MOLECULAR PHYLOGENETICS AND CONSERVATION ASPECTS OF ANTELOPES.

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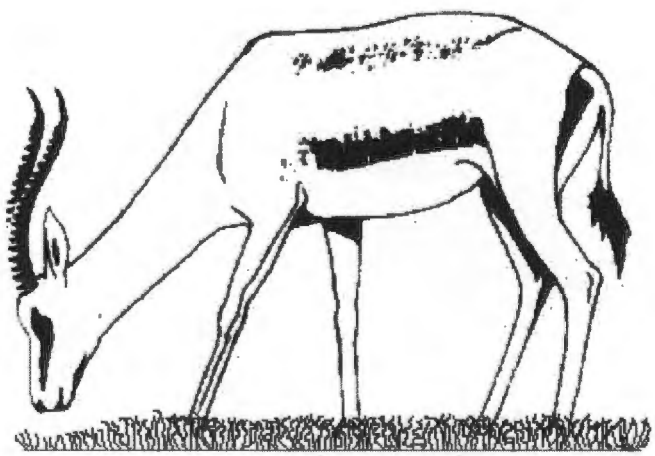
Voor Marjolein,

Omdat je zoveel meer voor me betekent dan je denkt !

I would do anything for you
I would climb mountains
I would swim all the oceans blue
I would walk a thousand miles
Reveal my secrets
More than enough for me to share

Education is an important keys - yes
But the good life's never won by degrees - no
Pointless passing through Harvard or Yale
Only window shopping - and strictly no sale



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ABSTRACT

This thesis concerns the molecular phylogenetics of three tribes of the family Bovidae, the Antilopini, Neotragini, and Tragelaphini. None of these tribes have been studied extensively with molecular techniques. The tribe Antilopini is one of the most speciose tribes (it includes 6 genera with 20 species) and the classification of several species of the genus *Gazella* is not clear. The tribe Neotragini is thought to be paraphyletic.

Mitochondrial sequences of the cytochrome c oxidase III and cytochrome *b* genes totalling 1083 base pairs have been determined for 52 taxa and used to determine phylogenetic relationships using cladistic and distance methods. Karyological analysis identified polymorphisms in several species (especially in *Gazella saudiya* and *G. subgutturosa*). Karyotypes of *G. dorcas pelzelni* and an XXY karyotype of a *G. dorcas* individual are shown for the first time.

The main conclusions are that the Antilopini and the Tragelaphini are monophyletic and that the tribe Neotragini is paraphyletic. There is a lack of phylogenetic resolution between tribes which is probably due to the rapid radiation of the different tribes about 20 million years ago. The genus *Taurotragus* in the tribe Tragelaphini is shown to be paraphyletic and it would be appropriate to incorporate these taxa in the genus *Tragelaphus*. The genus *Gazella* could be paraphyletic, due to the position of *Antilope cervicapra*, in which case the genus needs to be split into two genera or renamed as *Antilope*. It is also argued that the use of the subgenus Trachelocele should be discontinued and that its only species, *G. subgutturosa* should be included in the subgenus *Gazella*. *G. rufifrons* and *G. thomsonii* may be more appropriately considered as conspecific. Cytogenetic and sequence data reveal that the herd of *G. saudiya* in Al Areen Wildlife Park is hybridised with *G. bennettii* and it is argued that it is important to identify unhybridised *G. saudiya* in other collections, since this species is on the brink of extinction. This case study demonstrates the need to genetically screen individuals which are part of a captive breeding program, especially if they are intended for reintroduction into the wild.

Chapter 1: INTRODUCTION TO THE BOVIDAE.

1.1 Fossil record.

The Bovidae evolved in Eurasia between 17 and 25 million years ago, with the earliest known bovid *Eotragus artenensis* from France (Lowenstein, 1986b; Savage and Russell, 1983). Although antelope fossils are abundant in many localities and the identification of the different species in the paleontological record is relatively uncomplicated, the phylogenetic analysis of African fossil Bovidae is not far advanced (Vrba, 1985).

The Antilocaprinae of North America have been diagnosed as the ancestors of the Antilopinae, the Rupricaprinae, the Ovicaprinae and the Ovibovinae (Von Zittel, 1925). The first Antilocaprinae appeared in the fossil record 20 to 17 million years ago (Hemingfordian, early Miocene). According to this theory, the Antilopini would have evolved less than 20 million years ago. It is believed that the Antilopini are not monophyletic, but it is not indicated how the tribe could be split up (Vrba, 1985). The first recorded fossils of Antilopini appear in the early Miocene in North Africa during the Rusingan period, 19 - 17.5 million years ago (Savage and Russell, 1983). These fossils were found in Gebel Zelten (Libya) (Savage and Hamilton, 1973), and are labelled "*Gazella*" since is it difficult to establish which species they were. The first Antilopini fossils found in Europe belong to the Astaracian (15 - 13 million years ago) and are designated "*Gazella stehlini*". During the Turolian (late Miocene, 10 - 5 million years ago) Antilopini were abundant in Eurasia, whereas in Africa Antilopini only became abundant in the middle Pliocene (4 - 2 million years ago). The late appearance of Antilopini in Africa may be due to the "Hominid gap", which corresponds to the period between 5 and 10 million years ago during which fossils of Hominidea (and Antilopini) are lacking. During this period Africa might have had an environment which was not conducive to fossilisation, since the fossil record of this period is absent. Because of this contradiction between first appearance in the fossil record and period of apparent abundance it is still not clear whether the Antilopini originated in Eurasia or in Africa (Vrba, 1985). Gentry (1978) presented a tentative phylogeny for the Antilopini (Figure 1). He recognised two different

lineages within the genus *Gazella*: one ancestral to *G. dama, G. granti,* and *G. soemmerringii,* the other one ancestral to *G. thomsonii, G. rufifrons,* and *G. leptoceros.*

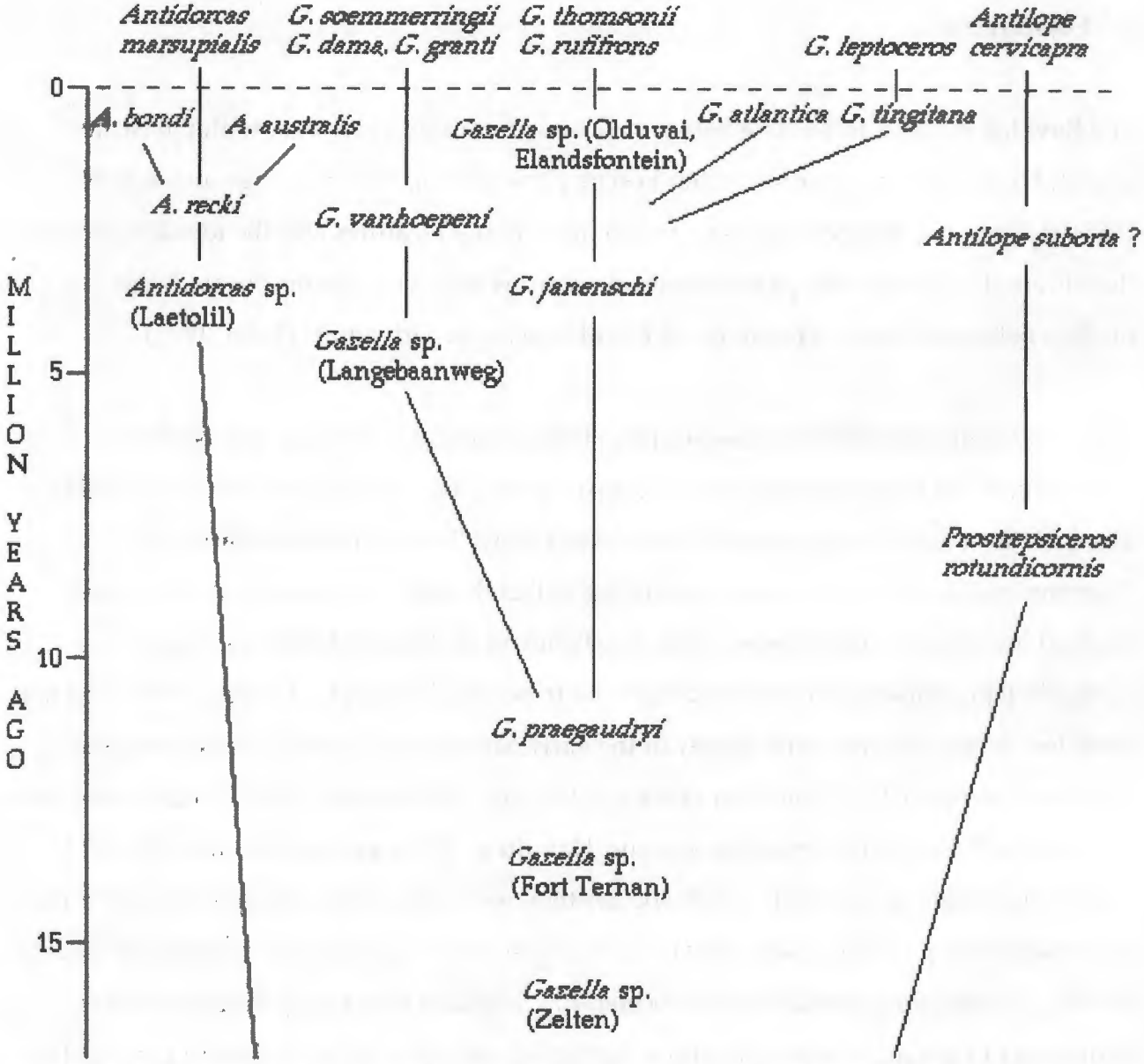


Figure 1: Tentative phylogeny of the Antilopini, according to Gentry (1978).

The earliest Neotragini (*Homoiodorcas*) lived about 12 million years ago, so the tribe evolved shortly after the Antilopini moved into Africa from Eurasia (Gentry, 1992; Vrba, 1985). These fossils are hard to distinguish from Antilopini fossils (Gentry, 1992). The most ancient genus of the Neotragini for which there are current extant members was the genus *Madoqua* and showed up in the fossil record about 8 to 5 million years ago in Kenya (Savage and Russell, 1983). The two genera *Raphiceros* and *Oreotragus* appeared relatively soon afterwards (Savage and Russell, 1983). Fossils of *Pelea capreolus* occur at at least two sites in the Transvaal province in South Africa (Kromdraai and Swartkrans), which date

back to the lower Pleistocene, less than 1.8 million years ago (Vrba, 1975). Fossil data suggest that the Antilopini are ancestral to the Neotragini (Savage and Russell, 1983).

The oldest Tragelaphini fossils were found in Kenya and consisted of teeth only. These teeth belonged to animals from the middle to late Miocene and are therefore approximately 15 million years old (Gentry, 1978). There are no known fossils of either *T. buxtoni, T. eurycerus* or their immediate ancestors (Gentry, 1978). For all other Tragelaphini recent fossil ancestors are known, as shown in a provisional phylogeny of the Tragelaphini (Gentry, 1978; Figure 2).

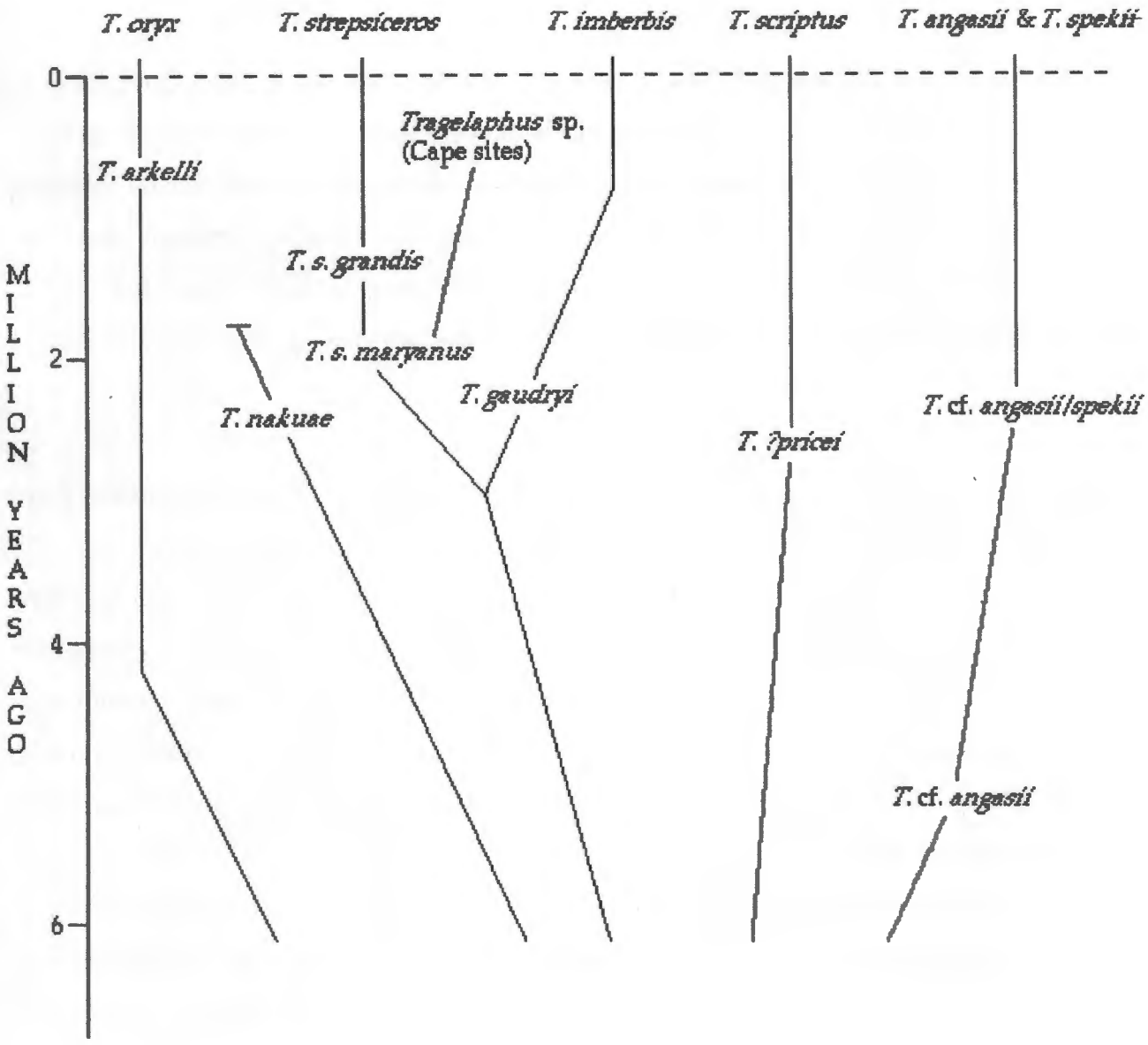


Figure 2: Tentative phylogeny of the Tragelaphini, according to Gentry (1978).

1.2 Morphological classification.

The order Artiodactyla consists of 10 families with their subfamilies (Table 1). At the family level there is hardly any doubt that the current classification is a valid categorisation. In the family Bovidae however, there has been discussion about the arrangements of genera into tribes and subfamilies (Allard et al., 1992; Gatesy et al., 1992; Gentry, 1978; Gentry, 1992; Georgiadis et al., 1990; Vrba, 1985). The family is represented by 127 species occurring in Eurasia, Africa, and North America (Corbet and Hill, 1991). In all but a single case, notably *Gazella saudiya*, the classification according to Corbet and Hill (1991) is followed in this thesis for all members of the Bovidae.

An extensive morphological phylogenetic study of the Bovidae was published recently (Gentry, 1992). Both cladistic and phenetic methods were used and both methods show slightly different trees. One significant difference between the two methods is the splitting up of the Bovinae clade into two distantly related clades in the cladistic analysis. Another difference is that the Neotragini are ancestral to the Antilopini in the cladistic analysis, whereas the phenetic analysis implied that the two tribes are sister groups (Figures 3a and 3b).

The subfamily Antilopinae is subdivided in two tribes: Neotragini (dwarf antelopes) and Antilopini (gazelles). Within the tribe Antilopini the genus with the most species is *Gazella*, which is subdivided in three subgenera: Nanger, Gazella, and Trachelocele. There is one species in Trachelocele: *Gazella subgutturosa*, three in Nanger: *G. granti*, *G. dama*, and *G. soemmerringii*, the others belong to the subgenus Gazella. See Table 1 for the listing of all Antilopini species. The Antilopini are small to medium-sized animals with slender legs and rather long necks. The back is straight but they are capable of great speed. Horns are usually present in both sexes, simply curved and strongly ringed, but they may be reduced or absent in the female. They are mostly browsers of succulent vegetation and inhabit open dry country, extending into deserts, and many are water-independent. They weigh between 20 and 80 kg and have a lifespan of 12 to 14 years (Jones, 1993).

There are no morphological phylogenetic studies available which include all Antilopini species. Despite this, the idea has been put forward that the genus *Gazella* and the tribe

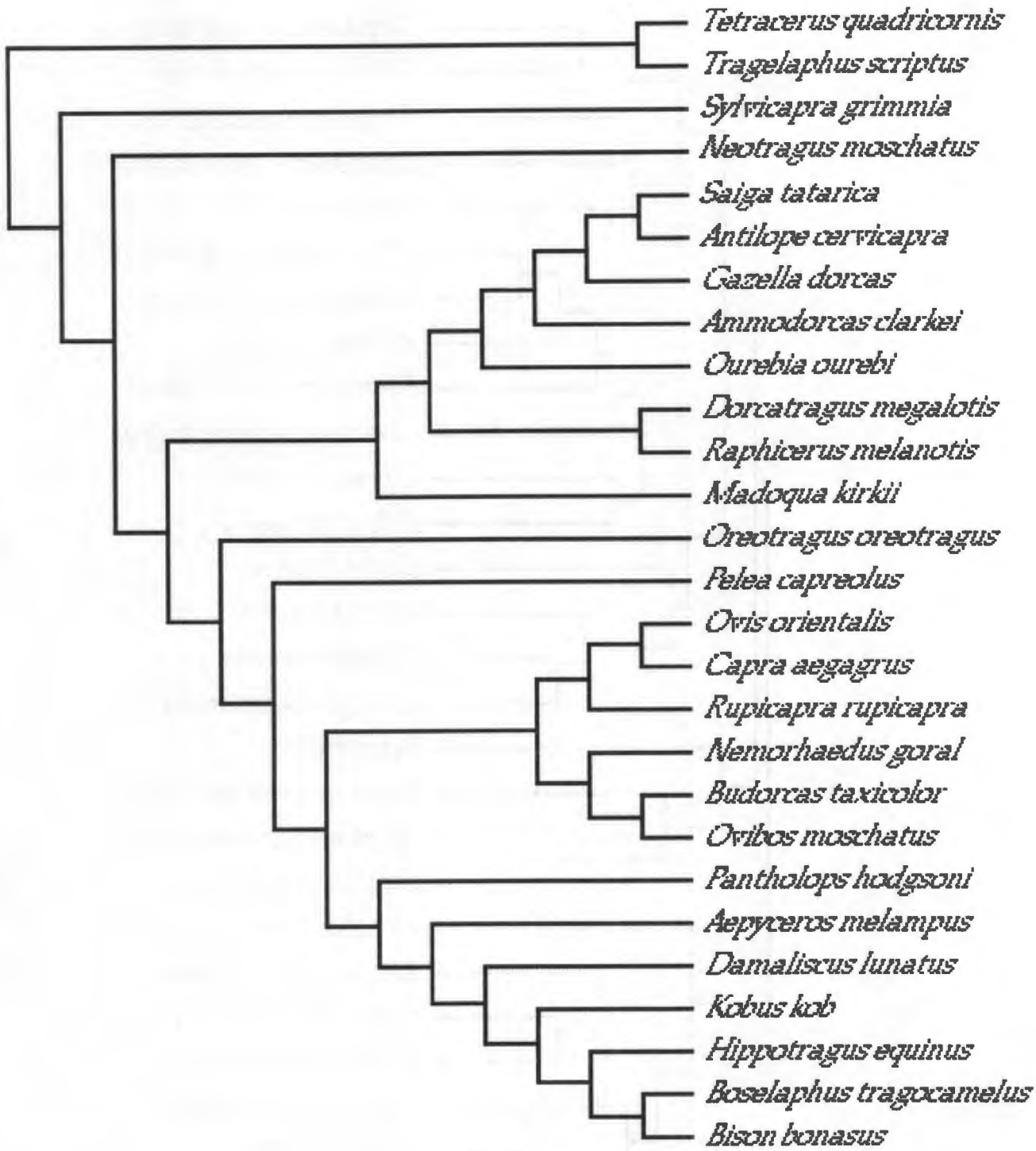


Figure 3a: Phylogeny of the Bovidae based on morphological characters. Cladogram after Gentry (1992).

Antilopini might not be monophyletic (Groves, 1985; Vrba, 1985). The classification of the species which belong to the five genera other than *Gazella* (*Antidorcas, Antilope, Ammodorcas, Litocranius,* and *Procapra*) is unambiguous. However, classification within the

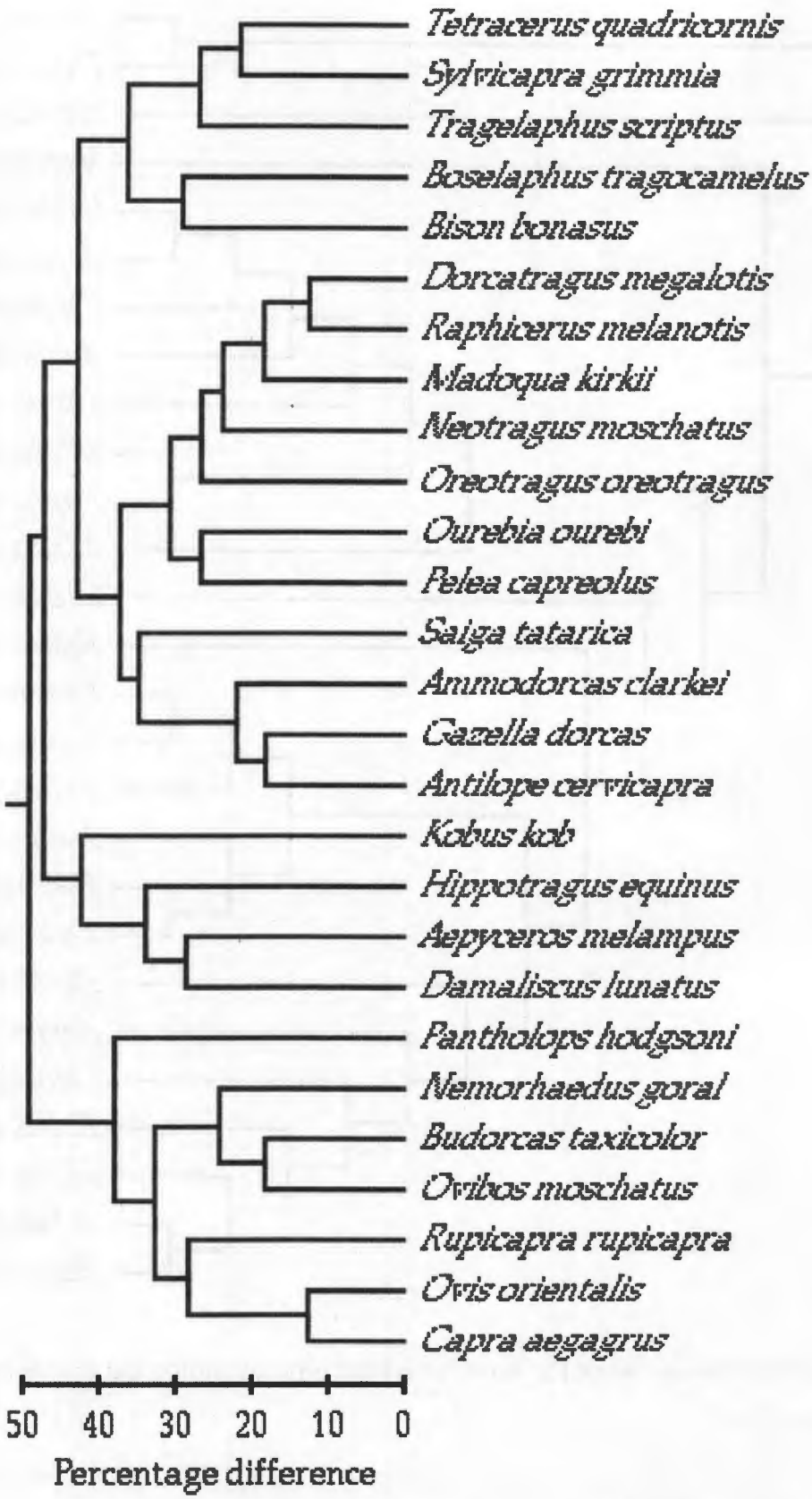


Figure 3a: Phylogeny of the Bovidae based on morphological characters. Phenogram after Gentry (1992).

genus *Gazella* is very problematical, the slight differences between species being obscured by considerable geographical variation within species. The most recent view is that the tribe Antilopini contains 20 species, of which 13 belong to the genus *Gazella* (Table 1). The taxonomy of Antilopini has long been a source of controversy, and as a result many publications use different classifications (Benirschke and Kumamoto, 1988; Corbet, 1978; Corbet and Hill, 1991; Groves, 1969; Groves, 1985; Groves, 1988; Grzimek, 1968; Haltenorth and Diller, 1988; Honacki et al., 1982; Le Berre, 1990; O'Regan, 1984; Spinage, 1986; Vrba, 1985; Walther et al., 1983). See Groves (1969) for a historical review. Descriptions of pelage coloration and measurements of skulls and bones are common features used as a basis for classification (Groves, 1969). In Antilopini these features often overlap, leading to different classifications by different authors and it is not clear which species are closely related to each other. Even today, subspecies and species are being reclassified, because many disagreements about taxa are of the subspecies versus species type. For instance, it has been reported that *G. pelzelni* could be a subspecies of *G. dorcas*, and should therefore be renamed *G. dorcas pelzelni* (Gentry, 1964; Groves, 1981). It has also been reported that *G. thomsonii* and *G. rufifrons* are close relatives (Gentry, 1964; Groves, 1985). All these uncertainties are responsible for the fact that the phylogenetics of this tribe show little common agreement.

There are six genera with 13 species in the tribe Neotragini (Table 1). In the genus *Raphiceros R. melanotis* and *R. sharpei* are generally considered to be separate species, but have been reported as conspecific (Haltenorth and Diller, 1988). The classification of the genus *Madoqua* is even more confusing. Some authors regard *M. phillipsi* and *M. swaynei* to be synonyms for *M. saltiana* and recognise *M. piacentinii* as a separate species (Ansell, 1971; Corbet and Hill, 1991). Other authors treat *M. phillipsi, M. swaynei* and *M. saltiana* as separate species and do not recognise *M. piacentinii*, placing it with *M. swaynei* (Haltenorth and Diller, 1988; Smithers, 1983). Occasionally the six genera are separated into five different subfamilies, the Neotraginae, Madoquinae, Raphicerinae, Dorcatraginae, and Oreotraginae, effectively creating separate subfamilies for almost all genera except *Raphiceros* and *Ourebia* (Haltenorth and Diller, 1988).

Only one morphological phylogenetic study on Bovidae included all six Neotragini genera (Gentry, 1992). Data from 112 characters were used for both phenetic and cladistic

analysis. The cladistic analysis showed that the Neotragini clustered with the Antilopini, and that they were ancestral to the Antilopini, which is incongruent with the fossil record data. The phenetic analysis showed that the Neotragini and the Antilopini were sister groups, and it included *P. capreolus* in the Neotragini. It was also argued that the Neotragini were paraphyletic and that their name would disappear eventually.

The subfamily Bovinae consists of three tribes; the Bovini (cattle and buffaloes), the Boselaphini (nilgai and four-horned antelope), and the Tragelaphini (kudu, bushbuck etc.). The tribe Tragelaphini is generally considered to consist of one genus (*Tragelaphus*) with a total of nine species (Table 1), although some authors assign *T. eurycerus* and *T. oryx* to a separate genus, *Taurotragus* (Gentry, 1978; Georgiadis et al., 1990; Haltenorth and Diller, 1988; Vrba, 1975; Vrba, 1985). All species are large to medium-sized, with heights varying from 70 to 180 cm. There seems to be general consensus that this tribe is monophyletic (Gentry, 1978; Gentry, 1992).

Several species from two different families (Bovidae and Suidae) have been used for outgroup comparison with the three tribes of Bovidae (Antilopini, Neotragini, and Tragelaphini) which are the main focus of this project. See Table 1 for the species which were used as outgroups. The species used for outgroup comparison were studied opportunistically as samples became available, but they were purposefully selected from different subfamilies in the Bovidae. *P. capreolus*, although generally placed in its own subfamily Peleinae, was specifically chosen for its possible close relationships with Neotragini (Gentry, 1978; Gentry, 1992). The reason for choosing several outgroups within the family Bovidae was to avoid the effects that too few outgroups can have, since it has been shown that outgroups can have significant effects on the way the ingroups are arranged (Adachi and Hasegawa, 1995).

The classification of most of the species which were used as outgroups in this study has never been problematic, except for two of them, *Pelea capreolus* and *Aepyceros melampus*. *P. capreolus* has on occasion been put in the tribe Neotragini (Gentry, 1978; Gentry, 1992), in the tribe Reduncini (Simpson, 1945), in the subfamily Reduncinae (Haltenorth and Diller, 1988), and in its own subfamily Peleinae (Smithers, 1983; Vrba, 1985). *A. melampus* has been put in the tribe Alcelaphini (Gentry, 1978; Gentry, 1992), in the tribe Antilopini

(Simpson, 1945), in the tribe Reduncini (Ellerman et al., 1953), and in its own subfamily Aepycerotinae (Ansell, 1971; Haltenorth and Diller, 1988; Smithers, 1983; Vrba, 1985). *Saiga tatarica* (Saiga) has been classified as a member of the Antilopini, the Caprini, as well as a member of a separate tribe, the Saigini (Gentry, 1978; Gentry, 1992; Georgiadis et al., 1990; Vrba, 1985).

Order Artiodactyla		
Family Suidae		
Subfamily Suinae		
	Sus scrofa	*+
Family Tayassuidae		
Family Hippopotamidae		
Family Camelidae		
Family Tragulidae		
Family Moschidae		
Family Cervidae		
Family Giraffidae		
Family Antilocapridae		
Family Bovidae		
Subfamily Reduncinae		
Subfamily Hippotraginae		
Subfamily Caprinae		
Tribe Saigini		
	Saiga tatarica	
Subfamily Cephalophinae		
	Cephalophus natalensis	*+
Subfamily Peleinae		
	Pelea capreolus	*+
Subfamily Aepycerotinae		
	Aepyceros melampus	*+
Subfamily Alcelaphinae		
	Damaliscus lunatus	*+
Subfamily Bovinae		
Tribe Bovini		
	Bos taurus	*+
	Synceros caffer	*+
Tribe Tragelaphini		
	Tragelaphus derbianus	
	Tragelaphus oryx	*
	Tragelaphus eurycerus	*
	Tragelaphus angasii	*
	Tragelaphus buxtoni	*
	Tragelaphus imberbis	*
	Tragelaphus scriptus	*
	Tragelaphus spekii	*
	Tragelaphus strepsiceros	*

Tribe Boselaphini		
	Boselaphus tragocamelus	
	Tetracerus quadricornis	
Subfamily Antilopinae		
Tribe Neotragini		
	Dorcatragus megalotis	
	Madoqua guentheri	*
	Madoqua kirkii	
	Madoqua piacentinii	
	Madoqua saltiana	
	Neotragus batesi	
	Neotragus moschatus	*
	Neotragus pygmaeus	
	Oreotragus oreotragus	
	Ourebia ourebi	*
	Raphiceros campestris	*
	Raphiceros melanotis	*
	Raphiceros sharpei	
Tribe Antilopini		
	Ammodorcas clarkei	
	Antidorcas marsupialis	*
	Antilope cervicapra	*
	Litocranius walleri	*
	Procapra gutturosa	
	Procapra picticaudata	
	Procapra przewalskii	
Subgenus Nanger		
	Gazella dama	*
	Gazella granti	*
	Gazella soemmerringii	*
Subgenus Trachelocele		
	Gazella subgutturosa	*
Subgenus Gazella		
	Gazella bennettii	*
	Gazella cuvieri	*
	Gazella dorcas	*
	Gazella gazella	*
	Gazella leptoceros	*
	Gazella rufifrons	*
	Gazella saudiya	*
	Gazella spekei	*
	Gazella thomsonii	*

Table 1: Classification of extant Artiodactyla, with names according to Corbet and Hill (1991). Species with an "*" behind their name are included in this study. Outgroups are marked with a "+".

1.3 Zoogeography.

Most extant Antilopini species live in Africa, some live in the Middle East and a few exist in Asia. See Figure 4 for their distribution maps. *G. dorcas* and *G. subgutturosa* are the most widely distributed, with most species only inhabiting relatively small areas (Grzimek, 1968; Haltenorth and Diller, 1988). Quite a few species are classified as endangered since their small range is associated with small population size. All Neotragini and Tragelaphini are found in the sub-Saharan region (Figures 5 and 6). Most Neotragini species are inhabitants of steppe or savannah, and species belonging to the genus *Neotragus* are the only thicket or forest dwelling species of the tribe. Most Tragelaphini inhabit forests, dense bush or bushy savannah, and are seldom found in open savannah (Haltenorth and Diller, 1988). Apart from *B. taurus* and *S. scrofa*, which are cosmopolitan due to dispersal by humans, all species used as outgroups in this study are African species (Figure 7). All shown distribution maps are based on published information (East, 1988; East, 1989; East, 1990; Grzimek, 1968; Haltenorth and Diller, 1988; Honacki et al., 1982; MacDonald, 1984; O'Regan, 1984).

