Evolutionary Relationships among Hares (*Lepus* spp.) from Ethiopia: Multivariate Morphometry, Molecular Phylogenetics and Population Genetics

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LIST OF ABBREVIATIONS

AMOVA- Analysis of Molecular Variance

ANCOVA- Analysis of Covariance

BA-Bayesian Analysis

DA-Descriminate Analysis

DF- Discriminate Function

IAM- Infinite Allele Model

MCMC- Markov Chain Monte Carlo

MtDNA- Mitochondrial DNA

NJ- Neighbour Joining

NuDNA- Nuclear DNA

PCA- Principal Component Analysis

PCR- Polymerase Chain Reaction

PCR-RFLP-Polymerase Chain Reaction Fragment Polymorphism

RSA- Republic of South Africa

SMM- Stepwise Mutation Model

SSM- Single Strand Mis-paring

SSR- Simple Sequence Repeat

STR- Short Tandem Repeat

TPM- Two Phase Model

UCO- Unequal Crossing-over

VNTR-Variable Number of Tandem Repeat

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Zelalem Gebremariam Tolesa: Addis Ababa University, Christian Albrecht's University of Kiel and Vienna University of Veterinary Medicine

ABSTRACT

Three *Lepus* species are currently considered to occur in Ethiopia: *Lepus habessinicus* Hemprich and Ehrenberg 1832 (Abyssinian Hare*), L. fagani* Thomas 1903 (Ethiopian Hare) and *L. starcki* Petter 1963 (Ethiopian Highland Hare) based on limited morphologic and cytogenetic data. Analyses of morphological characters, 13 microsatellite loci, one mitochondrial (ATPase sub-unit 6 sequence) and five nuclear sequences (Transferrin (TF), Thyroglobulin (TG), Uncoupling protein two (UCP2), Uncoupling protein four (UCP4) and Thyroid stimulating hormone beta (TSHB)) were made to provide an initial comprehensive data on the evolutionary relationship among the three hare species collected from twenty six localities in Ethiopia and identified by their external phenotypes, dental and skull characters. In addition, other *Lepus* species: *L. capensis*, *L. saxatilis*, *L. europaeus*, *L. timidus* and *L. victoriae* were used for phylogenetic comparison. Multivariate skull morphometric analyses (PCA & DA) of seven *Lepus* species (n = 327) indicated clear distinction among the species. *Lepus habessinicus* had a distinct morpho shape space relative to *L. capensis*. *Lepus fagani* had a distinct morpho shape space relative to *L. saxatilis* and *L. victoriae*. *Lepus starcki* had a distinct morpho shape space relative to *L. europaeus* and *L. capensis*. Microsatellite analysis based on hares (n = 107) collected from Ethiopia resulted in clusters of hare species in accordance with population origin and collection sites. Individuals of *L. habessinicus* and *L. fagani* were substructured in accordance with population of origin and collection localities with some degree of SSR introgression among each other. Microsatellite introgressions were documented also between species. In all analyses (structure, FCA and NJ) *L. starcki* individuals were consistently clustered into a single group. All phylogenetic reconstruction methods used (Neighbour Joining, Maximum Likelihood, Bayesian Analysis and Median Joining Networks) by and large resulted in similar topologies for mitochondrial and nuclear sequences, respectively. The three hare species from Ethiopia were not monophyletic for both mitochondrial and nuclear sequences, but showed signs of both unidirectional and bidirectional introgressions among each other. *Lepus habessinicus* carried mtDNA distinct from South and North African *L. capensis*, with no sign of introgression, contrary to earlier suggestions to include *L. habessinicus* under *L. capensis*. Similarly, *L. starcki* had distinct mtDNA relative to *L. capensis* and *L. europaeus* as opposed to previous suggestions to include *L. starcki* either under *L. capensis* or *L. europaeus*. Despite the morphological distinctness of *L. fagani*, it shared a substantial proportion of mtDNA and nucDNA with hare species from Ethiopia and *L. saxatilis* from South Africa. The nuclear sequences revealed phylogenetically and geographically meaningful clusters, albeit with a fair amount of indications of recent or historical introgresions among the species. In line with ATP, the nuclear sequences indicated close phylogenetic relationships among the three *Lepus* species from Ethiopia, with *L. fagani* being surprisingly tightly connected to *L. habessinicus*. Furthermore, the microsatellite analysis indicated low levels of genetic differentiation (G'_{ST} and D_{est} 0.384 and 0.334, respectively) between *L. habessinicus* and *L. fagani*. Moreover, the nuclear sequences suggested a closer evolutionary relationship of Cape hare from north and south Africa to hare species in Ethiopia. Although the nuclear sequence analyses supported the North American ancestry of *Lepus*, the results of the present analysis also suggested Africa as a potential ancestral area of *Lepus*. Overall, both multivariate skull morphometric and molecular analyses suggested a specific status of *L. habessinicus*, *L. fagani* and *L. starcki*.

Keywords: Ethiopia, introgression, *Lepus habessinicus*, *Lepus fagani*, *Lepus starcki*, microsatellite, mtDNA, nucDNA, phylogenetics, phylogroup and skull morphometry.

1. Introduction

The genus *Lepus* (Lagomorpha; Mammalia) has a worldwide distribution and comprises 24 to 30 species of jackrabbits and hares (Corbort and Hill, 1980; Flux and Angermann, 1990; Hoffmann and Smith, 2005; Alves, 2008). Currently, three *Lepus* species (*Lepus habessinicus* Hemprich & Ehrenberg, 1833, Abyssinian hare; *L. fagani* Thomas, 1903, Ethiopian hare and *L. starcki* Petter, 1963, Ethiopian highland hare) are identified in Ethiopia on the basis of limited morphological, dental and cytogenetic characters (Angermann, 1983; Flux and Angermann, 1990; Hoffmann and Smith, 2005).

The distribution of *L. habessinicus* covers from most parts of Somalia and eastern parts of Ethiopia, northeastern Eritrea and probably northern Kenya (Angermann, 1983; Yalden *et al*., 1986, 1996; Flux and Angermann, 1990; Azzaroli–Puccetti *et al.,* 1996; see Fig.1). The Ethiopian hare is distributed along the plateau in the north and west Ethiopia and at high altitude in Kenya (Yalden *et al*., 1986, 1996; Azzaroli-Puccetti, 1987a, b; Flux and Angermann, 1990; see Fig.1). The Ethiopian highland hare is distributed in the central highland of Shoa and mountains of Bale regions, from 2500 to 4000 m above the sea level (Angermann, 1983; Azzaroli-Puccetti, 1987a,b; Flux and Angermann, 1990; Azzaroli–Puccetti *et al*., 1996; see Fig. 1).

However, the evolutionary relationships among these taxa and their phylogenetic positions in relation to other species in the genus *Lepus* worldwide in general and in Africa in particular are not well understood. Previous works based on limited morphological and dental characters had placed *L. habessinicus* as a subspecies of *L. capensis* (Angermann, 1983; Azzorali-Puccetti, 1987a; Flux and Angermann, 1990). In contrary, Azzaroli-Puccetti (1987a, b) accepts the subspecific status of the taxon and pointed out the absence of interparietal bone in

Berbera population in north Somalia. The hypothesis that *L. habessinicus* is a subspecies of *L. capensis* (Cape hare) is controversial because of the widespread distribution of *L. capensis* and that Cape hare is a polytypic species (about 80 subspecies) (Flux and Angermann, 1990; Hoffmann and Smith, 2005). Cape hare, in broad sense (*L. capensis* sensu lato), is distributed in southern, eastern, and northern Africa, the Mediterranean, Israel, Arabian peninsula, Iran, Pakistan, north India, southern Russia, and most of north China (Azzorali-Puccetti, 1987a, b; Flux and Angermann, 1990; Hoffmann and Smith, 2005; see Fig. 1). However, Cape hare in narrow sense (*L. capensis* sensu stricto) is confined to South Africa (Cape Province) (Hoffmann and Smith, 2005; Robinson and Matthee, 2005; Palacios *et al.*, 2008).

Determining the phylogenetic position of hares that are grouped under Cape hare (*L. capensis* sensu lato) is hard because of its polytypic nature. Conflicting results were found by different authors and accordingly hares once grouped under *L. capensis* were classified into different taxa (Azzaroli-Puccetti, 1987a,b; Flux and Angermann, 1990; Hoffmann and Smith, 2005). A good example on the incongruity in Cape hare systematics is the work of Palacios *et al.* (2008) that splitted the Cape hare *(L. capensis)* in South Africa in two morphotypes namely *L. capensis* (i.e. confined to Cape province) and *L. centralis* (i.e. distributed in the remaining ranges of Cape hares), based on extensive skull morphometric data. The diagnostic features of *L. centralis* are a deep groove and more abundant cement relative to *L. capensis* in the first upper incisor, and the internal lobe is squared while that of latter species is rounded (Palacios *et al.*, 2008). In addition, *L. capensis* has stronger maxillae and more robust rows of teeth, but *L. centralis* has larger tympanic bullae. Nevertheless, Suchentrunk *et al.* (2009) challenged the hypothesis that Cape hares are confined to only Cape province (Palacios *et al.*, 2008) and showed that all Cape hares in the Republic of South Africa (RSA) are conspecific based on their microsatellite and mtDNA analyses. Several such analyses have shown the incongruence in Cape hare (*L. capensis* sensu lato) systematics and put under question the suggestion that *L. habessinicus* is a subspecies of Cape hare.

The phylogenetic position of *L. fagani* in the genus *Lepus* is not clearly known and it has remained a species rather neglected by specialists (Azzaroli-Puccetti, 1987a,b). It is considered as subspecies of the *L. victoriae*/*L. saxatilis* complex (Flux and Angermann, 1990; Hoffmann and Smith, 2005). However, Azzaroli-Puccetti (1987a,b) has put it into a distinct species on the basis of more materials as it has uniform characters in its range. Furthermore, comparisons of specimens collected from Kenya, South Sudan, and Uganda with the type specimen showed similar pelage and cranial characters (Azzaroli-Puccetti, 1987a,b).

Lepus saxatilis (scrub hare) is distributed in most of Cape Province, RSA, excluding grassland and dry desert (Robinson, 1982; Robinson and Dippenaar, 1987; Flux and Angemann, 1990; Kryger, 2002; Kryger *et al*., 2004; see Fig.1). The geographic distribution of *L. victoriae* (savanna hare) is most of Africa below Sahara and north of South Africa (RSA; Flux and Angerman, 1990; see Fig. 1). Apart from *L. saxatilis,* no molecular work was carried out on both *L. fagani* and *L. victoriae*, and the molecular evolutionary relationship of the later two species needs to be elucidated by further work using various nuclear and cytoplasmatic molecular markers.

Although the Ethiopian highland hare has a well established species status (Angermann, 1983), its phylogenetic position relative to other taxa in the genus is questionable. Using some morphological, dental and cytogenetic characters *L. starcki* was categorized as a subspecies of *L. europaeus* (Azzaroli-Puccetti *et al.*, 1996) or *L. capensis* (Petter, 1963). *Lepus europaeus* (brown hare/European hare) is distributed over most of Europe to 6^0 N, except Ireland, the

Mediterranean region and Scandinavia, where introduced populations occur (Flux and Angermann, 1990; Hoffmann and Smith, 2005; see Fig. 1).

The statement that *L. starcki* is a subspecies of *L. europaeus* is controversial and about 30 subspecies are categorized under brown hare/European hare and all with variable status (Palacios, 1996; Pierpaoli *et al*., 1999; Riga *et al*., 2001). The brown hare itself used to be considered a subspecies of *L. capensis* (Flux and Angermann, 1990). Taxonomic uncertainties in relation to *L. europaeus* were: the specific status of *L. corsicanus* (Italian hare) (Pierpaoli *et al*., 1999; Riga *et al*., 2001) and that of *L. granatensis* (Bonhomme *et al*., 1986; Palacios, 1989). In both cases species status is now accepted (Bonhomme *et al*., 1986; Alves *et al*., 2003; Estonba *et al.,* 2006). Similarly, the proposition that *L. starcki* is a subspecies of the Cape hare (Petter, 1963) is contentious because several Cape hare subspecies are invalid and some of them could be distinct species (e.g. *L. c. granatensis*; Flux Angermann, 1990).

Although several molecular evolutionary genetic investigations have been carried out in leporid species (Alves *et al*., 2000, 2006; Ben Slimen *et al*., 2005; Melo-Ferreira *et al*., 2005, 2008, 2009, 2011, 2013; Kriegs *et al*., 2010), there is currently one molecular phylogenetic study on *L. habessinicus* (n = 1) and *L. starcki* (n = 1; Pierpaoli *et al*., 1999) but for *L. fagani* the first molecular data was generated herein. Further studies of nuclear and mitochondrial DNA markers plus additional morphological and dental characters too are necessary to further resolve the phylogenetic uncertainties in *Lepus* and to determine molecular evolutionary relationships among hare taxa in Ethiopia as well as their respective position within the genus.

1.1 Objectives

General Objective

- To investigate the morphological/molecular systematics and population genetics of hare species from Ethiopia (*Lepus habessinicus, L. fagani* and *L. starcki*).

Specific Objectives

- To study the systematics of *L. habessinicus* and to clarify whether or not *L. habessinicus* is a subspecies of *L. capensis.*
- To study the systematics of *L. fagani* and to clarify whether or not *L. fagani* is a subspecies of the *L. victoriae* / *L. saxatilis* complex.
- To study the systematics of *L. starcki* and to clarify whether or not *L. starcki* is a subspecies of *L. europaeus* or *L. capensis.*
- To study the distribution of genetic diversity within each of the species.

2. Literature Review

2.1. Lagomorph Distribution and Systematics

Lagomorphs are distributed worldwide except in Antarctica as native or introduced species (Chapman and Flux, 2008). All are herbivores, their size ranges from that of rodents "small mammals", to that of ungulates "large mammals". The smallest lagomorph weighs less than 100 g and the largest weighs about 4 kg. However, there are cases were lagomorphs weigh as much as over 4 kg (e.g. 4.5 kg e.g. in Scandinvian hares and 7 kg in some Austrian rabbits (Suchentrunk, personal communication). Compared to rodents, the number of extant lagomorph species is small, but evolutionarily and ecologtically, they may be considered quite adaptive when considering the diversity of their habitats and environments where populations are able to dwell successfully (Chapman and Flux, 2008). They occur from sea level up to over 5, 000 m, from the equator to 80^0 N, and in a variety of habitats including tundra, steppe, swamps, and tropical forest.

Generally, there are only few of lagomorphs species (78) relative to rodents (1,685) or ungulates (172). This could probably be due to lagomorphs' slow and conservative diversification in early geological times, leading to bounding locomotion in the rabbit and hares and small limb size in alpines pikas (Chapman and Flux, 1990). Several studies have been undertaken to understand the differentiation and phylogenetic relationships between the different groups of lagomorphs. These studies used a range of markers such as: morphological characters (e.g. Hibbard, 1963; Dawson, 1981; Angermann, 1983; Corbet, 1983, 1986; Robinson, 1987; Palacios, 1996; Suchentrunk *et al.*, 1994, 2000; Suchentrunk and Flux, 1996; Riga *et al.*, 2001; Suchentrunk, 2004), cytogenetic (e.g. Robinson *et al.*, 1981, 1983a,b, 2002) and molecular (e.g. Halanych *et al.*, 1999; Pierpaoli *et al.*,1999; Alves *et al.*, 2000, 2003, 2006; 2007; 2008; Waltari and Cook, 2000; Wu *et al*., 2000, 2005; Ben Slimen *et al*., 2005, 2007, 2008a,b; Matthee *et al*., 2004; Kasapidis *et al.,* 2005).

In spite of these investigations the phylogenetics of lagomorphs still exhibits many gaps to be filled. Previously, Lagomorphs were classified under Rodentia and it was not until 1912 that the order Lagomorpha is recognized as a distinct order in the class Mammalia. The diagnostic anatomic feature that separates Lagomorphs from other mammal groups is the presence of a second set of incisors (peg teeth) which are located directly behind the first upper incisors (Chapmann and Flux, 1990, 2008; Robinson and Matthee, 2005). Pikas have 26 (dental formula i.2/1, c. 0/0, p. 3/2, m. 2/3) while rabbits and hares 28 teeth (dental formula i.2/1, c. 0/0, p. 3/2. m.3/3). Another diagnostic feature common to all lagomorphs is an elongated rostrum of the skull reinforced by a lattice work of bone (fenestration)(Chapman and Flux, 2008). The order Lagomorpha consists of two families: the monotypic Ochotonidae (pikas) and Leporidae (hares and rabbits with 11 genera) (Robinson and Matthee, 2005).

2.2. Origin of Lagomorphs

Two different ancestral regions were proposed for the origin of Lagomorphs, based on fossil records: an Asian development (Hibbard, 1963; Dawson, 1981; McKenna, 1982; Asher *et al*., 2005, Rose *et al.,* 2008) and a North American origin (Dawson, 1981; White, 1991). But the lack of a complete fossil record in the early and middle Eocene had made the situation complicated (Dawson, 1981). The hypotheses that leoporids expanded from North America (Dawson, 1981), was supported by the discovery of two fossils in North America (White, 1991; Voorhies and Timpreley, 1997).

Matthee *et al. (*2004) carried out dispersal-vicariance analyses and found that the most parsimonious solution to reveal the present geographic distribution and North American or Asian origin scenarios needs at least nine dispersal and five vicariance events. Probably, at least three intercontinental exchanges might have occurred between North America and Asia via the Bering Strait and about three independent dispersal events could be identified that occurred into Africa (Matthee *et al*., 2004). Furthermore, by applying a relaxed Bayesian molecular clock to the seven of the loci they had used in the super matrix construction; they found an indication that most intercontinental exchanges occurred between 14 and 9 million years ago and this period is broadly coincidental with the onset of major Antarctic expansions causing the land bridges to be exposed (Matthee *et al*., 2004). Wu *et al*. (2005) suggested that *Lepus* originated in North America and spread to Eurasia via Bering Land and finally spread to Africa. In addition, their Brook parsimony analysis revealed that dispersal events followed by speciation processes occurred in other geographic areas as well and resulted in adaptive radiation and speciation of *Lepus*. *Lepus* have arisen approximately 10.76 MYA (±0.86 MYA) with most speciation events occurring during the Pliocene epoch (5.56 ± 1.15MYA ~ 1.12 ± 0.47; Wu *et al*., 2005).

Despite both fossils and molecular evidence suggesting a North American origin of Lagomorphs, recent fossil evidence supports Asian origin of Lagomorphs. The discovery of a new fossil in Early Miocene in India which is much older than the fossils discovered in North America strongly supported the hypothesis that Lagomorphs originated in Asia (Rose *et al*., 2008). These authors discovered small fossil ankle bones (calcaneus and talus) from Early Eocene deposits (middle Ypresian ca. 53 MYA) of Gujarat, west-central India, which predate the oldest earlier known Lagomorph record by many million years and extend the evidence of fossil record of legomorphs in India by 35 Myr (Rose *et al*., 2008). The evidences found from these were mosaics of derived cursorial adaptations typical in gracile leporids (rabbits and hares) and primitive traits characteristics of extant Ochontanidae (pikas) and more robust leporids. The fossils discovered indicate that the diversification within crown lagomorphs and possibly the divergence of family Leporidae were already underway in the early Eocene (Rose *et al.*, 2008). Suggestions that the Lagomorphs originated in Asia were also supported by a fossil (*Gomphos elkema*) described from the deposit close to the Paleocene-Eocene boundary at Tsagan Khushu, Mongolia (Asher *et al.*, 2005). This fossil shares a suite of cranioskeletal characters with extant rabbits, hares and pikas. However, it retains a primitive dentation and jaw compared to its modern relatives.

2.3. Leporidae Sytematics

Based on the pattern of enamel folding of P^2 and P^3, the family Leporidae can be divided into three subfamilies, namely, Palaeolaginae, Archaeolagine and Leporinae (Dice, 1929). The work of Hibbard (1963) was among the earliest in the investigation of evolutionary relationship among the leporids and based on pattern of enamel folding of the P^2 and P^3 molar teeth. Other works include morphological markers (Corbert, 1983), cytogenetics (Robinson *et al.*, 2002), mitochondrial sequences (Halaynch and Robinson, 1999; Yamada *et al.,* 2002) and DNA supermatrix approach (Matthee *et al.*, 2004).

Despite the fact that there is a strong conservative chromosome number in the species of leporids, the diploid (2n) chromosome number in the family ranges from $2n = 38$ to $2n = 52$ (Robinson *et al.*, 1983a). Chromosomal banding pattern studies made for some taxa indicated the tendency of retaining the supposed ancestral state ($2n = 48$; Robinson *et al.*, 1981). The diploid chromosome number of all karyotyped species is $2n = 48$ (Robinson *et al.*, 1983b;

Azzaroli-Puccetti *et al*., 1996). Robinson *et al.* (2002) found out that at least 18 fusions and six fissions are needed to differentiate the extant karyotype from the presumed ancestral state (2n = 48). They also pointed out that the riverine rabbit, *Bunolagus monticularis*, as having the most derived karyotype, differing by 7 fusions and fissions from the ancestral state. This data is in accordance with the proposed Palaoelaginae/Leporinae dichotomy in lagomorphs. The result contributed in resolving the structural changes that have shaped the evolutionary history of lagomorphs and put under question the rapid evolutionary scenarios implicated by mitochondrial markers (Robinson *et al.,*2002).

Matthee *et al*. (2004) constructed a molecular super matrix to see intergeneric relationship between Leporidae using five nuclear and two mitochondrial markers. The analyses of each nuclear fragment separately and in combination with mtDNA markers have failed to show strong statistical intergeneric relationships. But the combined nuclear DNA topology based on 3602 characters greatly increased the phylogenetic resolution among leporid genera. The result was supported by the 95 % confidence interval and the number of significantly supported nodes. The final molecular super matrix, composed of 5463 analysed genetic characters consistently recovered the same topology across a range of 6 arbitrarily chosen model specifications (Matthee *et al*., 2004). Robinson and Matthee (2005) have suggested four evolutionary lineages on the bases of the Hibbard/Dawson phylogenetic scheme but with no resolution among them and potentially conflicting morphological data.

2.4. Application of Morphological Markers in leporid Systematics

The use of morphological markers in leoprid systematics goes back to the work of Hibbard (1963) who found similar enamel folding pattern in *Nekerolagus progressus* and extant leporid genera (e.g. *Lepus, Oryctolagus* and *Sylvilagus*). The major problem in the application of morphological characters in *Lepus* taxonomy is lack of diagnostic characters (Angermann, 1983). In extant hare species, the interspecific morphological differentiations are small in comparison with individual and geographical variation. Robinson (1987) applied univariate and multivariate analyses to assess the phylogenetic relationship between *Lepus* and the monotype *Bunolagus;* however, he was not able to differentiate them using morphometric methods. But *Bunolagus* is recognized as a genus from karyotypic studies (Robinson, 1987).

Petter (1963) was the first to use morphological characters for phylogenetic analyses of hares from the Horn of Africa. Five species of hares (*L. capensis* Linnaeus 1757, *L. habessinicus* Hemphric & Ehreberg 1833, *L. crawshayi* de Winton 1899, *L. fagani* Thomas 1903, and *L. starcki* Petter 1963) were distinguished on the bases of the color and texture of the fur, skull morphomerics and dental characters in the Somalian lowland and Ethiopian plateau (Azzaroli-Pucceti, 1987a). Some of the recent studies using morphological characters in Leporidae and hares are works of (Palacios, 1996; Riga *et al*., 2001) which dealt with the phylogenetic position of *L. corsicanus* relative to that of *L. europaeus*. Both studies found that the Italian hare is phylogenetically distinct from the brown hare. Other similar works include morphometic analyses of eastern Africa Cape hare (*L. capensis*) and the savanna hare (*L. victoriae*) by Suchentrunk *et al*. (2007), and morphological analyses of the South African Cape hare (*L. capensis*) by Palacios *et al.* (2008*).*

Dental occlusal character analysis of Israeli hares, some East African hares and the brown hare by Suchentrunk *et al*. (2000) had confirmed that Israeli hares are closer to eastern African hares. The result also confirmed that dental occlusal variations are influenced by phylogenetic factors than ecological and hares from Israel probably belong to a single species that encompasses two closely related geographical populations with a probable area of overlap. The of work of Suchentrunk (1993-*L. europaeus*; 2000- Israeli hare; 2004- *Lepus nigricollis* and *L. peguensis*) and Suchentrunk and Flux (1996-*Lepus capensis* and *L. victoriae)* are among the studies using dental characters for *Lepus* systematics.

2.5. Application of Cytogenetic Markers in Leporid Systematics

Several comparative karyological studies indicate that there is a degree of karyotypic conservatism in the family Leporidae (Robinson, 1980; Robinson *et al.,* 1981, 1983a,b, 2002; Azzaroli-Puccetti *et al*., 1996). It is suggested that the major factor in karyotypic evolution in leporids could be fusion but not fission (Robinson, 1980; Robinson *et al*., 1984). Analyses of patterns of G- and C- banding in species of *Sylivilagus* and *Lepus* indicated that chromosomal evolution seems mainly driven by the change in the amount of hetrochromatin and fixation of Robertsonian fusion (Robinson *et al.,* 1984). The patterns of G- and C- banding in six species of *Lepus* have shown that absence of gross chromosome morphology change or banding pattern indicating speciation in this widespread genus has not involved karyotypic changes (Robinson *et al*., 1983b). Robinson *et al.* (2002) using 22 rabbit chromosome specific stainings derived from flow sorted chromosomes revealed that at least 18 fusion and six fission differentiate the extant karyotype from the presumed ancestral state (2n = 48).

2.6. Application of Protein Markers in leporid Systematics

The utilization of allozyme markers in lagomorphs by biochemical systematicists was low compared to Rodents and Primates (Grillitsch *et al*., 1992). Robinson and Osterhoff (1983) made analyses of 9 protein coding loci of two leporid genera (*Lepus* and *Pronolagus*) and found that only three of the loci (Transferrin, TF; Esterase-I, Es-I and esterase-II, Es-II) are variable within species. Furthermore, they found only carbonic anhydrase-I (CA-I) to provide useful information for species identification in *Lepus*. Enzyme electrophoretic analyses indicated that hares on the Iberian Peninsula belong to three distinct species: *L. europaeus* in the northeast, *L. castroviejoi* in the range of the the upper Cantabaric Cordilera and *L. granatensis* in the remainder of the Peninsula (Bonhomme *et al*., 1986). Similarly, Hartl (1987) using enzyme electrophoresis and isoelectronic focusing (i.e. of 28 enzyme systems on two species *O. cuniculus* and *L. europaeus*) found indices of genetic variation to be similar to average values for numerous mammalian species. Unlike the previous electrophoretic analyses, Grillitsch *et al*. (1992) studied more loci (i.e. 38 isozyme systems) and a more representative set of lagomorph species (e.g. *L. europaeus, L. timidus, O. cuniculus* and *O. rufescens*). The numerical dendrograms revealed a constant picture of lagomorph relationships, which was in accordance with the conclusions drawn from other sources. Furthermore, estimates of divergence time was in good agreement with paleontological data (0.49 myr between *Lepus* species, 3.65 myr between *Lepus* and *Oryctolagus*, 37.5 myr between *Leporidae* and *Ochotonidae*), but only when calculated in different ways at low and high taxonomic levels (Grillitsch *et al*., 1992).

The use of allozyme data in the evolutionary genetics of leporids is not only limited to biochemical systematics but is also common in population genetics, see Hartl and Höger (1986- *O. cuniculus*), Hartl *et al*. (1989- *L. europaeus*; 1990- *L. europaeus;* 1992- *L. europaeus;* 1993- *L. europaeus; 1995- L. europaeus)*, Hartl (1991-*L. europaeus*), Peterka and Hartl (1992- *O. cuniculus*), Hartl and Ferrand (1993- L. *europaeus*) for examples.

2.7. Application of mtDNA Markers in leporid Systematics

Over the last three decades, mitochondrial DNA (mtDNA) has been the most popular marker of molecular diversity, because of a combination of technical ease-of-use considerations, and the supposed biological and evolutionary properties of clonality, near-neutrality and clock like nature of its substitution rate (Galtier *et al.,* 2009). Zardoya and Meyer (1996) have classified protein coding genes of mtDNA into three groups based on their resolution power in recovering the expected phylogenetic relationships of tetrapods and mammals: good (ND4, ND5, ND2, cytb, and COI), medium (COB, COIII, ND1, and ND6) and poor groups (ATPase 6, ND3, ATPAse 8, and NDL4).

Although proportions of mtDNA evolve extremely rapidly, a significant portion of the molecule is under strong selective constraint (Ballard and Kreitman, 1995; Ballard and Whitlock, 2004). In addition, there are several evidences against the clonally inheritance and neutrality of mtDNA (Dowling, 2008; Eyre-Walker *et al*., 1999; Piganeua *et al*., 2004). Pitfalls in the use mtDNA in phylogenetic reconstruction are a potential discordance between history reconstructed from nuclear and or morphological data and from mtDNA data (McGuire *et al*., 2007). Furthermore, it was also found that one of the drawbacks in the use of mtDNA in animal sytematics is frequent introgressive hybridization (Thulin *et al*., 1997a,b, 2000, 2006; Halanych and Robinson, 1999; Halanych *et al., 1999;* Alves *et al.,* 2003*;* Ben Slimen *et al., 2008*; Liu *et al.,* 2011*;* Melo-Ferreira *et al*., 2012). There are some evidences suggesting the lack of correlation between mtDNA variations and species abundance, history, ecology and ability to adapt (Bazin *et al*., 2006).

Despite all the aforementioned drawbacks, phylogenetic analyses based on mtDNA markers and their use in animal systematics are widespread. Some of the pioneering works using mtDNA in lagomorph molecular systematics and population genetics were: Perez-Suarez *et al*. (1994- *L. casteroviejoi*/ *L.europaeus*/ *L. granatensis* / *L. capensis*), Matthee and Robinson (1996-*Pronolagus rupestris*), Thulin *et al*. (1997a,b- *L. timidus*/ *L. europaeus*), Halanych and Robinson (1997- *Sylvilagus*; 1999- Leporidae), Halanych *et al*. (1999- *Lepus*, Lagomorpha) and Pierpaoli *et al*. (1999-*L. corsicanus*).

Did the use of mtDNA refine the Leporidae/Lagomorph phylogeny**?** The answer to this question could be "yes" or "no". Studies made in the last 20 years show that mtDNA studies have contributed a lot to resolve parts of Leporidae phylogeny (Perez-Suarez *et al., 1994;* Yamada *et al., 2002;* Niu *et al*., 2004; Alves and Melo-Ferreira*, 2007;* Kinoshita *et al.,* 2012*;* Melo-Ferreira *et al*., 2012). However, it has also been confounded by problems like multiple substitutions, incongruence with nucDNA (nuclear DNA) or morphological phylogenies, and frequent introgressive hybridization (Thulin *et al*., 1997a,b, 2000, 2006; Halanych and Robinson, 1999; Halanych *et al., 1999;* Alves *et al.,* 2003*;* Ben Slimen *et al., 2008*; Liu *et al.,* 2011b*;* Melo-Ferreira *et al*., 2012).

Halanych and Robinson (1997) carried out a comparative analysis of 12S rDNA and cytogenetic data to investigate the congruence of the two datasets and to determine the phylogenetic position of *Sylvilagus.* They found that 12S rDNA data resulted in a robust phylogeny, supported by different phylogeny reconstruction methods. Moreover, the results were consistent with the then available cytogenetic data. Besides, 12S rDNA suggested a separate generic status of Pygmy rabbit, *Brachylagus idahoensis*, as is warranted based on its phylogenetic position and sequence divergence value. Similarly, the analysis of cytochrome b

(Cytb) sequences of North American hares and jackrabbits have suggested that *Lepus* taxa in North America are not monophyletic and the phylogenetic status of some species needs reassessment (Halanych *et al*., 1999). Pierpaoli *et al*. (1999) analysed the mtDNA control region and Cytb sequences to determine the phylogenetic position of *L. corsicanus* (Italian hare) and wether or not it is a distinct taxon, They found that the Italian hare and brown hare to be distinct species. Furthermore, the Italian hare probably had isolated refuge in southern Italy during the last glaciations. Yamado *et al*. (2002) analysed 12S rRNA and Cytb of three leporid species (*Pentalagus furnessi*, *L. brachyurus* and *L. timidus*) from Japan. Their 12S rRNA results suggested that the lineage of *P. furnessi* diversified during the generic radiation of leporids, which is estimated to have occurred in the Middle Miocene. *Lepus brachyurus* might have branched off in the Pliocene as revealed by Cytb sequence result (Yamado *et al*., 2002). Analyses of partial sequences of Cytb and transferrin of five species (*L. europaeus*, *L. timidus*, *L. granatenis*, *L. corsicanus* and *L. castroviejoi*) indicated that *Lepus* is a monophyletic group (Alves *et al*., 2003).

Wu *et al.* (2005) analysed the complete mtDNA Cytb gene, the 12S gene, parts of DN4 and a fragment of the control region to examine the phylogenetic relationship of Chinese hare and other leporids. Their results suggested that hares from China do not form a monphyletic group. However, the genus *Lepus* is monophyletic with three unique species groups occurring in North America, Eurasia, and Africa (Wu *et al.,* 2005). Ramirez-Silva *et al*. (2010) analysed complete Cytb sequences to assess the phylogenetic relationships of Mexican jackrabbits and found that Mexican hare species are a monophyletic entity that evolved independently of other American members of *Lepus*. In addition, they identified two apparent main clades in the Mexican forms: one comprising *L. alleni, L. callotis* and *L. flavigularis,* the other including *L. californicus* and *L. insularis*. Koh and Jang (2010) analysed nuclear

Thyroglobline (TG) gene and mtDNA control region seqence of *L. coreanus (*Korean hare). They found that the Korean hare is a distinct taxon vis-à-vis *L. timidus*, which contrasts earlier suggestions by Wu *et al*. (2005).

Several studies provided evidence for an introgression of *L. timidus* (mountain hare) mtDNA into *L. granatensis*, *L. europaeus*, *L. arcticus*, *L. castroviejoi*, *L. corsicanus*, *L. oiostolus*, *L. othus* or *L. townsendii* (Thulin *et al*., 1997a,b, 2003; Alves *et al*., 2003, 2006, 2008; Jansson *et al*., 2007; Liu *et al*., 2011b; Melo-Ferreira *et al*., 2012). Thulin *et al*. (1997a) checked the transmission of mtDNA between brown hare and mountain hare, and among 18 brown hares, six individuals did possess two haplotypes similar to that of *L. timidus*, indeed suggesting introgression mtDNA from the latter to former taxon. Alves *et al*. (2003) reported introgression of mtDNA from *L. timidus* into as many as 8 species (i.e. *L. granatensis*, *L. europaeus, L. arcticus*, *L. castroviejoi*, *L. corsicanus*, *L. oiostolus*, *L. othus* and *L. townsendii)*. Similar widespread events of mtDNA introgressions have recently been documented between different pairs of hare species from China (from *L. sinesis* into *L. capensis, L. mandshuricus* and *L. timidus; from L. timidus* into *L. mandshuricus*, *L. capensis*; from *L. yarkandensis* into *L. capensis*; from *L. capensis* into *L. oiostolus*; Liu *et al*., 2011b). Melo-Ferreira *et al*. (2012) analysed 14 nucDNA and two mtDNA fragments in 11 species of hares from Eurasia, North America and Africa to determine the extent of mtDNA introgression. They applied the isolation with migration model to the nuclear data on a subset of species and found very limited gene flow from *L. timidus* into four temperate European species, but not in the American boreal ones. Further, the simulation of mtDNA evolution under the species history inferred from nuclear genes did not support the hypothesis of mtDNA introgression from *L. timidus* into the American *L. townsendii* but did suggest introgression from *L.timidus* into four temperate European species. The results support the earlier findings of a complete replacement of the aboriginal mtDNA of *L. castroviejoi* and *L. corsicanus* (Melo-Ferreira *et al.*, 2012).

The mtDNA introgression is almost always in one direction from the cold–adapted species into more temperate ones. The absence of the reverse event is still not well understood (Melo-Ferreira *et al.*, 2012). Introgression from *L. timidus* into temperate species could then correspond to a phase of climate warming, when the latter species would be favored and replace the former. According to Melo-Ferreira *et al.* (2007), the most recent introgrssive event in the Iberian Peninsula could correspond to the last major episode of climate warming at the end of the last glaciation. They also estimated the more ancient mtDNA introgression from *L. timidus* into the common ancestor of *L. castroviejoi* and *L. corsicanis* to have occurred about 550, 000 years ago, during the Pleistocene glacial cycles. Liu *et al.* (2011b) suggested that driving forces for mtDNA introgression among Chinese hares could be: species abundance asymmetries, mating preference, range expansion and low divergence between species.

Although the use of mtDNA data in phylogenetic studies of leporid species used to be a common trend (Preze-Saurez *et al.*,1994; Halaych *et al.*, 1997, 1999; Pierpaoli *et al.*, 1999; Waltari *et al.*, 2004; Waltari and Cook, 2005; Wu *et al.*, 2005), combined analyses of mtDNA and nucDNA are recommended (Matthee *et al.*, 2004; Robinson and Matthee, 2005; Alves *et al.*, 2003, 2006; 2008; Ben Slemen *et al*, 2005, 2007, 2008; Suchentrunk *et al.*, 2006, 2008; Liu *et al.*, 2011a,b; Melo-Ferreira *et al.*, 2012). It is recommended to include evidence from the nuclear gene pool when systematic inferences are made within a genus; even when there is no clue for the existence of mitochondrial introgression (Ben Slimen *et al.*, 2008b). Alves *et al.* (2003) found that as many as 8 species have mtDNA introgression from *L. timidus* and therefore phylogenetic conclusions should not be drawn solely from mtDNA without including information from other sources. By analyzing 5 nuclear and two mitochondrial fragments, Matthee *et al.* (2005) found that a combined nuclear DNA topology based on 3601 characters greatly increased phylogenetic resolution among leporid genera.

2.8. Microsatellite Markers in *Lepus* Evolutionary Genetic Studies

Microsatellites /simple sequence repeats (SSR)/ variable numbers of tandem repeats (VNTR)/ short tandem repeats (STR) are tandem repeats of 1–6 bp found at higher frequencies than expected from a random distribution of the nucleotides in the genome. Microsatellite repeats could consist of different motif sizes ranging from mono-, di-, tri- and tetra-nucleated repeats that are distributed in the genome (Tautz and Renz, 1984; Epplen *et al.*, 1993; *Schlötterer, 2004*). SSR can be classified as perfect, imperfect, interrupted, and composite. In a perfect microsatellite, the repeat is not interrupted by any base not belonging to the repeat motif (e.g. TATATATATATATA), imperfect ones include a base pare not being part of the repetitive motif (e.g. TATATATACTTATA), interrupted microsatellites (e.g. TATATA*CTGT*TATATA) and in composite microsatellites the motif contains two adjacent distinctive sequences (e.g. ACACACGTGTGTGT) (Olivera *et al.*, 2006).

