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Genetic variation of Kappa-casein in South African goats

by

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The experimental work described in this dissertation was carried out in the Department of Animal and Wildlife Sciences, University of Pretoria, Pretoria, from January 2006 to June 2008, under the supervision of Dr. Estè van Marle-Köster and Mrs. Carina Visser.

I declare that the dissertation, which I hereby submit for the degree MAGISTER SCIENTIAE AGRICULTURAE at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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ABSTRACT

Milk protein polymorphisms have a significant influence on milk quantity and composition. Kappa-casein is of special interest due to its known relationship with milk quality. In goats, a number of allelic variants have been identified, primarily classified into two groups. Group B^{IEF} alleles (D, E, K, and M) have been shown to have a positive effect on milk yield and technological properties, while group A^{IEF}, the remaining alleles, have a less positive influence on milk composition. The aim of this study was to investigate genetic variation in the kappa-casein genotype of South African goats. PCR-RFLP and DNA sequencing were performed on 68 and 77 samples, respectively. In addition, 84 milk samples were analyzed for milk composition. RFLP analysis revealed that the A and/or B alleles were the most frequent in the populations studied. A frequency of 0.00 was observed for the B^{IEF} variants using DNA sequencing. In all goat types included, the B allele was the most common, with frequencies ranging from 60% in SA Boer goats to 100% in Saanens. The B' allele had lower frequencies of 0.357 and 0.207 in SA Boer goats and local goat types, respectively. The H allele was present at low frequencies in local goat types (10.3%) and in SA Boer goats (3.6%), but was absent in Saanens. AMOVA results indicated that most of the total variation occurred within populations (80.66%) with the remainder of the variation ($F_{ST} = 0.1934$; $p < 0.01$) occurring due to genetic differences between populations.

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LIST OF ABBREVIATIONS

Ala	Alanine
αs1-casein	alpha-s1-casein
αs2-casein	alpha-s2-casein
AMOVA	Analysis of Molecular Variance
ARC	Agricultural Research Council
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
AS-PCR	allele specific-Polymerase Chain Reaction
β-casein	beta-casein
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	complementary deoxyribonucleic acid
CMP	caseinomacropeptide
CSN3	kappa-casein
Cys	Cysteine
DADIS	Domestc Animal Diversity Information System
DM	dry matter
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetra-acetic acid
g	gram
Gln	Glutamic acid
Glu	Glutamine
Gly	Glycine
IEF	iso-electric focusing
Ile	Isoleucine
κ-casein	kappa-casein
kg	kilogram
l	litre
Leu	Leucine
Lys	Lysine

MCT	medium chain triglycerides
MEDUNSA	Medical University of Southern Africa
Met	Methionine
$MgCl_2$	Magnesium Chloride
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
NAMC	National Agricultural Marketing Council
NDA	National Department of Agriculture
ng	nanogram
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction Restriction Fragment Length Polymorphism
Phe	Phenylalanine
pmol	picamol
Pro	Proline
RFLP	Restriction Fragment Length Polymorphism
s	second
SA	South Africa
SE	standard error
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
µl	microlitre
USDA	United States Department of Agriculture
Val	Valine

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In many respects, milk can be considered to be the food most complete and balanced in nutritional content (Haenlein, 2002). It is a key component of a balanced diet, comprising an excellent source of energy, protein, carbohydrates, calcium, phosphorous and several other minerals and vitamins (Lopez-Aliaga *et al.*, 2000; Belewu & Aiyegbusi, 2002). In developing countries, goats may be a more appropriate source for this nutrient compared to cows due to the relatively lower cost of keeping goats. Particularly in developing countries such as South Africa, where poverty, malnutrition, and an ever-growing human population are the order of the day, milk will become an increasingly important source of high quality protein to reduce malnutrition, especially in the most vulnerable groups – children, pregnant or nursing mothers, and those suffering from HIV/AIDS (Devendra, 1980; Masika & Mafu, 2004; Ahuya *et al*., 2005; Peacock, 2005). Goat milk and the products prepared from it such as yoghurt, cheese, and powder, have twofold significance in human nutrition. Firstly, goat milk feeds more emaciated and malnourished people in the developing world than cow milk (Guo *et al*., 2004; Haenlein, 2004; Peacock, 2005). Secondly, goat milk may be used as an alternative to cow milk for people suffering from cow milk allergies and gastrointestinal disorders (Guo *et al*., 2004; Haenlein, 2004; Degen, 2007). The potential of goats to contribute to improve the livelihoods of the resource-poor was recognized in Kenya as early as the 1980s (Ahuya *et al.*, 2005).

Research indicates that milk protein polymorphisms have a great influence on the quantity, composition, and technological properties of milk (Caroli *et al*., 2001; Jann *et al*., 2004; Reale *et al*., 2005), and that certain alleles may even be linked to milk protein allergy (Kusza *et al*., 2007). Caseins comprise about 80% of the total protein content in milk and, even though kappa-casein is the least abundant of the three main groups of caseins present in ruminant milk, it has been extensively studied in bovines because of its role in micelle stabilization and in cheese-making (Calavia & Burgos, 1998; Yayhaoui *et al.*, 2001; Haenlein, 2002; Jann *et al.*, 2004; Otaviano *et al*., 2005). Given the associations between specific casein variants and superior milk composition and processing properties along with the fact that the alleles are inherited according to Mendelian laws, it is necessary to screen goat breeds further for the kappa-casein locus in order to routinely incorporate milk protein variants as additional selection criteria in future (Medrano & Cordova, 1990; Ng-Kwai-Hang & Grosclaude, 1992;

Fitzgerald, 1997; Caroli *et al*., 2001; Biase *et al.*, 2005). Currently, countries such as Austria, Switzerland, and Italy breed for the kappa-casein B allele specifically to improve milk quality for cheese-making (Fitzgerald, 1997), and Canada has already incorporated kappa-casein genotype information as selection criteria in their dairy cattle breeding programs (Glimm *et al*., 1996). There is a lack of relevant research in developing countries on local animals, which justifies investigations into this gene (Jann *et al*., 2004; Biase *et al*., 2005).

Literature was reviewed to examine the available scientific literature regarding the value of goats and milk proteins and their relevance in human nutrition and cheese-making in South Africa. The present study seeks to investigate genetic variation in the kappa-casein genotype of South African local goat types using two techniques – PCR RFLP and DNA sequencing.

1.2 Why goats?

Goats were among the first farm animals to be domesticated by man for the production of meat, milk, skins, and fibre. Archaeological evidence suggests that domestication took place as early as 7000BC in the Near East, and that the common ancestor of domesticated goats was *Capra aegagrus* (Mason, 1981). Today the goat (*Capra hircus*) is still present in all aspects of civilization – religion, economy and nutrition. Recently, the overall appreciation of this long-underestimated species has grown, boosting its significance in the livestock sector, especially as a tool for the economic growth and social enhancement of less developed rural populations (Ayalew *et al*., 2003; van Marle-Köster *et al*., 2004; Boyazoglu *et al*., 2005; Peacock, 2005; De Vries, 2008). In Southern Africa, goats are primarily kept for meat or mohair production, with milk serving as a by-product only. For example, in impoverished areas, they may be kept mainly for meat production, but also milked as the opportunity arises on a seasonal basis (Donkin, 1997; Degen, 2007).

In just over a decade, the world's population has increased from 4.4 to 5.5 billion, with the greatest increases (29%) occurring in developing countries like Asia, Africa, and Latin America, resulting in a corresponding increase in requirement for food, including milk and milk products (Knights & Garcia, 1997). Given the current rate of population growth, the effect of increased food demand means that agricultural production has to increase at a rate of more than 4% per year in order for the demand to be met (Ahuya *et al.,* 2005). Goats may be more suited to fulfil this need than cattle, for several reasons.

Attempts to increase milk production by importing high milk-producing cattle may not be feasible for smallholdings due to poor quality forages or low levels of management. The higher demand for milk and milk products in developing tropical countries might be easier met by increasing the population and efficiency of small ruminants, particularly goats (Donkin, 1997; Knights & Garcia, 1997; Haenlein & Caccese, 2003; Ahuya *et al*., 2005; Simela, 2005).

In both temperate and tropical agricultural systems, sheep and goats form the second most significant group of milk-producing animals, after dairy cattle (Knights & Garcia, 1997; Morand-Fehr & Boyazoglu, 1999; Olivier *et al*., 2005; Pandya & Ghodke, 2007). However, according to Devendra (1980), goat milk may be more important than sheep milk in less-developed countries as they contribute 69% (versus 47%) of the total world supply. In addition, goats have a longer lactation period than sheep -300 versus 250 days (Iaschi *et al*., 2004; Degen, 2007), and can produce up to three times as much milk (Garrine, 2007).

Tables 1.1 and 1.2 show the continuous and rapid increase in goat populations and products, especially in developing countries, which suggests that this animal might provide the tool required to meet some of the animal protein needs associated with the continuous increase in human populations (Boyazoglu & Morand-Fehr, 2001; Gomez-Ruiz *et al*., 2004; Boyazoglu *et al*., 2005). As indicated in Table 1.1, the total world goat population has increased by 26.4% over a ten year period, the most noteworthy of which has occurred in Africa and Asia, with increases of 24.1% and 27.1%, respectively. In 1984, world production of goat milk represented 1.52% of total milk production by dairy cattle, buffalo, sheep and goats. Ten years later, the contribution of goat milk to total milk production had increased to 2.00% and, although the total contribution only increased marginally, individual increases in developing countries were significant (Knights & Garcia, 1997; Morand-Fehr & Boyazoglu, 1999; Pandya & Ghodke, 2007). It is clear from Table 1.2 that milk production from goats is increasing all over the world. It is significant to note that although developed countries only show an increase of 28.2%, developing and low-income countries show increases of between 47.8% and 94.2%.

Table 1.1 Total world goat populations between 1993 and 2003 (in thousands) (Boyazoglu *et al*., 2005)

Region/country	**Years**		
	1993	**2003**	**Change (%)**
Africa	176996	219736	**24.1**
Asia	370269	487588	**27.1**
Europe	18940	18425	-2.7
Americas	37652	37940	0.7
Oceania	871	817	-6.2
World	604727	764510	26.4

Table 1.2 Goat milk and meat products in developed and developing countries, 1983-2003 (Boyazoglu *et al*., 2005)

Countries/production	**1983**	**2003**	**Change (%)**
Developed countries:			
Goat milk production (x1000 tonnes)	1980	2539	**28.2**
Goat meat production (x1000 tonnes)	161.2	187.8	16.5
Developing countries:			
Goat milk production (x1000 tonnes)	6276	9278	**47.8**
Goat meat production (x1000 tonnes)	1754	3904	122.0
Low income countries:			
Goat milk production (x1000 tonnes)	4002	7772	**94.2**
Goat meat production (x1000 tonnes)	1116	1891	69.4
Least developed countries:			
Goat milk production (x1000 tonnes)	2230	3792	**70.0**
Goat meat production (x1000 tonnes)	398	718	80.4

Approximately 96% of the world's goats are found in developing countries, but when marketed, goat products are mainly consumed in developed countries due to recognition of their superior health qualities (NAMC, 2005). South Africa is a relatively small goat producing country and possesses only approximately 3% of Africa's goats and less than 1% of the world's number of goats.

A recent survey found that there were 42 commercial diary goat farmers in South Africa (Kyozaire *et al*., 2005). In the developing areas of South Africa, small ruminants (especially goats) form an integral part of animal farming systems. Three recent surveys in the Eastern Cape, Free State, and Northern provinces of South Africa found that goats were mainly kept for food security and cultural reasons (Lusweti, 2000; Masika & Mafu, 2004; Lehloenya *et al*., 2007). However, reliable statistics regarding goat numbers and distribution are, to a large extent, absent (Coetzee, 1998; Simela, 2005). Prior to South African Democracy, goat numbers were often included in statistics figures for sheep. According to the National Department of Agriculture (May, 2004) goat numbers in South Africa totalled just over six and a half million. Almost 60% of the total number of goats in South Africa is found in the Eastern Cape and Kwa-Zulu Natal, and these consist mainly of Angora goats kept for mohair production. The Western Cape and North West province each contain 10%, and the remaining 20% is found in Limpopo, Gauteng, and Mpumalanga. However, these statistics only show total goat numbers in each province, and probably only indicate those goats that are involved in the commercial sector, i.e. Angora goats producing fibre, dairy goats like the Saanen, and SA Boer goats for meat production. The informal sector consists mainly of local goat types kept by small farmers and households not using formal marketing channels and often disregarded during routine census (Coetzee, 1998; Dubeuf *et al*., 2004; van Marle-Köster *et al.*, 2004; Roets & Kirsten, 2005; Simela, 2005). Historically, indigenous goats were primarily utilized for traditional and religious purposes and the emphasis was not placed on maximizing the commercial potential of the animals (Roets & Kirsten, 2005).

Devendra (1980) stated that goats, together with sheep, are probably the most neglected livestock species that are of value to humans, especially in less-developed countries. Today, more than 25 years later, the picture still does not look all that different. Limited research and false perceptions about environmental degradation, biases, and inadequate official support are probably the major constraints that detract from sustainable goat production (Devendra, 1980; Dubeuf *et al*., 2004; Webb & Mamabolo, 2004; NAMC, 2005; Degen, 2007). Until recently, in southern Africa there has been an official bias against the goat in southern Africa as a destroyer of vegetation (Donkin, 1997; Webb & Mamabolo, 2004) yet goats can only damage vegetation when animal densities are too high and when they roam freely without supervision (Boyazoglu *et al.*, 2005). In developed countries goats are now used more frequently to actively and positively modify vegetation cover by clearing areas thick in bushes and thorny vegetation so that sheep and cattle can graze on better vegetation afterwards. At the same time, removal of undergrowth reduces the risk of forest fires (Boyazoglu *et al.*, 2005).

From a production point of view, goats have many advantages over cattle and sheep. Goats are generally more prolific and easier to manage than sheep for individuals with limited animal experience. This provides a significant advantage in rural communities where culture often dictates that women and children act as herders (Devendra, 1980; Johnson *et al*., 1986; Donkin, 1997; Webb & Mamabolo, 2004; Peacock, 2005).

In tropical and arid environments, goats are generally agreed to be more efficient digesters of low-quality roughage than other domesticated ruminants (Knights & Garcia, 1997; Webb & Mamabolo, 2004; NAMC, 2005). The browsing ability of goats allows them to change their diet according to seasonal availability and growth rate of plants (Knights & Garcia, 1997; Olivier *et al.*, 2005).

Anatomically, goats show variation in body size, feet and ear length, coat colour, body surface, and possess the ability to reduce and stabilise their body weight. Physiologically, goats exhibit lower water turnover rates, as well as the abilities to desiccate faeces, concentrate and reduce urine volume, and reduce evaporative water loss. Goats also exhibit higher heat tolerance, reduced susceptibility to respiratory alkalosis and other metabolic disorders compared to cattle and sheep. (Knights & Garcia, 1997; Donkin & Boyazoglu, 2000; Webb & Mamabolo, 2004; Boyazoglu *et al*., 2005; De Vries, 2008).

Goats have a lower maintenance requirement compared to cattle (Greyling *et al.*, 2004). Goats produce energy and protein in milk more efficiently than cattle or sheep and 20 goats can be kept on the same piece of land that would be adequate for only two dairy cattle, which makes them ideal animals for milk production by small-scale farmers and rural households (Knights & Garcia, 1997; NAMC, 2005).

1.3 Why goat milk?

Despite a number of anecdotal experiments regarding the nutritional and medical benefits of goat milk, few technical studies have been conducted and published in refereed journals (Haenlein, 2004). It is interesting to note that one of the first scientific reports to suggest that goat milk may have health benefits was published in the South African Medical Journal in 1956 (Mowlem, 2005). Despite the early interest in the health benefits of goat milk, there is still a deficiency of scientific literature on the significance of goat milk in human nutrition, allergy, dietetics, paediatrics, and medicine (Park, 1994; Haenlein, 2004; Boyazoglu *et al*., 2005).

Goat milk differs from cow or human milk in that it has superior digestibility, alkalinity, buffering capacity, and certain therapeutic values in medicine and human nutrition (Haenlein & Caccese, 2003; Park *et al*., 2007). The goat has been described as the universal foster mother because its milk is particularly easy to digest by the young of many species (Mowlem, 2005). The chemical composition of goat milk is shown in Table 1.3, as determined by Guo *et al.* (2004).

Table 1.3 Chemical composition of bulk goat milk samples (Guo *et al*., 2004)

Milk component	**Mean ± SD**
Fat (%)	3.61 ± 0.47
Lactose (%)	4.47 ± 0.15
Crude protein (%)	3.47 ± 0.21
Casein (%)	2.57 ± 0.15
Total solids (%)	12.38 ± 0.71
Ash (%)	0.83 ± 0.04
Specific gravity	1.02 ± 0.0007

Goat milk may have reduced allergenicity when compared to cow milk (Donkin, 1997; Haenlein, 2004; Pandya & Ghodke, 2007). Resistance to digestion is one of the key determinants of a protein's allergenicity. Some researchers have found beta-lactoglobulins to be the milk proteins most resistant during digestion (Park, 1994; Haenlein, 2004; El-Agamy, 2007). Although goat milk contains similar levels of beta-lactoglobulin compared to cow milk, these proteins in goat milk are digested faster, resulting in less intact protein in the intestine. Bovine milk is also rich in alpha-s1- and alpha-s2-caseins, which are not produced by human beings. Therefore, the advantage of goat milk for the allergic consumer may be attributed to the low amount of these caseins determined by particular casein haplotypes (Marletta *et al*., 2004b; Kusza *et al.*, 2007; Bozkaya *et al*., 2008).

