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Characterisation of selected *Culicoides* (Diptera: Ceratopogonidae) populations in South Africa using genetic markers

by

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ABSTRACT

Culicoides (Diptera: Ceratopogonidae) are small (<3mm) blood feeding flies. These flies are biological vectors of viruses, protozoa and filarial nematodes affecting birds, humans, and other animals. Among the viruses transmitted those causing bluetongue (BT), African horse sickness (AHS) and epizootic haemorrhagic disease (EHD) are of major veterinary significance. *Culicoides* (*Avaritia*) *imicola* Kieffer, a proven vector of both AHS and BT viruses, is the most abundant and wide spread livestock-associated *Culicoides* species in South Africa. Field isolations of virus and oral susceptibility studies, however, indicated that a second *Avaritia* species, *C. bolitinos* Meiswinkel may be a potential vector of both BT virus (BTV) and AHS virus (AHSV). Differences in oral susceptibility, which are under genetic control, of populations from different geographical areas to viruses may be an indication of genetic differences between these populations, which may be the result of limited contact between these populations. A good knowledge of the distribution, spread and genetic structure of the insect vector is essential in understanding AHS or BT disease epidemiology.

In the present study, an effort was made to gather field specimens of both *C. imicola* and *C. bolitinos* from different areas within their natural distribution in South Africa. The aim was to partially sequence two mitochondrial genes from these specimens and to analyse the sequence data making use of phylogenetic trees to clarify the genetic relationships between individuals or groups collected from geographically distinct sites. The two species were collected from four geographically separated areas in South Africa viz. Gauteng Province, Eastern Cape Province, Western Cape Province as well as the Free State Province. DNA was extracted from a total of 120 individual midges of the two *Culicoides* species using DNA extraction kits. Extracted DNA was analysed using PCR, sequencing as well as phylogenetic methods.

A total of 117 mitochondrial DNA COI and 104 mitochondrial 16S ribosomal RNA *Culidoides* sequences were analysed. DNA sequence polymorphism and

phylogenetic relationships of various groups of *C. imicola* and *C. bolitinos* midges were determined. The results of the phylogenetic analysis of *Culicoides* populations using mitochondrial COI gene fragment showed that, at least one subpopulation of *C. imicola* and two distinct genotypes of *C. bolitinos* species do exist in South Africa, and further analysis is necessary. This study showed that COI has the potential to separate *Culicoides* midges based on their geography.

KEY WORDS: African horse sickness, bluetongue, *Culicoides*, mitochondrial DNA, sequences, cytochrome oxidase subunit I (COI), 16S rRNA, *C. imicola*, *C. bolitinos*, South Africa

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LIST OF ABBREVIATIONS

AHS	African horse sickness
AHSV	African horse sickness virus
bp	base pair
BT	Bluetongue
BTV	Bluetongue virus
COI	Cytochrome Oxidase subunit I
COII	Cytochrome Oxidase subunit II
ºC	degree Celsius
DNA	Deoxyribonucleic acid
DnaSP	DNA Sequence Polymorphism
dNTP	deoxynucleoside triphosphate
dsRNA	double stranded Ribonucleic acid
EDTA	Ethylene di-amine-tetra-acetate
EE	Equine encephalosis
EEV	Equine encephalosis virus
EHD	Epizootic hemorrhagic disease
EHDV	Epizootic hemorrhagic disease virus
Fig	figure
g	gram
Hd	haplotype diversity
ITSI	Internal transcribed spacer I
ITS2	Internal transcribed spacer 2
km	kilometre
µg	microgram
µl	microlitre
µM	micromolar
ml	millilitre
mm	millimetre

mM	millimolar
mtDNA	mitochondrial DNA
MEGA	Molecular Evolutionary Genetic Analysis
ng	nanogram
Pi	nucleotide diversity
OIE	Office International des epizooties
OVI	Onderstepoort Veterinary Institute
pmol	picomole
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA marker
rRNA	Ribosomal Ribonucleic acid
s	seconds
ss	senso stricto
SSCP	single stranded conformation polymorphism
SA	South Africa
sp	species
SD	standard deviations
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
UV	ultraviolet
U	unit
V	Volt
v	volume
W	Watt
W/V	Weight per Volume
16S	16 subunit

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Culicoides biting midges are small blood feeding flies 1 to 3 mm in size (reviewed by Meiswinkel *et al.* 2004a) which play an important role as vectors of several disease agents such as nematodes, protozoa and viruses (Linley 1985; Meiswinkel *et al.* 2004a). *Culicoides* females feed on a broad spectrum of hosts including reptiles, mammals, birds, man (Meiswinkel *et al.* 2004a) and will even feed on blood-engorged mosquitoes (Wirth & Hubert 1989). They are regarded as pests of great veterinary importance (Ludivik *et al.* 2005). Bites from some species of *Culicoides* can cause summer seasonal recurrent dermatitis referred to as sweet-itch (Braverman 1988). Orbiviruses are the most important pathogens vectored by *Culicoides* species.

Genetic analysis has become a vital tool in vector and vector borne disease research. It can help to clarify the taxonomy of vectors including systematics and classification. It can assist in phylogenetics and population genetic studies as well as determining the geographical distribution and dispersal of midge species. Defining the genetic differences and similarities between vectors species and populations will help to clarify vector dispersal which might be linked to viral spread, vector capacity and vector competence. A good knowledge of the latter can make the combat of the diseases spread by *Culicoides* vectors more efficient.

1.2 Species Classification

The genus *Culicoides* belongs to the Ceratopogonidae family within the Order Diptera. Worldwide there are more than 1 400 described *Culicoides* species grouped into at least 38 subgenera (Borkent & Wirth 1997). The most important vectors are found within the *Culicoides Imicola* complex of the subgenus *Avaritia*

Fox, 1955 which comprises of at least 13 species of which four are yet undescribed. The nine described species are *Culicoides imicola* ss (senso stricto) Kieffer 1913, C*ulicoides brevitarsis* Kieffer 1917, *Culicoides pseudopallidepennis* Clastier 1958, *Culicoides nudipalpis* Delfinado 1961, *Culicoides bolitinos* Meiswinkel 1989, *Culicoides miombo* Meiswinkel 1991, *Culicoides loxodontis* Meiswinkel 1992, *Culicoides* sp.# 107 (= *C. kwagga*, Meiswinkel, unpublished thesis 1995), and *Culicoides tuttifrutti* Meiswinkel, Cornet and Dyce 2003.

1.3 Geographical distribution of *Culicoides*

Culicoides midges have a worldwide distribution and are found in all countries on every continent except Antarctica, New Zealand and Hawaii (Mellor *et al.* 2000). Specific species have been identified as dominant vectors in broad geographical regions of the world: In southern Europe *Culicoides* (*Avaritia*) *obsoletus* Meigen and *C. imicola*, northern Europe *C. obsoletus*, North America *Culicoides* (*Monoculicoides) sonorensis* Wirth & Jones, South America *Culicoides* (*Hoffmania*) *insignis* Lutz, Australia *Culicoides* (*Avaritia*) *wadai* Kitaoka and *Culicoides* (*Avaritia*) *brevitarsis* Kieffer*,* and the most widely distributed species in Africa is *C. imicola* (Tabachnick 2004).

Apart from being widespread in Africa, *C. imicola* is also present in the Near, Middle and Far East as far as southern China, Laos and Vietnam (Meiswinkel 1989; Meiswinkel *et al.* 2004a). Although morphological studies have identified *C. imicola* from Italy, Spain, Portugal, Israel and South Africa as being a single species (Meiswinkel 1989; Meiswinkel & Baylis 1998), it has been shown that *C. imicola* populations from different geographic locations could be distinguished genetically (Nolan *et al.* 2004). A subdivision was observed between *C. imicola* populations from the western and eastern Mediterranean using COI sequences (Nolan *et al.* 2008).

1.3.1 South Africa

Of the more than 112 *Culicoides* species identified in South Africa over the past 35 years (Meiswinkel *et al.* 2004b), *C. imicola* is the only proven vector of the viruses that cause AHS and BT. Nevill (1971) has shown that *C. imicola* was the most abundant livestock associated species in the Onderstepoort area. Subsequent light trap surveys confirmed *C. imicola* to be the most abundant livestock-associated *Culicoides* species in the summer rainfall areas, especially in the warm frost-free areas of the country (Meiswinkel 1989; Venter *et al.* 1996). It was also dominant in the winter rainfall areas of the country (Venter *et al.* 2006b). *Culicoides imicola* cannot be regarded as the only vector of orbiviruses because it is relatively uncommon in warm/dry and cool/wet areas (Venter 1991; Venter *et al* 1996). Members of the *Culicoides* (*Remia*) *schultzei* group and *Culicoides* (*Hoffmania*) *zuluensis* de Meillon were the most abundant species in the latter areas (Venter 1991; Venter *et al.* 1996). Some of the abundant and widely distributed *Culicoides* species might be of less importance as potential vectors of livestock viruses due to their host preference for birds and/or restricted breeding site preferences (Nevill *et al*. 1992). Although the genetic relationships between populations of single species from different parts of the world were established (Nolan *et al.* 2004; Nolan *et al.* 2008), the genetic relationship between midges of the same species from different geographic localities within a single country is not known. *Culicoides imicola* and *C. bolitinos* are the most widespread and abundant members of the Imicola complex in South Africa.

1.3.1.1 *Culicoides* (*Avaritia*) *imicola* Kieffier, 1913

In South Africa *C. imicola* is the only member of the Imicola complex which was successfully used in transmission of an orbivirus (Du Toit 1944; Wetzel 1970) and is therefore the only proven vector of orbiviruses in the country. *Culicoides imicola* can be found in low numbers in the cooler regions of the country (Baylis *et al*. 1998; Meiswinkel *et al.* 2004b) (Fig. 1.1). *Culicoides imicola* can be extremely variable in prevalence and abundance ranging from being totally absent (Meiswinkel 1997) to being widespread and super abundant in the vicinity

of livestock (Meiswinkel 1998) (Fig. 1.1). As an abundant and important vector species, its distribution has been well studied (Venter *et al*. 1996; Baylis *et al.* 1998;1999; Meiswinkel *et al.* 2004b). The distribution and abundance of *C. imicola* is affected by climatic factors such as temperature, aridity, topographic slope (inducing water run-off), soil type (slow or rapid drainage) and soil fertility (presence of specific microorganisms) (Baylis *et al.* 1998; 1999).