Dieringer and Schlötterer (2003) documented the distribution of microsatellites with long repeats to be more frequent than SSRs with short repeats in 10 of the completely sequenced genomes so far. Furthermore, they reported that the pattern of microsatellites could neither be explained by base substitutions nor by a combination of length dependent slippage and base substitutions. However, in the eukaryotic genome, the distribution of perfect or near perfect tandem iteration of repeats is extremely high. In the human genome, one can find tandem repeats at hundreds of thousands of sites on a chromosome. The distributions of the different motifs are not even in all genomes studied so far. For instance, with 66% dinucleotide repeats are more frequent in the genome of *Drosophila melanogaster* than trinuclotide repeats (30%) and tetranucleotide repeats (4%) (Schug *et al.,* 1998). Microsatellites make up for about 3% of the human genome (IHGSC, 2001), with dinucleotids contributing the highest (0.5%) share. In our genome the most frequent dinucleotide is (CA)n 50%, followed by (AT)n 35%, (GA)n 15% and (GC)n 0.5 %. However, in plant genomes, the abundance of SSR in organelle genomes vis-à-vis the nuclear genome is extremely rare (Wang *et al.,* 1994). These authors also found that the $(AT)_n$ motif is the most abundant followed by $(A)_n.(T)_n$, $(AG)_n$, $(CT)_n$, $(AAT)_n.(AAT)_n$, $(ACC)_n.(GTT)_n$, $(AGC)_n.(GCT)_n$, $(AAG)_n.(CCT)_n$, $(AATT)_n.(TTAA)_n$,$(AAAT)_n.(ATTT)_n$ and $(AC)_n.(GT)_n$ sequences. The rareness of microsatellite repeats in organelle genomes is not only a feature of plant genomes but it is also documented in some mammalian species (Mayer and Kerth, 2005).

Compared to the degree of polymorphism shown by most of the sequences found in the genome, microsatellites show a considerable degree of polymorphism. They show a high degree of hetrozygosity and multiple alleles. Features like hypervariability and ubiquitous occurrence in the genome implicate the usefulness of microsatellite markers but on the contrary these properties raise several different questions (Ellegren, 2004; Schlötterer*, 2004).* Some of these questions are: why are microsatellites so abundant? Why are they polymorphic? and by what mechanisms do they mutate? What are the factors controlling the intricate balance between the frequent genesis and expansion of simple repetitive arrays?

Microsatellite loci are mostly used in the analysis of the genetic structure of both natural and artificial populations. Our understanding of how genetic variation is distributed within and among populations havs a tremendous influence in purely academic as well as in applied fields of biological sciences such as evolutionary biology, ecology, conservation biology, medical genetics, and plant and animal breeding (Epplen *et al*., 1993; Chambers and MacAvoy, 2000; Balloux and Lungon-Moulin, 2002; Mayer and Kerth, 2005). The utilization of microsatellites in different areas of biological sciences emanates from their high degree of variability, codominant inheritance, reproducibility, high resolution level, and their being PCR based markers (Olivera *et al.,* 2006).

Despite the wider application of microsatellites in population genetics, forensics and medical genetics, the mechanisms of mutation and patterns of evolution are not well understood (Schlötterer, 2000). The evolutionary mechanisms of microsatellite have been debated among scientists (Li *et al*., 2002). There are two competing mechanisms of evolution that are responsible for the increase or decrease of repeat size in microsatellite loci (Eisen, 1999). The proposed mechanisms of evolution are: unequal crossing-over (UCO) and slip-strand mispairing (SSM). Unequal crossing-over is a consequence of recombination between homologous chromosomes that are not perfectly aligned, whereas, SSM is the result of misalignments of DNA strands during replication, having escaped the proof reading of DNA polymerases. Levinson and Gutman (1987) suggested that intrahelical slip strand misparing is much more responsible for the initial spread of short tandem repeats and the further expansion of these short motifs is probably aggravated by unequal crossing-over or other interhelical events because of their propensity to mispairing.

Another fundamental question one needs to answer for utilzing the tremendous potential of microsatellites in various disciplines is concerned with finding the appropriate model of evolution (Balloux and Lugon-Moulin, 2002; Ellegrem, 2004). Population geneticists have developed two extreme models of evolution: the infinite allele model (IAM, Kimura and Crow, 1964) and the stepwise mutation model (SMM; Kimura and Otha, 1978). In the IAM, each mutation creates a novel allele at a given rate, *u* (Balloux and Lugon-Moulin, 2002; Ellegrem, 2004). Consequently, this model does not allow for homoplasy. Identical alleles share the same ancestry and are identical-by-descent (IBD). However, the stepwise mutation model (SMM), adds or subtracts one or few repeat motifs from the strings of repeats at some constant rate to mimic the process of errors during DNA replication that generates mutations, creating a Gaussian shaped allelic frequency distribution (Ellegren, 2004).

2.9. Population Genetics of Hares

Stamatis *et al*. (2009) analysed RFLP-PCR products of Cytb/CR, COI, and 12S-16S rRNA and their results grouped the brown hare into five well defined phylogeographic regions in Europe. The identified phylogeographic regions are: southeastern Europe haplogroup, Anatolian/Middle eastern group, European haplogroup one, European haplogroup two and intermediate haplogroup. Haplotypes from the Anatolian/Middle eastern group were those detected in the large overlap region in Bulgaria and northeastern Greece indicates the presence of gene flow from Anatolian to Europe across the late Pleistocene Bosporus land bridge. This suggests that the brown hare distributions are partitioned into different phyletic groups.

Ben Slimen *et al*. (2008a) analysed mtDNA (mtHV-1), microsatellites and allozymes of the Cape hare (*L. capensis)* from North and South Africa indicating the absence of correspondence at individual level within either population. The mtHV-1 result suggested a high degree of divergence at individual level between the two populations whereas no significant level of divergence was detected in the nuclear data between the two populations.

Therfore, evidence from the nuclear gene pool should be included when systematic inferences are made within a genus; even when there is no clue for the existence of mitochondrial introgression (Ben Slimen *et al*., 2008b). Stamatis *et al*. (2008) compared $\mathrm{tRNA}^{\mathrm{Thr}}$ and $\mathrm{tRNA}^{\mathrm{Pro}}$ of brown hares from different geographical regions and detected different substitutions. Their phylogenetic reconstructions based on mitochondrial tRNA and mitochondrial control region resulted in a similar phylogenetic clustering. Their finding indicates the potential use of mitochondrial tRNA as auxiliary phylogenetic markers.

Scandura *et al*. (2007) analysed a 461 bp fragment of the mitochondrial CR-1 of hares in the Sardinian region in Europe in and different parts of the Old World (e.g. North Africa, South Africa and East Asia). They found that Sardinian hares form a monophyletic group with hares from North Africa. These findings support the hypothesis that hares in the Sardinian region and North Africa can be grouped under the super species *L. capensis*. Ben Slimen *et al.* (2007) analysed 415 bp HV-1 of mtCR for hares from north-central Tunisia and northwest Egypt to test the hypothesis that hares from north Africa belong to *L. capensis* and all phylogenetic reconstruction methods concordantly grouped all North African Cape hares into one monophyletic clade. The Cape hares (*L. capensis* from South Africa, North Africa and China) have their haplotypes clustered into separate groups according to their origin, but the haplotypes from China formed a subclade within a major clade mainly containing mountain/Arctic hare type sequences in addition to other sequences from Palaeartic and Neartic species. Besides, some *L. capensis* haplotypes from China clustered in the *L. comus* clade, which suggests the existence of introgression and or shared ancestral polymorphism. The presence of such evolutionary scenarios implies the application of nuclear markers in addition to mtDNA inferring phylogenetic relationships in *Lepus* (Ben Slimen *et al*., 2007).

Cytb and Y-chromosome analyses were applied to assess sex specific gene flow in the brown hare by Mamuris *et al*. (2010). The markers showed no interspecific variation indicating that they are of limited use for investigating *Lepus* phylogeny. Ben Slimen *et al*. (2006) carried out restriction fragment polymorphism (RFLP) analyses of mtDNA in hares from North Africa, South Africa, and Europe, which revealed a marked substructuring of mtDNA into three phylogroups: an African, a Central European and an Anatolian group. However, allozyme analyses showed less level of overall differentiation in spite of the occurrence of a relatively large number of population specific private alleles (Ben Slimen *et al*., 2006). Based on the analysis of 473 bp in the mitochondrial DNA control region of Arctic hares (i.e. *L. arcticus, L. othus and L. timidus*) Waltari and Cook (2005) suggested that the Arctic species are closely related. Furthermore, all *L. othus* individuals formed one clade, individuals of *L. arcticus* clustered into two separate clades, while those of *L. timidus* were scattered over the tree. Suchentrunk *et al.* (2009) analysed 13 microsatellite loci and CR-1 in Cape hares *L. capensis* from South Africa, which proved to be monophyletic. This result is in contrast to recent suggestions by Palacios *et al*. (2008) to split the Cape hare into two species *L. capensis* and *L. centralis* on the basis of intensive skull morphometric data. Ben Slimen *et al*. (2008b) used 11 polymorphic loci to asses that Cape hare and brown hare are nonspecific or distinct species have revealed higher genetic variation (14.7 alleles per locus) although the distribution of alleles and genotypes was not homogeneous across the locations**.** Furthermore, the finding supported the degree of differentiation between populations belonging to the two different taxa was not higher than within the taxa.

2.10. Hare Species in Africa

In Africa, currently there are more than five species of *Lepus* (e.g. *L. capensis*, *L. saxatilis, L. victoriae*, *L. habessinicus*, *L. fagani* and *L. starcki*) that could be identified based on morphological data and these morphotypes are thought to have an allopatric distribution due to ecological isolation (Flux and Angerman,1990; Yalden *et al.,* 1996; Hoffmann and Smith, 2005).

2.10.1 *Lepus capensis* Linnaeus, 1757, Cape Hare

Its distribution ranges from southern, eastern, and northern Africa, the Mediterranean, Israel, Arabian peninsula, Iran, Pakistan, north India, southern Russia, to most of north China (Robinson, 1982; Azzorali-Puccetti, 1987; Flux and Angermann, 1990; Hoffmann and Simth, 2005; see Fig. 1). The Cape hare is not only a widespread species, but also a polytypic taxon (i.e. it comprises about 80 subspecies), and all these groups need revision.

Hence, determining the phylogenetic position of hares that are grouped under *L. capensis* is hard because of its polytypic nature and conflicting results were found by different authors. Accordingly, hares once grouped under *L. capensis* are presently classified into different taxa (Azzaroli-Puccetti, 1987; Flux and Angermann, 1990; Hoffmann and Smith, 2005). For instance, Palacios *et al.* (2008) partitioned Cape hares *(L. capensis)* in South Africa in two species, namely *L. capensis* (i.e. confided to Cape Province) and *L. centralis* (i.e. distributed in the remaining ranges of Cape hares based on intensive skull morphometric data. However, Suchentrunk *et al.* (2009) have challenged the suggestion that Cape hares are confined to only Cape Province (Palacios *et al*., 2008) and shown that all Cape hares in the Republic of South Africa (RSA) are conspecific based on their microsatellite and mtDNA analyses.

2.10.2 *Lepus saxatilis* F. Cuvier 1823, Scrub Hare

The diagnostic features of *L. saxatilis* are large body size (2-4 kg), long ears, large skull long and long rostrum **(**Angermann, 1983, Flux and Angermana, 1990; Smith and Hofman, 2005**).** *Lepus saxatilis* (scrub hare) is distributed in most of the Cape Province (see Fig. 1), RSA excluding grassland and dry desert (Robinson, 1982; Robinson and Dippenaar, 1987; Flux and Angemann, 1990; Kryger, 2002; Kryger *et al*., 2002, 2004; Hoffmann and Smith, 2005).

2.10.3. Lepus habessinicus Hemprich and Ehrenberg, 1833, Abyssinian Hare

Abyssinian hare *Lepus habessinicus* is a small hare with a body weight of about 2 kg, with dorsal side of cineraceous color marked with black or grizzled black and buff (Flux and Angermann, 1990). The tail is black above, white below and it has ears with narrow black edges and moderate length. About half of the skull has interparietal bone, this bone is normally fused in *Lepus*, nevertheless, this bone commonly occurs in *L. capensis* (Angermann, 1983). Although Angemann (1983) considered *L. habessinicus* a subspecies of *L. capensis,* there is now unresolved consensus about the species status of this taxon. Authors like Azzorali-Puccetti (1987) probably accept the subspecies status of *L. habessincus*; however, they pointed out the absence of the interparietal bone in the Berbera population of northern Somalia. Flux and Angermann (1990) thought that it is a subspecies of Cape hare, widespread and abundant in its range.

Lepus habessinicus is recorded in Somalia and Ethiopia/Eritrea and probably northern Kenya (Azzorali-Puccetti, 1987; Flux and Angermann, 1990; Yalden *et al.,* 1996; see Fig.1).

Abyssinian hares are recently recorded from the Mount Kaka area in southeastern Ethiopia (Girma *et al*., 2012). The preferred habitats are open grassland, steppe, savanna, and desert areas (Hoffmann and Smith, 2005). In this area, it is replacing the Cape hare (Azzorali-Puccetti, 1987; Flux and Angerman, 1990; Yalden *et al.,* 1996; Hoffmann and Smith, 2005). It needs scattered shrubs for shade and hiding from predators. However, thicker shrubs have been taken over by other hare species like the *L. saxatils/L. victotiae* complex. Little is known about their reproductive biology, ecology, population biology and genetics. Similar to the Cape hare it is spreading due to overgrazing of domestic stock (Hoffmann and Smith, 2005).

2.10.4 *Lepus victoriae* Thomas 1893, Savanna Hare

The savanna hare is a medium sized hare of about 2 kg in body weight. It can be identified by a grayish brown back, rufous breast, sides and legs, and white below. But in East Africa where the Savanna hare and the Cape hare coexist, they are almost identical in color (Flux and Flux, 1983). The geographic distribution of *L. victoriae* (savanna hare) is most of Africa below Sahara except the small isolated population in West Algeria (Flux and Angerman, 1990; Hoffmann and Smith, 2005; see Fig.1). However, *L. microtis*, Heuglin, 1865 and *L. crawshayi*, De Winton, 1899, are considered synonymous to *L. victoriae* (Hoffmann and Smith, 2005). *Lepus crawshayi* has a superficial resemblance to *L. starcki*, the fur having a similar texture and the ear tips showing a prominent black patch on the ear tips like in the latter species (Yalden *et al*., 1986). In *L. starcki* the grooves of the the first upper incisor are not cement filled whereas in *L. crawshayi* each has a pair of faint, cement filled grooves on the anterior surface (Angermann, 1983; Yalden *et al*., 1986). Robinson and Dippenaar (1987) have considered *L. crawshay* as smaller clinal variation of *L. saxatilis*.

2.10.5. *Lepus fagani* Thomas, 1903, Ethiopian Hare

This hare is of medium size with long, dense and harsh fur (Flux and Angermann, 1990). The dorsal part is buff ochrasraous brown, the sides, nape and chest are tawny. The tail has large dorsal stripes and and is white below, the belly is white. It is distributed in northern and western Ethiopia on the plateau and in Kenya at high altitude (Fig. 1).

The Ethiopian hare is closely related to the *L. victoriae/L. saxitils* complex and considered part of it (Flux and Angermann, 1990; Hoffmann and Smith, 2005). Azzorali–Puccetti (1987) separated it into a distinct species on the basis of more materials since it has uniform characters in its range. But the taxonomic position of this hare is still not well understood and some authors (Azzorali–Puccetti, 1987; Flux and Angerman, 1990) consider it based on few morphological data as subspecies of the *L. victoriae/L. saxatilis* complex, yet the mountain populations are isolated geographically. Little is known about the reproductive biology, ecology, habitat and behavior of this hare (Hoffmann and Smith, 2005).

2.10.6. *Lepus starcki* Petter, 1963, Ethiopian highland hare

The Ethiopian highland hare weighs about 2.3 kg on the average. Hair on the dorsal side is thick, soft and tawny and mixed with black color turned into grayish along the rump. The ventral side is white, and the breast, sides, nape, and legs are tawny. The dorsal side of the tail is fully white in the Shoa plateau but the populations in the Bale Mountains has a black stripe on the dorsal side (Azzaroli-Puccetti, 1987a; Flux and Angermann, 1990). The ear of this species is multicolored, with brownish grey leading to the edge, the larger portion of the dorsal part of the ear is pale grey. There is a conspicuous large black patch towards the ear tip and this is a unique feature that is not found in other hares in eastern Africa (Azzaroli-Puccetti, 1987a; Flux and Angermann, 1990).

Different authors have tried to determine the phylogenetic position of the Ethiopian highland hare. Among them were Petter (1963), who described it as subspecies of *L. capensis* (Cape hare), and Angermann (1983) who considered it a valid species. But it is also considered a subspecies of *L. europaeus* dating from late Quaternary (Azzaroli-Puccetti, 1987). Based on karyotypic and chromosome banding patterns Azzaroli-Puccitti *et al.* (1996) also confirmed that *L. starcki* is a relict species of brown hare. It is distributed in the central highland of Shoa region and in the mountains of Bale region, from 2500 to 4000 m above sea level. All of these areas are located between 6^0 *50′* and 9^0 35′N*,* 38^0 *to* slightly 40^0 E (Angermann*,* 1983; see Fig. 1*).* The ecology, habitat, and biology of this hare is poorly understood (Hoffmann and Smith, 2005). Mekonnen *et al.* (2011) made fecal analysis and direct observations to determine the materials consumed by *L. starcki.* They found that the diet consisted of 27 plant species (21 herbs, 4 grass and 2 shrubs). Monocots are the most frequent plant species eaten by the Ethiopian highland hare.

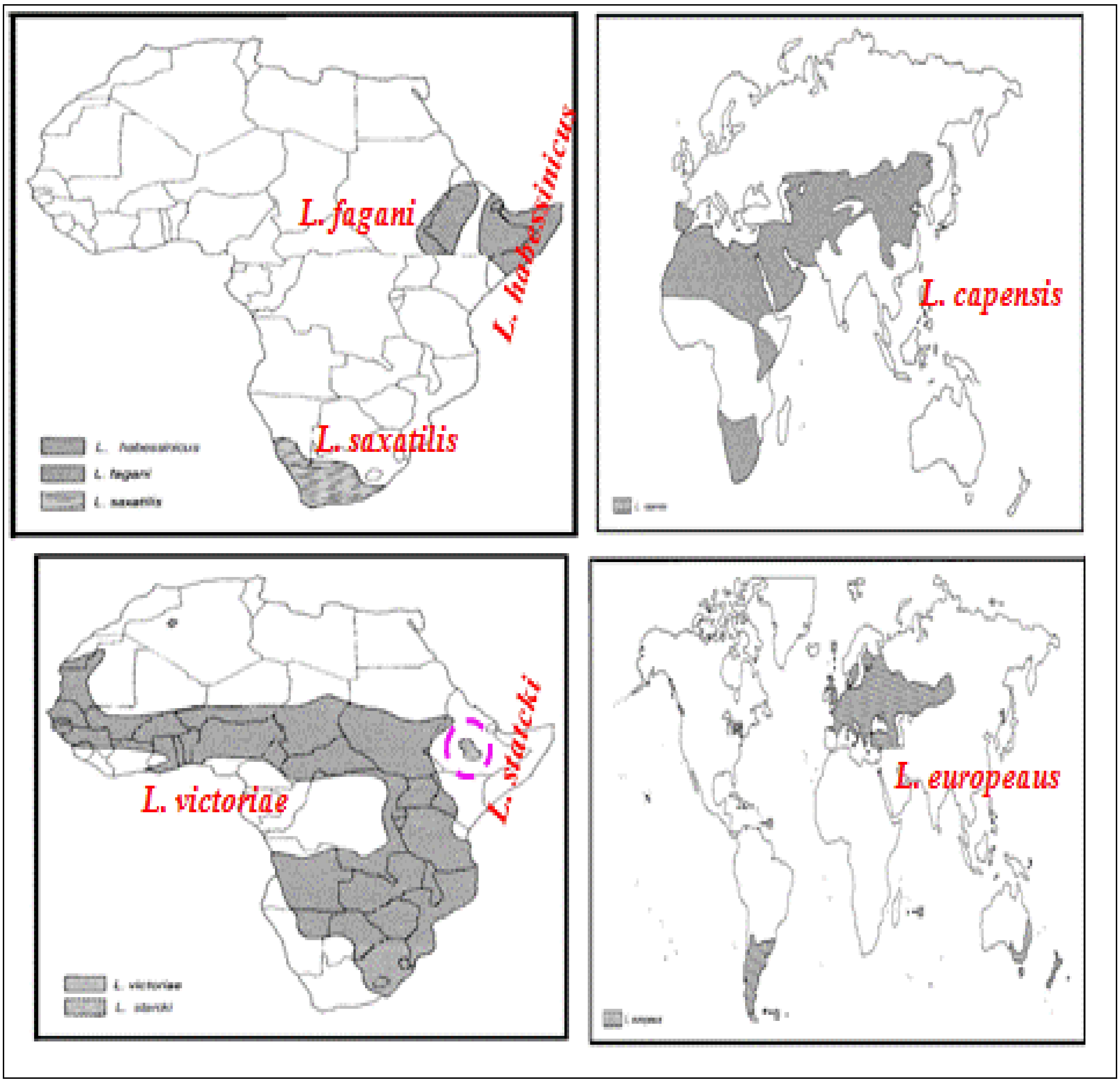

Figure 1. The distribution of *Lepus* species used in the present study (Flux and Angermann, 1990).

3. Materials and Methods

Sample hares were collected from 26 localities in Ethiopia (Fig. 2 and Table 1). Species identification was made on the basis of morphological features: coat color, diagnostic features on the posterior surface of the ear, ossification level of the interparietal bone, cement filling pattern in the first upper incisor.

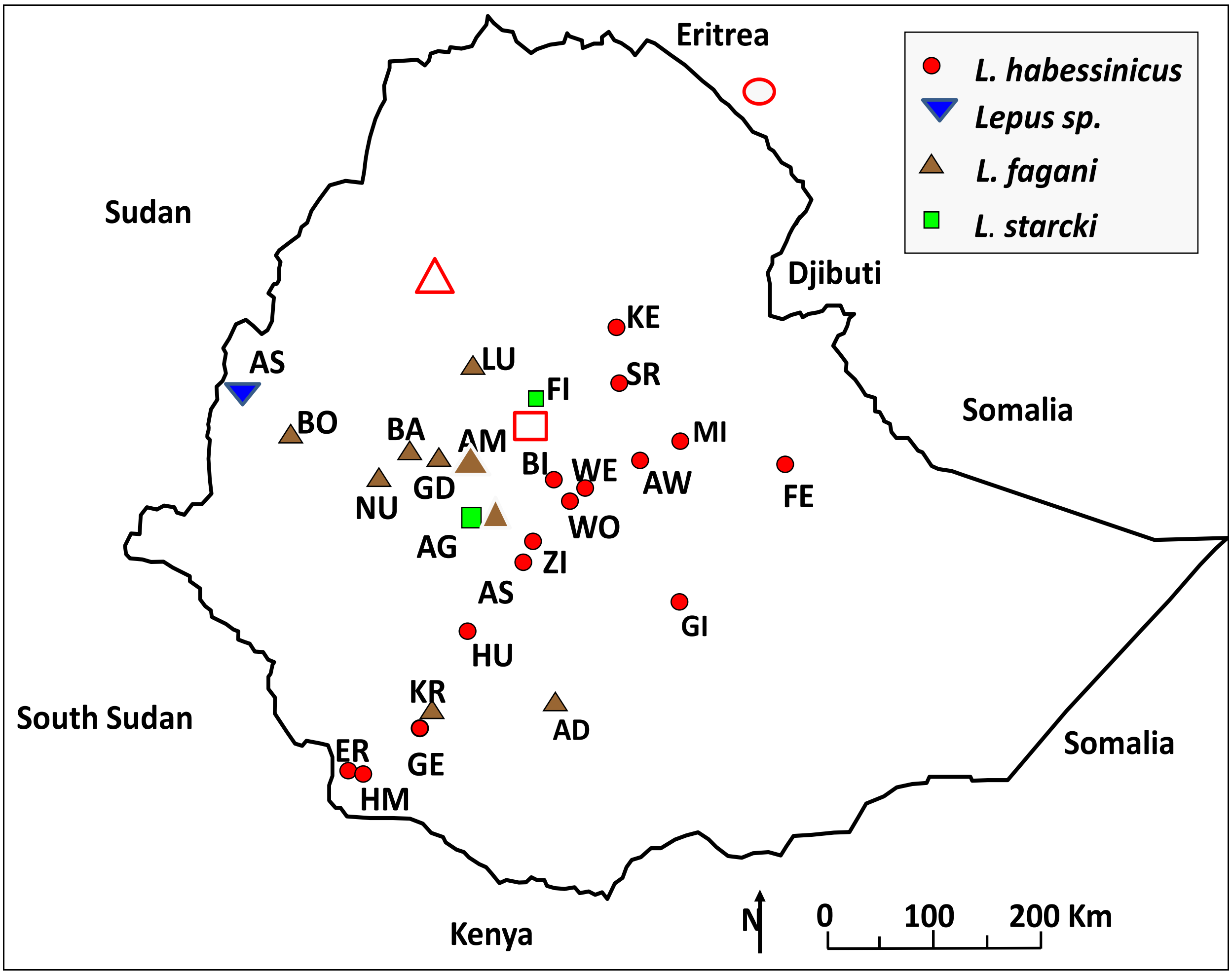

Figure 2. Map of Ethiopia showing hare species collection sites. Open symbols indicate holotype localities of the respective species. Full names of collection sites are given in Table 1.

Table 1. Samples collected in this study in Ethiopia

Species	Common Name	Collection Localities	Sample size	Region
L. habessinicus	Abyssinian Hare	Bishoftu/ Debrezeit (BI)	4	Rift valley
		Wonji(WO)	3	Rift valley
		Wellenchiti(WE)	6	Rift valley
		Andido/Afar (Aw)	4	Rift valley
		Miesso (MI)	7	East Ethiopia
		Fedis(FE)	11	East Ethiopia
		Ginnir (GI)	1	Southeast Ethiopia
		Shoa robit (SR)	2	North Ethiopia
		Kemise (KE)	1	North Ethiopia
		Ziway (ZI)	17	Rift valley
		Abijata Shalla NP(AS)	3	Rift valley
		Humbo/Wolaita (HU)	4	Southwest Ethiopia
		Gewada/Konso(GE)	1	Southwest Ethiopia
		Erbore/ S. Omo (ER)*	1	Southwest Ethiopia
		Hamer/S.Omo (HM)*	1	Southwest Ethiopia
L. starcki	Ethiopian Highland Hare	Fiche/ Oromia (FI)	26	Central Ethiopia
		Agena /Gurage (AG)	1	Central Ethiopia
L. fagani	Ethiopian Hare	Ambo(AM)	1	Central Ethiopia
		Gedo(GE)	3	West Ethiopia
		Bako(BA)	1	West Ethiopia
		Nunnu Kumba (NU)	1	West Ethiopia
		Boji Chokorsa (BO)	1	West Ethiopia
		Adolla/ Guji (AD)	9	South Ethiopia
		Lumama/ Gojam (LU)	2	Northwest Ethiopia
		Kerkerty / Konso (KR)	7	Southwest Ethiopia
		Agena /Gurage (AG)	1	Central Ethiopia
Lepus sp.		Assosa (AS)	1	North west Ethiopia

*S. Omo (South Omo)

Species Identification

According to our present collection, we identified three different morphological types and the three groups are distinct from one another. One individual from Assosa could not be clearly identified on the basis of morphological characters. Diagnostic features for *L. habessinicus* are a black rim at the posterior margin of the ear, relatively long ears, a deep, cement filled groove on the anterior surface of the first upper incisor, the fur is silky to touch and the sides of the tail are white (Yalden *et al*., 1986; Azzaroli-Puccetti, 1987; Flux and Angermann, 1990). The external morphological features can be seen in Fig. 3.

Figure 3. Phenotype of *L. habessinicus* collected from Bishoftu (Debrezeit) area, Oromia Regional State. Photo taken during field trips of this study.

The diagnostic features for Ethiopian hare are very short ear length, small tuft white hair on the forehead and brownish prominent coloration of some of the extremities, the fur is rough in texture relative to that of *L. habessinicus*, there is a slightly dark margin on the posterior surface of the ear (Yalden *et al*., 1986; Azzaorili-Puccetti, 1987a, b; Flux and Angermann, 1990). See Fig. 4 for external morphological features.

Figure 4. Phenotype of *L. fagani* collected from Adolla district Guji zone/Oromia Regional State. Photo taken during field trips of this study.

Features such as the presence of a larger blackish patch on the ear tip and the fully white color of the dorsal part of the tail (i.e. in Shoa region, but tails in populations in Bale Mountains are not fully white), and the grooves in the upper incisor are not cement filled are diagnostic features of Ethiopian highland hare (Yalden *et al*., 1986; Angermann, 1983, Azzaroli-Puccetti, 1987a,b). See Fig. 5 for external morphological features.

Figure 5. Phenotype of *L. starcki* collected from Fiche (North Shoa) Oromia Regional State. Photos taken during field trips of this study.

3.1. Analyses of External Morphological, Dental and Non-metric Skull Characters

For most samples (*L. habessinicus*: n = 43, *L. fagani*: n = 9, *L. starcki*: n = 24) colleted in the present study, we took measurements of seven external body characters: tail length (TL, from root to tip, without hairs), hind foot length (HFL, without nails), ear length (EL, to the tip, without hairs), body weight (BW), total body length curved (TBLC: along the dorsal outline from the tip of the nose to the root of the tail), and total body length straight (TBLS, in natural lateral position, from nose tip to root of the tail); for the remaining samples, however, only heads or genetic samples were available. The whole external morphological statistical analyses were performed using the SPSS v.10.0.1. A generalized linear model (GLM, type three sum squares, 2-way analysis of variance (ANOVA) with sex and species as a fixed factor was done for each one of the external body measurements. In addition a 2-way analysis of covariance (ANCOVA) (GLM, type three sum squares) with sex and species as fixed factors and HBLC as covariate was done for each external measurement. A comparison of relative body proportions of external body measurements (i.e. vi/HBLC) was carried out to check for relationships between external body measurements and both elevation and local temperature, and to test some ecogeograpic rules (e.g. Bergmann's rule and Allen's rule). Presence and absence of cement filling of the groove of the first upper incisor were checked under a dissecting microscope. A binary logistic regression analysis was performed for testing the presence/absence of cement filling in relation to sex, species and as a fixed factor. In addition, the extent of ossification of interparietal bone sutures was assessed macroscopically and in unclear cases this was followed by a control under the dissecting microscope.

3.2. Morphometric Analyses of Skull

A multivariate morphometric analysis of adult-sized (i.e. sub adult or adult) skulls was performed as was done by Suchentrunk *et al*. (2007) for Cape hares and savanna hares from East Africa and Canu *et al*. (2012) for Sardinian hares. We used the following seven species from diverse geographical regions: *L. habessinicus* (Ethiopia & Somalia; n = 55), *L. fagani* (Ethiopia; n = 10), *L. starcki* (Ethiopia; n = 49), *L. capensis* (Kenya, RSA, North Africa; n = 128), *L. europaeus* (Europe; n = 37), *L. saxatilis* (RSA; n = 21) and *L. victoriae* (Kenya; n = 27). Some of the skulls were collected during the present study and the remainder were museum specimens from the Zoological Museum of the Humbold University, Berlin (Germany), the Natural History Museum, Zoological Section "La Specola", University of Florence (Italy), the earlier collection of Dr. J.E.C. Flux, now housed at the Te Papa Museum, Wellington, New Zealand, the Transvaal Museum, Pretoria, R.S.A., mammal collection at the

Smithsonian Institution, Washington, D.C., U.S.A., and the private collection of Dr. Franz Suchentrunk, Vienna.

Thirty one (Fig.6 A-E) cranial and mandibular linear measurements were taken for each skull using digital calipers with an accuracy of 0.01 mm**.** The linear measurements were: basal length (BL), cndylobasal length (CBL), profile length (PL), rostrum length (RL), cranium length (CL), upper tooth row length (UTL), diastema length (DIA), upper molar row length (UML), nasal length (NL), zygomatic arch length (ZAL), rostrum width (RB), choane width (CHB), maxillae width (MB), zygomatic arch anterior (ZYGA), meatus accusticus (MEB), post orbital width (POB), brain case height (BCH), rostrum height (RH), mandible length (ML), mandible diastema length (MDL), lower molar row length (LML), mandible height (MH), zygomatic arch posterior (ZYGP), palatal bridge (PB), fenestera insisivo width (FIB), nasal breadth (NB), frontal width (FB), tympanic bullea width (TBB), distance between the first premolar (DUFP) and total skull length (TSL).

The whole morphometric statistics were performed using the SPSS v.10.0.1 software. Missing values, which did occur due to partly damaged skulls in less than one percent of the total data set, were estimated by means of stepwise regression models, separately for each species. Each of the original variables was calculated as percentage of the associated CBL, which produced a shape (proportion) variable without any size siganl (see e.g. Suchentrunk *et al*. 2006; Canu *et al*. 2012).

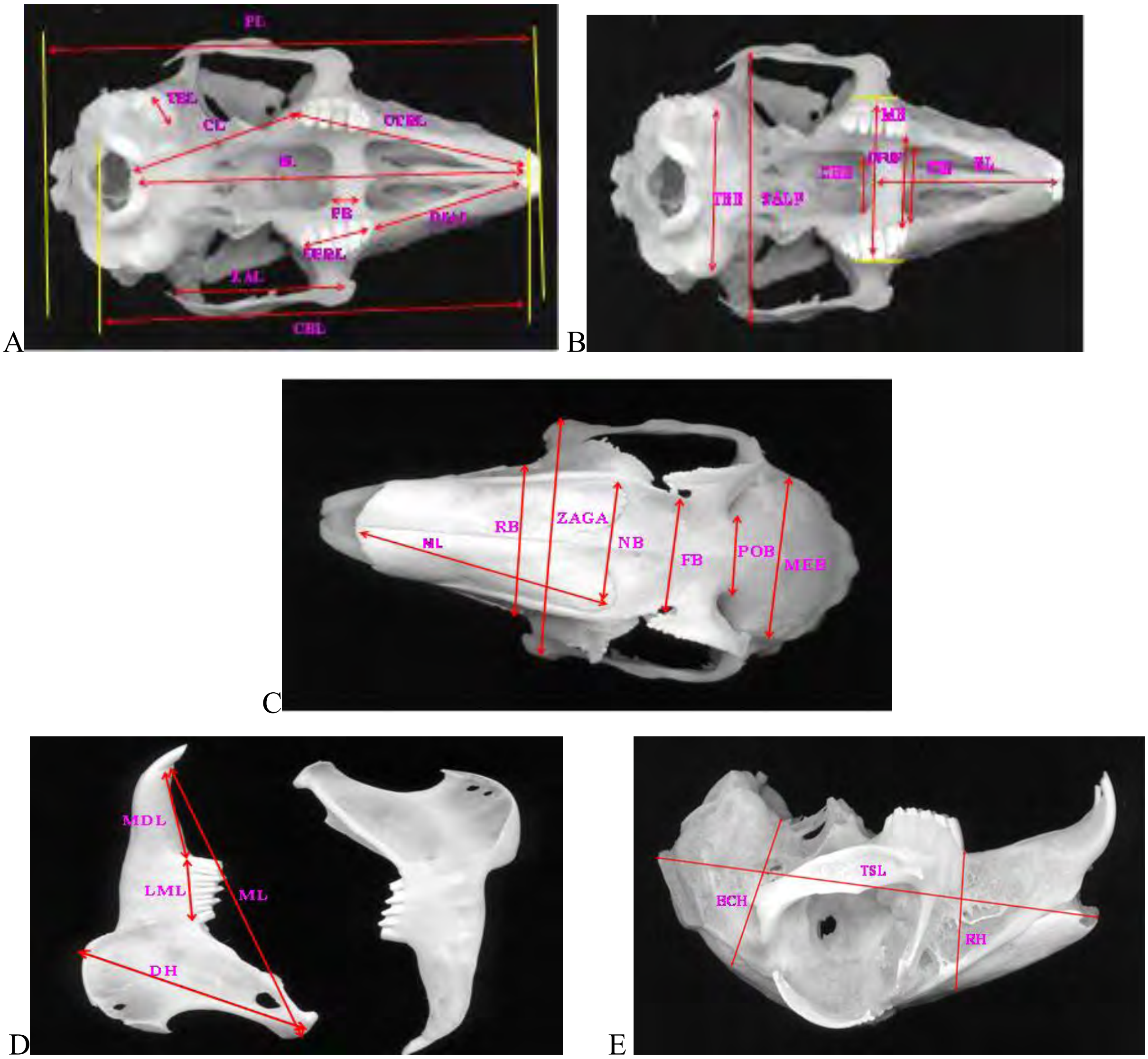

Figure 6. A-E Metric skull variables used in morphometric analyses.

Resulting ratios (v_i/CBL_i) were transformed as $v_t = ln(arcsine\ (v_i/CBL_i)^{0.5}) \times 100)$ to maintain normality and to stabilize variable variances. Transformed variables were subjected to discriminate analysis (DA) with simultaneous entry (e.g McGarigal *et al.*, 2000), based on Wilk's lambda statistics, equal prior group probabilities of group membership, and within group covariance matrices. Normality of measurements (i.e. variables) was tested with Kolmogorov-Smirnov tests (e.g. Gotelli and Ellison, 2004). Non singularity of variables were achieved since no variable was defined as a linear combination of other. Equality of covariance matrices, which is the prerequesites of DA, was tested by a Box's M-tst. A subset of variables was selected for discriminate analysis based on the power of discriminating among groups. A multi-group principal components analysis (PCA) was performed onto the transformed variables in order to summarize the morphometric variation as multivariate variables, i.e., principal components (PCs). A generalized linear model (GML, type three sum square, 3-way ANOVA) with species, sex and age as fixed factor was done for each metric variable followed with post-hoc tests. In addition, 3-way ANCOVAs (GLM, type three sum squares) of each metric variable with sex, species and age as fixed factor and CBL as covariate, to control for possible size dependence was done.