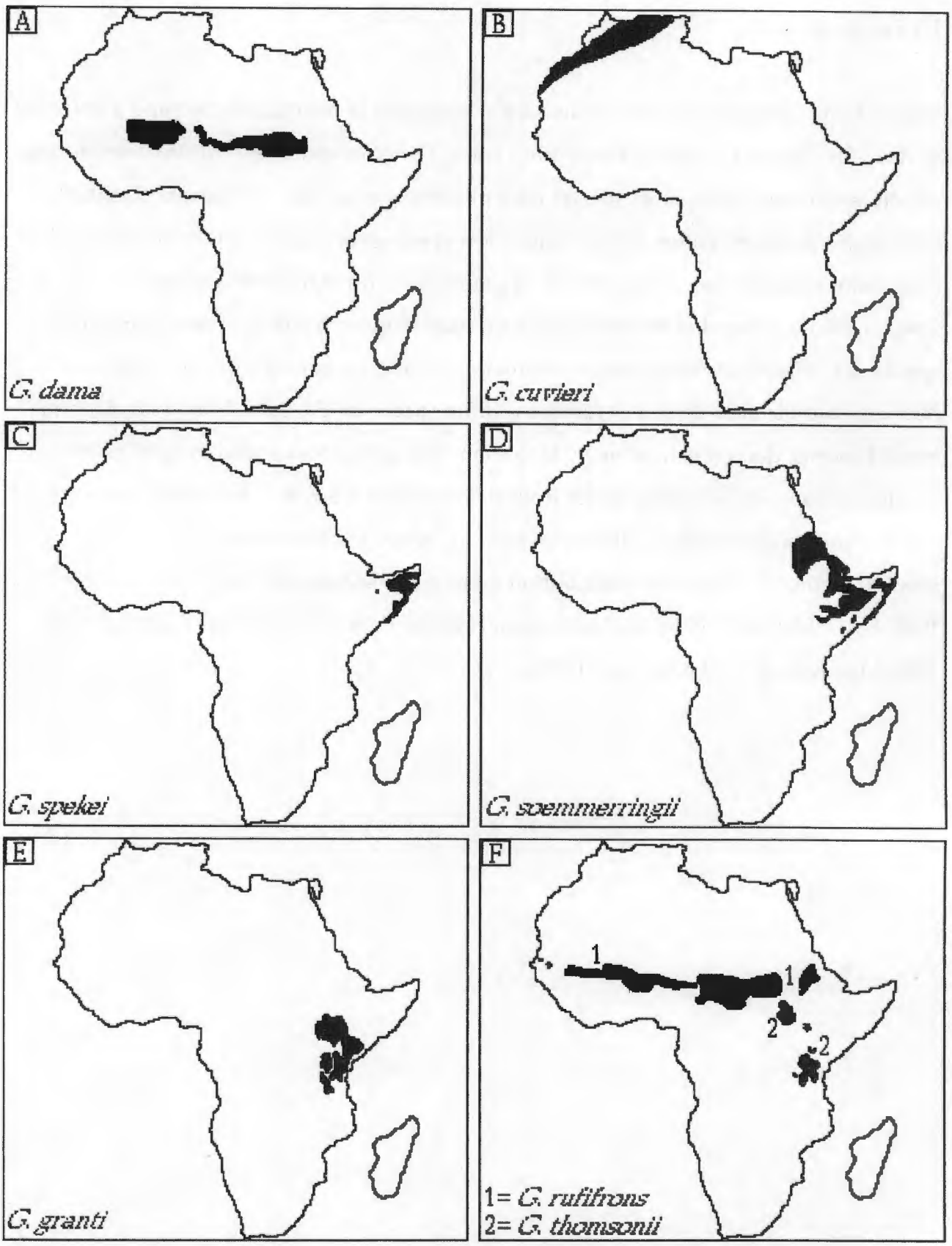
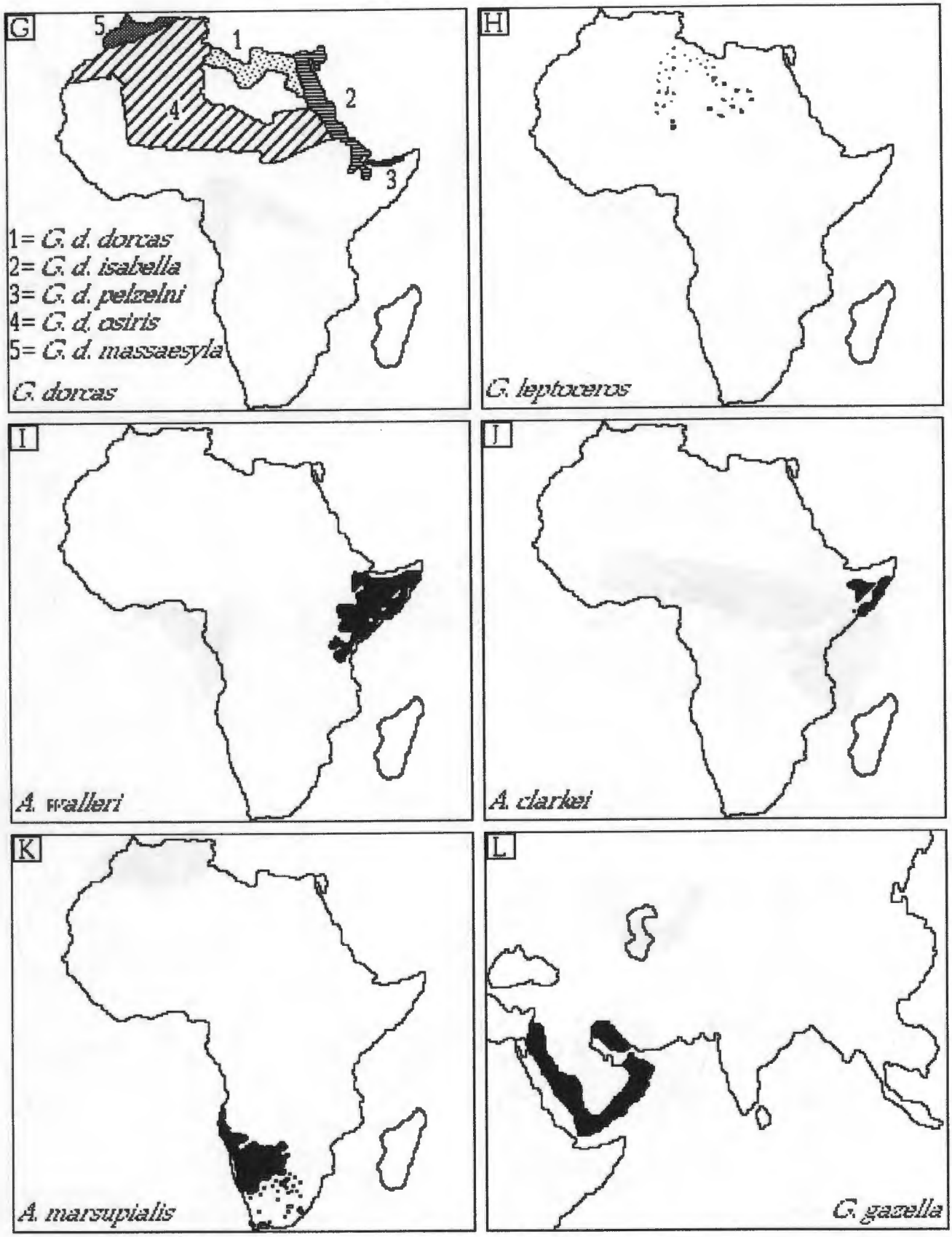


Figure 4: The distributions of the Antilopini, after East (1988, 1989, 1990).



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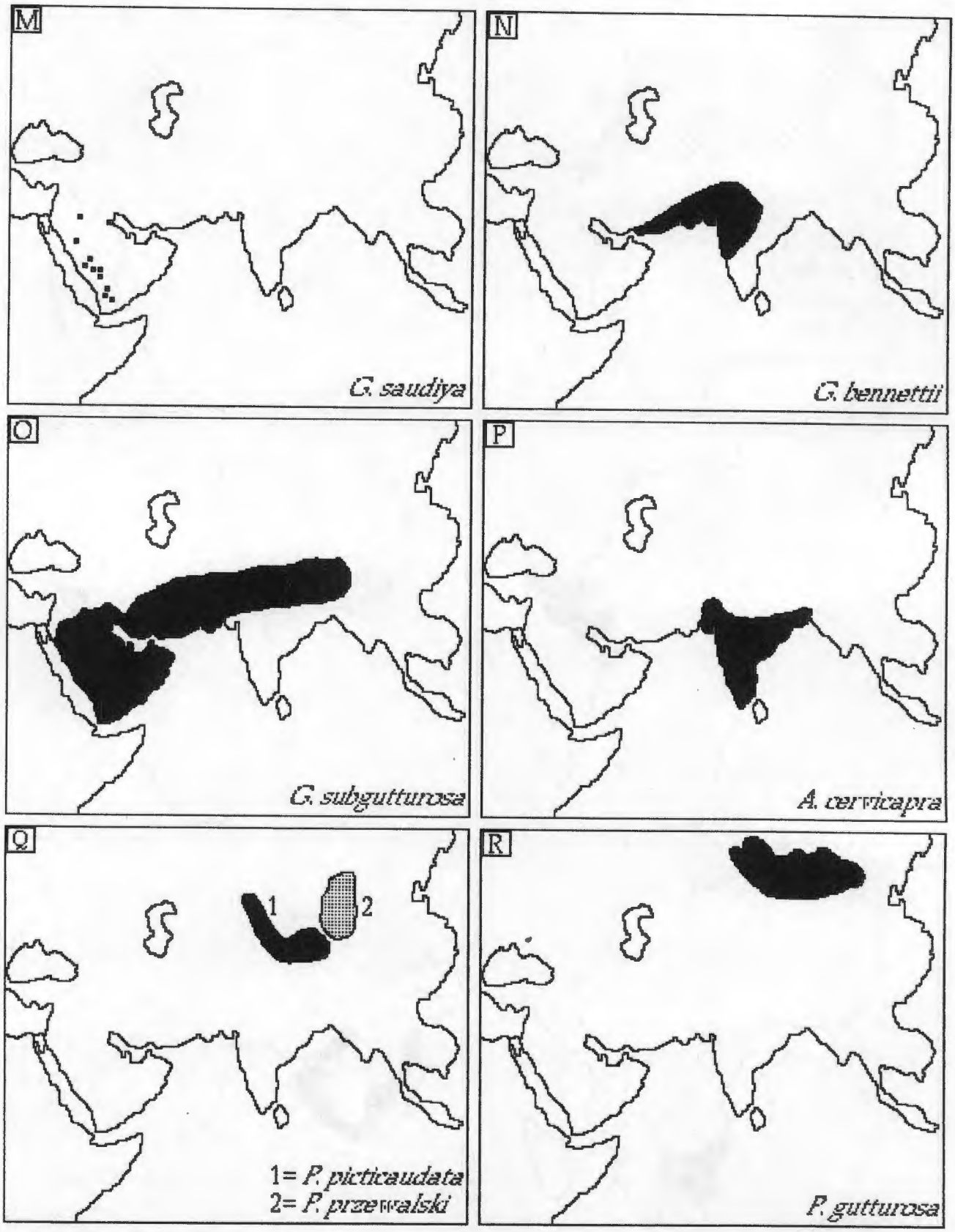

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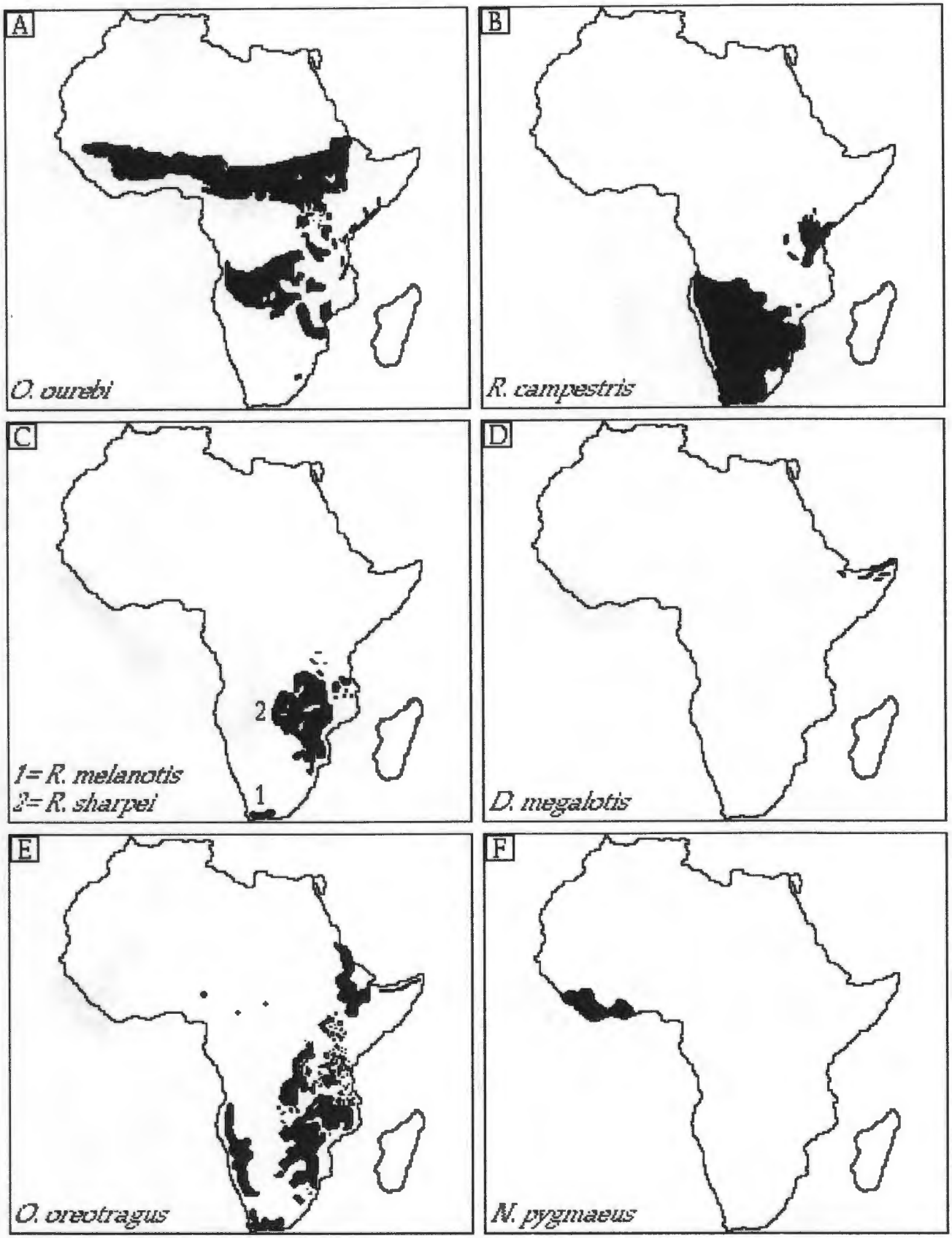
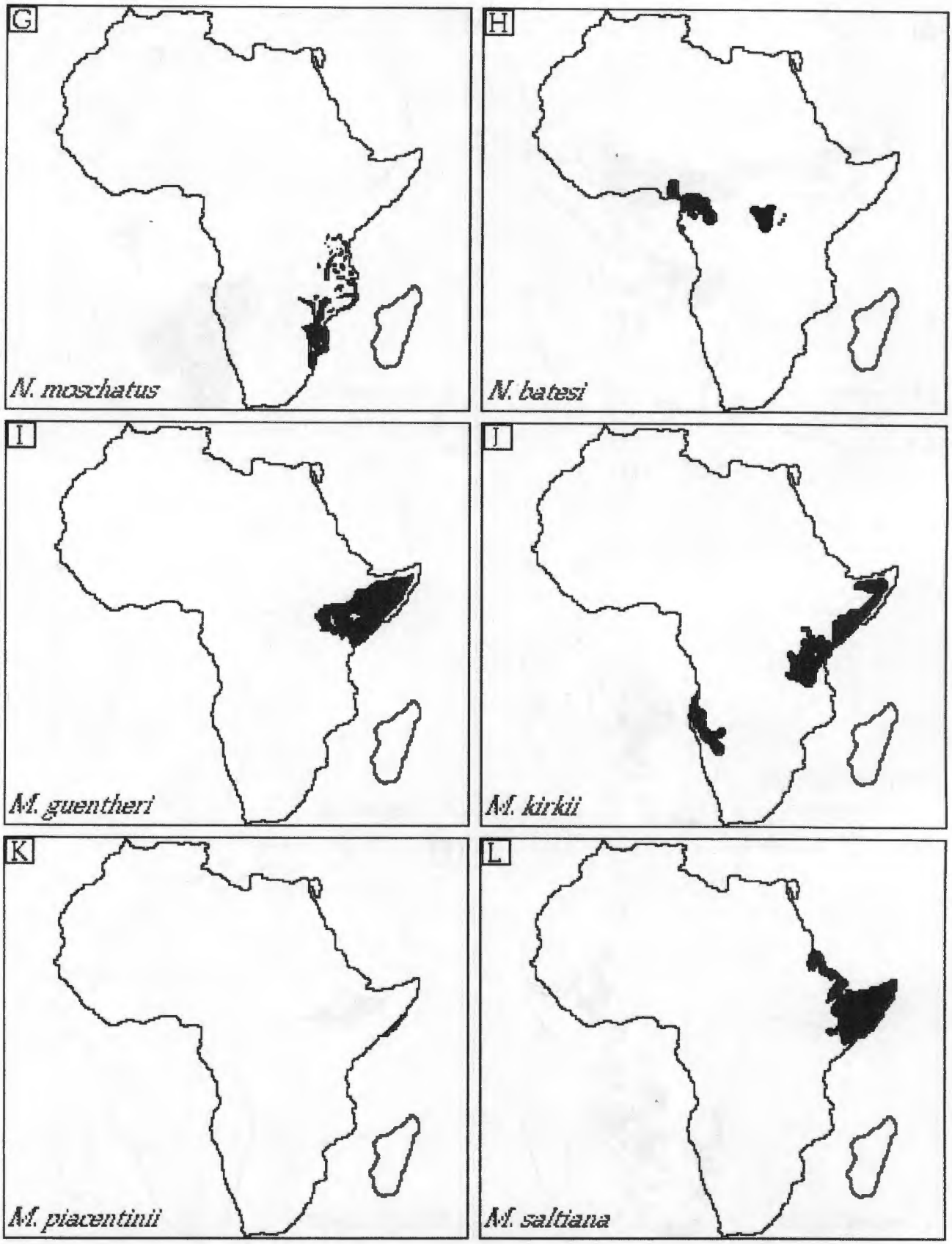


Figure 5: The distributions of the Neotragini, after East (1988, 1989, 1990).



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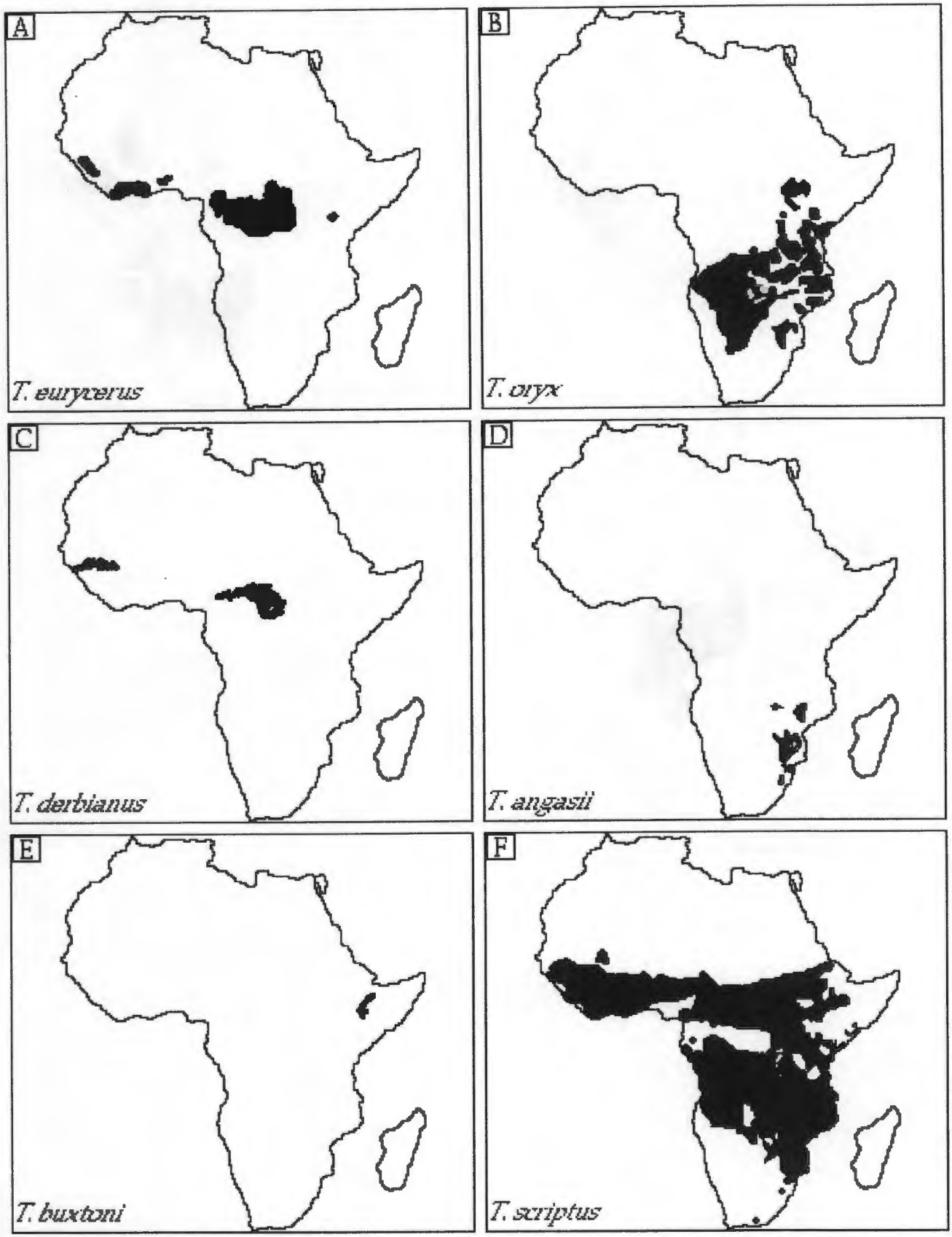
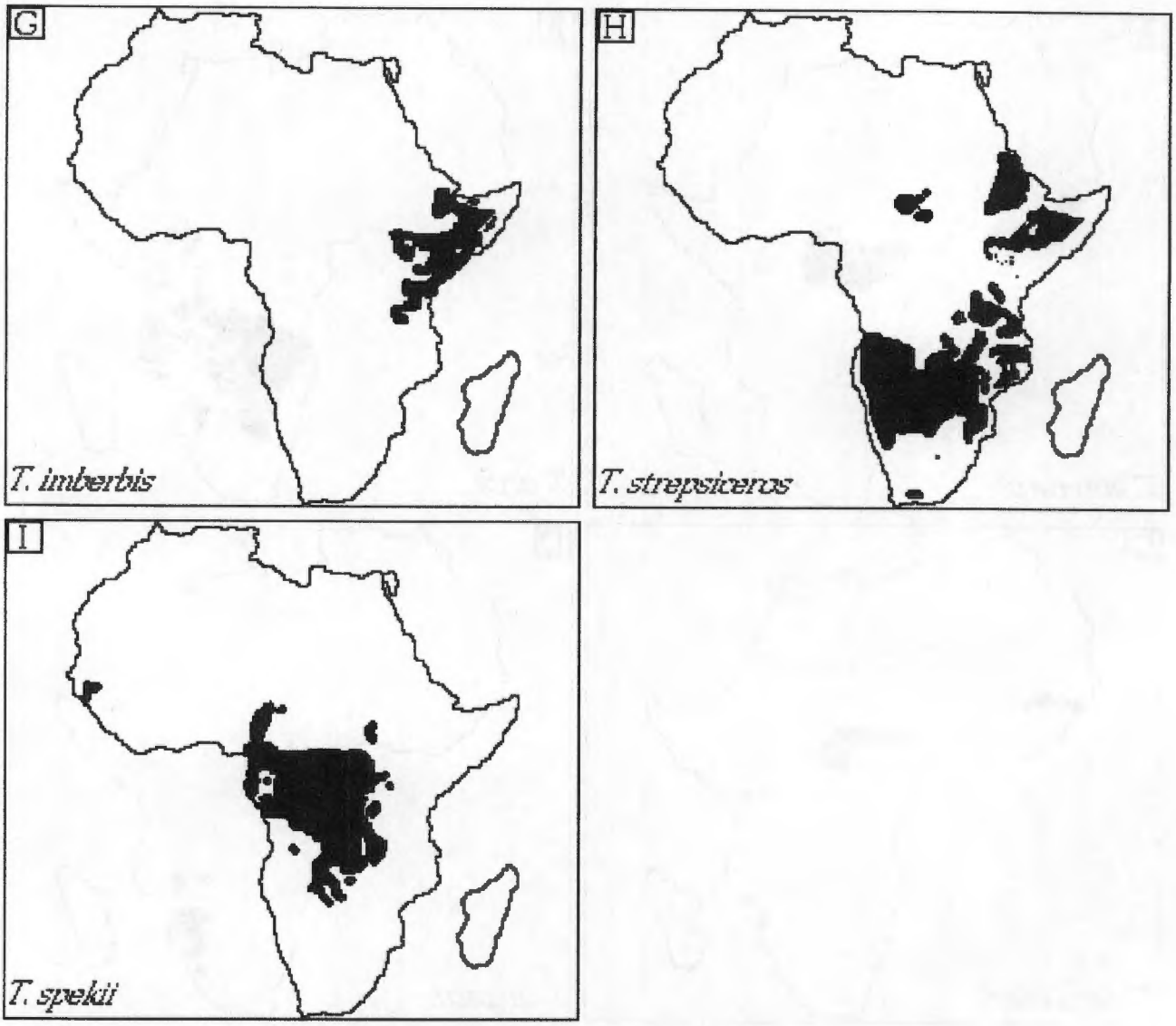


Figure 6: The distributions of the Tragelaphini, after East (1988, 1989, 1990).



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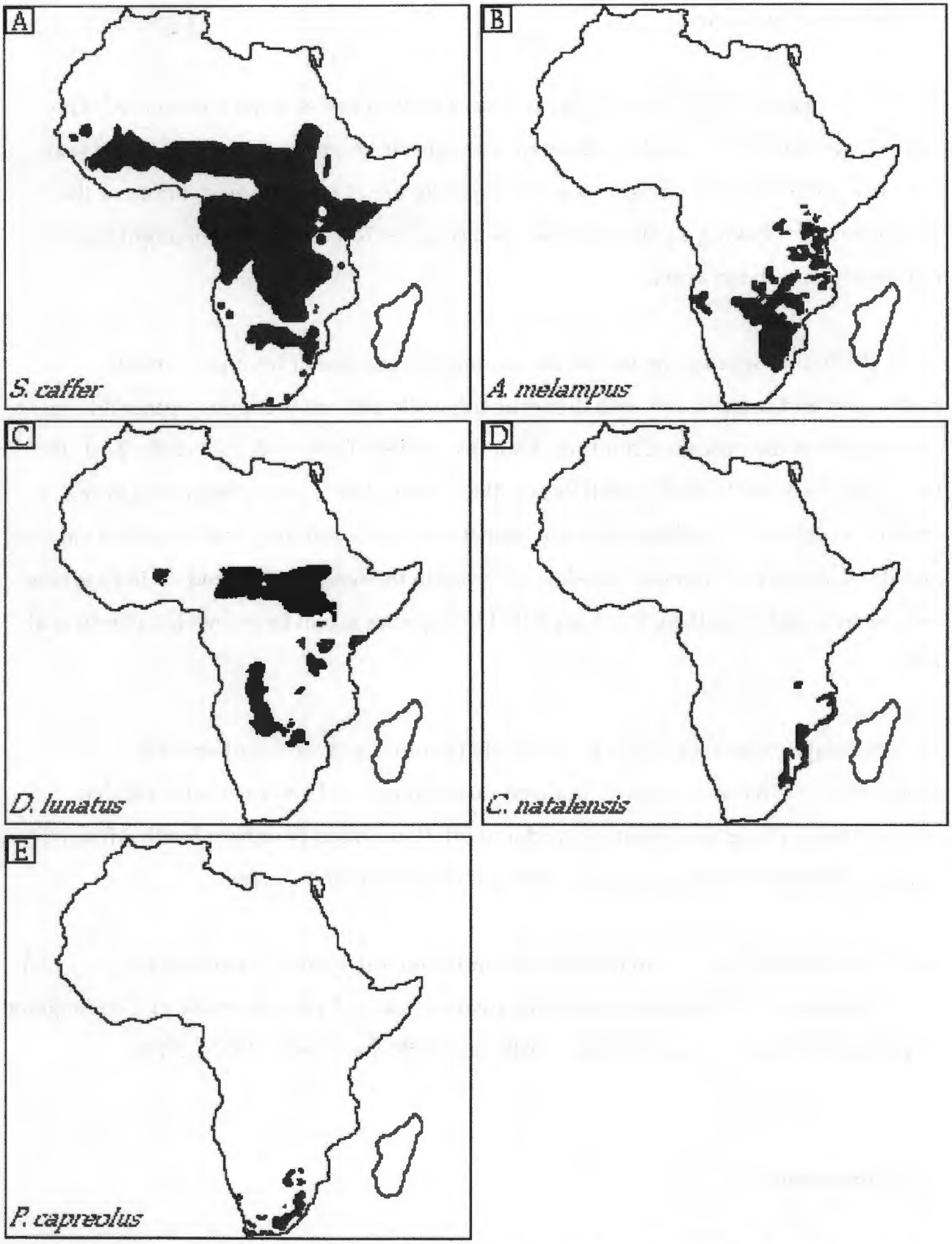

Figure 7: The distributions of some Bovidae species used as outgroups, after East (1988, 1989, 1990).

1.4 Conservation status.

S. scrofa, *B. taurus*, *S. caffer*, and *A. melampus* are widespread and not endangered. The same can be said for *D. lunatus*, although four subspecies are known, of which several have restricted ranges. *C. natalensis* and *P. capreolus* are not endangered, but have the disadvantage of having a patchy distribution (Figures 7d and 7e), which might lead to inbreeding in isolated areas.

Many Antilopini species are decreasing in numbers because of hunting, habitat destruction and competition with domestic livestock, and are becoming vulnerable or endangered in the process (Cloudsley-Thompson, 1992; East, 1988; East, 1989; East, 1990; East, 1992; East, 1993; IUCN, 1988; Ryder, 1987; Saleh, 1987). At the beginning of this century, *G. rufina* from Algeria became extinct because of hunting. Individuals of another gazelle, *G. bilkis* from Yemen, were last collected in the wild in 1951, and no individuals have been found since then. It is feared that this species might be extinct too (Greth et al., 1993).

Most Neotragini species seem to be relatively common and have widespread distributions. Three species have localised distributions and are vulnerable for that reason. These species are (Figures 5c, 5d and 5f): *R. melanotis* (southern South Africa), *D. megalotis* (northern Somalia), and *N. pygmaeus* (southern West Africa).

Most Tragelaphini species are widespread and common, although two species, *T. derbianus* and *T. buxtoni*, have a limited distribution and are vulnerable and endangered respectively (Figures 6c and 6e; East, 1988; East, 1989; East, 1990; IUCN, 1988).

1.5 Cytogenetics.

The Bovidae have not been studied extensively with cytogenetic techniques, and only when more species have been studied cytogenetically it could become feasible to infer the phylogeny of this family (Gallagher and Womack, 1992). Although some subfamilies have been studied thoroughly enough to ascertain the phylogenetic relationships within the

subfamily (see below), for most subfamilies more data need to be obtained. However, the karyotypes of most of the species included in the present study have been studied, although they have not been used in a phylogenetic context (Basrur and Gilman, 1964; Gallagher and Womack, 1992; Hard, 1969; Ulbrich and Fischer, 1967; Wallace and Fairall, 1967a). A species which has not been studied is *P. capreolus*, and it would be very interesting to study this species in the light of the different classifications used (see section 1.2). In *A. melampus* a polymorphism (a simple fusion between two acrocentrics) has been found, which does not seem to have any deleterious effects (Wallace and Fairall, 1967a). Many translocations have been described in *B. taurus* and *S. scrofa*, but this will be because many individuals have been studied in these species (Gustavsson, 1980). Most of these are Robertsonian translocations which are considered to have little or no deleterious effects on the individuals or their reproduction (Gustavsson, 1980).

Many cytogenetic studies have been carried out on Antilopini (Benirschke et al., 1984; Effron et al., 1976; Furley et al., 1988; Granjon et al., 1991; Kingswood and Kumamoto, 1988; Kumamoto and Bogart, 1984; Kumamoto et al., 1995; Rebholz et al., 1996; Rebholz et al., 1991; Vassart et al., 1993; Vassart et al., 1995a; Vassart et al., 1995b; Vassart et al., 1996; Wurster, 1972). Three studies have shown phylogenetic trees of Antilopini based on G-banded karyotypes (Benirschke and Kumamoto, 1988; Effron et al., 1976; Vassart et al., 1995b; Figure 8). These publications did not include all Antilopini species nor did they arrive at a single phylogeny. The major difference between these publications is the position of *G. rufifrons* and *A. cervicapra* in the trees. Two publications suggest that *G. rufifrons* is distantly related to the Nanger group (*G. dama, G. granti,* and *G. soemmerringii*), and that *A. cervicapra* is closely related to that group (Benirschke and Kumamoto, 1988; Effron et al., 1976), whereas Vassart et al. (1995b) suggest the opposite.

Only five species of the Neotragini have been studied cytogenetically, and a proper phylogenetic study has never been undertaken on this tribe (Benirschke and Kumamoto, 1987; Chandra et al., 1967; Seluja et al., 1985; Wallace and Fairall, 1967b).

All Tragelaphini species except *T. buxtoni* and *T. derbianus* have been karyotyped and the data used for phylogenetic analysis (see Benirschke et al. (1980) for a review). Figure 9 shows the phylogenetic tree as published by Benirschke et al. (1980). It shows that

T. spekii, *T. strepsiceros*, and *T. oryx* form a monophyletic group. The positions of *T. scriptus* and *T. eurycerus* in the tree are unresolved. That study also showed that *Boselaphus tragocamelus* (Nilgai) should by karyotypic criteria be included in the Tragelaphini. However, studies which used other techniques have placed it in a separate tribe, the Boselaphini (Allard et al., 1992; Gatesy et al., 1992; Gentry, 1978; Gentry, 1992; Lowenstein, 1986a; Lowenstein, 1986b; Vrba, 1985).

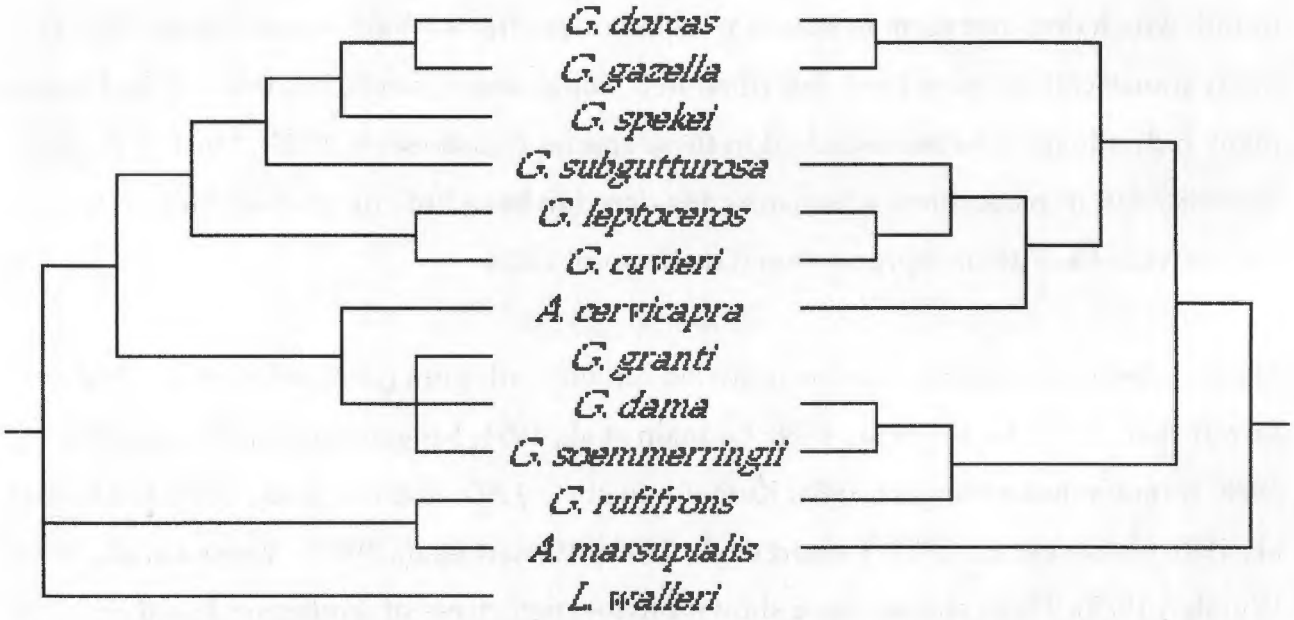

Figure 8: Phylogenetic tree for the Antilopini based on karyotypes, as published by Effron et al. (1976) and Benirschke et al. (1988), both on the left, and Vassart et al. (1995b) on the right.