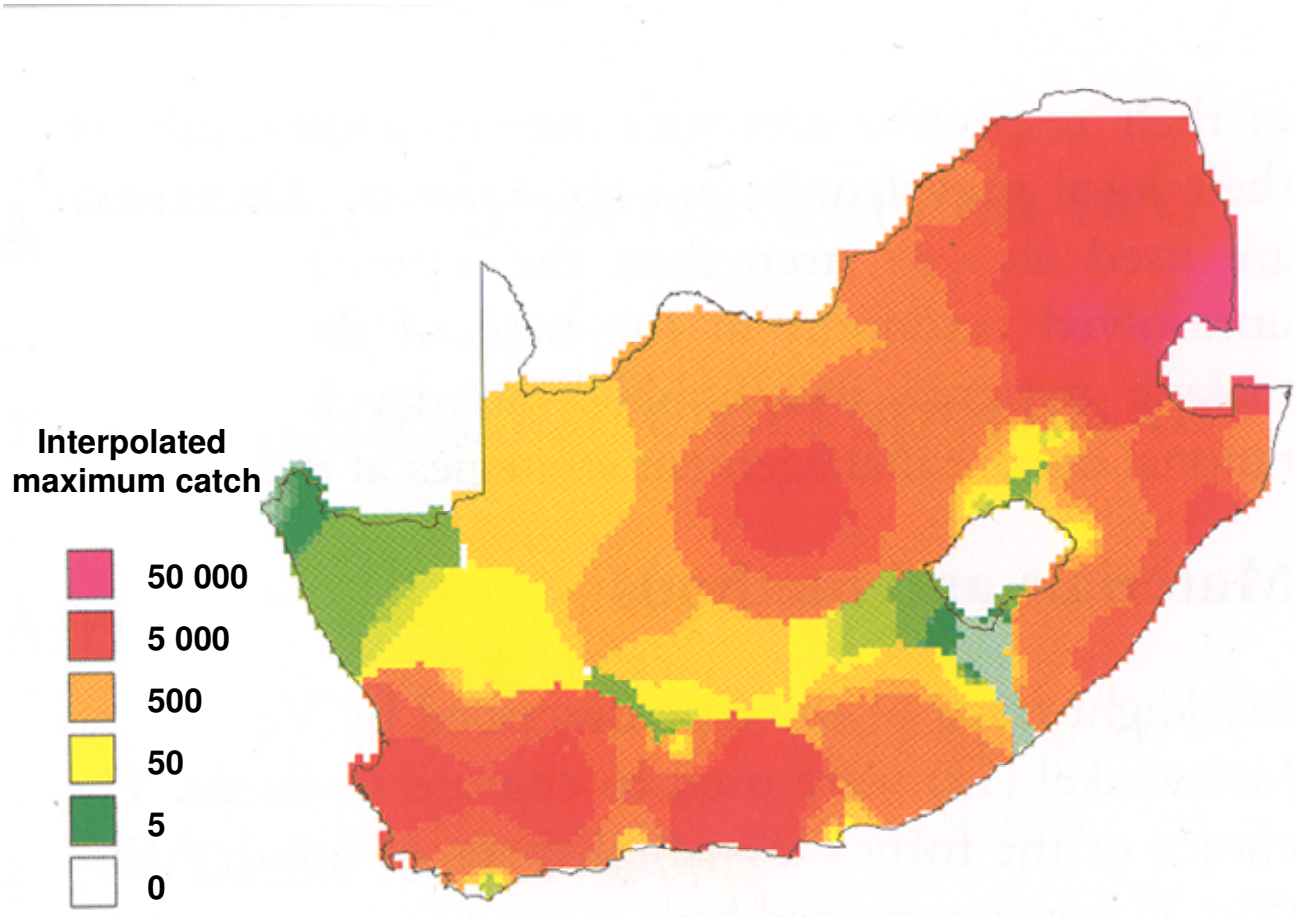


Figure 1.1: Inverse distance weighing interpolated maximum catches of *Culicoides imicola,* using a search radius of 200 km. The results are based on weekly light trap collections made at 40 sites from September 1996 to August 1998 (Meiswinkel *et al.* 2004b)

1.3.1.2 *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel, 1989

Another species of the Imicola Complex, *C. bolitinos,* has emerged in the last decade as a potential vector of bluetongue virus (BTV) and African horse sickness virus (AHSV) in South Africa, especially in higher-lying parts where cooler conditions prevail and *C. imicola* are less abundant (Meiswinkel & Paweska 2003). Its role as a field vector is supported by laboratory oral susceptibility studies (Venter *et al.* 1998; Venter *et al.* 2000). However, it is not yet proven that it can transmit any virus.

Culicoides bolitinos was described in 1989 after it was initially confused with both *C. imicola* and *C. brevitarsis* (Meiswinkel 1989). It was only distinguished from *C. imicola* after it was found to breed in African buffalo (*Syncerus caffer*) dung in the Kruger National Park in South Africa (Meiswinkel & Dyce 1989). *Culicoides bolitinos* is restricted to the tropical and subtropical regions of Africa south of the Sahara desert. This includes South Africa, Botswana, Zimbabwe, Lesotho, Malawi, Kenya, Nigeria, the Ivory Coast, Gambia, Madagascar and Mauritius (Meiswinkel 1989; Meiswinkel *et al.* 2004a). It is widespread in South Africa and has been found in most areas where *C. imicola* occurs (Meiswinkel *et al.* 2004a) (Fig. 1.2). It is believed that *C. bolitinos* will concentrate in areas where there are bovines as its main breeding site is bovine dung. Its prevalence and seasonal abundance has only been studied in South Africa (Meiswinkel 1989) and it was determined that *C. bolitinos* is about 10 times less abundant than *C. imicola* (Figs 1.2 & 1.3). Due to its wide temperature stable breeding habitat, bovine dung, it can be more abundant than *C. imicola* in the cooler parts of the country (Meiswinkel *et al.* 2004b).

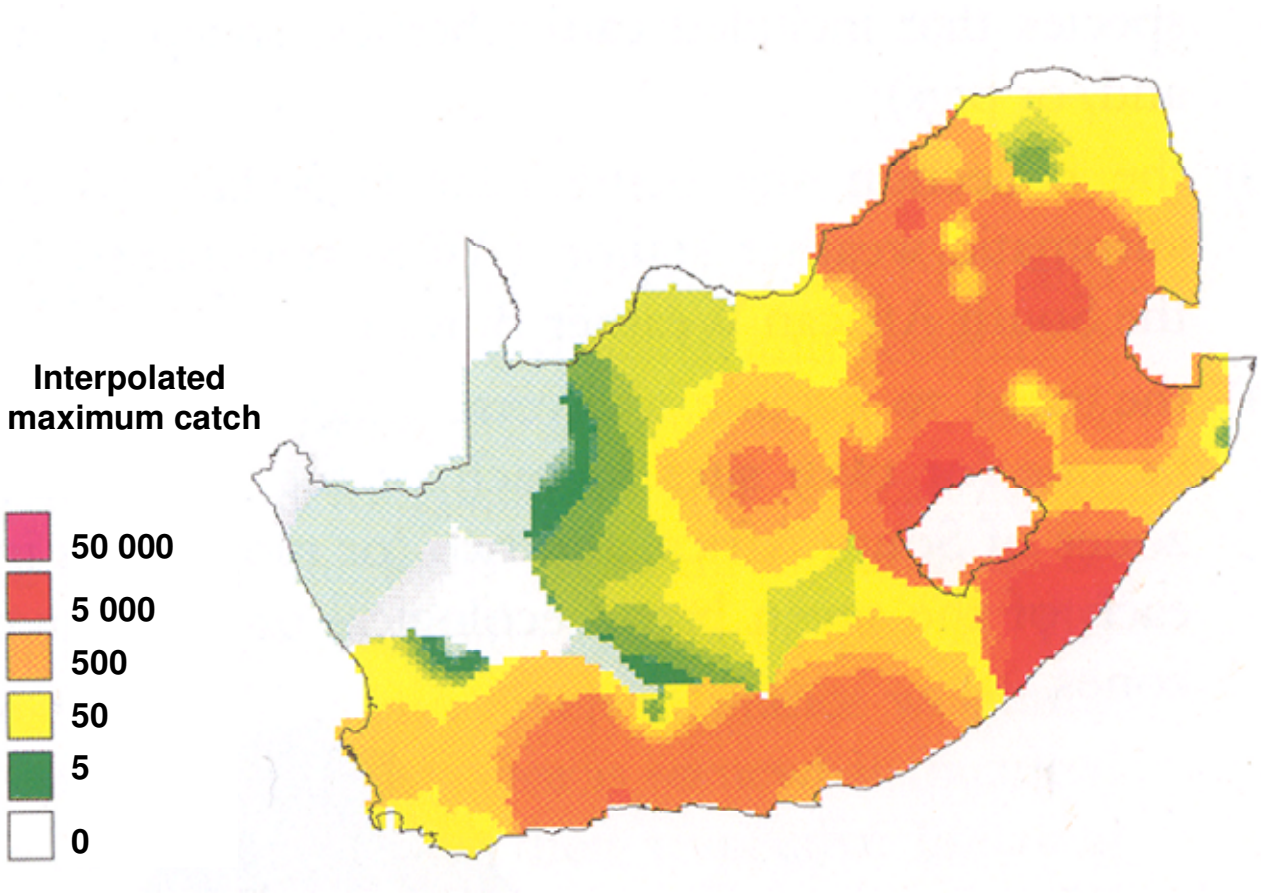


Figure 1.2: Inverse distance weighing interpolated maximum catches of *Culicoides bolitinos,* using a search radius of 200 km. The results are based on weekly light trap collections made at 40 sites from September 1996 to August 1998 (Meiswinkel *et al.* 2004b)

1.4 Disease transmission

Of the more than 1 400 described *Culicoides* species, about 96% are obligatory blood feeders on mammals and birds (Meiswinkel *et al.* 2004a). Fewer than 50 of these species are involved in disease transmission and less than 10 are proven vectors of viruses (Mellor *et al.* 2000; Meiswinkel *et al.* 2004a). More than 75 viruses were isolated from *Culicoides* worldwide and 23 are from species of the Imicola complex of the subgenus *Avaritia* (Meiswinkel *et al.* 2004a). The geographical distribution and seasonal incidence of the disease causing viruses

are partly determined by the distribution and the biology of competent *Culicoides* vectors.