3.3 Microsatellite Screening

Individuals of *L. habessinicus (n* = 59), *L. starcki* (n = 26), and *L. fagani* (n = 22) were screened for allelic variation for the following thirteen microsatellite loci with different levels of polymorphism: Sol08, Sol28, Sol30 (Rico *et al*., 1994*),* Sol33 (Surridge *et al*., 1997), Sat 2, Sat 8, and Sat 12 (Mougel *et al*., 1997), Lsa1, Lsa2, Lsa3, Lsa6 and Lsa8 (Kryger *et al*., 2002) and INRACCDDV0001(N4) (Chantry-Darmon *et al*., 2005). The details of the SSR primers used are given in Table 2. The GenElute^{TM} Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) was used to extract total genomic DNA from ear tissue. DNA amplification (i.e. polymerase chain reaction, PCR) was performed in 25 µl reaction volumes with the following components: 0.3 µl of genomic DNA, 0.2 µl of each primer, 2.5µl each dNTP, 0.5µ $MgCl_2$ (with MgCl2 for Lsa1 and Lsa2 primers), 0.12 µl Taq DNA polymerase and 2.5 µl 10x reaction buffer ($MgCl_2$ free). After a preliminary denaturation step at 95 ^{0}C for 5 min (3 min for Sol333 and all Lsa primers), PCR amplification was performed for 35 cycles (30 cycles for Sol33 and all Lsa primers) of 40 s denaturing at 95 ^{0}C, 40 s of annealing at locus- specific temperatures (53 ^{0}C for Sol33 and all Lsa primers; 57 ^{0}C for Sol28 and Sol30 primers; 61 ^{0}C for Sol08 primer) and 1 min extension at 72 ^{0}C, with final 10 min extension step at 72 ^{0}C. For the Sat primers amplficafication was performed in 25 µl reaction volumes with the following components: 1 µl genomic DNA, 0.2 each primer, 2.5 µl of mM dNTP mix, 0.5 µl MaCl2, 0.1µl Taq DNA polymerase and 2.5 µl 10x reaction buffer. After a preliminary denaturation step at 95 ^{0}C for 4 min, PCR amplification was performed for 30 cycles of 30 s denaturing at 95 ^{0}C, 30 s annealing at locus-specific temperatures (55 ^{0}C for Sat2 and Sat12 primers, and 60 ^{0}C Sat8 primer) and 30 s extension at 72 ^{0}C, with a final 10 min extension step at 72 ^{0}C. PCR products were electrophoresed on a LI-COR 4200 automated sequencer along with fluorescently labelled size standard (30-350 bp sizing standard; LI-COR® Biotechnology Division) and allele lengths were determined by the Gene ImageIR v. 3.52 software (LI-COR, Inc., dr 1990).

Table 2. SSR primers used, GenBank Acc. Nr., Repeat array and References

Locus	Acc. Nr.	Primer (5'-3')	Repeat array	Refereces
Sol08	X79217	F: GGATTGGGCCCTTTGCTCACACTTG R:ATCGCAGCCATATCTGAGAGAACTC	(TG)19(N)15(TG)5	Rico *et al*., 1994
Sol28	X79216	F: ATTGCGGCCCTGGGGAATGAACC R: TTGGGGGATATCTTCAATTTCAGA	(TC)23(N)3(TC)4	Rico *et al*., 1994
Sol30	X79215	F: CCCGAGCCCCAGATATTGTTACCA R: TGCAGCACTTCATAGTCTCAGGTC	(TC)14A(T)4(TC)5	Rico *et al*., 1994
Sol33	X94683	F: GAAGGCTCTGAGATCTAGAT R: GGGCCAATAGGTACTGATCCATGT	(TG)3CG(TG)16	Surridge *et al*., 1997
Sat2	M77195	F: GCTCTCCTTTGGCATACTCC R: GCTTTGGATAGGCCCAGATC	(TC)15(TG)10	Mougel *et al*.,1997
Sat8	X99889	F: CAGACCCGGCAGTTGCAGAG R: GGGAGAGAGGGATGGAGGTATG	(CT)14(GT)8TT(GT)5	Mougel *et al*.,1997
Sat12	X99891	F: CTTGAGTTTTAAATTCGGGC R: GTTTGGATGCTATCTCAGTCC	(CTAT)10	Mougel *et al*.,1997
N4	AJ874368	F: CATTCGCTGTCTCAATCCAA R: ACATGGGTACATGCCAACTG	(CA)14	Chantry-Darmon *et al*., 2005
Lsa1	AF491762	F: CCTTGCAGGTTTTCAGCCTC R: GCTGTAGAAAATGAGAGGGAC	(CA)9CT(CA)2	Kryger *et al*., 2002
Lsa2	AF491763	F: GGTACTCTATTAGGGAACCCG R: GCTAGTTGCCATTAGCTCCC	(AC)13	Kryger *et al*., 2002
Lsa3	AF491764	F: TGCCTTAAAGGCTGATTCTG R: GGATATCAAAGAACATGCCC	(AC)10	Kryger *et al*., 2002
Lsa6	AF491766	F: CCTAAGATGAAATGGATAAGTT R: CTCTTCTGTTTTCTGGAGCA	(AC)10	Kryger *et al*., 2002
Lsa8	AF491768	F: AAGGTATTAAATTGGGCACTC R: GACTGAAATTGATGTGCTACC	(GT)11	Kryger *et al*., 2002

3.3.1 Allelic Variability

For all statistical analyses 107 individuals with full set of genotypes or maximally one locus missing (due to insufficient DNA quality) were considerd. The Micro-Checker v.2.2.3 (Van Oosterhout *et al*., 2004) program was used to test for the possibility of the presence of null alleles, possible genotyping errors due to stuttering and large allele dropout. Also, each locus was tested, separately for each species, for deviation from Hardy-Weinberg equilibrium (HWE) and pairs of loci, separately for each species, were tested for linkage disequilibrium (LD), using the Markov chain method implemented in GENEPOP v.4.0 (Rousset 2007). Tests were run with default parameter settings and the strict Bonferroni method (at $\alpha = 0.05$) was used to account for multiple tests (Rice, 1989). The latter correction method was also applied to all further series of tests. Allele frequencies, mean number of alleles (A), observed (Ho) and (unbiased, corrected for small sample sizes) expected (He) heterozygosity for each locus and species were calculated with GENETIX v.4.05.2 (Belkhir 2004). The FSTAT v.2.9.3.2 (Goude, 2002) program was used to calculate species-specific allelic richness (Rs) by a rarefaction approach, to account for different sample sizes. As allelic diversity appeared to be somewhat lower in *L. starcki* than in the other two species from Ethiopia, one-tailed Wilcoxon sign rank tests, as implemented in BOTTLENECK v. 1.2.02 (Cornuet and Luikart, 1997), were used to test (10.000 iterations) for reduced effective population sizes/bottlenecks in the recent past. Thereby, the stepwise mutation model (SMM), the infinite allele model (IAM), and a two-phased model (TPM., with default settings) of microsatellite evolution was employed. Finally, overall and species-specific Weir and Cockerham (1984) estimators of F_{IS} (population-level inbreeding coefficient) and associated significance higher than zero (by permutation tests 10.000 permutations) were calculated by using GENETIX v.4.05.2 (Belkhir 2004).

3.3.2 Genetic Differentiation

GENETIX v.4.05.2 (Belkhir 2004) was used to calculate pairwise Cavalli-Sforza and Edwards (1967) chord (CSE) distances between population and associated 95% confidence intervals by randomizations (10.000) and to perform a ten-factorial correspondence analysis (FCA) based on individual multilocus genotypes. The F_{ST} statistics and associated significance levels (10000 randomizations) as calculated in FSTAT were used to estimate pairwise relative genetic differentiation among the three species. As F_{ST} values might give lower levels of differentiation under high levels of heterozygosity, we additionally calculated pairwise Jost´s D distances (estimator of actual differentiation, D_{est}, Jost, 2008) and standardized measure of genetic differentiation (G'_{ST}, Hedrick, 2005) using GenAlex v. 6.5 (Peakall and Smouse, 2012). Furthermore, an analysis of molecular variance (AMOVA) model with one structure level was calculated using Arlequin v.3.11 (Excoffier *et al.*, 2005), to evaluate the amount of partitioning of relative genetic (allelic) variance due to the three species (*L. fagani*, *L. habessinicus*, *L. starcki*). Further, the MSA program (Dieringer and Schlötrer, 2003) was used to calculate Cavalli-Sforza and Edwards (1967) chord (CSE) distances between individuals with 1000 bootstraps replication. An NJ tree based on CSE distances was constructed by using the "NEIBHBOR" routine as implemented in the Phylip v.3.695 software package (Felsenstein, 2013).

The GENECLASS2 v.2.0 program (Piry *et al.*, 2004) was used to estimate the likelihood of an individual's multilocus genotype to be assigned to the species from which it was sampled. Specifically, specimen assignments were conducted by the Bayesian method of Rannala and Mountain (1997) and by computing probabilities following the resampling algorithm of Paetkau *et al* (2004). In addition, the STRUCTURE v.2.3.1 software (Pritchard *et al.*, 2000) was used to assess the most likely number of population groupings (i.e., genetic clusters, k) compatible with the observed genotypic distribution across all samples, under the assumption of no linkage disequilibrium and according to multi-locus Hardy Weinberg equilibrium. STRUCTURE was run for the entire genotype data set (107 hares from Ethiopia) without and with prior population (i.e., species) information under admixture models with correlated allele frequencies. The likelihood when assuming different numbers of populations (k = 1 to 10) was calculated by 150.000 MCMC following a birn-in of 50.000, and 10 iterations per k. Mean and standard deviation of likelihood values were calculated for each k and plotted together with Evanno *et al*. (2005) ad hoc statistic by using the STRUCTURE HARVESTER on-line option (Earl and vonHoldt, 2012). The STRUCUTRE analysis was repeated with the above specifications, but by leaving out the Lsa2 locus, as it revealed significant LD (Linkage disequilibrium) with loci Sol8 and Sat12 in *L. habessinicus*, which contradicts the modelling assumptions for STRUCTURE. Eventually, MIGRATE v.3.2.1 (Beerli, 1998; Beerli and Felsenstein, 1999; Beerli and Felsenstein, 2001) was used for a coalescent-theory based maximum likelihood estimates of migration rates between the three species, as additional indicators of levels of current interspecific hybridization.

3.4. PCR Amplification and Sequencing of Mitochondrial DNA (mtDNA) and Nuclear DNA (nucDNA) Sequences

Mitochondrial DNA Sequence

PCR amplification was done as carried out by Smith *et al.* (2010) to amplify a 416 bp segment of the mitochondrial ATPase sub-unit 6 (ATP6, from site 8142 to 8594; see also Arnason *et al*., 2002 for the mitochondrial genome of the brown hare, *L. europaeus*). The species and the number of individuals used for ATPase sub-unit and the nuclear sequences are given in Table 3. PCR amplification was carried out in 25 µl volume with the following components: 200mM each dNTP, 0.2mµM of each primer and 0.5 units of Taq polymerase. Samples were first denatured for 5 min at 94 ^{0}C followed by 35 cycles of 95 ^{0}C for 45 s, 53 ^{0}C for 45 s and 72 ^{0}C for 60 s and a final extension step of 72 ^{0}C for 7 min.

Nuclear DNA Sequences

PCR amplifilification was done for five nuclear sequences: transferrin (TF), thyroglobulin (TG), uncoupling protein two (UCP2), uncoupling protein four (UCP4) and thyroid stimulating hormone beta (TSHB). For the nuclear sequences details of primers, conditions and references are given Table 4. Amplification was done for transferrin (TF, between exon 6 and 7) fragment was performed as carried out by Alves *et al.* (2003). For the rest of nuclear sequences, namely, thyroglobulin (TG) fragment amplification was done as performed by Mathee *et al*. (2004) and UCP2, UCP4 and TSHB PCR was carried out as done by Melo-Fereirra *et al*. (2012). In addition, wild rabbits (*Oryctolagus cuniculus*) were sequenced for all nuclear fragments for outgroup comparison. However, our primers did not yield satisfactory results when applied to *Oryctolagus cuniculus* to amplify ATPase sub unit 6 fragments. There were no available sequences in the GenBank for either *Oryctolagus* or *Sylvilagu*s for ATPase 6 fragment. PCR product purification was accomplished through the enzymatic clean-up process using Exonuclease I and Shrimp Alkaline Phosphatase (Werle *et al*., 1994).

Table 3. Species name, Locus name (mtDNA or nucDNA) and sample size used for each sequences fragments

	Locus name and sample size					
Species name	ATPase 6 (n = 155)	TF (n = 159)	TG (n = 112)	UCP2 (n = 113)	UCP4 (n = 87)	TSHB (n = 97)
L. habessinicus	63	60	38	52	39	36
L. starcki	27	27	19	20	19	11
L. fagani	15	23	15	20	15	18
Lepus sp. ***	1	0	0	0	0	0
L. capensis (RSA)	15	6	5	3	4	11
L. capensis (North Africa)	13	11	12	9	7	12
L. Saxatilis	5	15	14	8	0	4
L. europaeus	14	10	6	1	2	1
L. timidus	2	2	2	0	1	2
O. cuniculus	0	5	1	0	0	2

*** *Lepus sp.* Collected from Asosa (Ethiopia)

Cycle sequencing of both strands was done in an ABI 3130xl genetic analyzer in both directions. Sequences were edited using the BioEdit v. 7.1.3.0 (Hall, 1999) program and sequences and alignments were checked by eye. We used composite sequences as obtained from both strands for phylogenetic analyses. We used the Phase 2.1.1 algorithm (Stephens *et al*., 2001; Stephens and Donnely, 2003), as implemented in DnaSP v. 5. (Labrado and Rozas, 2009), to reconstruct haplotypes (e.g. Flot *et al*., 2006; Garrick *et al*., 2010) for nuclear sequences with ambiguity.

The available sequences were downloaded from GenBank: TF (Alves *et al*., 2003; Liu *et al*., unpublished), TG (Mathee *et al*., 2004; Liu *et al*., 2011), UCP2, UCP4 and TSHB (Melo-Fereirra *et al*., 2012) for phylogenetic comparison. The downloaded nuclear sequences used for both the ingroups and outgroups are found in Appendix 2 (Tables 1-5).

Table 4. Names of nuclear gene fragments used in this study. PCR annealing temperature, MgCl2 concentration, primers used and References

		PCR			PCR Primers	References
Name	Symbol	MgCl2	ATb	Ec	Forward/Reverse (5'-3')	
Transferrin	TF	1.8mM	57	30"	GCCTTTGTCAAGCAAGAGACC	Wallner *et l.*,2001
					CACAGCAGCTCATACTGATCC	Wallner *et l.*,2001
Thyrogloblin	TG	2.5mM	55	45"	GCATTGCAGGACAATGAACCCA	Matthee *et al.,* 2004
					CCACTGTTCATACCACTCGAAG	Matthee *et al.,* 2004
Uncoupling protein 2	UCP2	1.8mM	61	45"	TGCCGAGCTGGTCACCTAT	Melo-Ferreira *et al.*, 2012
					CCCAGGGCAGAGTTCATGTAT	Melo-Ferreira *et al.*, 2012
Uncoupling protein 4	UCP4	1.8mM	61	45"	GCTTCTATTCTGGGAACACCA	Melo-Ferreira *et al.*, 2012
					TGAACAGCCTGAATCAAGCA	Melo-Ferreira *et al.*, 2012
Thyroid stimulating hormon beta	TSHB	1.8mM	64	60"	CATCAACACCACCATCTGTGC	Melo-Ferreira *et al.*, 2012
					CACTTGCCACACTTACAGCT	Melo-Ferreira *et al.*, 2012

3.4.1 Phylogenetic Analyses of MtDNA (ATP6) and Nuclear Sequences

DnaSP v.5 (Labrado and Rozas, 2009) was used to calculate numbers of variable sites, phylogenetic informative sites, singletons, haplotype diversity (hd), nucleotide diversity (π) and mean numbers of pair wise differences (k) as indices of levels of polymorphism. MEGA v. 5.0 (Tamura *et al*., 2011) was used to reconstruct phylogenetic relationships among haplotypes with the Neighbor Joining (NJ, Saitou and Nie, 1987; Tamura *et al*., 2004) and the Maximum Likelihood (ML, Kimura, 1980) approaches. Prior to NJ analyses, both the ATP6 and the nuclear sequences (after phasing) we checked for suitability by calculating average Jukes Cantor (JC) distances (average pair wise JC distance < 1.0; Nei and Kumar, 2000; Hall, 2011). To construct NJ trees for both mtDNA (ATPase 6) and nuclear sequences the Maximum Composite Likelihood model was used, as recommended by Kumar 1993 (see Hall, 2011), and assessed confidence in resulting nodes by 1000 bootstrap replicates (Felsenstein, 1985). For ML analyses, the most appropriate evolutionary model based on BIC values (see Posada, 2009) was used, according to the model test option in MEGA v. 5.0. Again, confidence in nodes was based on 1000 bootstrap replicates. Furthermore, the same software was used to calculate mean between group uncorrected p-distances between populations (species) for ATP6 and nuclear sequences.

In addition, MrBayes v. 3.2 (Huelsenbeck and Ronquist, 2001) was used, which is based on Bayesian statistical principles and estimates phylogenetic relationships by seeking the most likely tree given the actual sequence data and the chosen substitution model. The Bayesian inference (BI) was started random starting trees and was ran it for 5 million generations, with Markov chains sampled every 500 generations. The average standard deviation of split frequencies was checked for parameter convergence. The first 1.25 million generations were excluded as burn-in. The BA was conducted twice to ensure that the analyses were not trapped in local optimum (Huelsenback and Bollback, 2001; Leache and Reeder, 2002). The remaining trees from both analyses were used to create a majority rule consensus tree, where the percent of samples recovering the same clade represented the posterior probability (PP) value of that clade. For tree viewing and editing FigTree v. 1.4.0 (Rambaut, 2012) was used.

Alternative to all tree-generating phylogenetic approaches, phylogenetic haplotype networks were constructed for both sets of sequences by using the median-joining algorithm (with default parameter settings) (and by leaving out indels, if revealed in certain alignments) implemented in the Network software, v.4.6.1.1 (Bandelt *et al*., 1999). Contrary to tree-based inference, this approach enables to uncover possible alternative phylogenetic pathways for haplotypes (i.e., depiction of evolutionary relationships among haplotypes are not reduced to bifurcating events) and may facilitate inference on the presence of shared ancestral polymorphism or recent introgression, particularly for clusters with low bootstrap support. Such networks may be particularly useful to infer intraspecific evolutionary patterns, and if reticulate evolutionary scenarios are anticipated (Moulton and Huber, 2009; Hall, 2011, Morrison, 2012).

4. Results

4.1. External Morphology

Seven external measurements were taken for hare species collected from different localities in Ethiopia. The descriptive statistics for the respective species are given in Table 5. Two-way ANOVA for body weight of subadult and adult individuals, with “sex” and “species” as fixed factors indicated a significant “species” [$F_{2,48} = 39.72$, $p < 0.005$] and “sex” [$F_{2,48} = 4.26$, $p = 0.044$] effects, with females having heavier body weights in general (see Appendix 1 Fig.1). Post-hoc comparisons using Scheffè tests revealed significantly higher body weights for *L. starcki* compared to *L. fagani* ($p < 0.005$) and *L. habessinicus* ($p < 0.005$) (Fig. 7). But there was no significant difference in body weight between *L. fagani* and *L. habessinicus*. The two-way ANOVA for curved head body length (HBLC) of adult-sized individuals revealed significantly bigger values for females [$F_{1,55} = 3.28$, $p = 0.016$] and a significant variation among the three species [$F_{2,55} = 16.806$, $p = 0.002$]. The three-way ANCOVA of EL with sex, age class (adult vs. subadult) and species as fixed factors and correcting for HBLC values (co-variate) resulted in no significant sex and age effect but a significant species effect ($p <0.005$). The two-way ANCOVA of hind foot length (HFL) of adult-sized individuals resulted in a significant sex difference [$F_{1,63} = 7.430$, $p = 0.008$] and significant variation [$F_{2,63} = 21.296$, $p < 0.005$] among the three species. The three-way ANCOVA for tail length (TL) with sex, age (adult vs. subadult) and species as fixed factors and HBLC as co-variate showed a significant age [$F (1, 59) = 6.275$, $p = 0.015$] and species [$F (2, 59) = 17.132$, $p = 0.005$] but no sex [$F (1, 59) = 3.004$, $p = 0.088$] effect. Three way ANOVA of the relative hind foot length (HFL/HBLC) to the main effect of sex, age (adults and subadults) and species indicated no significant sex [$F (1, 60) = 0.014$, $p = 0.906$] and species [$F (2, 60) = 2.496$, $p = 0.091$] but significant age effect [$F (1, 60) = 8.551$, $p = 0.005$]. Three way ANOVA of relative ear length (EL/HBLC) to the main effect of sex, age (adult and subadults) and species showed a significant age [F (1, 61) = 9.830, p = 0.003] and species [F (2, 61) = 18.826, p = 0.005] (Fig. 7) but no sex [F (1, 61) = 3.628, p = 0.062] effect. Post-hoc test (Scheffe test) indicated *L. fagani* had shorter relative ear length relative to the other two taxa (p < 0.005). Two way ANOVA of the relative body weight (BW/HBLC) to see the main effect of sex and species indicated a significant species [F (2, 47) = 29.829, p = 0.000] but no sex [F (1, 47) = 3.578, p = 0.065] effect. The post-hoc test indicated *L. habessinicus* has a smaller relative body weight compared to the other two species (Table 5). The scatter plots of EL versus HFL (Fig.8) indicated that *L. fagani* has relatively shorter ear length compared to the other two taxa.

Table 5. Descriptive statistics of external morphological measurements of adult-sized individuals in the three species L. *habessinicus, L. starcki* and *L. fagani*

	L. habessinicus			*L. starcki*			*L. fagani*		
Item	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD
BW	1415	2440	1815.14±254.76	1692	2963	2426.92±371.69	1704	2278	2058.33±234.17
EL	9.5	12	10.68±0.57	10	11.9	10.75±0.45	6	9.5	8.34±0.97
TL	5	11.1	9,14±1.16	9.5	13	10.91±0.86	7	10.7	8.55±1.08
HFL	9.5	11.4	10.45±0.47	10.5	12.3	11.35±0.42	9	11	10.13±0.76
HBLS	36.5	46.5	42.09±2.11	39.5	47	43.48±1.64	32.5	42.5	37.70±3.25
HBLC	43.5	53	48,63±2.31	44	54	51.04±2.29	42	50	46.50±3.24

BW = Body Weight; EL = Ear Length; TL = Tail Length; HFL = Hind Foot Length; HBLS = Head Body Length Straight and HBLC = Head Body Length Curved.

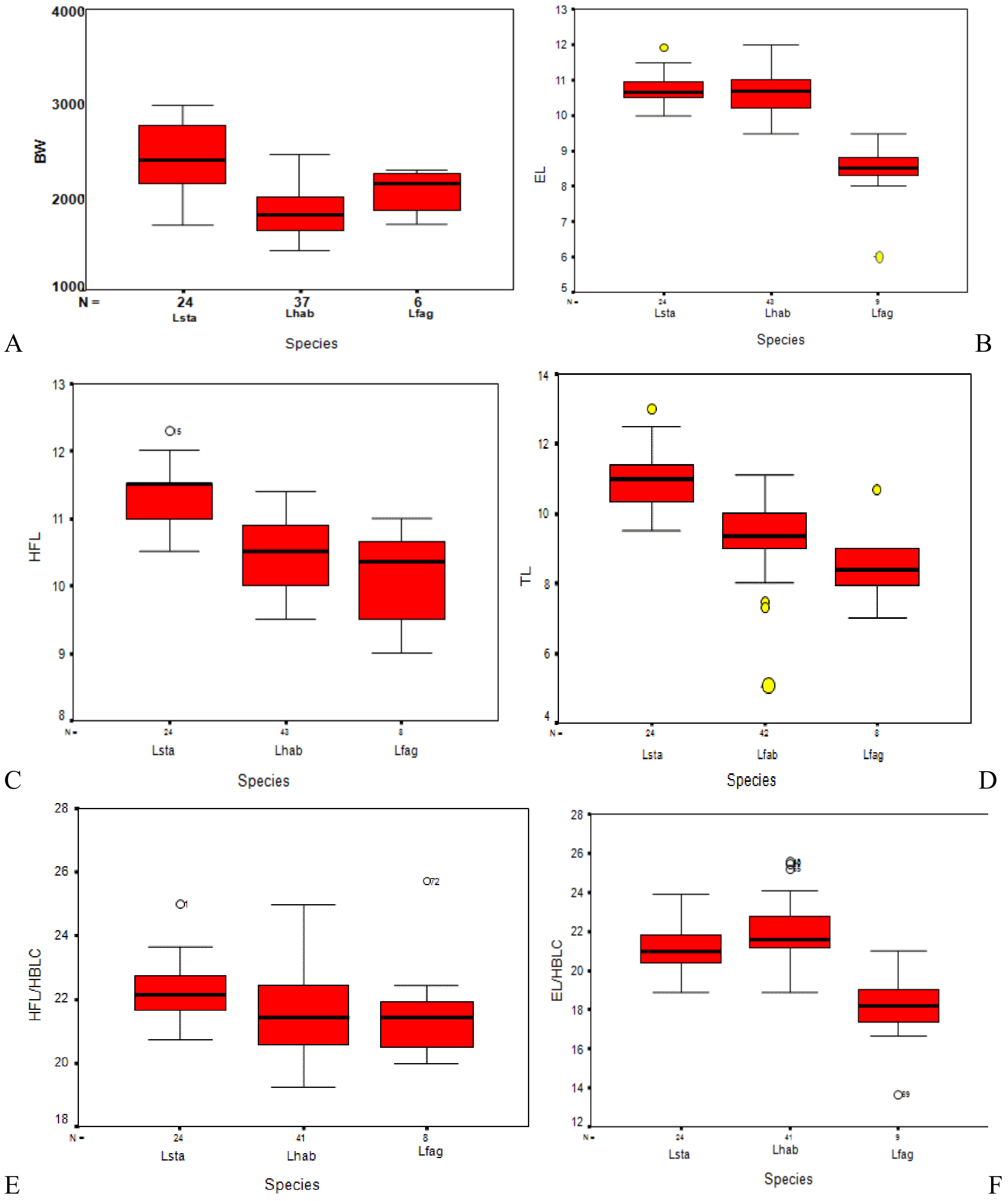

Figure 7A-F. Box plots of external measurements for *L. starcki* (Lsta), *L. habessinicus* (Lhab) and *L. fagani* (Lfag): Body Weight (BW), Ear Length (EL), Hind Foot Length (HFL), Tail Length (TL), HFL/HBLC and EL/HBLC. Each box shows the median, quartiles and extreme values within species.

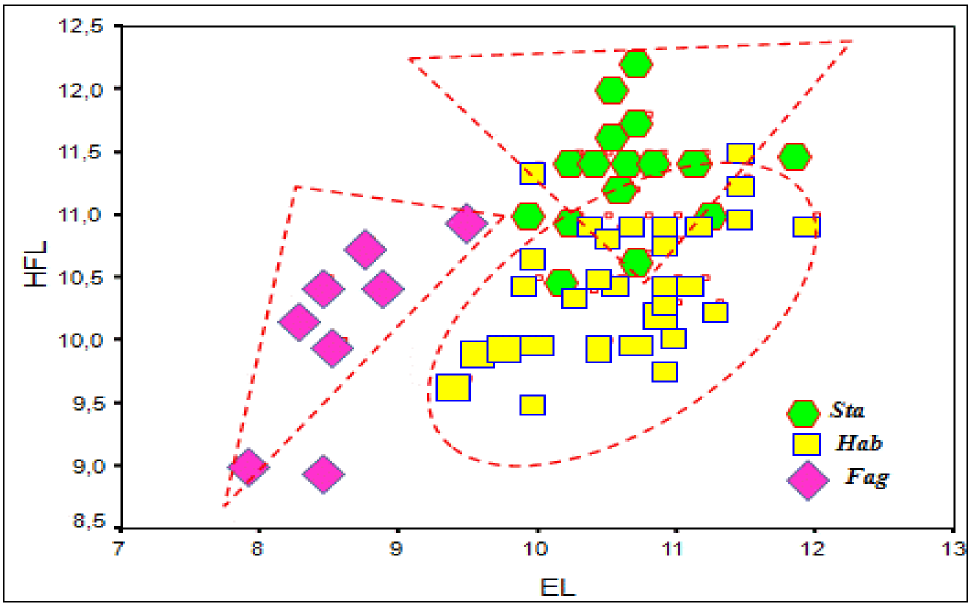

Figure 8. Scatter plots of Ear Length (EL) versus Hind Foot Length for *L. fagani* (Lfag), *L. habessinicus* (Lhab) and *L. starcki* (Lsta).

4.2. Non-metric Dental and Skull Characteristics

The logistic regression analysis for the cement filling ("present" vs. "absent") in the labial groove of the first upper incisors yielded significant species ($p < 0.005$) and age class (i.e. adult vs subadults) effects ($p < 0.005$), but no significant sex effect ($p > 0.005$). In particular *L. habessinicus* had more cement filling relative to *L. starcki* but there was no difference between *L. starcki* and *L. fagani*. When sex was not taken as a predictor variable, there was a significant effect ($p < 0.005$) of both species and age. Furthermore, *L. starcki* was significantly different from the other two taxa. Logistic regression analysis of osinterparietale ("presence" or "absence") based on sutural ossification level (i.e. class two, class three and class four) and age for *L. starcki* indicated no significant effect for both level of ossification and age. For *L. habessinicus,* the same analysis resulted in a significant effect ($p < 0.005$) of the level of ossification and no significant effect age effect. But we if left out the level of ossification there was a significant age effect for *L. habessinicus*.

4.3. Skull Morphometric Characteristics

All transformed metric skull variables were normally distributed according to the Kolmogorov-Smirnov test results, when accounted for multiple testing. The multiple-group PCA based on the variance-covariance matrix of the transformed metric variables resulted in the extraction of seven principal components (PCs) that conveyed altogether 78.07% character variation (PC1: 27.9%, PC2: 15.4% and PC3: 12.8%). Tests for correlations (overall sample set and separately for each species) between CBL and BL shows concordantly a highly significant positive correlation; indicating that these two skull size (length) variables can effectively be substituted by each other as skull length index. Nevertheless, there is a slightly negative sigignificant loading of BL into PC1, which indicates that PC1 is not a general component of skull size (length). Also, PC1 has various positive or negative loadings of variables that should otherwise show more or less strong positive loading into PC1, if this component was interpreted as general skull size (length) factor. Moreover, our variable transformations prior to PCA (by expressing variables as percent of CBL) have removed the CBL-skull length (size) effect. From the above arguments it follows that the correlations found between PC1, PC2, PC3 and PC6 scores and CBL (skull size) are indicating allometry, respectively. In general, there was no siginificant positive correlation between CBL and individual PC1. This is in line and confirms the above conclusion that PC1 was not a general skull size factor. However, we observed a slight tendency towards of a positive correlation between CBL and PC1 scores for *L. habessinicus* and *L. starcki*; corresponding to the somewhat higher level of positive correlation as observed for the overall morphometric data set across all species.

The multivariate morphometric analysis indicated that *L. europaeus* was distinct from the hare species from Africa. This is evident from PCA results, which separate the brown hare from the other hares included in the multivariate skull analysis (Appendix1 Fig. 2 A-B). The PCA results indicated that *L. starcki* was distinct from *L. europaeus* this was evident from the scatter plots PC1 vs. PC2 (Fig. 10E). *Lepus habessinicus* and *L. capensis* were different in shape as seen from PC2 box plot (Fig. 9B) and scatter plot (Fig. 10 A-B). The two species also shared some shape component to some extent (Fig. 9A).

For discriminate analysis a set of variables was selected based on the discriminating power and to minimize the degree of variance among groups. Box's M test was not significant ($P > 0.05$). Seven variables were used for DA between *L. starcki* and *L. europaeus* and four of them had discriminate loading > 0.3. The discriminate function had correctly classified 97.3 % and 91.8 % of *L. europaeus* and *L. starcki,* respectively. The scatter plot CBL vs. DF1 (Fig. 10 F) revealed multivariate skull differentiation between the two taxa. Six variables were used for DA between *L. habessinicus* and *L. capensis* from east Africa. The selection of *L. capensis* from east Africa was to reduce the amount of within group variance. Most variables had discriminated loading > 0.3 and 80.6 % and 85.5 % of *L. capensis* and *L. habessinicus,* respectively, were correctly classified. The distinction between the two taxa was also evident from the scatter plot of CBL vs. DF1 (Fig.10B).

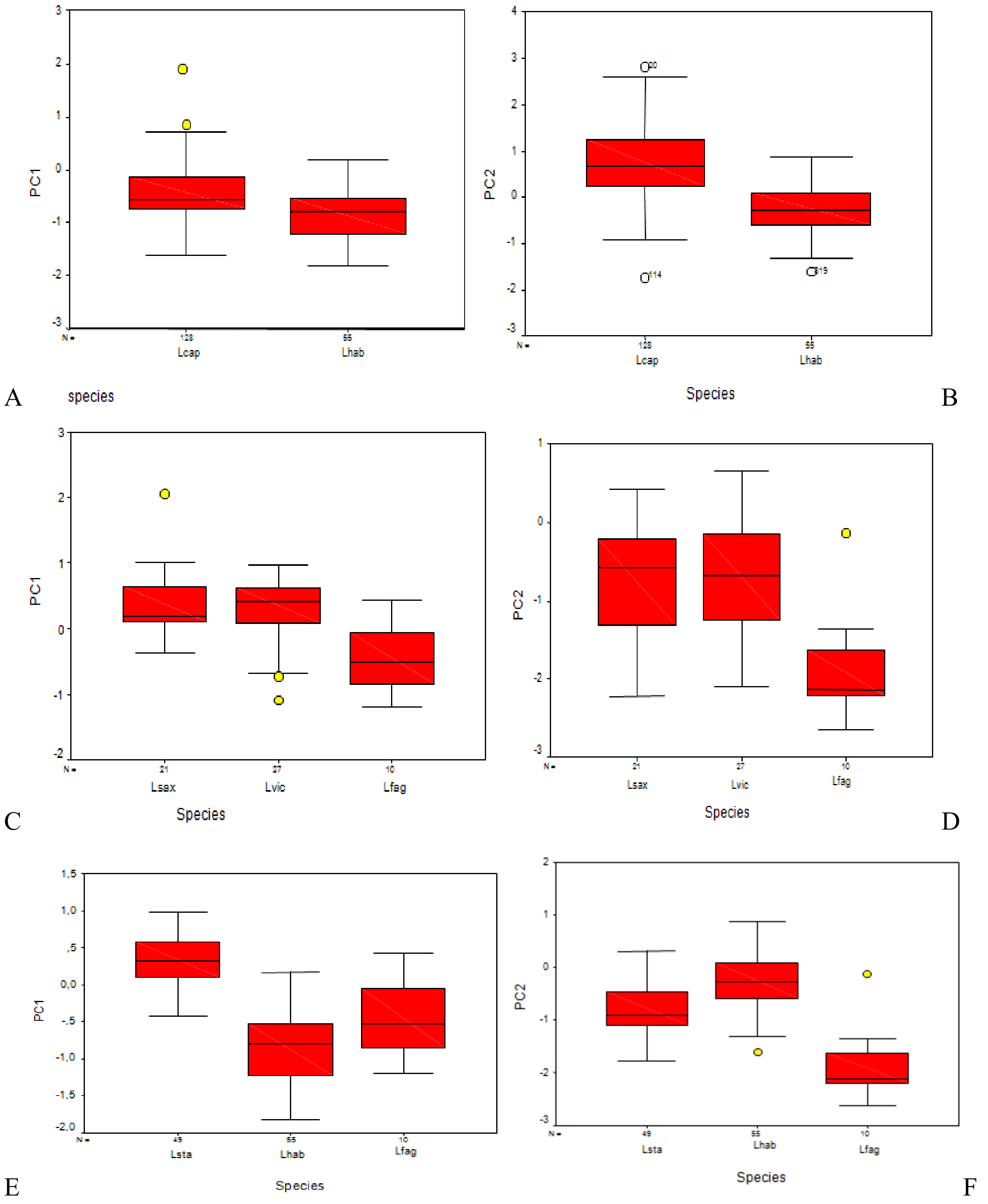

Figure 9. A-F Box plots of skull morphometric data for *L. capensis* (Lcap), *L. habessinicus* (Lhab), *L. saxatilis* (Lsax), *L. victoriae* (Lvic), *L. fagani* (Lfag) and *L. starcki* (Lsta): principal component 1 (PC1) and principal component 2 (PC2). Each box shows the median, quartiles and extreme values within species.

The multivariate as well as series of univariate (GLM; 3-way ANCOVA taking CBL as a covariate) analyses indicated that *L. fagani* is distinct from both *L. victoriae* and *L. saxatilis.* Five variables were selected with good discriminating power for the distinction between *L. fagani* and *L. victoriae*. Box's M test was not significant (P > 0.05). All the variables have discriminate loading > 0.3. The discriminate function had allocated correctly 81.1 % and 70.0 % of *L. victoriae* and *L. fagani*, respectively. *Lepus fagani* had distinct morpho shape spaces relative to both *L. saxatilis* and *L. victoriae*, but that of the latter two species was very much overlapping. The multivariate skull differentiation between *L. fagani* and both *L. saxatilis* and *L. victoriae* was evident from box plots (Fig. 9 C-D) and scatter plots (Fig. 10 C-D). In addition, *L. fagani* had a different skull shape relative to the taxa from Ethiopia (Fig. 9 E-F).