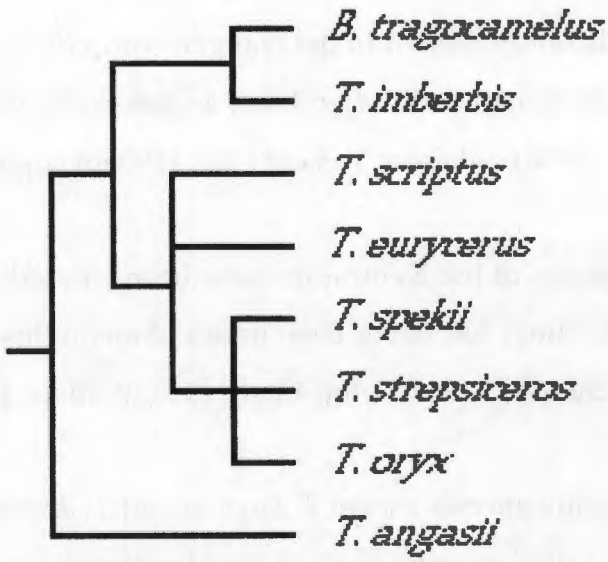

Figure 9: Phylogenetic tree for the Tragelaphini based on karyotypes, as published by Benirschke et al. (1980).

1.6 Molecular genetics.

Several authors doubt whether the family Bovidae is a monophyletic group (Gatesy et al., 1992; Irwin et al., 1991; Miyamoto and Goodman, 1986). The difficulty in establishing whether the Bovidae are monophyletic or not could be due to the rapid radiation of the Bovidae about 20 million years ago and the absence of continuous successions in the fossil record (Vrba, 1985). Some of the Bovidae species used as outgroups for this project have been studied before on the molecular level, but mostly on an ad hoc basis as representatives for their respective subfamilies (Allard et al., 1992; Gatesy et al., 1992; Georgiadis et al., 1990; Lowenstein, 1986b). It is therefore difficult to compare the results from this project with the results published previously.

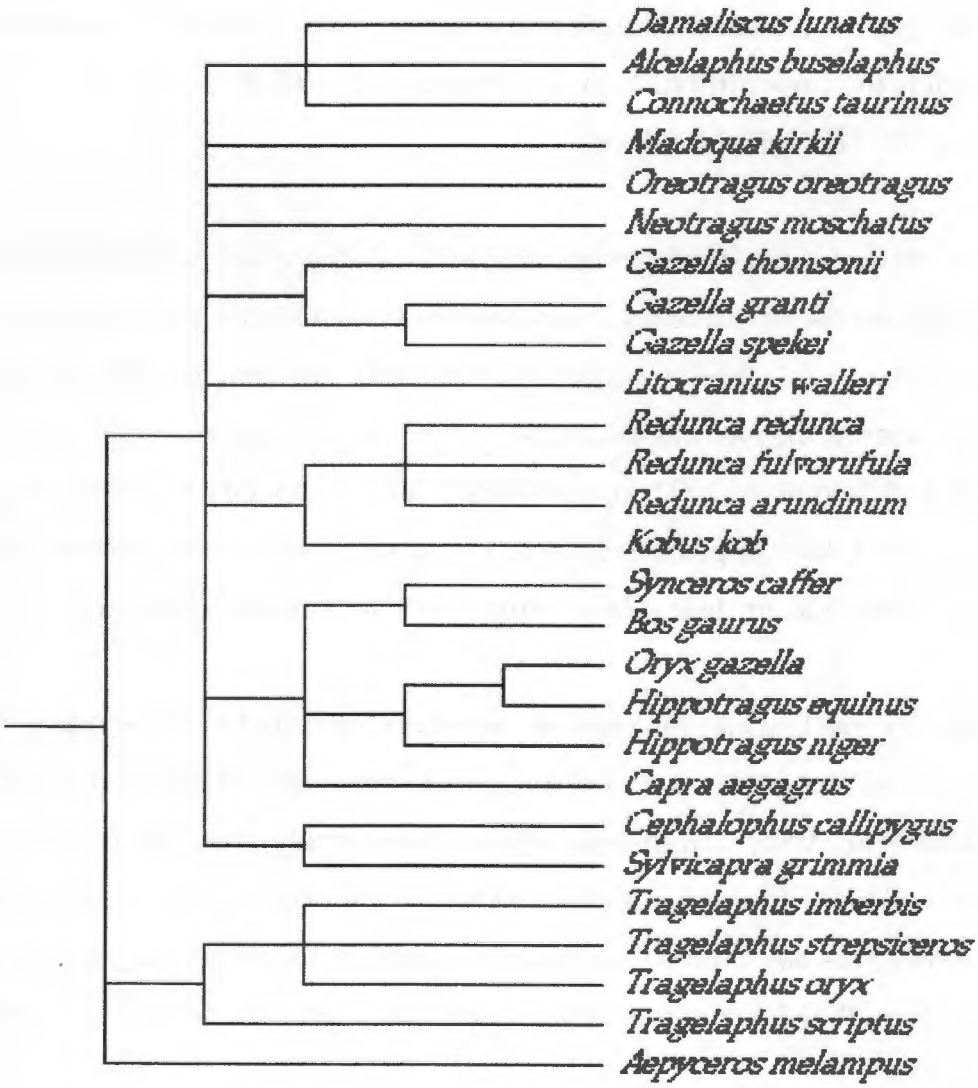

Figure 10: Phylogeny of some Bovidae based on allozymes, after Georgiadis et al. (1990).

The phylogenetics of the Bovidae have been studied in some detail with allozyme data (Georgiadis et al., 1990). Seven Antilopinae were included: *A. walleri*, *M. kirkii*, *O. oreotragus*, *N. moschatus*, *G. thomsonii*, *G. granti* and *G. spekei* (Figure 10). Only the three *Gazella* species were closely related. The relationships between several subfamilies and tribes (Antilopini, Neotragini, Cephalophinae, Reduncinae, Hippotraginae etc.) were unresolved. Immuno-distances have also been used to study the phylogenetics of the Bovidae (Lowenstein, 1986b). Five Antilopinae species were included in that study: *A. marsupialis*, *A. cervicapra*, *O. ourebi*, *G. dorcas* and *G. thomsonii* (Figure 11). Of these, *G. thomsonii* and *A. cervicapra*, appeared to be the most closely related species. *O. ourebi* stood apart from the other four species. It is difficult to make firm conclusions based on the results of the allozyme and immuno-distance studies mentioned above, because the overlap in studied species is small. The main difference between studies which use molecular genetic data and those which use morphological data for studies which include Antilopini is the close affiliation of *G. thomsonii* with either *A. cervicapra* or *G. dama* (Gentry, 1978; Lowenstein, 1986b).

There are no molecular phylogenetic studies available which include the majority of the Neotragini species, only a few species have been included in phylogenetic studies of the Bovidae (Allard et al., 1992; Georgiadis et al., 1990; Lowenstein, 1986b). Where only a single member of the Neotragini has been used, it came out as a sister species to the Antilopini (Allard et al., 1992; Lowenstein, 1986b). In a study where three members of the tribe were used, they appeared either to be paraphyletic in both phenetic and cladistic analyses of the data, or their relationships were unresolved (Georgiadis et al., 1990).

Although several Tragelaphini species have been included in molecular genetic studies at the tribal level, the group as a whole has not been studied before (Gatesy et al., 1992; Georgiadis et al., 1990; Lowenstein, 1986b). In one study, four species were included, *T. imberbis*, *T. oryx*, *T. scriptus*, and *T. strepsiceros* (Georgiadis et al., 1990). That particular study, which was based on allozyme data, concluded that the Tragelaphini are monophyletic, that *T. scriptus* was basal and that *T. oryx* was basal to *T. imberbis* and *T. strepsiceros*.

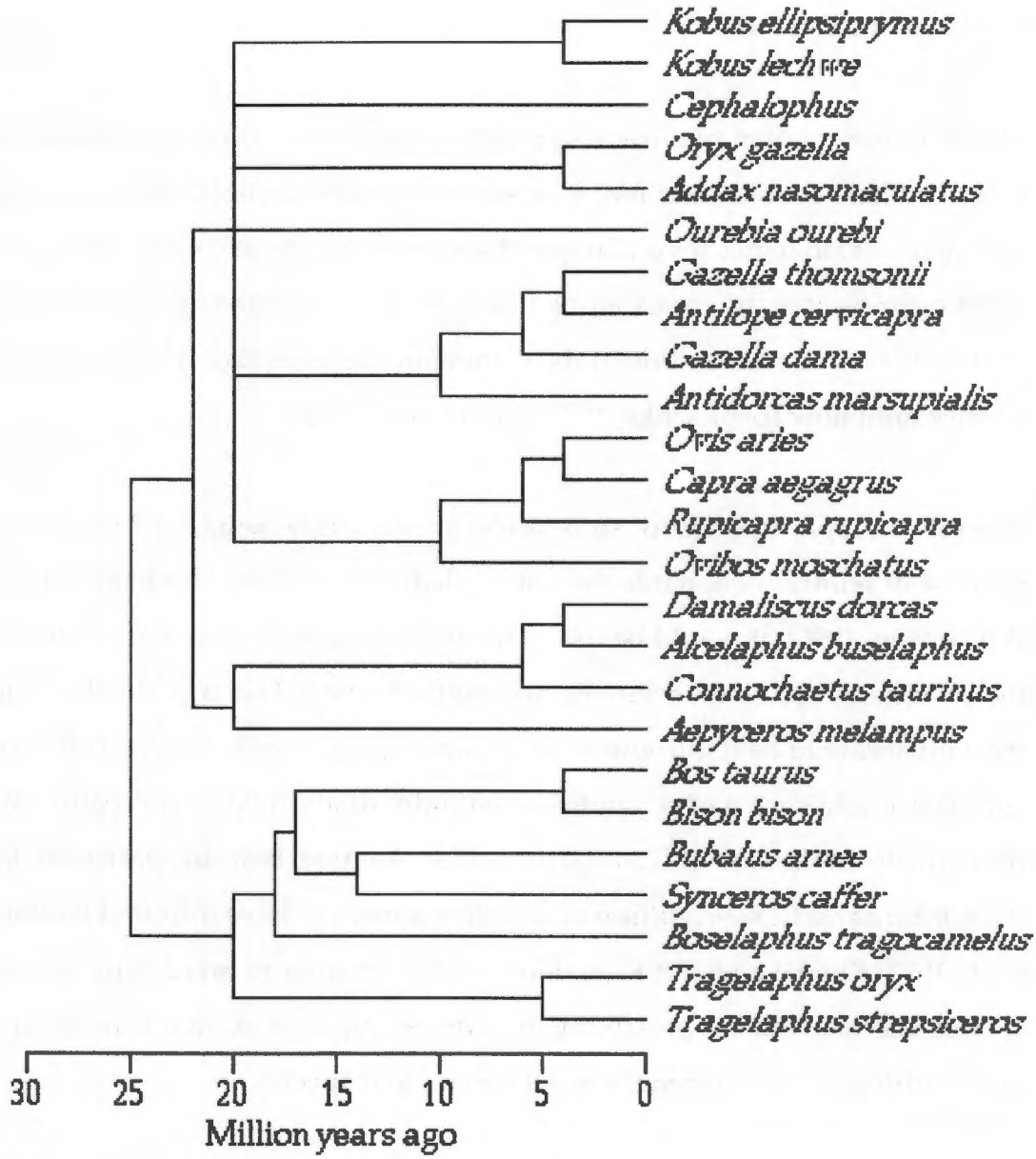

Figure 11: Phylogeny of some Bovidae based on immuno-distances, after Lowenstein (1986).

1.7 Aims of this study.

Effective conservation requires a sound taxonomic basis, therefore the results of the work described here should be of major importance for the implementation of conservation management strategies for endangered species of the three studied tribes. Molecular genetic approaches, by contributing to a definitive phylogenetic history of the three tribes, should assist in the resolution of the taxonomic status of taxa which have been difficult to classify until now (Benirschke, 1977; Furley et al., 1988).

Several Antilopini species (or subspecies) are currently being bred in captivity for the purpose of reintroduction into the wild (Alados et al., 1988; Dunham et al., 1993; Williamson, 1990). It would be useful to study the genetic make-up of these species with the techniques described below before reintroduction. This is particularly important when individuals are to be reintroduced in an area which already has a resident population of such species. In such a case, reintroduced individuals must be compatible with resident individuals. In the case of *G. subgutturosa* for example, two subspecies are known (*G. s. marica* and *G. s. subgutturosa*) and they appear to have different karyotypes (Granjon et al., 1991; Kingswood and Kumamoto, 1988). In order to breed pure subspecies and pure species and to avoid the possibility of outbreeding depression it is necessary to know the genetic differences between those subspecies and species.

The main aim of this study is to solve the following questions:

1) What are the phylogenetic relationships between species in the tribes Antilopini, Neotragini, and Tragelaphini ?

2) What is the timing of evolution of extant species of these tribes, and are these evolutionary patterns in accordance with the fossil record ?

3) Are the Neotragini paraphyletic, as suggested by several authors, and is *P. capreolus* a member of the Neotragini (Gentry, 1992; Georgiadis et al., 1990) ?

4) Is the tribe Neotragini the sister group to the Antilopini ?

5) **Which consistent divisions of species and subspecies are there in the tribes Antilopini and Tragelaphini ?**

6) **What is the taxonomic status of the endangered *G. saudiya* ?**

7) **Is the use of two genus names in the tribe Tragelaphini justified ?**

Chapter 2: MATERIAL AND METHODS.

2.1 **Samples.**

Samples from 172 live animals were collected for this project. From some animals both blood and skin samples were taken, but from most of them either blood or skin samples were taken. The samples were obtained from 13 different collections world-wide. Thirty-six dried skin samples from three species (seven subspecies altogether) were also obtained from three different collections. Appendix 1 contains all samples which were collected, although only a selection of them have been used for this project. Corbet and Hill (1991) has been used as a general guide for species names, except for two cases, *G. saudiya* and *G. thomsonii*. Subspecific names were used according to Groves (1985) and Groves(1988).

2.2 **Karyotyping.**

Before blood was collected, the animal's skin was disinfected with 70% alcohol. Then 5 to 10 ml blood was collected from the jugular vein in a sterile heparin vacuum tube. The tube was inverted a few times to mix heparin and blood well. All samples were kept refrigerated (- 4 °C), and cell cultures were mostly set up within 48 hours, to ensure that the white blood cells were still viable and to eliminate the chances of bacterial and fungal contamination of the samples.

To set up the cell cultures, 5 to 10 ml of blood was centrifuged for 10 to 15 minutes at 1500 rpm. In a sterile cabinet, 1.0 ml of the buffy coat was added to each polystyrene culture flask, containing: 8.5 ml Ham's F-10 medium (Gibco), 0.2 ml antibiotics (Gibco, penicillin 1000 U/ml, streptomycin 1 mg/ml), 0.3 ml pokeweed mitogen (Gibco, strength unknown) and/or 0.2 ml phytohaemagglutinin M (Difco, a 1% solution), using sterile syringes and needles for all solutions and blood. Pokeweed mitogen worked best for ungulates, because of a higher yield of dividing cells and it caused less agglutination than phytohaemagglutinin. The culture flasks were put in a CO_2 incubator at 37 °C for three days. After three days the medium was changed, but no extra mitogens added.

The fourth day 0.05 ml colcemid (Gibco, 10 µg/ml, final conc. 0.05 µg/ml) was added to the cultures. A hypotonic solution (0.075 or 0.067 M KCl) was put in a waterbath (at 37 °C) to warm up. One hour later, the contents of the culture flasks were poured in centrifuge tubes and they were spun down (1000 rpm for 10 minutes). After centrifuging, the medium was aspirated from the tubes, but a small quantity of medium was left on top of the pellet, and the pellet was gently resuspended. Five ml of the warm hypotonic solution was added to lyse the white blood cells and retain the cell nuclei. Centrifuge tubes were put in the waterbath for 10 minutes. After 10 minutes three drops of fixative were added and the tubes were spun down again (1000 rpm for 5 minutes). Afterwards the supernatant was aspirated from the centrifuge tubes, but a small quantity of medium was left on top of the pellet. The pellet was gently resuspended with a pasteur pipette and sucked up in the pipette, then 5 ml of a cold fixative mixture of methanol and acetic acid (3:1) was poured in the tube. The cell suspension was slowly dropped into the fixative and mixed well. The suspension was kept at 4 °C for at least half an hour. Prolonged fixation in the cold increased average chromosome length. The suspension was centrifuged (1000 rpm for 5 minutes) and the fixative was aspirated off, leaving a small amount of fixative on top of the pellet. The fixative was changed at least three times before making slides. The fixed cell suspension was used to make microscope slides. Fixed cell suspensions were stored at - 20 °C in closed cryotubes. This procedure was derived from the protocol in Belterman and De Boer (1984).

Slides were cleaned with ethanol and dipped in distilled water just before two drops of resuspended cell suspension were dropped on the slides. When almost dry, the slides were washed in water again and a few drops of fixative were put on the slide and left to dry. The chromosomes on the slide were stained for a few minutes in Giemsa (5% in a phosphate buffer) and then the slides were washed in phosphate buffer and distilled water. When the slides were dry a few drops of xylene and Malinol were put on the slide and then a coverslip was put on. Afterwards the chromosomes on the slide were photographed and the individual chromosomes were cut out from the photograph, arranged pairwise and glued on a chromosome card.

2.3 DNA extraction.

2.3.1 DNA extraction from fresh samples.

Both blood and skin samples (1.5 ml of fresh blood or 100 mg of skin) were used for DNA extraction.

Fresh or frozen blood was used. Blood was collected in vacuum tubes and 1.5 ml of that blood was added to 1.5 ml TNE with 150 µl 1M Tris HCl pH 7.5 , 80 µl of 25 % SDS, and 10 µl of 10 µg/ml proteinase K. Recipes for all buffers used for this study are given in Appendix 2. After every step in this procedure the mixture was mixed thoroughly. The mixture was incubated for 1 hour or more at 40 °C to lyse all cells. After incubation, an equal volume of phenol/TE pH 7.5 was added to remove haemaglobins and other proteins. This was then centrifuged for 10 minutes at 3000 rpm. The supernatant was taken off and phenol/chloroform (24:1) was added in equal volume to the sample, to remove the proteins completely. The sample was mixed for 5 minutes and centrifuged for 10 minutes at 3000 rpm. The supernatant was taken off and an equal volume of chloroform/iso-amylalcohol (24:1) was added to it to complete deproteination and remove the phenol. This was then centrifuged for 10 minutes at 3000 rpm and the top layer was taken off. To this top layer twice its volume of 100 % ethanol was added and mixed gently to precipitate the DNA. If it did not immediately precipitate, 100 µl of a saturated NaCl solution was added. The sample was centrifuged for 3 minutes at 3000 rpm and the ethanol decanted. The pellet was washed with 70% ethanol, and centrifuged for 3 minutes at 3000 rpm. The ethanol was poured off and the DNA pellet was left to dry overnight. The next day the DNA was dissolved in TE buffer and stored at - 20 °C.

A small piece of tissue (10 mm^2) was used for DNA extraction. This was cut up into smaller pieces and put in a 1.5 ml eppendorf tube with 600 ml TNES buffer and 20 µl of proteinase K (20 mg/ml). This was then incubated overnight at 55 °C. The next day, 166.7 µl of a 6M NaCl solution was added and the sample was shaken vigorously for 15 seconds. The sample was then centrifuged for 5 minutes at 12.000 - 14.000 g. The

supernatant was taken off and 1 volume of cold 95% ethanol was added. The DNA could be seen to precipitate out when the sample was gently inverted. Again the sample was centrifuged for 5 minutes at 12.000 - 14.000 g, and the pellet was rinsed with 95% ethanol. The pellet was dried with the tube inverted for 10 - 15 minutes and then resuspended in 100 - 200 µl TE buffer.

2.3.2 **Ancient DNA extraction.**

The following skin samples from museums were also used to extract DNA:

G. saudiya	40.315 female (British Museum of Natural History)
G. g. acaciae	M 6585 male (Tel-Aviv University)
G. d. isabella	M 4865 male (Tel-Aviv University)

The dried skin samples (approx. 1 gram) were first soaked in sterile water for 48 hours at 55 °C to make sure that all water soluble preservatives were leached out of the samples. The water was changed once after 24 hours. At that stage it was clearly visible that the water had changed in colour. After 48 hours the samples were cut up in smaller pieces and transferred to eppendorf tubes which contained TNES buffer and proteinase K, just as for fresh samples. The samples were incubated overnight at 55 °C and extracted using equal volumes of phenol/chloroform. After centrifuging this mixture, the supernatant was taken off, an equal volume of chloroform was added and the mixture was shaken well before centrifuging. The supernatant was then put into Centricon30 tubes and the total volume of the samples was made up to 2 ml with TE buffer. The Centricon30 tubes were spun for half an hour at 4500 g, according to the manufacturer's instructions. The samples were washed twice with 2 ml TE buffer and spun again. After the second wash the Centricon30 tubes were inverted and the 40 µl samples collected by centrifuging it for 2 minutes at 500 g. The samples were diluted to 400 µl with TE buffer and then used for PCR. Whenever the samples had to be exposed to air, this was done in a sterile hood in a laboratorium which has never been used to extract Antilopini samples, to prevent the samples from becoming contaminated.

2.4 PCR.

Part of the mitochondrial cytochrome *b* gene and the whole of the cytochrome c oxidase III (COIII) gene were amplified from the genomic DNA using the PCR (polymerase chain reaction) technique. The primers used for both PCR and sequencing, with nucleotide positions numbered according to the 3′ end of the cow sequence (Anderson et al., 1982) in brackets, were:

cytochrome *b*:

L (14491)	5′-TGATATGAAAAACCATCGTTG-3′
H (14917)	5′-CCTCAGAAAGATATTTGTCCTC-3′
L (15175)	5′-ATAGACAAAATCCCATTCCA-3′
H (15342)	5′-GATCGTAGGATTGCGTATGC-3′

cytochrome c oxidase III:

L5 (8945)	5′-CCTATGTATTCACTCTCCTAGTCAG-3′
L1 (8947)	5′-ATTCACTCTCCTAGTCAGCC-3′
L3 (9148)	5′-(CT)ACAATATA(CT)CAATGATGACGAG-3′
L2 (9151)	5′-ATACCAATGATGACGAGACG-3′
H2 (9618)	5′-GTTGTCGGAAAAAACATACG-3′
H3 (9638)	5′-(CT)TAGAGGT(AG)AAGTG(AG)AATTTT-3′
H1 (9781)	5′-GAAACTAGCTGATTGGAAGTC-3′

The cytochrome *b* gene primers were conserved primers modified from (Kocher et al., 1989). The two cytochrome *b* primers L (15175) and H (15342) were used to amplify 167 base pairs from three museum samples (see section 2.3.2). Primers in the flanking regions of the COIII gene were specifically designed for this project. They were designed with sequences from six vertebrate species of which the whole mtDNA was known (Table 2). A wide range of species was used to ensure that the chosen sites would be as conserved as possible. From the relatively conserved sites between all species the cow sequences were taken to design primers. Antilopini specific and Tragelaphini/outgroup specific internal primers were designed with sequences from several Antilopini and Tragelaphini/outgroups respectively when those sequences became available (Tables 3

and 4). The Tragelaphini/outgroup specific primers were made because the internal Antilopini specific primers did not work well for the Tragelaphini and the outgroups. All primers were tested for compatibility with the PC program Oligo (version 3.4, National Biosciences Inc., Plymouth MN, USA) and screened for their GC content.

```
Cow           TTGCAGTAGCTATAATCCAAGCCTATGTATTCACTCTCCTAGTCAGCCTA L1
Cow           TTGCAGTAGCTATAATCCAAGCCTATGTATTCACTCTCCTAGTCAGCCTA L5
White rhino   .C..C.....CC.G..T........C..G..T.....T.....A.....C
Fin whale     ....T..C..CC....T.....T..C.....T..C..T.....A......
Human         .C..T..C..CT............C..T.....A..T.....A.....C
Mouse         ..........AT....T........C........C........A......
Toad          .C..T.....A...........A..C......GTCT.A...T.A.....T

Cow           TATCTGCATGACAACACATA<-COIII-gene->CCTATTC-TTTTAGTA
White rhino   ..C..A...........C..<-COIII-gene->.......-........
Fin whale     ..C..T............<-COIII-gene->....G..C........
Human         ..C.....C.........<-COIII-gene->.T..C..-........
Mouse         .....A.....T..T.....<-COIII-gene->.T..C..CC-......
Toad          .....A..A..A...GTC..<-COIII-gene->.A..C.TTC-......

Cow           TTAACTAGTACAGCTGACTTCCAATCAGCTAGTTTCGGTCTAGTCCGAAA  H1
White rhino   ....AC......AT............ATC..C......AA.AC......
Fin whale     .....A......A.............T..........G..CC......
Human         .-..A......C.T.A.........T.A.......T.ACAACA.T.A...
Mouse         .-..T..A..T.A..............TAGA..CT.AATA.AC..AG..
Toad          .....C......CG............CAA...C.TA..TAGAAT.T..G
```

Table 2: Sequences of the six species that were used to design primers bordering the COIII gene. Dots were nucleotides identical to the top sequence.

```
              1         1         1         1         1
              4         5         6         7         8
              01234567890123456789012345678901234567890123456789

G. dorcas     TAACAACAAATATACTTACTATATACCAATGATGACGAGACGTAGTCCGA  L2
G. dama       ..............C.............................A.....
G. granti     ..........C.....C.............................A.....
G. gazella    ..................C..........................A.....
G. cuvieri    ........................T......................G
G. leptoceros ........................T......................G
G. bennettii  ..................C.....T................GA.....
G. saudiya    ........................T................GA....G
L. walleri    ..................C.....T..............A.CA.....
```

```
                1         2                                   6
                9         0                                   2
                0123456789012345-----------------4567890123456789

G. dorcas       GAAAGCACCTTTCAAG-rest-of-the-gene-CTTCATGTTATTATTG
G. dama         .C......T..C....-rest-of-the-gene-..C..C.....C....
G. granti       ........A..C....-rest-of-the-gene-..C........C....
G. gazella      ..............G.-rest-of-the-gene-..C.............
G. cuvieri      ................-rest-of-the-gene-..C.............
G. leptoceros   ................-rest-of-the-gene-..C........C....
G. bennettii    ..G.............-rest-of-the-gene-..C.............
G. saudiya      ................-rest-of-the-gene-..C.............
L. walleri      .....T..T.......-rest-of-the-gene-..C..C........C.

                6         6         6         6         6
                3         4         5         6         7
                01234567890123456789012345678901234567890123456789

G. dorcas       GATCCACTTTCCTAATCGTATGTTTTTTCCGACAACTAAAATTCCACTTT  H2
G. dama         ....A..C.....................................C
G. granti       ....A..C.....................................C
G. gazella      .......C.....................................T...
G. cuvieri      .......C...T.............................T.....C
G. leptoceros   .......C...T.............................T.....C
G. bennettii    .......C...T.........................G.....T.....C
G. saudiya      .......C.....................................T.....C
L. walleri      ......A...T.......C.....C..................T..T....
```

Table 3: Internal primers (underlined sequences) which were specifically designed for Antilopini. Numbers above the sequences were the base pair positions in the COIII gene. Nucleotides were numbered from the start of the COIII gene. Dots were nucleotides identical to the top sequence.

```
                  1         1         1         1
                  5         6         7         8
                  012345678901234567890123456789012345678901234567890123456789

T. eurycerus      CATACTTACAATATACCAATGATGACGAGACATCATTCGA  L3
T. scriptus       ............G.....................C..
T. oryx           ...G..............G.............C...
T. buxtoni        T..................................C...
T. angasii        T..G..C..............G.........G.C...
T. strepsiceros   ...................................C...
T. spekii         ..............T....................
T. imberbis       T.....G........T.............T..T..C...
S. caffer         T.............T.....G........T..T..C...
A. melampus       T.....C........T.............T..T..C...
P. capreolus      T.....C........T.............T..T..C...
C. natalensis     ..............T................TG.C...
D. lunatus        T.....C.....G..T..G...........T..T..C...
S. scrofa         A.CTT.A..........G..G...........T..C...

                  1         2                   6         6
                  9         0                   5         6
                  01234567890------------------01234567890

T. eurycerus      GAGAGCACCTT-rest-of-the-gene-TCTGCTTTTTC
T. scriptus       ..........-rest-of-the-gene-..........
T. oryx           ..........-rest-of-the-gene-....T......
T. buxtoni        ..........-rest-of-the-gene-.T.........
T. angasii        ..A..T.....-rest-of-the-gene-.........T
T. strepsiceros   ..A........-rest-of-the-gene-....T......
T. spekii         ..A........-rest-of-the-gene-.T.........
T. imberbis       ..........-rest-of-the-gene-.A.........
S. caffer         ..A.....T..-rest-of-the-gene-....T..C...
A. melampus       ..A.....T..-rest-of-the-gene-.T..T.....T
P. capreolus      ..A..T..T..-rest-of-the-gene-..........
C. natalensis     ..A..T.....-rest-of-the-gene-.......C...
D. lunatus        .....T.....-rest-of-the-gene-.......C..T
S. scrofa         ..A.....T..-rest-of-the-gene-.G.....AC.A
```

```
                    6         6         6         6         7
                    6         7         8         9         0
                    12345678901234567890123456789012345678 90

T. eurycerus        CGCCAACTAAAATTTCACTTTACCTCTAGTCATCACTTTG  H3
T. scriptus         ..T..........C.............................
T. oryx             ..............C.....C........C.....T....
T. buxtoni          ..............C.....C........C.....T....
T. angasii          ..............C.................C.......
T. strepsiceros     ..............C.....C..............T....
T. spekii           ................................C.......
T. imberbis         ..........G.....T..........C..C.......
S. caffer           ..T..........C..T..C.......AC..C.....C.
A. melampus         ..T..........C.....C..T.....C..C.......
P. capreolus        ..A.........................AC..C.....C.
C. natalensis       ......T.......C............AC..C..T....
D. lunatus          .....G..........T..C.....C.CC.....T....
S. scrofa           ..A..........C.....C..A..C.AC..C.....C.
```

Table 4: Internal primers L3 and H3 (underlined) which were specifically designed for Tragelaphini/outgroups. Double underlined nucleotides were degenerate sites (C/T or A/G). Nucleotides are numbered from the start of the COIII gene. Dots are nucleotides identical to the top sequence.

For all studied specimens 425 base pairs for cytochrome *b* and all 783 base pairs of COIII were amplified in the PCR reactions. PCRs were set up in 0.5 ml or 1.5 ml eppendorf tubes. Reaction volumes were 100 µl per tube and set up as follows:

	per 100 µl	final concentration
dNTPs (50 mM each)	4 µl	0.2 mM
primers (25 pM/µl)	2 µl	0.5 µM
10 x reaction buffer	10 µl	
(KCl 500 mM)		50 mM
(Tris-HCl 100 mM)		10 mM
$MgCl_2$ (25 mM)	12 µl	3 mM
H_2O	65.6 µl	
Taq DNA polymerase (5 U/µl)	0.4 µl	2 U/100 µl
DNA (0.1 x stock solution)	4 µl	

Initially the optimal $MgCl_2$ concentration was determined by adding different volumes of $MgCl_2$ to the reactions, with final concentrations ranging from 1 - 7 mM. In almost all experiments with samples from different individuals it was found that a final concentration of 3 mM $MgCl_2$ was optimal. Even with DNA polymerases from different suppliers ("Taq" - Stratagene, Promega; "Thermostable" - Advanced Biotechnologies LTD) and their slightly different PCR reaction buffers a concentration of 3 mM $MgCl_2$ always proved to be optimal. All PCR reactions were overlaid with three drops of mineral oil to prevent evaporation of the liquid. For the PCR reaction a Hybaid or Techne thermocycler was used and a typical PCR experiment used the following conditions:

30 cycles:	- 94 °C for 60 seconds	(dissociation of the dsDNA strands)
	- 55 °C for 60 seconds	(annealing of the primers to the ssDNA)
	- 72 °C for 60 seconds	(extension of the PCR product)
end with:	- 72 °C for 5 minutes	(final extension of all PCR products)
	- hold at 15 °C.	(end of the reaction and cooling down)

Several times a re-amplification step was done, when the initial PCR product was fainter than normal. The original PCR product was run on a gel, and a pipette tip was inserted into the gel where a band was seen. The piece of agarose in the pipette tip was put in 1 ml of water and left to dissolve for at least half an hour. From this, 4 µl was used for the re-amplification PCR. In the re-amplification PCR either the original primers were used again, or internal primers were used to amplify a smaller product. A typical re-amplification PCR was as follows:

15 cycles:	- 94 °C for 60 seconds
	- 55 °C for 60 seconds
	- 72 °C for 60 seconds
end with:	- 72 °C for 5 minutes
	- hold at 15 °C.

Three tubes of amplified products (a total of 300 µl) were concentrated with 100 µl of 10 % ammonium acetate and 800 µl of 100 % ethanol. This mixture was vortexed and put in a - 20 °C freezer overnight after which it was spun in a cooled centrifuge (- 20 °C) for 15

minutes at 15000 rpm. The supernatant was taken off and 1 ml of 100 % ethanol was poured on top of the pellet. After another round of centrifuging at 15000 rpm for 10 minutes, the supernatant was taken off and the pellet was left to dry for about 15 minutes. The pellet was not dried out completely, as it is difficult to dissolve dry DNA. The pellet was reconstituted in 40 µl of TE buffer and run on a 20 cm 0.6% agarose gel. The PCR product band was cut out and subsequently cleaned from residual primers and nucleotides using the Wizard PCR preps DNA purification system. The purification worked as follows:

An agarose slice of approximately 300 mg was put in an eppendorf tube, 1 ml of resin was added and then it was incubated at 65 °C for 5 minutes. When the agarose was melted, the solution was pipetted into a minicolumn (a 3 ml syringe with a Wizard filter unit attached) and gently filtered. After that 2 ml of 80 % isopropanol was pipetted into the syringe to wash the column. The filter unit was then put in a 1.5 ml eppendorf tube and centrifuged for 20 seconds at 12.000 g to dry the resin. Warm 20 µl TE buffer (70 °C, pH > 7.5) was added to the filter and after 1 minute the filter in the eppendorf tube was centrifuged for 20 seconds at 12.000 g to elute the bound DNA fragment from the resin. These 20 µl of purified PCR product were then stored at - 20 °C until they were used for sequencing.

The typical recovery with this method was (see booklet in the Wizard PCR prep kit):

dsDNA size (bp):	50	75	200	300	1500	3200
recovery (%):	2	3	69	99	96	≥ 60

2.5 DNA sequencing.

2.5.1 Sequencing.

The cleaned PCR products were sequenced with the chain-termination reactions (Sanger, 1981; Sanger et al., 1977) using a USB sequencing kit . For each sequencing reaction 5 µl of DNA template was added to a cocktail consisting of:

1.65 µl	H_2O
1.25 µl	primer (at 25 pM/µl)
1.0 µl	10x reaction buffer
0.75 µl	DMSO

This mixture was heated up to 95 °C for 3 to 5 minutes to denature the double stranded DNA, and then it was put in a - 20 °C freezer for 5 minutes to have the primers anneal to the template before the two strands could come together again. The sequencing itself was done in microtitre plates which were placed on a 40 °C heating block. To each reaction 4 µl of the following mixture was added:

1.0 µl	H_2O
0.35 µl	DMSO
0.45 µl	^{35}S-dATP
1.0 µl	0.1M DTT
0.2 µl	dG label mix
1.0 µl	Sequenase (8x diluted in enzyme dilution buffer)
4.0 µl	

The tubes were incubated at room temperature (approx. 20 °C) for 3 to 5 minutes. At that time the microtitre plate with the termination mixes (2.5 µl of each ddGTP, ddATP, ddTTP, ddCTP) were put on the heating block for 1 minute. To each well (with one of the four termination mixes) 2 µl of the above reaction template was added. After 5 minutes 2 µl Chase (see Appendix 2) was added to each well, to make sure that every reaction would be completed. After the next 5 minutes 4 µl of "stop mix" from the USB sequencing kit was added to stop the sequencing reaction. The microtitre plate was then wrapped in parafilm and Saran wrap and stored in a - 20 °C freezer until the samples were run on a sequencing gel.