Due to the high degree of sequence homology among ruminant milk proteins, it is suggested that patients with cow milk allergy should also react to goat milk (Vereda *et al*., 2006). Haenlein (2006) however reported that approximately 40% of individuals that are sensitive to cow milk proteins can tolerate goat milk proteins, which substantiates Park (1994) that only one in 100 infants that are allergic to cow milk do not thrive well on goat milk.

Malnutrition is common among children in the developing world and, often, cow milk is not affordable or available in sufficient quantities. Goat milk may be cheaper to produce and more readily available (Donkin, 1997; Haenlein, 2004; Mowlem, 2005). In 1952, studies by Mack (as cited by Haenlein, 2004), prescribed goat milk as an alternative to cow milk in 38 children during a five-month period and they had improved weight gain, height, and skeletal mineralization compared to the group on cow milk. These results were supported by further studies done by Razafindrakato in 1993 and Sabbah in 1997 (as cited by Haenlein, 2004). In both cases, goat milk was recommended as a valuable substitute for cow milk in the rehabilitation of undernourished children.

Belewu & Aiyegbusi (2002) compared the mineral content and biological value of human, cow, and goat milk and found that the mineral content of goat milk is 7-10 times higher than that of cow milk but similar to that of human milk (Table 1.4). They concluded that, from a human nutrition standpoint, goat milk is to be preferred to cow milk due to the higher content of most minerals. In addition, Lopez-Aliaga *et al*. (2000) reported that micronutrients like calcium, copper, and iron can be absorbed more efficiently from goat milk than from cow milk. The only significant deficiency found in goat milk is that of folic acid (Pandya & Ghodke, 2007).

Table 1.4 Mean mineral composition of human, cow, and goat milk (Belewu & Aiyegbusi, 2002)

Mineral	**Human milk content (ppm)**	**Cow milk content (ppm)**	**Goat milk content (ppm)**
Sodium	150.00	51.92	210.41
Potassium	1.60	1.30	1.55
Calcium	6.26	4.03	5.56
Magnesium	3.33	1.03	2.30
Phosphorous	1.50	0.92	1.20
Iron	1.40	1.07	1.30
Zinc	2.95	0.11	0.80
Copper	0.34	0.25	0.56
Manganese	5.19	1.59	3.29
Ca:P	4.20	4.40	4.63

Goat milk fat composition differs significantly from that in cow milk (Appendix A), being much higher in short and medium chain fatty acids (Park, 1994; Haenlein, 2004; Haenlein, 2006; Park *et al.*, 2007). This difference may contribute to more rapid digestion of goat milk fat, since lipase attacks ester linkages of such fatty acids more readily than those of longer chains (Park, 1994). In addition, medium chain triglycerides (MCT) have become of considerable interest to the medical profession because of their unique ability to provide direct energy instead of being deposited in adipose tissue (Park, 1994; Haenlein, 2004; Haenlein, 2006). MCT have become established treatments for an array of clinical disorders, including malabsorption syndrome, intestinal resection, hyperlipoproteinaemia, coronary diseases, cystic fibrosis, and gallstone problems (Haenlein, 2004). In recent years, the intake of *trans*-fatty acids has been associated with the risk of coronary heart disease. The main source of *trans*-unsaturated fatty acids consumed by humans is partially hydrogenated vegetable fats and oils, although these compounds also occur naturally in milk. However, the mean *trans*-C18:1 content of caprine milk fat is 2.12%, which is lower than the 3.8% reported for bovine milk fat (Alonso *et al.,* 1999; Park *et al.*, 2007). Goat milk exceeds cow milk in monounsaturated, polyunsaturated and medium chain fatty acids, all of which are known to be beneficial for human health, especially for cardiovascular conditions (Haenlein, 2004).

Goat milk proteins are similar to the main cow milk proteins in their general classifications of alpha-, beta-, and kappa-caseins, alpha-lactalbumin, and beta-lactoglobulin, but they differ in genetic polymorphisms and the amounts of each in goat milk. According to official USDA tables of the average amino acid composition of goat and cow milk, goat milk contains higher levels of six of the ten essential amino acids: Thr, Ile, Lys, Cys, Tyr, and Val (Appendix B). The adult daily dietary nutrient recommendations for essential amino acids would be met or exceeded by a 0.51 goat milk consumption compared to cow milk (Haenlein, 2004). Goat milk proteins may be digested more readily and their amino acids absorbed more efficiently than those of cow milk. Goat milk is considered to form a softer, more friable curd when acidified in the stomach, which would be attacked more rapidly by stomach proteases (Park, 1994).

Enzymatic hydrolysis during digestion and/or processing of milk proteins can release fragments able to exert specific biological activities (called bioactive peptides) such as antihypertensive (angiotensin converting enzyme inhibitory peptides), antimicrobial (lactoferrin and caseins), or antithrombic (kappa-casein) properties (Boland *et al*., 2001; Park *et al.*, 2007).

1.4 Milk proteins

There are two types of proteins found in milk, namely whey and casein (Figure 1.1). The caseins comprise approximately 80% of the total milk proteins (Martin & Grosclaude, 1993; Karatzas & Turner, 1997; Moioli *et al*., 1998; Rando *et al*., 2000; Yahyaoui *et al*., 2000). They are the only coagulable milk proteins and determine cheese yield and quality. Besides their nutritional function of supplying amino acids to the neonate, the caseins also act as sequestrants of calcium phosphate, with the dual function of safe transportation of an insoluble calcium salt through the mammary gland and calcium and phosphorous nutrition for the neonate (Sawyer *et al*., 2002).

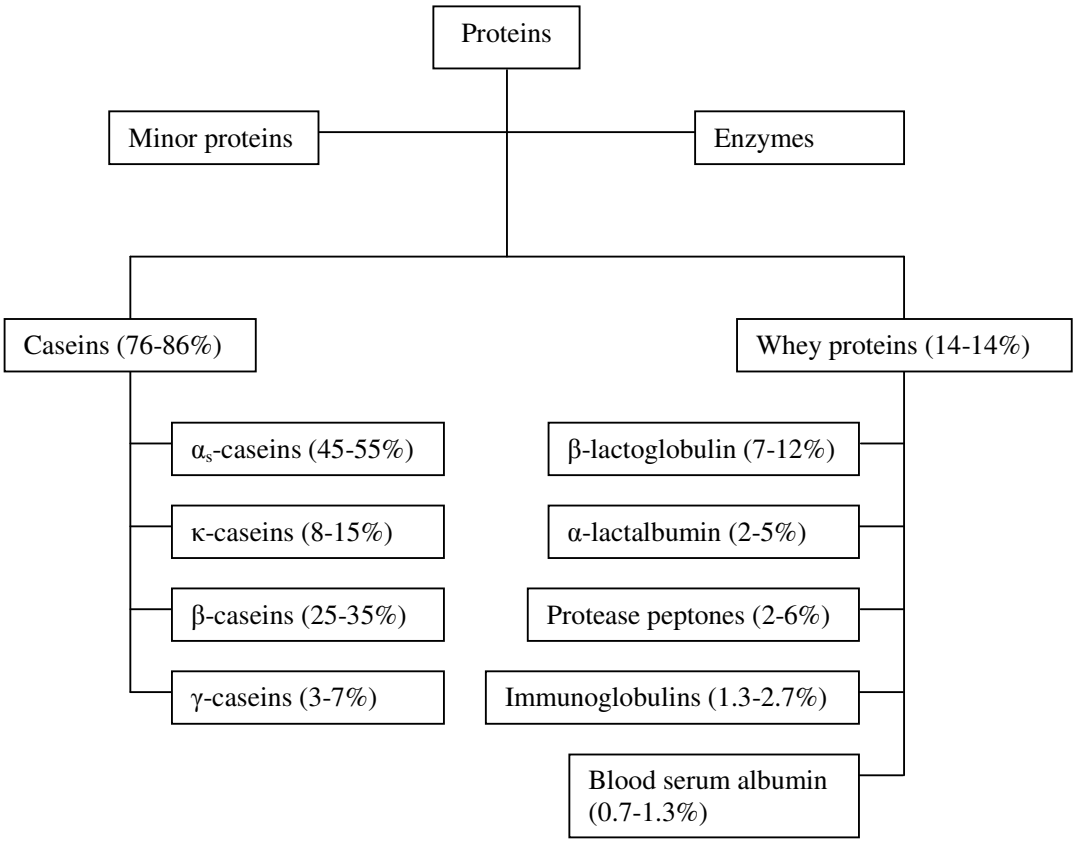


Figure 1.1 Milk protein composition (adapted from Fox & McSweeney, 1998)

There are four types of caseins: alpha-s1-, alpha-s2-, beta-, and kappa-casein, and the genes coding for the four are closely linked (Moioli *et al*., 1998; Rando *et al*., 2000; Sulimova *et al.*, 2007). These caseins are characterized by specific properties such as a low solubility at pH 4.6 and an organization into protein chain clusters called micelles. Furthermore, three of the four caseins (alpha-s1-, alpha-s2-, and beta-casein) are sensitive to calcium precipitation (Ng-Kwai-Hang & Grosclaude, 1992; Ramunno *et al*., 2004). γ -caseins are excluded from further discussion as they are proteolytic products of β-casein (Tziboula & Horne, 1999).

Whey proteins make up the remaining 20% of total milk protein. Their biological function is not yet fully known. These proteins consist of alpha-lactalbumin, which is involved in lactose synthesis, and beta-lactoglobulin, which is a possible carrier of hydrophilic molecules (Moioli *et al.,* 1998; Rando *et al*., 2000).

Caseins appear to be a rapidly evolving gene family, seemingly due to the minimal structural requirements for function. However, in contrast to the calcium-sensitive alpha-s1-, alpha-s2- and beta-caseins, the kappa-casein portion plays a critical role in the formation, stabilization, and aggregation of the casein micelles and thus influences the technological and nutritional properties of milk, especially when processed into cheese (Yahyaoui *et al.*, 2001; Jann *et al*., 2004; Prinzenberg *et al*., 2005; Reale *et al.,* 2005). Therefore, the kappa-casein gene is unlikely to be entirely free of selective constraints. Interspecies comparisons have established that CSN3, especially the caseinomacropeptide portion, possesses the highest degree of conservation among the casein genes (Ceriotti *et al*., 2004; Jann *et al*., 2004; Prinzenberg *et al*., 2005).

Cheese-making is based on the cleavage of the kappa-casein Phe_{105}-Met_{106} peptide bond by enzymes (chymosin) or heat. This produces two protein subunits, an insoluble (para-kappa-casein: amino acids 1-105) and a soluble glycopeptide (caseinomacropeptide/CMP: amino acids 106-171), and leads to micelle coagulation (Martin *et al*., 1999; Yahyaoui *et al*., 2000; Jann *et al*., 2004; Veress *et al*, 2004), as shown in Figure 1.2. In certain varieties of cheese (e.g. Cheddar), the casein fraction may represent up to half of the dry matter (DM) that is present in the cheese. The quantity of casein in raw milk is thus a critical factor in influencing cheese yield and nutritional quality (Cerbulis & Farrell, 1974; Karatzas & Turner, 1997, Marletta *et al*., 2005).

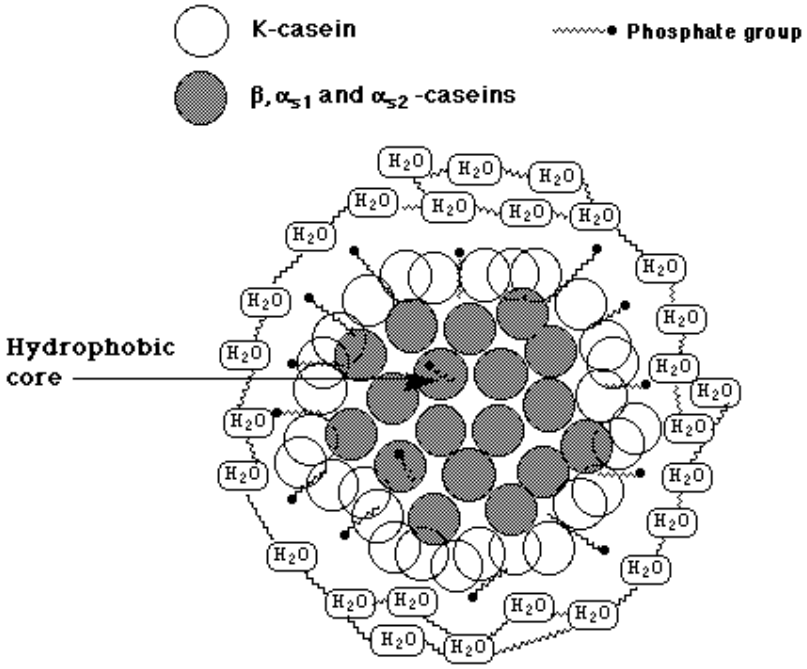


Figure 1.2 Casein micelle organization and the roles of the different casein fractions (Ford, 2000)

Haenlein (2002) reviewed the relationship of genetic variants of milk proteins with processing parameters and indicated that the best κ-casein genotype for cheese-making was the BB type. Fitzgerald (1997) listed many benefits of the kappa-casein B variant in cheese-making, including increased cheese yield, faster curd formation, shorter coagulation time, firmer curd, and greater heat stability. This may be due to the fact that the kappa-casein B type is associated with higher kappa-casein content, and consequently higher protein content in milk (about 10%) (Fitzgerald, 1997; Rando *et al*., 2000; Chiatti *et al*., 2005).

1.5 Polymorphism

Individual differences in milk proteins have a great influence on the composition and technological properties of milk. These protein polymorphisms, in turn, are the result of differences at the DNA level, according to the central dogma of molecular biology (Ordas *et al*., 1997; Moioli *et al*., 1998; Ceriotti *et al*., 2004; Moioli *et al*., 2007; Thomann *et al*., 2008).

At DNA level, polymorphisms can either be due to point mutations (single nucleotide substitutions) or to DNA rearrangement phenomena (including insertions, deletions and inversions of DNA fragments). These polymorphisms can occur in the coding region of the gene, which is transcribed into mRNA, or in DNA regions where gene expression is promoted and regulated (Moioli *et al.*, 1998).

Milk protein polymorphism describes the phenomenon where two or more forms of a particular protein exist in nature (Fitzgerald, 1997). Milk protein genes can be subject to a deletion or a substitution of one or more bases in the nucleotide sequence. This leads to changes in the amino acid composition of the protein, resulting in a new genetic variant (Ng-Kwai-Hang & Grosclaude, 1992; Martin & Grosclaude, 1993; Fitzgerald 1997; Moioli *et al*., 1998).

These differences can be detected through a variety of analytical techniques, including electrophoresis of milk, isoelectric focusing (IEF), and DNA analyses. In the case of point mutations, differences between two individuals can be detected using restriction enzymes which cut DNA only at a specific DNA sequence, after which different alleles can be distinguished on an electrophoresis gel by bands of different lengths (Restriction Fragment Length Polymorphism technique) (Ng-Kwai-Hang & Grosclaude, 1992; Moioli *et al*., 1998). If the point mutation is not recognized by any endonucleases, allele-specific primers can be constructed for the two different variants and used independently in a Polymerase Chain Reaction (PCR) with a second common primer to amplify the region where that mutation has occurred. (Ng-Kwai-Hang & Grosclaude, 1992; Fitzgerald, 1997; Moioli *et al.*, 1998).

In the past, genetic polymorphisms that affect milk protein phenotypes were inferred from milk electrophoresis experiments, which, of course, are only possible for lactating females (Rando *et al*., 2000). The value of analyzing animal samples at the DNA level instead of the milk protein level is that analyses may be carried out on both sexes, independent of animal age. This means that animals carrying economically important casein variants can be identified shortly after birth and thus used more effectively to improve the milk characteristics of certain animals or breeds (Medrano & Cordova, 1990; Ordas *et al*., 1997; Marletta *et al*., 2005).

Genetic polymorphism of milk proteins was first recognised after the discovery of beta-lactoglobulin variants A and B by Aschaffenberg and Drewry in 1955 (as cited by Ng-Kwai-Hang & Grosclaude, 1992; Moioli *et al.*, 1998). Since then, numerous studies have been performed to investigate milk protein polymorphism, especially those that influence milk quantity, composition, and technological properties (Ng-Kwai-Hang & Grosclaude, 1992; Caroli *et al*., 2001; Chessa *et al.,* 2003; Jann *et al*., 2004; Reale *et al.,* 2005). Although most of the work on the genetic polymorphism of milk proteins has been performed on cattle, several studies have recently been performed in an attempt to characterize these polymorphisms in other species including goats (Ng-Kwai-Hang & Grosclaude, 1992; Ramunno *et al*., 2001; Chessa *et al*., 2003).