The ability of a vector to successfully transmit a disease depends on factors such as vector competence and vector capacity. Vector competence is defined as a measure of the number of midges that become infective and successfully transmit a virus after feeding on a viraemic host and is dependant upon the genetic makeup of the vector and external environmental influences (Tabachnick 1991; Wellby *et al.* 1996; Wittmann *et al.* 2001). The vector capacity of a species can be expressed by the average number of infective bites that will be delivered by a vector feeding on a single host in one day (Dye 1992). Low biting rates and/or survivorship can render a highly competent vector to have a low vectorial capacity and more efficient virus transmission may occur in a more abundant vector with low competence status. The Australian species *C. brevitarsis* has a low competence for BTV, but could transmit the virus effectively due to its high biting rate, whereas, due to lower abundance and limited geographical distribution, the more competent *C. fulvus* has a lower vectorial capacity (Standfast *et al.* 1985).

It was demonstrated through a series of studies that *C. bolitinos* is highly susceptible to infection with BTV, and has the ability to replicate strains to higher virus titres more than *C. imicola* (Venter *et al.,* 1998, 2004, 2006a, 2007). In addition it was found that *C. bolitinos* can replicate BTV serotype 1 to higher levels than *C. imicola* at lower temperatures over shorter incubation periods (Paweska *et al.* 2002). Oral susceptibility studies for *C. imicola* populations from Clarens, Stellenbosch and Onderstepoort revealed significant differences in the vector competence of Clarens and Onderstepoort populations for specific isolates of AHSV2 and AHSV9 (Venter *et al*, 2009). This suggests that genetically distinct populations of *C. imicola* may be present in South Africa.

Viruses isolated from field collected *C. imicola* include BTV, AHSV, EHDV and EEV (Nevill, *et al.* 1992; Meiswinkel *et al.* 2004a). Oral susceptibility studies have shown that BTV, EHDV, AHSV and EEV can infect and replicate in this species (Venter *et al*. 1999; 2002; Paweska & Venter 2004). The role of *C. bolitinos* as a potential vector of BTV and AHSV is highlighted by the fact that isolates of both viruses were made from field collected midges (Meiswinkel & Paweska 1998). Viruses isolated from field collected *C. bolitinos* include BTV, AHSV and EEV (Nevill, *et al.* 1992; Meiswinkel *et al.* 2004a).

The relationship between the virus, the insect vector, the vertebrate host, and the environmental conditions determines whether the successful transmission of the virus from an infected to a susceptible host will occur or not (Hardy *et al.* 1983).

1.4.1 Viral diseases associated with *Culicoides* species

Of the 75 arboviruses isolated from *Culicoides* species worldwide, most belong to the *Bunyaviridae* (20), *Reoviridae* (19) and *Rabdoviridae* (11) families (Mellor *et al*. 2000; Meiswinkel *et al*. 2004b). The AHSV, BTV, EEV and EHDV are the most well known viruses that cause the diseases AHS, BT, EE and EHD respectively.

1.4.1.1 African horse sickness

African horse sickness is an infectious, non contagious disease that affects equids. AHS disease remains essentially African but incursions have occurred into Saudi Arabia, Portugal and Spain (Howell 1963; Mellor *et al.* 1990). The disease is enzootic in sub-Saharan Africa and it has a mortality rate of more than 90% in susceptible horses (Maurer & McCully 1963; Coetzer & Erasmus 1994).

1.4.1.2 Bluetongue

Bluetongue is an infectious viral disease that affects sheep and other ruminants. It is characterized by inflammation, haemorrhage, excoriations and erosion, coronitis, torticollis, oedema of the head and neck and cyanosis of the mucous membranes of the oronasal cavity (Verwoerd & Erasmus 2004). Bluetongue is of such international significance that it has been classified as a notifiable disease by the OIE.

1.4.1.3 Equine encephalosis

Equine encephalosis is an infectious, non-contagious, disease of Equidae. The etiological agent, EEV, belongs to the genus *Orbivirus* (Calisher & Mertens 1998) and it was isolated in 1967 from horses in South Africa (Erasmus *et al.* 1970; 1978). EEV has only been isolated in southern Africa and no vaccine is available against this disease. While high seroprevalence in horse and donkey populations were reported in South Africa (Paweska *et al.* 1999; Venter *et al*. 1999) only sporadic cases or localised disease outbreaks occurred in the country. It does indicate that EEV infections are mostly asymptomatic. The involvement of *Culicoides* species in the epidemiology of EEV in the field (Erasmus *et al.* 1970; Theodoridis *et al.* 1979, Nevill *et al*. 1992) were confirmed when it was shown that *C. imicola* and *C. bolitinos* are susceptible to infection by this virus (Venter *et al*. 1999; 2002; Paweska & Venter 2004).

1.4.1.4 Epizootic hemorrhagic disease

Epizootic hemorrhagic disease is an often-fatal hemorrhagic disease in North American white-tailed deer (*Odocoileus virginianus*) with bluetongue-like symptoms in cattle and subclinical infections in domestic sheep (Pasick *et al.* 2001). In South Africa, evidence shows that EHDV can be dated back to 1932 (Bekker *et al.* 1934, Barnard *et al.* 1998). The involvement of *Culicoides* species in the epidemiology of EHD in South Africa has been demonstrated by the isolation of EHDV from field collected *Culicoides* species (Nevill, *et al.* 1992).

1.5 Role of *Culicoides* in disease spread

Vector dispersal and the rate of dispersal of infected vectors can play a critical role in the spread of a disease and the implementation of effective control measures. The dispersal of *Culicoides* midges can be influenced by factors such as temperature, wind speed, wind direction, distance to be travelled from endemic areas, increasing altitude, urban areas and mountain ranges acting as physical barriers (Bishop *et al.* 2004).

Very little is known about the dispersal of *C. imicola*. It is believed that *Culicoides* species can be transported for hundreds of kilometres by prevailing winds (Sellers *et al.* 1977; Mellor *et al.* 2000) and inadvertently through the transportation of livestock (Meiswinkel 1998). It cannot be explained whether the presence of *C. imicola* on the islands off the African east and west coast such as Madagascar, Reunion, Mauritius and the Cape Verde resulted from wind transport or whether it was transported simultaneously with the livestock (Meiswinkel 1995). The dispersal of *C. bolitinos* is similar to that of *C. imicola*, but *C. bolitinos* is closely associated with African buffalo and can disperse, over relative short distances, with slow moving herds (Meiswinkel 1995).

In Europe, BT has expanded northwards between 1998 and 2004 with outbreaks of the disease over 800 km further north than had previously been recorded in southern Europe (Purse *et al.* 2005). Expansion of *C. imicola* northwards into the southern Europe has been ascribed to the recent climate change (Wittmann *et al.* 2001; Purse *et al.* 2005). Outbreaks of BTV serotype 8, in the absence of *C. imicola,* have been confirmed as far north as the Netherlands, Belgium, Germany and northern France in September 2006 (OIE 2006; Thiry *et al*. 2006).

In South Africa, it has been postulated that the transportation of viraemic horses from endemic areas lead to outbreaks of AHS in the Western Cape Province (Bosman *et al.* 1995; Bell 1999; Guthrie 1999). In addition to the movement of viraemic hosts, the occurrence of AHS in the Eastern and the Western Cape

Provinces over the past 5-10 years could be attributed to the movement or dispersal of infected vectors. However, the region in the middle of the country is dry and it is believed to create a barrier that prevents *C. imicola* from the north (e.g. Onderstepoort) to interbreed with the population from the south (i.e. Western and Eastern Cape Provinces). If infected vectors are transported, by any method, it follows that this might be a possible mechanism for the introduction of disease into new areas. The identification and genetic characterization of *Culicoides* populations in South Africa may help to predict outbreaks and explain the epidemiology of AHSV and BTV in the country.

1.6 Genetic analysis of *Culicoides*

Mitochondrial genes are often targeted for genetic analysis of insects because they evolve faster than nuclear genes (Watanabe *et al.* 1999). They are ideal for measuring genetic variations because: they are abundant in cells, can easily be amplified by PCR using conserved primer sets, are maternally inherited and have specific regions that are highly variable (Simon *et al.* 1994). The cytochrome oxidase I (COI) and 16S ribosomal RNA genes are the most commonly used mitochondrial sequences in genetic analysis of insect vectors (Shouche & Patole 2000; Linton *et al.* 2002). They have several advantages which include high resolution in distinguishing between species of the same and different genera as well as revealing subpopulations in certain species (Shouche & Patole 2000, Dallas *et al.* 2003; Ouma *et al.* 2005). These two mitochondrial sequences were used in the current study for the genetic analysis of midges from different locations in South Africa.

The COI gene is one of the fastest evolving mitochondrial genes that have often been used in insect genetic studies. An example was the use of mitochondrial COI and cytochrome oxidase II (COII) genes in *Papilio* butterflies phylogenetics to examine patterns of gene evolution across a broad taxonomic range of these taxa (Caterino & Sperlings 1999). Their results have shown that COI had an excellent performance, in resolving Papilionidae relationships at species and

species group level (Caterino & Sperlings 1999). Similarly, COI sequences could successfully distinguish between the east and the west population of *C. imicola* in the Mediterranean basin (Dallas *et al*. 2003; Nolan *et al.* 2008). The COI primer sequences as described by Dallas *et al*. (2003) were used in this study for the analysis of the South African populations of *C. imicola* and *C. bolitinos*.

The present study will provide a basis for midge genetic analysis of geographically closer and separated populations of the same species from the same country. Several studies have been done to characterise different *Culicoides* species and midges of the same species from different countries (i.e. geographically well separated study areas), but very little on individuals of a single species from one country (i.e. relative close geographic locations). For example, mitochondrial COI sequences were successfully used to distinguish between five species of the *C. imicola* complex (Linton *et al.* 2002). Their results suggested that BTV vector competence could be an ancestral character in this complex. Furthermore, *C. imicola* populations from Portugal, Rhodes and Israel were analyzed using COI sequences to determine their matrilineal structure and to phylogenetically characterize them, and its reliability has been shown by the ability to differentiate between the western and the eastern populations (Dallas *et al.* 2003). Analyses of the haplotype diversity of the mitochondrial COI gene in *C. imicola* from 88 sites in the Mediterranean and outgroups showed extreme differentiation across the Mediterranean basin, with four common haplotypes each predominating in different areas. Eastern and western areas characterized by distinct BTV incursions accounted for most of the molecular variance in haplotype composition (Nolan *et al.* 2008).