2.5.2 Sequencing gels.

Sequencing gels were made of 40 grams urea, 10 ml of a 50% acrylamide solution, and

16 ml 5x TBE buffer for a 80 ml gel solution. The urea was first dissolved in TBE, during which care was taken not to let the solution heat up to more than 37 °C, otherwise it could polymerise too soon. The solution was then filtered through a number 540 Whatman filter and 10 ml acrylamide solution was poured on the same filter. After adding the acrylamide, the total volume was 80 ml. The acrylamide solution contained 380 grams acrylamide and 20 grams N,N'-methylene-bis-acrylamide per 600 ml.

One side of each glass plate was carefully cleaned with detergent and subsequently with ethanol, after which one of the plates was sprayed with Acrylease, to prevent the gel from sticking to both plates when taking the plates apart after the run was completed. The glass plates were treated with Acrylease every fifth time they were used. Gel spacers were used to make a 0.25 mm thin gel.

Just before pouring the sequencing gel, 400 µl of a 10% ammonium persulphate solution and 35 µl TEMED was added, to start the polymerisation. After the gel was poured a comb was inserted into the top part of the gel and the poured gel was allowed to polymerise for at least 60 minutes at room temperature. Gels were generally used within a day, but on some occasions gels were stored for up to 2 days before they were used. In those cases, the top of the gel was covered with paper towels that had been soaked in 1x TBE after polymerisation was complete. The top of the gel was covered in Saran wrapped and stored at room temperature.

Gels were normally run at approximately 2000 V, 50 mA, and at constant power (80 W) for approximately two hours after warming up for half an hour first. When the gel was warmed up, 2.5 µl of the sample (which was heated at 94 °C for 5 minutes) was loaded on the gel. After two to three hours the electrophoresis was terminated and one of the plates was taken off while the other plate with the gel attached to it was fixed for ten minutes in a solution of 10 % methanol, 10 % acetic acid, and 5 % glycerol. After fixing, the plate was taken out of the fixing solution and the gel was carefully dried with paper towels. The gel was taken off of the plate with 3MM Whatman chromatography paper, and then dried for about one hour on a vacuum gel drier.

2.5.3 Autoradiography.

The dried gels were screened with a Geiger counter to estimate how much radioactivity was present, so that the length of time of autoradiographic exposure could be more accurately estimated. Two types of film were used: Hyperfilm-ßmax and Hyperfilm-MP, which both gave good results. As a rule of thumb film was left down for a week when the Geiger counter registered less than 10 counts per minute (cpm). When it registered up to 20 cpm the film was left down on the gel for three days at least, but when it registered 50 cpm or more, the film was developed after one day.

2.6 Analysis of DNA sequencing results.

The autoradiographs were read manually and the sequencing data were entered into GeneJockey (Taylor, 1990). The sequences were aligned with Clustal V (Higgins et al., 1992) and the alignment was checked by eye. The aligned data were then put in a PAUP (Swofford, 1993) file and analysed. (See Chapter 4 for more detailed explanation of the analyses). MacClade (Maddison and Maddison, 1992) was used to see whether slightly longer trees would give results found by other authors. Treeview (Page, 1996) was used for the drawing of the trees. These five programs are all Macintosh programs and were executed on a Macintosh Quadra 650.

For the PC the programs DAPSA (Harley, 1996), RNA (Farris, 1994), and MEGA (Kumar et al., 1993) were used to align the sequences (where necessary) and generate phylogenetic trees respectively.

Chapter 3: CYTOGENETICS.

3.1 **Introduction.**

The make-up of the chromosomes is relatively characteristic for each species, and banding techniques enable identification of individual chromosomes, which is useful for comparing chromosomes within and between species. Chromosome banding patterns have been used to establish phylogenetic trees, since translocations and other chromosomal changes can be strong characters for assessing the relationships between different species. The changes that occur are inversions, deletions, additions, fissions, and fusions. They are of variable nature; they may reduce or increase the chromosome number (but not the gene content), and they may have the effect of inducing post-mating reproductive isolation and thus enhance speciation. Translocations of whole chromosomes, or portions thereof, occur spontaneously and have been detected in most species for which large numbers of individuals have been studied. See Ryder (1986) for a review of chromosomal polymorphisms in non-domestic mammals. Chromosome bands typically consist of 25 to 100 genes, depending on whether the chromosomes are in prophase or metaphase. If banding patterns do not change too often over time (so that homologues are still recognisable between species), it is relatively easy to assess relationships between species and construct phylogenetic trees, as shown for the Arvicolids (Modi, 1987). By comparing banding patterns on chromosomes of different species one can also establish the origin of changed chromosome parts. If two species carry identical banding patterns, not present in other species, both are likely to be derived from a common ancestor. Robertsonian translocations are strong phylogenetic informative characters (Gallagher and Womack, 1992). In special cases, for example when two species have identical metaphase banding patterns, high-resolution banding of prometaphase chromosomes can be used to look for differences not detectable in metaphase bands. See Sumner (1990) for a review of banding techniques and their applications.

Cytogeneticists still argue about whether chromosome fusions or fissions are the driving force behind chromosome evolution (Benirschke and Kumamoto, 1991). Some believe that chromosome fusions are the major influence in the evolution of the Bovidae and that the

basic or more primitive karyotype is represented by a diploid number of 60, 58 of which are acrocentric autosomes with X chromosomes being either large acrocentrics or metacentrics (Gallagher and Womack, 1992; Wurster and Benirschke, 1968). Others believe that chromosome fissions are more important in the evolution of the Bovidae, and their primitive chromosome number is believed to be 14 (Todd, 1975). However, it might be possible that both chromosome fusions and fissions played a major role in the evolution of chromosomes (Qumsiyeh, 1994). Both processes have their advantages: chromosome fissions lead to higher diploid numbers, which then leads to increased recombination and increased variability (Qumsiyeh, 1994). Fusions on the other hand, lead to decreased recombination and the fixation of new mutations that increase the potential for speciation (Qumsiyeh, 1994).

The Antilopini is a good group with which to study these hypotheses, because it is highly speciose and the diploid chromosome numbers vary between 30 and 60. Especially now that the group has been studied using morphometrics (Groves, 1969; Groves, 1985; Groves, 1996; Groves and Harrison, 1967), cytogenetics (Benirschke and Kumamoto, 1988; Effron et al., 1976; Vassart et al., 1995b) and molecular genetics (see Chapter 4) it is possible to combine all data and see whether there is directionality in the evolution of the chromosome numbers.

Robertsonian translocations (chromosome fusions or fissions) of autosomes have been found in several Antilopini species (*A. cervicapra, G. dama, G. soemmerringii, G. subgutturosa, G. bennettii,* and *G. saudiya*), and mainly in species of which quite a few individuals have been studied. Chromosomal rearrangements can easily go undetected when only a small number of individuals per species are studied. Most species in the Antilopini and the Tragelaphini have fusions between autosomes and X and/or Y chromosomes, which resulted in the XY_1Y_2 system (Wurster, 1972). This accounts for the differences between chromosome numbers found in females and males in most Antilopini species: males have one chromosome more than females. Diploid chromosome numbers in Antilopini vary from 30 (*G. granti, G. dorcas, G. subgutturosa, A. cervicapra*) to 60 (*L. walleri*). See Kingswood and Kumamoto (1996) for an up-to-date review of all published Antilopini karyotypes. Most species have chromosome numbers in the low 30's and only three species studied so far have high chromosome numbers (56 - 60; *G. thomsonii,*

A. marsupialis, G. rufifrons; Table 5). Only a few extensive banding studies on Antilopini have been published (Benirschke and Kumamoto, 1988; Effron et al., 1976; Vassart et al., 1995b), and although they generally support the taxonomy based on morphological characters, they also suggest that the subgenus Trachelocele, which only contains *G. subgutturosa*, is not a valid subgenus (Figure 8 and Table 1).

	2n
Antidorcas marsupialis	56
Antilope cervicapra	30 - 33
Gazella bennettii	49 - 52
G. cuvieri	32, 33
G. dama	38 - 40
G. dorcas	30, 31
G. gazella	34, 35
G. granti	30, 31
G. leptoceros	32, 33
G. saudiya	46 - 53
G. soemmerringii	34 - 39
G. spekei	32, 33
G. subgutturosa	30 - 33
G. thomsonii	58
Litocranius walleri	60

Table 5: All karyotyped species and their diploid chromosome numbers.

3.2 Results.

Twenty-two individuals belonging to six species have been karyotyped for this study. The species were: *G. dorcas isabella* (1.1), *G. dorcas* ssp. (2.2), *G. dorcas pelzelni* (2.2), *G. saudiya* (2.1), *G. gazella cora* (1.1), *G. thomsonii* (0.1), *G. subgutturosa marica* (3.2), and *Antilope cervicapra* (0.1) (Table 6). The numbers in brackets represent the number of males and females respectively. Blood samples from these species were obtained from three different collections:
King Khalid Wildlife Research Centre (KKWRC, Thumamah, Saudi Arabia), The Al Wabra collection (Qatar), and Whipsnade Wild Animal Park (Dunstable, England). The methods used to obtain karyotypes from the samples of these animals are described in Chapter 2.2. Conventional Giemsa staining was used for this study.

	Sex	ID	2n	Origin
Antilope cervicapra	female	F274	31	India
G. dorcas isabella	female	A76	30	Sudan
G. dorcas pelzelni	female	591	30	Somalia
	male	594	31	Somalia
G. dorcas ssp.	male	577	31	Saudi Arabia
	male	578	31	Saudi Arabia
	female	579	30	Saudi Arabia
G. gazella cora	male	G112	35	Saudi Arabia
G. thomsonii	female	T56	58	Kenya ?
G. subgutturosa marica	male	R633	31	Saudi Arabia
	male	R230	32	Saudi Arabia
	female	R284	31	Saudi Arabia
	male	R235	33	Saudi Arabia
G. saudiya	male	D1	50	Qatar ?
	male	D3	51	Qatar ?
	female	D4	47	Qatar ?

Table 6: Specimens used for karyotyping.

The following results were obtained from these samples:

In a female *A. cervicapra* (2n = 31) a heterozygous translocation was found (Figure 12, the three chromosomes). Only banding studies can reveal which chromosomes are involved in the translocation. Although a similar translocation was also found in other specimens (Effron et al., 1976), it is not certain whether the translocation in that publication is the same as the one shown here. Most other published karyotypes are homozygous for the fusion (Effron et al., 1976; Vassart et al., 1995b; Wurster et al., 1968). A heterozygous translocation has not been shown before, probably due to the fact that only a few animals have been studied.

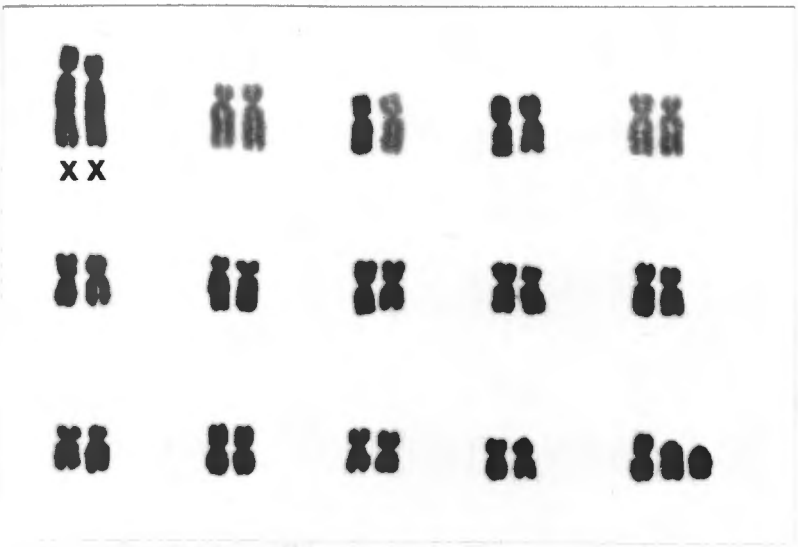

Figure 12: Karyotype of a female Blackbuck (*A. cervicapra*). The diploid chromosome number (2n) is 31.

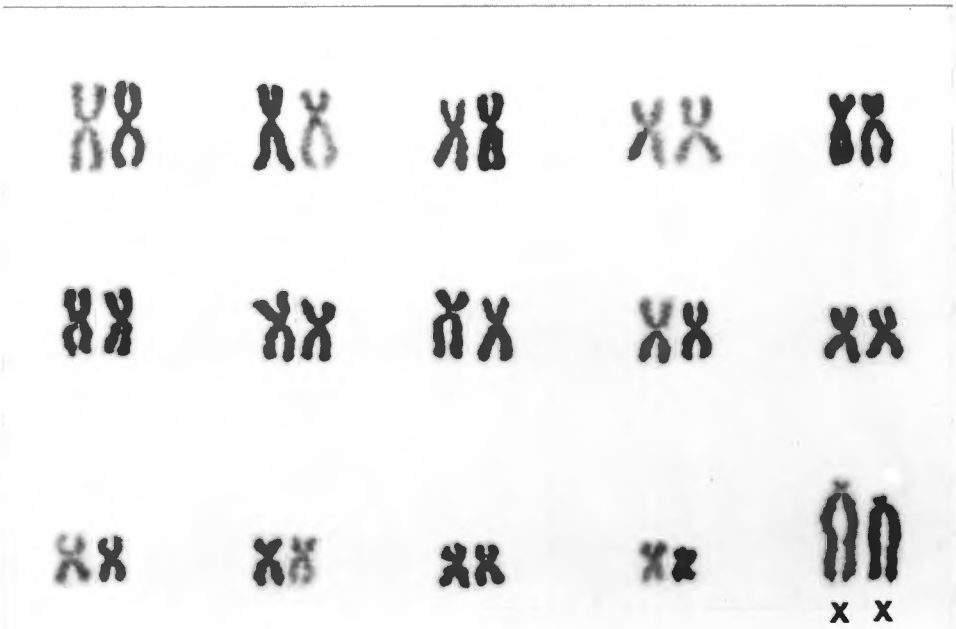

Figure 13: Karyotype of a female Dorcas gazelle (*G. dorcas isabella*). The diploid chromosome number (2n) is 30.

In the genus *Gazella*, *G. dorcas* is the only species with acrocentric X chromosomes, and are therefore readily distinguishable. The karyotype of the subspecies *G. dorcas isabella* (Figure 13) was identical to published karyotypes (Hsu and Benirschke, 1974b; Wurster, 1972). Karyotypes of the subspecies *G. dorcas pelzelni* are shown here for the first time. They are of the typical *G. dorcas* type; 2n = 30 and 31 for females and males respectively. All autosomes are metacentric, and X chromosomes are acrocentric (Figures 14a and 14b). The karyotypes from *G. dorcas pelzelni* cannot be distinguished from other *G. dorcas* subspecies. Several individuals which were found to belong to *G. dorcas*, could not be assigned to a particular subspecies, since they have not been studied morphologically. These individuals were probably caught in Saudi Arabia. A male from this *G. dorcas* ssp. group was found to have an XXY karyotype (Figures 15a and 15b), a pattern which is associated with Klinefelter syndrome in humans. All cells from that animal were of the XXY type. There is no doubt about the identification of the sex of the animal, which was assessed at the time of sampling. Two other animals from the same breeding group showed karyotypes which are typical for *G. dorcas*, and neither of them had a chromosomal abnormality (Figures 15c and 15d).

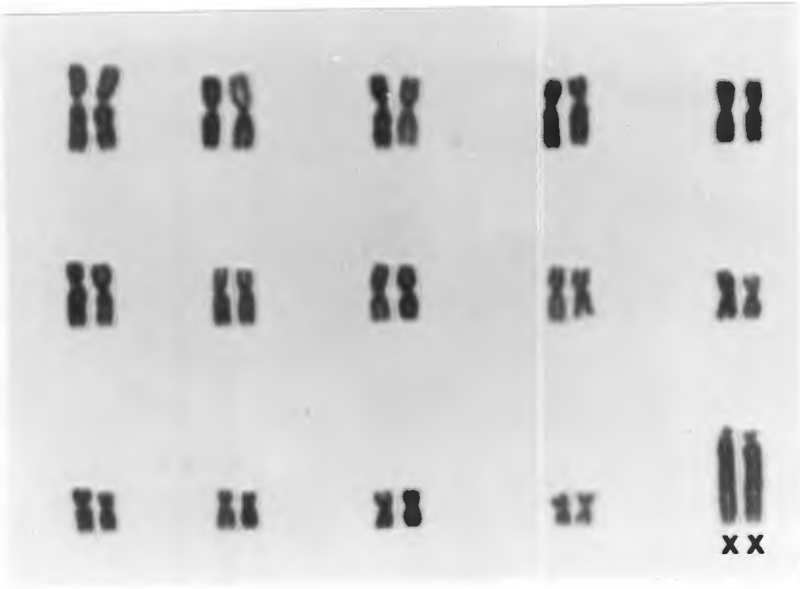

Figure 14a: Karyotype of a female Pelzeln's gazelle (*G. dorcas pelzelni*). The diploid chromosome number (2n) is 30.

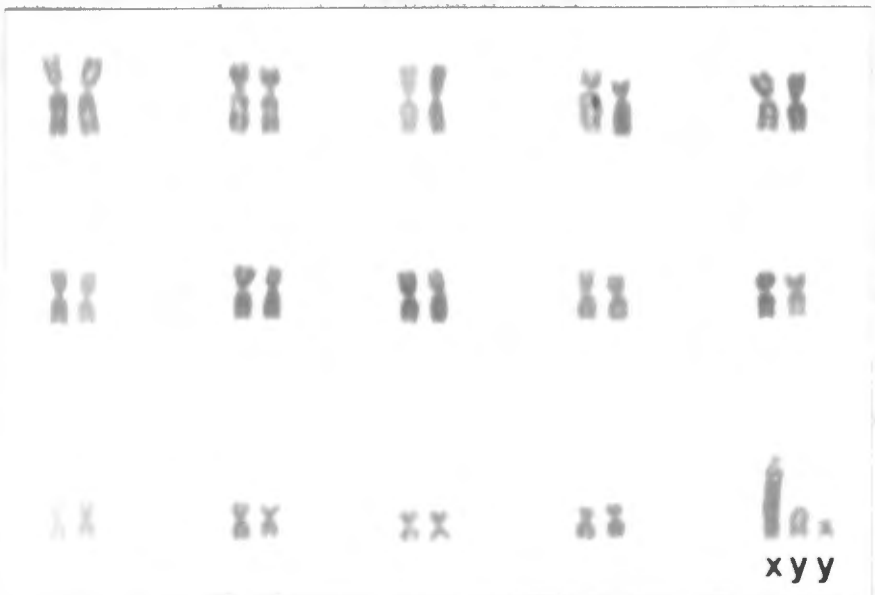

Figure 14b: Karyotype of a male Pelzeln's gazelle (*G. dorcas pelzelni*). The diploid chromosome number (2n) is 31.

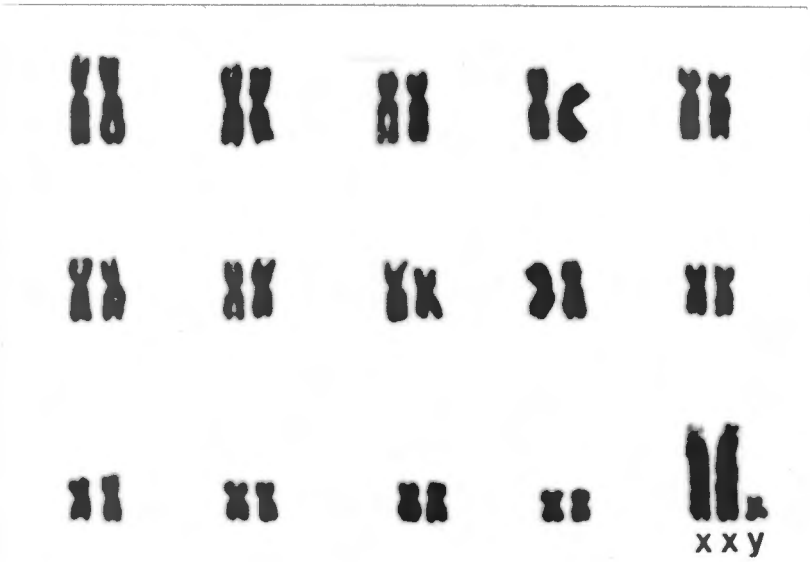

Figure 15a: Karyotype of a male Dorcas gazelle (*G. dorcas* ssp.) with a sex-chromosome abnormality. The diploid chromosome number (2n) is 31. The same individual as Figure 15b.

Figure 15b: Karyotype of a male Dorcas gazelle (*G. dorcas* ssp.) with a sex-chromosome abnormality. The diploid chromosome number (2n) is 31. The same individual as Figure 15a.

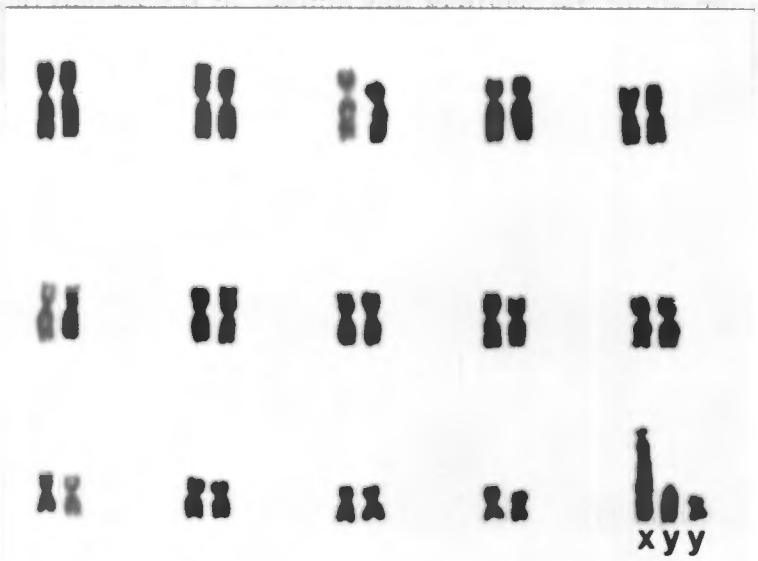

Figure 15c: Karyotype of a male Dorcas gazelle (*G. dorcas* ssp.). The diploid chromosome number (2n) is 31.

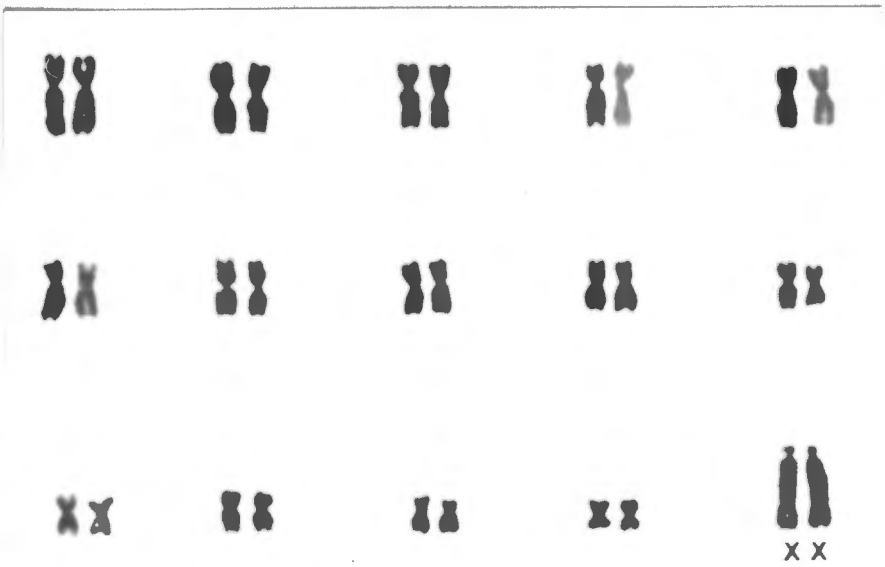

Figure 15d: Karyotype of a female Dorcas gazelle (*G. dorcas* ssp.). The diploid chromosome number (2n) is 30.

The karyotype of *G. g. cora* (Figure 16) is identical to karyotypes published elsewhere (Vassart et al., 1995a; Vassart et al., 1995b) which have 2n = 34, 35 for males and females respectively. Little is known about the origin of this animal, as it was part of a private collection in Saudi Arabia.

Figure 16: Karyotype of a male Mountain gazelle (*G. gazella cora*). The diploid chromosome number (2n) is 35.

Only a few karyotypes of *G. thomsonii* have been published before (Effron et al., 1976; Nelson-Rees et al., 1967; Vassart et al., 1995b) and that of the female presented here (Figure 17) is identical to the published karyotypes. Vassart et al. (1995b) differ with the other published karyotypes in that they conclude that the large acrocentric chromosomes are the X chromosomes. The male *G. thomsonii* karyotype published by Nelson-Rees et al. (1967) does not show conclusively which chromosomes are the sex-chromosomes.

In *G. s. marica* from KKWRC a Robertsonian translocation involving chromosome pair 1 was found (Figures 18a to 18d). This is a simple fission (or fusion) of one or both of the chromosomes of this particular pair. Such simple translocations occur in other Bovidae too and do seem to have a slightly negative effect on the fecundity of the carriers (Gustavsson, 1980).

The karyotypes of three *G. saudiya* individuals have been described in Chapter 5 (Figures 19a, 19b, and 19c). Karyotypes and sequences show it to be likely that some of these animals are hybrids between *G. bennettii* and the original *G. saudiya* (Kumamoto et al., 1995; Rebholz et al., 1991).

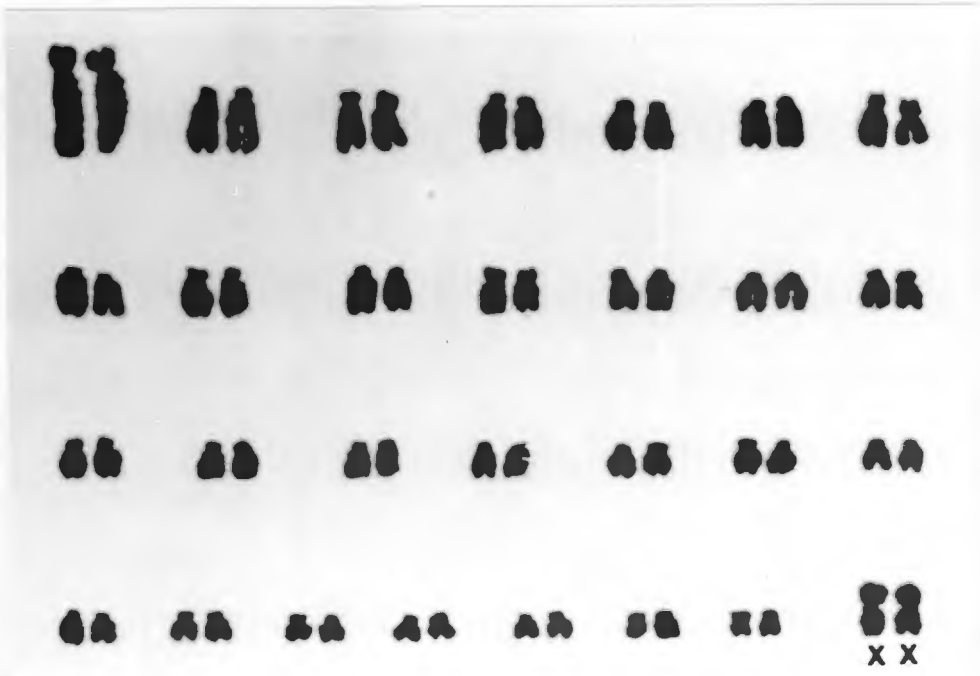

Figure 17: Karyotype of a female Thomson's gazelle (*G. thomsonii*). The diploid chromosome number (2n) is 58.

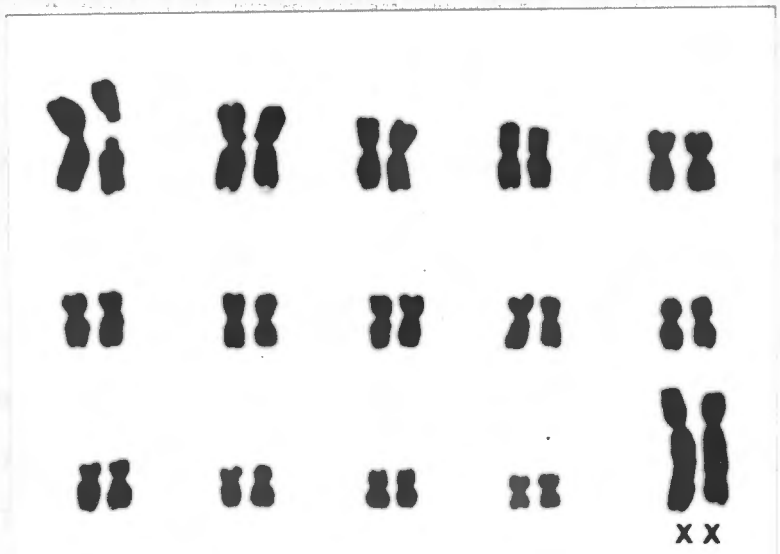

Figure 18a: Karyotype of a female Sand gazelle (*G. subgutturosa marica*) with pair 1 consisting of one metacentric and two acrocentric chromosomes. The diploid chromosome number (2n) is 31.

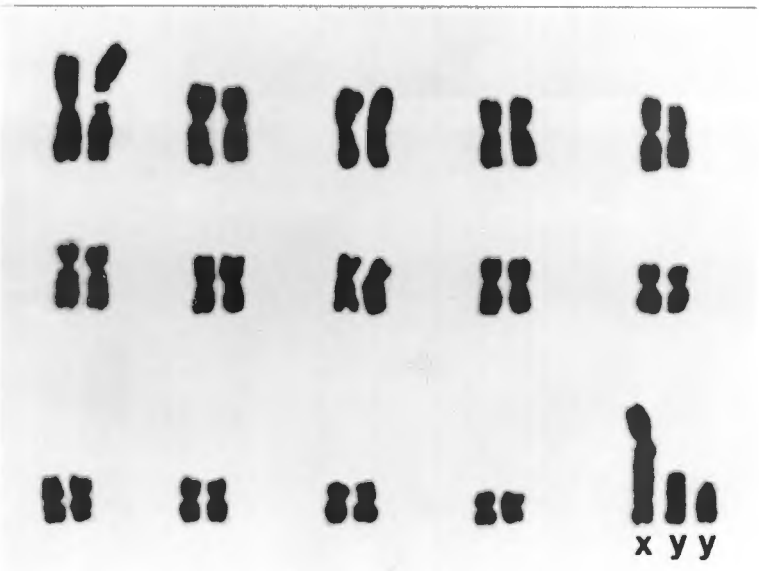

Figure 18b: Karyotype of a male Sand gazelle (*G. subgutturosa marica*) with pair 1 consisting of one metacentric and two acrocentric chromosomes. The diploid chromosome number (2n) is 32.

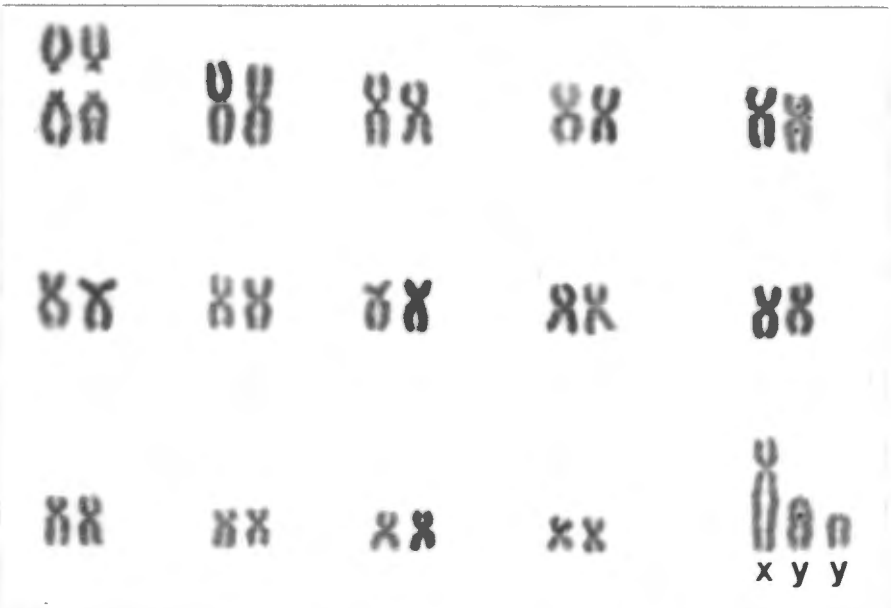

Figure 18c: Karyotype of a male Sand gazelle (*G. subgutturosa marica*) with pair 1 consisting of four acrocentric chromosomes. The diploid chromosome number (2n) is 33.

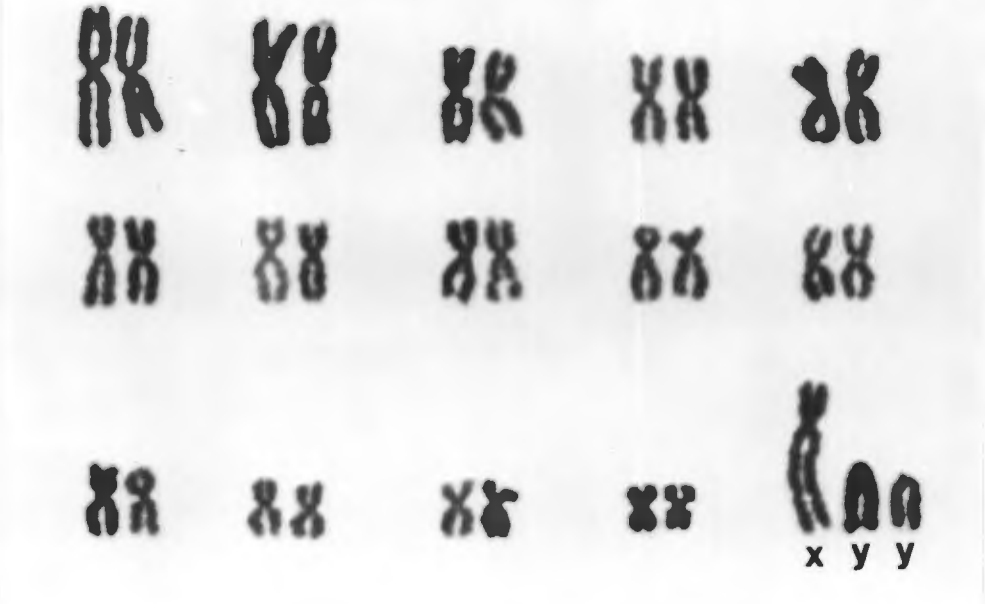

Figure 18d: Karyotype of a male Sand gazelle (*G. subgutturosa marica*) with pair 1 consisting of two metacentric chromosomes. The diploid chromosome number (2n) is 31.

3.3 Discussion.

The results presented here are consistent with published results. The karyotypes of *G. dorcas* ssp., *G. d. isabella*, *G. saudiya*, *G. g. cora*, *G. thomsonii*, *G. s. marica*, and *A. cervicapra* are identical to previous publications (Benirschke and Kumamoto, 1987; Effron et al., 1976; Granjon et al., 1991; Hsu and Benirschke, 1974a; Hsu and Benirschke, 1974b; Kingswood and Kumamoto, 1988; Kumamoto et al., 1995; Nelson-Rees et al., 1967; Vassart et al., 1995a; Vassart et al., 1995b; Vassart et al., 1996; Wurster, 1972; Wurster et al., 1968). *G. d. pelzelni* is the only taxon which has not had its karyotype described before, and their karyotypes are identical to all other studied *G. dorcas*. This is fully congruent with the idea that *G. d. pelzelni* is not a separate species, but a subspecies of *G. dorcas*, as described by Gentry (1964) and Groves (1969). It is not known whether banding patterns of the different subspecies differ at all, since this is the first study to include several different subspecies, and there are no banding studies available for *G. dorcas* subspecies.