Alpha-s1 (αs1) casein

Most research thus far has been done on this protein. The nucleotide sequence of the goat αs1-casein-encoding gene plus 1973 nucleotides at the 5' flanking region and 610 nucleotides at the 3' flanking region has been determined (GenBank accession no. AJ504710). The gene is spread over 16.7 kb and consists of 19 exons and 18 introns (Rammuno *et al.*, 2004).

At least 18 genetic variants of caprine αs1-casein have been identified to date (Tziboula & Horne, 1999; Clark & Sherbon, 2000; Rammuno *et al*., 2004; Sztankoova *et al*., 2006; Kusza *et al.*, 2007). These variants can be grouped into four quantitative categories based on the level of synthesis of the protein in the milk: high type variants (A, B1, B2, B3, B4, C, H, L, and M) are associated with higher amounts of αs1-casein (approximately 3.6 g/l); intermediate type variants (E and I) are associated with intermediate amounts (approximately 1.6 g/l); weak variants (D, F, G) are associated with low amounts (approximately 0.6 g/l) and the null type variants (O1, O2, and N) are associated with the absence of αs1-casein in milk (Moioli *et al.*, 1998; Clark & Sherbon, 2000; Gomez-Ruiz *et al*., 2004; Zullo *et al*., 2005; Caroli *et al*., 2006; Barillet, 2007; Cosenza *et al*., 2008).

The mutations that characterize the different alleles are quite diverse, ranging from single nucleotide substitutions to large insertions or deletions or even interallelic recombination (Martin *et al*., 1999; Sztankoova *et al*., 2006).

The alpha-s1 A, B1, B2, B3, B4, C, E, H, I, and L variants are composed of 199 amino acid residues and differ from each other only because of a few amino acid substitutions (Moioli *et al.*, 1998; Rando *et al.*, 2000). Variant D shows a deep structural difference to the other variants, consisting of an internal deletion of 11 residues (Moioli *et al*., 1998). Allele E

contains a 457 bp insertion within the 19th and last untranslated exon, corresponding to a truncated long interspersed repeated element (LINE), highly repeated in the goat genome (Moioli *et al*., 1998; Martin *et al*., 1999; Rando *et al*., 2000). Variant F appears to have an internal deletion of 37 residues, leading to the loss of a hydrophilic cluster of five contiguous phophoseryl residues: SerP64-SerP-SerP-SerP-SerP-Glu-Glu70 (Moioli *et al*., 1998; Martin *et al*., 1999; Rando *et al*., 2000). The G allele is characterized by a G→A transition in the 5' splice site consensus sequence of intron 4 causing exon 4 to be skipped during the course of pre-mRNA processing (Moioli *et al*., 1998; Martin *et al*., 1999; Rando *et al*., 2000). The mutational origin of the αs1-casein null allele is a large deletion (approximately 8.5 kb), starting from nucleotide 181 of intron 12, and spanning the last seven exons and the 3' region of the gene (Moioli *et al*., 1998; Sztankoova *et al*., 2006; Cosenza *et al*., 2008). Sztankoova *et al*. (2006) proved that the AS-PCR method could be used to identify carriers of the goat αs1-casein null allele, as milk obtained from such goats could be of use to individuals with milk intolerance.

Genetic variants of αs1-casein are associated with milk yield, composition and processing properties. Goat milk with high levels of αs1-casein has been found to have better milk composition, including total solids, fat, protein, casein, phosphorous and lower pH, as well as faster coagulation rate and firmer curd than milk with low levels of this protein (Clark & Sherbon, 2000; Barillet, 2007). The αs1-casein BC genotype is associated with the shortest firming time and the best curd firmness (Haenlein, 2002). Goat milk with A/A type αs1-casein has a higher total nitrogen and higher fat level than milk with the O/O type (Clark & Sherbon, 2000). In addition, Zullo *et al*. (2005) showed that the low type variants like the F variant negatively influence the cheese-making properties of milk.

Approximately 80% of caseins in milk are associated with each other and with calcium phosphate to form casein micelles (Tziboula & Horne, 1999). This is the physiological mechanism by which the passage of milk in the digestive tract is slowed so that it can be properly assimilated. This phenomenon also forms the basis of the cheese-making process (Martin *et al*., 1999). The mean size of casein micelles in goat milk appears to be correlated to the αs1-casein variant present in the milk. Variants producing high αs1-casein levels (A, B, C variants) are associated with lower mean sizes and those producing low levels (E, F, O variants) with higher mean sizes of casein micelles. As micelle size increases, the proportions of individual caseins also change: αs2- and β-casein increase and the proportion of κ-casein decreases (Pierre *et al*., 1999; Tziboula & Horne, 1999).

Kusza *et al*. (2007) reported that the weak F allele is the most frequent in local Hungarian Milking goats as well as in Alpine and Saanen goats, followed by the strong B allele in Hungarian goats and the intermediate E allele in Alpine and Saanen breeds, respectively. This is partly in agreement with Caroli *et al.* (2006), who found the F allele to be the most frequent in the Verzasca and Orobica breeds, but the B allele to be rare in Lombardy breeds. In both cases the O allele was present in certain breeds at relatively high frequencies of 0.11-0.20. This type of genotype information could be utilised in selection schemes for milk protein content. The low frequency of strong variants in certain breeds suggests that selection for these alleles should be an important breeding objective to improve milk composition, and breeds with a high incidence of null alleles might be exploited for the production of milk with specific nutritional properties (Caroli *et al.*, 2006; Kusza *et al*., 2007).

Alpha-s2 (αs2) casein

The αs2-casein gene is 18.5 kb long and composed of 18 exons (Rando *et al*., 2000). This locus is characterized by the presence of eight alleles, among which A, B, C, E and F are associated with normal levels of αs2-casein in milk (approximately 2.5 g/l), while the D allele is associated with intermediate, decreased levels and the O allele with the absence of αs2-casein in goat milk (Gomez-Ruiz *et al*., 2004; Marletta *et al*., 2004a; Bozkaya *et al*., 2008).

The B and C variants differ from the A variant by single nucleotide substitutions: a G→A transition at the tenth nucleotide of exon 4 that produces a single amino acid substitution, Glu→Lys at position 64, and an A→T transversion at the fifth nucleotide of exon 16 that produces an amino acid substitution of Lys→Ile at position 167, respectively (Bouniol *et al*., 1994; Ramunno *et al*., 2001). The D allele is characterized by a 106 bp deletion, involving the last 11 bp of exon 11 and the first 95 bp of the following intron (Marletta *et al*., 2004a; Bozkaya *et al*., 2008). A mutation in the 28th codon of exon 16 is responsible for the C→G transversion that characterizes the E allele (Ramunno *et al*., 2001). The mutation that characterizes the O allele is a G→A transition in exon 11 that produces a premature stop codon in position 110 (Rando *et al*., 2000; Marletta *et al*., 2004a).

Literature has reported that the A allele is the most frequent, followed by the F allele (Ramunno *et al*., 2001; Marletta *et al*., 2004a; Marletta *et al*., 2005). The frequency of the O allele in different populations varies between 0.000 and 0.146, and the frequency of the D allele between 0.000 and 0.019 (Rando *et al*., 2000; Marletta *et al*., 2004a; Bozkaya *et al.,* 2008). These alleles may be significant in production of more humanized milk, since α-s2-casein is absent in milk produced by humans (Rando *et al*., 2000; Marletta *et al*., 2004b).

Beta (β) casein

In goats, fewer research studies have been conducted on the β-casein gene compared to the other caseins. This gene consists of nine exons ranging in size from 24-492 bp (Cosenza *et al*., 2005). To date, seven alleles (A, A1, B, C, D, O1, and O2) associated with contents of β-casein in milk varying from 0 to 5 g/l have been reported (Cosenza *et al*., 2005; Marletta *et al.,* 2005; Caroli *et al*., 2006).

Cosenza *et al*. (2005) described a silent mutation consisting of a C→T transition at nucleotide 180 of the ninth exon named A1. The DNA and protein sequences of the B allele remain undetermined. The C allele is characterized by a single nucleotide substitution (C to T) at position 404 of the seventh exon, creating an Ala→Val substitution at position 177 of the mature protein. Beta-casein D is characterized by the amino acid substitution at position 207 of Val→Asn (Cosenza *et al*., 2005). The O1 allele has been characterized at the nucleotide level and shown to exhibit a single nucleotide deletion in the seventh exon of the gene that creates a premature stop codon at position 58 (Marletta *et al*., 2005). Some studies have reported the presence of a second null allele that has a premature stop codon at position 182 (Rando *et al*., 2000). These alleles also influence the technological properties of goat milk, causing longer renneting time, weaker curd firmness, and decreased cheese yield (Moioli *et al.*, 1998; Martin *et al*., 1999).

Kappa (κ) casein

The first attempts to describe polymorphisms in this protein were performed more than 40 years ago by Grosclaude in 1965 using electrophoretic methods (as cited by Prinzenberg *et al.*, 2003). At the DNA level, the nucleotide sequence of the goat kappa-casein gene was established by Coll *et al*. (1993). The kappa-casein gene consists of five exons, with more than 90% of the coding region for the mature protein occurring in the fourth exon (Yahyaoui *et al*., 2000).

Since the discovery of two kappa-casein variants (A and B) in goats by Di Luccia in 1990 (as cited by Moioli *et al.*, 1998), additional polymorphisms have been detected by DNA analysis (Yahyaoui *et al*., 2000; Yahyaoui *et al*., 2001; Angiolillo *et al*., 2002; Chessa *et al*., 2003; Yayhaoui *et al*., 2003; Jann *et al*., 2004), bringing the total number of alleles identified in domesticated goats to 16: 13 protein variants and three silent mutations involving 15 polymorphic sites in kappa-casein exon 4 (Chiatti *et al*., 2005; Prinzenberg *et al*., 2005; Moioli *et al*., 2007). In previous studies, the A and B alleles have been found to be more frequent in cattle populations (Malik *et al*., 1997; Biase *et al.,* 2005; Otaviano *et al*., 2005).

By isoelectric focusing (IEF) of milk samples, all kappa-casein variants identified in domesticated goats cluster into two groups on the basis of their isoelectric point (IP): D, E, K, M (B^{IEF} IP= 5.66) and A, B, B', B'', C, C', F, G, H, I, J, L (A^{IEF} IP = 5.29) (Chiatti *et al*., 2005; Prinzenberg *et al*., 2005; Caroli *et al*., 2006). Relevant research lists many benefits of the kappa-casein B^{IEF} variant in cheese-making, including increased cheese yield, faster curd formation, shorter coagulation time, firmer curd, and greater heat stability. The kappa-casein B^{IEF} type has been suggested to be associated with up to a 10% higher κ-casein content, and consequently higher protein content in milk (Rando *et al*., 2000; Chiatti *et al*., 2005).

Simultaneous publication of different papers describing new κ-casein alleles has led to inconsistency in the literature, due to assignation of the same letter to different variants (Chessa *et al*., 2003; Prinzenberg *et al*., 2005). New nomenclature has been proposed, based primarily on the GenBank chronological order of the variants (Prinzenberg *et al*., 2005).

1.6 Aim of the study

Literature has been reviewed regarding the value of goats and milk proteins and their relevance in human nutrition and cheese-making in South Africa. It is clear that goats play a significant role in the socio-economic well being of people South Africa in terms of nourishment and income (Peacock, 2005; Lehloenya *et al*., 2007; Kosgey *et al*., 2008).

Genetic variation for milk protein genes has been studied in bovine dairy herds in the northern hemisphere (Kusza *et al*., 2007; Cosenza *et al*., 2008). Research papers have been published as much as ten years ago explaining the possibility of exploiting existing genetic variation in the dairy cattle population and thereby altering milk composition to benefit the dairy industry (Glimm *et al*., 1996; Yahyaoui *et al*., 2000). To date, no attempt has been made to study these polymorphisms in the milk protein genes of local goat types in South Africa.

The quantity and type of casein, especially κ-casein, in raw milk is a critical factor in influencing nutritional quality and cheese yield (Karatzas & Turner, 1997). Haenlein (2002) indicated that the best κ-casein genotype for cheese making was the B^{IEF} type, which includes the D, E, K, and M alleles. Fitzgerald (1997) listed increased cheese yield, faster curd formation, and shorter coagulation time as benefits of the kappa-casein B^{IEF} type.

It is assumed that Saanen goats, having been bred over centuries for improved milk characteristics, would have a higher frequency of favourable kappa-casein alleles. If the desired alleles are frequent in local goat populations, the opportunity arises to select them for improved cheese-making ability, thereby creating an additional source of income for local farmers. In addition, the proven presence of null alleles for other casein genotypes such as αs1-, αs2-, and β-casein (Park *et al*., 2007) presents the possibility that a null allele also exists for kappa-casein. These null alleles have been associated with an absence of the specific casein in milk, which may be correlated to decreased allergenicity (Kusza *et al*., 2007).

In this study, the first of its kind in South Africa, the purpose was to investigate genetic variation in the kappa-casein genotype of South African local goat types, SA Boer goats, and Saanen goats representing different geographical locations. The SA Boer goat was developed from genetic pooling of local types with Indian and European breeds (Casey & van Niekerk, 1988; Ramsay & Donkin, 2000), and is expected to share some genetic similarities with local goat types. Saanen goats were included as a control group, as they are expected to have a higher frequency of favourable milk protein alleles. Two techniques were employed in this investigation – PCR RFLP and DNA sequencing.

CHAPTER 2
MATERIALS AND METHODS

2.1 Introduction

In this study, the purpose was to describe the genetic variation of the kappa casein gene in SA Boer and local goat types in South Africa, using Saanen goats as a control group. Blood and milk were sampled from various populations of local goat types, SA Boer goats, and Saanen goats. An additional 15 DNA samples of known genotype were received from the Department of Veterinary Science and Food Technology at the University of Milan in Italy as control samples for different kappa-casein variants. Two methods were employed in order to deduce the amount of variability present in the kappa casein gene – PCR-RFLP and DNA sequencing. Milk samples were analysed for milk protein, fat, and lactose composition, as well as somatic cell count and total casein.

2.2 Materials

There are a variety of different goat breeds and types in South Africa. Some, like the SA Boer goat, are naturally better adapted to extensive farming conditions. Other breeds, such as the Saanens, were imported from Europe and have been selected over generations for improved milk production and are more adapted to intensive farming conditions. When attempting to establish a herd of goats in remote rural areas, both of these characteristics (milking performance and adaptability) will need to be taken into account. Especially in rural or subsistence farming conditions, breeds that require intensive management and facilities may not be practical. The SA Boer goat and local goat types are the most abundant and freely available animals in the rural areas (Greyling *et al*., 2004), which is why it is these goats that are the focus of the current study.

The South African Boer goat (Figure 2.1) was developed in South Africa in the early 1900's for meat production, with the SA Boer Goat Breeders' Association being formed in July of 1959 (Campbell, 2003; NAMC, 2005). Considered in the light of the health-consciousness that prevails on a worldwide basis, the SA Boer goat yields lean meat of a high quality. Although the exact origin of the SA Boer goat is unclear, it is believed to be the result of a genetic pooling of African indigenous or local goat types, Indian goats, Angora goats, and some European dairy goats (Casey & van Niekerk, 1988; Ramsay & Donkin, 2000).

Figure 2.1 A SA Boer goat ram (www.arc.agric.za)

The Boer goat is well-adapted to a great variety of climatic and pasture conditions and is consequently fit for conditions varying from extensive to intensive production. The SA Boer goat is white with a red head and ears, with an evident white blaze on the forehead (http://www.boergoats.co.za/).

"Indigenous goat" is a collective term used for all the local varieties of South African goat breeds. Breed names are usually given according to the geographical area in which they occur, or taken over from the nations or tribes that own them. The indigenous goat populations in South Africa exhibit quite distinct phenotypic variation in their size and colour, as indicated in Figure 2.2 (Visser *et al*., 2004; NAMC, 2005).

Figure 2.2 An indigenous goat type (NAMC, 2005)

Indigenous goats have been subjected to very limited artificial selection. They are known to be remarkably hardy, being able to subsist on the poorest of vegetation and survive periodic droughts and harsh temperatures. They also seem to be less susceptible to diseases such as Heartwater compared to the commercial goat breeds (Donkin & Boyazoglu, 2000). This unimpressive goat could be one of nature's most useful animals due to the fact that it can be used for meat, milk, fibre, skins and manure. Owing to its great adaptability, it can survive almost anywhere in South Africa (www.arc.agric.za; Campbell, 2003; NAMC, 2005).

According to the National Saanen Breeders Association, Saanen goats derived their name from the Saanen valley in the south of Canton Berne, Switzerland (www.ansi.okstate.edu/breeds/goats/saanen/). During the late 1890's, the Cape Agricultural Department imported three Saanen males and twelve females from Switzerland but, unfortunately no immediate and official attempt was made to keep them pure in South Africa.