Mitochondrial ribosomal genes can also be effectively used for genetic studies of insects. Ribosomal genes have been extensively used for phylogenetic studies in a wide range of species due to their universal occurrence, abundance, as well as sequence and structural conservation (Hamby & Zimmer 1992). The mitochondrial rRNA genes are less complex than the nuclear rRNA genes which

have about five different subunits that might be duplicated as tandem arrays, separated by transcribed and non transcribed spacer regions (Brown *et al*. 1979; 1982; Watanabe *et al.* 1999). The rate of evolution of ribosomal RNA genes is known to vary with the length of the molecule and its sequences have been widely used for phylogenetic studies and for determination of sequence differences that reflect strain variation in hypervariable regions (Shouche & Patole 2000).

Sequences of the mitochondrial 16S rRNA gene have been used to study black flies, mosquitoes as well as midge species, and this gene has proven to be a useful tool to construct insect phylogenies (Xiang & Kochar 1991; Shouche & Patole 2000; Ouma *et al.* 2005). The diversity of this molecule has allowed a comparative analysis of eubacterial, archaebacterial and eukaryotic kingdoms, chloroplasts as well as mitochondria (Noller *et al.*1985). Due to its potential as a phylogenetic marker, it became important that this gene be considered for use in the current *Culicoides* study.

Very little is known about the population structures of *Culicoides* species in South Africa, taking into account the size, geographical diversity and the presence of potential natural barriers. The geographical distribution of *C. imicola* and *C. bolitinos* is well known in South Africa. What is not clear is if these populations are dispersed homogenously throughout South Africa or if they are static with limited gene flow between different geographically isolated populations. If populations can indirectly be shown to be isolated, dispersal of the virus over large distances through insect dispersal is unlikely. On the other hand, if the genetic analysis shows that there is a high level of inbreeding between these populations it might be possible that viruses may be transmitted along with individual dispersing insects.

However, due to the presence of natural barriers which range from semi desert areas and mountain ranges, it is believed that subpopulations of midges might

have become established over time in South Africa with the biggest possibility of a north-south split. This notion is supported by oral susceptibility studies indicating that the susceptibility of different geographical populations of *C. imicola* and *C. bolitinos* may differ for identical isolates of AHSV (Venter *et al.* 2009). Furthermore a study done using random amplified polymorphic DNA (RAPD) markers has indicated that some bands are population specific as observed on the agarose gel, with *C. imicola* populations from Onderstepoort and Skukuza distinguished from Tshipise, Phalaborwa and Mtunzini populations (Sebastiani *et al.* 2001). Microsatellites genes can be helpful in the genetic characterisation of midges, however, more markers need to be developed for *Culicoides* species to the extent that it can provide meaningful data.

1.6.1 Methods used in phylogenetic analysis

DNA sequence assembly is the first essential step in phylogenetic analysis which combines the two DNA sequences generated from the same DNA sample using the reverse and forward primers. The forward and the reverse sequences are assembled together to generate the consensus sequence. The Gap4 Staden software package is the preferred and easy to use method for this purpose due to its ability to handle sequence data produced by variety of sequence instruments and can handle data entered using digitiser or typed by hand (Bonfield & Staden 1995).

Consensus sequences generated using the Gap4 are used for multiple sequence alignment process. The process aligns the most closely related sequences first and groups them together. Software packages that are being used to perform multiple sequence alignments include T-Coffe, MAFFT, DNAMan, Clustal X, e.t.c. Clustal X is a widely used and preferred method due to its ability to provide integrated system for performing multiple sequence alignment, profile alignment and it allows one to view results by providing algorithms to refine and improve the alignment. The multiple sequence alignment generated using Clustal X can also

be transferred to BioEdit software as an additional tool to help with the editing process of the sequences. BioEdit can be defined as a mouse-driven, easy-to-use sequence alignment editor and sequence analysis program with four modes of manual alignment such as select and slide, grab and drag, gap insert and delete by mouse click with on screen typing that behaves like a text editor (Hall 1999). This method allows for the trimming of the sequence terminals to generate a desired sequence length. The edited sequences are then transferred back to Clustal X for re-alignment. Clustal X then converts aligned multiple sequences to a PHB file that could be opened using Neighbor-joining (NJ Plot) method for phylogenetic tree construction. NJ method is widely used for the construction of the phylogenetic trees due to its high computational speed and its ability to incorporate large amount of data. NJ method produces phylogenetic trees where the sum of the number of mutations along evolutionary path for each taxon within the cluster is represented by the distance since their divergence is from common ancestory (Saitou & Nei 1987).

1.7 Problem/ Hypothesis

Very little is known about the genetic variation within *Culicoides* species and no particular genetic markers have been identified for use in intra-species analyses. Due to geographical separation of midge populations in the southern and northern regions of the country, together with phenotypic variations that have been observed, it is hypothesized that genetic differentiations do exist within *Culicoides* species in South Africa. Can COI and 16S rRNA nucleotide sequences that have been used previously be used to distinguish between closely related species or specimens from different countries also be used with success to indicate genetic variations within *C. imicola* and *C. bolitinos* in South Africa?

1.8 Aims and Objectives

The aim is to identify suitable genetic markers that can reveal genetic variation between geographically separated populations of *C. imicola* and *C. bolitinos*, if they exist.

The specific objectives are to:

1. Gather field specimens of both *C. imicola* and *C. bolitinos* from different areas within their natural distribution in South Africa.
2. Partially sequence two mitochondrial genes from these specimens.
3. Analyse the sequence data and make use of phylogenetic trees to clarify the genetic relationships between individuals or groups collected from geographically distinct sites.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

All the materials and methods that were used to carry out the experimental work described in Chapters 3, 4 and 5 are described in this chapter. Any diversions in subsequent chapters will be noted where applicable.

2.2 Midge collections

Onderstepoort 220 V ultraviolet downdraught suction light traps equipped with a 23 cm 8 W black light tube (Fig. 2.1) were used to collect midges at each site. Traps were hung close to livestock and operated from dusk to dawn (Fig. 2.3). Insects were collected into 200-300 ml of a 1% v/v water solution of Savlon® (Johnson and Johnson, South Africa) (containing Chlorhexidine gluconate 0.3 g/100 ml and Cetrimide 3.0 g/100 ml). Savlon breaks the surface tension of the water so that the insects sink to the bottom of the beaker and also prevents bacterial growth which can cause rotting of the insects. The removal of insects from the traps was done in the morning, and the insects were taken to the laboratory for morphological identification of species.

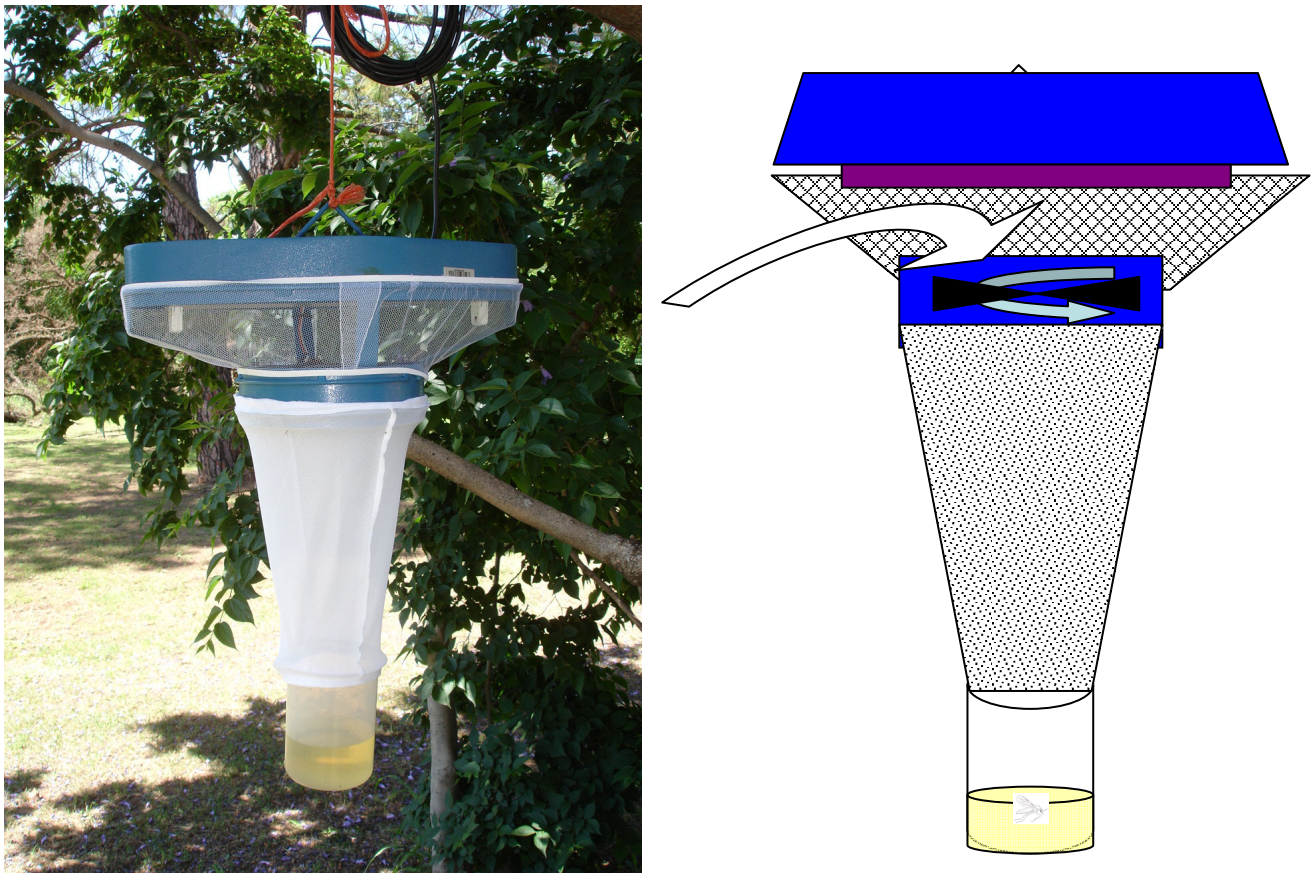


Figure 2.1: A 220 V ultraviolet suction light trap equipped with an 8 W black light tube