Chromosome translocations are known from six Antilopini species, *A. cervicapra* (Effron et al., 1976; Hsu and Benirschke, 1974a), *G. dama* (Arroyo Nombela et al., 1990; Benirschke, 1985; Effron et al., 1976), *G. soemmerringii* (Benirschke et al., 1984), *G. subgutturosa marica* (Benirschke and Kumamoto, 1987; Granjon et al., 1991; Kingswood and Kumamoto, 1988; Kingswood et al., 1994; Vassart et al., 1993), *G. bennettii* (Kumamoto et al., 1995), and *G. saudiya* (Kumamoto et al., 1995). Especially in *G. soemmerringii* it is clear that the large variety of chromosome numbers can lead to outbreeding depression, as the hybridised animals suffer from increased perinatal mortality (Benirschke et al., 1984). It is not certain that the original imported *G. soemmerringii* belonged to different subspecies. However, a male and a female of a breeding group in Qatar were found to have identical karyotypes with 2n = 34 (Rebholz, unpublished data). This supports the conclusion that in this species the diploid chromosome numbers for males and females within subspecies are identical, and that all subspecies have slightly different karyotypes, probably with diploid numbers between 34 and 40. Different subspecies of *Madoqua kirkii* (Neotragini) have shown to have considerable chromosomal differences, which can lead to reproductive isolation between the subspecies (Kumamoto et al., 1994). Results shown here and elsewhere indicate that cytogenetic research can have a major impact on conservation (Benirschke and

Kumamoto, 1987; Benirschke and Kumamoto, 1988; Benirschke and Kumamoto, 1991; Robinson and Elder, 1993).

Two of the species studied here (*G. s. marica* and *A. cervicapra*) show chromosome translocations, which were found in other animals by other authors. It is not known whether the translocations have a negative effect on the fecundity of these animals, because they have not been monitored specifically.

Of the animals studied here, one individual (*G. dorcas* ssp., Figures 15a and 15b) was shown to have an XXY karyotype (equivalent to the Klinefelter syndrome in man), which is the first time that such an aberration has been reported for a non-domestic species. This is the first Antilopini species to be reported with an abnormality of the sex-chromosomes. This condition leads to testicular hypoplasia with azoospermia and aspermatogenesis in a variety of domestic animals (cattle, horse, pig and cat; see Gustavsson (1980) for a review). If the same applies to Antilopini then this animal should be sterile. It is not known whether this particular animal has these features, because the set-up of the collection in question does not allow for the animals to be monitored closely.

Combining the phylogenetic trees produced from molecular data (see Chapter 4), the phylogenetic trees based on cytogenetic data (Benirschke and Kumamoto, 1988; Effron et al., 1976; Vassart et al., 1995b), and the diploid chromosome numbers for each species, it is not clear whether fusions or fissions played a major role during the evolution of the Antilopini. Despite this, it is clear that four not very closely related clades have relatively high chromosome numbers: *L. walleri/A. marsupialis*, *G. dama/G. soemmerringii*, *G. rufifrons*, and *G. bennettii/G. saudiya*. About half of the karyotyped species have chromosome numbers in the low 30's, and they are as evenly distributed over the tree as the species with the high chromosome numbers are. It seems that there is not a clear trend in the evolution of the Antilopini towards either chromosome fusions or fissions, which supports the idea that both processes are important for speciation (Qumsiyeh, 1994).

The published phylogenetic analyses based on chromosomes (Figure 8) differ slightly from each other, since they do not agree on the identification of certain chromosomes (Effron et al., 1976; Gallagher and Womack, 1992; Vassart et al., 1995b). The best solution

to this problem would be to re-examine the species in question using high resolution banding studies, which makes identifying the chromosomes easier since they show more bands per chromosome (Sumner, 1990).

Chapter 4: DNA SEQUENCING.

4.1 Mitochondrial DNA.

The main features of mitochondrial DNA (mtDNA) in which it differs from nuclear DNA are its maternal inheritance, the absence of mutation repair and recombination, its relatively small size, its relatively rapid evolution, and the presence in each somatic cell of thousands of copies (Brown et al., 1979; Hutchison et al., 1974; Irwin et al., 1991; Wilson et al., 1985). Mitochondrial DNA (Figure 20), which is approximately 16500 bp in size in the Bovidae, evolves up to 10 times faster than most nuclear DNA (Wilson et al., 1985). Not all mtDNA genes evolve at identical evolutionary rates. The fastest evolving part is the control region, or D-loop, which is a non-coding region. Some genes, notably the tRNA and rRNA genes, evolve slower than the D-loop region and most of the other coding regions (Irwin et al., 1991; Kocher et al., 1989; Meyer, 1994; Miyamoto and Cracraft, 1991; Miyamoto et al., 1990). Consequently, different genes are valuable for estimating evolutionary relationships at different levels (Irwin et al., 1991; Lowenstein, 1986b). Mitochondrial DNA tends to evolve at a constant rate, at least at smaller divergences, which can be very helpful for establishing the time of divergence between species (Brown et al., 1979; Moritz et al., 1987; Wilson et al., 1985). However, calibrating the mtDNA clock is not a simple procedure because of inaccuracies in the data used for calibration, such as fossil record data (Moritz et al., 1987; Springer, 1995). A complicating factor for estimating divergence times accurately is that the rate of mtDNA evolution is not identical for distantly related or even for closely related taxa (Britten, 1986; Li et al., 1987; Moritz et al., 1987). Even within a single species different rates of mtDNA evolution can exist, as has been discovered recently for Kirk's dikdik (Zhang and Ryder, 1995).

Cytochrome *b* is a gene which shows a linear rate of evolution for up to 10 million years, and is therefore useful for assessing evolutionary divergence times in this range (Brown et al., 1979; Irwin et al., 1991). The overall evolutionary rate of mtDNA as estimated using the restriction fragment length polymorphism (RFLP) technique is about 2 % per million years (Brown et al., 1979; Krajewski and King, 1996; Moritz et al., 1987). Most extant species of the Antilopini, Neotragini, and Tragelaphini are thought to have evolved from

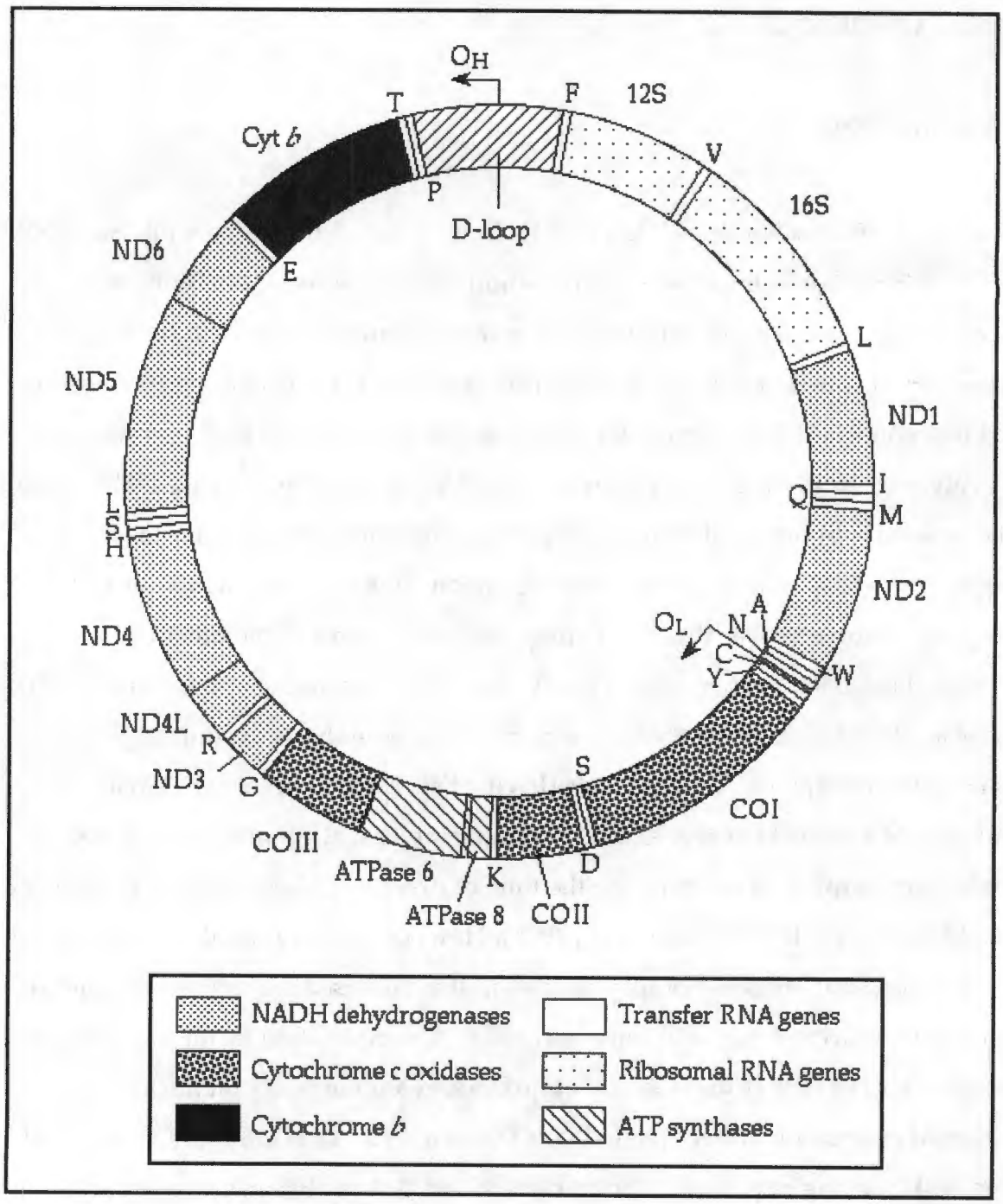

Figure 20: Structure and gene order in mammalian mitochondrial DNA.

the common ancestors of each of these tribes relatively recently (see Chapter 1). Therefore, cytochrome c oxidase III (COIII) and cytochrome *b* sequences should be appropriate choices of genes to clarify phylogenetic relationships in these tribes (Avise and Nelson, 1989; Baker et al., 1995; Brown et al., 1982; Graybeal, 1993; Mouchaty et al., 1995; Spradling DeWalt et al., 1993; Stanley et al., 1994; Watanabe et al., 1985).

The three codon positions of coding genes do not evolve at identical rates. Most third codon position changes do not result in amino acid changes, and are called silent or synonymous substitutions. Synonymous substitutions occur more frequently than non-synonymous substitutions, since the former have little or no selection against them (Brown et al., 1979). A few nucleotide substitutions at the first codon position are synonymous too, whereas none of the substitutions at the second position are synonymous (Irwin et al., 1991). This has the effect that third codon positions change faster than first codon positions, which change faster than second codon positions. Data should be checked for third codon position saturation, to identify instances where saturation is being approached, which would contribute to "phylogenetic noise". In the case of large sequence divergences it may be beneficial to exclude such data when performing phylogenetic analyses by selecting only the first and second codon position changes, or by using the amino acid sequence data.

4.2 Phylogenetic analysis.

The most common approaches for phylogenetic analyses of sequence data are cladistic (parsimony), distance based (neighbor-joining and UPGMA), and maximum likelihood methods. These methods handle the data in different ways.

Parsimony analysis is based on the assumption that the best explanation of a taxonomic data set is the one that requires the least number of character state changes (Farris, 1986; Felsenstein, 1983). The underlying assumption is that character state changes are unlikely events and should therefore be minimised on the trees. This means that the most parsimonious trees are the shortest trees for a particular data set. The strength of parsimony analysis is that it uses shared derived characters (synapomorphies) to define monophyletic groups (Stewart, 1993). The main disadvantage of parsimony analysis it that it performs less well if character state changes are frequent in the data set, and thereby give rise to homoplasies (parallel changes and back mutations). Parsimony also performs not very well when the evolutionary rates of different branches are very dissimilar (Felsenstein, 1978).

Distance methods use overall similarity between taxa as a measure of relatedness. These methods compute pairwise differences between taxa and construct a distance matrix. Depending on the assumptions one has about the evolutionary processes, different parameters are used to calculate the pairwise distances and to take into account multiple changes at a site. For instance, the Jukes-Cantor distance parameter assumes that substitutions are equally distributed over all four nucleotides, and that the ratio of transitions/transversions does not deviate too much from 0.5 (Jukes and Cantor, 1969). The advantage of distance methods is that they allow the distance values to be corrected for superimposed substitutions and they do not require a lot of computation time (Swofford et al., 1996). Methods such as neighbor-joining (Saitou and Nei, 1987) are also remarkably robust to differing rates of change along lineages. UPGMA performs poorly in this regard, probably because its main assumption is that the evolutionary rate is identical between different lineages. The claimed disadvantage of distance methods is that they do not use all the information in the original sequence data in the way which parsimony does (Quicke, 1993; Swofford et al., 1996). However, they do make full use of autapomorphic data. For morphological data the complete lack of any relation between amount of change and time renders distance methods quite inappropriate. The presence of a molecular clock renders molecular data more appropriate for use of distance methods which are a function of rate variation, base composition, and mutation bias (Felsenstein, 1984).

The principle underlying maximum likelihood methods is that the tree which fits the data best for a given model of the evolutionary process is the most likely tree (Felsenstein, 1981; Quicke, 1993; Swofford et al., 1996). Maximum likelihood methods require too much computing time for the analysis of the large amount of taxa and characters used in this project and are therefore not used. In the foreseeable future it could be possible to analyse the data with maximum likelihood methods as computers and algorithms become faster.

The debate about the best method for recovering correct phylogenies is still continuing, and it will probably continue for some time to come. Since both parsimony and neighbor-joining have advantages and disadvantages (Farris, 1985; Felsenstein, 1978; Felsenstein, 1983; Felsenstein, 1984), both were used to analyse the data. The parsimony and neighbor-joining methods, as implemented by PAUP (Swofford, 1993), MEGA (Kumar et al., 1993), and RNA (Farris, 1994) have been used to analyse the sequence data. These two are the

most widely used analysing methods and require reasonable amounts of computing time. The Kimura 2-parameter model was used for the neighbor-joining method, as suggested in the MEGA manual for data sets with p-distances between 0.05 and 0.30 and transition/transversion ratios higher than 2. The Kimura 2-parameter model takes transition/transversion ratios into account when calculating the distances, and these ratios vary considerably in the Bovidae data set (Figure 21).

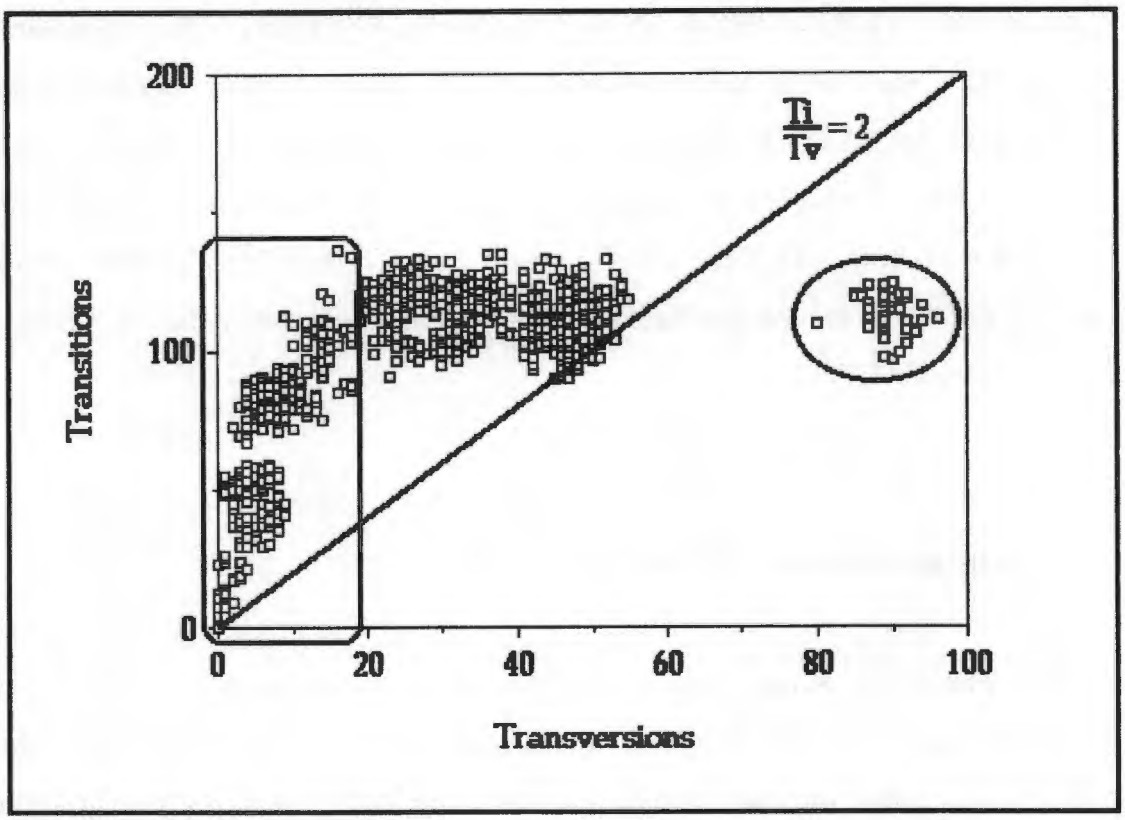

Figure 21: Transitions versus transversions in pairwise comparisons between all taxa. The line indicates where the transitions/transversions ratio equals 2. The data points in the oval are pairwise comparisons between pig (*S. scrofa*) and all other taxa. The data points in the square are pairwise comparisons within each of the three tribes.

The bootstrap method has been introduced to phylogenetic analyses to put confidence limits on nodes in phylogenetic trees (Felsenstein, 1985). This is the most widely used method for assessing the confidence in a branching order. It is based on resampling with replacement, and results in subsamples with the same number of characters as the original data set. This is essentially identical to random differential weighting of characters, with weights from zero upwards and the total of the weights being equal to the amount of characters in the original data set. Several different subsamples are produced and for each

of them the shortest trees are produced. At the end of the procedure, a consensus tree is produced in which clusters that occur in 50 percent or more of the trees are retained. Parsimony bootstrap analyses were performed with heuristic searches, since branch and bound searches were too time consuming for the large data sets used. Tests on smaller data sets showed that in all cases heuristic searches resulted in the same trees as branch and bound searches. All bootstrap analyses were based on one thousand bootstrap replicates. Neighbor-joining bootstrap analyses were performed with the Kimura 2-parameter. PAUP (Swofford, 1993), RNA (Farris, 1994), and MEGA (Kumar et al., 1993) are all able to implement bootstrap analyses. Initially, parsimony bootstraps were performed with PAUP. RNA does bootstrap analyses in a fraction of the time used by PAUP. To test whether the two programs give similar bootstrap results, the data sets were analysed with both PAUP and RNA. Figures 26e, 27d, and 28c show that some parsimony analysis bootstrap values generated with RNA are significantly different from those produced by PAUP.

4.3 **Results applicable to all three tribes.**

The complete data set used here was split up into sequences of cytochrome *b* and COIII to assess the base composition for each of these two genes. The analyses show that both genes have similar amounts of adenine, cytosine and thymine, but low guanine contents (Figure 22a). This has been shown to be normal for many mammals (Irwin et al., 1991; Meyer, 1994). The analysis of the combined data set showed that there were no significant differences in base composition between the three tribes (Figure 22b). However, when the complete data set was divided according to codon positions a different picture was shown. The results showed that for the first codon position all base pairs are present in similar amounts, but for the second codon position there was a significant excess of thymine (Figure 22c). For the third codon position adenine and cytosine were present in high amounts, whereas guanine was present in very low amounts, which accounts for the low amount of guanine in the complete data set. The low frequency of guanine at silent positions (mostly third codon positions) has also been reported for rodents, birds and fish (Kocher et al., 1989; Krajewski and King, 1996). Although several hypotheses have been

postulated for this phenomenon, the exact reason behind the strong bias against guanine in mtDNA genes is not completely clear (Jermiin et al., 1994; Martin, 1995).

At low levels of sequence difference most of the changes occur at the third codon positions. At high sequence difference levels many third codon positions are saturated (homoplasies become abundant) and first and second codon positions become more

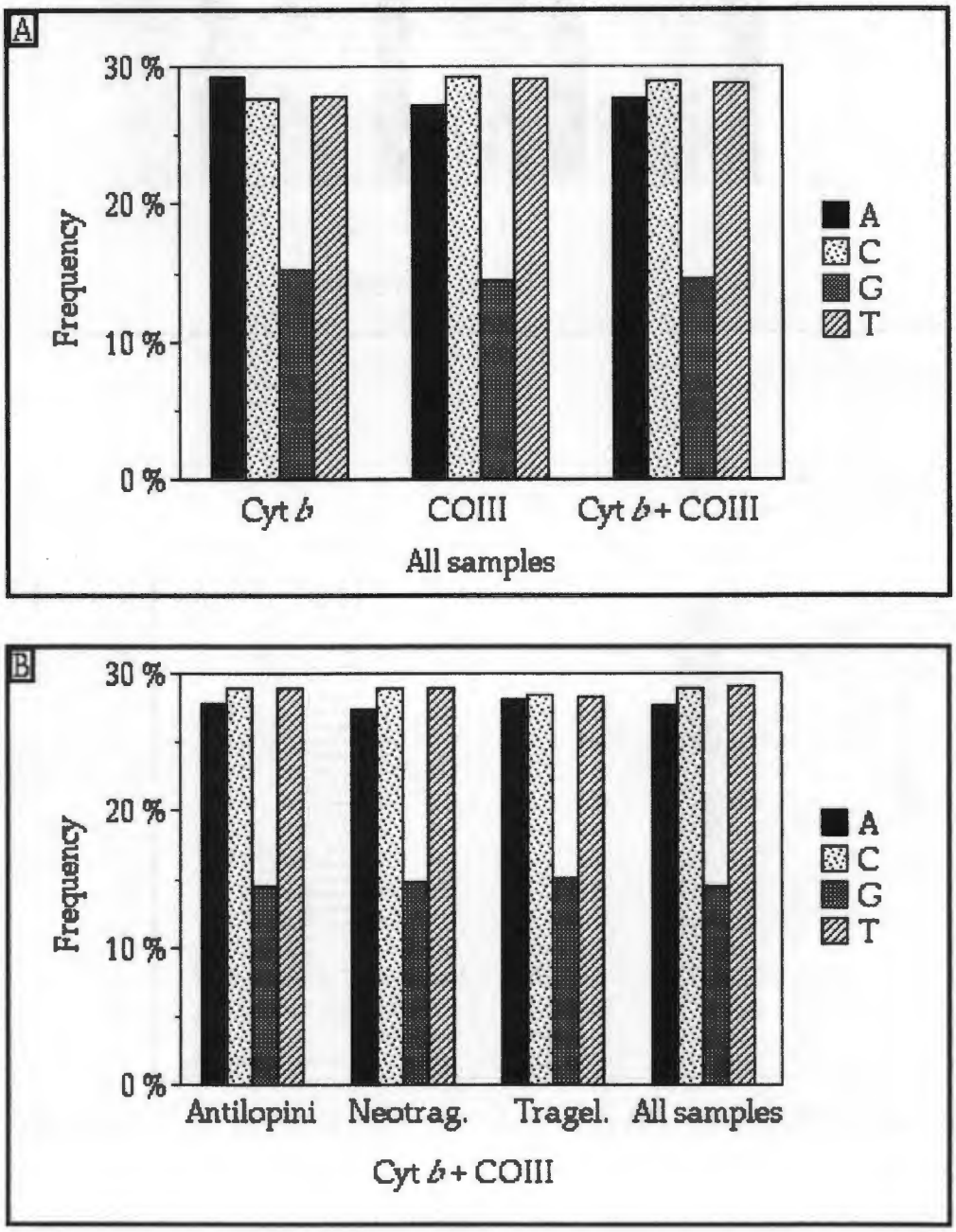

Figure 22: Nucleotide distributions for the three tribes, for the two genes, and for each codon position.

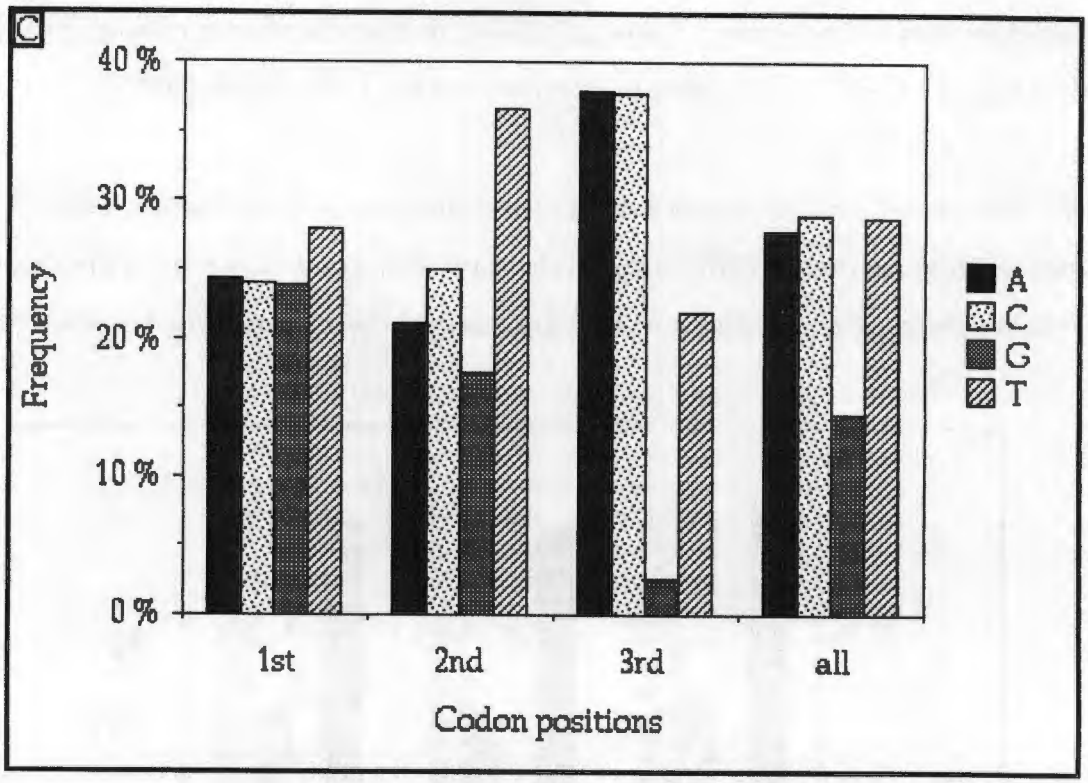

Figure 22: Continued.

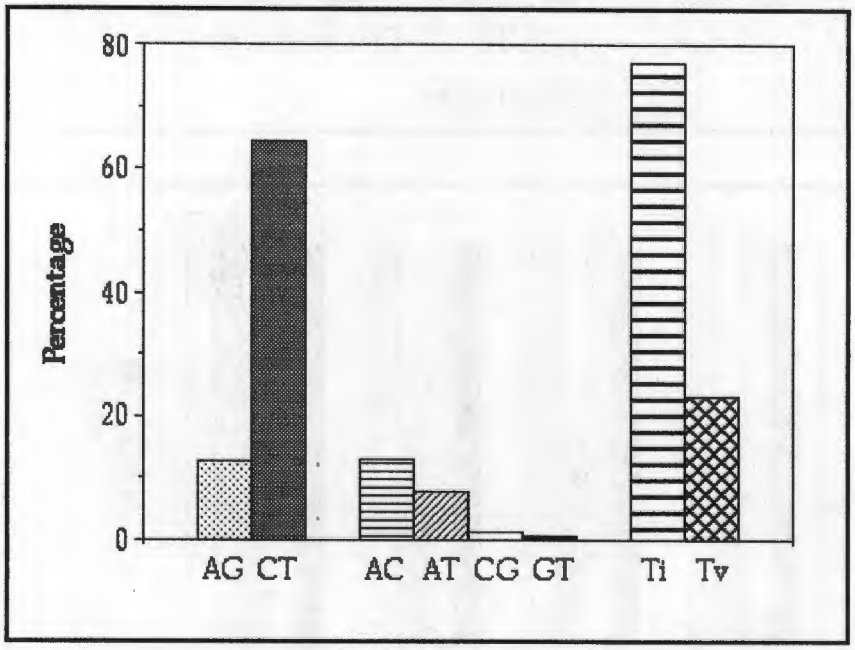

Figure 23: The contribution of the different nucleotide changes to the total sequence differences.

informative. A disadvantage of this is that at intermediate levels third codon positions are starting to become saturated and the other two positions have not accumulated enough changes to be informative (Meyer, 1994). The average transition/transversion ratio is close

to 4, and most transitions are of the CT type (Figure 23). For p-distances between 0 % and 10 % the number of transitions increase steadily (Figure 24). At p-distances higher than 15 %, which includes all distances between *S. scrofa* and the other taxa, the number of transitions decrease, which suggests that transition saturation becomes an important factor (Figure 24). It was therefore decided to exclude the *S. scrofa* sequences from the analyses. Figure 21 shows that in all pairwise comparisons there is a strong transition bias, with most comparisons having a transition/transversion ratio larger than two, which is relevant for the choice of the Kimura 2-parameter model with the neighbor-joining analyses (Kumar et al., 1993). The transition/transversion ratios between *S. scrofa* and all other taxa are less than two (Figure 21), which is not ideal for analyses with the Kimura 2-parameter model. Figure 25 shows that most changes in the two genes occur at the third codon positions, and that the first two codon positions are not very informative up to 20 % overall sequence difference.

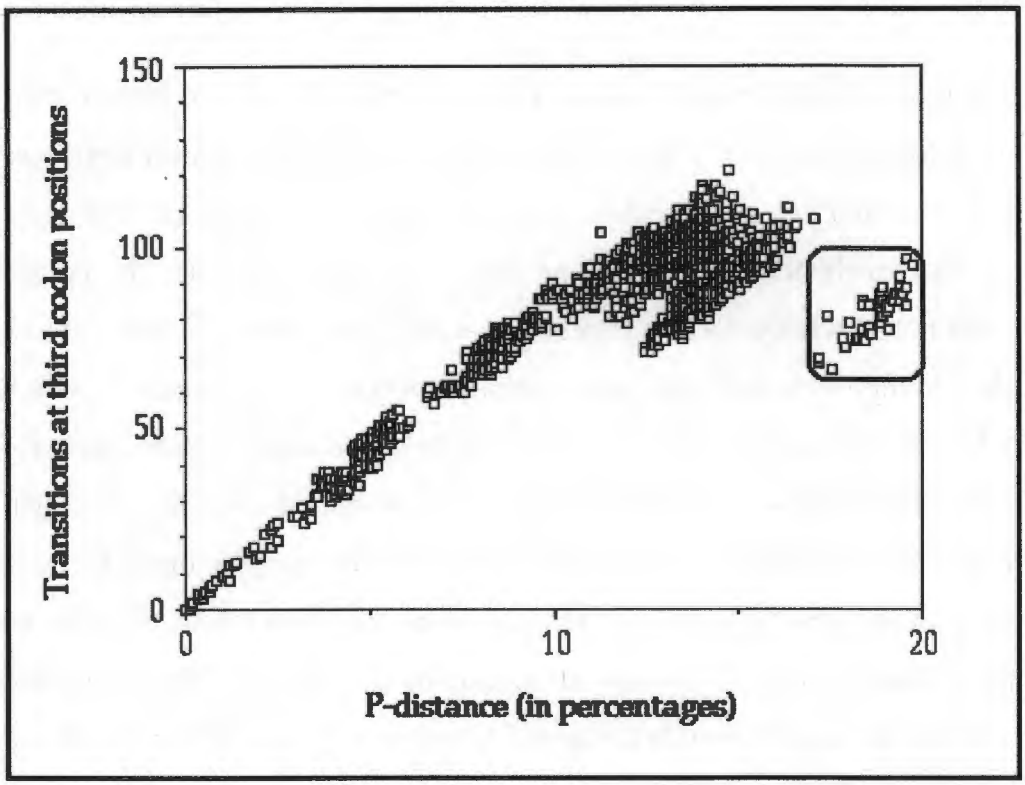

Figure 24: The number of transitions for third codon positions at different p-distances. The data points in the square are pairwise comparisons between pig (*S. scrofa*) and all other taxa.

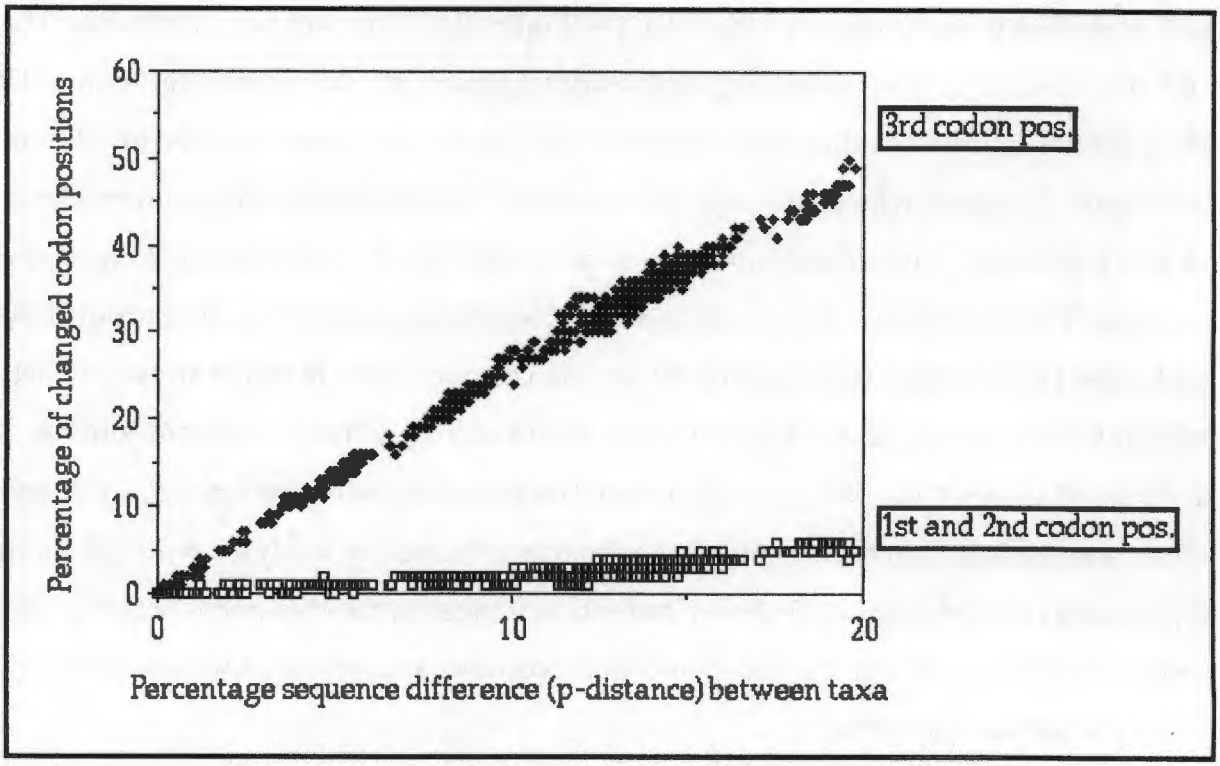

Figure 25: Percentages of the transitions and transversions for all pairwise comparisons for all taxa.

The sequence differences between the species of Bovidae which comprise the data set used here are 16 % or less. This means that many third codon positions in the ingroup are not saturated yet, so all codon positions could be used for phylogenetic analyses. A similar conclusion has been found elsewhere (Meyer, 1994). The Tragelaphini data set was used to test whether a few homoplasies at third codon positions would make an impact on the phylogenetic analysis. A parsimony analysis was carried out with this data set in which third codon positions with three or four character states were excluded. Figure 28a show that there is no significant difference in bootstrap values between the phylogenetic tree generated with all available nucleotides and the phylogenetic tree generated from unsaturated positions only. A similar result has been found with the analysis of cytochrome *b* data of a variety of mammals (Irwin et al., 1991). All this indicates that for the data set used here it is not sensible to exclude several third codon positions, to avoid relatively few homoplasies.