Figure 2.3 A Saanen doe (www.studbook.co.za/Society/Milch/general.htm)

The present day Saanen (Figure 2.3) in South Africa originated from one male and one female imported from Germany in 1923, as well as from other imports from Switzerland, England and Germany until 1946. The Saanen is the most popular breed of dairy goat in the world and has been bred by careful selection for improved milk characteristics (www.studbook.co.za/Society/Milch/general.htm).

The goats included in this study were randomly selected, the main aim being to avoid narrow genetic relationships among them. Animals of various ages were sampled for milk and blood; the only prerequisite being that they be in the same stage of lactation (approximately 63 ± 38 days) in order for milk samples to be comparable.

Three populations of local goat types (n = 32) were included (Figure 2.4 and Table 2.1). The first population was sampled and consisted of mostly longhaired, white or spotted goats kept by farm workers on a farm in Vrede (Fig. 2.4D) in the Free State Province. The second population was kept by a farmer in the Colesberg area (Fig. 2.4E) of the Free State Province who donated samples to the Department of Animal & Wildlife Sciences for a genetic diversity project. This population consisted of typical local goat types with very specific colour types. The third population was kept at the Mara Research Station (Fig. 2.4B) in the Limpopo Province, and was mainly chosen for their phenotypic differences from the other two populations. Samples from this population were also donated for research purposes. Samples were collected from two South African Boer goat populations (n = 30), a stud herd in Louis Trichardt (Fig. 2.4A), and a commercial herd in the Free State Province (Fig. 2.4D). Finally, samples were collected from two Saanen goat herds (n = 19) to be included as control samples. The first herd consisted mainly of does and was kept at the University of Pretoria's Experimental Farm (Fig. 2.4C), and the second was a commercial herd in Louis Trichardt (Fig. 2.4A).

Figure 2.4 Collection of blood and milk samples of goats from five different geographical areas in South Africa

A Louis Trichardt, Limpopo province (23º03'S; 29º55'E); **B** Mara Research Station, Limpopo province (23º03'S; 29º55'E); **C** University of Pretoria Experimental Farm, Gauteng province (25º43'S; 28º13'E); **D** Vrede, Free State province (27º40'E; 29º34'S); **E** Colesberg, Free State province (30º43'S; 25º05'E)

Table 2.1 Blood sample collection from Saanen, SA Boer, and local goat types representing five geographical areas of South Africa

	Area	SA Boer goat (n)	Local goat type (n)	Saanen goat (n)
A	Louis Trichardt	16		16
B	Mara Research Station (donation)		8	
C	UP Experimental Farm			3
D	Vrede	14	15	
E	Colesberg (donation)		9	
	Total	**30**	**32**	**19**

Milk samples for compositional analysis were only sampled from goat herds in Vrede and Louis Trichardt. Therefore, the sample sizes and origins of blood and milk analyses do not correspond.

2.3 Methods

Collection of milk samples

Milk samples were collected from 84 goats for compositional analysis – 35 SA Boer goats, 33 Saanen goats, and 16 local goat types. These samples only represent the Vrede (Fig 2.4D) and Louis Trichardt herds (Fig 2.4A), as they were physically sampled on the farms.

The night before milk collection, kids were removed from their mothers to ensure that sufficient milk was available the next morning. All the animals were milked to drain the udder and the total milk sample was mixed so that a representative 50 ml sample could be collected, however, some of the local goat types were unable to deliver that much, probably due to nutritional and management constraints. Milk samples were collected in bottles received from Lactolab at the Agricultural Research Council (www.arc.agric.za) containing Microtabs (a Bronopol-based preservative). The milk samples were stored at 4ºC and transported to the laboratory within 24 hours. Milk samples were analysed for milk protein, butterfat, and lactose composition, as well as somatic cell count. Samples that were large enough were also analysed for casein content. The different protein fractions (αs1-, αs2-, β-, and κ-casein) in goat milk can unfortunately not be determined in a laboratory in South Africa.

Collection of blood samples

Blood samples for this study included 81 samples representing five different geographical areas in South Africa, as indicated in Figure 2.4 and Table 2.1. Blood samples (5 ml) were collected from each animal by jugular venipuncture into vacutainer tubes containing the anticoagulant ethylenediaminetra-acetic acid (EDTA). Blood samples were stored at 4ºC and transported to the Department of Animal & Wildlife Sciences at the University of Pretoria.

DNA extraction

Blood was transferred into 1.5 ml eppendorf tubes and stored at –15ºC. DNA was extracted from whole blood with the use of a GFX® Genomic Blood DNA Purification Kit (Amersham Biosciences UK Ltd., England), following the standard protocol for whole blood samples. To estimate the DNA concentrations, samples were visualised by electrophoresis in a 1% agarose gel stained with ethidium bromide. The estimated sample concentrations were also verified using a Nanodrop® ND 1000 UV-vis Spectrophotometer (www.nanodrop.com) in the Department of Genetics at the University of Pretoria.

Amplification of the caprine kappa-casein gene

One set of primers was used to amplify a 645 bp fragment containing exon 4 of the gene (Table 2.2). Amplification of the DNA region encoding the mature kappa-casein protein (Figure 2.5) was performed using the protocol of Yahyaoui *et al.* (2003).

Table 2.2 Primer sequence set for amplification of exon 4 of the kappa-casein gene

Name	Primer sequence	Primer design	Reference
Forward I3F	5'- TCC CAA TGT TGT ACT TTC TTA ACA TC-3'	designed over introns 2 and 3 of the caprine kappa-casein gene	Yahyaoui *et al.* (2003)
Reverse Kb2	5'- GCG TTG TCC TCT TTG ATG TCT CCT TAG-3'	designed relative to the caprine cDNA	Coll *et al.* (1993)

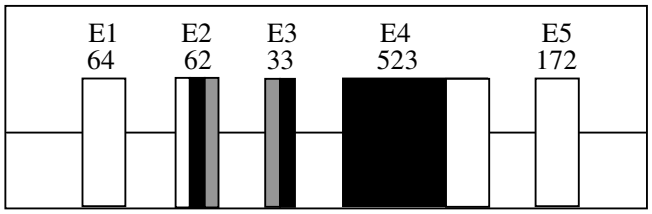


Figure 2.5 Structure of the caprine kappa-casein gene. Exons are depicted schematically as boxes, with numbers and sizes indicated above. White boxes indicate non-coding regions, grey indicates signal peptides, and black boxes indicate the mature protein (adapted from Yahyaoui *et al*., 2001).

The PCR reaction was performed in a 50 µl final volume containing 1.25 U GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1X PCR buffer, 1.5mM $MgCl_2$, 10 mM dNTP's, 10pmol of each primer, and 100 ng of goat genomic DNA. Thermal cycling conditions were as follows: 95ºC for 5 min, 10 cycles of 97ºC for 15 s, 63ºC for 1 min and 72ºC for 1 min 30 s, followed by 25 cycles of 95ºC for 30 s, 63ºC for 1 min and 72ºC for 1 min 30 s, with a final extension at 72ºC for 5 min and subsequent cooling to 4ºC in a GeneAmp® PCR System 9700 (Applied Biosystems, Chesire, UK).

PCR products were visualized and sized using a 50 bp DNA Step Ladder (Promega, Madison, WI, USA) on a 3% agarose gel stained with ethidium bromide. To ensure that the correct fragment of the genome was amplified, two samples were sequenced. The sequencing results were submitted to the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/), which finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. In this manner, it was confirmed that the samples showed 98% homology to *Capra hircus* mRNA for kappa casein (EMBL accession number X60763) (Figure 2.6).

```
>emb|X60763.1|CHKAPPA  C.hircus mRNA for kappa casein
Length=826

 Score =  942 bits (510),  Expect = 0.0
 Identities = 516/522 (98%), Gaps = 0/522 (0%)
 Strand=Plus/Plus

Query  66   TGCTGTGAGAAAGANGAAAGATTCTTYGATGACAAAATAGCCAAATATATCCCAATTCAG  125
            ||||||||||||||| |||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  144  TGCTGTGAGAAAGATGAAAGATTCTTCGATGACAAAATAGCCAAATATATCCCAATTCAG  203

Query  126  TATGTGCTGAGTAGGTATCCTAGTTATGGACTCAATTACTATCAACAGAGACCAGTTGCA  185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  204  TATGTGCTGAGTAGGTATCCTAGTTATGGACTCAATTACTATCAACAGAGACCAGTTGCA  263

Query  186  CTAATTAATAATCAATTTCTGCCATACCCATATTATGCAAAGCCAGTTGCAGTTAGGTCA  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264  CTAATTAATAATCAATTTCTGCCATACCCATATTATGCAAAGCCAGTTGCAGTTAGGTCA  323

Query  246  CCTGCCCAAACTCTTCAATGGCAAGTTTTGCCAAATACTGTGCCTGCCAAGTCCTGCCAA  305
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  324  CCTGCCCAAACTCTTCAATGGCAAGTTTTGCCAAATACTGTGCCTGCCAAGTCCTGCCAA  383

Query  306  GACCAGCCAACTACCCTGGNACGNCACCCACACCCACATTTATCATTTATGGCCATTCCA  365
            |||||||||||||||||| ||| ||||||||||||||||||||||||||||||||||||
Sbjct  384  GACCAGCCAACTACCCTGGCACGTCACCCACACCCACATTTATCATTTATGGCCATTCCA  443

Query  366  CCAAAGAAAGATCAGGATAAAACAGAAATCCCTGCCATCAATACCATTGCTAGTGCTGAG  425
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  444  CCAAAGAAAGATCAGGATAAAACAGAAGTCCCTGCCATCAATACCATTGCTAGTGCTGAG  503

Query  426  CCTACAGTACACAGTACACCTACCACCGAAGCAATAGTGAACACTGTAGATAATCCAGAA  485
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  504  CCTACAGTACACAGTACACCTACCACCGAAGCAATAGTGAACACTGTAGATAATCCAGAA  563

Query  486  GCTTCCTCAGAATCGATTGCGAGTGCATCTGAGACCAACACAGCCCAAGTTACTTCAACC  545
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  564  GCTTCCTCAGAATCGATTGCGAGTGCATCTGAGACCAACACAGCCCAAGTTACTTCAACC  623

Query  546  GAGGTCTAAAAACTCTAAGGAGACATCAAAGNGGACAACGCA  587
            |||||||||||||||||||||||||||||| ||||||||||
Sbjct  624  GAGGTCTAAAAACTCTAAGGAGACATCAAAGAGGACAACGCA  665
```

Figure 2.6 BLAST results for PCR product

RFLP analysis

A total of 68 samples (10 Saanen, 24 local type, 30 SA Boer, four control samples) were genotyped by the PCR-RFLP method, following the protocol of Yahyaoui *et al*. (2003). In the case of point mutations, differences between two individuals can be detected using restriction enzymes, which cut DNA only at a specific DNA sequence (Table 2.3, Figure 2.7, and Appendix C), after which different alleles can be distinguished on an electrophoresis gel by bands of different lengths (Ng-Kwai-Hang & Grosclaude, 1992; Moioli *et al*., 1998).

The nucleotide sequences of all the kappa casein variants have been deduced (Table 2.5) and were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) (Prinzenberg *et al*., 2005). All the alleles were entered into the CLC Combined Workbench v3 software program (www.clcbio.com) and scanned to find any enzymes other than those used by Yahyaoui *et al*. (2003) that might have recognition sites within these variants, however, none were found.

Table 2.3 Properties of the three restriction endonucleases used in this study (www.fermentas.com)

Restriction enzyme	Source	DNA recognition site
HaeIII	*Bacillus subtilis* R	5'…G G ↓ C C…3'
(BsuRI)		3'…C C ↑ G G…5'
BseNI	*Bacillus species* N	5'…A C T G G N ↓…3'
		3'…T G A C ↑ C N …5'
Alw44I	*Acinetobacter Iwoffi* RFL44	5'…G ↓ T C C A C…3'
		3'…C A C G T ↑ G…5'

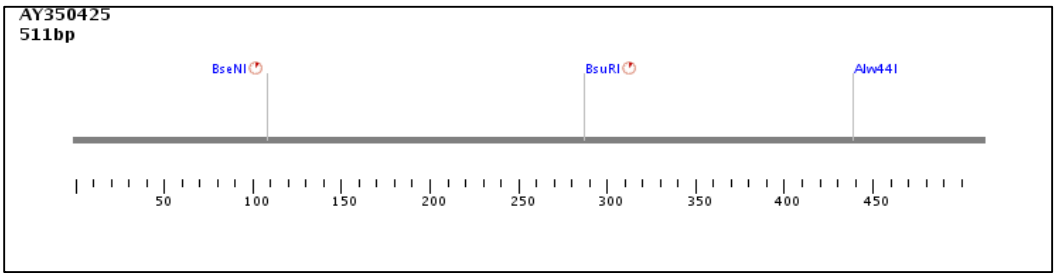


Fig 2.7 Restriction sites of the three enzymes used in RFLP analysis indicated schematically on the caprine kappa-casein C allele (GenBank acc. no.AY350425)

Ten microlitres (µl) of the PCR product were digested with 10 units (1 µl) of the restriction endonucleases *Alw44I* and *HaeIII* (Fermentas, www.fermentas.com) at 37ºC for 16 hours and with 10 units (1 µl) of *BseNI* (Fermentas) at 65ºC for 6 hours. The three enzymes chosen for use have specific properties (Tables 2.3 and 2.4), and do not create restriction sites for each other. The resultant DNA restriction fragments were then separated by electrophoresis in a 2% agarose gel stained with ethidium bromide. The fragments were compared to a size standard (50 bp DNA Step Ladder, Promega, Madison, WI, USA) and photographed. Table 2.4 indicates the expected size of restriction fragments for the different variants.

Table 2.4 Expected restriction endonuclease fragment results per allele

Allelic variant	*HaeIII*	*BseNI*	*Alw44I*
A	230; 415	51; 235; 359	645
B	230; 415	51; 235; 359	645
C	230; 415	235; 410	79; 566
D	230; 415	235; 410	79; 566
E	50; 230; 365	51; 235; 359	645
F	230; 415	51; 235; 359	79; 566
G	230; 415	235; 410	79; 566

As seen in Table 2.4, PCR-RFLP methodology does not distinguish between all seven kappa-casein alleles. Only the E and F alleles can be conclusively identified, whereas A and B group together, and C, D, and G group together. Allele E can be distinguished with *HaeIII* because this allele is the only one with guanine at position 242. Similarly, *BseNI* allows discrimination of alleles A, B, E, and F from others, whereas *Alw44I* distinguishes only A, B, and E alleles. Consequently, the F allele can be differentiated by combining these two endonucleases (Yahyaoui *et al*., 2003).

Lambda DNA (Promega, Madison, WI, USA) has a known number of recognition sites in all three these enzymes (*Alw44I* – 4; *HaeIII* – 149; *BseNI* – 110) and was used as a random control to monitor that the enzymes had not degraded (Figure 2.8).

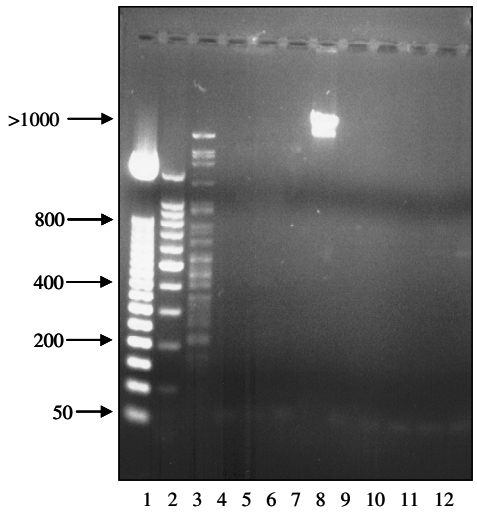


Figure 2.8 Agarose gel electrophoresis of lambda DNA digested by all three enzymes.
Lane 1: 50 bp DNA Step Ladder (Promega, Madison, WI, USA), Lane 2: *HaeIII*, Lane 3: *BseNI*, Lane 7: *Alw441*

DNA samples of known genotype received from the Department of Veterinary Science and Food Technology at the University of Milan (Italy) were included as control samples. Genotypes AA and BB could be confirmed, but, unfortunately, the other samples were of insufficient quantity to be included in the analysis.

DNA sequencing

After RFLP analysis, random samples (n = 60 consisting of 10 Saanen, 27 SA Boer, 21 local goat type, and two control samples) were chosen for DNA sequencing to confirm the results obtained by the PCR-RFLP method. In addition, a further 17 samples that were not subjected to RFLP analysis were also sequenced by the following method.

PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The concentration and purity of purified PCR products was estimated using two methods – agarose gel electrophoresis and spectroscopy. The sequencing reaction was performed in a 10 µl final volume containing 2 µl Therminator mix (2.5X), 1 µl sequencing buffer (5X), 3.2 pmol primer, and 64.5 µg PCR product (BigDye® Terminator v3.1 Kit, Applied Biosystems®, Chesire, UK). Thermal cycling conditions were: 94ºC for 1 min, 25 cycles of 94ºC for 10 s, 50ºC for 5 s and 60ºC for 4 min, with subsequent cooling to 4ºC, using a GeneAmp PCR System 9700 (Applied Biosystems, Chesire, UK). Samples were then purified by ethanol precipitation and frozen at –20ºC before being submitted to the Forestry and Agricultural Biotechnology Institute (FABI©) at the University of Pretoria for a sequencing run on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Chesire, UK).