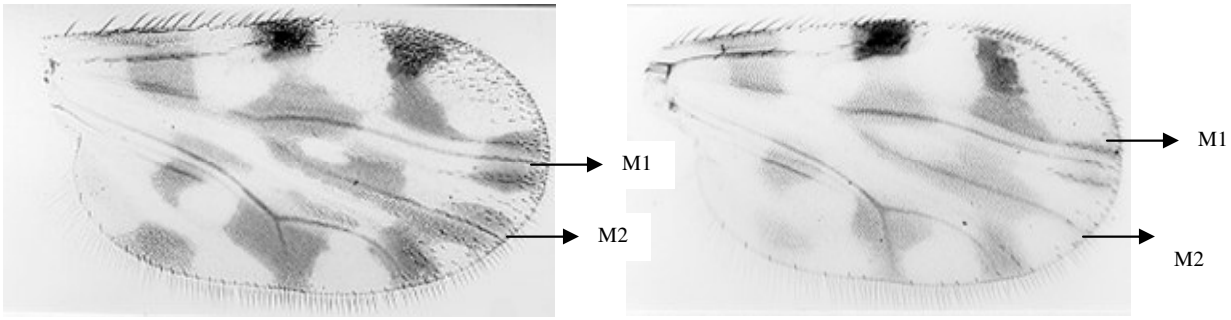
Figure 2.2: Light traps hung close to sheep on Koeberg farm at Clarens in the Free State Province

2.3 Identification

Light traps are non-specific and collect a variety of night flying insect species that are small enough to go through the surrounding gauze (mesh size 2-3 mm). Upon arrival in the laboratory, the contents of the beaker was washed by filtering it through fine netting and rinsed gently three or four times with distilled water. Rinsing cleans the midges from any extraneous moth-scales that may adhere to the wings as this can make identification difficult once preserved in alcohol (Meiswinkel 1995). A key to the adults of nine of the 13 species of the Imicola complex is provided by Meiswinkel (1995; 2003).

Identification of the *C. imicola* and *C. bolitinos* was done under a dissecting microscope using wing morphology (Fig. 2.3) as described by Meiswinkel (1995). Ten character states are used for morphological identification in *Culicoides* species. *Culicoides imicola* can be distinguished from *C. bolitinos* using the

single most diagnostic character called the preapical excision, which is the juxtaposition of pale and dark areas on the anterior distal third of vein M2. The diagnostic features of the female *C. imicola* are only based on patterns of the wings, whereas *C. bolitinos* is identified based on the median third of both the posterior and anterior margins of vein M2 that has extreme darkness. These margins taper and fade simultaneously as they leave the apex of the vein (Fig. 2.3) (Meiswinkel 1989).



A – *Culicoides imicola* B – *Culicoides bolitinos*

Figure 2.3: A – B, Wing patterns used in distinguishing *Culicoides* species (Meiswinkel 1995)

The two species were stored separately in 1.5 ml epperndorf tubes in 80% ethanol. The tubes were labelled with species name, collection date and site. Individual midges were used for molecular analysis. Only nulliparous females and males were used for DNA extraction. Midges were either used immediately after trapping or stored at -20˚C. Parous midges were not considered for DNA extraction as the host DNA might contain similar targeted mitochondrial genome that could be amplified together with midge DNA due to the universal nature of the primers. Nulliparous and parous midges were separated based on the presence or absence of pigment in the abdomen following the methods described by Dyce (1969).

2.4 DNA extractions

Total genomic DNA was extracted from individual midges using the QIAamp DNA Micro kit (QIAgen, Germany) and NucleoSpin tissue kit (Macherey-Nagel, Germany). Individual midges were homogenized in a 1.5 ml microcentrifuge tube containing the lysis buffer. The manufacturer‘s instructions for the isolation of genomic DNA from tissues were followed with slight modification (i.e. midges were homogenized using a battery operated microtissue grinder (Kontes, Vinelan, NY) and micro-pestle in a lysis buffer before the addition of proteinase K). DNA was eluted in 30-60 µl of elution buffer and stored at -20ºC for subsequent amplification reactions.

2.5 Polymerase Chain Reaction (PCR)

DNA amplification was carried out in a programmable thermocycler (GeneAmp PCR System 2700, Applied Biosystems). The PCR reaction mixtures were performed in a total volume of 50 µl which contained 5 µl of 10 X PCR buffer, 4 µl of 2.5 mM dNTP mixture, 10 pmol of each of the forward and the reverse primers, 5 U of Ex-Taq DNA polymerase (Takara) and 34.5 µl of distilled water. A volume of 5 µl DNA was added to each PCR reaction. Samples without DNA were included in each amplification to check for contaminations. Two sets of primers (i.e. cytochrome oxidase I (COI) and 16S rRNA primer sets) were used separately with the same PCR parameters. Thermal cycling conditions were as follows: denaturation at 94ºC for 2 minutes, followed by 94ºC for 30s, annealing at 55ºC for 30s and extension at 72ºC for 30s, for 40 cycles followed by final extension at 72ºC for 4 minutes.

The success of the DNA amplification was determined by loading an aliquot (4 µl) of the PCR product mixed with 1 µl loading dye (0.25% bromophenol blue; 40% sucrose) on a 1.2% (W/V) agarose gel containing 0.25 µg/ml ethidium bromide in 1 x Tris-borate EDTA (TBE) buffer. A 50 bp DNA molecular ladder (Roche Diagnostic, Germany) was loaded alongside the PCR samples and

electrophoresed at 90 V for 45 minutes. Amplified DNA was visualized using UV fluorescence and the size of the product was verified.

2.6 Purification and sequencing of the PCR amplicons

PCR products were purified using the QIAquick PCR purification kit (QIAgen, Germany) or a MSB Spin PCRapace kit (Invitek, Germany). A 250 µl binding buffer was added to the PCR sample and vortexed for 1 minute. The sample was completely transferred onto a spin filter and centrifuged for 3 minutes at 12.000 rpm. The DNA was eluted in 50 µl of the elution buffer. The purified DNA was quantified using spectrophotometer and loaded alongside 50 bp DNA ladder on 1.2% ethidium bromide stained agarose gel prior to nucleotide sequencing. Approximately 5-20 ng of the purified PCR product was sequenced using the same forward and the reverse primers that were used for the PCR amplification.

2.7 Data analysis

Nucleotide sequences obtained from the sequencing laboratory were edited by assembling together the forward and the reverse sequences of each individual midge using Gap4 of the Staden software package (Bonfield *et al.* 1995) to generate a consensus sequence. Multiple alignments of the consensus sequences were performed using Clustal X (Higgins & Sharp 1989; Higgins 2003). Editing of the multiple alignments was done using BioEdit and the sequence terminals of the consensus sequences were trimmed at each end to produce sequences with a length of 472 bp and 450 bp for COI and 16S rRNA respectively. The data generated from Clustal X was used to construct distance matrices for the phylogenetic trees using the Neighbor-joining method (Saitou & Nei 1987). The sequences were also imported for construction of the phylogenetic trees into Mega 4.0 (Tamura *et al.* 2007). The significance of the branching pattern was evaluated using 1 000 replicates with bootstrap statistical support values. Values of 70% or bigger were regarded as evidence that the groupings are true reflection of the real relationship (Hillis & Bull 1993). The sequence data was then imported for further analysis into DNA sequence

polymorphism (DnaSP) software package to determine DNA polymorphism (Rozas *et al*. 2003).

CHAPTER 3

FIELD COLLECTIONS OF *CULICOIDES* SPECIES AT DIFFERENT LOCALITIES IN SOUTH AFRICA

3.1 INTRODUCTION

Midges from geographically separated location in South Africa were collected using 220 V light traps as described in Chapter 2. Midges were identified to species level based on wing morphology and DNA was extracted as described in Section 2.3 and 2.4. The extracted DNA was used for the analysis described in Chapter 4 and 5.

3.2 RESULTS

3.2.1 Study areas

Culicoides imicola were collected from three geographically separated areas in South Africa viz. Gauteng Province (Onderstepoort, Pretoria), Eastern Cape Province (Kirkwood, Port Elizabeth) and the Western Cape Province (Stellenbosch, Cape Town). As reflected in Fig. 3.1 and Table 3.1, midges were collected at more than one site within each of these broad geographical areas.

Stellenbosch is situated 1 500 km southwest of Onderstepoort and is a winter rainfall area. Winters at Onderstepoort and Stellenbosch are relatively frost-free. The names of the farms where midges were collected are given in Table 3.1.

