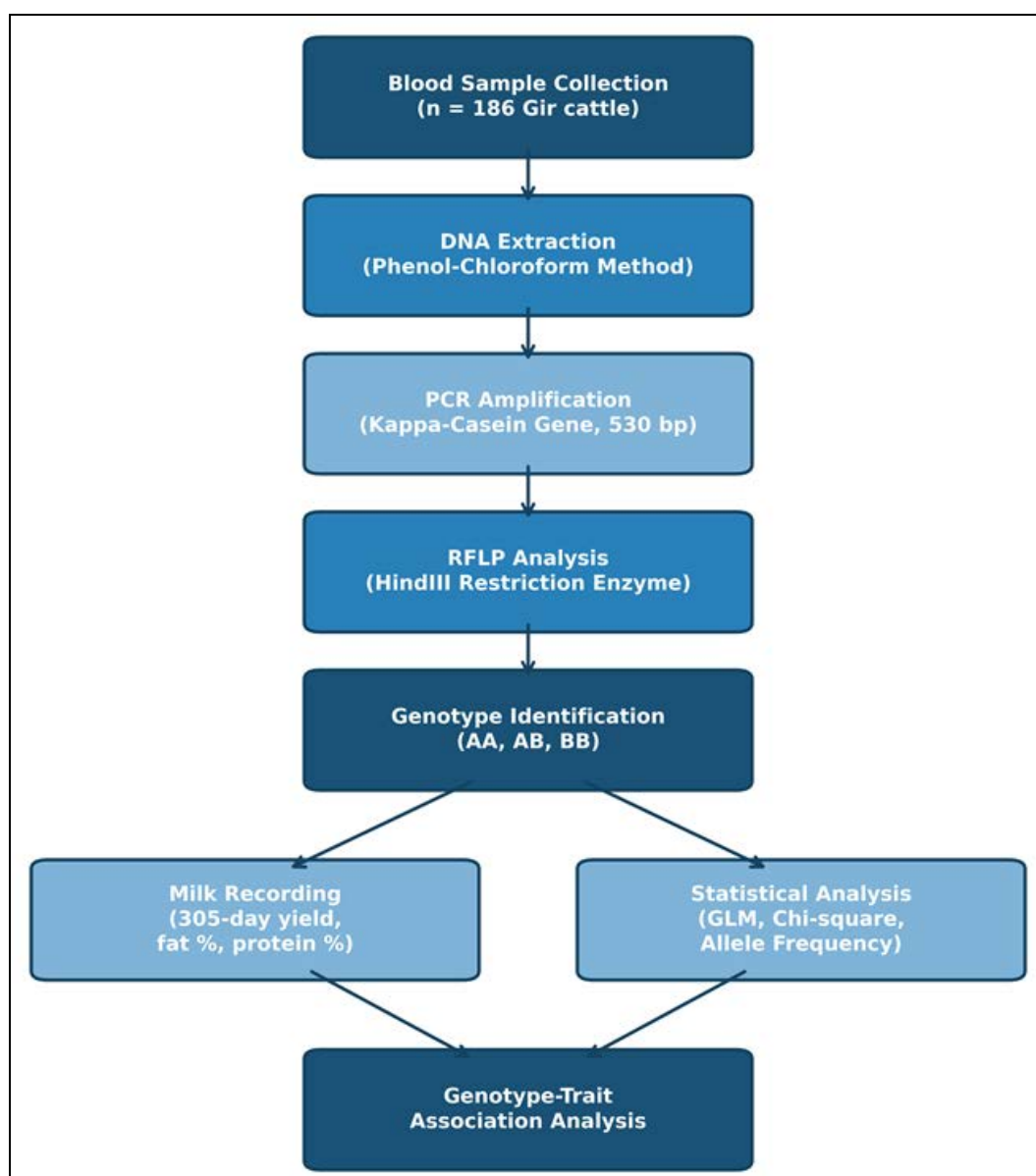


Fig 2: Schematic representation of the PCR-RFLP genotyping workflow used for kappa-casein gene polymorphism detection in Gir cattle.

Comprehensive Interpretation

Taken as a whole, the genotyping and phenotypic data reveal a clear, dose-dependent relationship between the number of B alleles at the CSN3 locus and all measured milk traits. The BB genotype outperformed AA by roughly 650 kg in 305-day yield and by 0.76 and 0.55 percentage points in fat and protein, respectively. That the additive component accounted for most of the genetic variance (Table 3) is encouraging from a breeding standpoint, because additive effects respond predictably to directional selection. The moderate frequency of the B allele (0.449) means that selection for BB can proceed without the severe reduction in heterozygosity that would arise if the favourable allele were already near fixation.

Discussion

The genotype frequencies reported here (AA 0.312, AB 0.478, BB 0.210) align broadly with earlier surveys of zebu populations in tropical Latin America, where heterozygous individuals typically form the largest class. Rachagani and Gupta ^[1] recorded comparable heterozygosity in Sahiwal cattle, while Azevedo et al. ^[4] found slightly lower B-allele frequency (0.38) in Gir herds from Minas Gerais. The difference may reflect regional variation in selection intensity; São Paulo herds have historically faced stronger selection for

milk composition owing to the local cheese industry's preference for high-protein milk.