2.4 Sequencing analysis

DNA sequence results were edited using the ContigExpress application of Vector NTI Advance v.10.3.0 (www.invitrogen.com/vectorNTIcommunity). ContigExpress was applied for assembling and editing sequencing fragments in the form of chromatograms from automated sequencers into longer contiguous sequences or "contigs". Firstly, the background noise of the first and last nucleotides was deleted and the sequence chromatogram was scanned for manual calling of inconsistent base naming. Then the forward and reverse sequences were aligned to form a contig, using the assembly feature of the program. Contigs were then exported in FASTA format to a Word document for further analysis.

Known kappa-casein sequences (nomenclature from Prinzenberg *et al.,* 2005) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) and imported into the BioEdit Sequence Alignment Editor v.7.0.0 (Hall, 1999) for basic nucleic sequence alignment, manipulation and analysis. The specific positions at which nucleotide differences allow allele discrimination are indicated in Table 2.5 and Figure 2.9.

It was decided to exclude two of the known kappa-casein sequences from this analysis. The sequences of the B1 and B2 alleles (GenBank acc. numbers AF485340 and AF434988) and the D1 and D2 alleles (GenBank acc. numbers AY027868 and AY090465) were found to be identical, except that in each case one was longer. Thus, only the longer B1 and D2 alleles were included based on length.

Table 2.5 Kappa-casein gene (CSN3) variants in the domesticated goat according to Prinzenberg's modified nomenclature (2005)

CSN3	**IEF**	**GenBank Acc. No.**	**Nucleotide position**														
			170	245	247	274	284	290	298	309	384	385	471	509	550	583	591
A	A^{IEF}	X60763	C	T	A	A	G	C	A	G	G	A	G	A	T	C	T
B	A^{IEF}	AF485340											A				
		AF434988											A				
B'	A^{IEF}	AY166706	T										A				
B''	A^{IEF}	AY166707						T					A				
C	A^{IEF}	AY350425		C			A			A			A			T	C
C'	A^{IEF}	AF485341		C			A			A			A	G		T	C
D	B^{IEF}	AY027868		C	G					A			A				C
		AY090465		C	G					A			A				C
E	B^{IEF}	AF486523										G	A				
F	A^{IEF}	AY090466		C									A				C
G	A^{IEF}	AY090467		C						A			A				C
H	A^{IEF}	AF521022				G							A				
I	A^{IEF}	AY166710								A			A				
J	A^{IEF}	AY166711							G				A				
K	B^{IEF}	AY166709			G								A				
L	A^{IEF}	AY166708								A			A				C
M	B^{IEF}	AY428577		C							A		A		C		C

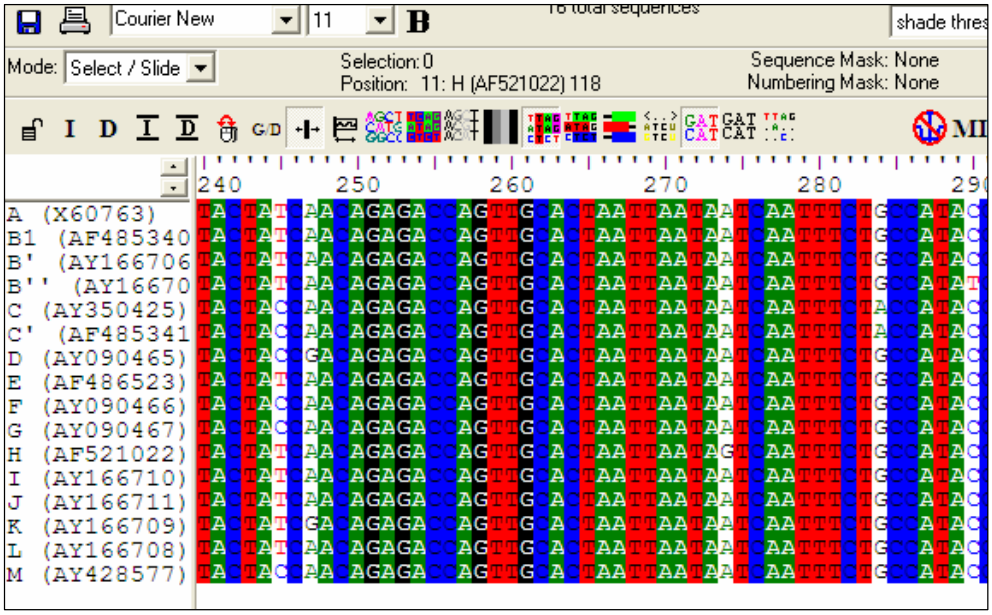


Figure 2.9 Known kappa-casein sequences aligned in BioEdit to indicate nucleotide positions 245, 247, 274, 284, and 290 that allow allele discrimination

FASTA formatted contig sequences were individually imported into the BioEdit file containing the known kappa-casein sequences. The ClustalW 1.4 Multiple Alignment application (Higgins *et al.,* 1994) of BioEdit was used to perform a multiple alignment of the DNA sequences. Multiple alignments were carried out in three stages. Firstly, a pairwise alignment was performed in which all pairs of sequences were aligned separately in order to calculate a distance matrix giving the divergence of each pair of sequences. Then a guide tree was constructed from the distance matrix, and finally, the sequences were progressively aligned according to the hierarchy in the guide tree. This application was used to align sample contigs with known allele sequences, after which known allele sequences that did not align with the sample at the nucleotide positions indicated in Table 2.5 and Figure 2.9 were individually eliminated until only one remained.

2.5 Statistical analysis

Allele and genotype frequencies of samples were estimated by direct counting. An Analysis of Molecular Variance (AMOVA) was performed using the molecular information (DNA sequences) gathered to investigate the genetic differentiation of the sampled populations using Arlequin (Excoffier *et al.,* 2005). In AMOVA, significance is tested via a permutational approach, eliminating the need of normal distribution that is required for analysis of variance but inappropriate for molecular data (Mengoni & Bazzicalupo, 2002).

CHAPTER 3
RESULTS

3.1 Milk composition

A total of 84 milk samples composed of 16 local goat types, 35 SA Boer goats, and 33 Saanens, were analysed for butterfat, protein, lactose, and casein composition, as well as somatic cell count (Table 3.1). See appendix D for complete results per sample.

Table 3.1 Summary of results obtained from milk compositional analysis

Sample group	Butterfat %	Protein %	Lactose %	SCC	Casein %
SA Boer goats	3.08 ± 1.32	4.02 ±0.50	4.71 ± 0.25	693.50 ± 1158.60	3.23 ± 0.33
Local goats	5.73 ± 1.47	4.02 ± 0.42	4.81 ± 0.29	14.60 ± 1.47	3.51 ± 0.48
Saanen goats	4.32 ± 2.03	2.77 ± 0.26	4.37 ± 0.29	1226.40 ± 2738.10	2.28 ± 0.27
Average	**4.07 ± 1.91**	**3.53 ± 0.73**	**4.40 ± 0.33**	**773.54 ± 1906.72**	**2.85 ± 0.60**

Butterfat percentage averaged 4.07 ± 1.91 over all goat samples analyzed. It is interesting to note that the local goat types had the highest butterfat percentage (5.73 ± 1.47), followed by the Saanen goats (4.32 ± 2.02) and the SA Boer goats (3.08 ± 1.32). The local goat types and SA Boer goats had the highest protein percentages (4.02 ± 0.42 and 4.02 ± 0.50, respectively), with the Saanen goats having a lower protein percentage (2.77 ± 0.26). The total casein percentage averaged 2.85 ± 0.60 for all goats sampled, with local goat types again having the highest average (3.51 ± 0.48), followed by SA Boer goats (3.23 ± 0.33) and the Saanen goats (2.28 ± 0.27). The average lactose percentage for all samples analyzed was very similar to the protein percentage (4.60% ± 0.33), with the local goat types having a slightly higher lactose percentage (4.81 ± 0.29) than the other breeds analyzed. The analysis for somatic cell count indicated the highest counts for the Saanen goats (1226.36 ± 2738.10), followed by the SA Boer goats (693.55 ± 1158.56), with the local goat types having the lowest counts (14.60 ± 1.47).

3.2 RFLP analysis

RFLP results were obtained for 68 samples consisting of 10 Saanen, 24 local type, 30 SA Boer goats, and four control samples (Appendix E). RFLP analysis does not allow for simultaneous genotyping of all known genetic variants (Prinzenberg *et al*., 2005), but the three enzymes chosen to be used in this analysis (*HaeIII*, *BseNI*, and *Alw411*) are the three that allow for optimum differentiation between the possible alleles. The enzyme restriction sites on the sequence of kappa-casein allele C (GenBank Acc. no AY350425) were shown in Fig.2.7. Results obtained by RFLP digestion of the 645 bp PCR product containing exon 4 of the kappa-casein gene are shown in Table 3.2.

Table 3.2 Summary of results obtained by RFLP digestion of the 645 bp PCR product containing exon 4 of the CSN3 gene

No. of samples	***HaeIII* fragment results**	***BseNI* fragment results**	***Alw411* fragment results**	**Final allele deduction (based on Table 2.4)**
SA Boer goats				
27 samples	230; 415	51; 235; 359	645	A and/or B
3 samples	230; 415	645	645	Unusual result
Local goats				
23 samples	230; 415	51; 235; 359	645	A and/or B
1 sample	230; 415	645	645	Unusual result
Saanen goats				
10 samples	230; 415	51; 235; 359	645	A and/or B
Control DNA				
4 samples	230; 415	51; 235; 359	645	A and/or B

Exon four of kappa-casein contains one natural restriction site for *HaeIII* at approximately 230 bp, which causes six of the seven possible alleles (A, B, C, D, F, and G) to show two fragments of 230 and 415 bp after digestion. The E allele creates an additional restriction site at approximately 280 bp with an A to G substitution, creating three fragments of 50, 230, and 365 bp (Yahyaoui *et al*., 2003). As indicated in Fig. 3.1, all samples subjected to H*aeIII* digestion showed two fragments upon agarose gel electrophoresis, eliminating the E allele as a possibility.

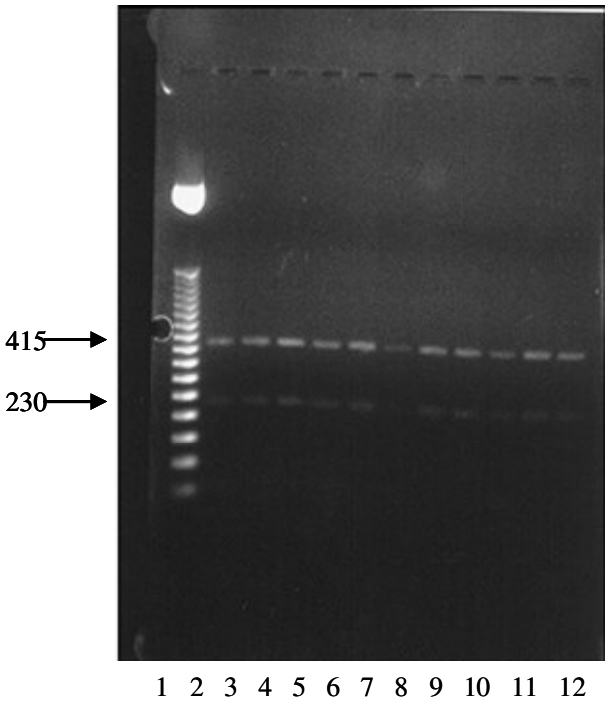


Figure 3.1 DNA electrophoretic patterns obtained after digestion of the 645 bp PCR product containing exon 4 of the CSN3 gene with *HaeIII* endonuclease, indicating two fragments of approximately 230 and 415 bp. Lane 1: 50 bp DNA Step Ladder (Promega, Madison, WI, USA); Lanes 2 – 12: 11 DNA samples

The single nucleotide substitution at position 309 (G to A) affects the *BseNI* target site which is present in alleles A, B, E, and F, but not in the C, D, or G alleles (Yahyaoui *et al.*, 2001). Therefore, digestion produces three fragments of 51, 235, and 359 bp for the former alleles and two fragments of 235 and 410 bp for the latter. In 64 of the 68 samples analyzed in this study, digestion of samples with *BseNI* produced three fragments of 51, 235, and 359 bp (Fig. 3.2), indicating that the C, D, and G alleles could be eliminated as possibilities.

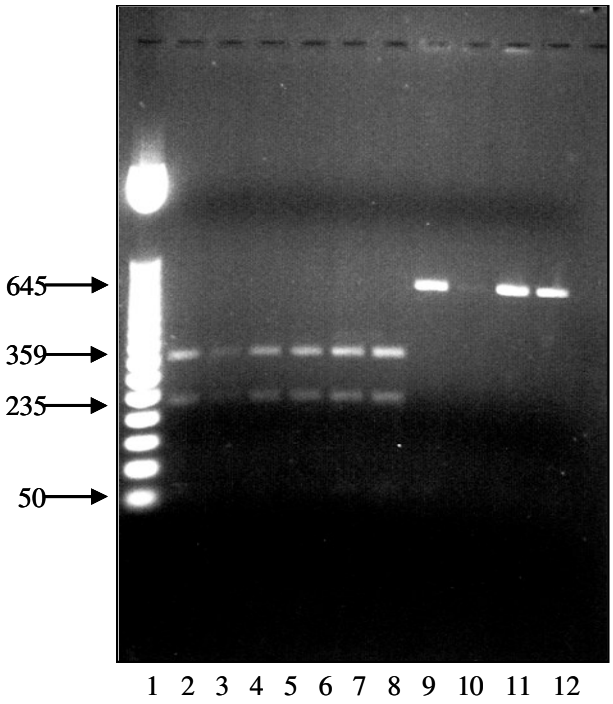


Figure 3.2 DNA electrophoretic patterns obtained after digestion of the 645 bp PCR product containing exon 4 of the CSN3 gene with *BseNI* endonuclease, indicating three fragments of approximately 50, 235 and 359 bp in lanes 2-8. Lanes 9-12 indicate one undigested fragment of approximately 645 bp. The 50 bp DNA Step Ladder (Promega, Madison, WI, USA) is shown in lane 1

The remaining four samples (3 SA Boer and 1 local goat type) produced unusual results when digested with *BseNI* (Fig. 3.2). Digestion with *HaeIII* resulted in two fragments of 230 and 415 bp (Fig. 3.1), indicating the presence of A, B, C, D, F, and G alleles, and *Alw44I* digestion resulted in an undigested fragment of 645 bp (Fig 3.3), ruling out the C, D, and F alleles. However, digestion with *BseNI* resulted in an undigested fragment of 645 bp, which does not correspond to any of the seven possible alleles or to previous studies cited.

Finally, the polymorphism at position 591 (T to C) results in the appearance of an *Alw44I* recognition site that allows discrimination of the A and B alleles from the remaining G allele because the $CSN3^{A}$ and $CSN3^{B}$ alleles show an undigested fragment of 645 bp, and the remaining $CSN3^{G}$ allele would show two fragments of 79 and 566 bp. In all samples analyzed in this study, digestion with *Alw441* resulted in an undigested fragment of 645 bp (Figure 3.3). Thus, most of the samples analyzed, including the control (AA and BB) samples, were discriminated down to the A and/or B level, eliminating the other five alleles.

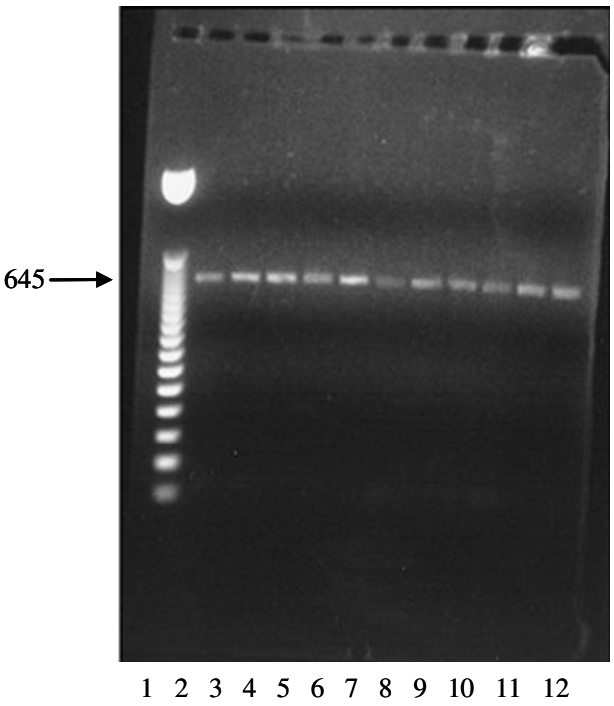


Figure 3.3 DNA electrophoretic patterns obtained after digestion of the 645 bp PCR product containing exon 4 of the CSN3 gene with *Alw44I* endonuclease, indicating one undigested fragment of 645 bp. Lane 1: 50 bp DNA Step Ladder (Promega, Madison, WI, USA); Lanes 2 – 12: 11 DNA samples

Since RFLP analysis was only able to distinguish between alleles A, B, C, D, E, F, and G, and not the other eight known kappa-casein alleles, DNA sequencing was performed for further investigation.