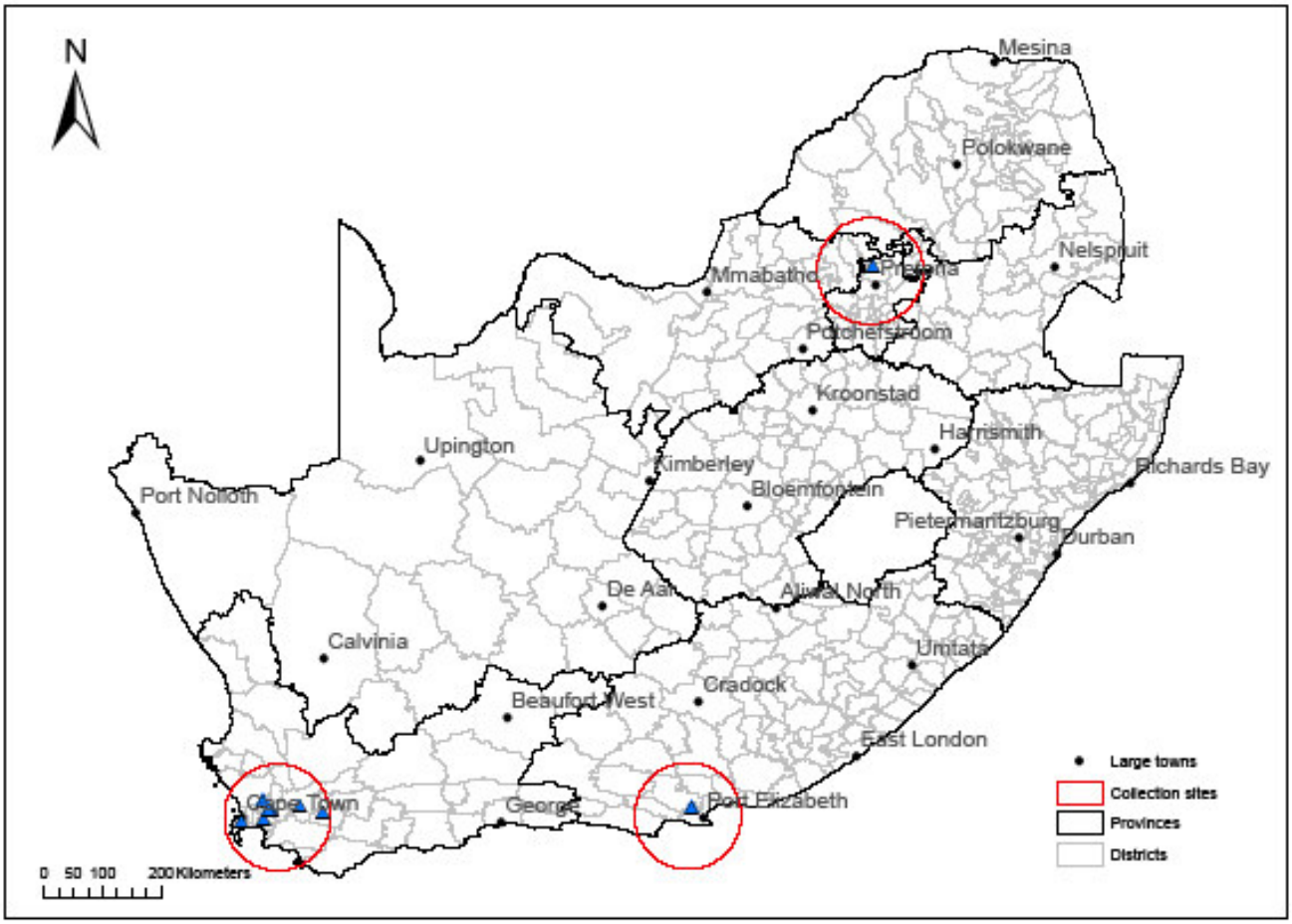


Figure 3.1: Map showing areas in South Africa where *Culicoides imicola* were collected (Chantel De Beer, ARC-OVI). Light trap collection sites are represented by blue triangles inside the red circles.

Table 3.1 *Culicoides* abundance and *Culicoides imicola* representation as determined with light traps at the collection sites where *C. imicola* were collected for phylogenetic analysis (unpublished data Venter & Labuschagne, ARC-OVI)

Sample Name	Locality	Farm (No. of light trap collections)	Collection date	Average No. of *Culicoides* collected/night
	Gauteng Province			
iOPV1-3 & 8	Onderstepoort	OVI (1)	July 2007	***63** (56)
iOPV4 & 9	Onderstepoort	OVI (1)	May 2007	**110** (103)
iOPV5 & 10	Onderstepoort	OVI (1)	February 2007	**720** (657)
iOPV6 & 11	Onderstepoort	OVI (1)	November 2006	**1 329** (1 316)
iOPV13-22	Onderstepoort	OVI (1)	February 2007	**846** (846)
iOPV7& 12,23-32	Onderstepoort	Kaalplaas (1)	March 1996	**1 054 620** (1 048 292)
iKP1 & 2	Onderstepoort	Kaalplaas (1)	August 2007	**145** (141)
	Western Cape Province			
iStelBea1 & 2	Stellenbosch	Beaumont (6)	April 2007	**14 590** (14 451)
iStelTr1 & 2	Stellenbosch	Trough End (6)	April 2007	**3 774** (3 662)
iStelRiv1 & 2	Stellenbosch	Riverworld (5)	April 2007	**247** (203)
iStelWod1 & 2	Stellenbosch	Woodhill (4)	May 2007	**7 519** (7 136)
iStelKun 1 & 2	Stellenbosch	Kunnenberg (5)	May 2007	**374** (290)
iStelBon1-5	Stellenbosch	Bona Vista (1)	Jan 2006	**1 280** (922)
iStelKen1-8	Kenilworth	Quarantine Station (1)	October 2007	**41** (35)
iStelRob1-6	Robertson	Nerina Guest Farm (2)	January 2007	**3 572** (3 237)
	Eastern Cape Province			
iUITWic1-20	Kirkwood	Wicklow Trust (2)	March 2007	**661** (196)

Key = OVI – Onderstepoort Veterinary Institute

*The total numbers of *Culicoides* specimen collected at each site are indicated in bold and the numbers of catches for *C. imicola* are shown in brackets.

Culicoides bolitinos were collected from four areas, viz eastern part of the Free State Province (Clarens), eastern (George) and western (Stellenbosch) Western Cape Province and Eastern Cape Province (Port Elizabeth) (Fig. 3.2). Midges were collected at more than one collection site within each of the four areas. Clarens is 266 km north-east of Bloemfontein and 500 km south of Onderstepoort (Pretoria) and is a summer rainfall area. At the higher lying Clarens, heavy frost and occasional snow can be found in winter.

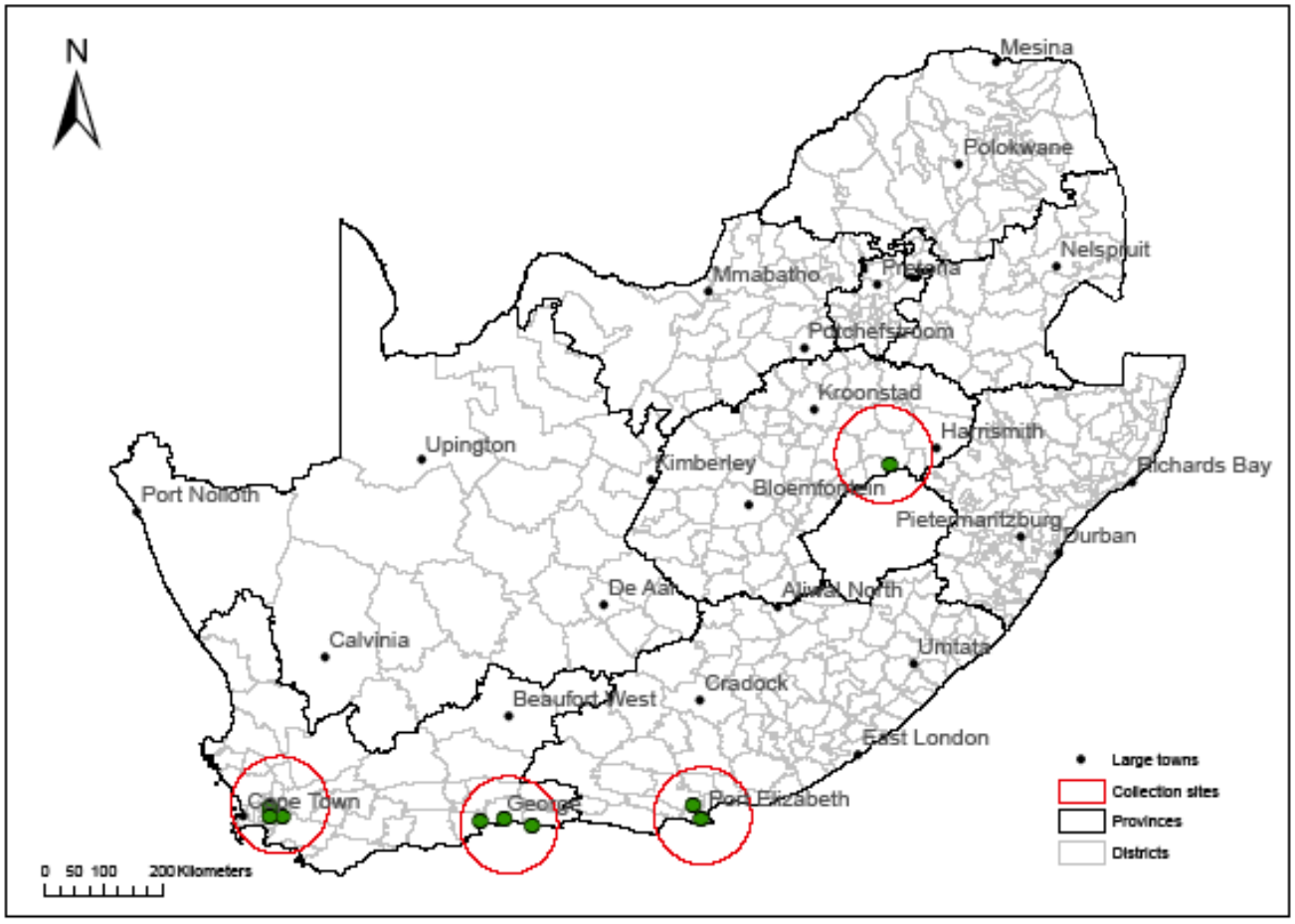


Figure 3.2: Map showing areas in South Africa where *Culicoides bolitinos* were collected (Chantel De Beer, ARC-OVI). Light trap collection sites are represented by small green circles inside the big red circles.

Table 3.2 *Culicoides* abundance and *Culicoides bolitinos* representation as determined with light traps at the collection sites where *C. bolitinos* were collected for phylogenetic analysis (unpublished data Venter & Labuschagne, ARC-OVI)

Sample Name	Locality	Farm (No. of light trap collections)	Collection date	Average No. of *Culicoides* collected/night
	Free State Province			
bCLA1-2	Clarens	Koeberg (1)	March 2001	***715** (266)
bCLA3-5	Clarens	Koeberg (9)	March 2007	**1 140** (1 082)
bCLA6-15	Clarens	Koeberg (2)	January 2007	**880** (640)
	Western Cape Province			
Geo1	George	Outeniqua (9)	November 2006	**4 114** (3 316)
Geo2-4	George	Kidbuddie (3)	October 2007	**66** (37)
Geo8-15	George	Riding Club (1)	November 2007	**358** (277)
bStel1-3	Stellenbosch	Robertsvallei (10)	April 2007	**111** (47)
bStel4-7	Stellenbosch	Bona Vista (1)	January 2007	**1 178** (1 178)
bStel8-15	Stellenbosch	Rozendal (1)	February 2007	**1 590** (885)
	Eastern Cape Province			
bPE1-3	Port Elizabeth	Ascot Stud (5)	January 2007	**700** (669)
bPE4-7	Port Elizabeth	Ascot Stud (1)	April 2007	**739** (602)
bUIT8-15	Kirkwood (PE)	Wicklow Trust (2)	March 2007	**661** (228)

*The total numbers of *Culicoides* specimen collected at each site are indicated in bold and the numbers of catches for *C. bolitinos* are shown in brackets.