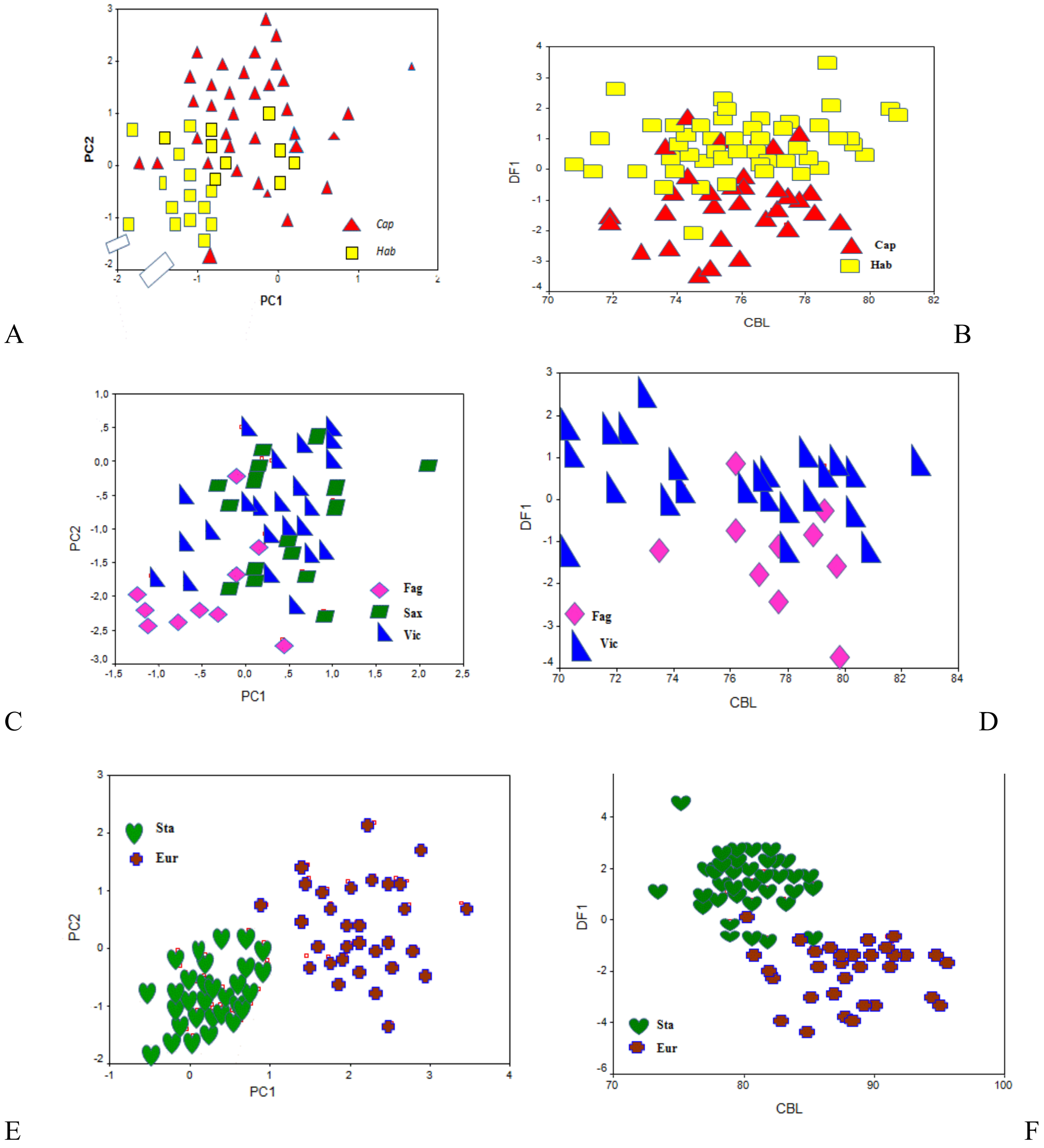

Figure 10. A-F Scatter plots of skull morphometric data for *L. capensis* (Cap), *L. habessinicus* (Hab), *L. fagani* (Fag), *L. saxatilis* (Sax), *L. victoriae* (Vic), *L. starcki* (Sta) and *L. europaeus* (Eur): principal component 1 (PC1), principal component 2 (PC2), discriminant function 1 (DF1) and condylobasal length (CBL).

4.4 Nuclear Microsatellite Analyses

Allelic variation at 13 microsatellite loci was determined for three species of *Lepus* collected from Ethiopia. Locus Lsa2 showed signs of genotypic disequilibrium with the loci Sol8 and Sat12 it was excluded from all subsequent analyses. Some loci showed signs of null alleles but only a few had null allele frequencies greater than 20 %. Thus, null alleles were not considered problematic for further analyses (Chapuis and Estoup, 2007; Carlsson, 2008). Errors due to stuttering were documented for some loci as evident from deficits of heterozygous genotypes. But no sign of large allele dropout was found.

Overall, 146 alleles were found and on average 12.17 alleles were recorded per locus. The minimum and maximum numbers of alleles were four and 22 for locus Lsa6 and Sol28, respectively. The lowest number of private allele was found in *L. starcki* (Table 6). The lowest (55) and highest (101) number of alleles were recorded for *L. starcki* and *L. habessinicus*, respectively. A general heterozygote deficiency was detected in all the taxa. Number of alleles per locus (A), allelic size range (R), expected heterozygosity (He), observed heterozygosity (Ho), locus-specific allelic richness (Rs; calculated based on minimum sample size of 13 diploid individuals), number of private alleles as well as the F_{IS} value and the associated deviation from Hardy Weinberg equilibrium (HWE) are presented in Table 6. The locus specific allelic richness did not show a significance difference among species (p = 0.367). The observed level of heterozygosity was relatively low compared to the expected heterozygosity in the three groups. In particular *L. starcki* had lower level of variability compared to the other species.

Table 6. Number of alleles per locus (A), allelic size range in bp (R), expected heterozygosity (He), observed heterrozygosity (Ho), locus specific allelic richness (Rs) and inbreeding coefficient (F_{IS}). The numbers of private allele for each species are given in parentheses. Significant deviation from HWE*

		Locus Name												
Species		Sol08	Sol28	Sol30	Sol33	N4	Sat2	Sat8	Sat12	Lsa1	Lsa3	Lsa6	Lsa8	F_{IS}
L. habessinicus (26)	A	9	14	14	5	5	11	12	7	7	7	3	7	0.229*
	R	124-140	151-189	151-197	213-221	200-208	223-249	95-119	102-134	164-176	192-218	166-170	182-194	
	He	0.798	0.827	0.888	0.538	0.084	0.765	0.779	0.681	0.702	0.804	0.097	0.667	
	Ho	0.69	0.655	0.776	0.339	0.086	0.569	0.509	0.603	0.593	0.414	0.102	0.61	
	Rs	7.133	8.351	10.181	4.500	2.072	6.932	8.123	5.847	5.308	6.219	1.939	5.266	
L. starcki(11)	A	3	3	4	5	6	7	7	4	2	6	3	5	0.154*
	R	116-132	143-169	171-195	213-225	208-218	225-243	95-121	102-126	164-174	198-216	164-168	186-194	
	He	0.544	0.49	0.241	0.49	0.722	0.45	0.592	0.481	0.286	0.702	0.269	0.657	
	Ho	0.654	0.36	0.154	0.462	0.654	0.346	0.48	0.385	0.269	0.44	0.154	0.769	
	Rs	2.882	2.954	2.975	3.882	5.633	5.367	5.330	3.382	1.999	4.815	2.744	4.612	
L. fagani (31)	A	14	14	13	6	3	8	5	8	4	7	2	8	0.229*
	R	102-136	157-193	155-187	207-221	202-206	221-253	93-111	102-134	160-166	184-208	164-168	178-196	
	He	0.885	0.883	0.892	0.779	0.373	0.861	0.524	0.793	0.679	0.794	0.095	0.663	
	Ho	1	0.727	0.682	0.727	0.273	0.462	0.286	0.773	0.773	0.286	0.1	0.5	
	Rs	11.722	11.415	11.233	5.582	2.938	8.000	4.189	7.281	3.591	6.429	1.883	6.820	

Analyses based on heterozygote excess did not suggest the occurrence of genetic bottlenecks in the recent population history of *L. habessinicus* and *L. starcki* in the three mutation models (i.e. IAM, TPM and SMM; $p > 0.05$). However, there was a sign of a recent bottleneck for *L. fagani* in one of the mutation models (i.e. IAM), as there was a significant excess of heterozygote ($p < 0.05$). Migration analysis revealed a clear sign of migration asymmetry between *L. fagani* and the other two taxa (Table 7). A migration analysis with nine loci (i.e. excluding loci with signs of null alleles, namely Sol33, Sat8 and Lsa3) yielded almost similar patterns of migration per generation as that of 12 loci (Table 7).

Table 7. Pairwise F_{ST}, G'_{ST} and Jost's D values (upper diagonal; the 1st , 2nd and 3rd rows, respectively). Pairwise CSE distance (lower diagonal; 1st row). The 2nd and 3rd rows (i.e. lower diagonal) are the number of migrants per generation (2nd row receiving species are in the row and 3rd row receiving species are in the clomn).

		12 Loci		9 Loci		
		Lsta	Lfag		Lsta	Lfag
Lhab		0.350	0.142		0.385	0.110
		0.814	0.384		0.844	0.276
		0.761	0.334		0.792	0.232
Lsta	0.200		0.332	0.215		0.355
	1.600		0.795	1.658		0.808
	1.500		0.745	1.526		0.756
Lfag	0.138	0.232		0.115	0.229	
	2.447	2.019		2.902	1.575	
	0.311	0.323		0.233	0.158	

The AMOVA model results revealed more than twice as much of the total genetic variation to occur already within populations (72.34%) as compared to the extent of relative genetic differentiation between populations (27.66 %) (Table 8). A similarly higher level of within population variation was also found when loci showing signs of null alleles were excluded (data not shown). A neighbor-joining tree of 107 individuals based on Cavalli-Sforza and Edwards's chord distances (CSE) clustered the individuls according to population of origin (Fig.11). In addition, a multilocus factorial correspondence analysis (FCA) partitioned the taxa into three respective clusters (Fig.12).

Table 8. Analysis of Molecular Variance (AMOVA)* results of among populations and within population variance components of the 12 microsatellite loci

Source of variation	df	SS	Variance components	% variance
Among population	2	181.708	1.372	27.66**
Within population	211	756.754	3.587	72.34**
Total	213	956.754	4.959	

*df = degree of freedom, SS = sum of square; **P < 0.05

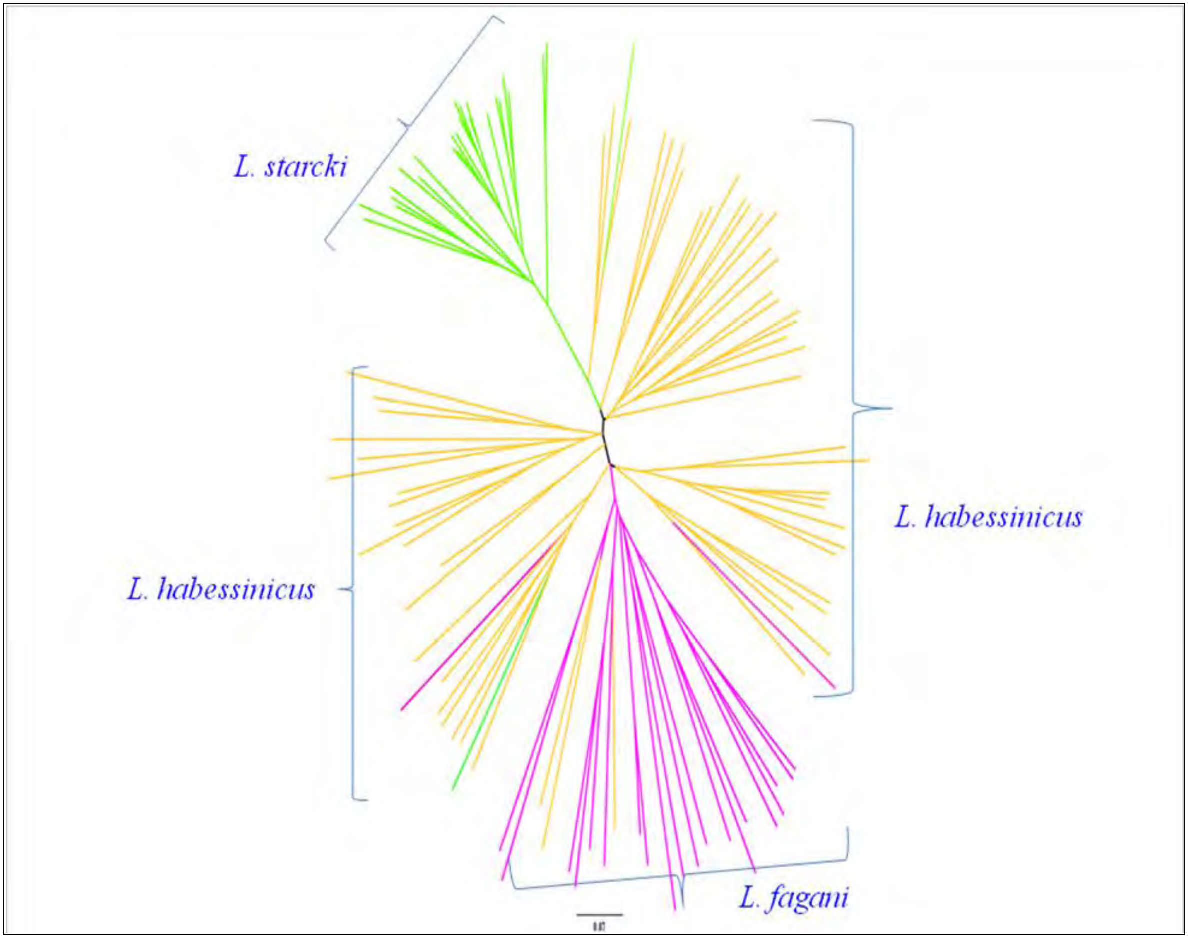

Figure 11. Neighbor-joining tree based on Cavalli-Sforza and Edwards Chord distances from 12 SSR loci genotype of 107 (i.e. *L. habessinicus* (59), *L. starcki* (26) and *L. fagani* (22)) from Ethiopia.

Various indices for population differentiation revealed a low genetic distance between *L. fagani* and *L. habessinicus* (Table 7). For instance, Cavalli-Sforza and Edwards's distance and Jost's D were highest between *L. fagani* and *L. starcki* (0.232 & 0.745) and lowest between *L. fagani* and *L. habessinicus* (0.138 and 0.334), respectively. However, the F_{ST} value was highest (0.350) between *L. starcki* and *L. habessinicus* and lowest (0.142) between *L. habessinicus* and *L. fagani*. The degree of population differentiation revealed by parwise F_{ST} was lower compared to that of both G'_{ST} and Jost's D (Table 7). All the pairwise distance estimates were significantly different from zero (P = 0.000). There was a significant ($p < 0.05$) negative correlation between the number allele per locus and the F_{ST} value of a respective locus, which supported the hypothesis of homoplasy. In addition, a comparison of the highly polymorphic loci (number of alleles per locus > 10) and the less polymorphic loci (number alleles per locus ≤ 10) revealed that the latter group of loci resulted in a relatively high level of differentiation (i.e. F_{ST}) among taxa (data not shown).

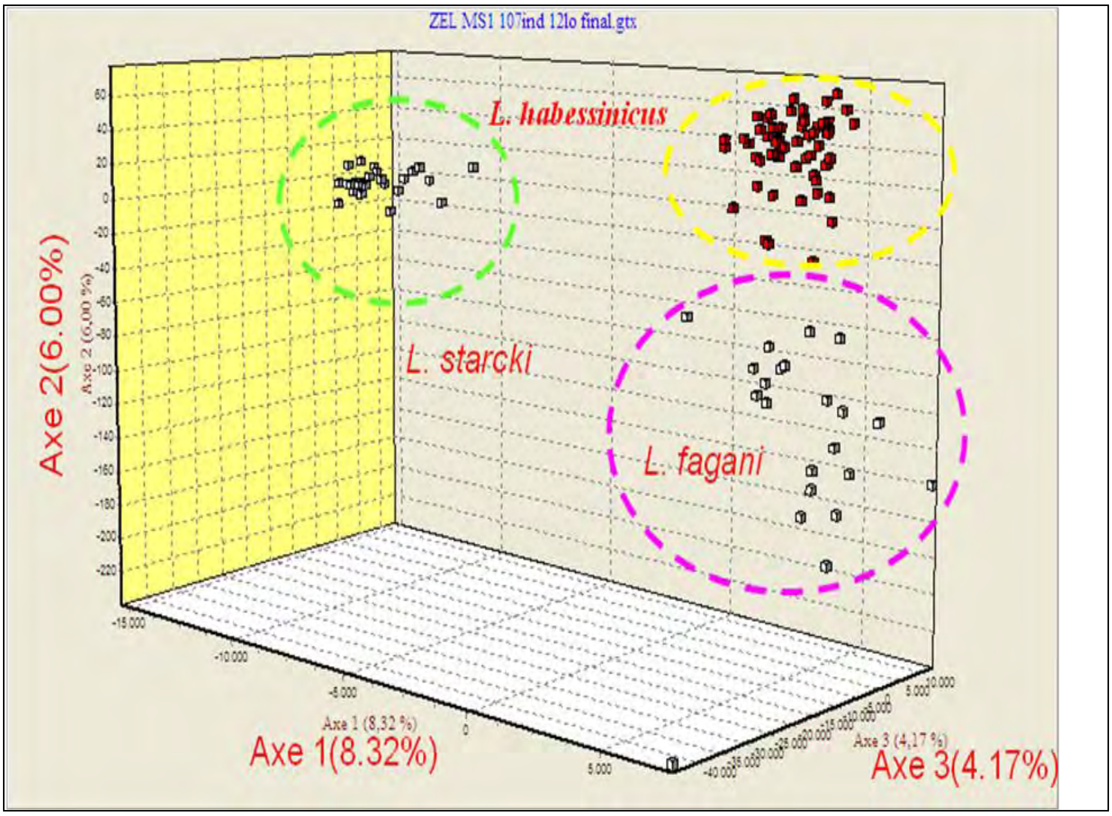

Figure 12. FCA plot 12 microsatellites of 107 individuals: *L. habessinicus* (59), L. *starcki* (26) and *L. fagani* (22) from Ethiopia.

A Bayesian multilocus test assigned all individuals to their respective population of origin with a high probability. However, two individuals of *L. habessinicus* were assigned to *L.*

fagani with a probability of 1.25 % and 6.60%, respectively. The structure analysis indicated that the best number of clusters is two (i.e. the *L. habessinicus / L. fagani* group versus *L. starcki*) (Fig.13).

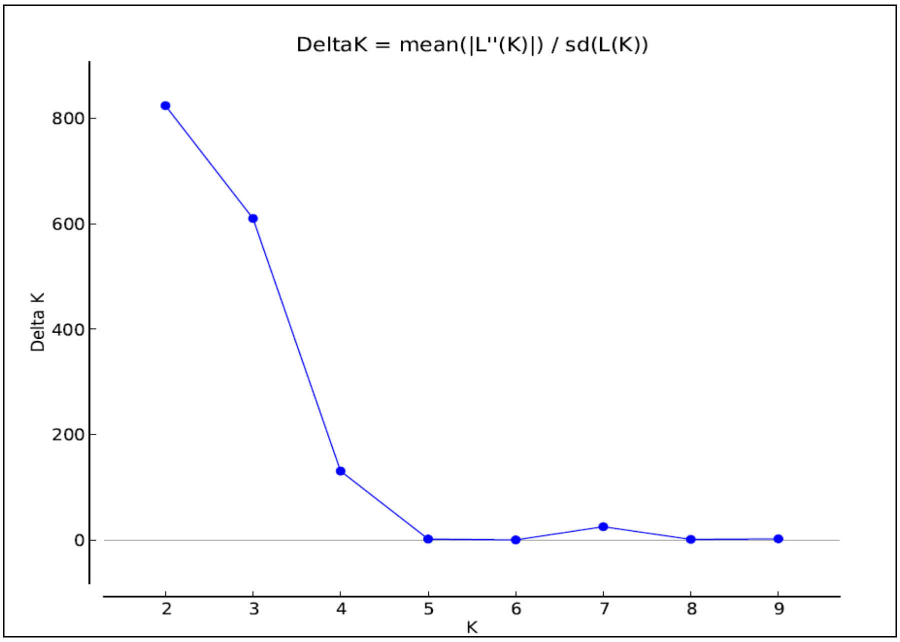

Figure 13. The best number of clusters estimated by Evano *et al.*(2005) method. Delta (Δ) K plot indicates the maximum peak at K = 2.

But we presented here other possible clusters together with one proposed by the Evano *et al.* (2005) method (Fig.14). By and large the results of our structure analyses reflect the clustering of individuals according to population of origin, with some evidence of SSR introgression between species and subgroups within species. At all levels of clustering *L. starcki* individuals formed a single phylogroup. In addition, one individual of *L. starcki* consistently showed a sign of SSR introgression from *L. habessinicus* (Fig. 14), again at all levels of clustering. At low levels of clustering (i.e. K= 2-4), we did not see any substructuring of *L. fagani* individuals. Nevertheless, *L. fagani* individuals started to substructure into three phylogeographic groups as K increases (Adolla, Kerkerty and western Ethiopia; Fig.14).

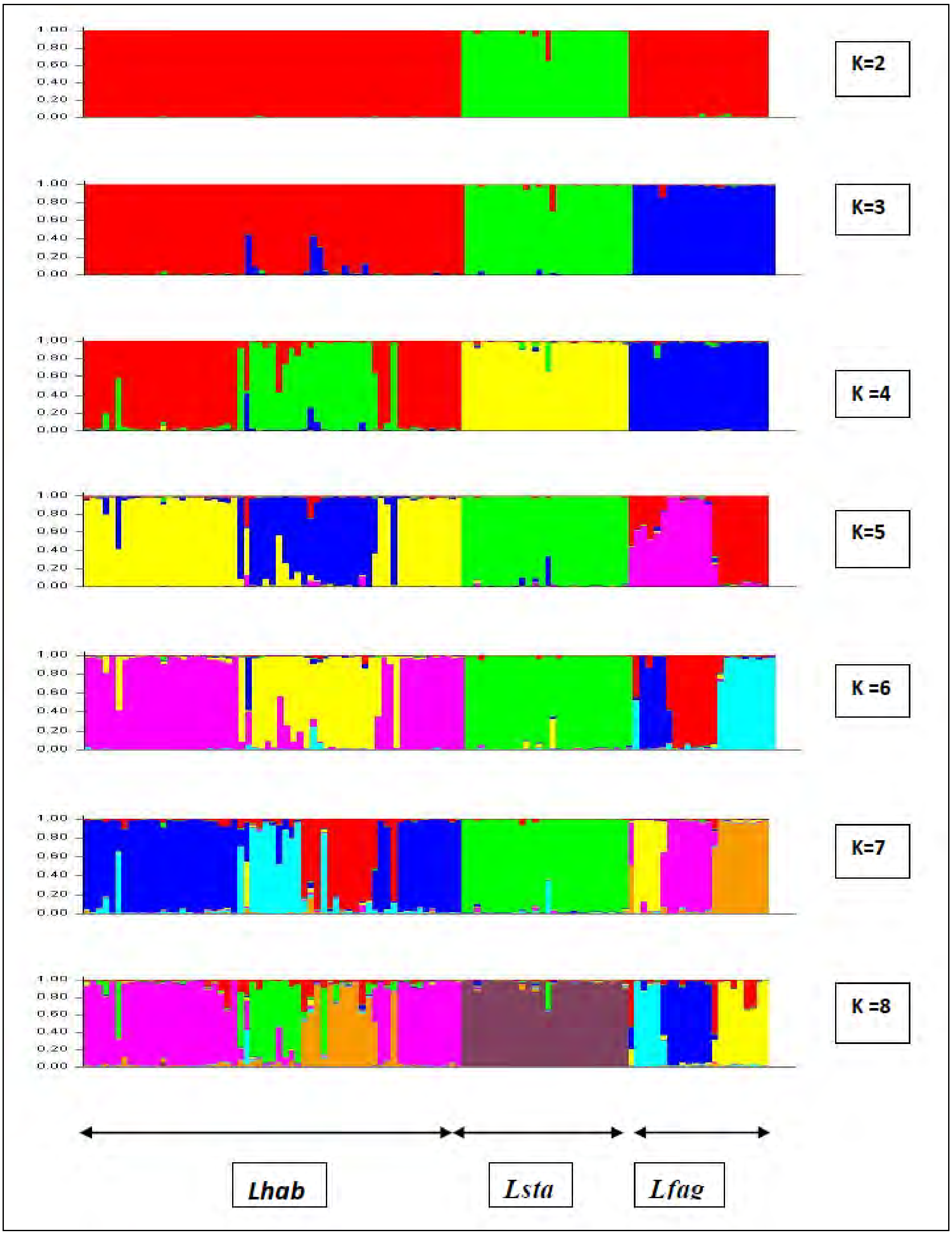

Figure 14. Bar plot of the structure analysis for K= 2-8 for 107 individuals (i.e. *L. habessinicus* (59), L. *starcki* (26) and *L. fagani* (22)) from Ethiopia.

The subgroups showed some level of SSR introgression among each other. However, *L. habessinicus* individuals started to substructure from K ≥ 4 into different phylogeographic groups. For instance, at K = 7, *L. habessinicus* was clustered into the following phylogeographic groups: Abijata Shalla Lakes National Park/ Ziway/ Wonji/ Humbo/ Bishoftu/ Andido in Afar/ Wellenchiti, Miesso/ Shoa Robit, and Fedis/ Kemise. The different substructures of *L. habessinicus* demonstrated a different level of SSR introgression among each other (Fig.14). According to the structure results, the microsatellite introgression patterns between *L. fagani* and *L. habessinicus* appear to be bidirectional (Fig.14). Few individuals of *L. habessinicus* have about 10-30% introgression from *L. fagani*; however, introgression in the reverse direction was not higher than 10%.

4.5. MtDNA (ATPase sub-unit 6) Sequences

A 416 bp fragment of ATPase 6 was sequenced. Numbers of haplotypes, numbers of variable sites, singletons, phylogenetically informative sites and other basic statistics are summarized in Table 9.

Table 9. Locus name, fragment size (FS), variable sites (VS), phylogenetically informative sites (PIS), singletons (Sig), number of haplotypes (NHP), haplotype diversity (Hd), nucleotide diversity (π), average number nucleotide differences (k), the best fit model (BfM) based on lowest BIC value.

Locus	FS	VS	PIS	Sig	NHP	Hd	π	k	BfM
ATP6	416 bp	125	108	17	64	0.952	0.054	22.36	HKY+G
TF	427 bp	110	77	33	92	0.942	0.02	8.15	K2+G
TG	464 bp	110	74	36	127	0.979	0.015	6.49	K2+G
UCP2	377 bp	61	53	8	66	0.955	0.015	4.51	K2+G
UCP4	471 bp	32	20	12	18	0.541	0.003	1.61	T92
TSHB	279 bp	38	28	10	38	0.883	0.014	4.04	T92 +G

All phylogenetic reconstruction methods produced an almost concordant tree topology (ML, Fig. 16 and Appendix1 Figs. 3 and 9). The average standard deviation of the split frequencies of the BI was 0.004 when run added. The lowest pairwise distance was between *L. europaeus* and *L. saxatilis* but the largest was between *L. capensis* and *L. timidus* (Table 10).

Table 10. Between groups mean distances of ATPase subunit 6 (mtDNA) computed as a p-distances.

		1	2	3	4	5	6	7
1	Lcap							
2	Lcapn	0.098						
3	Leur	0.100	0.090					
4	Lfag	0.106	0.098	0.024				
5	Lhab	0.106	0.098	0.032	0.030			
6	Lsax	0.099	0.091	0.018	0.026	0.032		
7	Lsta	0.113	0.101	0.042	0.036	0.029	0.036	
8	Ltim	0.140	0.125	0.115	0.119	0.122	0.119	0.131

Lcap = *L. capensis* (RSA), Lcapn = *L. capensis* (North Africa), Leur = *L. europaeus*, Lfag = *L. fagani*, Lhab = *L. habessinicus*, Lsax = L. *saxatilis*, Lsta = *L. starcki* and Ltim = *L. timidus*.

Two phylogroups were detected for *L. habessinicus* in the mtDNA dataset in which phylogroup one (Figs. 15-16) consisted individuals from central and eastern Ethiopia: Humbo/Wolaita, Welenchiti, Andido (Afar), Shoa Robit, Kemise, Mieso, Fedis and Ginnir and the second phylogroup (Figs. 15-16) consisted individuals from south western Ethiopia and Rift Valley areas (Hamer, Gewade, Erbore, Abijata Shalla LNP, Ziway and Wonji). In the second phylogroup also individuals from other taxa were found; for example *L. fagani*, *L. starcki* and *L. saxatilis*. Although the individuals of *L. habessinicus* form two major subgroups, some individuals (e.g. from Humbo/Wolaita and Bishoftu/Debrezeit) of this taxon were clustered within the *L. starcki* phylogroup (i.e. phylogroup six) (Fig.15).

All *L. starcki* individuals clustered into a single group (i.e phylogroup six) except for individual collected from Agena (Gurage Zone; Southern Nations, Nationalities and People's Regional State/SNNPRS) which clustered in the phylogroup two of *L. habessinicus*. Most individuals of *L. fagani* were clustered in phylogroup two of *L. habessinicus*. Furthermore, two *L. fagani* individuals clustered in the *L. starcki* group (phylogroup six) (Fig.15). Similar to *L. fagani*, the scrub hare (*L. saxatilis*) did not form a distinct group and was scattered over different phylogroups. *Lepus* species from Ethiopia were not monophyletic in all phylogenetic reconstruction methods.

In the mtDNA phylogeny, the Cape hare (*L. capensis*; sensu lato) from North Africa and South Africa formed a single monophyletic group (Fig.16) with two minor subclades: phylogroup three (Fig.15) from North Africa (Tunisia and Egypt) and phylogroup four (Fig.15) from South Africa (RSA). However, one individual of *L. capensis* from North Africa clustered within the Central Europe group of *L. europaeus* (phylogroup five) (Fig.15). Brown hares are also clustered into two groups, namely the Anatolian and the Central Europe group (Fig.15; phylogroup five).

In this study, signs of mtDNA introgression of *L. starcki* into *L. habessinius*, *L. fagani* and *L. saxatilis* were detected (Fig.15). Furthermore, introgression of *L. habessinicus* into *L. fagani* and vice versa was also observed (Fig.15). No introgression of mtDNA from *L. capensis* into *L. habessinicus* was recorded. Similarly, no introgression of mtDNA was found between *L. europaeus* and *L. starcki*.

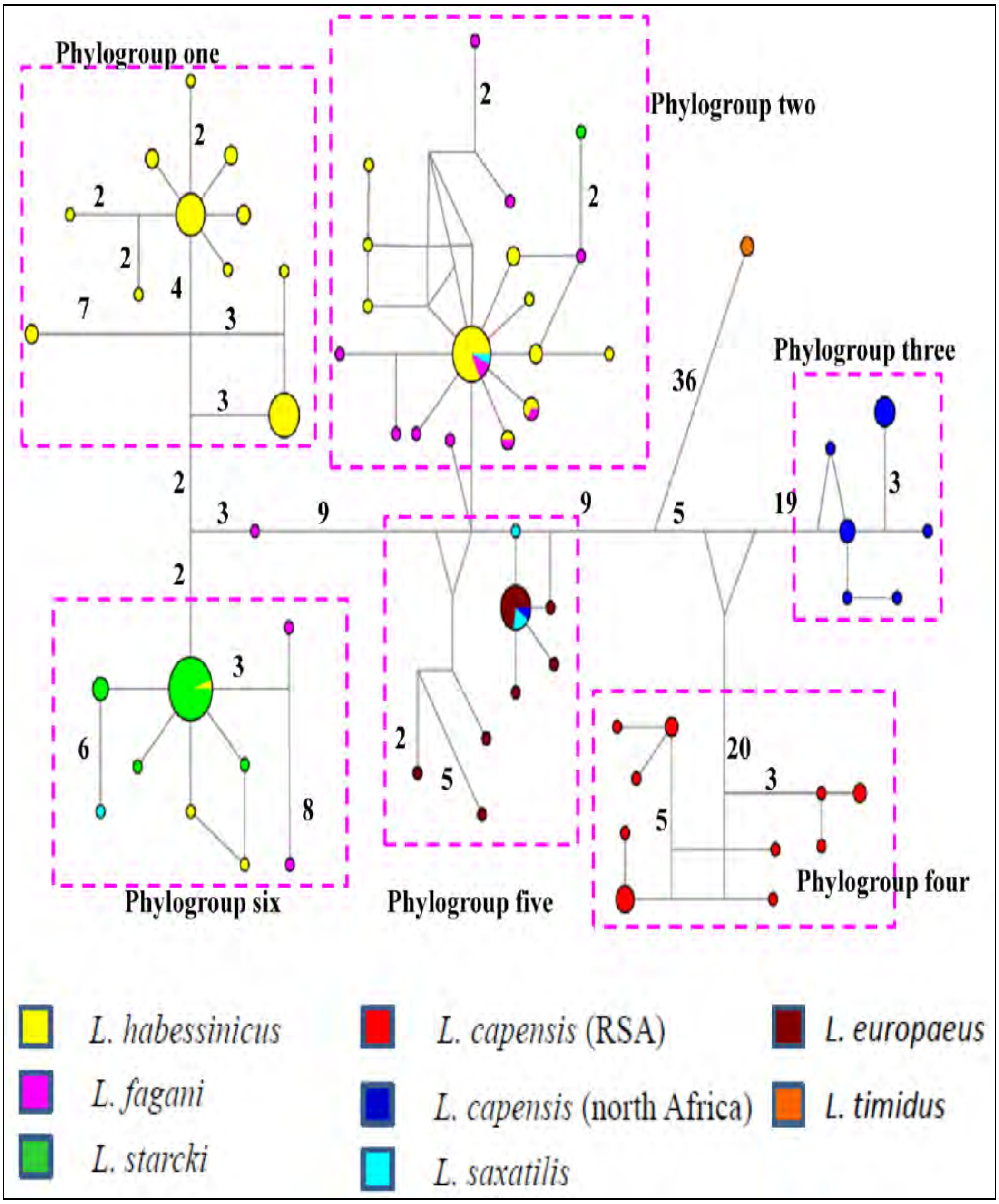

Figure 15. MtDNA median joining network. The size of the circles is proportional to the frequency of haplotypes. Numbers on the edges indicate the number of mutation steps if greater than one.

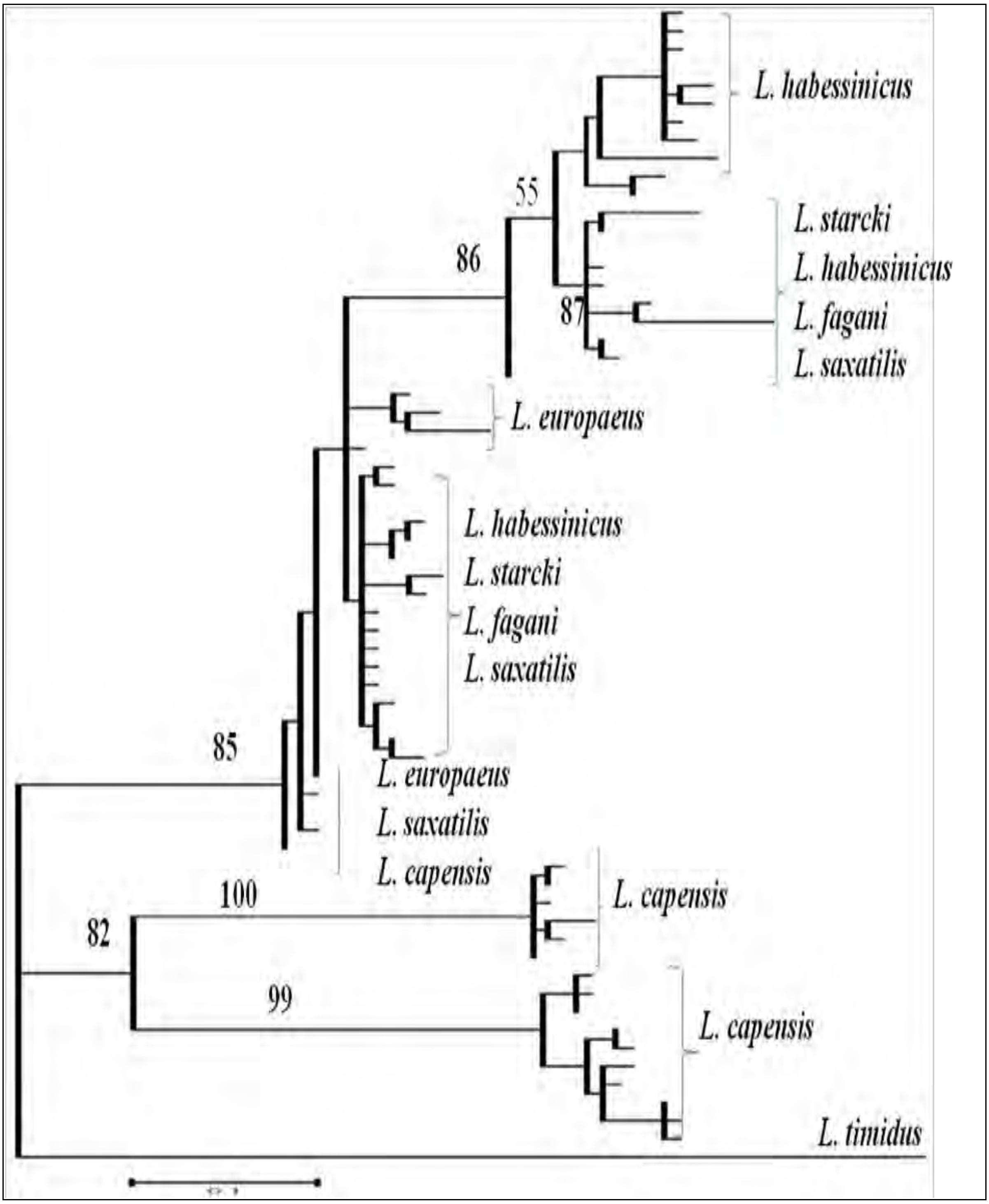

Figure 16. ATPase subunit 6 (ATP6) Maximum Likelihood (ML) tree. Numbers above the branch indicate bootstrap values greater than 50%.

4.6. Transferrin (TF) Sequences

A 427 bp fragment of transferrin was sequenced. Numbers of haploypes, variable sites, phylogenetically informative sites, singletons, haplotype diversity (hd), nucleotide diversity (π) and average number of nucleotide difference (k) are summarized in Table 9. The pairwise genetic differentiation (p-distances) among different groups was relatively low (Table 11). A unique InDeL (i.e. insertion/deletion) was found in four *L. starcki* individuals and also in individuals of other species (e.g. *L. towensendii*, *L. americanus*, *L. othus*, *L. arcticus*, *L. corsicanus*, *L. europaeus*, *L. comus*, *L. timidus*, *L. granatensis, L. castroviejoi, Oryctolagus cuniculus and Sylvilagus floridanus).* Except the four *L. starcki* individuals, all hares from Africa had a deletion at this site. In addition, all the taxa with this InDel either had an Adenine (A) or a Guanine (G) insertion at that site. All phylogenetic reconstruction methods produced almost concordant tree topologies (ML; Fig.18 and Appendix1 Figs. 4 and 10). The average standard deviation of the split frequencies was 0.007 for the BI when run added. Once again hare species in Ethiopia were not monophyletic in all phylogenetic reconstruction methods.

Three major phylogroups were found for the in group taxa (Figs.17-18). The groups by and large reflected the major geographic origin of the species. Phylogroup one (Fig. 17; Group one) contained species mainly from Ethiopia except *L. saxatilis* from RSA. Phylogroup two contained mainly taxa of African origin such as *L. capensis* (North and South Africa), *L. saxatilis*, *L. habessinicus*, *L. starcki* and *L. fagani.* But in the same group were also species of Eurasian origin (*L. timidus*, *L. yarkandensis*, *L. mandshricus, L. hainanus* and *L. sinensis)* and from North America (*L. californicus*).