3.3 DNA sequencing

The 645 bp region of exon 4 containing the major part of the coding sequence (159 amino acids out of a total of 171) for the goat kappa-casein gene was screened for polymorphism using DNA sequencing. Altogether, 15 polymorphic sites have previously been identified in this region. All are point mutations (base transversions) where C and A are substituted by T and G, respectively. Four sites are silent base pair substitutions, and the other 11 mutations produce codon changes (Appendix G).

A total of 77 samples were sequenced, consisting of 58 of the samples previously subjected to RFLP analysis, one each of the AA and BB control sequences, as well as 17 additional DNA samples. A summary of the results is shown in Table 3.3, for complete results per sample, please refer to Appendix F.

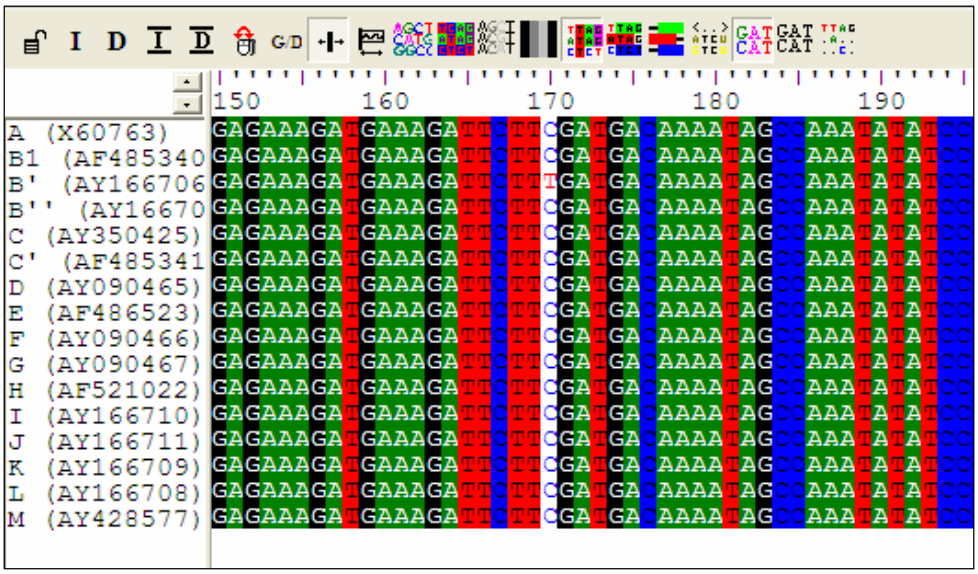
Table 3.3 Genotypes observed in the Saanen, SA Boer goat and local goat types analyzed in this study

Goat samples (IEF variant)	**N**	**AA** (A^{IEF})	**BB** (A^{IEF})	**B'B'** (A^{IEF})	**HH** (A^{IEF})	**BB'** (A^{IEF})	**BH** (A^{IEF})	**BB'H** (A^{IEF})
SA Boer goats	27	-	12	5	-	8	-	2
Local types	29	-	16	3	2	6	2	-
Saanen goats	19	-	19	-	-	-	-	-
Control	2	1	1	-	-	-	-	-
Total	**77**	**1**	**48**	**8**	**2**	**14**	**2**	**2**

In order to identify the sample sequences, DNA sequences were compared to known kappa-casein allele sequences downloaded from GenBank, as well as compared to the two control sequences (AA and BB). As indicated in Table 3.3, only three of the 15 known kappa-casein alleles were identified in this study, namely the B, B', and H alleles, all of which belong to the A^{IEF} variant.

The difference between kappa-casein B and B' involves only one nucleotide substitution from C to T at position 170 (Figure 3.4a). The kappa-casein H allele also only differs from the B and B' alleles at one position, with an A to G substitution at position 274 (Figure 3.4b).

(a)



(b)

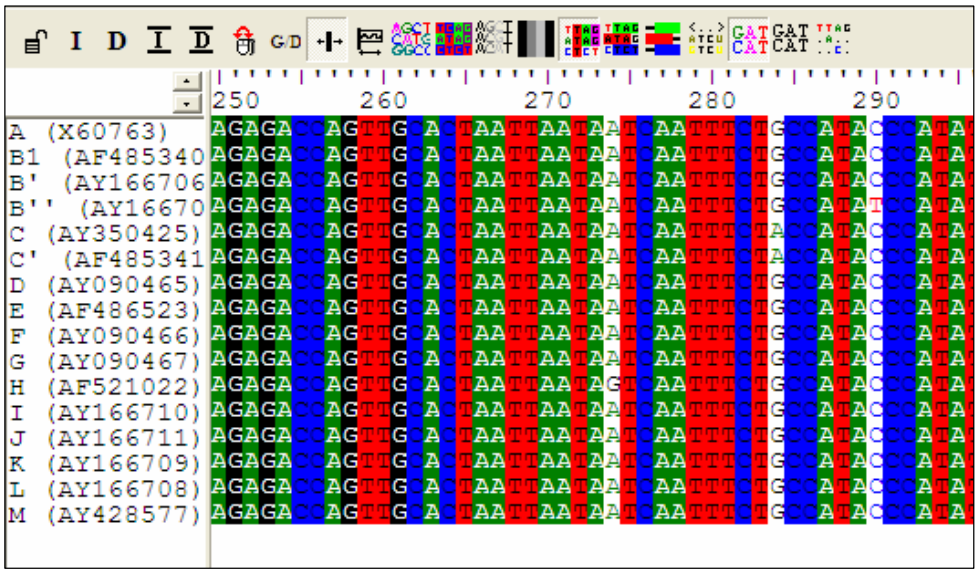
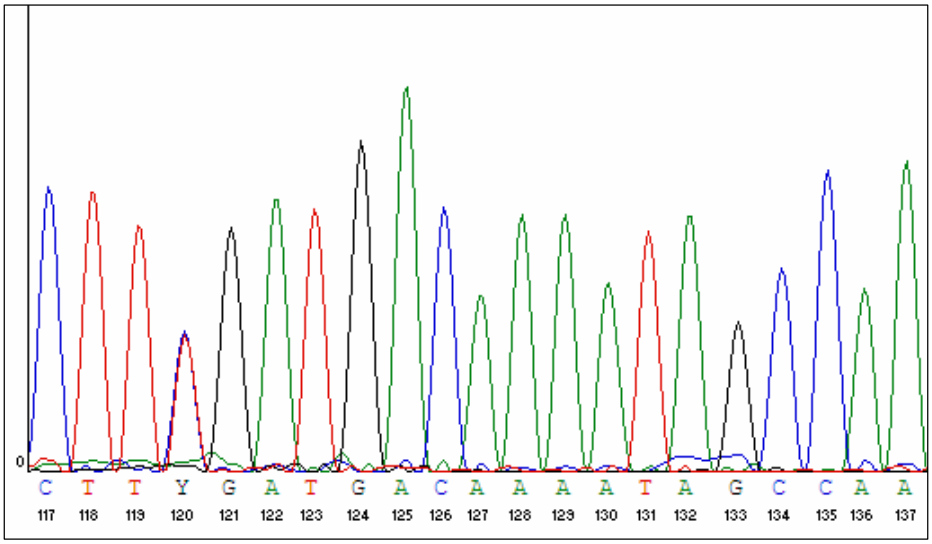


Figure 3.4 Known kappa-casein sequences aligned in BioEdit to indicate the nucleotide positions that allow discrimination of (a) Allele B' and (b) Allele H.

Heterozygosity was suspected when the chromatogram showed two nucleotides occurring at the same nucleotide position and if the peak was about half the strength of surrounding peaks (Figure 3.5).

(a)



(b)

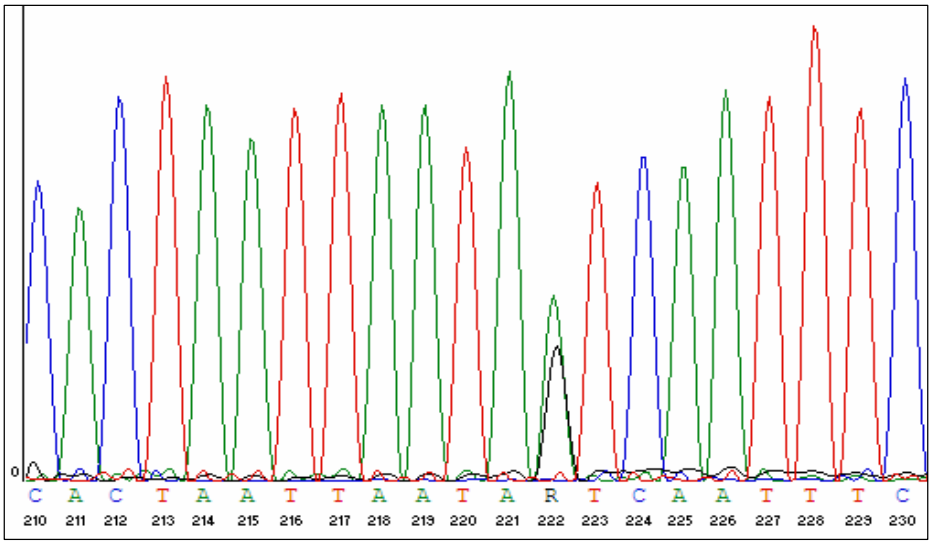


Figure 3.5 DNA sequence chromatograms showing heterozygosity at (a) position 170 (120 in this example) and (b) position 274 (222 in this example)

A small number of the samples were heterozygous either at nucleotide positions 170 (BB') or 274 (BH), with two samples showing heterozygosity at both these positions (BB'H). Samples suspected of heterozygosity were resequenced to confirm that results were not due to poor amplification or experimental error, and the phenomenon was only accepted if both the forward and reverse sequences indicated heterozygosity.

3.4 Statistical analysis

Frequencies of the kappa-casein alleles were calculated for the alleles and genotypes observed in the Saanen, SA Boer goat, and local goat types analyzed (Tables 3.4 and 3.5). Calculation was done by direct counting using the following formulae (Fairbanks & Andersen, 1999).

Allele frequency: $$p_A = \frac{\sum x}{2n}$$

Genotype frequency: $$p_G = \frac{\sum y}{n}$$

where x = number of occurrences of an allele, e.g. B
y = number of occurrences of a genotype, e.g. BB
n = sample size

Table 3.4 Frequencies of kappa-casein alleles in Saanen, SA Boer goat and local goat types analyzed (GenBank accession numbers are shown in brackets)

Allele	n	B (AF485340)	B' (AY166706)	H (AF521022)
SA Boer goat	27	0.607	0.357	0.036
Local goat types	29	0.689	0.207	0.103
Saanen	19	1	0	0

Table 3.5 Frequencies of kappa-casein genotypes in Saanen, SA Boer goat and local goat types analyzed.

Genotype	n	BB	B'B'	HH	BB'	BH	BB'H
SA Boer goat	27	0.552	0.103	0.069	0.207	0.069	0
Local goat types	29	0.444	0.185	0	0.296	0	0.074
Saanen	19	1	0	0	0	0	0

The B allele had the highest frequency in all the goats studied, followed by the B' allele, while the H allele was only observed in a few of the SA Boer goats and local goat types. As seen in Table 3.5, the homozygous BB genotype had the highest genotype frequency in all the goats analyzed, followed by the homozygous B'B' genotype, and then the heterozygous BB' genotype. The homozygous HH and heterozygous BH genotypes were only observed in the SA Boer goat, while the heterozygous BB'H genotype was only observed in the local goat types.

The sequence data were subjected to analysis of molecular variance (AMOVA) using the Arlequin 3.0 program (Excoffier *et al*., 2005). The different goats were grouped into categories by breed or type, i.e. SA Boer goats, local goat types, and Saanen goats. Results obtained for AMOVA analysis are shown in Table 3.6.

Table 3.6 Analysis of molecular variance (AMOVA) results for all goat populations studied, structured according to breed or type

Population	**SA Boer**	**Local type**	**Saanen**
n	27	29	19

Source	**df**	**SS**	**Estimated variance**	**%**	**FST**	**P value**
Among populations	2	1.180	0.02049	19.34		
Within populations	72	6.153	0.08546	80.66		
Total	74	7.333	0.10595		0.19338	0.00293

CHAPTER 4
DISCUSSION

4.1 Milk composition

In this study, milk samples were collected solely to analyze the milk composition, and no attempt was made to correlate milk composition with the kappa-casein alleles identified. The results of the milk analysis are only discussed as it pertains to differences between the SA Boer goat, local goat types, and the Saanen goats studied.

South African Boer goats and local goat types are not generally used for dairying purposes and thus very little is known regarding their milk production potential (Greyling *et al.*, 2004; Degen, 2007). A study in Malawi indicated that, although local goat types showed large variation in milk yield, it may be possible, with only minor modifications to traditional practices, to produce usable amounts of milk from them with no adverse effects on the doe's bodyweight or reproductive performance (Cooper & Banda, www.nda.agric.za). Production figures were not analyzed in this study due to the absence of production data for local goat types.

Protein in milk is composed of two types – whey and casein (Martin & Grosclaude, 1993; Karatzas & Turner, 1997; Moioli *et al*., 1998; Yahyaoui *et al*., 2000); of which the caseins comprise 80% of the total. The kappa-casein portion plays a critical role in formation, and stabilization of the casein micelles and thus influences the technological and nutritional properties of milk (Yahyaoui *et al.*, 2001; Jann *et al*., 2004; Prinzenberg *et al*., 2005; Reale *et al.,* 2005).

The average protein percentage of the milk over all samples in this study was 3.53 ± 0.73. This is in accordance with other studies in which averages of 3.20 - 3.51% in Alpine and Nubian does were reported (Park, 1991; Zeng & Escobar, 1996). Local goat types and SA Boer goats had higher protein percentages (4.02 ± 0.42 and 4.02 ± 0.5, respectively) compared to Saanen goats (2.77 ± 0.26). Notwithstanding differences in genetic potential that exist between breeds, protein content in milk is usually an indication of energy availability in feed. This leads to the assumption that Saanen goats should have higher milk protein contents. However, their protein percentage may be lower due to dilution of solids that occurs with higher milk yield (Greyling *et al*., 2004).

The average total casein percentage was 2.85 ± 0.60 over all goats sampled, with local goat types again having the highest average (3.51 ± 0.48), followed by SA Boer goats (3.23 ± 0.33), and then Saanen goats (2.28 ± 0.27). Total casein percentage would follow the same pattern as the total protein percentage, as the caseins make up 80% of the total protein content of milk.

Lactose is a disaccharide that consists of β-D-galactose and β-D-glucose fragments bonded through a β1-4 glycosidic linkage. In cattle, lactose content in milk is generally constant at approximately 4.9% (Welper & Freeman, 1992). Lactose percentages were very similar for all the goats analyzed, with the local goat types having a slightly higher lactose percentage. This concurs with Zeng & Escobar (1996), who found an average lactose percentage of 4.41 among 313 Alpine and Nubian does.

Protein and butterfat percentages are highly correlated in ruminant milk, with a correlation coefficient varying from 0.53 to 0.75 (Park, 1991; Zeng & Escobar, 1996). In this study, the butterfat percentage over all samples averaged 4.07 ± 1.91. Park (1991) found the average fat percentage among 72 goats sampled to be 3.94±1.21, which is again in agreement with the current study. It was interesting to note that local goat types had the highest butterfat percentage (5.73 ± 1.47) the Saanen goats in this case had a higher percentage (4.32 ± 2.02) than the SA Boer goats (3.08 ± 1.32).

Somatic cell count (SCC) is a quantitative index of mastitis condition of milk or for levels of glandular irritation in mammary glands (Park, 1991; Droke *et al.*, 1993). According to the Foodstuffs, Cosmetics, and Disinfectants Act (Act 54 of 1972), goat milk with a SCC higher than 750 000 cells per milliliter is not fit for human consumption (Kyozaire *et al.*, 2005), although Droke *et al.* (1993) stated that goat milk SCC standard is 1 x 10^6 cells/ml. According to Contreras *et al.* (1996), subclinical mastitis may be indicated with somatic cell counts as low as 500 x 10^3 cells/ml. In this study, the average SCC was 773.54 ± 1906.72. This is in agreement with Zeng *et al.* (1997), who studied Alpine goat milk and found that SCC was 887 ± 400 x 10^3. In this case, the Saanen goats had the highest counts (1226.36 ± 2738.10), probably because they are milked regularly. SA Boer goats had the second highest counts (693.55 ± 1158.56), with local goat types having the lowest counts (14.60 ± 1.47).

As discussed in the critical review, further research is necessary to correlate milk composition to the casein genotype of South African goats. The local goat types in this study had the lowest figures for SCC compared to the commercial dairy breed, and may be of interest for further investigation into genetic resistance to mastitis.