3.3 DISCUSSION

Culicoides imicola was abundant at all the collection sites from which specimens of this species were used for phylogenetic analyses (Table 3.1). Its representation varied from 29.7% at Wicklow Trust to 99.4% at Kaalplaas (Table 3.1). Except for the collections made at Wicklow Trust, *C. imicola* was the dominant species to be collected at all of these sites (Table 3.1). In the two collections made at Wicklow Trust, *C. bolitinos* represent 34.5% of a nightly average of 661 *Culicoides* midges collected (Table 3.1). The largest collection of 1 054 620 *Culicoides* of which *C. imicola* represented 99.4% was made in a single night at Kaalplaas in March 1996 (Table 3.1) (Meiswinkel *et al.* 2004a). Such large collections are not unusual during warm wind free nights during years of above average rainfall. The large numbers and dominance obtained in the present study (Table 3.1) confirms previous results which indicate C*. imicola* to be the dominant livestock associated *Culicoides* species in the warmer, frost-free summer and winter rainfall areas of South Africa (Meiswinkel 1989; Venter 1991; Venter *et al.* 1996; Meiswinkel *et al.* 2004a; Venter *et al.* 2006b).

Culicoides bolitinos was abundant at all the sites sampled for the phylogenetic analyses of this species (Table 3.2). *Culicoides bolitinos* is found in most areas where *C. imicola* is present (Meiswinkel *et al.* 2004a). Because *C. bolitinos* breeds in bovine dung (Meiswinkel & Dyce 1989; Meiswinkel 1989), it is generally accepted that *C. bolitinos* will be found in all areas where bovines are present. This close association with cattle probably increases the vectorial capacity of this species for BTV. Previous light trap surveys indicate that, due to its more stable temperature breeding habitat it can become more abundant than *C. imicola* in the cooler parts of the country (Venter & Meiswinkel 1994; Meiswinkel *et al.* 2004b), e.g. Clarens (Table 3.2). In some areas the abundance of these two species can vary according to the climatic and/or environmental conditions during a specific year or season. This was illustrated by the collections made at Bona Vista (Stellenbosch). In January 2006 *C. bolitinos* accounted for

72% of 1 280 of *Cuicoides* collected (Table 3.1). The next year, January 2007, *C. imicola* represented 100% of 1 178 *Culicoides* collected (Table 3.2).

The light trap collections made at the quarantine station in Kenilworth yielded the lowest number of *Culicoides* midges (Table 3.1). This can be attributed to the windy conditions and the sandy soil that is not ideal for *Culicoides* midges.

Lower numbers of midges were collected during winter and higher numbers during summer. Table 3.1 shows that low numbers were collected at Onderstepoort during May, July and August 2007 and that the largest collections were made between November 2006 and February 2007. This seasonal fluctuation in *Culicoides* numbers coincide with the seasonal occurrence of outbreaks of AHS in the country (Baylis *et al.* 1999).

The number of midges collected with light traps as reflected in the Tables 3.1 and 3.2 may represent less than 0.0001% of the total midge population in the area (Meiswinkel *et al.* 2004a). Comparisons of *Culicoides* numbers and especially the abundance of *C. imicola* in collections made in the presence and absence of cattle indicate that host animals will be the primary attraction for *Culicoides* midges and that light traps primarily sample midges already in the near vicinity of the host (Venter *et al.* in press).

CHAPTER 4

GENETIC ANALYSIS OF *CULICOIDES* POPULATIONS USING MITOCHONDRIAL CYTOCHROME OXIDASE I GENE FRAGMENT

4. 1 INTRODUCTION

The aim of the work done in this chapter was to extract DNA from individual *C. imicola* and *C. bolitinos* specimens, amplify specific fragment of the COI by PCR for sequencing. This data was then used in subsequent sequence and phylogenetic analysis according to the methods described in Chapter 2. The analysed data was compared with the homologous sequences from GenBank.

4.2 RESULTS

4.2.1 Primer selection

A set of oligonucleotide primers (C1-J and C1-N) from the conserved region of mitochondrial COI described by Dallas *et al.* (2003), were used to amplify DNA. These are the modified version of primers described by Linton *et al.* (2002). It was found that the original C1-J primer could form a 3' hairpin with a melting temperature of 54°C that could interfere with PCR priming and a 4 bp 3' intermolecular duplex that could promote primer-dimer formation, hence it was modified together with C1-N to ensure similar annealing temperatures (Dallas *et al*. 2003). Details of the primers used are shown in Table 4.1 and were synthesized by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). Upon arrival, the lyophilized oligo pellets were re-suspended and diluted to 100 µM using sterile TE buffer (10 mM Tris, Ph 8, 1 mM EDTA) and stored at -20°C. This primer concentration was diluted to 10 µM using nuclease-free water for direct use in all PCR reactions.

Table 4.1 Cytochrome Oxidase I primers, size of amplicon and their nucleotide sequences

Primer name	Orientation of primer	Amplicon size (bp)	Primer sequence	Reference
C1-J-1718	Forward	522	5'-GGAGGATTTGGAAATTGATTAGT-3'	Dallas *et al*, 2003
C1-N-2191	Reverse	522	5'-CAGGTAAAATTAAAATATAAACTTCTGG-3'	Dallas *et al*, 2003

4.2.2 Midge DNA extractions and PCR amplification

Genomic DNA was successfully extracted from whole individual midges as described in section 2.4. Approximately 10-40 ng of DNA, which was sufficient for both PCR and sequencing reactions, wAS extracted. Specimens older than one year and which were not properly stored in ethanol yielded the lowest DNA concentration of below 10 ng. PCR using the COI primers yielded an amplicon that corresponded to the predicted size of 522 bp (Fig. 4.1).

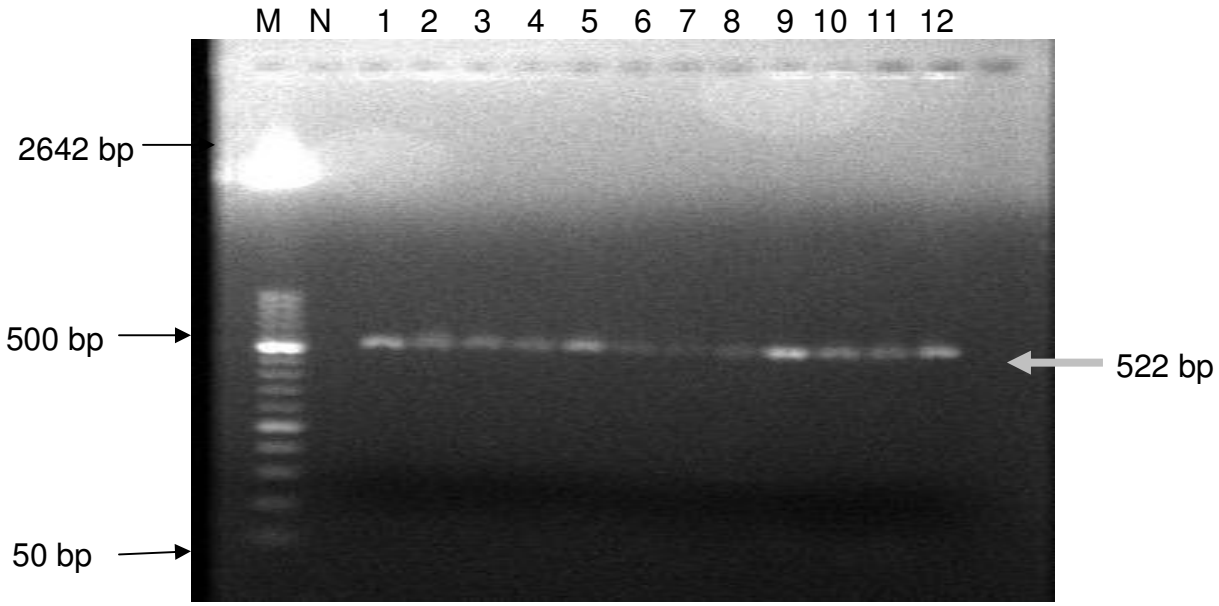


Figure 4.1: A 1.2% TBE agarose gel showing the COI amplification products from *C. imicola* individuals from various geographical regions of South Africa. Lanes: **M** = Molecular Weight Marker; **N** = negative control; **1-4** = Onderstepoort; **5-8** = Stellenbosch; **9-12** = Kirkwood

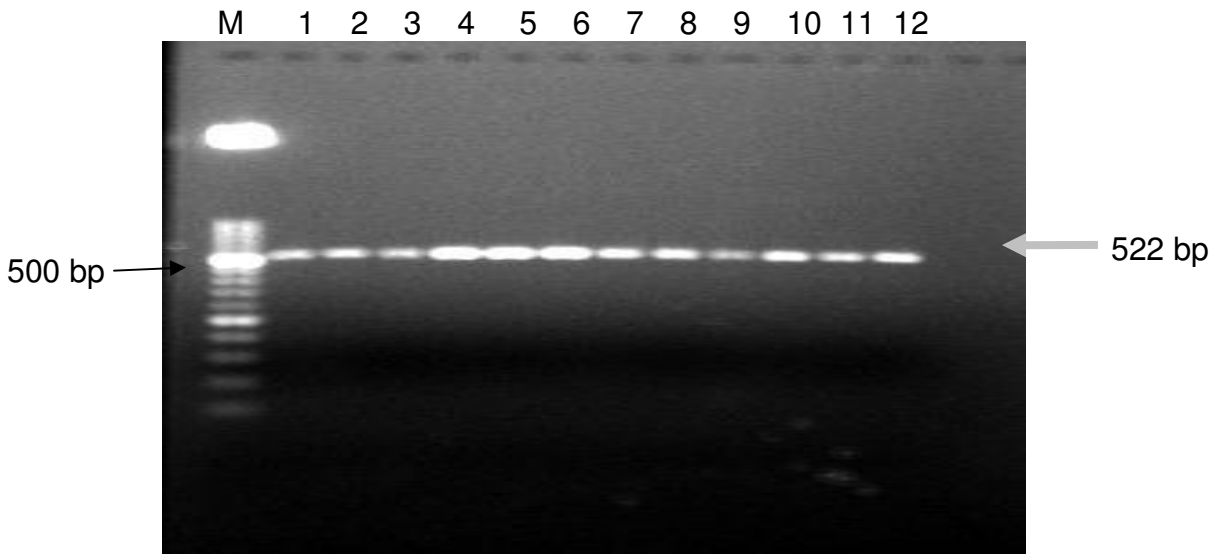


Figure 4.2: A 1.2% TBE agarose gel showing the COI amplification of *C. bolitinos* individuals from various geographical regions of South Africa. Lanes: **M** = Molecular Weight Marker; **1-3** = Clarens; **4-6** = George, **7-9** = Stellenbosch; **10-11** = Port Elizabeth.