The complete data set (52 taxa) gave the following amount of variable and phylogenetically informative sites.

	variable sites	phylogenetically informative sites
cyt *b*:	124	105
COIII:	300	260
cyt *b* + COIII:	424	365

Table 7: Variable and phylogenetically informative sites in cytochrome *b* and cytochrome c oxidase III.

These genes were not analysed separately, since the cytochrome *b* gene does not have enough characters with which to perform thorough analyses. When converted to amino acid data, the data set consisted of 51 variable sites, 28 of which were phylogenetically informative. Twenty-eight informative characters for 52 taxa are too few to generate reliable trees, so analyses of amino acid data were not considered.

4.4 Results for the Antilopini.

Figure 21b shows that the overall frequency of guanine in the sequences is half that of the other nucleotides. This is caused by the low guanine content of third codon positions as described above. Cytosine and adenine are the most prominent nucleotides at third codon positions. This is consistent with findings for the cytochrome *b* gene in other mammals, even though the 2.6 % guanine content at third codon positions is lower than the 3.7 % reported for *B. taurus* (Irwin et al., 1991; Kocher et al., 1989). As mentioned earlier, third codon positions which have three or four character states may represent saturated sites. However, when those third codon positions were to be excluded from a data set which includes several distantly related taxa many informative sites for closely related taxa would be lost. All p-distances for the Antilopini studied here are 11.5 % or less, and relatively few saturated third codon positions are present in these circumstances. It was therefore decided not to exclude any data from the analyses.

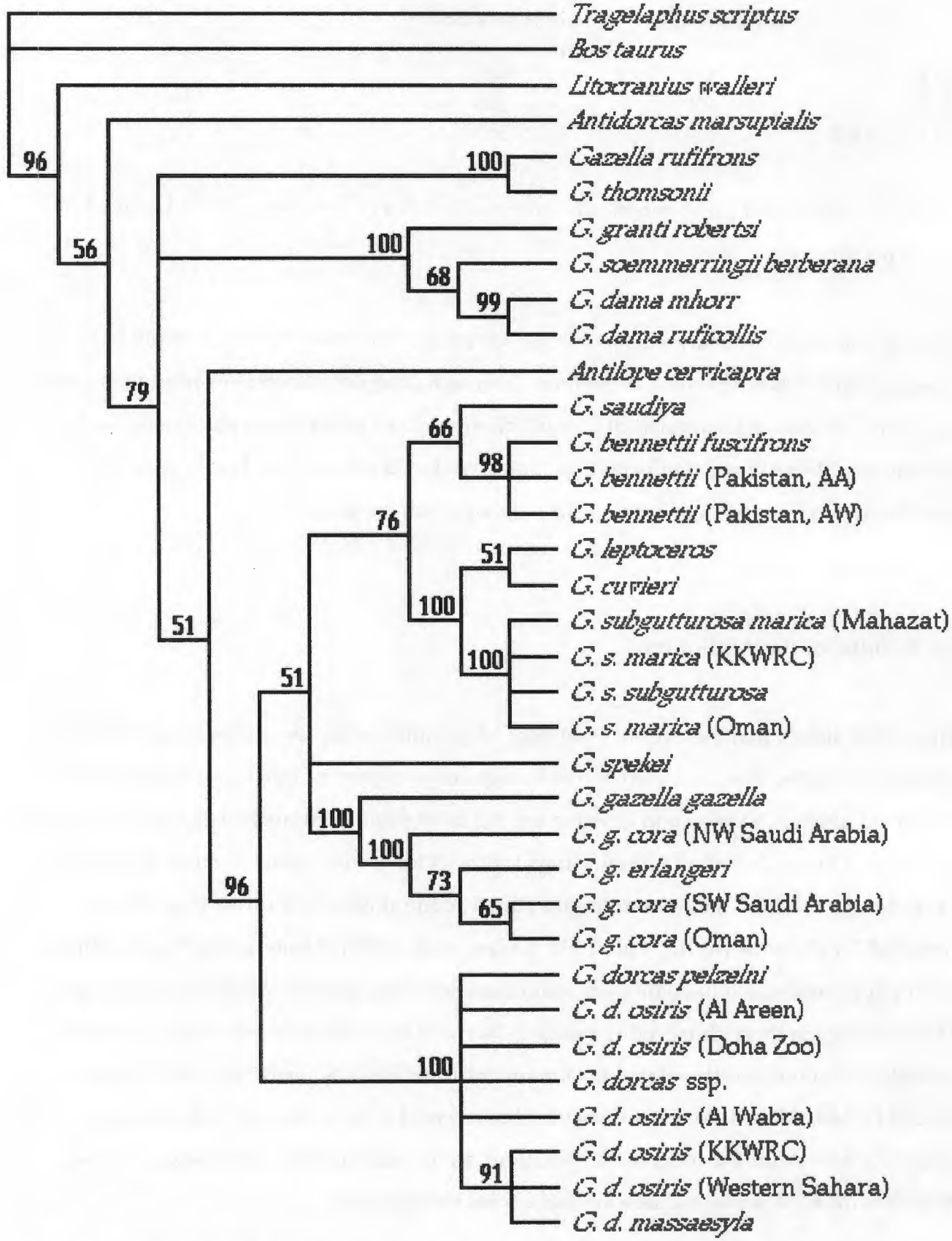

Figure 26a: Parsimony tree for the Antilopini with bootstrap values. AW= Al Wabra, AA= Al Areen, KKWRC= King Khalid Wildlife Research Center.

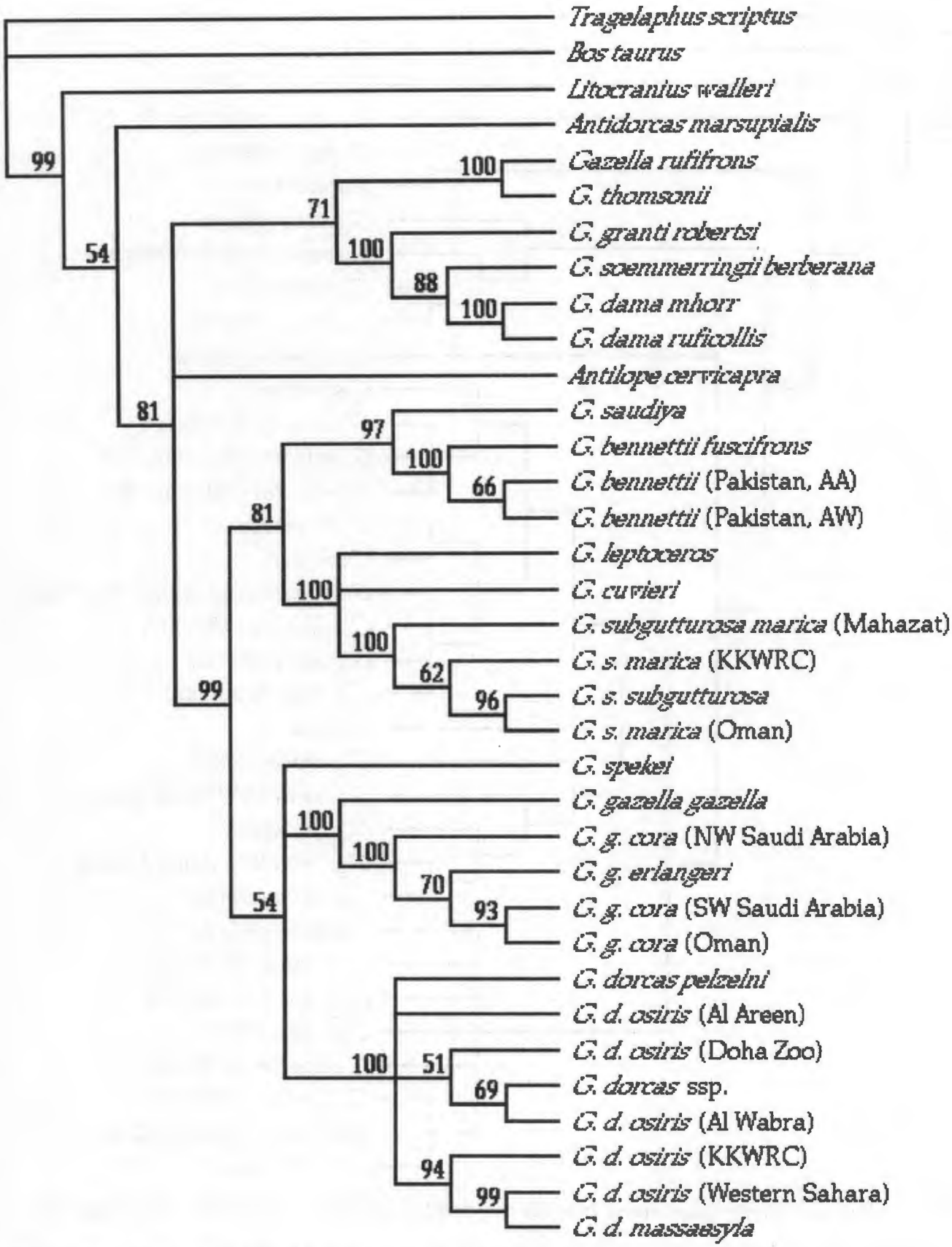

Figure 26b: Neighbor-joining tree for the Antilopini with bootstrap values.

AW= Al Wabra, AA= Al Areen, KKWRC= King Khalid Wildlife Research Center.

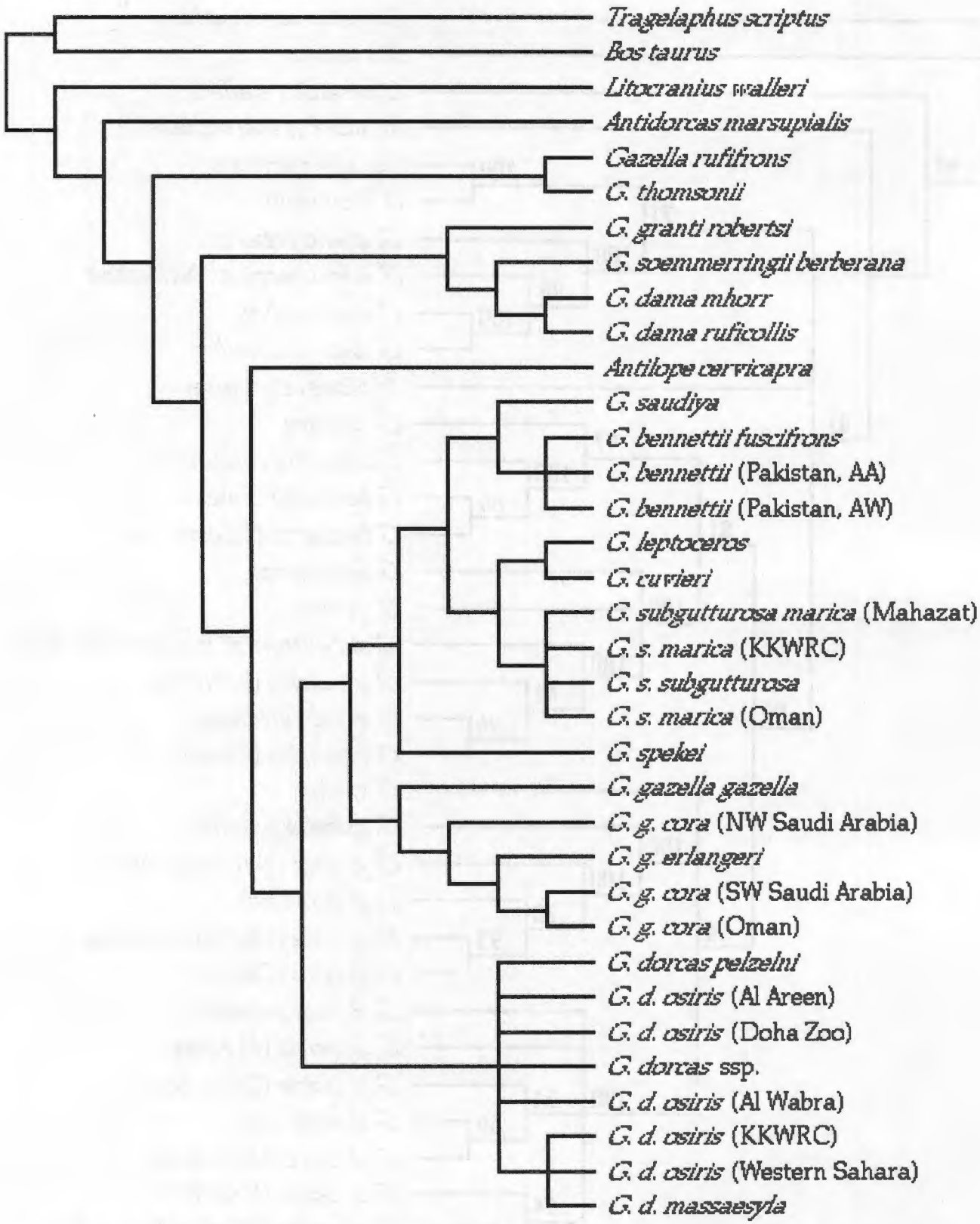

Figure 26c: Consensus parsimony tree for the Antilopini based on 8 trees with length 798, CI= 0.53, RI= 0.72, RC= 0.38, HI= 0.48. AW= Al Wabra, AA= Al Areen, KKWRC= King Khalid Wildlife Research Center.

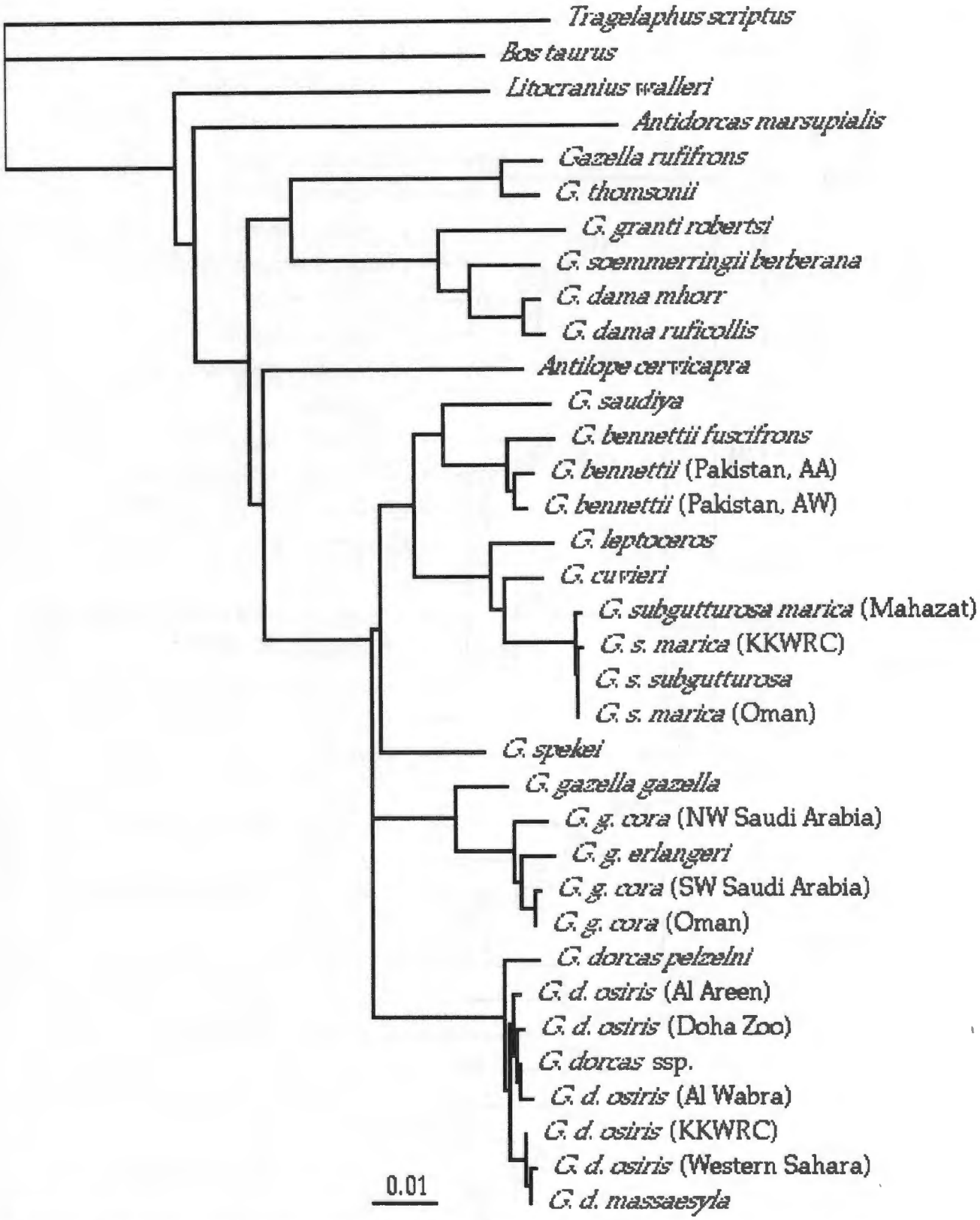

Figure 26d: Neighbor-joining tree for the Antilopini. AW= Al Wabra, AA= Al Areen, KKWRC= King Khalid Wildlife Research Center.

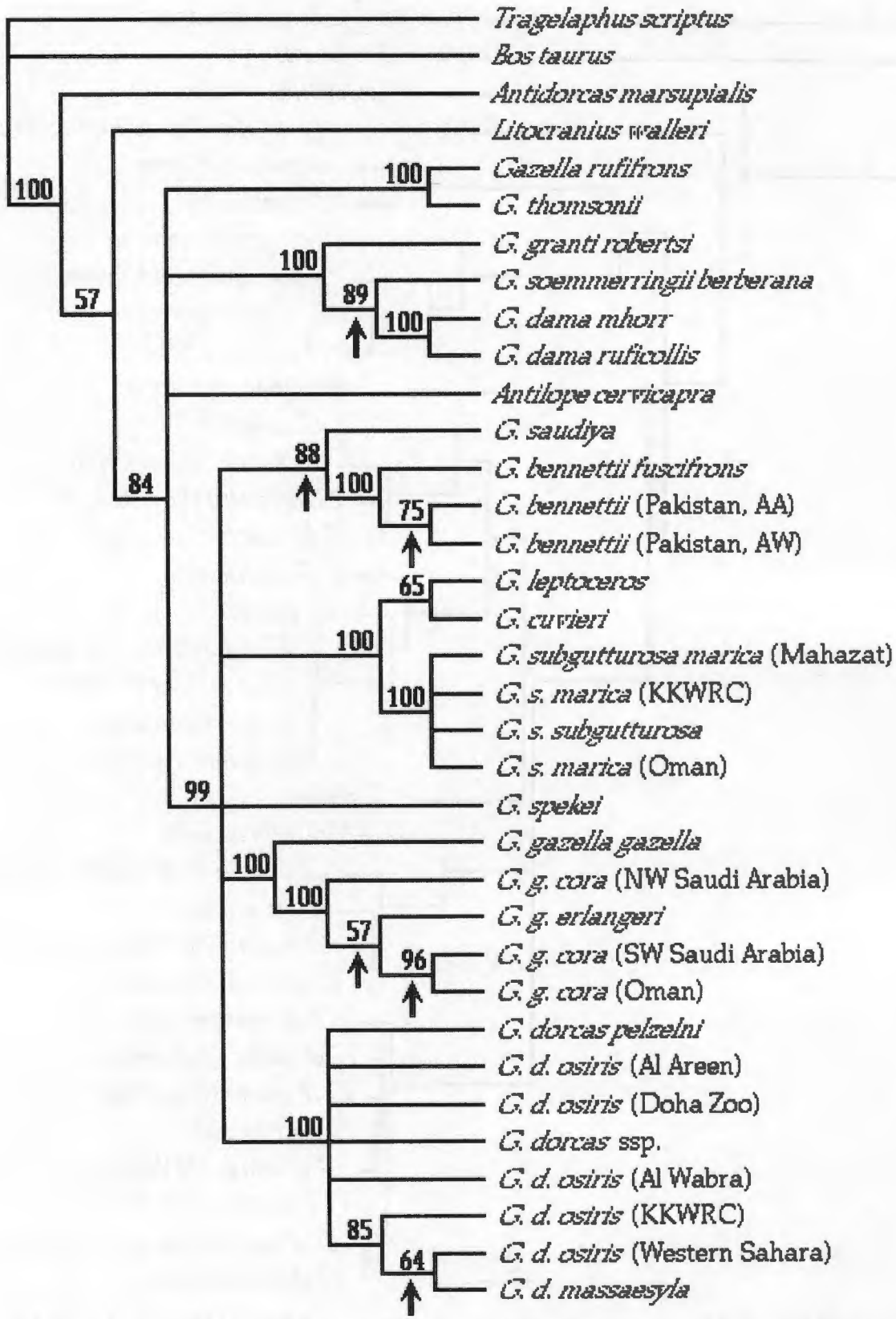

Figure 26e: Parsimony tree for the Antilopini with bootstrap values, generated with RNA. The arrows indicate nodes whose support values differ significantly from the same analysis with PAUP (Fig 26a). AW= Al Wabra, AA= Al Areen, KKWRC= King Khalid Wildlife Research Center.

The phylogenetic trees constructed with both the parsimony method and the neighbor-joining method, including bootstrap analyses, are shown in Figures 26 and 27. Figure 27a shows that there is strong bootstrap support for the monophyly of the tribe from neighbor-joining analysis, but not from parsimony analysis. When *A. marsupialis* was left out of the analysis, the parsimony bootstrap value went up from 75 % to 91 %, suggesting that this species was responsible for the low bootstrap value. Both methods show that all subspecies of a particular species cluster together and that they have 100 % bootstrap support. Most nodes have similar bootstrap support for both types of analysis. There are several differences between the two bootstrap analyses. *G. spekei/G. gazella* cluster with either *G. bennettii/G. saudiya/G. subgutturosa/G. cuvieri/G. leptoceros* (Figure 26a) or with *G. dorcas* (Figure 26b). This can be related to the low bootstrap values (51 % and 54 %) that support these nodes in both trees. Therefore, the position of *G. spekei* should be considered unresolved in both trees although it is associated with *G. gazella*. *G. spekei* and *G. gazella* are sister species in all analyses. In neighbor-joining and parsimony analyses *G. spekei* is associated with the cluster *G. saudiya/G. bennettii/G. cuvieri/G. leptoceros/G. subgutturosa* rather than with *G. dorcas* (Figures 26c and 26d). The neighbor-joining analysis shows a short branch connecting *G. spekei* to this cluster, which suggests that the association is not very robust. In these two analyses it is *G. gazella* that changes its association from *G. dorcas* to the cluster *G. saudiya/G. bennettii/G. cuvieri/G. leptoceros/G. subgutturosa*.

L. walleri and *A. marsupialis* are basal to the other Antilopini. There is weak bootstrap support in the parsimony analysis (51 %) for the position of *A. cervicapra* in the genus *Gazella*, which would render the genus *Gazella* paraphyletic. However, it takes seven extra steps (from the initial 798) to take *A. cervicapra* out of the *Gazella* clade. In the neighbor-joining analysis the cluster consisting of *G. rufifrons*, *G. thomsonii*, and the species of the subgenus Nanger (*G. dama*, *G. granti*, and *G. soemmerringii*) were basal to *A. cervicapra* (Figure 26d and 27c). In parsimony analyses the position of *A. cervicapra* either rendered the genus *Gazella* paraphyletic (Figure 26c) or its position was unresolved (Figure 27b).

The species belonging to the subgenus Nanger have a 100 % bootstrap support in both parsimony and neighbor-joining analyses. The species *G. thomsonii* and *G. rufifrons* cluster in both analyses and have a 100 % bootstrap support. They seem to be closely related to the species of the subgenus Nanger in the neighbor-joining method, although their

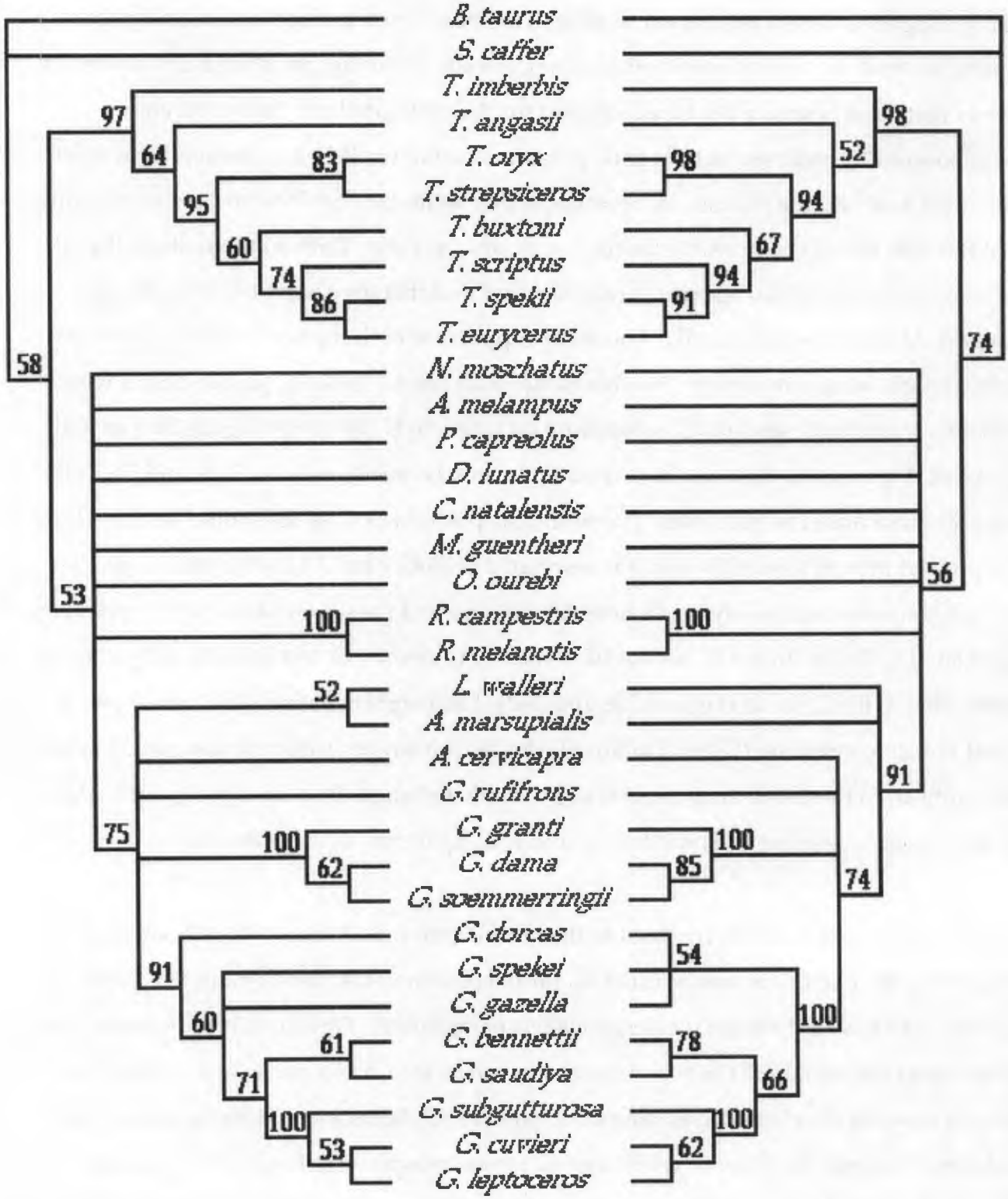

Figure 27a: Parsimony tree (left) and neighbor-joining tree (right) for all studied tribes with bootstrap values.

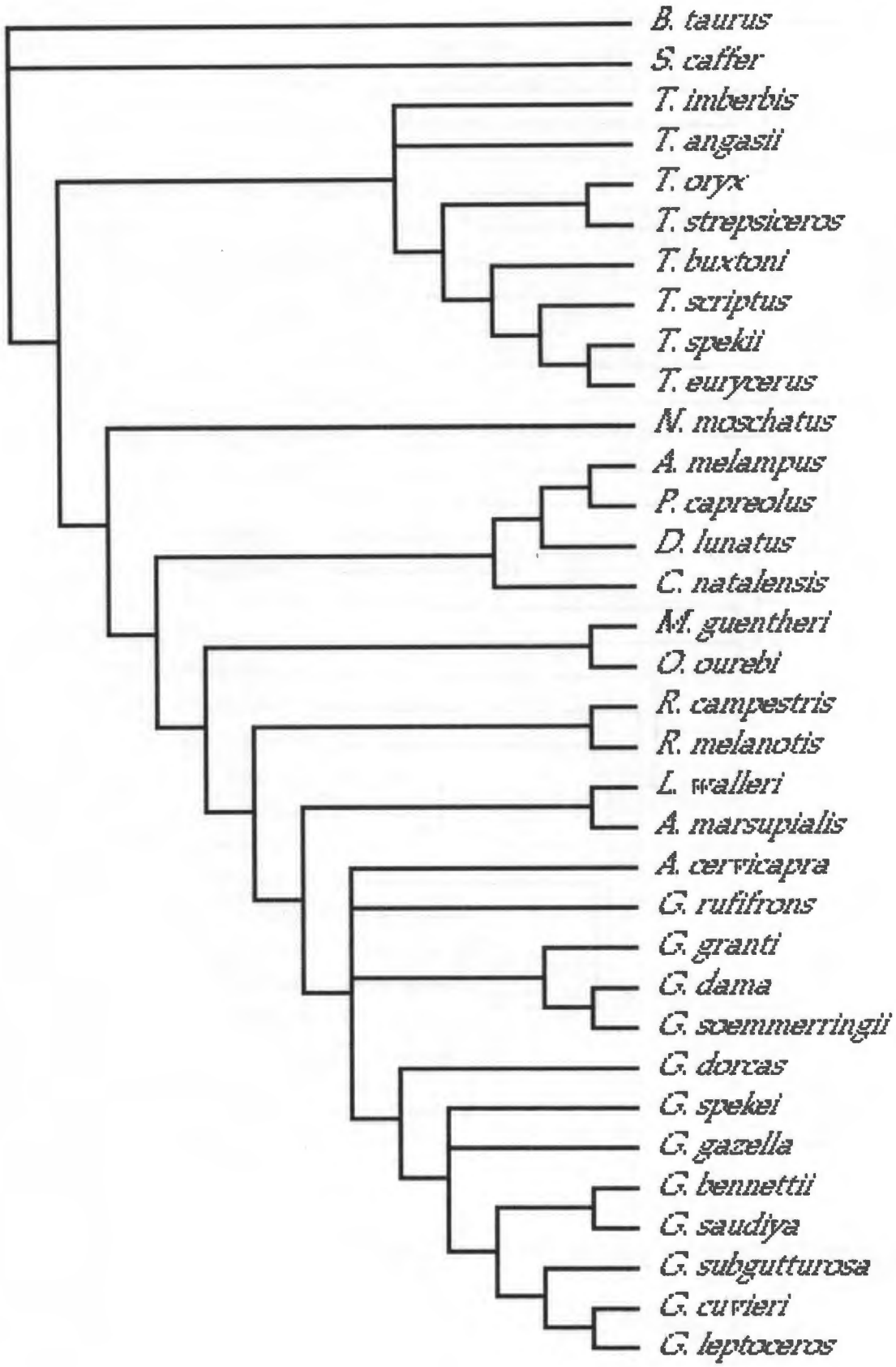

Figure 27b: Consensus parsimony tree for the Antilopini based on 6 trees with length 1897, CI= 0.30, RI= 0.51, RC= 0.16, HI= 0.70.

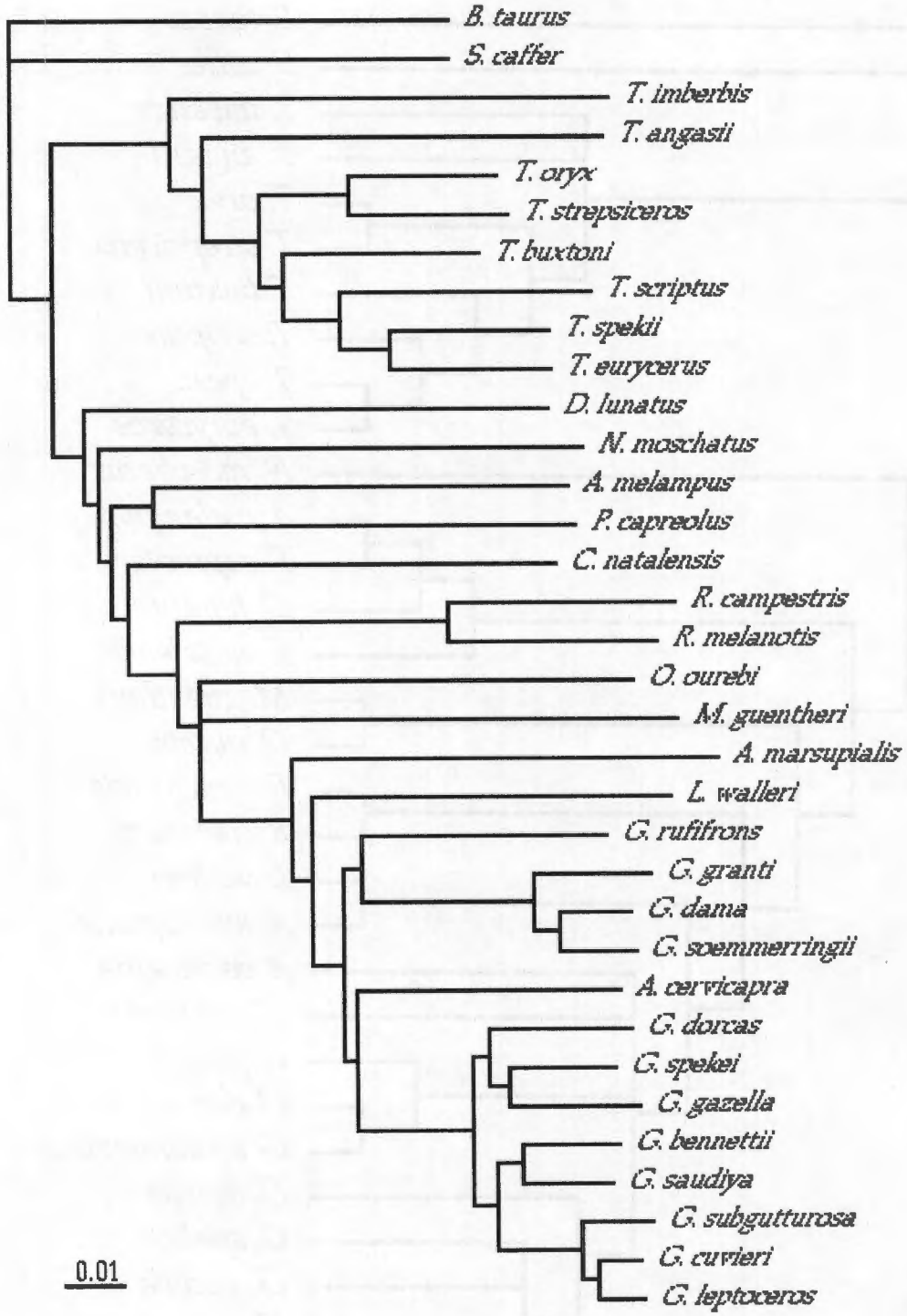

Figure 27c: Neighbor-joining tree for all studied tribes.