4.2 RFLP analysis

The use of DNA polymorphic markers allows the determination of individual genotypes at many loci and provides information on population parameters such as allele frequencies (Biase *et al*., 2005). The effectiveness of RFLP analyses in this context has long been recognised. Several studies have relied on this methodology for characterization of genetic variability in goat milk genes (Rammuno *et al*., 2001; Yahyaoui *et al*., 2001; Angiolillo *et al*., 2002; Cosenza *et al*., 2005; Kusza *et al*., 2005).

In this study, 68 goat samples were analyzed for kappa-casein variation using RFLP methodology. In order to confirm results obtained by RFLP analysis, and further characterize the kappa-casein polymorphism, a total of 77 samples were sequenced. The sample sizes for the different groups subjected to RFLP analysis (SA Boer goat n=30, local goat type n = 24, Saanen n = 10, control samples n = 4) are comparable to similar studies on kappa-casein variation where RFLP methodology was also applied (Yahyaoui *et al*., 2003; Jann *et al.*, 2004; Prinzenberg *et al.*, 2005; Kusza *et al*., 2006).

In this study, 64 of the 68 samples subjected to RFLP analysis were identified as carrying the A and/or B alleles. The remaining four samples remained unidentified due to unusual fragment results for the *BseNI* enzyme. These results are consistent with those of Veress *et al.* (2004) where kappa-casein variability in Hungarian milking goats (n = 109) was analyzed according to methodology of Yahyaoui *et al.* (2001). In that study, it was also not possible to distinguish between the A and B alleles. They reported a higher frequency of the A and/or B alleles (0.85 – 1) in six different breeds, with the C allele occurring at a frequency of 0.01 to 0.15. In the past, allele frequencies have been determined using IEF patterns, which does not allow discrimination of several alleles from the A allele, as performed by Caroli *et al.* (2001), who reported the A allele to be the most widely distributed in domestic goat breeds.

In a study by Yahyaoui *et al.* (2003) on ten goat breeds (n = 210) using the same PCR-RFLP methodology as this study, the found kappa-casein A and B alleles were the most common variants in the majority of the breeds with a high prevalence of the B allele. The B allele was also the only variant that was present in all analyzed breeds, with frequencies ranging from 0.4 to 0.7. The remaining alleles tested for were present in low frequencies (0.02 – 0.41) and seemed to be specific to certain breeds. The wild goat breed studied showed a frequency of 0.02 for the B allele and 0.98 for the F allele. This study did not find the F allele

in any of the breeds or types studied, but the local goat types and SA Boer goats did show the presence of the H allele, which was not present in the Saanen goats.

Sequencing data confirmed results obtained from RFLP analysis with the exception of four samples. Three of the four samples that exhibited unusual patterns in the RFLP analysis (T34, G101, and G102), were proven by DNA sequencing to present the B allele. Sample G85 was the only sample that exhibited unusual RFLP results that were later confirmed by DNA sequencing. Of the two local goat samples (G85 and G86) that were identified as the H allele, only one showed an atypical RFLP result. Since it was only at digestion with the *BseNI* restriction enzyme that these discrepancies occurred, and only four of the 68 samples tested were not confirmed by sequencing, it may be that these results were due to poor digestion by the specific enzyme, and not due to actual genetic differences between the animals. Each of the heterozygote samples (G88, W15, T25, and T29) showed the same RFLP pattern as the other samples that were deduced to be the A and/or B genotype

4.3 DNA sequencing

Restriction endonucleases have been important tools in studies of DNA primary structure, recombinant DNA technology and other fields of molecular genetics and molecular biology (http://www.fermentas.com). RFLP technology has been used extensively in similar studies and still has several advantages, including being a relatively inexpensive method. However, RFLP methodology is almost always used in conjunction with another method to ensure maximum discrimination of alleles, e.g. SSCP (Prinzeneberg *et al*., 2005). DNA sequencing is, however, becoming the method of choice due to higher specificity and accuracy (Martin *et al.,* 2006). Especially if funding is limited, researchers may use RFLP analysis for screening of the total sample set for polymorphism, after which, samples exhibiting different RFLP patterns are subjected to DNA sequencing analysis (Caroli *et al*., 2006).

In this study, a total of 77 samples were sequenced, consisting of 56 of the samples previously subjected to RFLP analysis, one each of the AA and BB control sequences, as well as 17 additional DNA samples. Only three of the 15 previously identified variants (Prinzenberg *et al*., 2005) were detected in this study. The B, B', and H alleles all belong to the A^{IEF} variant, which is the less favourable kappa-casein variant in terms of milk composition and technological properties (Fitzgerald, 1997; Rando *et al*., 2000; Haenlein, 2002). DNA sequencing did not detect any animal carrying one of the B^{IEF} alleles and indicates a frequency of 0.00 for $CSN3^{D}$, $CSN3^{E}$, $CSN3^{K}$, and $CSN3^{M}$ alleles.

Most variation was observed in the local goat types, with all three alleles present in varying frequencies. The Saanen goats showed the least variation, having only the B allele present. The SA Boer goat showed more variation than the Saanen goats, though less than the local goat types, with only four of the six possible genotypes being present. The local goat types showed marginally less heterozygous genotypes than the SA Boer goats. In the former group six of the 29 samples were B-B' heterozygous, and two samples were heterozygous for the B and H alleles. The latter group had eight samples that were heterozygous B-B' out of the 30 samples tested, and two of the SA Boer goats showed double heterozygosity between the three alleles because of heterozygosity observed at both of these positions.

In all goat types included in this study, the B allele was the most common, with frequencies ranging from 60% in SA Boer goats to 100% in Saanen goats. The B' allele was the second most frequent, with frequencies of 35.7% in SA Boer goats and 20.7% in local goat types, although this allele did not occur in Saanen goats. The H allele was present at a low frequency in local goat types (10.3%) and in SA Boer goats (3.6%), but was not found in Saanen goats.

The SA Boer goat was developed from local goat types in South Africa approximately 100 years ago. Thus, both the SA Boer goat and local goat types originated from the same gene pools and therefore they are expected to have some alleles in common. None of the additional alleles (B' and H) found in these two goat types were present in the Saanen goats studied.

These results are comparable to those obtained by Prinzenberg et al. (2005), who investigated kappa-casein variability in seven breeds (n = 233) using similar methods, which did not distinguish between the B and B' alleles, or between the D/I/K/L alleles. They reported a high frequency of the B/B' alleles (0.260 – 0.660) and for the A allele (0.151 – 0.414), with the other alleles all having much lower frequencies. The A and B alleles were present in all seven breeds analyzed. The M allele was only present in Red Sokoto goats, and the G allele was only present in Angora and Hair goats.

Recent studies by Yahyaoui *et al*. (2001), Angiolillo *et al.* (2002), and Jann *et al.* (2004) have indicated a high level of polymorphism in the kappa-casein gene. However, there have been conflicting results and it is difficult to compare results over all previous studies due to two main reasons. Firstly, it is important to note the difference between type and variant – two terms that many researchers have used interchangeably. There are two kappa-casein variants (A^{IEF} and B^{IEF}), identified by isoelectric focusing (IEF), and all kappa-casein alleles

(A to M) can be categorized into these two groups (Chiatti *et al*., 2005; Prinzenberg *et al*., 2005; Caroli *et al*., 2006). When discussing the association between polymorphism and characteristics of milk, most researchers refer to IEF type. In addition, simultaneous publication of different papers describing new kappa-casein alleles has led to inconsistency in nomenclature in the literature, caused by the assignation of the same letter to different variants (Chessa *et al*., 2003; Prinzenberg *et al*., 2005; Reale *et al*., 2005). To avoid confusion, in this study only the nomenclature as assigned by Prinzenberg *et al*. (2005) was used.

Chessa *et al.* (2003) reported that the D allele was the most frequent in four Italian goat breeds (0.699), with the A and B alleles occurring at a much lower frequency (0.178 and 0.038, respectively. They also did not identify the C or E alleles in any of the breeds investigated. However, upon closer inspection it is noted that their nomenclature is conflicting. The D allele identified corresponds to GenBank accession number AF485340, which is designated as the B allele in the current study, and the B allele they detected corresponds to GenBank accession number AY090465, our D allele. A study by Jann *et al.* (2004) reported similar results, also with conflicting nomenclature. They found the D allele (GenBank acc. no. 485340, which corresponds to the B allele in this and other studies) to be the most frequent among ten different breeds. Alleles D' (GenBank acc. no. AY166706, named B' here), D" (GenBank acc. no. AY166707, named B" here), K, and I were only detected in one animal each, suggesting a limited distribution of these mutations.

In cattle, the practical importance of typing IEF variants of *CSN3* has long been recognized, since the B^{IEF} variant is correlated with commercially valuable parameters of milk productivity like protein content and milk yield, and also improves the cheese yielding capacity (Barroso *et al*., 1998; Sulimova *et al*., 2007). In Russian cattle, Sulimova *et al*. (2007) found that the A type was much more common than the economically important B type (frequencies of approximately 0.7 versus 0.3). Results of this study indicate that there is little variation of the kappa-casein gene in South African Saanen goats. However, SA Boer goats and local goat types may have significant variation for this gene.

4.4 Genetic variation

AMOVA is a powerful statistical method for the analysis of variance of molecular data (Mengoni & Bazzicalupo, 2002). Differences between groups, among populations within groups, and among individuals within populations can be estimated using this technique (Mengoni & Bazzicalupo, 2002).

Results shown in Table 3.6 for the goat populations structured according to breed or type indicated that most of the total variation occurred within populations (80.66%) with the remainder of the variation significantly different from zero ($F_{ST} = 0.1934$; $p < 0.01$). In studies on cattle (Ciampolini *et al*., 1995) and pigs (Yang *et al*., 2003) using microsatellite markers, variation was also larger within breeds than between breeds.

The F_{ST} value measures the degree of genetic differentiation between populations. De Oliveira *et al.* (2007) stated that a value between 0 to 0.05 suggests little genetic variation, 0.05 to 0.15 indicates moderate differentiation, 0.15 to 0.25 a substantial degree of differentiation, and values above 0.25 a very large degree of differentiation. The F_{ST} values obtained here for variation mostly fall in the range of 0.15 to 0.25. The F_{ST} value of 0.18925 for structured population analysis indicates that 81.07% of the total variability resulted from high diversity between individuals within each population, while the remaining 18.925% was due to genetic differences between populations.

The variation between the goats in this study was significant ($P<0.01$) in terms of the kappa-casein gene. Studies on genetic diversity of goats with microsatellite markers also indicated a relatively high genetic variation (Visser *et al*., 2004). It could be concluded that there is sufficient variation in the breeds and further studies on the milk protein polymorphism could be recommended.

CHAPTER 5
CONCLUSION AND CRITICAL REVIEW

This study aimed to investigate the genetic polymorphism of the kappa-casein gene in South African goats using PCR-RFLP and DNA sequencing. Literature was reviewed regarding the value of goats and milk proteins and their relevance in human nutrition in South Africa.

The significance of small ruminants to the socio-economic well being of people in developing countries in terms of nourishment, income and intangible benefits (e.g. ceremonial and cultural purposes) cannot be overemphasized (Peacock, 2005; Lehloenya *et al*., 2007; Kosgey *et al*., 2008). Improvement programmes are therefore necessary to increase and sustain the productivity of small ruminants in these areas. The objective is either to improve the availability of milk for villagers, as in Asia or Eastern Europe, or to sell goat milk products and cheeses at a higher price to urban consumers, as in Morocco (Dubeuf, 2005).

Milk is a complex biological fluid that is often referred to as the "nearly perfect food". Molecular genetics has a legitimate place among other proven techniques, such as nutritional management and quantitative genetics, for the improvement of milk quality and quantity (Karatzas & Turner, 1997). The ultimate goal of molecular genetics applied to improvement of milk production is to identify mutations responsible for individual difference in the quali-quantitative characteristics of milk produced by domestic ruminants, which is used as food for man (Rando *et al*., 2000).

It has been stated that the low milk yield of indigenous goats in the tropics prevents development of goat milk production projects to facilitate food security and alleviate poverty through income generation (Masika & Mafu, 2004). In the past, the focus of development programmes in developing countries has been on introduction of higher-yielding, exotic breeds that were developed for high-input production settings, where adaptive traits are almost irrelevant (Ayalew *et al*., 2003). However, development of genetic improvement programmes for sheep and goats can be successful when accompanied by a good understanding of the different farming systems and when simultaneously addressing several constraints e.g. feeding, health control, general management, etc (Ahuya *et al*., 2005; Kyozaire *et al*., 2005; Lehloenya *et al*., 2007; De Vries, 2008; Kosgey *et al*., 2008). Rural farmers have proven that, when given appropriate technical support, they can be very efficient breeders, equitably sharing the limited genetic resources amongst themselves (Ahuya *et al*.,

2005). Goat milk may also be processed into cheese, opening up the possibility of development of indigenous products of interest for local niche markets (Roets & Kirsten, 2005).

Despite recent developments, the scientific investment in goats remains low in many countries (Dubeuf *et al*., 2004; Jann *et al*., 2004; Pandya & Ghodke, 2007). There are very few studies to date that have investigated gene constitution in local goat breeds in South Africa (Galal, 2005). Research indicates that milk protein polymorphisms have a great influence on the quantity, composition, and technological properties of milk (Caroli *et al*., 2001; Jann *et al*., 2004; Reale *et al*., 2005), and that certain alleles may even be linked to milk protein allergy (Kusza *et al*., 2007). Caseins comprise about 80% of the total protein content in milk (Calavia & Burgos, 1998; Otaviano *et al*., 2005). The known association between specific casein variants and superior milk composition and processing properties justifies investigations into milk protein polymorphisms (Medrano & Cordova, 1990; Ng-Kwai-Hang & Grosclaude, 1992; Fitzgerald, 1997; Caroli *et al*., 2001; Biase *et al*., 2005).

Samples for this study were collected from SA Boer goats, local goat types, and Saanens representing five different geographical areas of South Africa. Random subsets of these samples were subjected to milk compositional analysis, PCR-RFLP, and DNA sequencing. Results of the DNA analyses were interpreted using various genetic software programs, including VectorNTI v10.3.0 (www.invitrogen.com), BioEdit v.7.0.0 (Hall, 1999), Microsatellite Toolkit (Park, 2001), and Arlequin (Excoffier *et al*., 2005). RFLP analysis revealed very little genetic variation between the goat populations sampled. Most samples were determined to carry to the A and/or B alleles. DNA sequencing was performed in order to verify results obtained from RFLP analysis. Sequencing data indicated that most of the genetic variation was found in the local goat type, while the Saanen goats seemed to have the least genetic variation. AMOVA results revealed more genetic variation between individuals within a breed or type than between populations.

In this study, the main focus was on investigating local goat types. Only one commercial breed (the SA Boer goat) was included due to its historical relationship with local goat types. Samples from Saanen goats were included as a control group; however, these samples were only collected from one source since the Louis Trichardt and Pretoria samples originated from the same gene pool. The local goat types and SA Boer goats analyzed here showed more genetic variation than the Saanen goats. Molecular studies should seek to include a larger sample size than the current study in order to minimize sampling error and to accurately deduce population genetic structures.

The results obtained here reveal the potential of investigating goats in South Africa at molecular level. Future studies should concentrate on the association between genotype and milk composition characteristics in order for to make a useful contribution to upliftment of rural communities. Such studies have been performed in developed countries using high pressure liquid chromatography (HPLC) and capillary electrophoresis (Clark & Sherbon, 2000; Gomez-Ruiz, 2004), however, these technologies were unavailable for the present study.

In addition, the fact that caseins are encoded by a tight 250 kb cluster of four genes, in the order CSN1S1, CSN2, CSN1S2 and CSN3 (Caroli *et al*., 2006), future research should not concentrate on casein haplotypes and not on one casein gene alone (Jann *et al*., 2004; Bozkaya *et al.*, 2008).

In conclusion, although economically important kappa-casein alleles were not identified in the populations studied, this study represents the first investigation into the genetic polymorphism of the kappa-casein gene in South African goats. In most developing countries, including South Africa, goats play an indispensable role in the sustenance of rural families and contribute significantly to supplying their needs in animal proteins (Garrine, 2007). Knowledge of the genetic make-up of these breeds at the casein loci could present the opportunity to develop specialized dairy products to enhance the economic potential of these goats in the areas where they are bred.

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APPENDIX A

Average fatty acid composition (g/100g milk) in lipids of goat, cow, and human milk (Haenlein, 2004)

Fatty acid	Goat milk	Cow milk	Human milk
C4:0 Butyric	0.13	0.11	
C6:0 Caproic	0.09	0.06	
C8:0 Caprylic	0.10	0.04	
C10:0 Capric	0.26	0.08	0.06
C12:0 Lauric	0.12	0.09	0.26
Total MCT	**0.70**	**0.38**	**0.32**
C14:0 Myristic	0.32	0.34	0.32
C16:0 Palmitic	0.91	0.88	0.92
C18:0 Stearic	0.44	0.40	0.29
Total Saturated	**2.67**	**2.08**	**2.01**
C16:1 Palmitoleic	0.08	0.08	0.13
C18:1 Oleic	0.98	0.84	1.48
Total Monounsaturated	**1.11**	**0.96**	**1.66**
C18:2 Linoleic	0.11	0.08	0.37
C18:3 Linolenic	0.04	0.05	0.05
Total Polyunsaturated	**0.15**	**0.12**	**0.50**
Cholesterol (mg)	11	14	14
Total lipids	**4.14**	**3.34**	**4.38**

APPENDIX B

Average amino acid composition (g/100g milk) in proteins of goat, cow, and human milk (Haenlein, 2004).