Table 11. Between groups mean distances of Transferrin (TF)* sequences computed as a p-distance

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	Cap1																						
2	Cap2	0.013																					
3	Eur	0.035	0.031																				
4	Fag	0.014	0.012	0.032																			
5	Hab	0.012	0.009	0.031	0.005																		
6	Sax	0.012	0.010	0.031	0.003	0.003																	
7	Sta	0.013	0.010	0.025	0.010	0.008	0.008																
8	Tim	0.033	0.030	0.009	0.031	0.029	0.030	0.024															
9	Man	0.014	0.010	0.031	0.012	0.010	0.010	0.010	0.031														
10	Tow	0.030	0.026	0.004	0.028	0.026	0.026	0.020	0.005	0.028													
11	Cal	0.025	0.022	0.027	0.024	0.021	0.022	0.019	0.027	0.022	0.024												
12	Ame	0.030	0.027	0.013	0.028	0.026	0.027	0.022	0.014	0.029	0.009	0.024											
13	Oth	0.034	0.030	0.008	0.032	0.030	0.030	0.024	0.007	0.032	0.004	0.028	0.013										
14	Arc	0.028	0.024	0.013	0.026	0.024	0.024	0.020	0.011	0.026	0.009	0.025	0.016	0.012									
15	Cap3	0.012	0.008	0.030	0.010	0.008	0.008	0.008	0.030	0.009	0.027	0.020	0.028	0.031	0.024								
16	Cor	0.034	0.031	0.011	0.032	0.031	0.031	0.025	0.012	0.033	0.007	0.028	0.016	0.011	0.016	0.032							
17	Gra	0.032	0.032	0.012	0.033	0.031	0.032	0.026	0.013	0.033	0.008	0.029	0.017	0.012	0.016	0.032	0.011						
18	Oio	0.023	0.019	0.021	0.021	0.019	0.019	0.016	0.022	0.020	0.017	0.026	0.020	0.022	0.021	0.019	0.022	0.023					
19	Com	0.028	0.025	0.015	0.026	0.024	0.024	0.020	0.016	0.025	0.011	0.027	0.017	0.015	0.017	0.024	0.015	0.018	0.018				
20	Yar	0.013	0.011	0.025	0.013	0.010	0.011	0.010	0.025	0.010	0.022	0.021	0.023	0.026	0.022	0.010	0.027	0.026	0.018	0.022			
21	Hia	0.013	0.010	0.022	0.012	0.009	0.010	0.008	0.022	0.010	0.019	0.012	0.020	0.023	0.019	0.008	0.024	0.025	0.014	0.018	0.011		
22	Sin	0.013	0.010	0.031	0.012	0.009	0.010	0.010	0.031	0.010	0.028	0.021	0.029	0.033	0.026	0.007	0.033	0.034	0.021	0.025	0.011	0.009	
23	Cas	0.034	0.031	0.011	0.032	0.030	0.031	0.025	0.012	0.032	0.007	0.028	0.016	0.011	0.016	0.032	0.000	0.010	0.022	0.015	0.027	0.024	0.033

**L. capensis* (Cap1=RSA, Cap2= North Africa, Cap3= China*),* *L. europaeus* (Eur), *L. fagani* (Fag), *L. habessinicus* (Hab), *L. saxatilis*(Sax), *L. starcki* (Sta), *L. timidus* (Tim), *L. mandshricus*(Man), *L. townsendii* (Tow), *L. californicus* (Cal), *L. americanus* (Ame), *L. othus* (Oth), *L. arctincus* (Arc), *L. corsicanus* (Cor), *L. granatensis* (Gra), *L. oiostolus* (Oio), *L.comus* (Com), *L. yarkandensis* (Yar), *L. hainanus* (Hai), *L. sinensis* (Sin), *L.castroviejoi* (Cas).

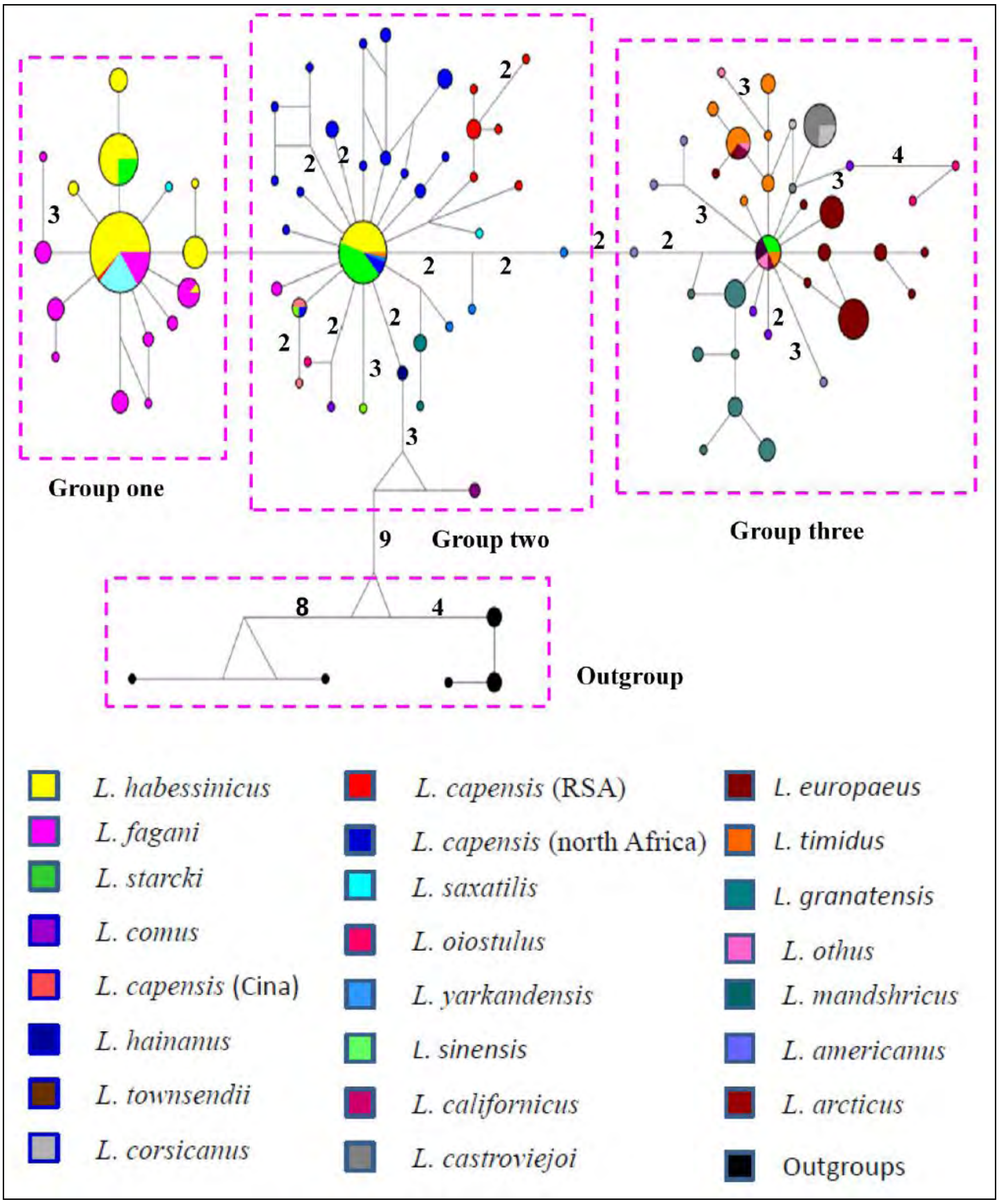

Figure 17. Transferrin (TF) median joining network. The size of the circle is proportional to the frequency of haplotypes. Numbers on the edges indicate the number of mutation steps greater than one.

But phylogroup three (Figs.17-18) consisted mainly of taxa of Eurasian and North American origin (*L. europaeus*, *L. timidus*, *L. garanatensis*, *L. castroviejoi, L. corsicanus*, *L. comus*, *L. oiostolus*, *L. americanus* and *L. othus*) except the four *L. starcki* individuals clustered at the core of the group. The Chinese species (*L. oiostolus* and *L. comus*) showed a close association. Furthermore, *Lepus othus* clustered in the *L. timidus* group.

Evidences of nuclear introgressive hybridization (i.e. ancient and or recent) among hare species were also documented. For example, a sign of recent introgression was documented among some species (e.g. between *L. habessinicus* and *L. starcki*, *L. fagani* and *L. habessinicus, L. corsicanus* and *L. castroviejoi*). Ancient introgressive hybridization was found between *L. habessinicus* and *L. saxatilis*, *L. starcki* and *L. saxatilis*, *L. fagani* and *L. saxatilis*, *L. capensis* and *L. habessinicus*. In addition, the nuclear introgressions could be both unidirectional and bidirectional. Furthermore, both the networks and trees indicated that the ancestral transferrin sequences could be from North America and or China.

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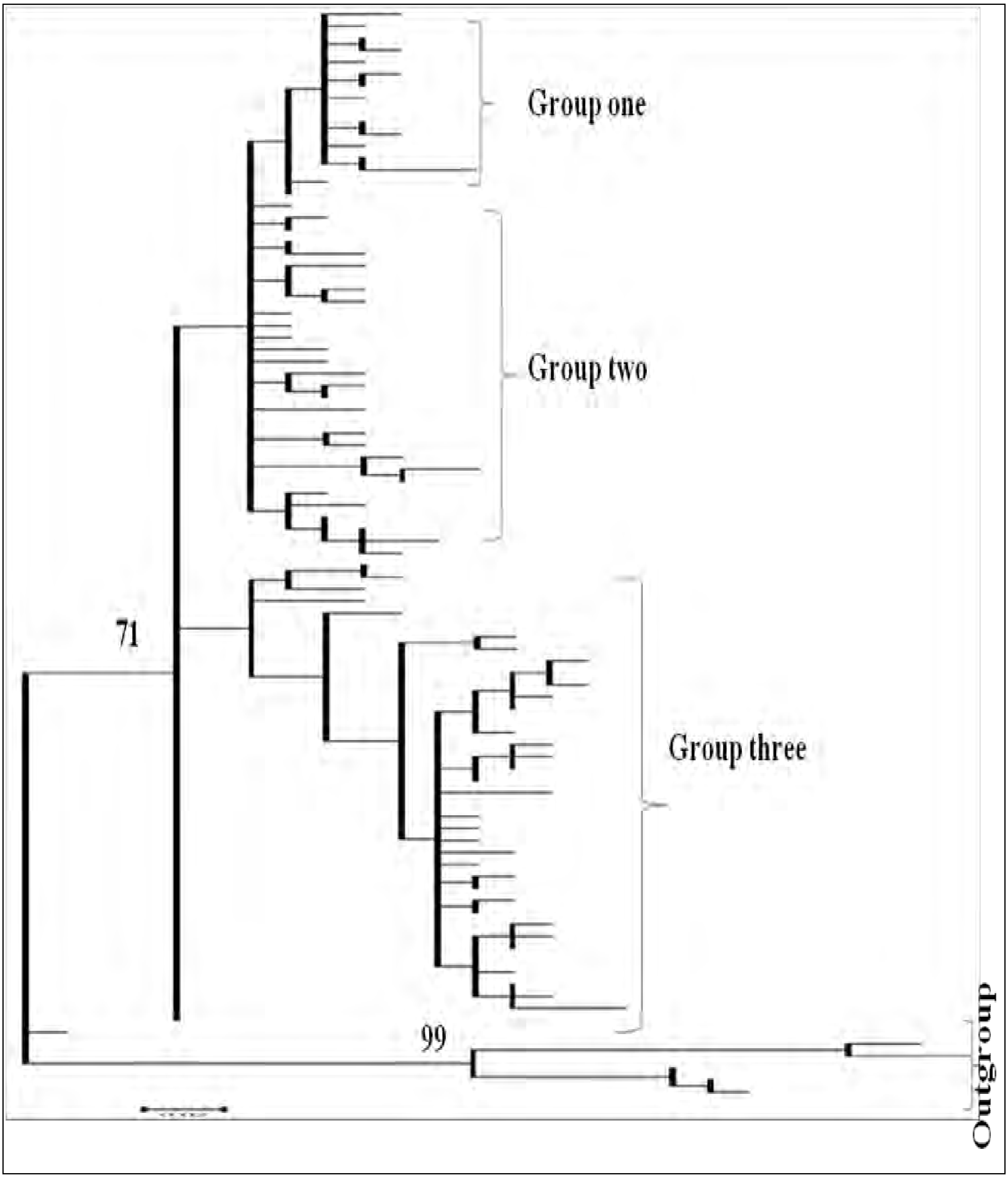

Figure 18. Transferrin (TF) Maximum Likelihood (ML) tree. Numbers above the branch indicate bootstrap values greater than 50%.

4.7. Thyroglobulin (TG) Sequences

A 464 bp fragment was sequenced in which at 17 sites there were gaps in some individuals. The gaps were excluded in all subsequent analyses. Numbers of haplotypes, the variable sites, phylogenetically informative sites, singletons, haplotype diversity (hd), nucleotide diversity (π) and average number of nucleotide difference (k) are summarized in Table 9. The pairwise genetic distances were relatively low (Table 12). All tree reconstruction methods resulted in concordant tree topologies (ML, Fig.20 and Appendix1 Figs. 5 and 11). The average standard deviation of the split frequencies was 0.011 for the BI when the runs were added. All hare species included in the TG sequence analyses were not monophyletic groups.

Two major phylogroups were detected: phylogroup one (Figs.19-20; African phylogroup) was mainly composed of species from Africa whereas the second (Figs.19-20; Chinese phylogroup) mainly contained species from China. In the African phylogroup were species such as: *L. capensis* (North Africa and RSA), *L. habessinicus*, *L. fagani*, *L. starcki* and *L. saxatilis*. Furthermore, in this group were also species from Eurasia (*L. europaeus*, *L. comus*, *L. oiostolus*, *L. sinensis, L. capensis* (China) and *L. yarkandensis*) and from North America (*L. townsendii)*. The Chinese phylogroup contained mainly species of Chinese origin (*L. timidus*, *L. capensis, L. mandshricus*, *L. yarkandensis*, *L. sinensis*, *and L. hainanus*). In addition, the Chinese phylogroup contained species such as: *L. saxatilis*, *L. fagani*, *L. habessinicus* and *L. europaeus.*

Table 12. Between groups mean distances of Thyrogrlobulin (TG)* sequences computed as a p-distance.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	Cap1																	
2	Cap2	0.008																
3	Eur	0.012	0.010															
4	Fag	0.011	0.009	0.013														
5	Hab	0.008	0.005	0.011	0.009													
6	Sax	0.027	0.025	0.027	0.021	0.025												
7	Sta	0.009	0.007	0.011	0.008	0.007	0.022											
8	Tim	0.020	0.018	0.020	0.015	0.018	0.013	0.016										
9	Yar	0.018	0.016	0.018	0.013	0.015	0.014	0.013	0.009									
10	Man	0.024	0.022	0.023	0.016	0.021	0.016	0.019	0.012	0.011								
11	Hai	0.015	0.013	0.016	0.012	0.013	0.019	0.012	0.014	0.012	0.016							
12	Oio	0.011	0.008	0.013	0.009	0.009	0.024	0.006	0.018	0.015	0.021	0.014						
13	Com	0.010	0.008	0.013	0.010	0.009	0.025	0.008	0.019	0.017	0.023	0.013	0.008					
14	Sin	0.019	0.017	0.020	0.015	0.016	0.018	0.016	0.014	0.013	0.017	0.016	0.017	0.019				
15	Cap3	0.021	0.019	0.021	0.016	0.018	0.017	0.017	0.012	0.010	0.014	0.015	0.018	0.021	0.013			
16	Ame	0.016	0.013	0.017	0.011	0.013	0.016	0.010	0.011	0.009	0.013	0.012	0.012	0.015	0.013	0.011		
17	Cal	0.010	0.009	0.010	0.012	0.010	0.029	0.010	0.022	0.020	0.026	0.017	0.012	0.012	0.020	0.023	0.017	
18	Tow	0.007	0.007	0.012	0.010	0.008	0.027	0.008	0.020	0.018	0.024	0.015	0.010	0.010	0.018	0.021	0.015	0.009

**L. capensis* (Cap;1 = RSA, 2 = North Africa, 3 = China*), L. europaeus* (Eur), *L. fagani* (Fag), *L. habessinicus* (Hab), *L. saxatilis*(Sax), *L. starcki* (Sta), *L. timidus* (Tim), *L. yarkandensis* (Yark), *L. mandshricus*(Man), *L. hainanus* (Hai), *L. oiostolus* (Oio), *L.comus* (Com), *L.sinensis*(Sin), *L.americanus*(Ame), *L.californicus*(Cal), *L.townsendii*(Tow).

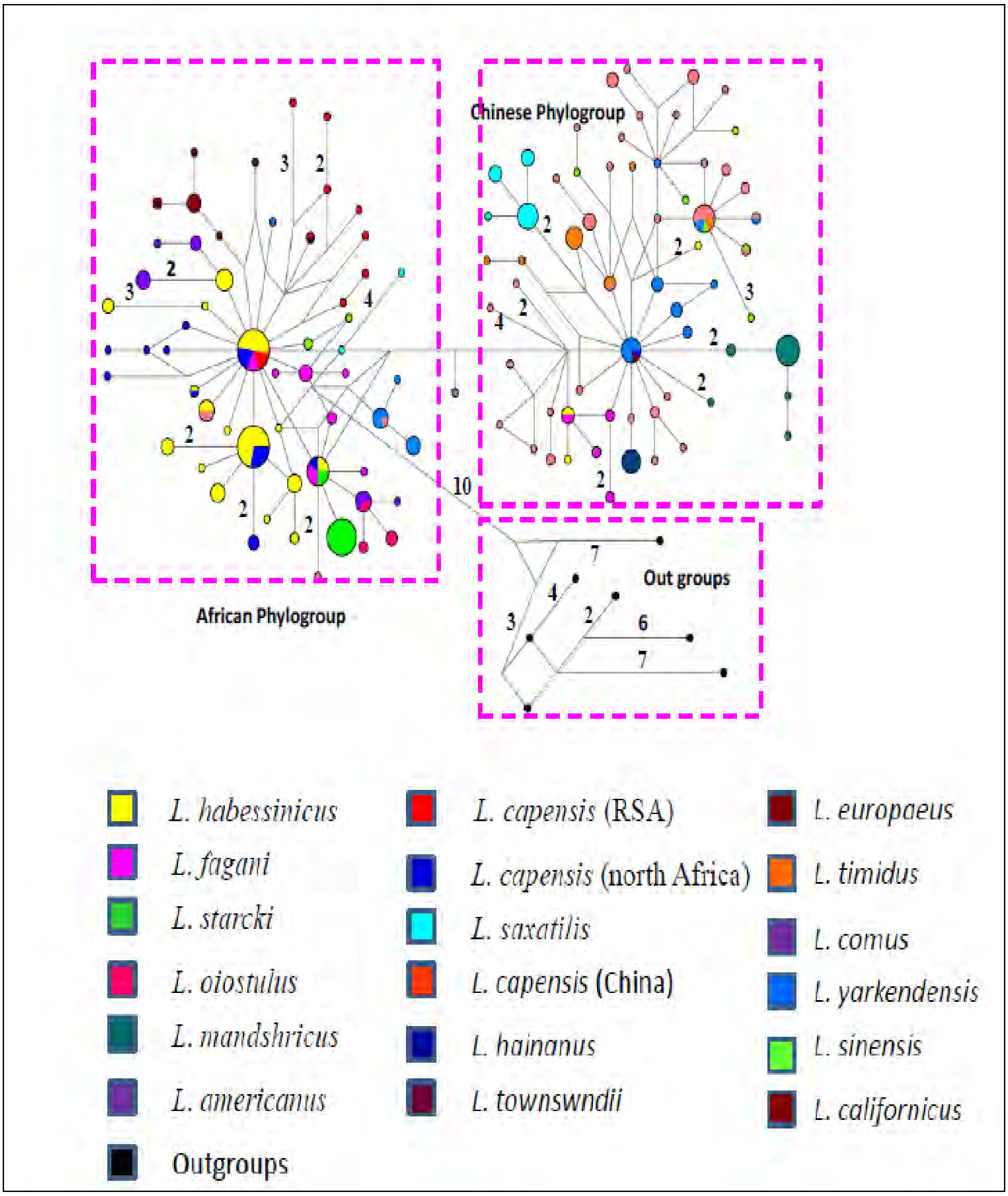

Figure 19. Thyroglobulin (TG) median joining network. The size of the circles is proportional to the frequency of haplotypes. Numbers on the edges indicate the number of mutation steps greater than one.

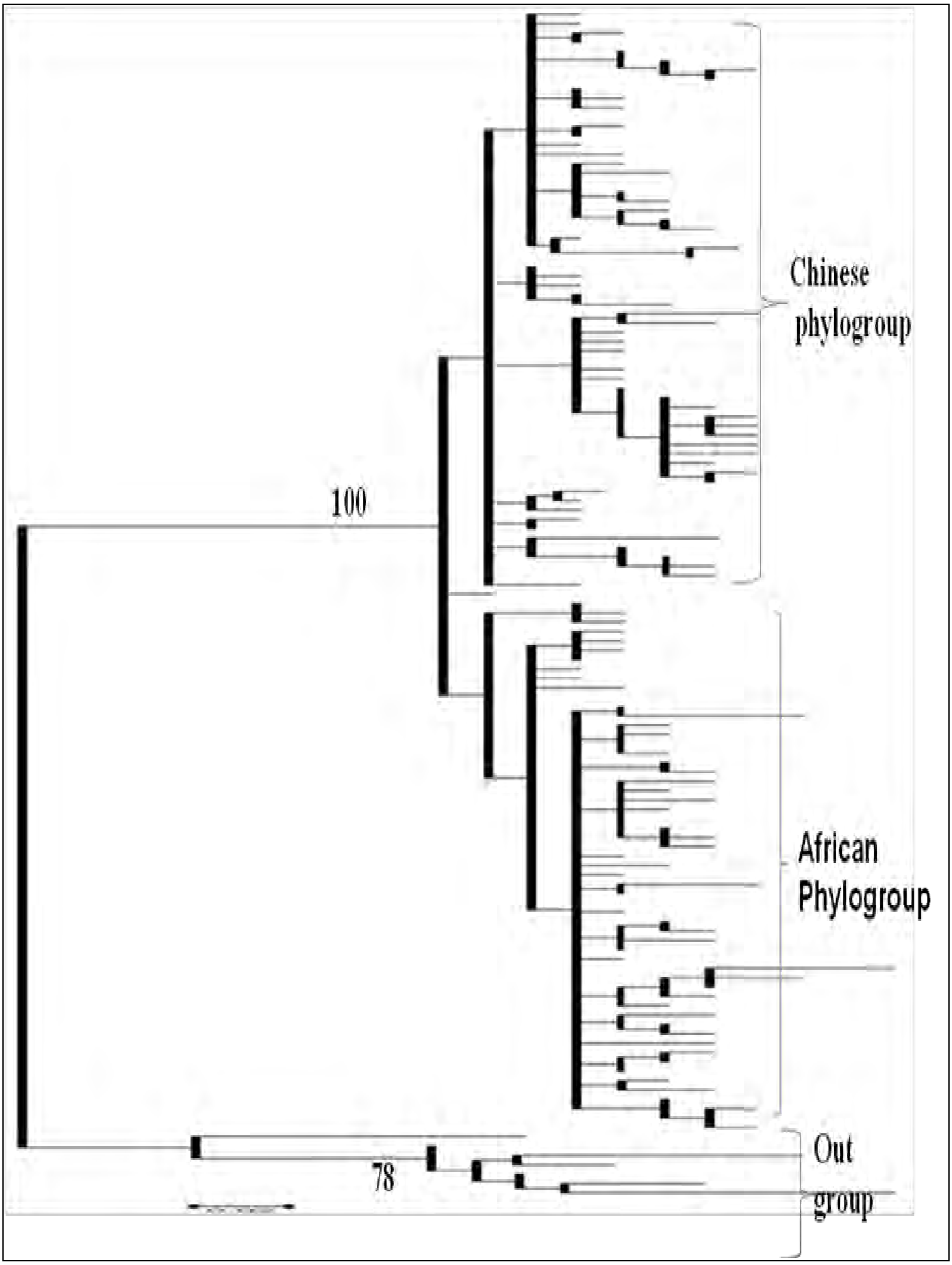

Figure 20. Thyroglobulin (TG) Maximum Likelihood (ML) tree. Numbers above the branch indicate bootstrap values greater than 50%.

Lepus capensis from Africa and China were distinct from one another forming separate phylogroups. In addition, Cape hares from Africa were more attached to *L. habessinicus* from east Africa than to *L. capensis* from China (Fig. 19). The Ethiopian highland hare and *L. europaeus* were distinct in the TG dataset as was evident from the median joining network and the different tree reconstruction methods (Figs.19-20). *Lepus fagani* did not form a distinct phylogroup; however, it did have a TG signal different from *L. saxatilis*.

Evidences of recent and or ancient introgressive hybridization were detected among species. For instance, a sign of recent introgressive hybridization was found between *L. habessinicus* and *L. fagani*, *L. habessinicus* and *L. starcki*, *L. fagani* and *L. starcki*, *L. capensis* (China) and *L. sinensis*, *L. comus* and *L. oiostolus*, *L. yarkandensis* and *L. capensis* (China). Signs of ancient introgressive hybridization were found among some species pairs: *L. capensis* (North Africa) and *L. habessinicus*, *L. capensis* (China) and *L. habessinicus*, *L. capensis* (RSA) and *L. habessinicus*, *L. capensis* (RSA) and *L. fagani* (Fig. 19).

4.8. Uncoupling Protein Two (UCP2) Sequences

Fragments of 377 bp were sequenced and at 72 sites there were gaps, which were excluded from all subsequent analyses. Numbers of haploypes, variable sites, phylogenetically informative sites, singletons, haplotype diversity (hd), nucleotide diversity (π) and average number of nucleotide differences (k) are summarized in Table 9. The pairwise genetic distances were relatively low (Table 13).

Table 13. Between groups mean distances of uncoupling protein two (UCP2)* sequences computed as a p-distance.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Cap2															
2	Eur	0.016														
3	Fag	0.011	0.015													
4	Hab	0.015	0.013	0.014												
5	Sax	0.010	0.015	0.007	0.014											
6	Sta	0.011	0.015	0.011	0.013	0.008										
7	Cap1	0.012	0.015	0.013	0.015	0.010	0.014									
8	Tow	0.018	0.013	0.018	0.013	0.017	0.017	0.015								
9	Cal	0.025	0.019	0.024	0.020	0.024	0.024	0.022	0.015							
10	Ame	0.018	0.014	0.017	0.013	0.016	0.017	0.015	0.012	0.016						
11	Oth	0.014	0.009	0.014	0.009	0.013	0.013	0.011	0.004	0.011	0.009					
12	Arc	0.015	0.009	0.014	0.010	0.014	0.014	0.012	0.005	0.011	0.009	0.001				
13	Cor	0.022	0.015	0.022	0.017	0.021	0.021	0.020	0.012	0.019	0.017	0.008	0.009			
14	Gra	0.018	0.011	0.016	0.012	0.016	0.017	0.015	0.007	0.011	0.011	0.003	0.003	0.011		
15	Tim	0.015	0.009	0.014	0.010	0.014	0.014	0.012	0.005	0.011	0.009	0.001	0.001	0.007	0.004	
16	Cas	0.019	0.012	0.018	0.014	0.018	0.018	0.016	0.009	0.014	0.013	0.004	0.005	0.004	0.007	0.004

**L. capensis* (Cap;1=RSA, 2= North Africa*), L. europaeus* (Eur), *L. fagani* (Fag), *L. habessinicus* (Hab), *L. saxatilis*(Sax), *L. starcki* (Sta), *L. townsendii* (Tow), *L. californicus* (Cal), *L. americanus* (Ame), *L. othus* (Oth), *L. arctincus* (Arc), *L. corsicanus* (Cor), *L. granatensis* (Gra), *L. timidus* (Tim), *L.castroviejoi* (Cat).

Hare species were clustered into three major groups: group one (*L. granatensis*, *L. timidus*, *L. europaeus*, *L. castroviejoi*, *L. corsicanus*, *L. othus* and *L. californicus*), group two (*L. habessinicus*, *L. starcki*, *L. europaeus, L. americanus* and *L. capensis*) and group three (*L. fagani*, *L. saxatilis*, *L. capensis* and *L. starcki*) (Fig.21).

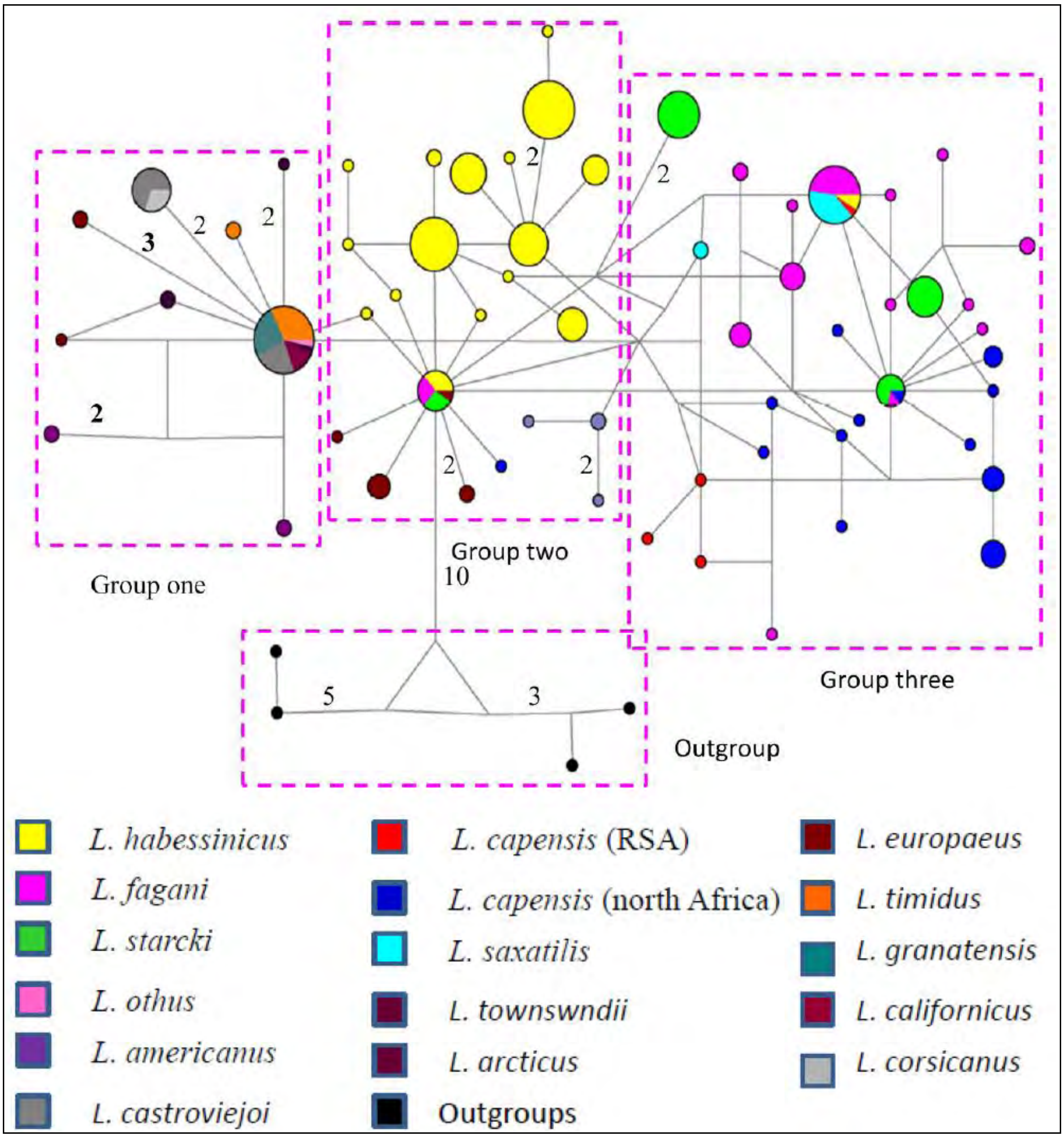

Figure 21. Uncoupling protein two (UCP2) median joining network. The size of the circles is proportional to the frequency of haplotypes. Numbers on the edges indicate the number of mutation steps greater than one.

Group two is mainly dominated by *L. habessinicus* but group three by *L. fagani* haplotypes. In addition, all hare species were not monophyletic. Individuals of *L. habessinicus* almost formed a separate group relative to *L. capensis* of both South and North African origin. All tree reconstruction methods resulted in concordant tree topologies (ML, Fig.22 and Appendix1 Figs. 6 and 12).

Signs of recent introgressive hybridizations were detected between some pair species (for instance; *L. habessinicus* and *L. fagani*, *L. fagani* and *L. starcki*, *L. habessinicus* and *L. starcki*). Signs of ancient introgressive hybridization were found between *L. starcki* and *L. europaeus, L. habessinicus* and *L. capensis, L. fagani* and *L. capensis, L. fagani* and *L. saxatilis, L. starcki and L. capensis.*

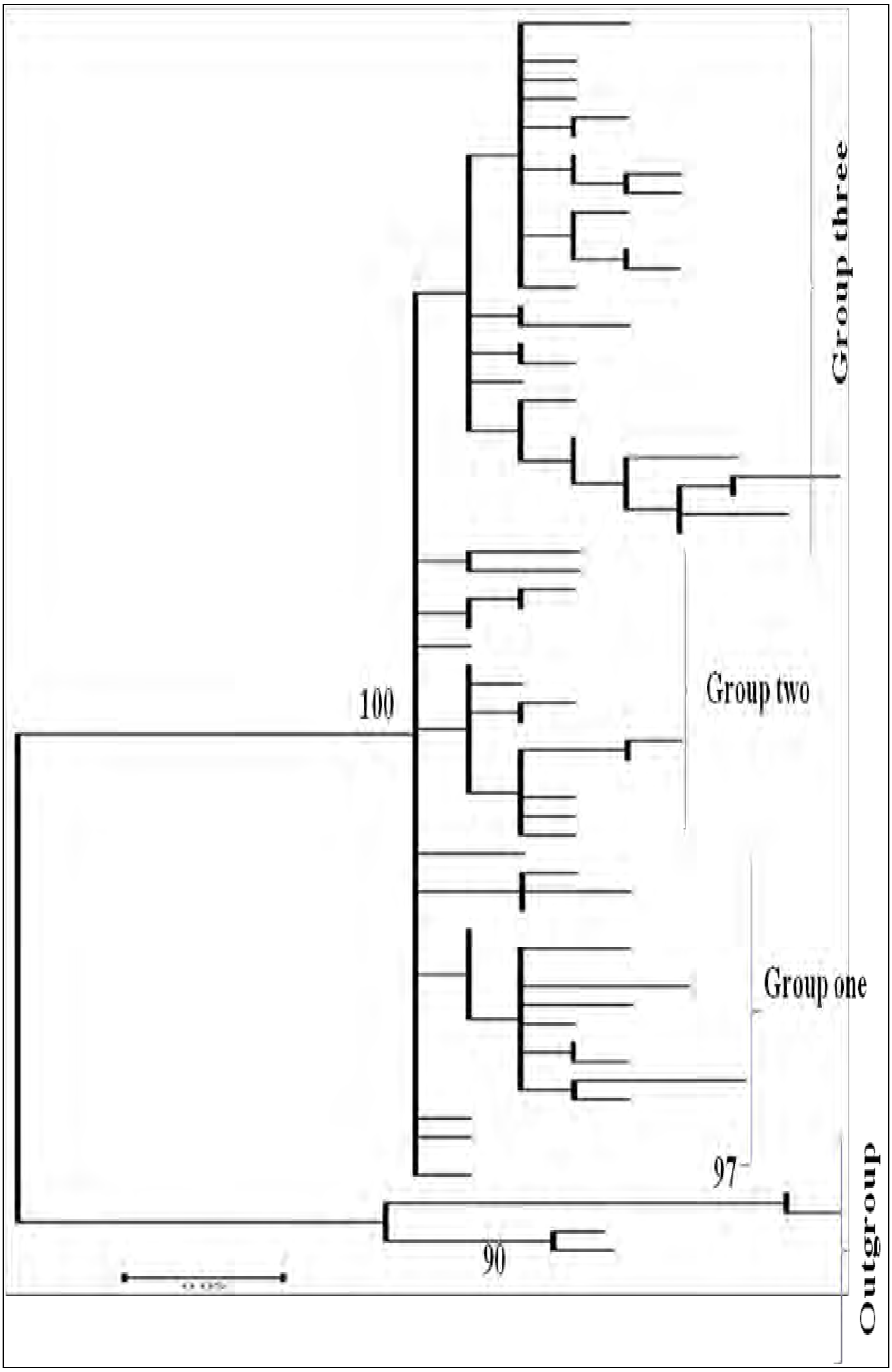

Figure 22. Uncoupling protein two (UCP2) Maximum Likelihood (ML) tree. Numbers above the branch indicate bootstrap values greater than 50%.

4.9. Uncoupling Protein Four (UCP4) Sequences

Fragments of 471bp were sequenced, 2 sites were gaps which were excluded in all subsequent analyses. Numbers of haploypes, variable sites, phylogenetically informative sites, singletons, haplotype diversity (hd), nucleotide diversity (π) and average number of nucleotide differences (k) are summarized in Table 9. The the pairwise genetic differentiation among species was very low (Table 14), suggesting a high degree of conservativism in UCP4 sequences.

Table 14. Between groups mean distances of uncoupling protein four (UCP4)* sequences computed as a p-distance.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Cap1														
2	Cap2	0.004													
3	Eur	0.002	0.002												
4	Fag	0.002	0.002	0.000											
5	Hab	0.002	0.002	0.000	0.000										
6	Sta	0.003	0.002	0.001	0.001	0.001									
7	Tim	0.003	0.002	0.001	0.001	0.000	0.001								
8	Town	0.002	0.001	0.000	0.000	0.000	0.001	0.000							
9	Cal	0.002	0.001	0.000	0.000	0.000	0.001	0.000	0.000						
10	Ame	0.002	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.000					
11	Oth	0.002	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000				
12	Arc	0.002	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000			
13	Cor	0.004	0.004	0.002	0.002	0.002	0.003	0.003	0.002	0.002	0.002	0.002	0.002		
14	Cas	0.004	0.004	0.002	0.002	0.002	0.003	0.003	0.002	0.002	0.002	0.002	0.002	0.000	
15	Gra	0.016	0.015	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.011	0.011

***L. capensis* (Cap;1=RSA, 2= North Africa*), L. europaeus* (Eur), *L. fagani* (Fag), *L. habessinicus* (Hab), *L. starcki* (Sta), *L. timidus* (Tim), , *L. townsendii* (Tow), *L. californicus* (Cal), *L. americanus* (Ame), *L. othus* (Oth), *L. arcticus* (Arc), *L. corsicanus* (Cor), *L.castroviejoi* (Cas), *L. granatensis* (Gra).