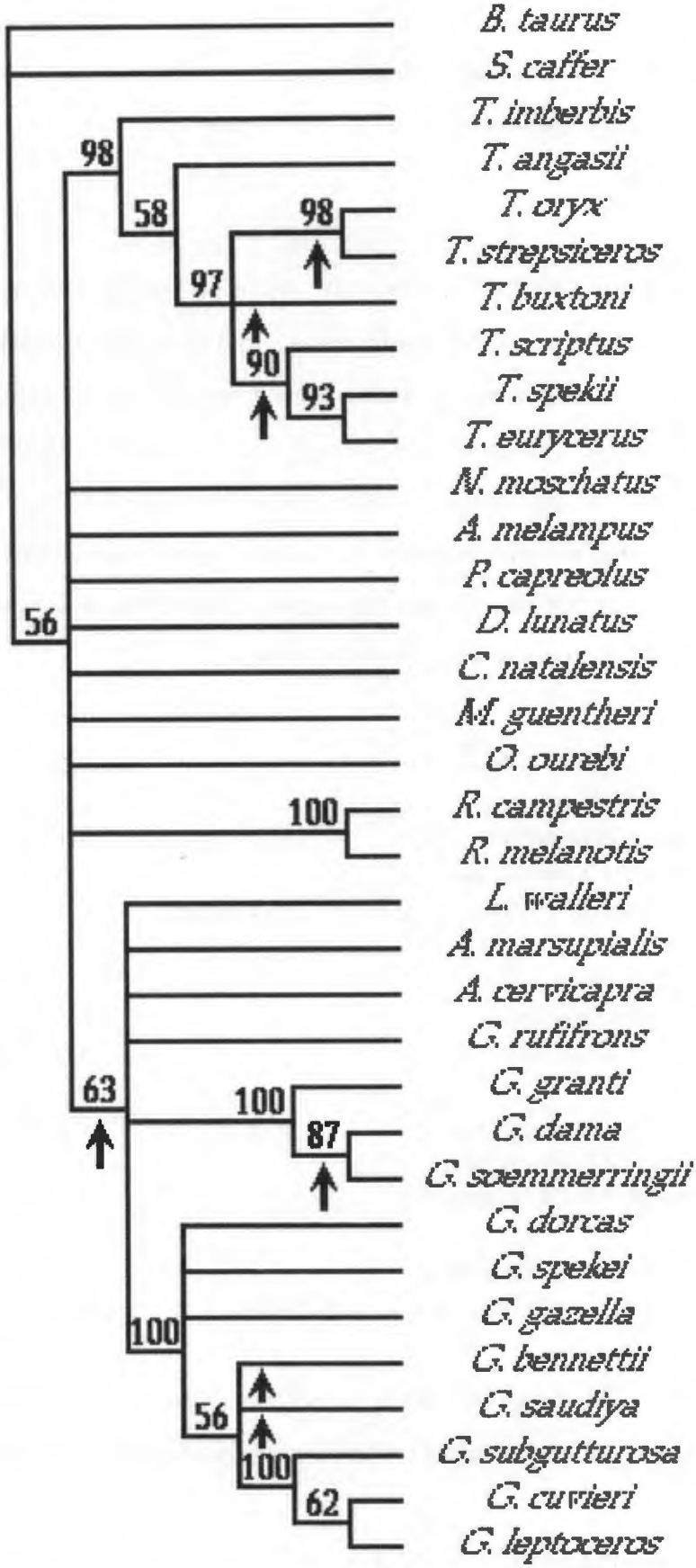

Figure 27d: Parsimony tree for all studied tribes with bootstrap values, generated with RNA. The arrows indicate nodes whose support values differ significantly from the same analysis with PAUP (Fig 27a).

position is unresolved with the parsimony method. The subgenus Trachelocele, which includes only *G. subgutturosa* (Table 1), also has a 100 % bootstrap support. It is situated within the subgenus Gazella and it clusters 100 % in all trees with *G. cuvieri* and *G. leptoceros*.

To find out what the intraspecific, interspecific, and intergeneric ranges of differences are the p-distances for the Antilopinae and Tragelaphini were analysed. Table 8 summarises some examples of these cases which demonstrate the range of difference found within each category. It shows that recognised subspecies differ by as much as 1.3 %, and species differences range from 1.4 to 12.5 %. Differences between genera range from 7.2 to 11.5 %. These ranges could be extended if more species are sequenced. The ranges overlap, which shows that there can be no rigid procedures to decide how much difference constitutes a subspecies, species, or genus difference.

G. subgutturosa subspecies	0.0 - 0.2 %
G. dama subspecies	0.6 %
G. dorcas subspecies	0.1 - 1.1 %
G. bennettii subspecies	0.5 - 1.2 %
G. gazella subspecies (excluding *G. g. gazella*)	0.1 - 1.3 %
Gazella species	1.4 - 9.5 %
Raphiceros species	6.6 %
Tragelaphus species	4.9 - 12.5 %
G. dorcas - *A. cervicapra*	7.2 %
G. granti - *A. marsupialis*	11.5 %
N. moschatus - *R. campestris*	14.1 %
G. rufifrons - *G. thomsonii*	1.2 %
G. g. gazella - other *G. gazella* subspecies	1.9 - 2.5 %

Table 8: The ranges of differences (p-distances) within species, between species, and between genera within the Antilopini, Neotragini, and Tragelaphini.

One individual from Doha Zoo in Qatar was misidentified as a *G. gazella* subspecies based on external morphological features, primarily coat colour and horn shape. Subsequent cytogenetic investigations suggested that that particular individual belonged to

G. bennettii (A. T. Kumamoto, pers. comm.). The sequence data for cytochrome *b*, as presented in Table 9, show that that sequence corresponds to that of *G. bennettii*. There can hardly be any doubt that the external features lead to a mistaken identity.

Doha Zoo individual	AACAAATTTTCCCTCTATATCA
G. bennettii (Pakistan 580)	.....
G. bennettii (Pakistan 8)	.....G.....
G. bennettii (Iran)	.....A.....
G. saudiya	G...TTC.C....C.C..GC..
G. gazella cora	.GTCT.CC.CT.T.T.GC..TG

Table 9: Variable sites in 300 base pairs of the cytochrome *b* gene, showing that the individual sampled at Doha Zoo was misidentified as a *G. gazella* subspecies.

The three museum samples used to extract DNA (*G. saudiya, G. g. acaciae* and *G. d. isabella,* as mentioned in Chapter 2) did not yield any PCR products for cytochrome *b*. Neither of the two cytochrome *b* primer sets (L14491/H14917 and L15175/H15342, which amplify 426 and 167 base pairs respectively) were able to give PCR products. Re-amplification also did not yield PCR products and therefore no sequence could be obtained for these samples.

4.5 Results for the Neotragini.

The four Neotragini genera used in this study do not cluster in either neighbor-joining or parsimony analysis (Figure 27a-c). However, the two species belonging to the genus *Raphiceros* (*R. campestris* and *R. melanotis*) do cluster and have 100 % bootstrap support (Figure 27a). *N. moschatus* is the only species which does not cluster with the other four Neotragini species. This species is associated with species that were used as outgroup species (*C. natalensis, P. capreolus,* and *D. lunatus*). *O. ourebi, M. kirkii,* and the two *Raphiceros* species do cluster in a neighbor-joining analysis (Figure 27c), although there is no bootstrap support for the clustering of these four species (Figure 27a).

4.6 Results for the Tragelaphini.

Whether parsimony or neighbor-joining methods were used, the analyses resulted in identical topologies. The Tragelaphini are always monophyletic with bootstrap values for the tribe in the high nineties, no matter which outgroups are being used (Figures 27a and 28a). Most nodes have slightly higher bootstrap values for the neighbor-joining method than for the parsimony method, and they are well supported. The single most parsimonious tree was recovered with the branch and bound algorithm and its topology was identical to the neighbor-joining tree of Figure 28b. The neighbor-joining tree without bootstrap values is shown in Figure 28b. Interestingly, the two species (*T. oryx* and *T. eurycerus*) which are sometimes put in a separate genus (*Taurotragus*), are separated in the phylogenetic tree. *T. imberbis* is basal to the other Tragelaphini species. It seems that the three species which occur at the eastern side of Africa, *T. imberbis*, *T. angasii*, and *T. buxtoni* are basal to the other species.

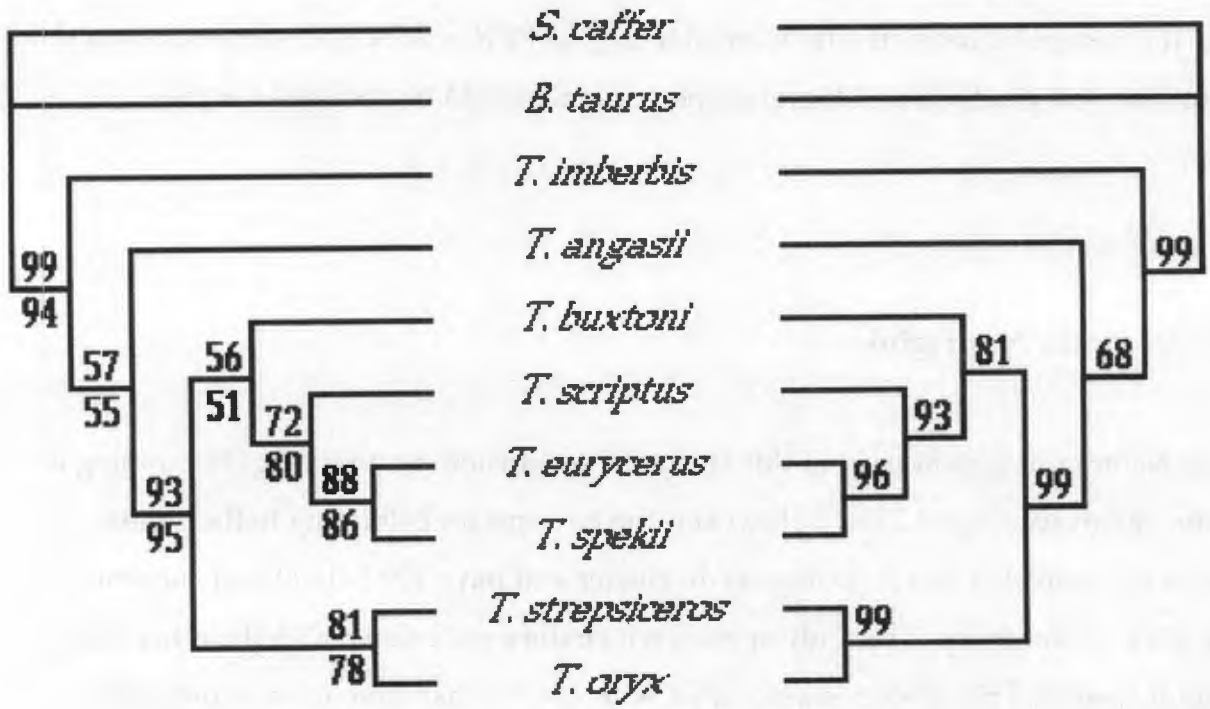

Figure 28a: Parsimony tree (left) and neighbor-joining tree (right) for the Tragelaphini with bootstrap values. Below the branches on the left are the bootstrap values for the data set when third codon positions with three or four states are excluded.

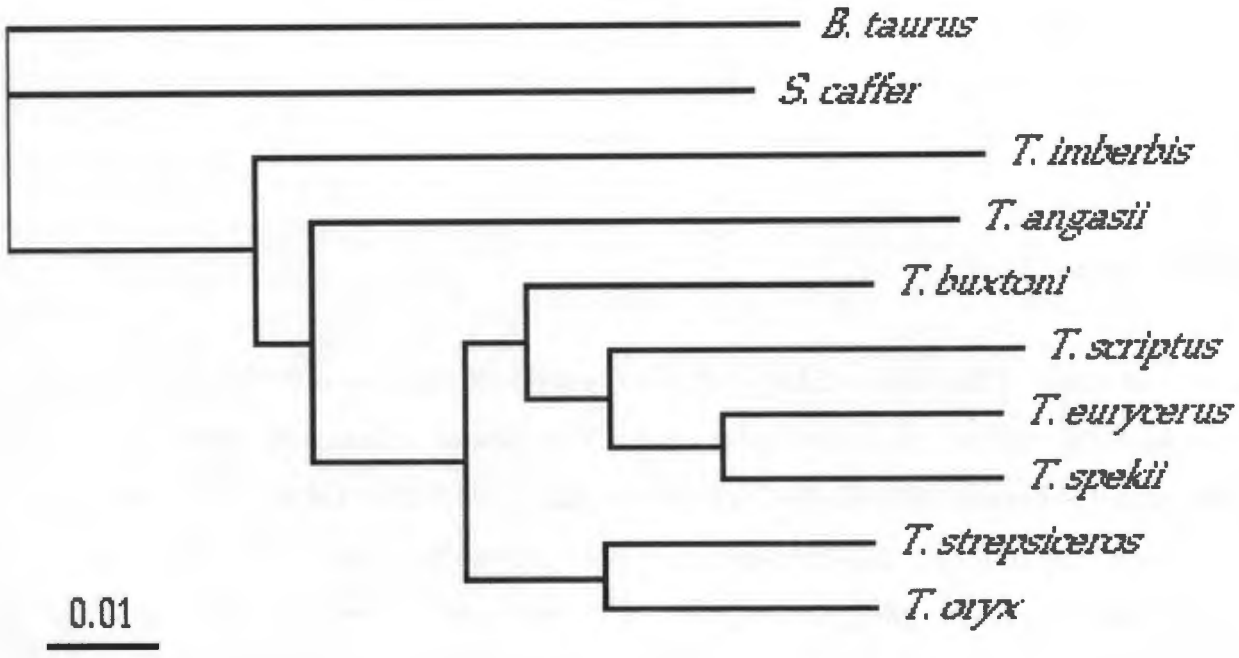

Figure 28b: Neighbor-joining tree for the Tragelaphini.

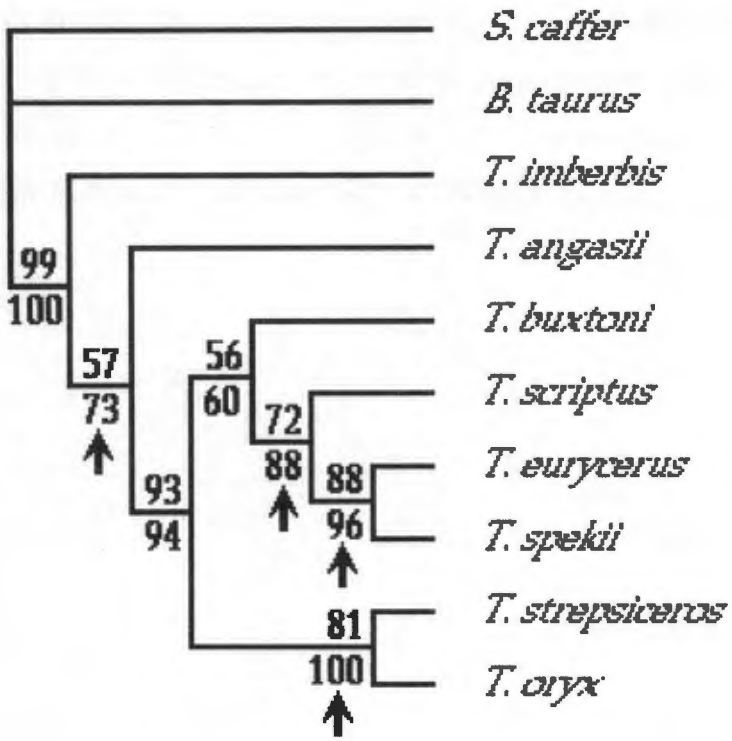

Figure 28c: Parsimony tree for the Tragelaphini with bootstrap values. The values above the branches were calculated with PAUP, the values below the branches were generated with RNA. The arrows indicate nodes whose support values differ significantly.

Chapter 5: HYBRIDISATION IN SAUDI GAZELLE: IMPLICATIONS FOR CONSERVATION MANAGEMENT.

5.1 **Introduction.**

The historical range of the now endangered Saudi gazelle (*Gazella saudiya*) is considered to have extended throughout the Arabian peninsula from Yemen to Iraq and Kuwait (Carruthers and Schwarz, 1935; Foster-Vesey-Fitzgerald, 1952; Lewis et al., 1965; see Figure 29 for a map of localities). This species is now probably extinct in the wild (Gasperetti and Gasperetti, 1981), unless some still survive in inaccessible areas in the north-western part of Saudi Arabia (Nader, 1989). There is doubt about the taxonomic status and origin of a herd of gazelles, which may be *G. saudiya*, on an island off the coast of the Northern Emirates (Gross, 1987). Less than a hundred putative *G. saudiya* survive in two larger collections (Al Areen Wildlife Park and Al Ain Zoo, see Figure 29 for locations), although there may be some present in small private collections on the Arabian peninsula. The numbers of *G. saudiya* in Al Ain Zoo have remained fairly constant for the last decade, but in Al Areen Wildlife Park they have declined drastically during the last few years.

Figure 29: Historical sample collection sites, historical sightings (after Thouless et al. (1991)), and the collections mentioned in the text.

The taxonomic position of *G. saudiya* has changed over the years. Morphometric analyses were originally used to claim that *G. saudiya* was the Arabian subspecies of *G. dorcas* (Groves, 1969; Groves, 1985; Groves and Harrison, 1967). Later morphometric and cytogenetic analyses proved it not to be a subspecies of *G. dorcas* (Furley et al., 1988; Groves, 1988; Rebholz et al., 1991). The cytogenetic data suggested that *G. saudiya* might be closely related to *G. bennettii*, which occurs from Iran to India (Rebholz et al., 1991).

Subsequent cytogenetic and molecular genetic studies of two *G. bennettii* subspecies and *G. saudiya* have raised the possibility that individuals of the Iranian *G. bennettii* subspecies have interbred with *G. saudiya* individuals, and at the same time excluded the Pakistani *G. bennettii* subspecies as the origin of the hybridisation (Kumamoto et al., 1995; Rebholz and Harley, 1997).

Data presented in this chapter show that one of the two principle captive populations of *G. saudiya* contains individuals which are likely to have hybridised with *G. bennettii*. For managers of these collections and other conservation orientated organisations to make firm commitments to a captive breeding program, it is highly advantageous to have information on the genetic make-up of these animals. These data will result in the provision of management recommendations for these two populations and suggestions for the type of work which needs to be done to ensure that only pure *G. saudiya* are included in a captive breeding program.

5.2 Cytogenetics.

5.2.1 Material and methods.

Blood samples used in this study were obtained from one female and two male *G. saudiya*, kept at King Khalid Wildlife Research Centre (KKWRC) in Thumamah, Saudi Arabia. KKWRC received these individuals in February 1990 from Al Areen Wildlife Park in Bahrain. Lymphocyte cultures were carried out as described in Chapter 2.2.

5.2.2 Results.

Pokeweed mitogen proved to be a better mitogen for *G. saudiya* lymphocytes than phytohaemagglutinin. Chromosome numbers of the three individuals studied were found to be 47, 50 and 51, the female having 2n = 47 (Figure 19a), the males having 2n = 50 (Figure 19b) and 2n = 51 (Figure 19c) respectively. Not only do chromosome numbers vary between individuals, but their karyotypes also differ. Variation in metacentric

chromosomes is conspicuous and their numbers are 11 (Figure 19c), 13 (Figure 19b) or 14 (Figure 19a) and some could not be paired, which is indicative of the presence of chromosome translocations. All other autosomal chromosomes are acrocentric and their numbers vary from 31 to 38. For the animals presented here banded chromosomes are not available. However, chromosome banding has been carried out on animals originating from the same Al Areen Wildlife Park collection (Kumamoto et al., 1995).

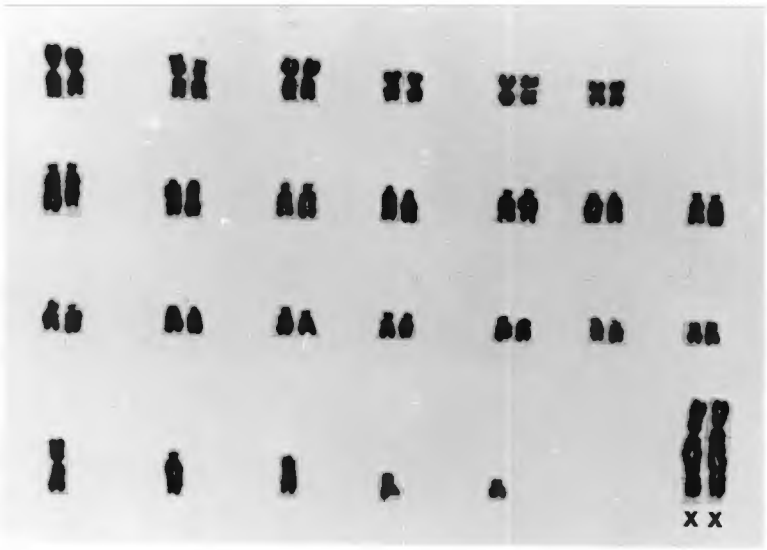

Figure 19a: Karyotype of a female Saudi gazelle (*G. saudiya*), with 2n = 47.

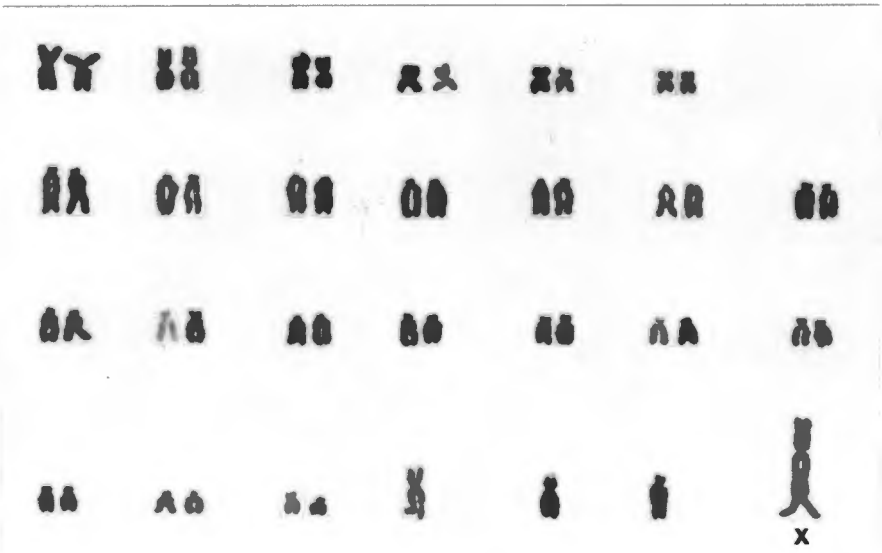

Figure 19b: Karyotype of a male Saudi gazelle (*G. saudiya*) with 2n = 50.

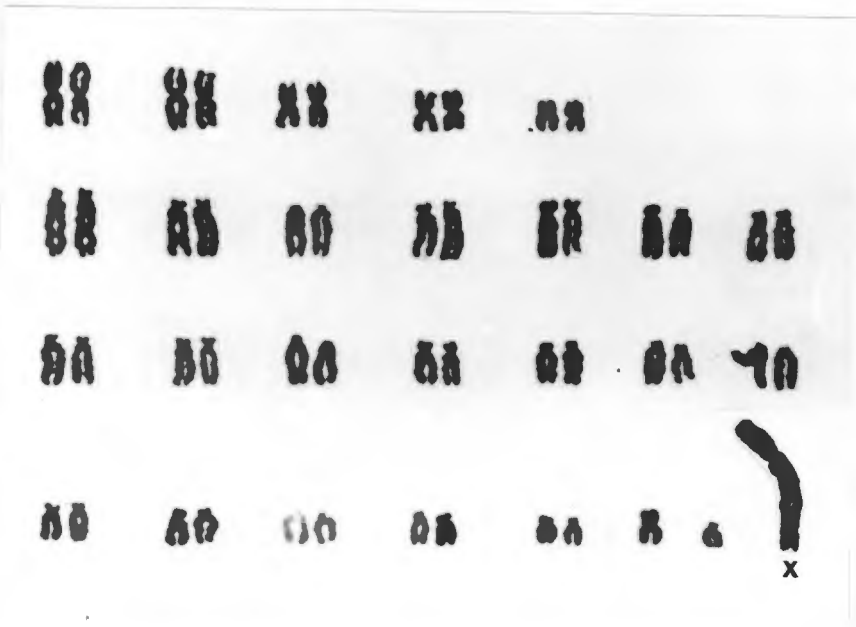

Figure 19c: Karyotype of a male Saudi gazelle (*G. saudiya*) with 2n = 51.

The submetacentric X chromosomes, dimorphic in the female, are double the size of the largest autosomes. This is the consequence of an X-autosome translocation, which is common in Antilopini. The absence of banded chromosomes makes it difficult to identify the Y chromosomes in these preparations, but recent banding studies have shown the Y chromosomes to be submetacentric (Kumamoto et al., 1995).

5.3 DNA sequencing.

5.3.1 Material and methods.

Blood and skin samples from nine *G. saudiya* and three *G. bennettii* individuals were used for DNA extraction, according to standard protocols (see section 2.3.1). Two individuals (*G. bennettii* 580 and 586) were from the Al Wabra collection in Qatar, all other animals were bred in Al Areen Wildlife Park and Reserve in Bahrain. Two *G. saudiya* individuals designated D1 and D4 were sent from Bahrain to King Khalid Wildlife Research Centre (KKWRC) in Thumamah, Saudi Arabia. The karyotypes of these two animals have been

described before (Rebholz et al., 1991), and see section 5.2 above. Karyotypes from the individuals designated SG1 to SG7 (except SG4) have been described by Kumamoto et al. (1995). Specimens from the British Museum of Natural History (BMNH) were used to characterise the species morphologically. Part of the mitochondrial cytochrome *b* gene (300 base pairs) was sequenced in both directions. The 300 base pairs fragment is the same as that used for all other animals as described in Chapter 4. The primers used for both PCR and sequencing, using slight modifications of standard protocols (Sanger et al., 1977; see sections 2.4 and 2.5) were:

L (14491) 5′-TGATATGAAAAACCATCGTTG-3′

H (14917) 5′-CCTCAGAAAGATATTTGTCCTC-3′

The nucleotide positions are in brackets and correspond to the 3′ end of the cow sequence (Anderson et al., 1982). Primer L (14491) is a modified version of the primer L14724 described in (Irwin et al., 1991), and primer H (14917) is a modified version of primer H15149, as described by Kocher et al. (1989). Sequence divergence values were calculated according to the Jukes-Cantor model (Jukes and Cantor, 1969).

5.3.2 **Results.**

Variable sites of the cytochrome *b* sequences are summarised in Table 10 and show that there are two distinct groups of mitochondrial DNA (mtDNA) in the nine studied *G. saudiya*. The first group contains three individuals (SG1, SG2, and SG7) which have identical mtDNA sequences (Table 10). The second group consists of six individuals with a significantly different sequence pattern. Four individuals (SG3, SG4, SG5, and SG6) in this group have identical mtDNA sequences, and two others (D1 and D4) differ by three and two nucleotides respectively from the previous four individuals (Table 10). The two groups differ from each other by between 7 and 10 nucleotides, corresponding to a 2.4 % to 3.5 % sequence divergence (Table 11). This is typical of values found between congeneric species (Irwin et al., 1991). The divergence between the three *G. bennettii* and the two *G. saudiya* groups, shown in Table 11, is 3.1 % to 3.4 % for the first group (SG1, SG2, and SG7) and 0 % to 1.0 % for the second group (SG3, SG4, SG5, SG6, D1, and D4). Although it would have been informative to include samples from all over the range of *G. bennettii* (Figure 4N), we only had access to *G. bennettii* samples from Iran and Pakistan.

	1 1 1 1 1 1 1 1 1 1 1
	4 4 4 4 4 4 4 4 4 4 4
	6 6 6 6 6 7 7 7 7 8 8
	0 4 5 5 8 4 4 6 9 0 2
	1 0 2 7 8 2 7 9 9 8 6
G. saudiya SG1	G T T C C C T C C G C
G. saudiya SG2	. . . . .
G. saudiya SG7	. . . . .
G. saudiya D1	A A A . T . . . T A T
G. saudiya D4	A A A T T . C T T A T
G. saudiya SG3	A A A T T A . T T A T
G. saudiya SG4	A A A T T A . T T A T
G. saudiya SG5	A A A T T A . T T A T
G. saudiya SG6	A A A T T A . T T A T
G. bennettii 586 (Iran)	A A A T T A . T T A T
G. bennettii 580 (Pakistan)	A A A T T . . T T A T
G. bennettii 8 (Pakistan)	A A A T T G . T T A T

Table 10: Variable sites in 300 base pairs of cytochrome *b* with their positions equivalent to the corresponding positions in the cow sequence. Sequence positions should be read from top to bottom (14601 etc.).

	1	2	3	4	5	6	7
1 *G. saudiya* SG1, SG2, SG7	300	3.4	2.4	3.5	3.4	3.1	3.4
2 *G. saudiya* SG3-SG6	10	300	1.0	0.7	0.0	0.3	0.3
3*G. saudiya* D1	7	3	300	1.0	1.0	0.7	1.0
4*G. saudiya* D4	10	2	3	289	0.7	0.4	0.7
5 *G. bennettii* 586 (Iran)	10	0	3	2	300	0.3	0.3
6 *G. bennettii* 580 (Pakistan)	9	1	2	1	1	300	0.3
7 *G. bennettii* 8 (Pakistan)	11	1	3	2	1	1	300

Table 11: Upper right half: percentage sequence divergence; lower left half: number of pairwise base pair differences; diagonal: numbers of base pairs sequenced.

5.4 Discussion.

There are no documented records of the origin of the individuals of the Al Areen collection, so information about the origin of *G. saudiya* individuals is anecdotal. However, the *G. saudiya* individuals were most likely acquired by Al Areen Wildlife Park after 1983 (F. A. Dean, pers. comm.). The lack of any history of subsequent introductions to the breeding herd since then implies that individuals with *G. bennettii* and *G. saudiya* haplotypes were present in the collection from its inception and most likely originated from a single collection. Hybridisation between *G. bennettii* and *G. saudiya* therefore is most likely to have taken place before the *G. saudiya* were transported to Al Areen Wildlife Park. At Al Areen Wildlife Park the *G. saudiya* individuals were originally referred to as "Qatari subgutturosa", indicating an origin in Qatar, but with an incorrect species designation. If these animals originated from Qatar, the chances are high that they came from an island off the north coast of Qatar, where the Ministry of Agriculture maintains a mixed gazelle population (F. Al-Timimi, pers. comm.).

The presence of two significantly different mtDNA haplotypes in the Al Areen *G. saudiya* herd strongly implies that the herd contains individuals from two species of gazelle. The sequence divergences between *G. bennettii* individuals and SG3 to SG6, D1, and D4 is 0 % to 1.0 %, so the mtDNA of the latter can therefore be concluded to originate from *G. bennettii*. There are three possibilities for the origin of the mtDNA haplotype found in the first three animals (SG1, SG2, and SG7) listed in Table 10:

1) the haplotype represents the genuine *G. saudiya* mtDNA,
2) it represents mtDNA from a still unrecognised species, or
3) it corresponds to mtDNA from a *G. bennettii* subspecies.

The second possibility seems unlikely and would require that the species has escaped recognition until now due to absence of specimens in museum collections or a lack of significant morphological characteristics. The third possibility requires acceptance that a mtDNA sequence divergence as large as 3.4 % is likely between two subspecies which are not widely separated geographically. Combined cytochrome *b* and cytochrome c oxidase III (COIII) sequences of the individual SG1 differ by between 3.0 % to 12.4 % from other species of Antilopini, including *G. bennettii* (Table 12). This indicates that the mtDNA from this individual represents the genuine *G. saudiya* mtDNA. This is supported by skull

measurements from a *G. saudiya* individual from the Al Areen collection, which assorted with museum specimens of *G. saudiya* (C. P. Groves, pers. comm.). Further support for this could be provided by sequencing mtDNA from *G. saudiya* museum specimens.

	G. saudiya		*G. saudiya*
G. bennettii (Pakistan, AW)	3.0 %	*G. dorcas osiris* (Almeria)	5.6 %
G. bennettii (Pakistan, AA)	3.1 %	*G. dorcas* ssp.	5.7 %
G. bennettii (Iran)	3.5 %	*G. dorcas osiris* (AW)	5.7 %
G. spekei	4.0 %	*G. g. cora* (Oman)	5.9 %
G. cuvieri	4.4 %	*G. g. cora* (SW Saudi Arabia)	6.0 %
G. leptoceros	4.5 %	*G. g. cora* (NW Saudi Arabia)	6.1 %
G. s. subgutturosa	5.1 %	*G. g. erlangeri*	6.1 %
G. s. marica (Oman)	5.1 %	*G. rufifrons thomsonii*	8.2 %
G. s. marica (KKWRC)	5.2 %	*A. cervicapra*	8.3 %
G. s. marica (Mahazat)	5.2 %	*G. dama mhorr*	8.4 %
G. g. gazella	5.4 %	*G. rufifrons leavipes*	8.5 %
G. dorcas osiris (KKWRC)	5.4 %	*G. dama ruficollis*	8.6 %
G. dorcas osiris (AA)	5.5 %	*G. soemmerringii*	8.9 %
G. dorcas osiris (Doha Zoo)	5.5 %	*G. granti*	9.1 %
G. dorcas massaesyla	5.5 %	*L. walleri*	9.3 %
G. dorcas pelzelni	5.6 %	*A. marsupialis*	12.4 %

Table 12: Percentages sequence divergence between *G. saudiya* individual SG1 and all other sequenced Antilopini species. Divergence values were calculated according to the Jukes-Cantor model and are based on 1083 base pairs of cytochrome *b* and COIII.

In *G. saudiya*, *G. bennettii*, and *G. dorcas*, only a few morphological characters support the separation of the three species, which is why the taxonomic position of *G. saudiya* has been so changeable. Originally, features of the nasofrontal suture were used to assign *G. saudiya* to an eastern subspecies of *G. dorcas*, and *G. bennettii* was considered an even farther eastern subspecies of *G. dorcas* (Groves and Harrison, 1967). Later analyses suggested that because *G. saudiya* was "so unlike its neighbour, *G. dorcas isabella*" it was doubtful whether it should persist as an Arabian subspecies of *G. dorcas* (Groves, 1985). Only very recently, an analysis of several skull measurements of museum specimens of *G. saudiya* , *G. bennettii* (both from the BMNH), and one recent specimen of *G. saudiya* from Al Areen resulted in the museum specimens of *G. saudiya* and the recent *G. saudiya* being interpreted as being identical and *G. saudiya* and *G. bennettii* interpreted as being separate species (Groves, 1996).

Since the diploid chromosome numbers of *G. dorcas* are 2n = 30 for females and 2n = 31 for males (Wurster, 1972), which is vastly different from 2n = 46 - 53 for *G. saudiya*, the two taxa cannot be subspecies of the same species. Apart from the difference in chromosome number, the sex-chromosomes of *G. saudiya* are completely different from those of *G. dorcas*. *G. dorcas* has metacentric autosomes only, whereas *G. saudiya* has both metacentric and acrocentric autosomes. It is therefore unlikely that *G. dorcas* and *G. saudiya* would produce fertile hybrids, if they could interbreed at all.

Karyotypes of *G. saudiya* appear to be very similar to those of *G. bennettii* (Furley et al., 1988; Kumamoto et al., 1995; Rebholz et al., 1991), the only difference between them being that *G. saudiya* has 10 to 14 metacentric autosomes whereas *G. bennettii* has 8 to 10. It has been shown that karyotypes of *G. bennettii* from Iran and *G. saudiya* are very similar, although the karyotypes of *G. bennettii* from Pakistan are distinctly different from either two (Kumamoto et al., 1995). It has also been shown that four chromosome translocations, (4;12), (8;14), (9;23), and (11;17) are present in *G. saudiya*, and three of these are also present in *G. bennettii* from Iran (Kumamoto et al., 1995). It is highly unlikely that *G. saudiya* is a subspecies of *G. bennettii* given the non-overlapping distributions of the two taxa (Groves, 1988).