Amino acids	Goat	Cow	Human
Essential amino acids			
Tryptophan	0.044	0.046	0.017
Threonine	0.163	0.149	
Leucine	0.314	0.322	0.095
Isoleucine	0.207	0.199	0.056
Methionine	0.080	0.083	0.021
Lysine	0.290	0.261	0.068
Cystine	0.046	0.030	0.019
Tyrosine	0.179	0.159	
Phenylalanine	0.155	0.159	0.046
Valine	0.240	0.220	0.063
Nonessential amino acids			
Arginine	0.119	0.119	0.043
Histidine	0.089	0.089	0.023
Proline	0.368	0.319	0.082
Alanine	0.118	0.113	0.036
Aspartic acid	0.210	0.250	0.082
Serine	0.181	0.179	0.043
Glutamic acid	0.626	0.689	0.168
Glycine	0.050	0.070	0.026

APPENDIX C

Restriction sites of the three enzymes used in RFLP analysis indicated on the nucleotide sequence of the caprine kappa-casein C allele (GenBank acc. no.AY350425), green indicates BseNI, yellow indicates HaeIII, and blue indicates Alw44I.

TGTGAGAAAG ATGAAAGATT CTTCGATGAC AAAATAGCCA AATATATCCC
ACACTCTTTC TACTTTCTAA GAAGCTACTG TTTTATCGGT TTATATAGGG

AATTCAGTAT GTGCTGAGTA GGTATCCTAG TTATGGACTC AATTACTACC
TTAAGTCATA CACGACTCAT CCATAGGATC AATACCTGAG TTAATGATGG

AACAGAGACC AGTT↓GCACTA ATTAATAATC AATTTCTACC ATACCCATAT
TTGTCTCTGG TC↑AACGTGAT TAATTATTAG TTAAAGATGG TATGGGTATA

TATGCAAAGC CAATTGCAGT TAGGTCACCT GCCCAAACTC TTCAATGGCA
ATACGTTTCG GTTAACGTCA ATCCAGTGGA CGGGTTTGAG AAGTTACCGT

AGTTTTGCCA AATACTGTGC CTGCCAAGTC CTGCCAAGAC CAGCCAACTA
TCAAAACGGT TTATGACACG GACGGTTCAG GACGGTTCTG GTCGGTTGAT

CCCTGGCACG TCACCCACAC CCACATTTAT CATTTATGG↓C CATTCCACCA
GGGACCGTGC AGTGGGTGTG GGTGTAAATA GTAAATACC↑G GTAAGGTGGT

AAGAAAGATC AGGATAAAAC AGAAATCCCT GCCATCAATA CCATTGCTAG
TTCTTTCTAG TCCTATTTTG TCTTTAGGGA CGGTAGTTAT GGTAACGATC

TGCTGAGCCT ACAGTACACA GTACACCTAC CACCGAAGCA ATAGTGAACA
ACGACTCGGA TGTCATGTGT CATGTGGATG GTGGCTTCGT TATCACTTGT

CTGTAGATAA TCCAGAAGCT TCCTCAGAAT CGATTGTGAG ↓TGCACCTGAG
GACATCTATT AGGTCTTCGA AGGAGTCTTA GCTAACACTC ACGT↑GGACTC

ACCAACACAG CCCAAGTTAC TTCAACCGAG GTCTAAAAAC TCTAAGGAGA
TGGTTGTGTC GGGTTCAATG AAGTTGGCTC CAGATTTTTG AGATTCCTCT

CATCAAAGAA T
GTAGTTTCTT A

APPENDIX D

Results of milk compositional analysis per breed

SA Boer goats						Saanen goats					
No.	**Fat B**	**Protein**	**Lactose**	**Cells**	**Casein**	**No.**	**Fat B**	**Protein**	**Lactose**	**Cells**	**Casein**
T12	1.46	3.73	4.76	954.00	2.89	T38	3.38	2.85	4.71	186.00	2.28
T13	1.82	4.61	5.15	222.00	3.69	T40	3.48	2.55	4.15	183.00	1.91
T14	3.39	4.50	4.53	1792.00	3.43	T41	2.70	2.69	4.15	3533.00	1.99
T15	6.31	4.31	4.52	2707.00	3.43	T42	2.76	2.96	4.42	489.00	2.28
T16	1.87	3.61	4.82	572.00	2.82	T46	3.24	2.51	4.33	323.00	1.97
T18	3.99	4.86	4.34	327.00	3.62	T49	1.61	2.59	4.25	371.00	1.94
T19	1.99	4.73	5.23	360.00	3.76	T50	3.17	2.74	4.52	1181.00	2.21
T21	2.54	3.89	4.74	40.00	3.04	T51	2.98	2.61	4.25	220.00	2.02
T22	3.02	4.61	5.03	1666.00	3.64	T52	4.47	3.30	4.76	44.00	2.80
T23	4.14	3.73	4.76	578.00	2.98	T53	3.48	2.57	4.65	178.00	2.10
T24	1.31	4.35	4.57	277.00	3.28	T54	3.93	2.96	4.52	301.00	2.39
T25	4.87	3.86	4.67	262.00	3.07	T55	4.33	2.53	4.25	641.00	2.02
T26	0.93	3.34	4.78	45.00	2.59	T56	3.34	2.89	4.13	1210.00	2.18
T27	2.24	5.15	4.91	360.00	3.97	T57	3.97	2.82	4.80	119.00	2.38
T28	3.59	4.36	4.48	1999.00	3.35	T58	2.54	2.89	4.17	1616.00	2.17
T29	2.10	4.42	4.63	4435.00	3.36	T59	3.79	2.78	4.55	967.00	2.26
T30	2.12	3.97	4.47	3951.00	3.00	T60	4.30	3.61	4.39	9762.00	2.85
T32	2.36	4.75	4.78	102.00	3.62	T61	4.47	2.72	4.60	235.00	2.25
T34	3.38	3.83	4.70	188.00	3.02	T62	2.96	2.49	4.19	1016.00	1.91
T35	1.95	3.57	4.54	2742.00	2.72	T63	2.49	2.51	4.38	1136.00	1.95
T36	1.61	3.37	4.68	403.00	2.61	T64	2.80	2.73	4.60	245.00	2.18
T37	1.91	4.38	4.71	132.00	3.37	T65	2.54	2.75	4.37	1694.00	2.09
G97	5.43	4.77	3.74	13.85	3.70	T66	5.20	3.27	3.18	12935.00	2.18
G98	4.33	3.90	4.99	13.34	3.43	T67	4.35	2.97	4.51	980.00	2.44
G99	3.73	3.87	4.63	12.34	3.30	T68	3.46	2.62	4.34	791.00	2.06
G101	5.28	3.38	4.77	13.53	3.09	P111	10.21	2.52	4.07	16.71	2.67
G102	2.88	3.59	4.84	11.49	3.11	P112	6.40	2.78	4.34	13.58	2.41
G103	4.09	3.81	4.66	12.66	3.30	P113	9.55	2.49	4.38	16.39	2.73
G104	2.94	3.85	4.74	11.68	3.29	P114	5.68	2.79	4.42	12.98	2.54
G105	3.60	3.57	4.80	12.12	3.11	P115	7.77	2.49	4.26	14.55	2.44
G106	2.03	3.70	4.53	10.43	2.78	P116	5.20	3.04	4.57	12.92	2.71
G107	3.91	3.78	4.75	12.55	3.28	P117	8.10	2.60	4.49	15.22	2.37
G108	2.04	3.27	4.77	10.30	3.05	P118	3.89	2.72	4.64	11.43	2.63
G109	3.92	3.78	4.83	12.65	3.29						
G110	4.74	3.42	4.94	13.22	3.15						
Ave	3.08	4.02	4.71	693.55	3.23	**Ave**	4.32	2.77	4.37	1226.36	2.28
Std Dev	1.32	0.50	0.25	1158.56	0.33	**Std Dev**	2.03	0.26	0.29	2738.10	0.27

Local goat types					
No.	**Fat B**	**Protein**	**Lactose**	**Cells**	**Casein**
G81	8.04	4.03	4.64	16.66	
G82	8.47	3.54	4.75	16.74	
G83	4.57	3.74	4.84	13.25	3.34
G84	4.99	4.41	4.37	13.77	
G85	7.35	3.80	4.62	15.75	
G86	7.00	5.09	5.19	17.25	4.44
G87	6.39	3.93	4.64	14.98	3.53
G88	4.41	4.30	4.83	13.61	3.71
G89	4.79	4.00	4.98	13.86	
G90	5.06	3.46	4.45	13.04	3.01
G91	5.64	4.11	4.98	14.78	
G92	4.23	3.87	5.11	13.35	3.07
G93	3.28	3.48	5.34	12.32	3.12
G94	5.30	3.94	4.76	14.06	
G95	5.19	4.07	5.13	14.49	
G96	6.90	4.53	4.39	15.76	3.83
Ave	5.73	4.02	4.81	14.60	3.51
Std Dev	1.47	0.42	0.29	1.47	0.48

APPENDIX E

Elimination of alleles according to fragment sizes after RFLP digestion per goat type

Control samples

Sample	*HaeIII* fragment results	*BseNI* fragment results	*Alw411* fragment results	Final allele deduction
C1 (AA)	230; 415	51; 235; 359	645	A and/or B
C2 (AA)	230; 415	51; 235; 359	645	A and/or B
C12 (BB)	230; 415	51; 235; 359	645	A and/or B
C13 (BB)	230; 415	51; 235; 359	645	A and/or B

Saanen goats

Sample	*HaeIII* fragment results	*BseNI* fragment results	*Alw411* fragment results	Final allele deduction
T38	230, 415	51, 235, 359	645	A and/or B
T40	230, 415	51, 235, 359	645	A and/or B
T41	230, 415	51, 235, 359	645	A and/or B
T46	230, 415	51, 235, 359	645	A and/or B
T49	230, 415	51, 235, 359	645	A and/or B
T50	230, 415	51, 235, 359	645	A and/or B
T51	230, 415	51, 235, 359	645	A and/or B
T52	230, 415	51, 235, 359	645	A and/or B
T53	230, 415	51, 235, 359	645	A and/or B
T54	230, 415	51, 235, 359	645	A and/or B

SA Boer goats

Sample	*HaeIII* fragment results	*BseNI* fragment results	*Alw411* fragment results	Final allele deduction
T12	230, 415	51, 235, 359	645	A and/or B
T13	230, 415	51, 235, 359	645	A and/or B
T14	230, 415	51, 235, 359	645	A and/or B
T16	230, 415	51, 235, 359	645	A and/or B
T17	230, 415	51, 235, 359	645	A and/or B
T25	230, 415	51, 235, 359	645	A and/or B
T26	230, 415	51, 235, 359	645	A and/or B
T27	230, 415	51, 235, 359	645	A and/or B
T28	230, 415	51, 235, 359	645	A and/or B
T29	230, 415	51, 235, 359	645	A and/or B
T30	230, 415	51, 235, 359	645	A and/or B
T32	230, 415	51, 235, 359	645	A and/or B
T34	230, 415	700	645	Unusual result
T35	230, 415	51, 235, 359	645	A and/or B
T36	230, 415	51, 235, 359	645	A and/or B
T37	230, 415	51, 235, 359	645	A and/or B
G97	230, 415	51, 235, 359	645	A and/or B
G98	230, 415	51, 235, 359	645	A and/or B
G99	230, 415	51, 235, 359	645	A and/or B
G100	230, 415	51, 235, 359	645	A and/or B
G101	230, 415	250, 375, 390	645	Unusual result
G102	230, 415	250, 375, 390	645	Unusual result
G103	230, 415	51, 235, 359	645	A and/or B
G104	230, 415	51, 235, 359	645	A and/or B
G105	230, 415	51, 235, 359	645	A and/or B
G106	230, 415	51, 235, 359	645	A and/or B
G107	230, 415	51, 235, 359	645	A and/or B
G108	230, 415	51, 235, 359	645	A and/or B
G109	230, 415	51, 235, 359	645	A and/or B
G110	230, 415	51, 235, 359	645	A and/or B

Local goat types

Sample	*HaeIII* fragment results	*BseNI* fragment results	*Alw411* fragment results	Final allele deduction
G82	230, 415	51, 235, 359	645	A and/or B
G83	230, 415	51, 235, 359	645	A and/or B
G84	230, 415	51, 235, 359	645	A and/or B
G85	230, 415	650	645	Unusual result
G86	230, 415	51, 235, 359	645	A and/or B
G87	230, 415	51, 235, 359	645	A and/or B
G88	230, 415	51, 235, 359	645	A and/or B
G89	230, 415	51, 235, 359	645	A and/or B
G90	230, 415	51, 235, 359	645	A and/or B
G91	230, 415	51, 235, 359	645	A and/or B
G92	230, 415	51, 235, 359	645	A and/or B
G93	230, 415	51, 235, 359	645	A and/or B
G94	230, 415	51, 235, 359	645	A and/or B
G95	230, 415	51, 235, 359	645	A and/or B
G96	230, 415	51, 235, 359	645	A and/or B
W10	230, 415	51, 235, 359	645	A and/or B
W15	230, 415	51, 235, 359	645	A and/or B
W18	230, 415	51, 235, 359	645	A and/or B
W20	230, 415	51, 235, 359	645	A and/or B
W23	230, 415	51, 235, 359	645	A and/or B
W25	230, 415	51, 235, 359	645	A and/or B
W31	230, 415	51, 235, 359	645	A and/or B
W33	230, 415	51, 235, 359	645	A and/or B
W36	230, 415	51, 235, 359	645	A and/or B

APPENDIX F

Results obtained by DNA sequencing of 80 goat DNA samples per goat type

Saanen goats

Sample ID	Homozygote	Heterozygote	Sample ID	Homozygote	Heterozygote
T38	B		T53	B	
T40	B		T54	B	
T41	B		T55	B	
T42	B		T59F	B	
T45	B		T61F	B	
T46	B		T63R	B	
T49	B		P111R	B	
T50	B		P114	B	
T51	B		P118	B	
T52	B				

SA Boer goats

Sample ID	Homozygote	Heterozygote	Sample ID	Homozygote	Heterozygote
T12	B		G97		B-B’
T13	B		G99		B-B’
T14	B		G100	B	
T16	B		G101	B	
T17		B-B’	G102	B	
T25		B-B’-H	G103	B’	
T27	B’		G104	B’	
T28	B		G105	B	
T29F		B-B’-H	G106	B	
T30F		B-B’	G107		B-B’
T32		B-B’	G108	B’	
T34	B		G110	B’	
T35	B				
T36		B-B’			
T37F		B-B’			

Local goat types

Sample ID	Homozygote	Heterozygote	Sample ID	Homozygote	Heterozygote
G82	B		W10	B	
G83	B’		W15		B-H
G84	B		W23	B	
G85	H		W25		B-B’
G86	H		W31	B	
G87	B		W33	B	
G88		B-H	M3F		B-B’
G89	B		M4		B-B’
G90		B-B’	M5		B-B’
G91	B		M14F		B-B’
G92	B		M17R	B	
G93	B’		M18	B’	
G94F	B		M20	B	
G95	B		M21	B	
G96	B				

APPENDIX G

Kappa-casein amino acid differences among domestic goats (Jann *et al*., 2004)

CSN3	GenBank Acc. No.	Amino acid position (para-kappa-casein)								Amino acid position (CMP)					
		18	**43**	**44**	**53**	**56**	**58**	**61**	**65**	**90**	**119**	**131**	**145**	**156**	**159**
A	X60763	Phe	Tyr	Gln	Asn	Leu	Tyr	Tyr	Val	Asp	Val	Thr	Val	Ala	Ser
B1	AF485340										Ile				
B2	AF434988										Ile				
B’	AY166706	*Phe*									Ile				
B’’	AY166707						*Tyr*				Ile				
C	AY350425		*Tyr*			*Leu*			Ile		Ile			Val	Pro
C’	AF485341		*Tyr*			*Leu*			Ile		Ile	*Thr*		Val	Pro
D1	AY027868		*Tyr*	Arg					Ile		Ile				Pro
D2	AY090465		*Tyr*	Arg					Ile		Ile				Pro
E	AF486523									Gly	Ile				
F	AY090466		*Tyr*								Ile				Pro
G	AY090467		*Tyr*						Ile		Ile				Pro
H	AF521022				Ser						Ile				
I	AY166710								Ile		Ile				
J	AY166711							Cys			Ile				
K	AY166709			Arg							Ile				
L	AY166708								Ile		Ile				Pro
M	AY428577		*Tyr*							Asn	Ile		Ala		Pro

Silent mutations are shown in italics