As can be observed, the median joining network, the UCP4 sequences are highly conserved and only 18 haplotypes were detected (Fig. 23). The founder haplotype was observed in individuals of diverse species (e.g. *L. habessinicus, L. fagani, L. starcki, L. timidus, L. capensis, L. californicus, L. europaeus, L. arcticus, L. townsendii* and *L. othus)* of different geographic origin ranging from Africa, Eurasia to the New World. This could reflect the presence of ancient and or recent introgressive hybridization among different species. All tree reconstruction methods resulted in concordant tree topologies (ML, Fig.24 and Appendix1 Figs. 7 and 13).

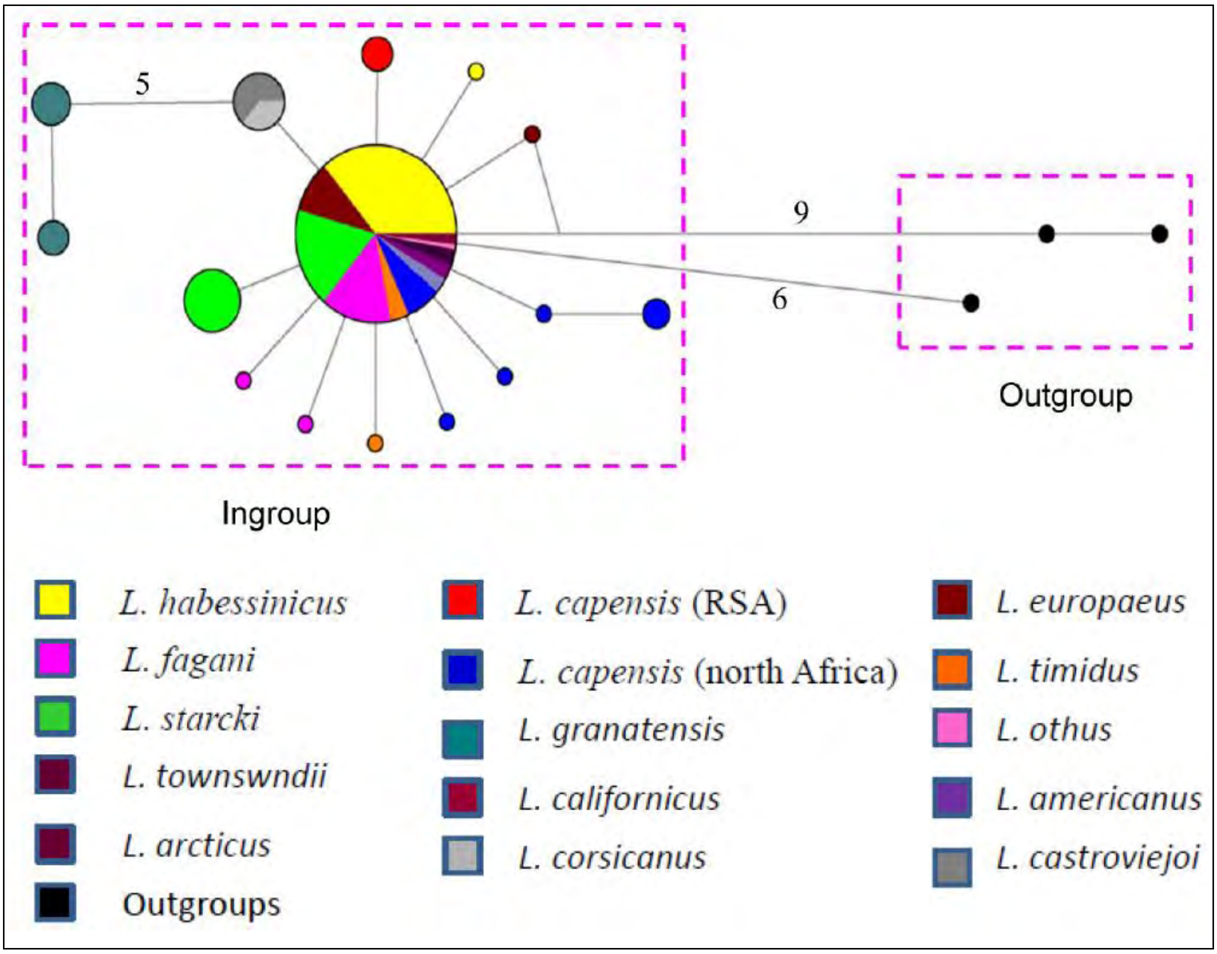

Figure 23. Uncoupling protein four (UCP4) median joining network. The size of the circles is proportional to the frequency of haplotypes. Numbers on the edges indicate the number of mutation steps if greater than one.

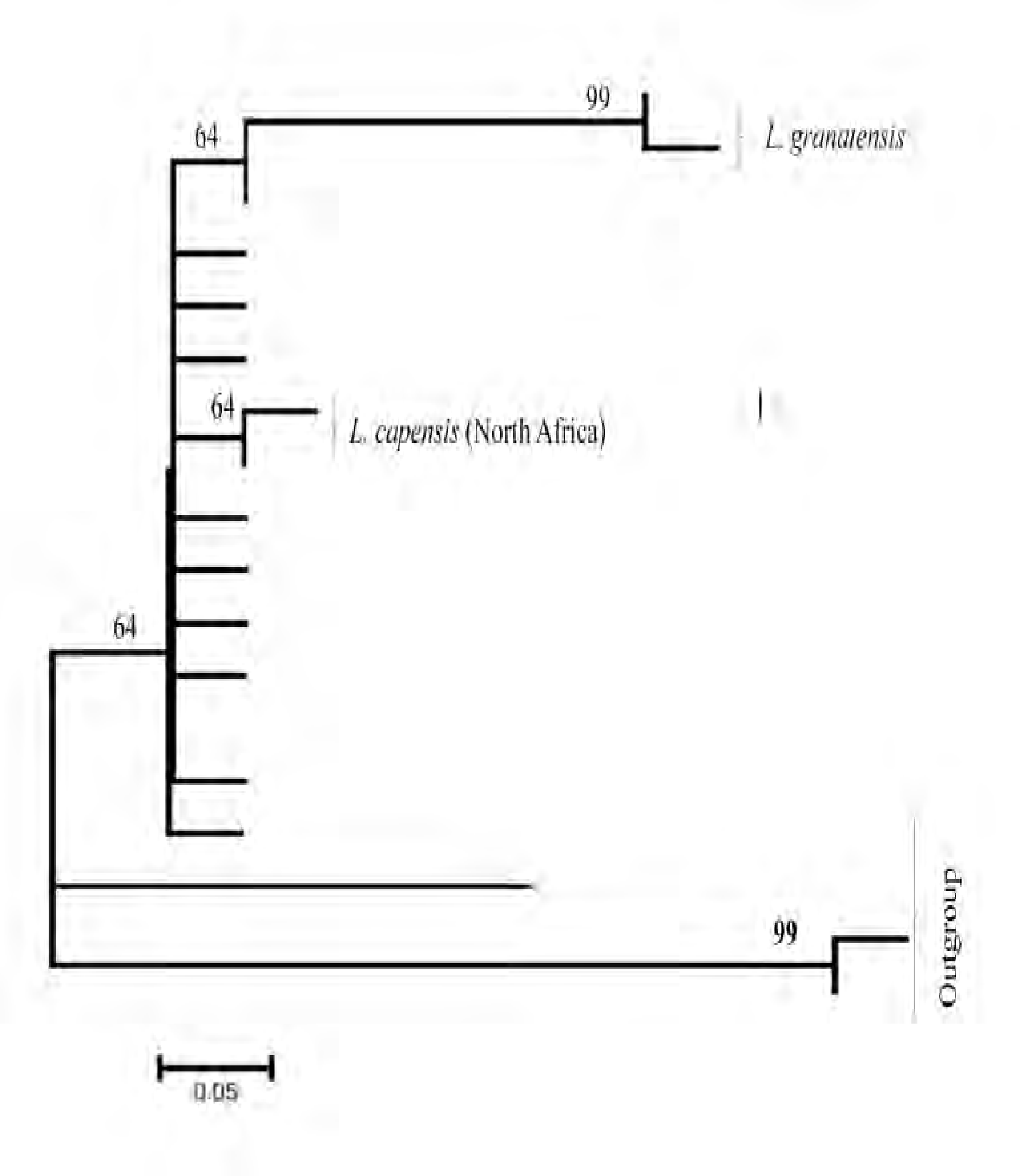

Figure 24. Uncoupling protein four (UCP4) Maximum Likelihood (ML) tree. Numbers above the node indicate bootstrap support greater than 50%.

4.10. Thyroid Stimulating Hormone Beta (TSHB) Sequences

A fragment of 279 bp was sequenced. Numbers of haploypes, variable sites, phylogenetically informative sites, singletons, haplotype diversity (hd), nucleotide diversity (π) and average number of nucleotide differences (k) are summarized in Table 5. The pairwise genetic distances were very low (Table 15) suggesting that TSHB sequences were conservative. All tree reconstruction methods resulted in concordant tree topologies (ML, Fig.26 and Appendix1 Figs. 8 and 14).

Hare species were partioned into two groups: group one contained (*L. capensis* (North Africa), *L. capensis* (South Africa), *L. saxatilis*, *L. fagani*, *L. starcki*, *L. habessinicus*, *L. europaeus*, *L. americanus*, *L. castroviejoi*, *L. corsicanus* and *L. californicus*) and group two contained (*L. habessinicus*, *L. fagani*, *L. starcki*, *L. granatensis*, *L. europaeus*, *L.timidus* and *L. saxatilis*) (Fig.25).

We documented signs of recent introgression (e.g. between *L. corsicanus* and *L. castroviejoi*, *L. habessinicus* and *L. starcki*) and ancient introgression (e.g. between *L. habessinicus* and *L. granatensis*, *L. europaeus* and *L. habessinicus*, *L. californicus* and *L. habessinicus*). As it is evident from the different tree reconstruction methods, hare species from North America appear to be ancestral species (Fig. 26). Furthermore, TSHB sequence support the hypothesis that the ancestral *Lepus* species are probably of North American origin. However, species of African origin also look ancestral to some extent.

Table 15. Between groups mean distances of thyroid stimulating hormone beta (TSHB)* sequences computed as a p-distance.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Cap1															
2	Cap2	0.002														
3	Eur	0.021	0.023													
4	Fag	0.002	0.004	0.022												
5	Hab	0.014	0.016	0.012	0.014											
6	Sax	0.004	0.005	0.019	0.005	0.014										
7	Sta	0.004	0.005	0.021	0.005	0.014	0.007									
8	Tim	0.014	0.016	0.008	0.015	0.008	0.013	0.015								
9	Tow	0.015	0.016	0.009	0.015	0.009	0.013	0.015	0.004							
10	Cal	0.006	0.007	0.020	0.007	0.015	0.008	0.008	0.013	0.015						
11	Ame	0.014	0.015	0.034	0.015	0.027	0.017	0.016	0.027	0.027	0.017					
12	Oth	0.015	0.016	0.007	0.015	0.008	0.013	0.015	0.001	0.002	0.014	0.027				
13	Arc	0.008	0.009	0.014	0.009	0.011	0.008	0.009	0.008	0.008	0.010	0.020	0.007			
14	Cor	0.015	0.016	0.035	0.016	0.028	0.018	0.018	0.028	0.029	0.020	0.020	0.029	0.022		
15	Cas	0.015	0.016	0.035	0.016	0.028	0.018	0.018	0.028	0.029	0.020	0.020	0.029	0.022	0.000	
16	Gra	0.019	0.020	0.008	0.019	0.006	0.016	0.018	0.005	0.006	0.018	0.031	0.004	0.011	0.033	0.033

**L. capensis* (Cap;1=RSA, 2= North Africa*),* *L. europaeus* (Eur), *L. fagani* (Fag), *L. habessinicus* (Hab), *L. starcki* (Sta), *L.saxatilis* (Sax), *L. timidus* (Tim), *L. townsendii* (Tow), *L. californicus* (Cal), *L. americanus* (Ame), *L. othus* (Oth), *L. arcticus* (Arc), *L. corsicanus* (Cor), *L.castroviejoi* (Cas), *L. granatensis* (Gra).

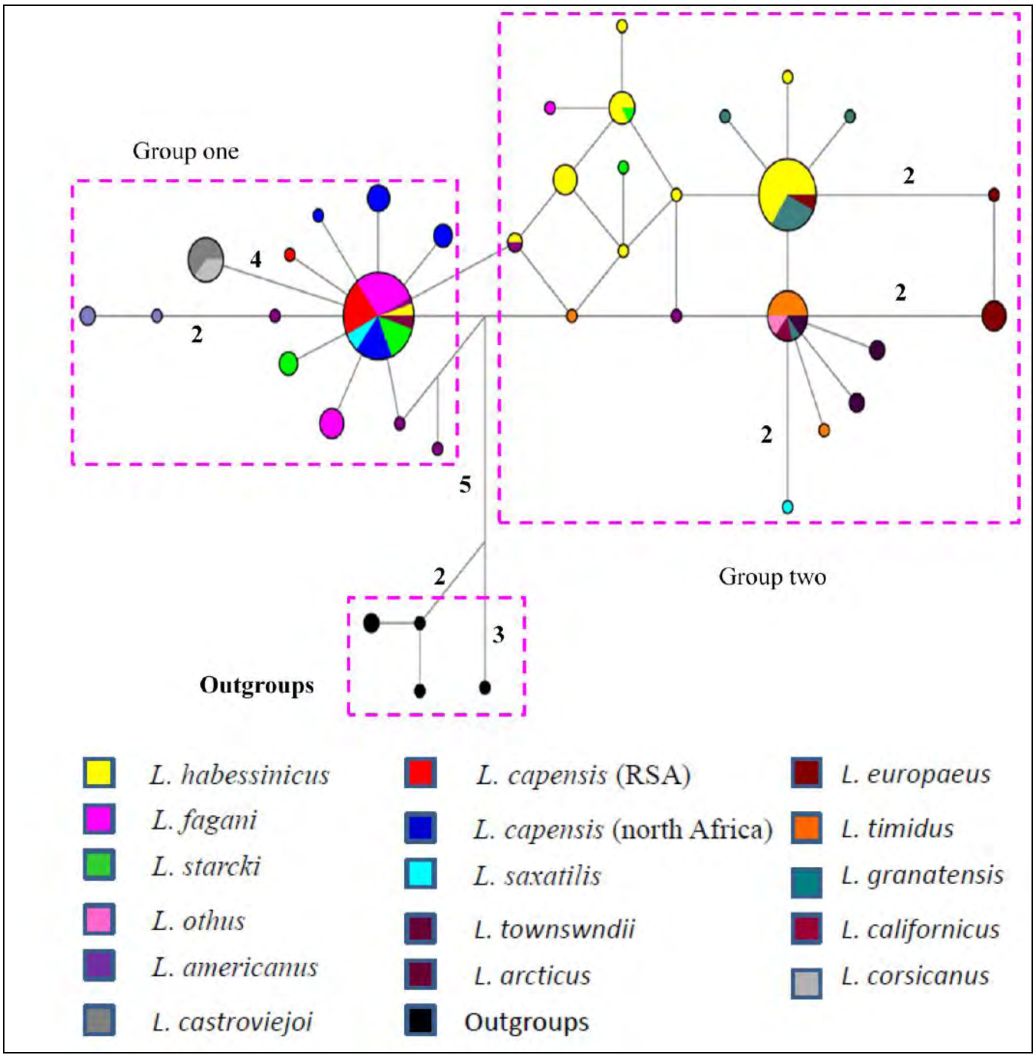

Figure 25. TSHB median joining network. The size of the circles is proportional to the frequency of haplotypes. Numbers on the edges indicate the number of mutation steps if greater than one.

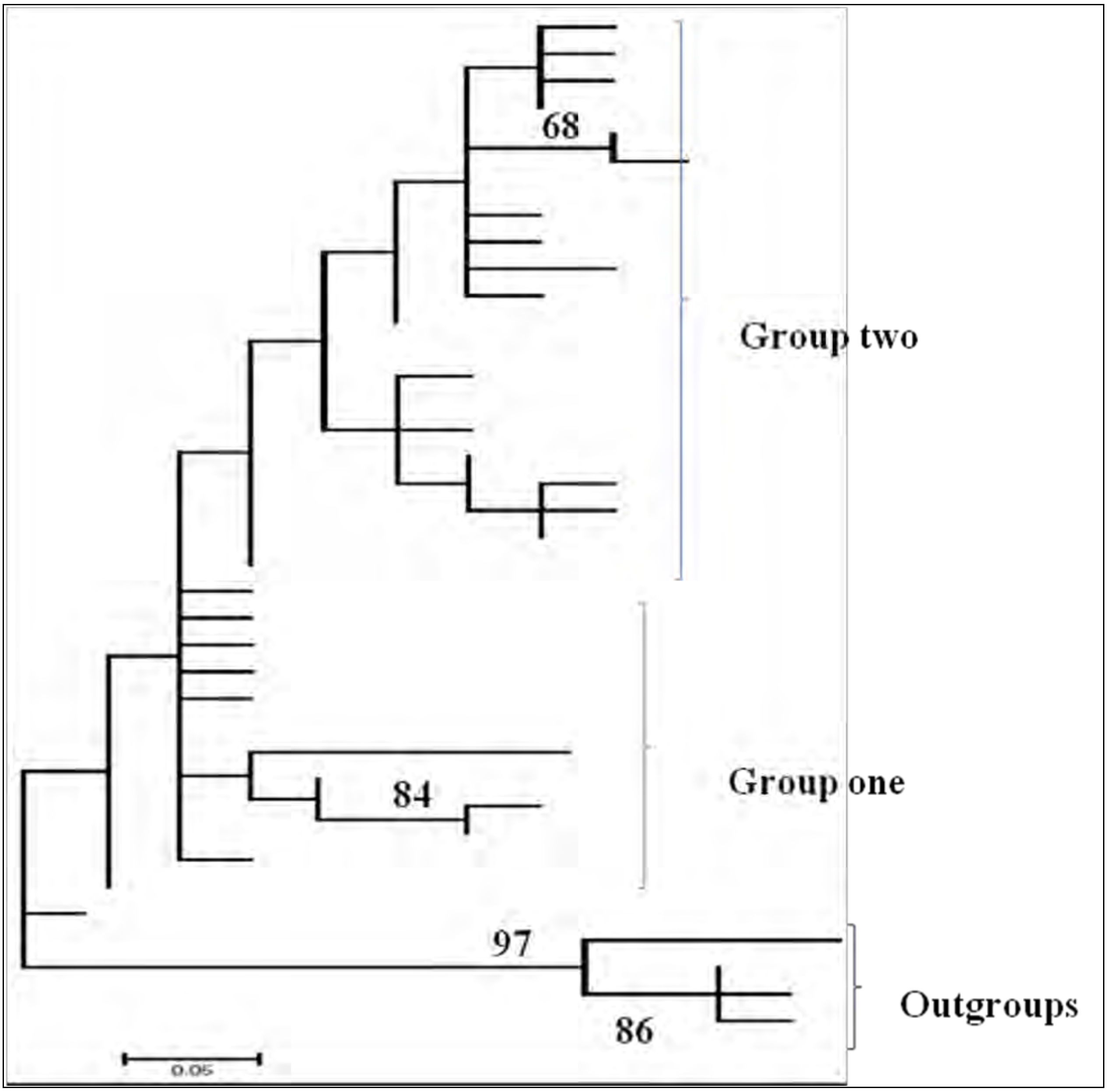

Figure 26. Thyroid stimulating hormone beta (TSHB) Maximum Likelihood (ML) tree. Numbers above the branch indicate bootstrap support greater than 50%.

5. Discussion

5.1. Morphological, Dental and Non-metric Skull Differentiation among Hare Species from Ethiopia.

Indications of sexual dimorphism were documented for three external measurements (BW, HBLC and HFL). Generally, females were heavier and had longer HBLC and HFL compared to males. The heavier female BW could be because some females were pregnant as we had found embryos in their uterus. However, no sexual dimorphism was found in ear length and hind foot length. Ethiopian highland hares have relatively heavier BW compared to the other two taxa. The heavier body weight was not only for the raw but also for the relative body weight. The heavier BW could be an adaptation to life to higher elevation and the ranges of *L. starcki* is in the highland areas in central and southeastern Ethiopia (Petter, 1963; Angermann, 1983; Yalden *et al.*, 1986; Flux and Angermann, 1990). This result is in line with Bergmann's rule (Bergmann, 1848) which states that organisms at higher latitude or elevation have larger body size (Ashton *et al.*, 2000; Ashton, 2002a,b; Meiri and Dayan, 2003). It was found that amphibians in general show an increase in body size at higher latitude and elevation (Ashton, 2002a). Similarly, Bergmann's rule is a valid ecogeographic rule for birds (Ashton, 2002b; Meiri and Dayan, 2003) and mammals (Ashton *et al.*, 2000; Meiri and Dayan, 2003).

The relatively wider body size variation of *L. habessinicus* could reflect clinal variation in body size due to a variety of habitats occupied by the species (for instance lowland in Afar vs. highland in the central Ethiopia). In addition, *Lepus habessinicus* had relatively lower body weight compared to the other taxa. However, Ethiopian hares had relatively shorter ear length compared to the other taxa as suggested by earlier workers (Yalden *et al.*, 1986; Azzaroli-

Puccetti, 1987a, b). The scatter plot of ear length versus hind foot length confirmed external morphological difference of *L. fagani* from other two species (Fig.8). Furthermore, a comparison of the relative ear portion among the three species indicated a significant species effect. However, there is no significant species effect on the relative hind foot length. *Lepus starcki* was significantly different from both *L. fagani* and *L. habessinicus* as suggested by Yalden *et al.* (1986) in the pattern of cement filling in the first upper incisor. From the pattern of cement filling, it was difficult to differentiate *L. habessinicus* from *L. fagani* and this character had also confused Petter (1963) in differentiating the two taxa. But there was no evidence of sexual dimorphism in pattern of cement filling.

5.2. Morphometric Skull Differentiation among Hare Species from Ethiopia

Multivariate morphometric skull analysis indicated that the three *Lepus* taxa in Ethiopia were morphologically different. Skull morphometry suggested that *L. europaeus* has a distinct skull shape relative to *Lepus* species from Africa. The separation is evident from the box plot of PC1 (Appendix 1 Fig. 2). Furthermore, *L. europaeus* had relatively longer CBL relative to *L. starcki*. The discriminate analysis had misclassified 2.7 % and 8.2 % of *L. europaeus* and *L. starcki,* respectively. The low level of misclassification suggests the discriminating power of the selected variables. The multivariate discrimination (i.e. PCA and DA) of *L. starcki* from *L. europaeus* is in line with mtDNA and nucDNA analyses done in this study. All nuclear fragments used in this study partitioned *L. europaeus* and *L. starcki* into distinct groups with little haplotypic sharing. Similarly, *L. starcki* had a different skull shape relative to *L. capensis* (Appendix 1 Fig.2) and was concordant with the results of the molecular analysis carriedout in this study. The skull morphometric results confirm the suggestion that *L. starcki* deserve the status of a valid species (Angerman, 1983). However, the results of our morphometric analysis are in contrast to the earlier suggestion by Petter (1963) considering *L. starcki* as a subspecies of *L. capensis* and that of Azzaroli-Puccetti *et al*. (1996) considering *L. starcki* as subspecies of *L. europaeus.* In addition*,* our skull morphometric analysis showed that *L. starcki* has a different skull shape relative to *L. habessinicus* or *L. fagani* (Fig. 9E-F). Both external morphological measurements and multivariate skull morphometric analyses revealed a clear discrimination of *L. starcki* from the other taxa from Ethiopia.

Principal component analysis (PCA) revealed that *L. habessinicus* had a different morphometric shape relative to *L. capensis* as seen from the PC2 box plot Fig. 9B. Although the shape space of *L. habessinicus* seems to be distinct from *L. capensis*, the two taxa share some shape component in the morpho space. In addition, *L. capensis* had wider morpho spaces which could be due to wider sampling areas (North Africa, East Africa and South Africa). As the wider morpho space could affect the within group variance we used Cape hares only from East Africa for the DA. Discriminate function one indicated that *L. capensis* (Cape hares) differs from *L. habessinicus* (Fig. 10B). In the morpho shape space, *L. habessinicus* was also different *L. saxatilis* (Appendix 1 Fig. 2). This is in contrast to the suggestion of Petter (1963) to consider *L. habessinicus* as a subspecies of *L. saxatilis.* Compared to other *Lepus* species from Ethiopia, *L. habessinicus* had a different morpho shape (Fig. 9 E-F). Further, the degree of morphological differentiation among hares in Ethiopia is also in line with the microsatellite data provided in this study (Figs.11-12). Our morphometric analysis is line with the earlier suggestion by Azzaroli-Puccetti (1987a, b) that *L. habessinicus* of north Somalia and central Ethiopia deserves species status.

Both multivariate and univariate analyses have shown that *L. fagani* was distinct from both *L. saxatilis* and *L. victoriae*. *Lepus fagani* had a distinct distinct skull shape space relative to either *L. saxatilis* or *L. victoriae*. However, *L. saxatilis* and *L. victoriae* had similar morpho shape space (Fig. 9C-D). This is in line with earlier suggestion that the savanna hare is a subspecies of the scrub (Robinson and Dippenaar, 1987; Flux and Angemann, 1990; Hoffmann and Smith, 2005). Further, DF1 discriminated *L. fagani* from *L. victoriae* but the CBL of *L. fagani* was within the range of latter species (Fig. 10 D). However, molecular analyses made in this study indicated some degree of haplotypic sharing of *L. fagani* with *L. saxatilis* for both mtDNA and nucDNA. This could be good evidence for incongruence between morphological and molecular markers. The haplotypic sharing (i.e. mtDNA and nucDNA) between *L. fagani* and *L. saxatilis* supports the earlier suggestion to include *L. fagani* in the *L. victoriae/L. saxatilis* complex (Flux and Angermann, 1990; Hoffmann and Smith, 2005). In addition in some of the nuclear sequences, *L. saxatilis* and *L. fagani* showed very low genetic differentiation. However, relative to other hare taxa in Ethiopia, *L. fagani* had a clear skull morphometric differentiation (Fig. 9E-F). The skull morphometric result is in line with the external morphological analysis for hares collected from Ethiopia. In addition, the SSR analysis made in the present study revealed a clear differentiation of *L. fagani* from other *Lepus* taxa found in Ethiopia. Therfore, we can conclude from external morphology and from the skull morphometric results that *L. fagani* deserves valid species status.

5.3. Microsatellite (SSR) Differentiation of *Lepus* in Ethiopia

Microsatellite analyses were made for 107 hares (i.e. of three species) collected from Ethiopia and the main results were: (1) hares clustered into three major groups, (2) *L. habessinicus* and *L. fagani* individuals were substructured into different phylogeographic groups according to populationsof origin, (3) *L. starcki* individuals consistently clustered in a single phylogroup, (4) signs of nuclear introgressions were found among species and (5) no evidence of recent bottlenecks was documented for *L. habessinicus* and *L. starcki.*

Estonba *et al*. (2006) analysed microsatellites to check levels of genetic differentiation among hare species on the Iberian Peninsula and found that there are clear differences among the three Iberian species (*L. europaeus*, *L. castroviejoi* and *L. granatensis*). Similarly in the present analysis, the three hare species in Ethiopia were clearly differentiated into three groups with some degree of introgressions. Individuals of the three species were clustered into respective species (Figs. 11-12 and 14). The clusters reflected the geographic origin and the morphological characters of the different species. Factorial correspondences analysis, structure analysis and Bayesian assignment revealed a clear differentiation among the three species. Although there were clear differences among the populations, the larger poroprtion of genetic variation was attributed to within population variation (Table 8**)**. Similarly, larger within population variation was also documented for hare species (Hamill *et al.*, 2006; Ben Slimen *et al.,* 2008). However, the within population variation found in the present analysis (70.56%) was low compared to that found by Hamill *et al.* (2006) (75.6%). Different indices of genetic differentiation revealed clear distinctions among the species. Levels of pairwise F_{ST} and G'_{ST} documented in the present study between *L. habessinicus* and *L. starcki* were larger compared to those between *L. capensis* and *L. europaeus* reported by Ben Slimen *et al.* (2008). In contrast, the extent of relative genetic differentiation found in the present study among hare species from Ethiopia is lower than the genetic differentiation ($F_{ST} > 0.4$) found between Scottish and Irish populations of *L. timidus* (Hamill *et al.*, 2006). The level of genetic diversity of SSR loci found in the current study is lower than that found by Ben Slimen *et al.* (2008) at 11 loci in two hare species from Africa and Europe. The relatively low level of genetic diversity could be due to a Wahlund effect for *L. habessinicus* and *L. fagani* as samples of these two species were combined from different localties. However, for *L. starcki* the low level of genetic diveristy could be due to a founder effect as most of the specimens came from a restricted area (i.e. 96.30% from Fiche, North Shoa /Oromia Regional State) and the rest from Agena (Guraghe Zone/SNNPRS). The level of microsatellite differentiation obtained in the present study was not incongruent with the morphological analysis done in this study. The multivariate skull morphometric analysis revealed a clear morphological distinction among the three hare species. The differentiation was also evident from the result of external morphological characters (e.g. body weight, ear length) and the pattern of cement filling in the first upper incisor, particulary between *L. starcki* and the other taxa.

Individuals of *L. habessinicus* and *L. fagani* were substructured according to population of origin and collection localities (Fig.14). Patterns of substructuring were in line with the phylogeographic patterns revealed by our mtDNA analysis, but with some degree of discordance (Fig.15). Discordance between microsatellite and mtDNA phylogroups are documented for other mammal species, for example in the genus *Rupicapra* (Rodriguez *et al.*, 2010). Although *L. habessinicus* individuals clustered according to population of origin there was a good degree of SSR introgression among the different substructures (Fig.14). Furthermore, individuals of *L. habessinicus* from different localities share haplotypes for the mtDNA and nucDNA sequences used in this study. The relatively higher degree of variability in *L. habessinicus* could be due the wider range of sampling that covered a wider geographic regions (Fig.2). Here, we documented a relatively higher number of private allele for *L. habessinicus* (26) which was more than two fold of that of *L. starcki* (11). *Lepus fagani* individuals were substructured into population of origin and collection sites. In addition, there was also signed of microsatellite introgression among the different substructures (Fig.14).

Unlike the mtDNA phylogeographic pattern where *L. fagani* individuals were scattered, here and there but in the microsatellite analysis they formed almost a single group (Figs. 11-12). *Lepus fagani* samples collected from Agena (Guraghe Zone/SNNPRS), Kerkerty (Konso Special Woreda/ SNNPRS) and Adolla (Guji Zone/Oromia Regional State) could be new collection sites as there were no previous records for the species from these localities. Therefore, this result showed the need for further exhaustive expedition of new populations of the taxon in other similar habitates.

Individuals of *L. starcki* consistently formed a single phylogroup in all level of clustering (Figs. 11-12 and 14). This is in line with the morphological analysis and earlier suggestion by Angermann (1983) that this taxon deserve species status. Similarly for mtDNA analysis made in the present study, *L. starcki* had clustered into a single group except a single individual collected from Agena (Guraghe Zone/SNNPRS). The pairwise genetic differentiation was highest between *L. starcki* and the other two taxa. In addition, the pairwise genetic differentiations were consistently higher between *L. starcki* and *L. fagani* (Table 7) and there was low microsatellite introgression among the two taxa (Fig. 14). The level of genetic differentiation (e.g. $G'_{ST;}$ Table 7) was high between *L. starcki* and *L. fagani* compared to the one reported by Ben Slimen *et al*. (2008) between *L. capensis* and *L. europaeus*. Although individuals of *L. starcki* form a single group, there is some evidence of microsatellite introgression from *L. habessinicus* into *L. starcki*. One individual of *L. starcki* showed sign of introgression from *L. habessinicus* samples from Mieso area, west Harerghe (Oromia Regional State; Fig. 2). In addition, migrate analyses indicated a good degree of exchanges of genetic materials between *L. habessinicus* and *L. starcki* (Table 7). Compared to the other two taxa, low genetic variation was found in *L. starcki* with lowest number of private allele and other basic population genetic statistics. Similarly, low nucleotide and haplotypic diversity was found in *L. starcki* for mtDNA analysis made in the present study (data not shown). This low degree of genetic variation could be attributed to narrower sampling area (about 96.30% samples are from North Shoa/Oromia Regional State; Fig.2) except the single *L. starcki* individual from Agena/Gurage Zone in SNNPRS. A similar low level of genetic (SSR) diversity was reported for Iberian hare relative to *L. europaeus* or *L. timidus* (Freitas, 2006). So far, there was no previous record about the occurrence of *L. starcki* in Agena area. Therefore, this result showed the need for further exhaustive expedition of new populations of the taxon in other similar habitates.

Although the level of microsatellite (SSR) introgression seems lower between *L. habessincius* and *L. fagani* at lower levels of K, there was increasing tendency at higher levels of K. Furthermore, the two taxa share larger number of alleles for both mtDNA and nucDNA done in the present study. The extent of haplotypic sharing and relatively low genetic differentiation reflects the closer association between Abyssinian hare and Ethiopian hare. Although the structure result revealed a good degree of introgression between *L. habessinicus* and *L. fagani*, migrate analysis result revealed low migration from *L. fagani* into *L. habessinicus* (Table 7). The present analysis had not revealed sign of recent bottleneck for both *L. habessinicus* and *L. starcki*, however, there was sign of recent genetic bottleneck for *L. fagani* for one of the mutation models (i.e IAM).

5.4. MtDNA Introgression among *Lepus* Species

An initial comprehensive molecular evolutionary genetic analysis was made for *Lepus* species from Ethiopia (i.e. *L. habessinicus*, *L. fagani* and *L. starcki*). The analysis revealed a complex molecular evolutionary relationship among the hare species with a large degree of reticulate evolution. The reticulate scenario was well depicted by both nuclear and mitochondrial markers that illustrate sign of either recent or ancient introgression among hares from Ethiopia and between hares from Ethiopia and other parts of the world (Figs. 15-26). MtDNA introgression among hare species is common (for example, from *L. timidus* into species like: *L. granatensis, L. europaeus*, *L. castroviejoi, L. capensis* and *L. mandshuricus* (Thulin *et al.,* 1997, 2003, 2006a; Thulin and Tegelström, 2002; Alves *et al*., 2006, 2008a,b; Fredsted *et al*, 2006; Melo-Ferreira *et al*., 2005, 2007, 2012; Liu *et al*., 2011b). Most of these introgressions are asymmetric and frequently from *L. timidus* into other species; however, rarely reciprocal introgressions have been documented from *L. europaeus* into *L. timidus* in the Alps (Suchentrunk *et al*., 2005) and in Russia (Thulin *et al*., 2006b).

However, introgressive hybridization among hares from Ethiopia was both unidirectional and or bidirectional as, for example, from *L. starcki* into *L. fagani* and from *L. starcki* into *L. habessinicus*. For instance, two *L. fagani* individuals were placed within *L. starcki* group in the mtDNA tree (Fig.16). The presence of *L. fagani* individuals within *L. starcki* lineage indicated asymmetric introgression of mtDNA from the later species into the former. Similarly, the presence of two individuals of *L. habessinicus* in the group of *L. starcki* reflected the introgression of mtDNA of the later species into the former (Fig. 16). Unlike the introgression between *L. fagani* and *L. starcki;* here we saw a bidirectional introgression because one *L. starcki* individual is placed in phylogroup two of *L. habessinicus* (Fig. 15). This could reveal the existence of recent introgressive hybridization among the two species. The probability of ongoing introgression among *L. habessinicus* and *L. starcki* could be large as the ranges of the two taxa overlaps in the central and southeastern highlands of Ethiopia (Agermann, 1983; Yalden *et al*., 1986; Azzaroli-Puccetti, 1987*;* Azzaroli-Pucceti *et al.,* 1996). The existence of *L. fagani* individuals in the *L. habessinicus* mtDNA phylogroup reflected of the presence of mtDNA introgression from Abyssinian hare into the Ethiopian hare (Figs.15-16). The presence of one *L. starcki* individual in the *L. habessinicus* phylogroup indicates the existence of mtDNA introgression from *L. habessinicus* to *L. starcki* (Figs.15-16). The introgressions between hare species in Ethiopia could be recent as the central highland the three species are sympatric (Yalden *et al*., 1986), and can contribute to recurrent gene flow among them. The existence of a *L. saxatilis* individual in the lineage of *L. starcki* mtDNA revealed the presence of introgressive hybridization that could be ancient because the probabilities of recent hybridization between the two species are negligible for the two species currently occur in two different corners of Africa (Flux and Angermann, 1990; Hofmann and Smith, 2005; see Fig.1). MtDNA introgression into *L. saxatilis* had also occurred from *L. habessinicus* which could be an evidence of ancient as the present day distribution of the two taxa are in South Africa and in the Horn of Africa, respectively. There was also sign of ancient mtDNA introgression from *L. europaeus* into *L. saxatilis.*

Different factors have been suggested as possible driving forces for introgressive hybridization between hare species. Among these, are species abundance asymmetry, mating preference, range expansion, low divergence between species and body size (Thulin and Tegelström, 2002; Jansson *et al*., 2007; Melo-Ferreira *et al*., 2005, 2007; Alves *et al*., 2008; Liu *et al*., 2011b). Females of rare species of hares (e.g. *L. timidus*) generally have a tendency to hybridize with males of more frequent species (e.g. *L. europaeus;* Thulin and Tegelström, 2002, Melo-Ferreirra *et al*., 2005, 2007; Alves *et al*., 2008). Similar results were also found among some Chinese hare species by Liu *et al*. (2011b); females of *L. timidus* (rare species) and males of *L. capensis* (common species) and between females *L. timidus* (rare species) and males of *L. mandshuricus* (common species). Introgression among hares from Ethiopia agrees to some extent with the hypothesis of mate preference between females of rare species and males of common species.

Introgression between females of *L. starcki* (rare species) and males of *L. habessinicus* (common species) agrees with the hypothesis species abundance asymmetry. MtDNA introgression in the reverse direction between *L. habessinicus* (females) and *L. starcki* (males) was against this hypothesis. However, introgression between females *L. stracki* (rare species) and males of *L. fagani* (common species) agrees with the hypothesis. Introgression between females of *L. habessinicus* (common species) and males of *L. fagani* (common species) is contrary to the hypothesis. MtDNA introgression between females of *L. habessinicus* (small bodied) and males *L. fagani* (large bodied) is in line with the suggestion of (Thulin and Tegelström, 2002) for asymmetric introgression. However, inconsistent results were found recently by Liu *et al*. (2011b), between females *L. timidus* (larger) and males of *L. capensis* (smaller) or *L. mandshuricus* (smaller) in China.