G. saudiya and *G. bennettii* are not the only known species with a large variation in chromosome numbers (2n = 46 - 53, 2n = 49 - 52 respectively). A similar degree of intraspecific karyotype variation (2n = 34 - 39) was found in captive *G. soemmerringii* (Benirschke et al., 1984). In all cases several chromosome translocations (4, 3 and 3 respectively) are responsible for the variation in chromosome numbers. Although there is no information about the perinatal mortality rate for *G. saudiya*, for *G. soemmerringii* it was 50 - 60 % (Benirschke et al., 1984). The presence of several chromosome translocations in the breeding group of *G. soemmerringii* and its consequent outbreeding depression could be responsible for this high perinatal mortality rate. If that was the case, it would mean that *G. saudiya* with its similar situation could experience a similar perinatal mortality rate. That would be quite disturbing, since there are not many *G. saudiya* individuals left in captivity, and that could increase the chances of the species going extinct in the near future.

A possibility which cannot be excluded on the present evidence is that the herd consists of two non-interbreeding populations of *G. saudiya* and *G. bennettii* individuals reproductively isolated by a pre-mating recognition mechanism. Although karyological features show that *G. saudiya* have not interbred with the *G. bennettii* subspecies from Pakistan, they do support interbreeding between *G. saudiya* and *G. bennettii* from Iran (Kumamoto et al., 1995). This finding is consistent with the sequencing data, which show complete identity between sequences for an individual *G. bennettii* (the Iranian subspecies) and four purported *G. saudiya* individuals. Geographical considerations also make it more likely that the Iranian *G. bennettii* have hybridised with *G. saudiya*. It is relevant in this context that the Al Areen's *G. bennettii* (maintained in a separate compound) belong to the Pakistani subspecies, which makes it unlikely that hybridisation occurred at Al Areen Wildlife Park. The Iranian *G. bennettii* studied by (Kumamoto et al., 1995) all came from the Al Wabra collection in Qatar, which has never sent any animals to Al Areen (F. Al-Timimi, pers. comm.).

Since mtDNA is strictly maternally inherited, the data presented here show that at least three different female *G. bennettii* have contributed to the Al Areen population of *G. saudiya*. The presence of these three different haplotypes of *G. bennettii* mtDNA in this one breeding population implies that *G. bennettii* females must either have been part of the original *G. saudiya* population in the wild or have been present in the captive population since about 1983.

The mtDNA studies have only been able to identify animals which are likely hybrid offspring of female *G. bennettii* and male *G. saudiya*. Identifying hybrid offspring of female *G. saudiya* and *G. bennettii* males requires screening for nuclear markers which show fixed differences for the two species. Since no living unhybridised *G. saudiya* have been identified yet, identifying fixed markers for *G. saudiya* poses a problem, which could only be solved by identification of a remnant wild herd or by analysis of museum specimens. It is therefore not possible at this stage to identify and remove prospective hybrids from the population.

Several cases of hybridisation between Antilopini in captivity have been documented (Gray, 1971). Although in most cases these are examples of hybridisation between subspecies of a single species, one case describes a stillborn hybrid between *G. gazella* and *G. rufifrons* (Gray, 1971). Karyotypes have shown that hybrids between *G. dorcas* and *G. gazella*, and between *G. subgutturosa* and *G. gazella* did exist in the above mentioned KKWRC collection (Badri and Flavell, unpublished data). These species occur in different habitats in the wild, so they will never meet naturally, but the examples show that it is possible for different species of Antilopini to hybridise in captivity.

Conservation management decisions are required urgently, because numbers of *G. saudiya* have been reduced to less than one hundred in captivity and they are probably extinct in the wild. Apart from Al Areen Wildlife Park there is another collection of purported *G. saudiya* in Al Ain Zoo (United Arab Emirates) and there is therefore a need for those animals to be studied using similar molecular techniques. According to the latest information (J. Boef and J. Samour, pers. comm.) only a few *G. saudiya* are left at Al Areen Wildlife Park, due to an outbreak of Clostridiosis. However, there are still some 70 - 80 *G. saudiya* individuals remaining at Al Ain Zoo and they seem to be more similar to the British Museum of Natural History types than the Al Areen Wildlife Park animals are (J. Boef, pers. comm.). Therefore, all efforts to conserve *G. saudiya* should be concentrated on the last significant population in Al Ain Zoo.

Hybridisation between genetically divergent taxa can result in decreased fitness and/or fertility in the offspring (outbreeding depression). The relatively low sequence divergence between *G. saudiya* and *G. bennettii* mtDNA (2.4 % to 3.4 %) and the lack of any evidence for production of sterile hybrids indicates that the two species are close relatives. However, the animals have never been studied specifically for evidence of decreased fertility. All *G. saudiya* individuals in Al Areen Wildlife Park should therefore be closely monitored to see whether animals with different mtDNA haplotypes are able to produce offspring which are as fertile as offspring from parents with identical mtDNA haplotypes. These animals should also be karyotyped and have their mtDNA analysed and their skull measurements taken after death, so that all analyses can be linked. In order to prevent further hybridisation, all animals from the two mentioned collections should be sampled and have their mtDNA haplotypes determined. Animals with different mtDNA

haplotypes can then be kept in separate breeding groups. After the separation of haplotypes, animals with *G. saudiya* mtDNA should be screened using nuclear markers, in order to assist in identifying and selecting *G. saudiya* individuals which are probably uncontaminated with *G. bennettii* DNA.

Another issue relevant to the conservation of *G. saudiya* is the possibility of inbreeding depression, if the numbers of animals maintained in captivity remain low. In a random-breeding population the heterozygosity falls by a factor of $(1-(1/2N_e))$ per generation; therefore, in each generation this decreasing heterozygosity will be compounded if identified hybrids were to be removed from the breeding population. The nuclear markers referred to above have the advantage of not only assisting in hybrid identification but also in providing a measure of current levels of genetic diversity. When this is achieved it may be possible to select *G. saudiya* individuals with the least genetic contamination by
G. bennettii, and from these expand the population rapidly to a size which will minimise loss of genetic diversity. For new mutations to balance loss of genetic diversity due to genetic drift requires that the term $4N_e\mu \approx 1$ (Crow, 1986), which is roughly equivalent to an effective population size of 25,000 animals, assuming a mutation rate (μ) of 1×10^{-5} per generation. This may not of course be practical, but represents the ideal which could be obtainable with reintroduction to the wild in suitable sized and protected reserves. In the meantime, it can readily be calculated how much diversity would be lost in captive breeding herds of varying sizes, and some figures to act as a practical guide are given in Table 13. This table not only applies to Antilopini but to all kind of species. It shows, for example, that in a herd of 100 animals 4.5 % of the genetic diversity would be lost in 10 generations, which for Antilopini would be equivalent to about 50 to 70 years. This assumes non-overlapping generations and random mating between equal numbers of males and females, but despite these somewhat unrealistic restrictions the figures provide a general point of departure for the conservation manager.

Population size	Number of generations	Heterozygosity remaining (%)
10	1	95.0
	10	59.9
	50	7.7
50	1	99.0
	10	90.4
	50	60.5
100	1	99.5
	10	95.5
	50	77.5
500	1	99.9
	10	99.0
	50	95.1

Table 13: Heterozygosity remaining after several generations with varying population sizes. Percentages are calculated with the formula $(1-(1/2N_e))$.

Chapter 6: DISCUSSION.

6.1 Methods and techniques.

The properties and use of mitochondrial DNA sequences for phylogenetic analyses have been discussed widely (Ballard and Kreitman, 1995; Harrison, 1989; Hutchison et al., 1974; Kocher et al., 1989; Moore, 1995; Moritz et al., 1987; Wilson et al., 1985; Zhang and Hewitt, 1996). Despite its limitations, if properly used, mtDNA sequences can be a powerful tool for inferring phylogenies, especially when used in conjunction with other data sets. Morphological data, cytogenetic data and molecular data have shown to be of value for phylogenetic inference. It is informative to compare phylogenetic trees constructed from these unrelated data sets, because if they show similar results it means that the trees produced are congruent and therefore more likely to represent the true phylogeny (Hillis, 1995; Miyamoto and Fitch, 1995). An alternative method which combines all available data sets is called "total evidence" (Bull et al., 1993; Chavarria and Carpenter, 1994; Huelsenbeck et al., 1996; Kluge, 1989). It is better to study a group of taxa with several data sets (whether combined or analysed separately) if they are available instead of relying on one data set, since every data set has its limitations. In the case of the Antilopinae and the Tragelaphini only limited data (other than molecular) are available.

One limitation of the use of mtDNA sequences is the possible saturation of sequence changes at third codon positions in protein-coding genes (Krajewski and King, 1996). In such cases there will be a high frequency of homoplasy at third codon positions. Homoplasies in the transitions at third codon positions seem to become quite prominent when the Bovidae species are compared with *Sus scrofa* (Figure 24). The exclusion of the *S. scrofa* sequence was therefore justified.

Although beyond the scope of this study, there is a vast amount of literature addressing the relative merits of the various methods of phylogenetic analysis (Adachi and Hasegawa, 1996; Backeljau et al., 1996; Felsenstein, 1981; Felsenstein, 1984; Hillis, 1996; Hillis et al., 1994: Farris, 1986; Russo et al., 1996; Sourdis and Nei, 1988; Stewart, 1993; Takezaki and Nei, 1994; Yang, 1995; Yang, 1996). The results here show that nodes which

are well supported with the parsimony bootstrap technique are generally also well supported with the neighbor-joining bootstrap technique. When a particular node is not well supported with the neighbor-joining bootstrap method, that node will generally be represented by a polytomy with the parsimony method. Although the bootstrap method is widely used, it is recognised that it is not a perfect measure of confidence in a topology (Dopazo, 1994; Efron et al., 1996; Felsenstein, 1985; Hillis and Bull, 1993; Zharkikh and Li, 1995). Although both PAUP and RNA parsimony bootstrap analyses lead to similar consensus topologies, some bootstrap values are quite different. Some of the bootstrap support values differ as much as 31 % (Figures 26a and 26e, the node including the two *G. g. cora*), which is too much to be explained as an artefact inherent to the bootstrap technique. With a few exceptions, RNA shows higher bootstrap support values than PAUP (see the arrows in Figures 26e, 27d and 28c). The unexpected finding that seemingly identical procedures in different programs give different results suggest that one has to be aware of the kind of algorithms used in each program. It seems sensible to test several of the most popular programs with identical data sets (Backeljau et al., 1996). It would be preferable if a particular procedure would give similar results for a given data set in all programs which allegedly use the same basic methodology, because otherwise it becomes difficult to compare different character sets analysed with different programs.

6.2 Discussion of results.

6.2.1 Antilopini.

It has been argued on the basis of morphological and paleontological data that the Antilopini are not monophyletic, although it has not been made clear which species or genera of the tribe could be responsible for its paraphyly (Groves, 1985; Vrba, 1985). However there are two genera, *Ammodorcas* and *Procapra* which have not been included in this study, and they could potentially make the tribe paraphyletic. The species which belong to the genus *Procapra* have been described as atypical gazelles (Groves, 1967). Sequence data for these genera need to be acquired before a conclusion can be reached as to whether the tribe is monophyletic or paraphyletic. The molecular data presented in Chapter 4 do not present strong support for the paraphyly of the tribe. In both parsimony

and neighbor-joining analyses the Antilopini always cluster. The bootstrap support for the monophyly of the tribe is high (91 %) in neighbor-joining analysis, but low (63 %) in parsimony analysis. The species which is responsible for this low parsimony bootstrap support is *A. marsupialis*. When this particular species was excluded, the bootstrap value was considerably higher.

Immuno-distance data suggest that *G. dama* is basal to *A. cervicapra* and *G. thomsonii*, and cytogenetic and morphological data imply that *G. dorcas* is basal to *A. cervicapra* and several species in the genus *Gazella* (Gentry, 1992; Lowenstein, 1986b; Vassart et al., 1995b). These results support the paraphyly of the genus *Gazella*. The suggestion that *A. cervicapra* is basal to *G. thomsonii*, which is based on immuno-distances (Lowenstein, 1986b), is not supported by molecular data nor by cytogenetic data (Vassart et al., 1995b; Chapter 4). Both parsimony and neighbor-joining analyses of sequence data show weak support for the paraphyly of the genus *Gazella*, but there is no significant bootstrap support for this from either analysis method. The analysis of more sequence data could perhaps lead to a firmer conclusion on the position of *A. cervicapra*. The molecular data presented here do not show conclusively whether the genus *Gazella* is monophyletic or paraphyletic, although the tendency is towards paraphyly. As discussed in Chapter 4, it only takes seven extra steps from the initial 798 to take *A. cervicapra* out of the *Gazella* clade, which shows that the position of *A. cervicapra* in the *Gazella* clade is not very robust. New morphological data (Groves in Vassart et al. (1995b)) suggest that *A. cervicapra* should be included in the *Gazella* clade, which supports the paraphyly of the genus *Gazella*. In view of these various results it would seem appropriate to revise the classification of the genus *Gazella*. Either all species of the genus *Gazella* should be put in the genus *Antilope*, or all species of the subgenus Nanger and *G. rufifrons*/*G. thomsonii* should be removed from the genus *Gazella*.

There are a few differences between the results of the molecular data presented here and the results of morphological studies, essentially those by Groves (Groves, 1985; Groves, 1988). None of the morphological studies have been used to construct a comprehensive a phylogeny for the Antilopini, but affinities between all species of the genus *Gazella* have been reported (Groves, 1985). The genus *Gazella* has been split up on this basis into five groups:

1) *G. dama, G.granti,* and *G. soemmerringii* (the subgenus Nanger)

2) *G. rufifrons* and *G. thomsonii*

3) *G. subgutturosa* and *G. leptoceros*

4) *G. cuvieri*

5) *G. dorcas, G. gazella, G. spekei, G. saudiya,* and *G. bennettii*

The close affinities deduced from morphological criteria within the subgenus Nanger and within the *G. rufifrons/G. thomsonii* group are confirmed by analyses of both cytogenetic and molecular data. These first two groups are closely related to each other, on the basis of both parsimony and neighbor-joining analysis, which is inconsistent with fossil data (Gentry, 1978). The fossil data suggest that *G. rufifrons* and *G. thomsonii* are more closely related to *G. leptoceros* than to the species from the subgenus Nanger. The fossil data are clearly at odds with the genetic data, and since the phylogeny based on fossil data was tentative, the two genetic data sets are probably a better indication of these relationships. There is no support from either cytogenetic data or molecular data to keep *G. cuvieri* in a separate group, because both cytogenetic and molecular data sets suggest that this species should be included in the clade *G. subgutturosa/G. leptoceros,* as indicated by Groves (1985). Support for the monophyly of the group *G. dorcas/G. gazella/G. spekei/G. saudiya/G. bennettii* is not evident from molecular data. *G. bennettii* and *G. saudiya* are more closely related to the cluster *G. subgutturosa/G. leptoceros/G. cuvieri* than to *G. dorcas* and *G. gazella,* in contrast to the suggestion by Groves (1985). Therefore, the genetically supported groupings are *G. dorcas/G. gazella/G. spekei,* and *G. bennettii/G. saudiya.* However, it is not conclusive from molecular data that the first three species constitute a monophyletic group. To summarise, the five groups of the genus *Gazella* advocated here are:

1) *G. granti, G. dama,* and *G. soemmerringii*

2) *G. rufifrons* (including *G. thomsonii*)

3) *G. subgutturosa, G. leptoceros,* and *G. cuvieri*

4) *G. saudiya* and *G. bennettii*

5) *G. dorcas, G. gazella,* and *G. spekei*

It seems that the groups 3 and 4 could constitute one group. Although anatomical data suggested a close relationship between *G. dorcas* and *G. gazella* (Groves, 1969), group 5 might have to be split up into two or even three separate groups, because there is no bootstrap support for a monophyletic clade from the molecular data. They are kept together here provisionally on the basis of the cytogenetic and morphological data, which do suggest a close relationship between them.

The results from the molecular data show conclusively that the subgenus Trachelocele (containing only *G. subgutturosa*) is not a valid subgenus. *G. subgutturosa* clusters strongly with *G. cuvieri* and *G. leptoceros* from the subgenus Gazella (Figures 26a-d), which is supported by cytogenetic data (Vassart et al., 1995b). Therefore, *G. subgutturosa* should be included in the subgenus Gazella and the subgenus Trachelocele should be abandoned.

Research done on *G. saudiya* (Chapter 5; Rebholz and Harley, 1997; Rebholz et al., 1991) showed that *G. saudiya* is not a subspecies of *G. dorcas*, in contrast to work published elsewhere (Groves, 1969; Groves, 1985; Groves and Harrison, 1967). The results from mtDNA data showed the *G. saudiya* in the Al Areen Wildlife Park and Reserve collection to be hybridised with *G. bennettii*. The population of *G. saudiya* in this collection has declined in numbers recently by disease. This population bottleneck will lead to a considerable loss of heterozygosity if the population does not recover soon. Consequently, from both the hybridisation and possible inbreeding aspects, it might not be worthwhile to investigate this collection any further. The focus of investigation should shift towards the other main population of *G. saudiya* in Al Ain Zoo. Another approach could be to investigate small private collections in the Arabian peninsula, where pure *G. saudiya* still could exist.

In the case of the molecular data concerning subspecies, p-distances vary between 0 % and 1.3 %, depending on the species in question (Table 8). If this is considered to be the typical range for subspecies differences, then *G. rufifrons* and *G. thomsonii* with a difference of 1.2 % show a value more typical of that between two subspecies. Chromosomal and morphological research also suggested that *G. rufifrons* and *G. thomsonii* should be regarded as being conspecific (Groves and Lay, 1985; Vassart et al., 1995b). These two taxa have been shown to hybridise in captivity (Gray, 1971). The ranges of intraspecific and

interspecific differences are very close to each other and probably will be found to overlap when more taxa are studied. In spite of this, three techniques lead to identical conclusions, which lends strong support to a subspecific designation in this particular case. A different situation exists for *G. g. gazella*. This Israeli subspecies of *G. gazella* is 1.9 % to 2.5 % different from the other *G. gazella* subspecies. This is considerably more than the differences between the other subspecies, which are between 0 % and 1.3 %. The range of p-distances between recognised Antilopini species is 1.4 % to 9.5 % (Table 8). This suggests that *G. g. gazella* is too different from the other *G. gazella* subspecies to be recognised as a subspecies of *G. gazella*. *G. g. gazella* is geographically and reproductively isolated from the other *G. gazella* subspecies, which occur in the Arabian peninsula. This is strong evidence to elevate *G. g. gazella* to species status.

The data for the Tragelaphini show that in that tribe interspecific differences are as high as 12.5 %, which is even more than the differences between Antilopini genera. It seems not sensible to extrapolate percentages sequence difference and the accompanying taxonomic difference from one tribe to another.

The clade for the *G. gazella* subspecies in Figure 26 could reflect the pattern in which the species invaded the Arabian peninsula. The species seems to have evolved first in Israel (*G. g. gazella*) and has subsequently moved from north-western Saudi Arabia (*G. g. cora*) down to south-western Saudi Arabia (*G. g. cora* and *G. g. erlangeri*) and Oman (*G. g. cora*) in the southeast. As the common name for this species indicates (Mountain gazelle), it is mostly restricted to mountains. Its expansion from Israel along the mountain ranges in the Arabian peninsula is therefore plausible. It would be interesting to investigate the biogeographic evolution pattern for *G. dorcas* subspecies. At present the clade is not resolved, because the two genes used here have not accumulated enough changes. Faster evolving DNA sequences such as the mitochondrial D-loop region, could be able to resolve such phylogenetic relationships (Brown et al., 1986; Wenink et al., 1993). This is only possible if the different subspecies evolved consecutively and over a relatively well spaced time period.

The genetic distances (calculated with the Kimura 2-parameter) between *G. dama* and *G. thomsonii* is 7.5 %, and that between *G. rufifrons* and *G. leptoceros* is 9.3 %. Since the

ancestors of these species are thought to have existed less than 5 million years and 10 million years ago respectively, this is consistent with the calibration of 2 % sequence divergence per million years (Brown et al., 1979). *G. dama* and *G. thomsonii* should on this basis have a common ancestor 3 to 3.5 million years ago, and *G. rufifrons* and *G. leptoceros* should have a common ancestor 4.5 to 5 million years ago. If this is true, then the common ancestor of *G. dama* and *G. thomsonii* is considerably more recent than that deduced from the fossil data, but similar to estimates from immuno-distance data (Gentry, 1978; Lowenstein, 1986b). The dating of the common ancestor of *G. rufifrons* and *G. leptoceros* is comparable to the dating from fossil data (Gentry, 1978). The maximum distance between species from the genus *Gazella* is 9.5 % (see Chapter 4), suggesting that the genus evolved only around 5 million years ago. Since cytochrome *b* and probably COIII only diverge linearly for up to 10 million years ago, no dates older than that are likely to be accurate, and even then it must be appreciated that the stochastic error on such estimates is high (Swofford et al., 1996).

One should be cautious about using only external features for identification, without having access to morphometric data since misidentification is possible in a genus with such close morphological similarities. Museum skins could also be a potential source of misidentification if no skulls are available for the same individual.

There is a possibility of misidentification with specimens of the species *G. g. erlangeri* and/or *G. g. cora*. In the phylogenetic tree in Figures 26a-d *G. g. erlangeri* resides inside the clade of *G. g. cora*. There are two possibilities: either *G. g. erlangeri* is a melanistic form of *G. g. cora*, or the *G. g. cora* from north-western Saudi Arabia is misidentified. It could belong to the subspecies *G. g. gazella*, *G. g. erlangeri*, or to an unidentified subspecies. The subspecies *G. g. erlangeri* seems to be sufficiently different morphologically from *G. g. cora* to be a separate subspecies, and separate species status has even been argued for *G. g. erlangeri* (Groves, 1996). The main reason for this is the overlap in range between *G. g. cora* and *G. g. erlangeri* without any signs of genetic exchange. If lineage sorting of mtDNA morphs has not taken place in this species, and *G. g. erlangeri* is to be given separate species status, then *G. g. gazella* and *G. g. cora* from north-western Saudi Arabia should be given separate species status as well (Moore, 1995).

Not all species of the tribe Antilopini were included in this study, notably all three species of the genus *Procapra* from China, Mongolia, and Tibet, and the African species *A. clarkei* have not been included. It would be desirable to include these species in future studies. The species *Saiga tatarica* should also be included in future studies, because an analysis of 300 bp of cytochrome *b* indicated a close relationship between *S. tatarica* and the subfamily Antilopinae (data not shown). However, 300 base pairs are not enough to reach a definitive conclusion in this case, and the COIII gene should be sequenced to obtain a better insight into the associations of *S. tatarica*. Only half of the 51 taxa (species and subspecies) in the tribe Antilopini (Groves, 1985) are included in this study. Although it is not necessary for the understanding of the species phylogeny of the tribe, it would be informative to include as many subspecies as possible in future studies. At the same time, these studies could focus on the validity of the many subspecies recognised in *G. dorcas* and *G. rufifrons*, since not all authors recognise the same subspecies (Groves, 1985; Groves, 1988; Grzimek, 1968; Haltenorth and Diller, 1988).

It has been shown that chromosomal polymorphisms exist in several of the Antilopini (Benirschke et al., 1984; Kumamoto et al., 1995; Rebholz et al., 1991). This demonstrates the need to include several unrelated individuals per species in future cytogenetic studies to maximise the chance of discovering intraspecific polymorphisms. This also applies to molecular data, where several slightly different mitochondrial DNA morphs can exist in a single species (Moore, 1995).

Several Antilopini species are endangered, and their numbers are still declining. However, it is reassuring to know that if these species are reintroduced into the wild and/or protected from hunting that they should increase in numbers and that such conservation measures are in progress (Dunham et al., 1993; Mendelssohn, 1993; Munton, 1988). This indicates that co-ordinated breeding programmes could be a way to save species from extinction. International and regional captive breeding programmes are in place for a variety of Antilopini species (Sausman, 1989; Sausman, 1993).

6.2.2 **Neotragini.**

The Neotragini appear not to be very closely related to each other since p-distances for individual genera are 13 % on average, with a maximum of 14.1 %. Although there seem to be affinities between *O. ourebi*, *M. guentheri* and the two species of the genus *Raphiceros*, these are distant relationships. *N. moschatus* is even more distantly related to the other four species, suggesting that the Neotragini are paraphyletic, which confirms previous suggestions (Gentry, 1992; Georgiadis et al., 1990). In all studies it is primarily *N. moschatus* that renders the Neotragini paraphyletic, so this suggests that consideration should be given for this species to be assigned to a different tribe, which could then make the other Neotragini monophyletic. However, two genera (*Dorcatragus* and *Oreotragus*) are not included in this study, which makes it difficult to reach firm conclusions at the present time.

It has been suggested that *Pelea capreolus* could be a member of the Neotragini (Gentry, 1992; Georgiadis et al., 1990). From the molecular data this species appears to be more closely related to *A. melampus* than to the Neotragini species, although statistical support for this is weak. *P. capreolus* is certainly not closely related to the group *Ourebia/Raphiceros/Madoqua*, but it could be closely related to *Oreotragus* and/or *Dorcatragus*, although there is no morphological evidence for that (Gentry, 1992). There is no reason to include *P. capreolus* in the Neotragini, especially since the Neotragini seem to be paraphyletic. It appears that the Neotragini, or at least the group *Ourebia/Raphiceros/Madoqua*, may be the sister group to the Antilopini, as advocated previously (Gentry, 1992; Savage and Russell, 1983). It would be valuable to include all Neotragini species, or at least representatives of all six genera, in a molecular study to assess their relationships. Then it would be possible to compare the results with the one morphological study that included all six genera (Gentry, 1992).

6.2.3 **Tragelaphini.**

The phylogenies produced with cytogenetic and molecular data (Figures 9, 27 and 28) provide good bootstrap support for the two species which are sometimes assigned to the

genus *Taurotragus* (*T. oryx* and *T. eurycerus*) not being sister species. Therefore there is no support for the retention of the genus name *Taurotragus* and all Tragelaphini would be more appropriately included in a single genus, *Tragelaphus*, as advocated by Vrba (1987).

The commonly held idea that the Tragelaphini is a monophyletic tribe is reinforced by the strong support for monophyly shown with the present molecular data. Cytogenetic data suggest that *Boselaphus tragocamelus* should be included in the Tragelaphini, although it is generally placed in the Boselaphini (Benirschke et al., 1980). This would make the Tragelaphini paraphyletic. In the present molecular study no Boselaphini were included. It could be informative to gather molecular data for the two Boselaphini species (*B. tragocamelus* and *Tetracerus quadricornis*) and test whether they are a sister tribe to the Tragelaphini or whether they force the Tragelaphini to be paraphyletic.

The phylogeny for the Tragelaphini based on fossil data is different from that produced by both cytogenetic and molecular data (Benirschke et al., 1980; Gentry, 1978). Fossil data suggest that *T. imberbis* is a recently derived species, which is closely related to *T. strepsiceros* and distantly related to *T. angasii*, while molecular and cytogenetic data imply that *T. imberbis* is closely related to *T. angasii* and that the two form a basal group in the tribe. Since the phylogeny based on fossil data was presented as provisional, the genetic phylogenies may be more reliable. Allozyme data have been used to suggest that *T. scriptus* is basal to *T. imberbis*, *T. strepsiceros*, and *T. oryx* (Georgiadis et al., 1990). The high bootstrap support for *T. imberbis* being basal instead of derived (93 % and 99%) and the support from cytogenetic data suggest convincingly that this relationship is reliable. There are two discrepancies between the cytogenetic and molecular phylogenies of the tribe. Firstly, cytogenetic data suggest that *T. angasii* is basal to *T. imberbis* while molecular data imply the opposite. This incongruence could be due to the rather low bootstrap support (57 % and 68 %) for this particular node with the molecular data. The cytogenetic data could be a better indication of the relationship between these two species. Secondly, molecular data show a high bootstrap support for the close relationships between *T. spekii*, *T. eurycerus* and *T. scriptus*, whereas cytogenetic data suggest that *T. spekii* is closely related to *T. strepsiceros* and *T. oryx*. The high bootstrap supports for the three nodes separating *T. spekii* and the cluster *T. strepsiceros*/*T. oryx* suggest strongly that the molecular data reveal the correct relationships of *T. spekii*.

6.2.4 Outgroups.

Several outgroup species were used since species sampling was shown to have an effect on the analysis (Adachi and Hasegawa, 1995; Lecointre et al., 1993). It has been shown that outgroup species which are close to 20 % different from the ingroups are not ideal, because the third codon positions start to become substantially saturated (Graybeal, 1993). It would be difficult to leave out the third codon positions if they were still informative for ingroups which are less than 15 % different. It is better to use outgroups which are 15 % or less different from the ingroup, if possible, so that all codon positions can be used informatively.

The rapid radiation during the early evolution of the Bovidae, which took place 20 million years ago (Lowenstein, 1986b; Vrba, 1985) could be responsible for the lack of bootstrap support in both parsimony and neighbor-joining analysis for the delineation between the outgroups and the Neotragini. The neighbor-joining analysis showed short branches at the tribal level, suggesting the same conclusion. It is unlikely that the amount of sequence data are insufficient for phylogenetic resolution at the tribal level, since there is good support for the monophyly of both the Antilopini and the Tragelaphini.

A. melampus and *P. capreolus* have been assigned to the Reduncini in the past (Ellerman et al., 1953; Simpson, 1945). Since no members of the Reduncini were included, these suggestions could not be tested. However, *A. melampus* has also been thought to belong either to the Antilopini or to the Alcelaphini (Gentry, 1992; Simpson, 1945), but molecular data presented here suggest otherwise. For the time being it appears to be best to keep *A. melampus* and *P. capreolus* in their own tribes, Aepycerotinae and Peleinae.

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Fresh blood and/or skin samples:

Al Wabra collection (Qatar):

Gazella dorcas ssp. (Saudi Arabia)	4.4.2
G. dorcas isabella	0.1.2
G. dorcas pelzelni	3.4
G. bennettii christii (Pakistan)	3.5
G. bennettii fuscifrons (Iran)	3.4
G. soemmerringii berberana	2.3
G. rufifrons leavipes	1.2
G. spekei	1.3
G. gazella erlangeri	1.0.1
G. subgutturosa subgutturosa	1.0

Parc Zoològic de Barcelona S.A. (Barcelona, Spain):

G. dorcas osiris	1.3.1

Los Angeles Zoo (Los Angeles, USA):

G. spekei	0.0.1

Diergaarde Blijdorp (Rotterdam, The Netherlands):

G. dama ruficollis	3.3

Al Areen Wildlife Park and Reserve (Bahrain):

G. bennettii ssp.	1.8
G. saudiya	1.6
G. dorcas osiris (or *G. d. isabella*)	0.0.4

Whipsnade Wild Animal Park (Dunstable, England):

Antilope cervicapra	0.1.2
G. thomsonii	0.0.2
Tragelaphus angasii	0.0.1

Estación Experimental de Zonas Aridas (Almería, Spain):

G. cuvieri	0.5
G. dorcas osiris	1.4
G. dama mhorr	2.3

San Diego Zoo (San Diego, USA):

Neotragus moschatus	0.0.1
Litocranius walleri	0.0.1
G. leptoceros	0.0.1
G. granti roosevelti	0.0.1
G. dama ruficollis	0.0.1
G. dama mhorr	0.0.1
G. cuvieri	0.0.1
Madoqua guntheri	0.0.1
Aepyceros melampus	0.0.1

White Oryx Project (Muscat, Oman):

G. gazella cora	0.0.3
G. subgutturosa marica	0.0.2

Parc Zoologique National de Rabat (Témara, Morocco):

G. dorcas massaesyla	3.2
G. cuvieri	2.3

King Khalid Wildlife Research Centre (Thumamah, Saudi Arabia):

G. thomsonii	0.1.3
G. gazella cora	1.1.5
G. subgutturosa marica	3.3
G. saudiya	2.2
G. dorcas isabella	1.1

Doha Zoo (Doha, Qatar):

G. dorcas isabella	0.1
G. bennettii ssp.	0.1

Nature Reserve Authority (Jerusalem, Israel):

G. gazella gazella	0.2

University of Cape Town (Cape Town, South Africa):

Antidorcas marsupialis	0.0.1
Cephalophus natalensis	0.0.1
Damaliscus lunatus	0.0.1
Ourebia ourebi	0.0.1
Pelea capreolus	0.0.1
Raphiceros campestris	0.0.2
Raphiceros melanotis	0.0.2
Sus scrofa	0.0.1
Synceros caffer	0.0.1
Tragelaphus buxtoni	0.0.1
T. eurycerus	0.0.1
T. oryx	0.0.1
T. scriptus	0.0.1
T. spekii	0.0.1
T. strepsiceros	0.0.1

Pretoria Zoo:

Tragelaphus imberbis	0.0.1

<u>Dried skin samples:</u>

British Museum of Natural History (London, England):

G. saudiya	2.5
G. gazella muscatensis ?	0.0.1
G. gazella erlangeri	0.0.5
G. gazella cora ?	0.0.3

Tel-Aviv University (Tel-Aviv, Israel):

G. gazella acaciae	3.1
G. gazella gazella	3.2
G. dorcas isabella ?	8.2

King Khalid Wildlife Research Centre (Thumamah, Saudi Arabia):

G. gazella cora ?	0.0.1

Appendix 1: The samples collected for this study. The digits correspond to the numbers of males, females and unknown sex respectively. Species names according to Corbet and Hill (1991). Subspecies names according to Groves (1985).

Buffer:	Final concentration:
TBE (pH 8.8)	
Tris-base	15.75 g/l
Boric acid	4.64 g/l
EDTA	0.91 g/l
TE (pH 7.8)	
Tris-base	10 mM
EDTA	1 mM
TNE (pH 7.5)	
Tris-HCl	50 mM
NaCl	100 mM
EDTA	5 mM
TNES (pH 7.5)	
Tris-base	50 mM
NaCl	400 mM
EDTA	100 mM
SDS	0.5 %
10 x PCR buffer (without $MgCl_2$):	
Tris-HCl (pH 8.8)	100 mM
KCl	500 mM
10x sequenase buffer:	
Tris-HCl (pH 7.5)	400 mM
$MgCl_2$	200 mM
NaCl	500 mM
Chase (100 µl):	
dH_2O	70 µl
dNTPs (at 5 mM each)	10 µl
DMSO	10 µl
10x sequenase buffer	10 µl

Appendix 2: Buffers.

AA	= Al Areen Wildlife Park and Reserve, Bahrain
AW	= Al Wabra animal collection, Qatar
BMNH	= British Museum of Natural History
bp	= Base pair(s)
cm	= Centimetre
CO_2	= Carbondioxide
COIII	= Cytochrome c oxidase III gene
Conc.	= Concentration
cyt b	= Cytochrome *b* gene
ddATP	= Dideoxyadenosine triphosphate
ddCTP	= Dideoxycytoosine triphosphate
ddGTP	= Dideoxyguanine triphosphate
ddTTP	= Dideoxytyrosine triphosphate
DMSO	= Dimethyl sulphoxide
DNA	= Deoxyribonucleic acid
dNTP	= Deonucleoside triphosphate
dsDNA	= Double Stranded DNA
DTT	= Dithiothreitol
g	= Gravitational constant
g/l	= Grams per litre
KKWRC	= King Khalid Wildlife Research Centre, Saudi Arabia
M	= Molar
µ	= Mutation rate
µg	= Microgram
µl	= Microlitre
mA	= MilliAmpere
mg	= Milligram
ml	= Millilitre
mM	= MilliMole
mm	= Millimetres
mtDNA	= Mitochondrial DNA
N_e	= Effective population size
PCR	= Polymerase chain reaction
Pers. comm.	= Personal communication
pH	= Acidity measure
pM	= PicoMole
rpm	= Rounds per minute
ssDNA	= Single stranded DNA
^{35}S-dATP	= Deoxyadenosine triphosphate, radioactively labelled with Sulphurus-35
Taq	= DNA polymerase derived from *Thermus aquaticus*
TEMED	= N,N,N',N'-tetramethylethylenediamine
Ti	= Transition
Tv	= Transversion
U	= Units
V	= Volts
W	= Watts

Appendix 3: Abbreviations.