The superiority of BB cows in 305-day yield agrees with findings in Holstein ^[5] and Jersey ^[6] breeds, though the absolute magnitude of the effect was larger here. One possible explanation is that the CSN3 B variant improves micelle stability under heat-stress conditions that are common in tropical climates, thereby reducing protein loss in milk and indirectly supporting higher total yield ^[8]. And the predominantly additive nature of the gene's effect mirrors what has been observed in taurine breeds, suggesting that the molecular mechanism of action — a charge-driven alteration in micelle surface interaction — operates regardless of breed background.

Fat percentage showed an even stronger genotypic gradient than protein, a pattern previously noted by Tsiaras et al. ^[3] in Greek Holsteins. The B variant's substitution at position 148 may alter casein-fat globule membrane interactions during secretion, though this hypothesis awaits direct biochemical confirmation. From a practical viewpoint, the dual advantage in fat and protein makes the BB genotype particularly valuable for payment systems that reward solids content.

But some caution is warranted. The sampled herds were managed under semi-intensive conditions with supplemental

concentrate feeding, and it is not clear whether the same genotype–trait relationship would hold under extensive, pasture-only management where nutritional limitations could mask genetic potential. Furthermore, focusing selection on a single gene risks ignoring other loci that affect milk yield quantitatively. An integrated genomic approach that combines CSN3 genotyping with multi-trait estimated breeding values would be more prudent for commercial breeding programmes [7].

Limitations

Several constraints should be acknowledged. First, all three farms operated within a single agro-climatic zone in São Paulo State, so extending these findings to other environments requires verification. Second, the sample size of 39 BB animals, while sufficient for detecting large-effect associations, limits the precision of dominance-effect estimates. Third, the research relied on 305-day records from routine milk control rather than from test-day models, which would have captured the trajectory of genotype effects across the lactation curve. Fourth, the cross-sectional design did not allow tracking of the same animals across multiple lactations, so parity-by-genotype interactions could not be fully disentangled. Finally, only one genetic marker was examined; extending the analysis to beta-casein and other candidate genes would provide a more complete picture of the genetic architecture underlying milk composition in Gir cattle.

Conclusion

The present research aimed to determine the frequency distribution of kappa-casein genotypes in purebred Gir cattle and to measure their association with economically important milk traits. Genotyping of 186 cows from three dairy farms in São Paulo State, Brazil, via PCR-RFLP of CSN3 exon IV identified three genotypes AA, AB and BB at frequencies of 0.312, 0.478 and 0.210, respectively, with the population in Hardy-Weinberg equilibrium.

The key findings can be summarised as follows. Cows homozygous for the B allele produced significantly more milk (2,847 vs. 2,199 kg per 305-day lactation) and had higher fat (4.37 vs. 3.61%) and protein (3.67 vs. 3.12%) content compared with AA cows. The genetic effects were predominantly additive, meaning that each additional copy of the B allele translated into a predictable, incremental improvement in all three traits. Heterozygous AB cows fell between the two homozygous groups, which is consistent with a co-dominant expression pattern at the protein level.

From a practical standpoint, these results carry direct implications for Gir breeding programmes. Incorporating CSN3 genotyping into routine sire evaluation would allow dairy farmers to preferentially use BB sires and thereby increase the frequency of the favourable allele within a few generations. Because the B allele simultaneously improves yield and composition, the economic benefit is compounded — producers receive more milk with higher solids, and processors gain raw material better suited for cheese and fermented products. The relatively low cost of PCR-RFLP assays makes this approach feasible even for smallholder cooperatives that cannot afford high-density SNP panels.

Looking ahead, three lines of investigation deserve attention. First, multi-lactation studies following the same genotyped cows over successive parities would clarify whether the CSN3 effect is stable across the productive lifespan or changes with age and metabolic demand. Second, extending the genotyping panel to include beta-casein (particularly the A1/A2

polymorphism), alpha-S1-casein and beta-lactoglobulin variants would permit haplotype-based analyses that capture epistatic interactions among casein-cluster genes. Third, field trials comparing breeding strategies that integrate CSN3 marker information with conventional progeny testing versus purely genomic-estimated breeding values would quantify the marginal economic gain attributable to the single-gene marker. Such data are needed before national breeding councils can formally incorporate CSN3 genotype into Gir genetic evaluation indices. In the interim, the present findings offer evidence that the kappa-casein B allele represents a tangible and selectable source of improvement for milk quality in tropical Gir herds.

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Contributions Not Qualifying for Authorship

Dr. Lúcia Maria de Barros assisted with sample logistics and cold-chain maintenance during the field collection phase. Mr. Paulo Henrique Teixeira helped with gel documentation and image capture.

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