This study did not find any mtDNA introgression between *L. habessinicu* and *L. capensis*. No sign of mtDNA introgression was detected from either Cape hare or brown hare into Ethiopian highland hare or vice versa. This is despite the common existence of mtDNA introgression among hare species (e.g. between *L. timidus* and other species: *L. granatensis* and *L. europaeus*, and between *L. corsicanus* and *L. castroviejoi*) (Thulin *et al, 1997,* 2003, 2006a; Melo-Ferreira *et al*., 2005, 2007; Alves *et al*., 2006, 2008a,b; Liu *et al*., 2011b). Therefore, it can be stated that *L. habessinicus* had distinct mtDNA relative to Cape hare as opposed to its earlier placement as a subspecies *L. capensis* (Angermann, 1983; Azzaroli-Puccetti, 19987a, b; Flux and Angermann, 1990). In a similar way, it can be said that *L. stracki* has distinct mtDNA relative to either *L. europaeus* or *L. capensis*.

5.5 Historical (Ancient) and or Recent Introgression of NucDNA among *Lepus* Species

Usually it is difficult to differentiate whether haplotypic sharing between taxa are due historical (ancient) and or recent introgressive hybridization. Here we documented sign of a historical and/or recent introgressive events among different species. For instance, 4 haplotypes of transferrin (TF) sequences (HP14, HP16, HP21 and HP22) were shared among different species. Haplotype HP14 was shared between *L. fagani* and *L. habessinicus* while HP21 shared between *L. starcki* and *L. habessinicus*. These two haplotypes could be taken as sign of recent nucDNA introgression. Since in some places range of these species overlap (e.g. Central highland) (Yalden *et al*., 1986; Flux and Angermann, 1990), hence, there could be chance for recurrent gene flow among them.

Haplotypes HP16 and HP22 were also shared with individuals from other species. For example, HP16 was shared among 4 taxa (*L. fagani, L. habessinicus, L. capensis* (RSA) and *L. saxatilis*) and HP22 shared among 5 taxa (*L. starcki, L. habessinicus, L. capensis* (North Africa), *L. timidus* and *L. yarkandensis*). These two haplotypes could be taken as evidence of histrocal introgression as the probability of ongoing gene flow among some of these species is rare. Some of these species occur in different corners of Africa (i.e. North Africa, East Africa and South Africa) (Flux and Angermann, 1900; Hoffmann and Smith, 2005) and others occur in different continents (e.g. *L. starcki* in Africa and *L. yarkandensis* in Asia) (Flux and Angermann, 1900; Hoffmann and Smith, 2005; see Fig.1). Additional TF haplotypes shared among different species were: HP5 (*L. capensis* (China), *L. sinensis* and *L. capensis* (North Africa)), HP28 (*L. starcki*, *L. timidus*, *L. townsendii*, *L. othus* and *L. arcticus*), HP34 (*L. timidus*, *L. townsendii* and *L. othus*) and HP54 (*L. corsicanus* and *L. castroviejoi*). Once again some of the haplotypes could be taken as evidence of ancient introgression (e.g. HP5, HP28 and HP34) or recent introgression (HP54). The hapltypic sharing between *L. timidus*, *L. othus* and *L. arcticus* (HP28 or HP34) support the closer association between these taxa as suggested by earlier works (Halanych *et al.,* 1999; Walteri *et al*., 2004; Walteri and Cook, 2005).

Similarly, sign of haplotypic sharing was also documented for Thyroglobulin (TG) and the remaining nuclear sequences (UCP2, UCP4 and THSB). Thyroglobulin haplotypes shared among different species include: HP2 (*L. capensis* and *L. townsendii*), HP3 (*L. habessinicus, L. fagani*, *L. capensis* (North and South Africa) and *L . timidus*), HP8 (*L. habessinicus* and *L. capensis* (North Africa)), HP10 (*L. habessinicus* and *L.capensis* (North Africa)), HP11 (*L. starcki*, *L. fagani*, *L. habessinicus* and *L. capensis* (North Africa)), HP15 (*L. yarkandensis*, *L. europaeus* and *L. capensis* (North Africa)), HP21 (*L. fagani* and *L. habessinicus*), HP30 (*L.capensis* (China) and *L. habessinicus*), HP58 (*L. comus* and *L. oiostolus*), HP65 (*L. timidus*, *L. capensis* (China), *L. yarkandensis* and *L. sinensis*), HP69 (*L. yarkandensis* and *L. capensis* (China)), HP72 (*L. capensis* and *L. sinensis*) and HP73 (*L. capensis* (China) and *L. yarkandensis*). Some of the haplotypes could indicate ancient introgression (e.g. HP2, HP3, HP8 and HP30) or recent introgressive hybridization (e.g. HP21, HP72 and HP73).

In the case of UCP2 sequences 5 haplotypes (HP6, HP13, HP14, HP45 and HP54) were shared among different species. Four haplotypes were shared among species of wider geographic origin: HP6 (*L. fagani*, *L. habessinicus*, *L. saxatilis* and *L. capensis* (RSA)), HP 13 (*L. habessinicus*, *L. fagani*, *L. starcki* and *L. europaeus*), HP14 (*L. starcki*, *L. fagani* and *L. capensis* (North Africa) and HP45 (*L. timidus*, *L. granatensis*, *L. castroviejoi*, *L. arcticus*, *L. townsendii* and *L. othus*). The extent haplotypic sharing species of wider geographic regions (different corner of a continent or in different continents) could implicate presences of ancient introgression among the species. Such scenarios have been documents among different species for nucDNA (Liu *et al*., 2011b; Melo-Ferreira *et al*., 2012).

Similarly, 6 haplotypes of TSHB (i.e. HP1, HP4, HP6, HP13, HP15 and HP30) were shared among species from wider geographic ranges. Two of the six haplotypes HP13 (*L. corsicanus* and *L. castroviejoi*) and HP30 (*L. habessinicus* and *L. starcki*) could indicate an ongoing recurrent introgressive hybridization. But the rest 4 haplotypes were shared among species of wider geographic origins (Africa, Eurasia and the New World). For instance, HP6 was shared among 7 species (*L. fagani, L. starcki, L. habessinicus*, *L. capensis* (North and South Africa), *L. saxatilis*, *L. californicus* and *L. arcticus*). Likewise, haplotypes HP4 (*L. habessinicus* and *L. californicus*), HP1 (*L. timidus, L. othus*, *L. arcticus*, *L. townsendii* and *L. granatensis*) and HP15 (*L. habessinicus*, *L. granatensis* and *L.europaeus*) were also shared among species originating from wider geographic ranges. Such events of haplotypic sharing among species of wider geographic origin could once again show existence of ancient introgression. Similar haplotype sharing was also documented for hare species (Waltari and Cook, 2005; Liu *et al*., 2011b; Melo-Ferreira *et al*., 2012). In all phylogenetic reconstruction, hare species from North America or China were basal compared to *Lepus* from other regions. Therefore, the nucDNA results support the earlier suggestion that the ancestral *Lepus* are from North America (Matthee *et al*., 2004; Wu *et al*., 2005; Ramirez-Silva *et al*., 2010).

5.6. Molecular Phylogeny of *Lepus* Species in Ethiopia

5.6.1 *Lepus habessinicus*

Our analysis revealed discordance between mtDNA and nucDNA phylogenies. But the discordance was not only between the two markers but also between nucDNA sequences.

Similar incongruence were also found by other works (Matthee *et al*., 2004; Liu *et al*., 2011b, Melo-Ferreira *et al*., 2012). Hares from Ethiopia were not monophyletic in both mtDNA and nucDNA datasets (Figs. 15-26). Similar non-monophyletic scenarios were documented for hares in the North America (Halanych *et al.,* 1999; Matthee *et al*., 2004; Ramirez-Silva *et al*., 2010) and *Lepus* species in China (Wu *et al.,* 2005; Liu *et al*., 2011b). Two phylogroups were detected for *L. habessinicus* in mtDNA dataset in which phylogroup one (Figs.11-12) contained of individuals from central and eastern Ethiopia: Humbo/Wolaita, Welenchiti, Andido (Afar), Shoa Robit, Kemise, Mieso, Fedis and Ginnir. The second phylogroup (Figs.11-12) contained individuals from southwestern Ethiopia and Rift Valley areas (Hamer, Gewade, Erbore, Abijata Shalla LNP, Ziway and Wonji). It can be stated from mtDNA dataset *L. habessinicus* is a paraphyletic taxon. The non-monophyletic event is not only for *L. habessinicus* but also for both *L. fagani* and *L. starcki* that exhibited similar scenario. Similar paraphyl were also recorded for other species of hares (e.g. *L. castroviejoi*) by Alves *et al*. (2003) and (e.g. *L. capensis* and *L. timidus*) by Liu *et al*. (2011b). In contrast, Cape hare formed a monophyletic group with two subclades, one from North Africa (Tunisia and Egypt) and second from South Africa (RSA) for mtDNA with 82 % bootstrap (Fig.16).

Although hare taxa from Ethiopia were not monophyletic, they are closer to each other than to other taxa included in this analysis. For instance we see a closer relationship between *L. habessincus* and *L. fagani* for both mtDNA and nucDNA results. Our argument is corroborated by the degree of haplotypic sharing between the two taxa and the low genetic differentiation (i.e. both mtDNA and nucDNA). Furthermore, compared to all hares included in our analyses, *L. capensis* (10.6% genetic differentiation) is the second distantly related to *L. habessinicus* next to *L. timidus* (12.2% genetic differentiation) based on pair wise mtDNA genetic distance (Table 10). The higher degree of differentiation between the two taxa agrees with earlier suggestion that *L. habessinicus* from north Somalia and central Ethiopia is considered a good species and should not be placed under *L. capensis* (Azzaroli-Puccetti, 1987b*).* Surprisingly, *L. saxatilis* from South Africa (RSA) was one of the most closely related species to *L. habessinicus* and had low genetic distance for mtDNA and nucDNA (e.g. TF), which is in line with the suggestion of Petter (1963) of closer relationship between the two taxa. Unlike the mtDNA, *L. habessinicus* individuals share haplotypes with *L. capensis* and other species for nucDNA (Figs.17-26). The extent of haplotypic sharing made the phylogenetic relationship between these species complicated. The median joining network of nucDNA (e.g. TF and TG) of *L. habessinicus* and *L. capensis* (North and South Africa) shared haplotypes. In addition, it looks that *L. habessinicus* haplotyppes were basal to that of *L. capensis*. In the case of the UCP2 sequences, *L. habessincus* individuals were almost clearly separated from *L. capensis* individuals with little haplotypic sharing. Therefore, the results of both molecular and morphological analysis of the present study suggest the specific status of *L. habessinicus*.

5.6. 2 *Lepus fagani*

Both mtDNA and nucDNA results revealed a closer affinity of *L. fagani* to *L. habessinicus* and *L. saxatilis.* This is evident from the degree of haplotypic sharing and low pair wise genetic differentiation. The low genetic differentiation between *L. saxatilis* and *L. fagani* is in line with earlier assumption that *L. fagani* belongs to the *L. saxatilis/L. victoriae* complex (Azzaroli puccetti, 1987; Flux and Angermann, 1990; Hoffmann and Smith, 2005). Furthermore, the ranges of *L. fagani* could overlap with that of the *L. saxatilis/L. victoriae* complex in western Ethiopia (see distribution map; Flux and Angermann, 1990). This could contribute to a recurrent gene flow in the overlap zone and result in low genetic differentiation between *L. fagani* and *L. saxatilis*. However, for TG dataset *L. fagani* had a distinct nucDNA relative to *L. saxatilis.* The closer relationship of Ethiopian hare with other hare species from Ethiopia could be plausible since the three species are sympatric in the central highland (Yalden *et al*, 1986, 1996; Flux and Angermann, 1990) and that could contribute for the recurrent gene flow in the overlap zone. A similar low genetic differentiation is documented for Chinese hares (Liu *et al*., 2011b).

Even though *L. fagani* shared more haplptypes with *L. habessinicus* and *L. starcki,* all our Ethiopian hare collections were morphologically distinct from other 2 taxa. The morphological features we had documented fit very well to descriptions made by Azzarolo-Puccetti (1987a,b). The lack of distinct nucDNA and mtDNA haplotypes could reflect an ongoing recurrent introgression in the overlapping zone that could be a recent or an ancient phenomenon (Thulin *et al*., 2003; Alves *et al.*, 2003, 2006, 2007, 2008; Ben Slimen *et al*., 2006, 2007; Liu *et al*., 2011; Melo-Ferreira *et al*., 2005, 2007, 2012). We can say that from the external morphological and skull morphometric characters, *L. fagani* is a distinct taxon relative to both *L. habessinicus* and *L. starcki*. In addition, the SSR analysis of the present study revealed a clear distinction between *L. fagani* and *L. starcki* with almost no sign of nuclear introgression. However, the mtDNA and nucDNA datasets did not completely rule out the earlier suggestion that *L. fagani* is part of the *L. saxatilis/L. victoriae* complex (Azzaroli-Puccetti, 1987a,b; Flux and Angermann, 1990; Hoffmann and Smith, 2005). Understanding the extent of gene flow and the degree of introgression from other hare species from Ethiopia will have a significant role in resolving specific status of *L. fagani*. In addition, there is a need to make further sampling of *L. victoriae* from East Africa to further deal with the evolutionary genetics of *L. fagani* and the *L. saxatilis/ L. victoriae* complexes.

5.6. 3 *Lepus starcki*

Ethiopian highland hare had a distinct mtDNA relative to both *L. europaeus* and *L. capensis* which is supported by the high degree of genetic differentiation and absence of introgression between *L. starcki* and the two taxa. For the nucDNA, *L. starcki* has low genetic distance with some Eurasian species: *L. yarkandensis* and *L. sinensis*. However, *L. starcki* and *L. europaeus* had larger genetic differentiation for nucDNA which is in contrast with previous suggestion that former taxon is subspecies to the latter (Azzaroli-Puccetti *et al*., 1996). In addition, in the mtDNA dataset, the two taxa have genetic differentiation of 4.2% and no sign of introgression and that reflect the mtDNA of *L. starcki* is distinct relative to that of European hare, which is in line with suggestion of Angermann (1983) that *L. starcki* deserve species status. Furthermore, the molecular phylogenetic genetic analysis is in line with the multivariate morphometric skull analysis that *L. starcki* and *L. europaeus* are distinct taxon. However, earlier cytogenetic work by Azzaroli-Puccetti *et al*. (1996) and molecular study by Pierpaoli *et al.* (1999) suggested a closer relationship between *L. starcki* and *L. habessinicus*. Both nucDNA and mtDNA analyses of the present study support closer relationship of *L. starcki* and *L. habessinicus* and this is evident from the extent of haplotypic sharing and low genetic differentiation. But we do not completely rule out previous suggestion that brown hare is a closer relative to *L. starcki*.

Astonishingly, four individuals of *L. starcki* had got similar InDeL in the transferrin dataset as that of *L. castroviejoi, L. comus, L. europaeus, L. oiostolus, L. timidus and L. yarkandensis.* Nevertheless, all individuals of hare species from Africa have deletion at this site. Further, the InDeL is also shared by the outgroups that could mean that the InDeL was ancestral. The presence of the InDeL in *L. starcki* and *L. europaeus* may reflect the plausibility of the suggestion that the two taxa are closely related (Azzaroli-Puccetti, 1987; Azzaroli-Puccetti *et al.,* 1996). In addition, the transferrin results support the suggestion of Pierpaoli *et al*. (1999) that hares from Africa could be ancestral to Eurasian hares.

As can be seen from transferrin network haplotype of *L. stracki* was at the core; however, haplogroups of some Eurasian species such as *L. castroviejoi*, *L. comus*, *L. corsicanus, L. europaeus*, *L. granatensis*, *L. oiostolus* and *L. timidus* are developments from this core. This scenario indicates the ancestral transferrin could be from eastern Africa and *L. starcki* could older than *L. europaeus*. Further TG and UCP2 data indicated that *L. stracki* had distinct nuclear genome relative to that of *L. europaeus*. Although most of nucDNA analyses support earlier suggestion that ancestral *Lepus* could be from North America (Matthee *et al*., 2004; Wu *et al*., 2005; Ramirez-Silva *et al*., 2010), some of the results of the current study indicated that Africa could also be a candidate for ancestral area of *Lepus*.

6. Conclusion and Recommendations

Our primary analysis indicated that hares from Ethiopia were not monophyletic based on both nuclear and mitochondrial fragments. The results of the present study contradict previous suggestions that include *L. habessinicus* under *L. capensis* and *L. starcki* under *L. capensis* or *L. europaeus*. The mtDNA dataset indicate that *L. habessinicus* has distinct mtDNA relative to *L. capensis*. In fact, our nuclear data suggest that *L. habessinicus* is closer to other hare species in Ethiopia. Similarly, the skull morphometric analyses results showed a distinction between *L. habessinicus* and *L. capensis*. However, the extent of nucDNA haplotypic sharing had also made difficult the determination of molecular phylogenetic relationship between *L. habessinicus* and *L. capensis*.

The results of both nucDNA and mtDNA revealed a degree of haplotypic sharing of *L. fagani* with other taxa in Ethiopia. The extent of haplotypic sharing had made the determination of molecular phylogenetic position of the taxon difficult. However, the results of external morphology, skull morphometrics and nuclear microsatellite and nuclear intron of some genes show that *L. fagani* is distinct relative to *L. habessinicus* and *L. starcki*. Although *L. fagani* has closer relationship with *L. habessinicus,* both morphological and molecular analyses support that the taxa are distinct groups. Further, multivariate morphometric skull analyses support specific status of Ethiopian hare relative to savanna hare or scrub hare.

Similarly, *L. starcki* has distinct mtDNA compared to that of *L. capensis* and *L. europaeus*. The nucDNA data set suggest, in contrast to earlier suggestion that *L. stracki* is subordinate *L. europaeus* and perhaps suggests the reverse scenario. In addition to molecular phylogenetic differentiation, the two taxa also showed a differentiation in the multivariate skull morph space. Both the morphological and molecular analyses of the current study support the earlier suggestion by Angermann (1983) that *L. starcki* deserve a status of fully fledged species. The results of the present study indicated the distinctness of *L. starcki* relative to either European hare or Cape hare.

Although the present study shades light on evolutionary genetic relationships among *Lepus* in Ethiopia based on multivariate morphometric and molecular data, there are still a lot of questions to be addressed. Among the issues that make our molecular evolutionary analyses difficult include: the degrees of haplotypic sharing among taxa, incongruence between mtDNA and nucDNA phylogenies and also incongruence among nucDNA phylogenies.

Some of the solutions could be to apply in the future analyses methods that may disentangle differences between ancient and or recent introgressive hybridization. In addition, the identification of hybrid zones and searching for intermediate phenotypes could tell us the extent of ongoing introgression and hybridization among species. The application of super tree analysis and DNA super matrix approaches could be alternative solutions in resolving the phylogenetic relationships among different species to some extent. There is also a need to collect some samples from the area we have not sampled so far for that could have great contribution on one hand to identify the species ranges and on the other hand help to reveal the molecular phylogeography of these species.

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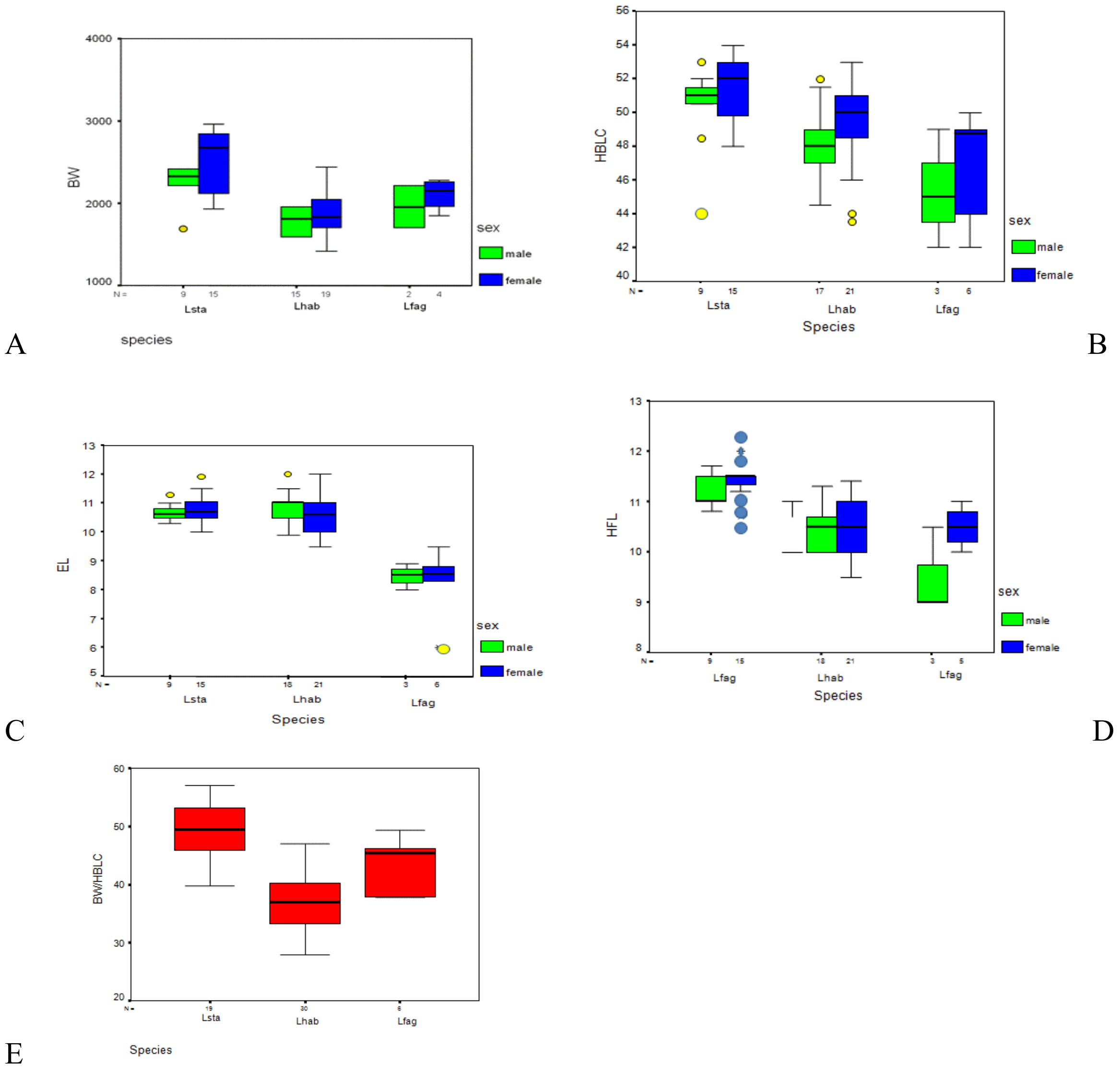
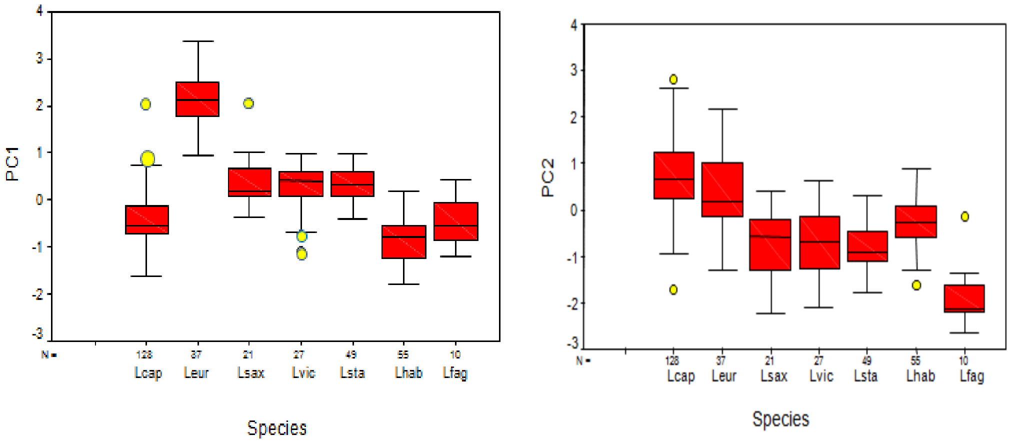
8. Appendices



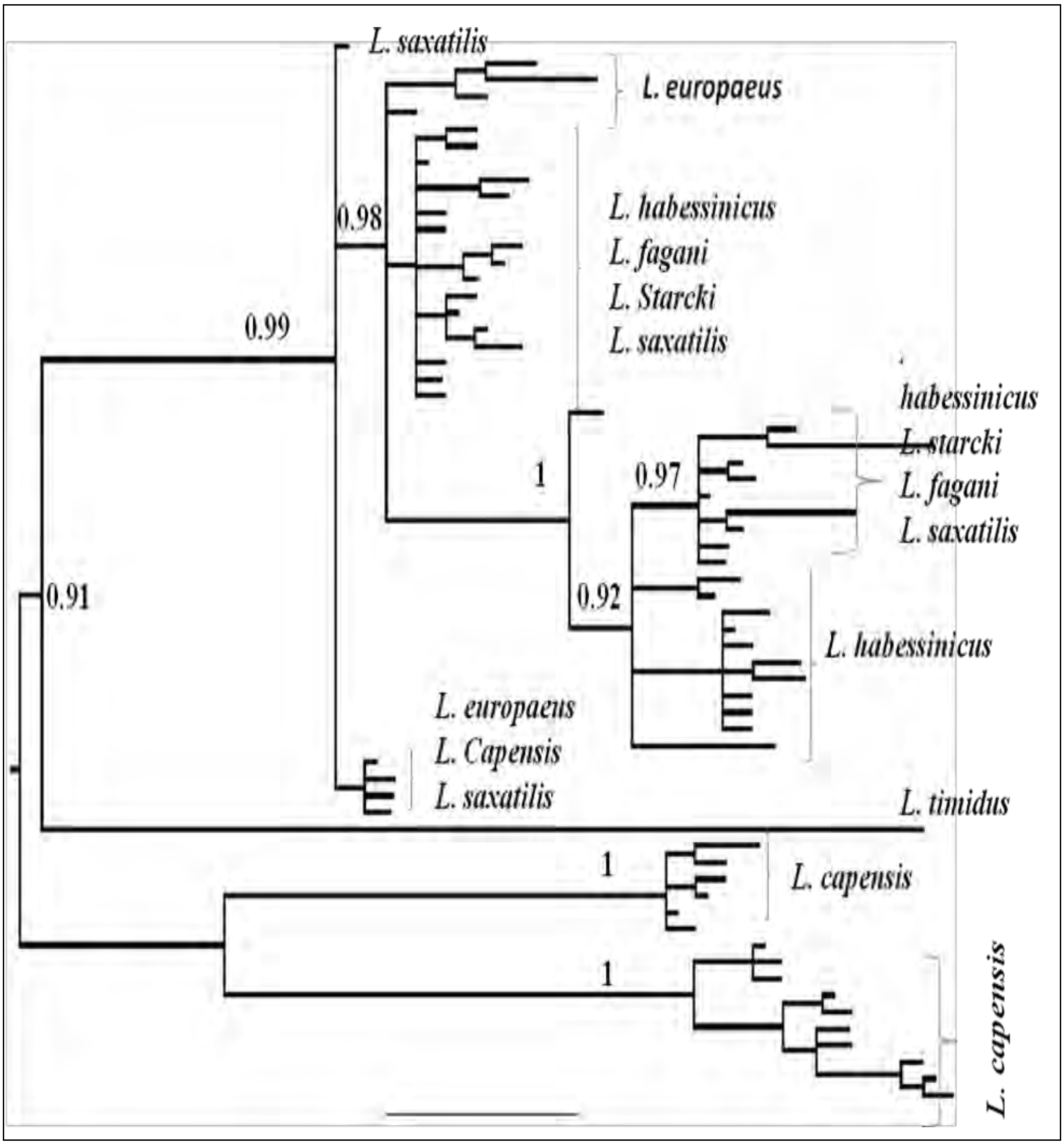
Appendix 1 Figure 1A-E. Box plots of external measurements for *L. starcki* (Lsta), *L. habessinicus* (Lhab) and *L. fagani* (Lfag): body weight (BW), head body length curved (HBLC), ear length (EL), hind foot length (HFL) and BW/HBLC. Each box shows the median, quartiles and extreme values within species.



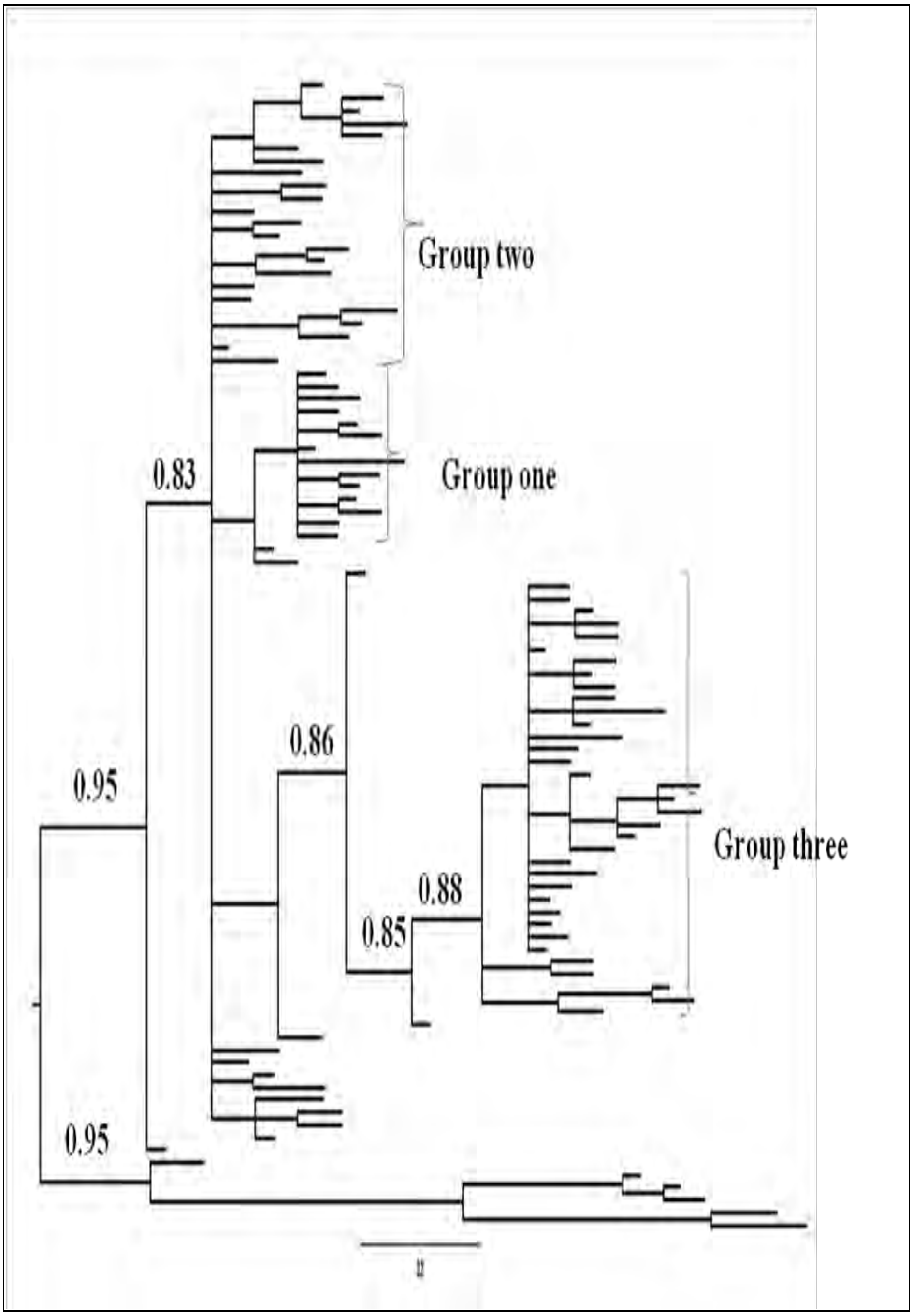
A

B

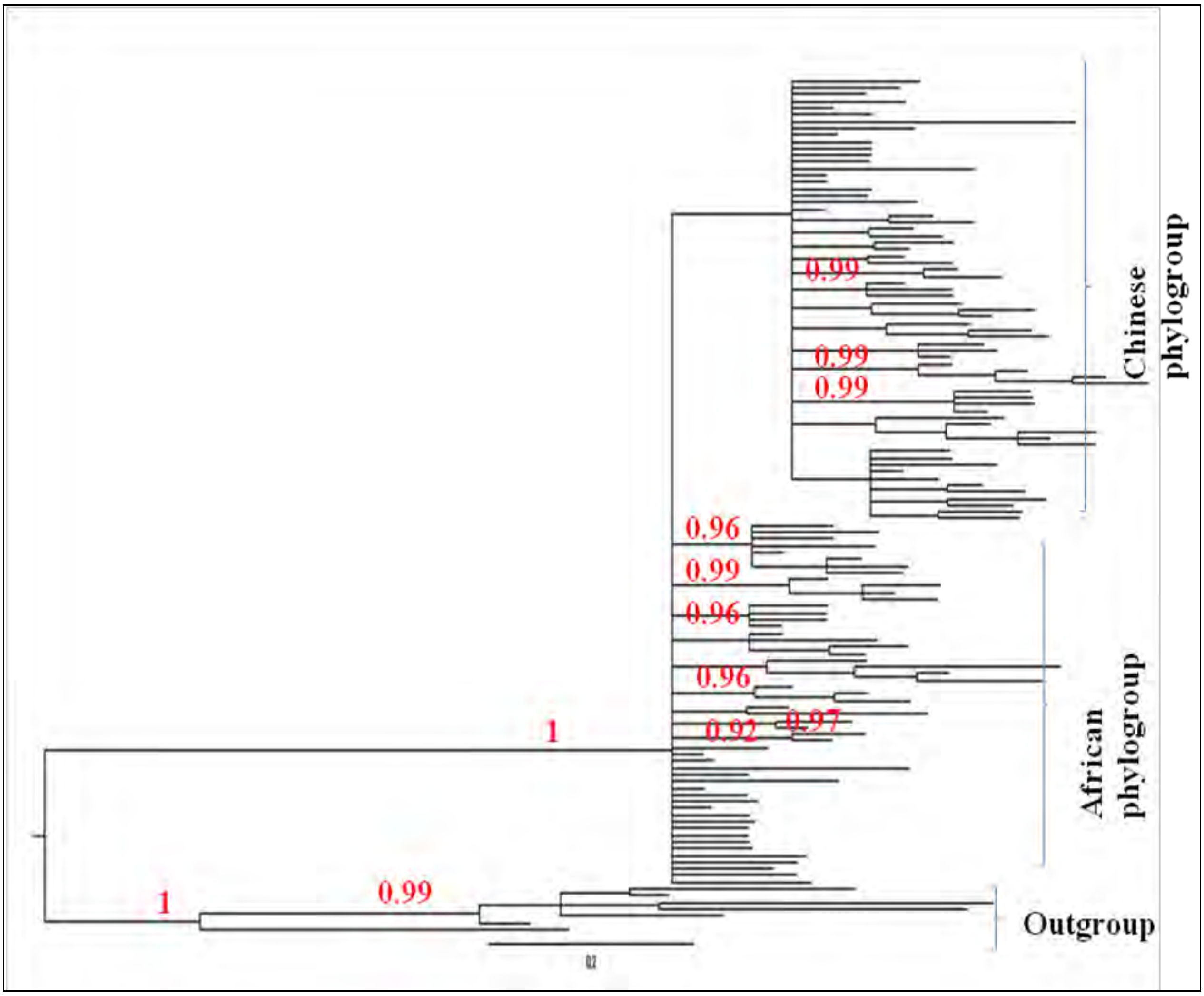
Appendix 1 Figure 2A-B. Box plots of skull morphometric for *L. capensis* (Lcap), *L. europaeus* (Leur), *L. saxatilis* (Lsax), *L. victoriae* (Lvic), *L. starcki*(Lsta), *L. habessinicus* (Lhab) and *L. fagani* (Lfag): principal component 1 (PC1) and principal component 2 (PC2). Each box shows the median, quartiles and extreme values within species.



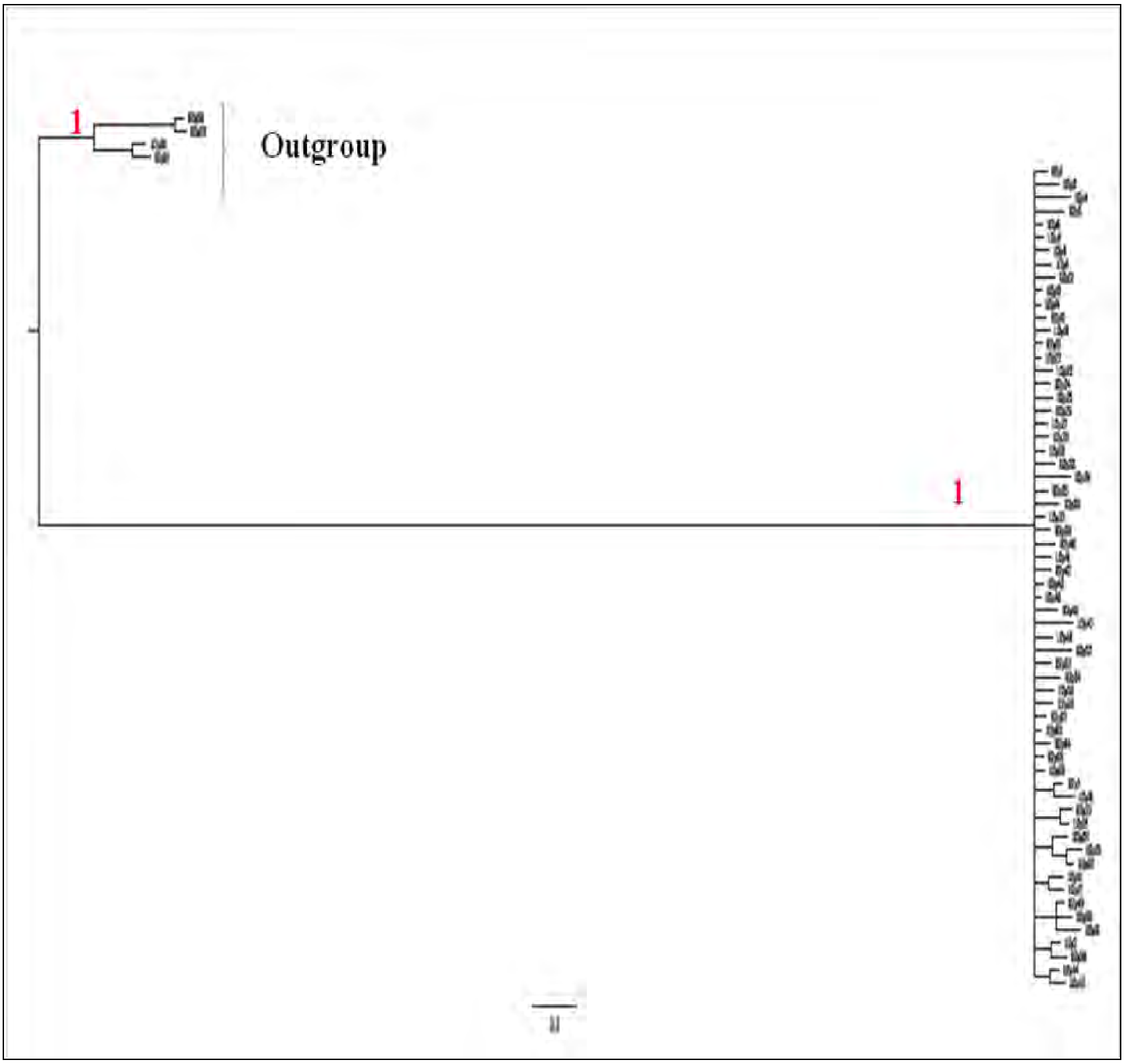
Appendix 1 Figure 3. ATPase subunit 6 (ATP6) fragment Bayesian tree. Numbers above the branch indicate posterior probabilities.



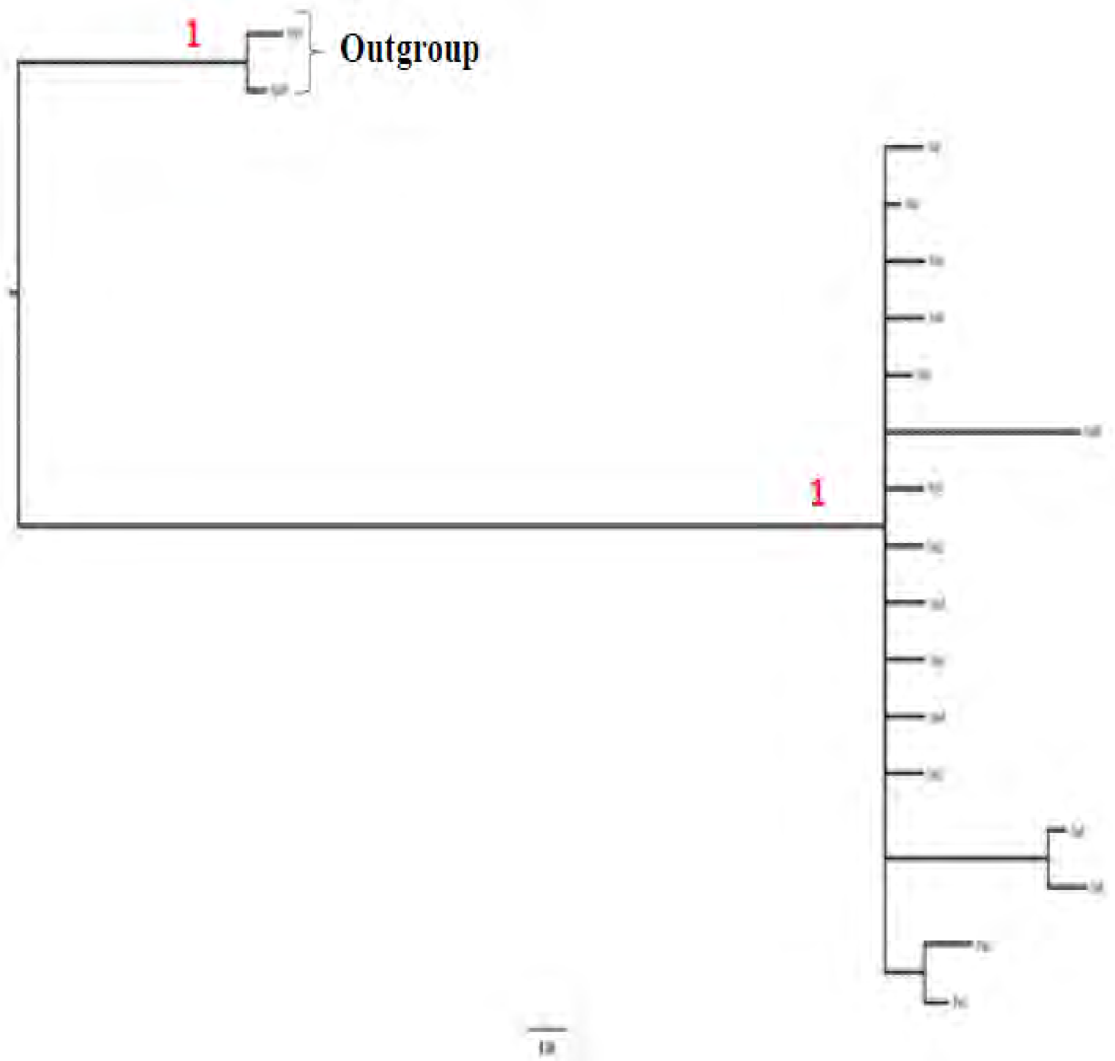
Appendix 1 Figure 4. Transferrin (TF) fragment Bayesian tree. Numbers above the branch indicate posterior probabilities.



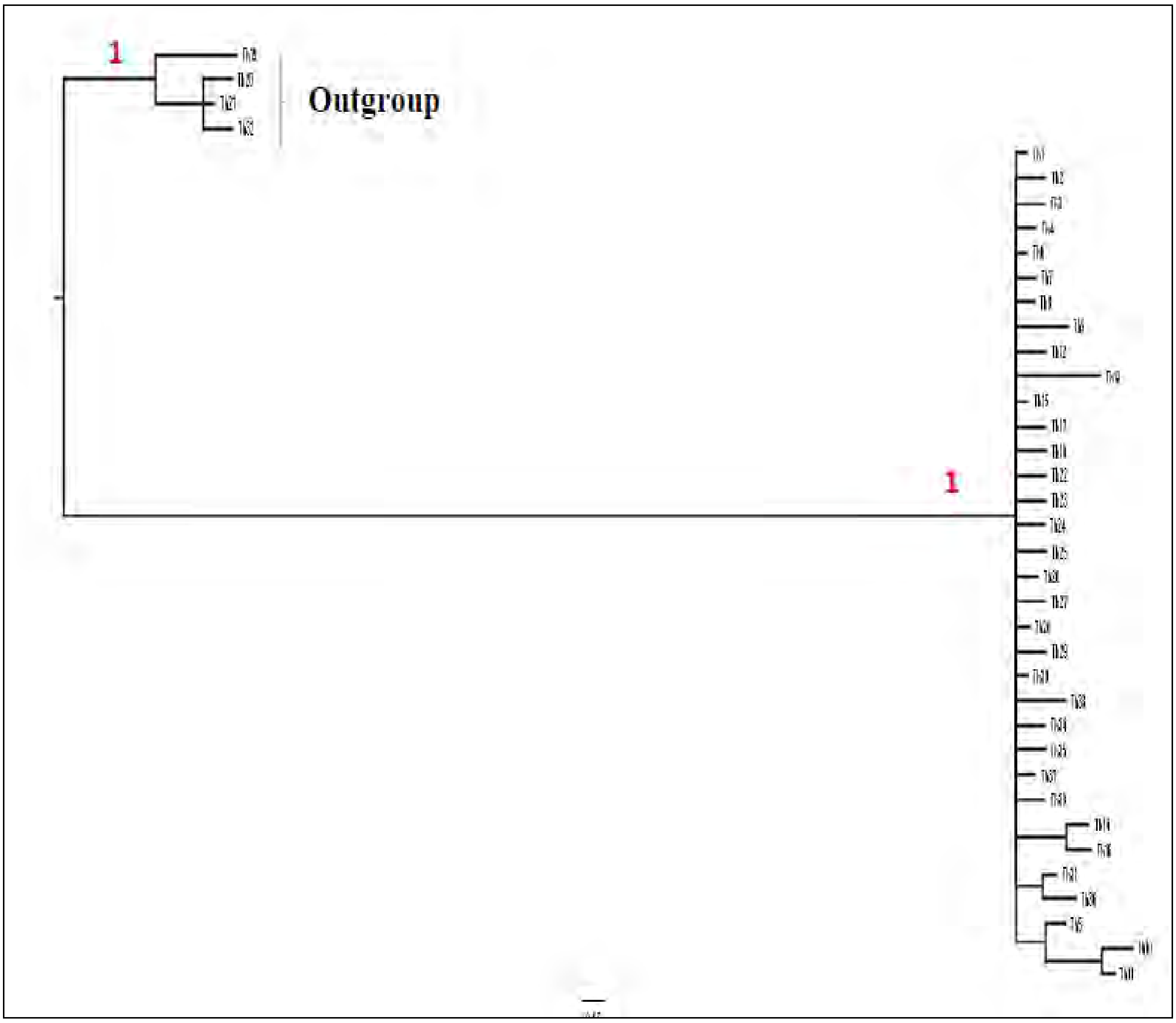
Appendix 1 Figure 5. Thyroglobulin (TG) fragment Bayesian tree. Numbers above the branch indicate posterior probabilities.



Appendix 1 Figure 6. Uncoupling protein two (UCP2) fragment Bayesian tree. Numbers above the branch indicate posterior probabilities.



Appendix 1 Figure 7. Uncoupling protein four (UCP4) fragment Bayesian tree. Numbers above the branch indicate posterior probabilities.



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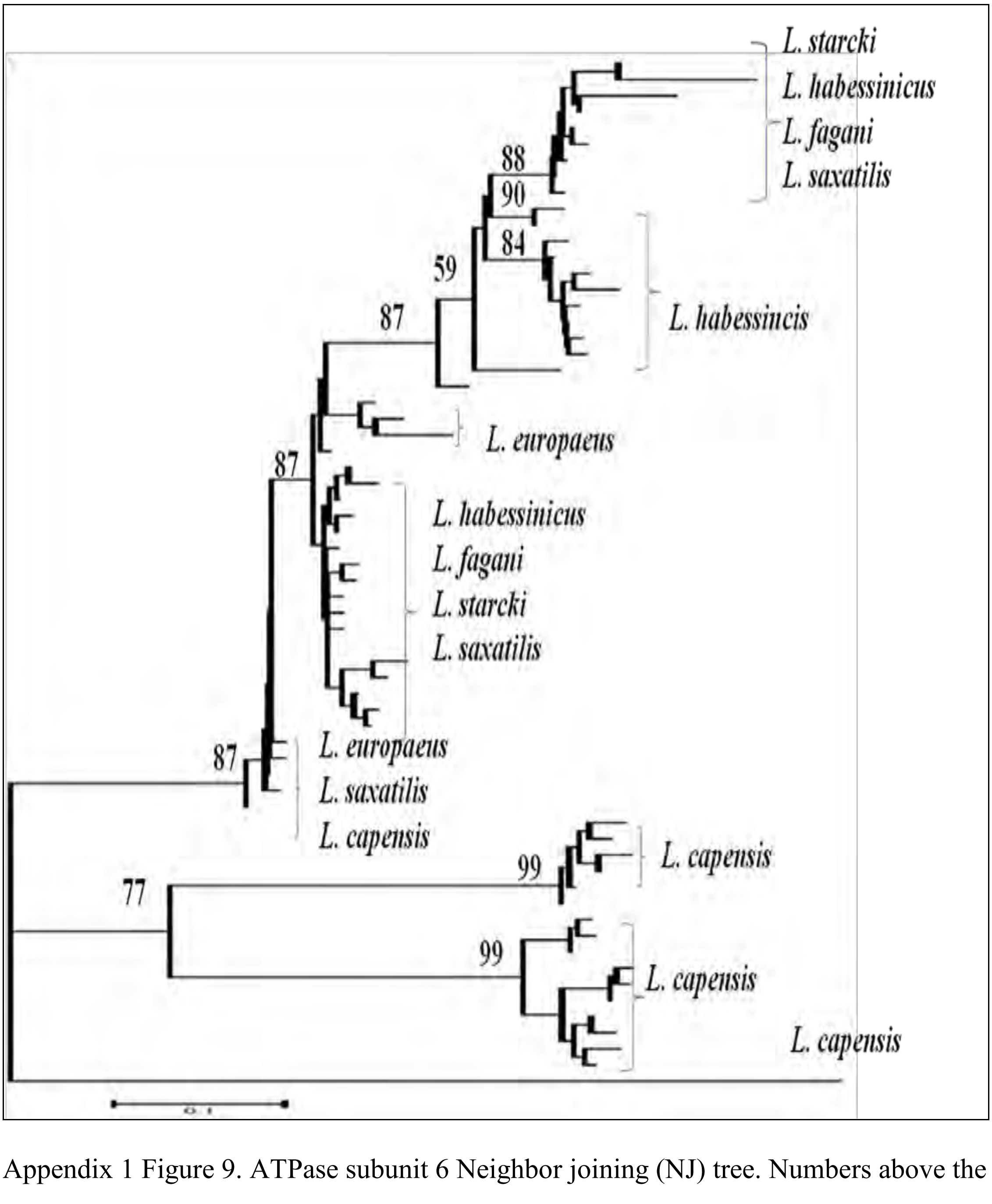
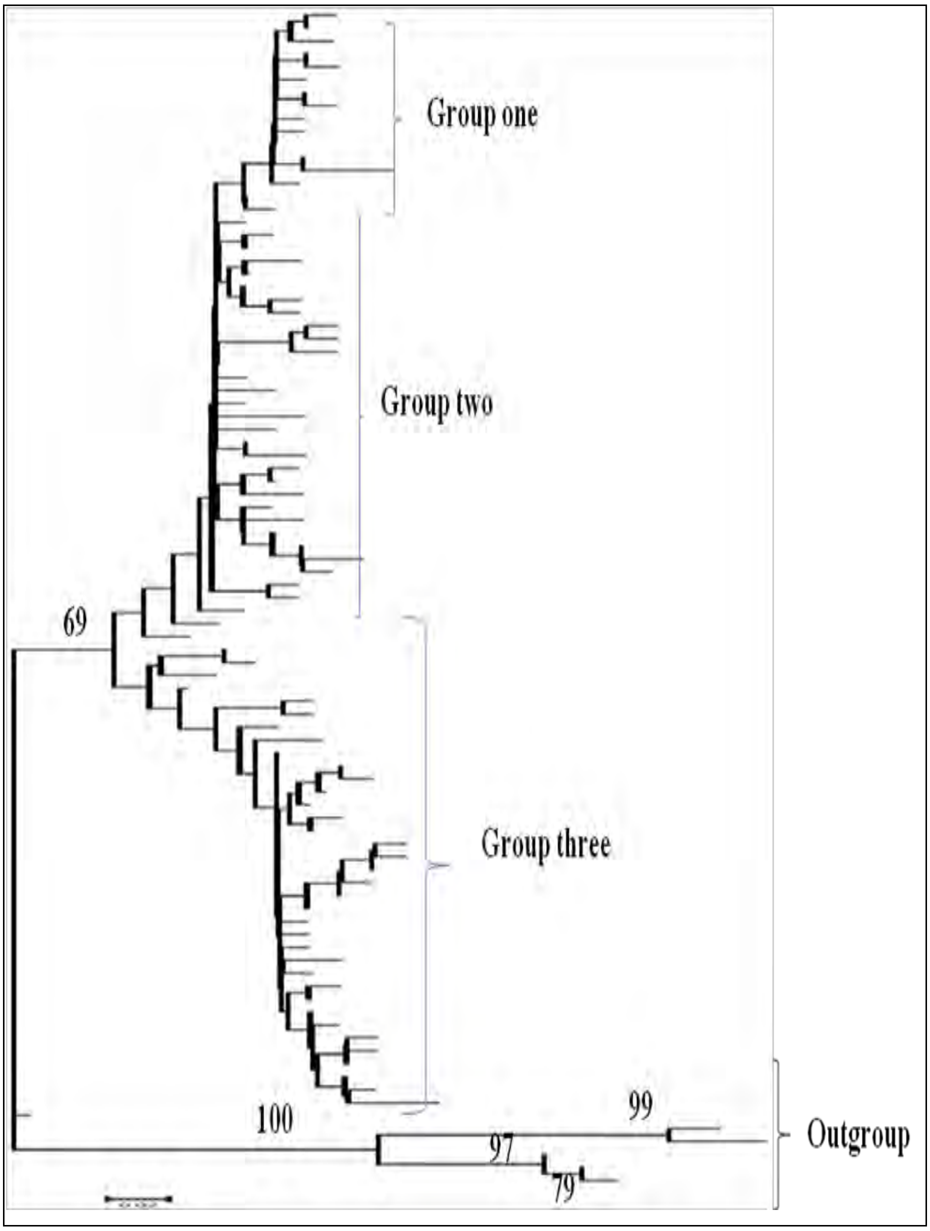
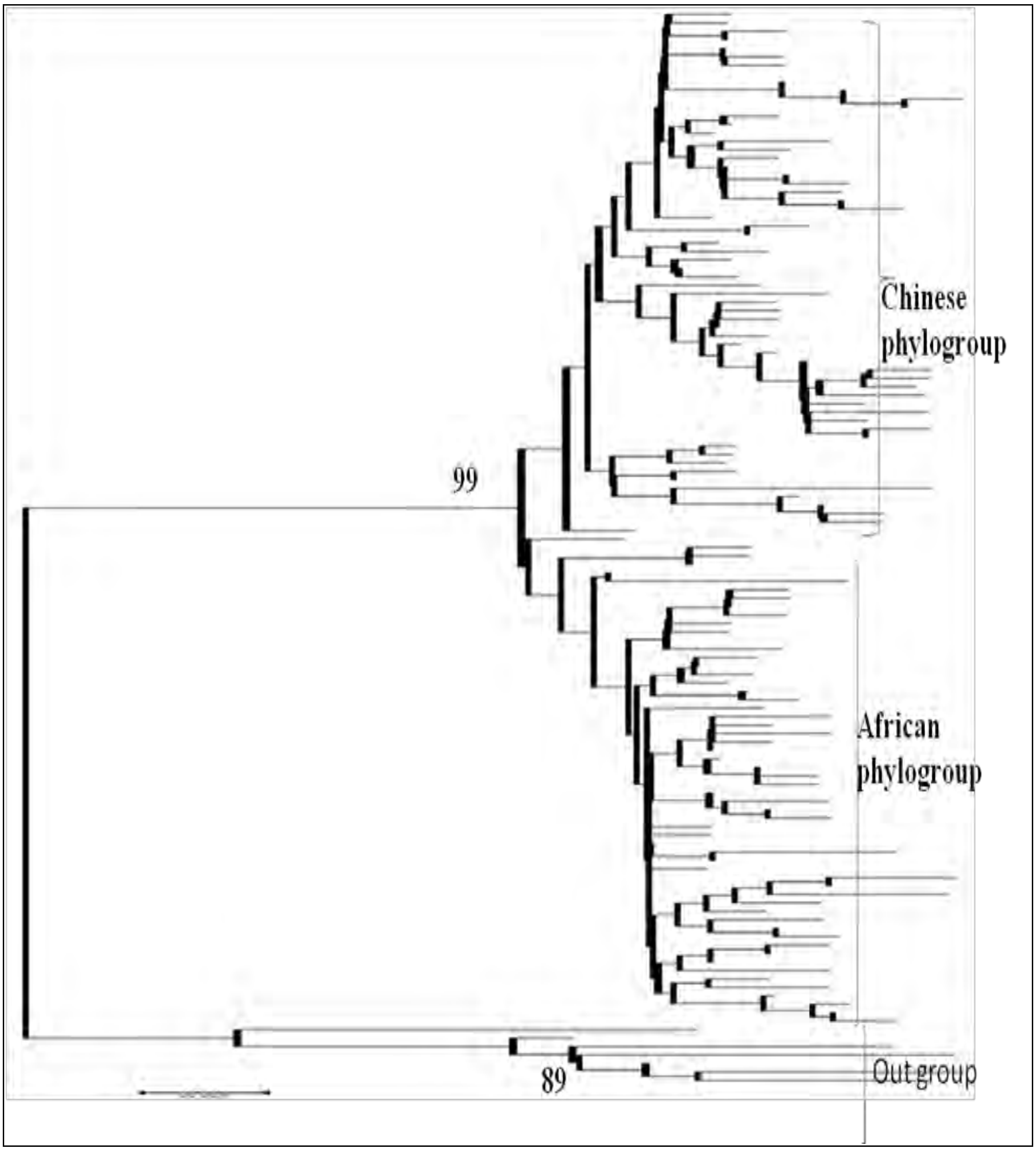
Appendix 1 Figure 8. Thyroid stimulating hormone beta (TSHB) fragment Bayesian tree. Numbers above the branch indicate posterior probabilities.



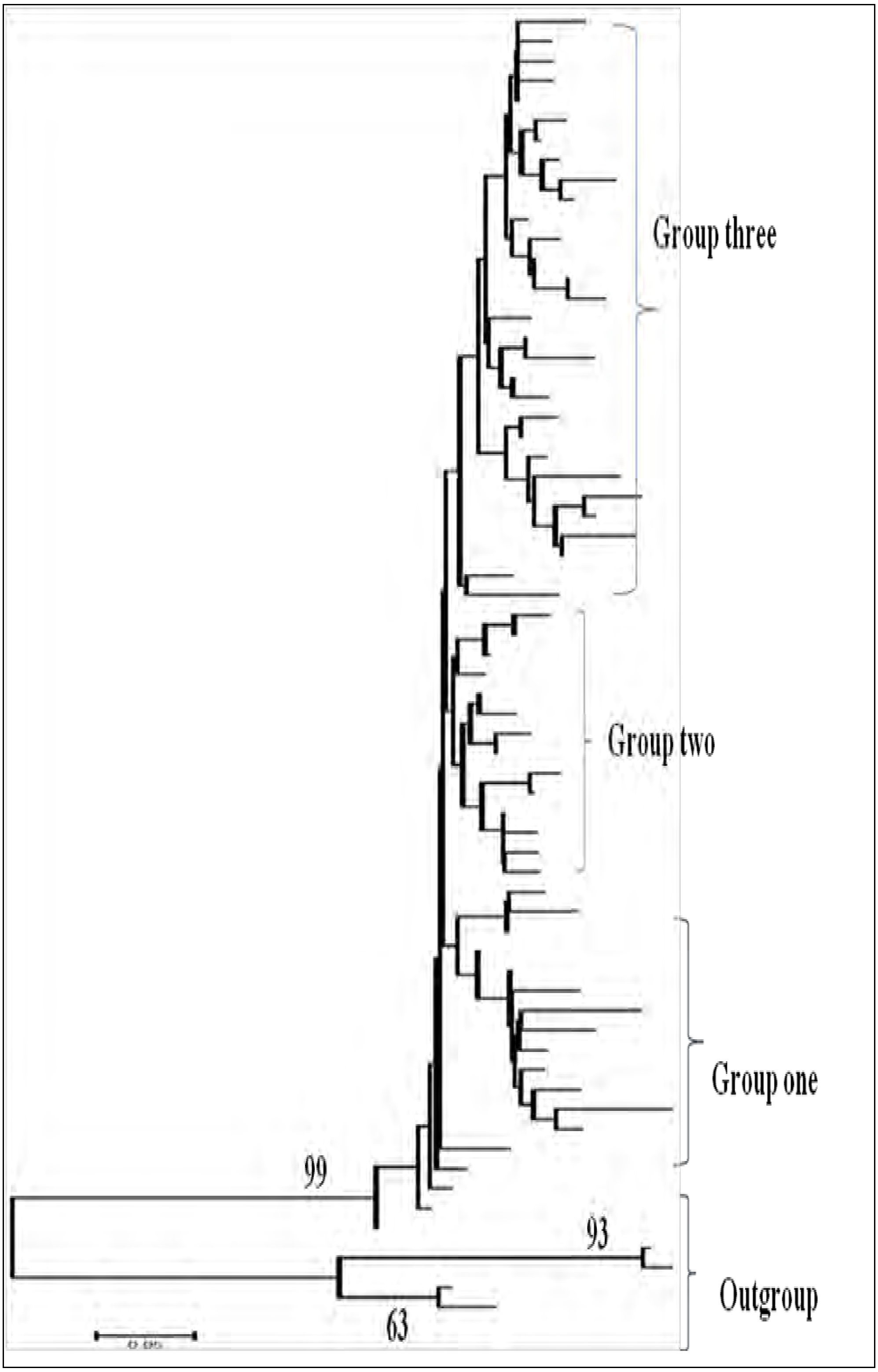
Appendix 1 Figure 9. ATPase subunit 6 Neighbor joining (NJ) tree. Numbers above the branch indicate bootstrap values greater than 50%.



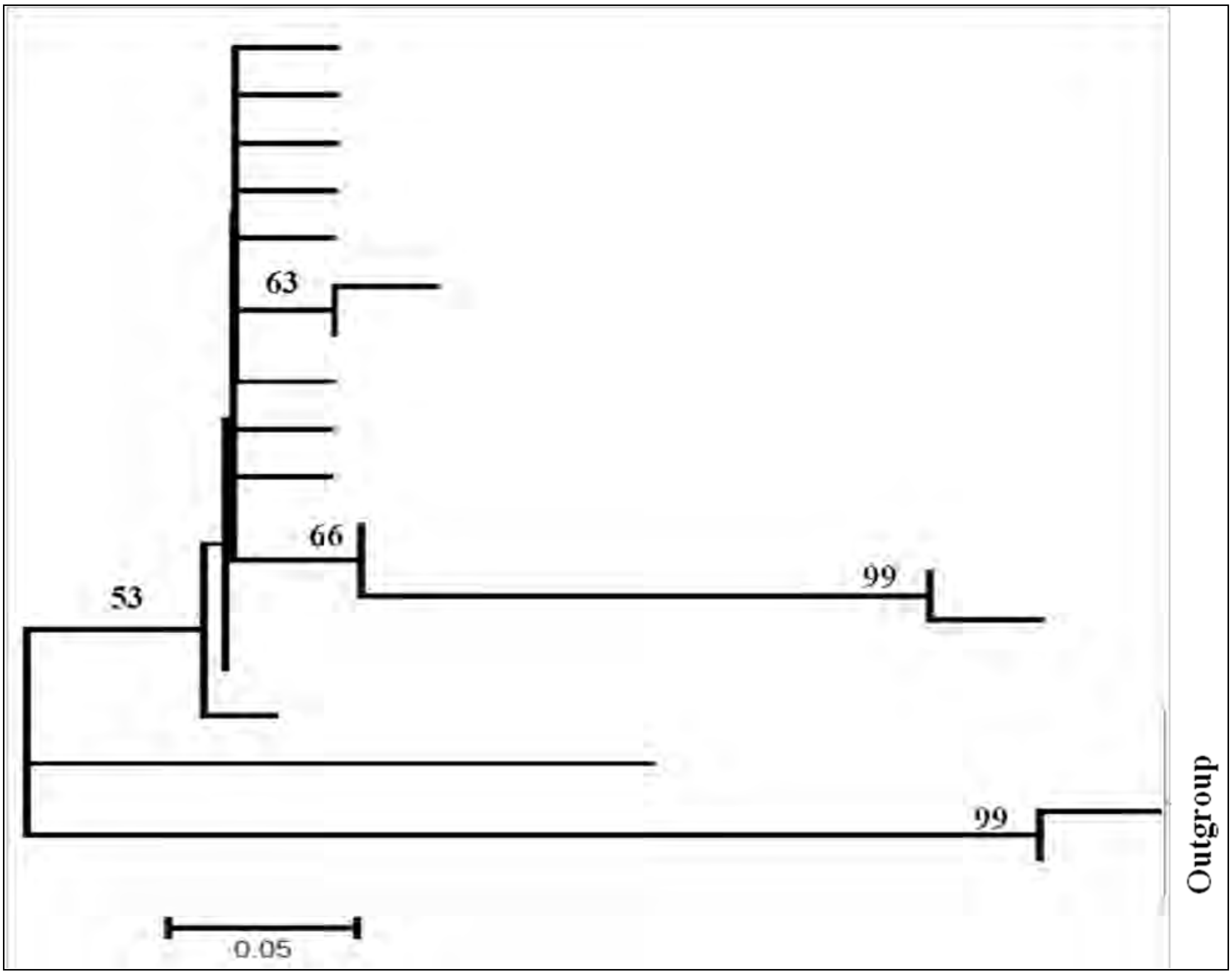
Appendix 1 Figure 10. Transferrin (TF) Neighbor joining tree. Numbers above the branch indicate bootstrap values above 50%.



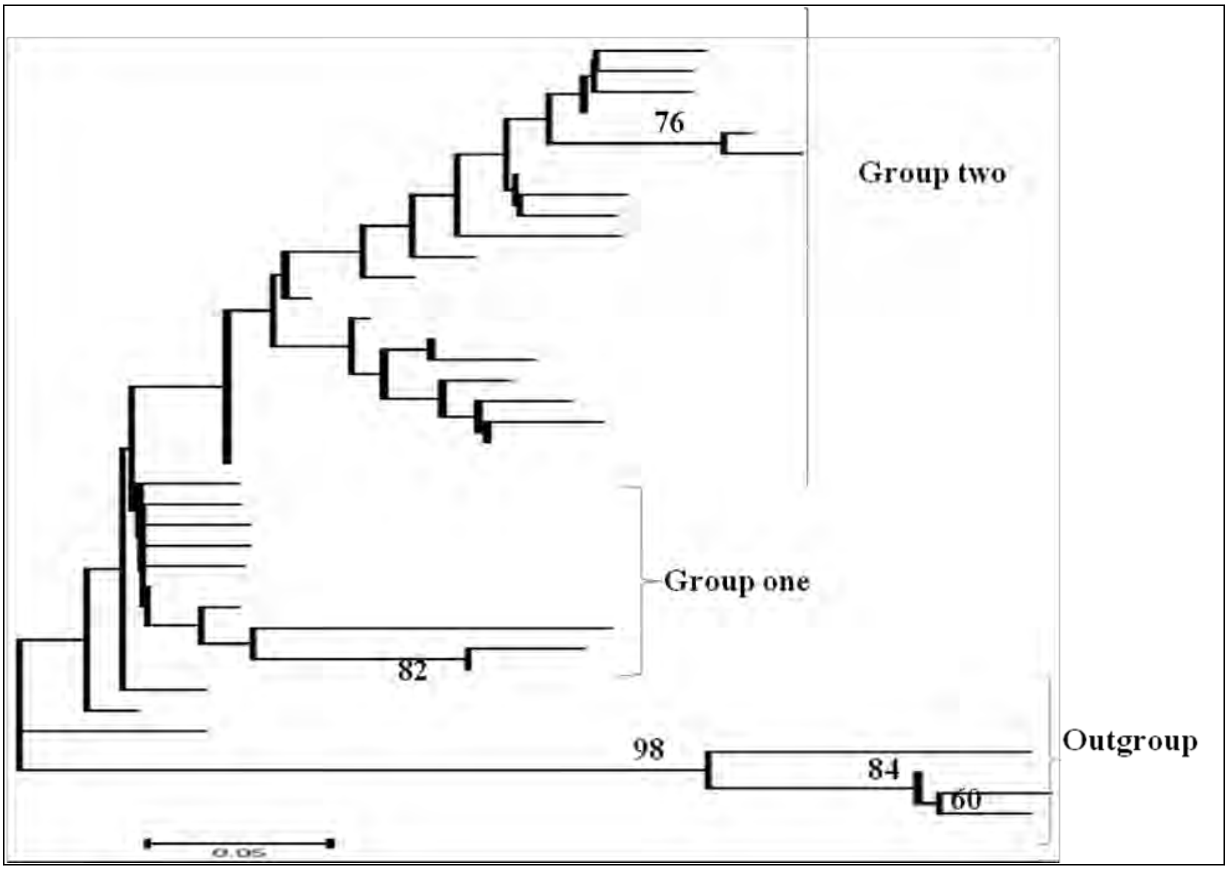
Appendix 1 Figure 11. Thyroglobulin(TG) Neighbor joining tree. Numbers above the branch indicate bootstrap values above 50%.



Appendix 1 Figure 12. Uncoupling protein two (UCP2) Neighbor joining tree. Numbers above the branch indicate bootstrap values above 50%.



Appendix 1 Figure 13. Uncoupling protein four (UCP4) Neighbor joining tree. Numbers above the branch indicate bootstrap values above 50%.



Appendix 1 Figure 14. Thyroid stimulating hormone beta(TSHB) Neighbor joining tree. Numbers above the branch indicate bootstrap values above 50%.

Appendix 2 Table 1 Downloaded Transferrrin (TF; n = 111) sequences *

HM233548	HM233547	HM233546	HM233545	JN037077	JN037076	JN037075	JN037073	JN037072.1	JN037071
JN037070	JN037069	JN037068.	JN037067	JN037066	JN037065	JN037064	JN037063	JN037062	JN037061
JN037060	JN037059	JN037058	JN037057	JN037056	JN037055	JN037054	HM233558	HM233557	HM233556
HM233555	HM233554	HM233553	HM233552	HM233551	HM233550	HM233549	HM233544	HM233543	HM233542
HM233541	HM233540	HM233539	HM233538	HM233537	HM233536	HM233535	HM233534	FJ811637	FJ811636
FJ811635	FJ811633	FJ811632	FJ811631	FJ811630	FJ811626	FJ811627	FJ811628	FJ811629	FJ811621
FJ811622	FJ811623	FJ811624	FJ811625	FJ811615	FJ811616	FJ811617	FJ811618	FJ811619	FJ811620
FJ811634	FJ811614	EU196169	EU196168	EU196167	EU196166	EU196165	EU196164	EU196163	EU196162
EU196161	AY176279	AY176278	AY176276	AY176277	AY176275	AY176274	AY176273	AY176272	AY176271
AY176270	AY176268	AY176267	AY176266	AY176265	AY176264	AY176263	AY176262	AY176261	AY176260
AY176259	AY176257	AY176256	AY176254	AY176253	AY176252	AY176251	AY176269	JN037079	JN037078
AY176280									

*(Alves *et al*., 2003; Liu *et al*., unpublished)

Appendix 2 Table **2** Downloaded Thyroglobulin (Tg; n = 171) sequences *

JF750710	JF750708	JF750706	JF750704	JF750702	JF750700	JF750698	JF750696	JF750694	JF750692
JF750690	JF750688	JF750709	JF750707	JF750705	JF750703	JF750701	JF750699	JF750697	JF750695
JF750693	JF750691	JF750689	JF750687	HM233484	HM233482	HM233480	HM233478	HM233476	HM233474
HM233472	HM233470	HM233468	HM233466	HM233464	HM233462	HM233460	HM233458	HM233456	HM233454
HM233452	HM233450	HM233448	HM233446	HM233444	HM233442	HM233440	HM233438	HM233436	HM233434
HM233432	HM233430	HM233428	HM233426	HM233424	HM233422	HM233420	HM233418	HM233416	HM233414
HM233412	HM233410	HM233408	HM233406	HM233404	HM233402	HM233400	HM233398	HM233396	HM233394
HM233394	HM233388	HM233390	HM233386	HM233384	HM233382	HM233380	HM233378	HM233376	HM233374
HM233372	HM233370	HM233368	HM233366	HM233364	HM233362	HM233360	HM233358	HM233356	HM233354
HM233352	HM233485	HM233483	HM233481	HM233479	HM233477	HM233475	HM233473	HM233471	HM233469
HM233467	HM233465	HM233463	HM233461	HM233459	HM233457	HM233455	HM233453	HM233451	HM233449
HM233447	HM233445	HM233443	HM233441	HM233439	HM233437	HM233435	HM233433	HM233431	HM233429
HM233427	HM233425	HM233423	HM233421	HM233419	HM233417	HM233415	HM233413	HM233411	HM233409
HM233407	HM233405	HM233403	HM233401	HM233399	HM233397	HM233395	HM233393	HM233391	HM233389
HM233387	HM233385	HM233383	HM233381	HM233379	HM233377	HM233375	HM233373	HM233371	HM233369
HM233367	HM233365	HM233363	HM233361	HM233359	HM233357	HM233355	HM233353	HM233351	AY292852
AY292851	AY292850	AY292849	AY292848	AY292847					

*(Matthee *et al*., 2004; Liu *et al*., 2011)

Appendix 2 Table 3 Downloaded UCP2 (n = 54) sequences *

JN037185	JN037184	JN037183	JN037182	JN037180	JN037179	JN037178	JN037177	JN037176	JN037174
JN037173	JN037172	JN037171	JN037170	JN037169	JN037168	JN037166	JN037167	JN037165	JN037163
JN037162	JN037161	JN037160	FJ811587	FJ811586	FJ811585	FJ811584	FJ811583	FJ811582	FJ811581
FJ811580	FJ811579	FJ811578	FJ811577	FJ811576	FJ811575	FJ811574	FJ811573	FJ811572	FJ811571
FJ811570	FJ811562	FJ811564	FJ811565	FJ811567	FJ811569	FJ811568	JN037186	JN037187	FJ811557
FJ811558	FJ811559	FJ811560	FJ811561						

*(Melo-Ferriera *et al*., 2012)

Appendix 2 Table 4 Downloaded UCP4 (n = 48) sequences *

JN037237	JN037236	JN037235	JN037234	JN037233	JN037232	JN037231	JN037230	JN037229	JN037227
JN037226	JN037225	JN037224	JN037221	JN037222	JN037223	JN037218	JN037219	JN037216	JN037214
JN037213	JN037212	JN037211	JN037210	JN037209	JN037208	JN037207	JN037206	JN037205	JN037204
JN037203	JN037202	JN037200	JN037201	JN037199	JN037198	JN037197	JN037196	JN037195	JN037194
JN037193	JN037191	JN037192	JN037238	JN037239	JN037188	JN037189	JN037190		

*(Melo-Ferriera *et al*., 2012)

Appendix 2 Table 5 Downloaded Thyroid stimulating hormone beta(TSHB; n = 52) sequences *

JN037129	JN037128	JN037127	JN037126	JN037125	JN037124	JN037123	JN037122	JN037121	JN037120
JN037119	JN037118	JN037117	JN037116	JN037115	JN037114	JN037113	JN037112	JN037111	JN037110
JN037109	JN037108	JN037107	JN037106	JN037105	JN037104	JN037103	JN037102	JN037101	JN037100
JN037099	JN037098	JN037097	JN037096	JN037093	JN037094	JN037095	JN037090	JN037091	JN037092
JN037089	JN037088	JN037087	JN037086	JN037085	JN037084	JN037083	JN037082	JN037081	JN037080
JN037131	JN037130								

*(Melo-Ferriera *et al*., 2012)

Declaration

I, the undersigned, declare that this Dissertation is based on my original work and that it has not been presented for a degree in any other university. All sources of materials have been duly acknowledged.

Zelalem Gebremariam

Signature:____

This Dissertation has been submitted for examination with my approval as supervisor of the Dissertation.

Professor Endashaw Bekele

Signature:____

Dr Kassahun Tesfaye

Signature:____

Professor Dr G. B. Hartl

Signature:____

Dr Abebe Getahun, AAU

Signature:____

Dr Franz Suchentrunk,

Signature:____