4.2.3 Purification of the PCR product and nucleotide sequencing

All the samples that could be visualised on the agarose gel after performing gel electrophoresis were purified as explained in section 2.5 and were cycle-sequenced using the same primers used for the PCR. The nucleotide sequencing yielded an average of approximately 492-522 bp readable bases from each reaction. Nucleotide sequences were aligned and trimmed on both ends to generate homologous stretches of 472 bp that were representative of all specimens. These 472 bp sequences were used for multiple alignments. The multiple sequence alignments are shown in Appendix 1, 2 and 3. *Culicoides imicola* and *C. bolitinos* GenBank sequences were included in the alignments to verify and compare with sequences generated in this study.

4.2.4 Phylogenetic analysis

The multiple sequence alignments generated in this study were used to construct phylogenetic trees using the Neighbor-joining (N-J) method. The N-J method was appropriate for the construction of the phylogenetic trees due to its high computational speed and the ability to incorporate a large amount of data. The tree in Fig. 4.3 was constructed using the multiple sequence alignment shown in appendix 1 to compare the relationship between the *C. imicola* and *C. bolitinos* and to confirm that individuals of the two species are grouped separately. The trees in Figs 4.4 and 4.5 were constructed using the sequence alignment shown in Appendix 2 and 3 respectively. This was done to determine phylogenetic relationship of midges of one species from different geographic localities.

Table 4.2 Details of the GenBank sequences included in the phylogenetic analysis

Species	**Origin**	**Isolate No.**	**Isolation date**	**GenBank accession No**.
C. imicola	Onderstepoort (S.A)	IMI 11	09 Jan 2006	AF069249
C. imicola	Onderstepoort (S.A)	IMI 15	09 Jan 2006	AF069232
C. imicola	Onderstepoort (S.A)	IMI 16	07 Jan 2005	AF069233
C. imicola	Spain: Constatina	SCON 7	12 July 1999	AF080537
C. imicola	Spain: Constatina	SCON 9	12 July 1999	AF080539
C. imicola	Portugal: Alter de Chao	ADC 19	03 Nov 1999	AF079975
C. imicola	Portugal: Evora	PEV 1	03 Nov 1999	AF079977
C. imicola	Israel: Bet Degan	IBET 1	03 Nov 1999	AF078097
C. bolitinos	Clarens (S.A)	BOL 14	09 Aug 2002	AF071928
C. bolitinos	Clarens (S.A)	BOL 17	09 Aug 2002	AF071929
C. bolitinos	Clarens (S.A)	BOL 20	09 Aug 2002	AF071930
C. bolitinos	Clarens (S.A)	BOL 22	09 Aug 2002	AF071931
C. tuttifrutti	Kimberly (S.A)	TUT 1	07 Jan 2005	AF069242
C. tuttifrutti	Kimberly (S.A)	TUT 3	07 Jan 2006	AF069244
C. tuttifrutti	Kimberly (S.A)	TUT 6	07 Jan 2006	AF069245
C. tuttifrutti	Kimberly (S.A)	TUT 7	07 Jan 2006	AF069246

Table 4.3: Explanations of the symbols used on the phylogenetic trees

Symbol	Explanation
	Species name
i	*C. imicola*
b	*C. bolitinos*
	Collection/study areas
OP	Onderstepoort (Gauteng Province)
Kp	Kaalplaas (Onderstepoort area)
V	Onderstepoort Veterinary Institute
PE	Port Elizabeth (Eastern Cape province)
Cla	Clarens (Free State province)
Geo	George (Western Cape province)
Stel	Stellenbosch (Western Cape Province)
	Farms in and around Stellenbosch
Bea	Beaumont
Tr	Troughend
Riv	River world
Kun	Kunnelberg
Bon	Bona Vista
Ken	Kenilworth racing course
Rob	Robertson (Nerina guest farm)
Wod	Wood hill
	Farm near Port Elizabeth
Wic	Wicklowtrust
	Meaning of numbers
1 – 20	Sample number from that area

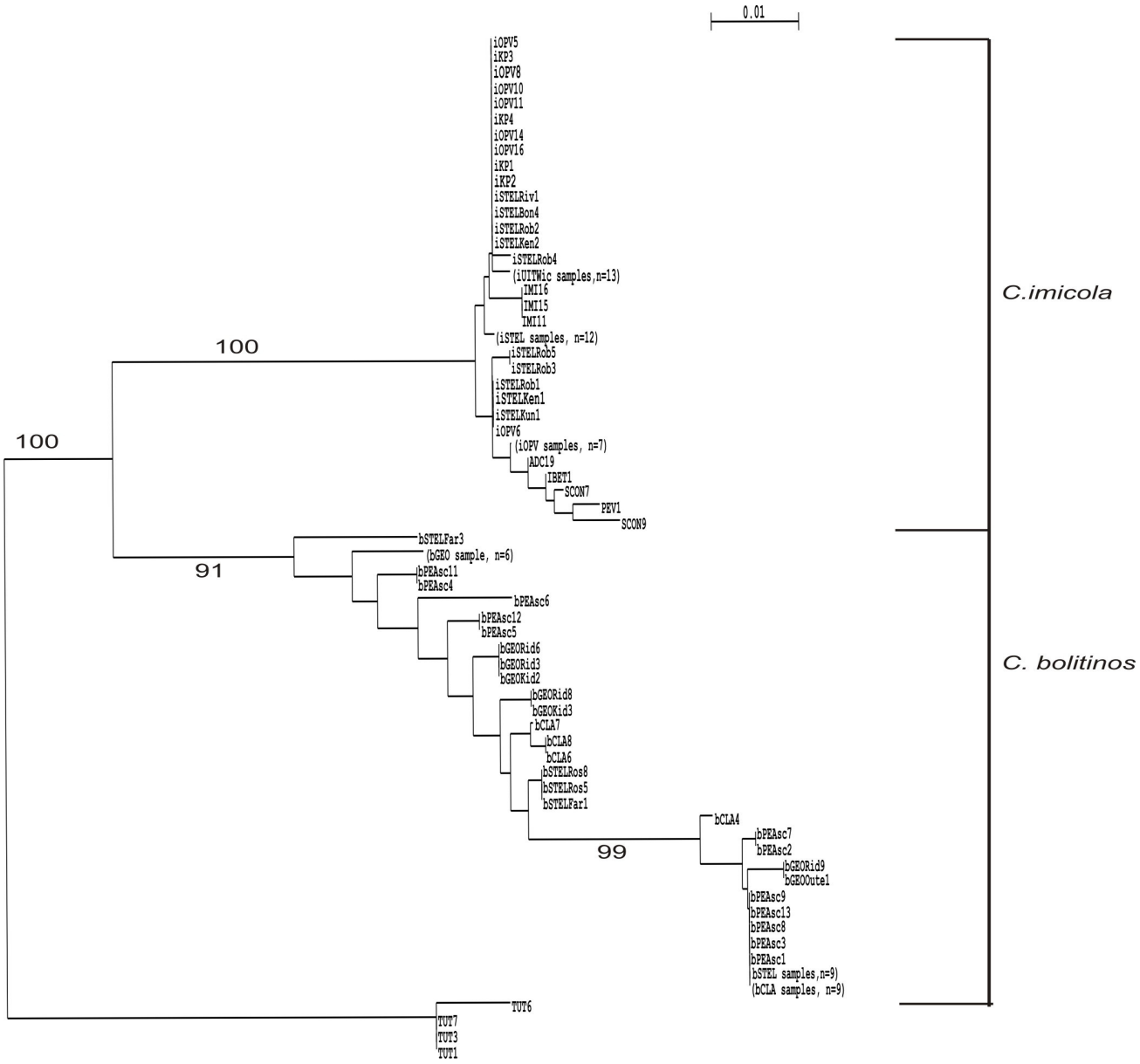


Figure 4.3: Neighbor-joining tree constructed from alignments of 472 bp partial nucleotide sequences of the mitochondrial DNA cytochrome oxidase subunit I (COI) gene of two members of the Imicola Complex. The horizontal branch lengths are proportional to the divergence between sequences within and between groups and vertical lines are for clarity purpose only. Letter n represents number of identical individuals from the same area. The species names corresponding to the abbreviations are listed in Tables 4.2 & 4.3.

Table 4.4 The 14 COI haplotypes for *C. imicola* populations from different geographic areas

Population	Site code	Number of specimens	Number of haplotypes	IMICOI haplotype
Onderstepoort	OVI KP	17 4	4	01, 02,03,04
Stellenbosch	Bea Bon Ken Kun Riv Rob Tri Wod	1 5 3 1 2 5 2 2	5	03,04,05,06,07
Kirkwood	Wic	13	2	08,09
Spain: Constantia	SCON	2	5	10,11,12,13,14
Portugal: Alter de Chao	ADC	1		
Israel: Bet Degan	IBET	1		
Portugal: Evora	PEV	1		

IMI11 was used as a reference sequence to determine haplotype structures of various *C. imicola* individuals.

Table 4.5 The COI diversity of *C. imicola* populations from different geographic areas

Population	**Nucleotide Diversity (*Pi*)**	**Standard Deviation (SD)**	**Haplotype Diversity (Hd)**	**No. of Polymorphic Sites (S)**
Onderstepoort	0.00407	0.044	0.655	5
Stellenbosch	0.00203	0.063	0.655	3
Kirkwood	0.00078	0.091	0.369	1
Overall results	0.00524	0.0147	0.864	8

Explanations: Nucleotide diversity is the average number of nucleotide differences per site between any two DNA sequences chosen at random from the population. It is used to measure the degree of polymorphism or genetic variation within a population. Haplotype diversity measures the uniqueness of a certain haplotype in a population. Standard deviation is a measure of dispersion or variation of a statistical data set and shows how much variation there is from the mean. The values above were calculated using DnaSP 4.0 (Rozas *et al.* 2003).

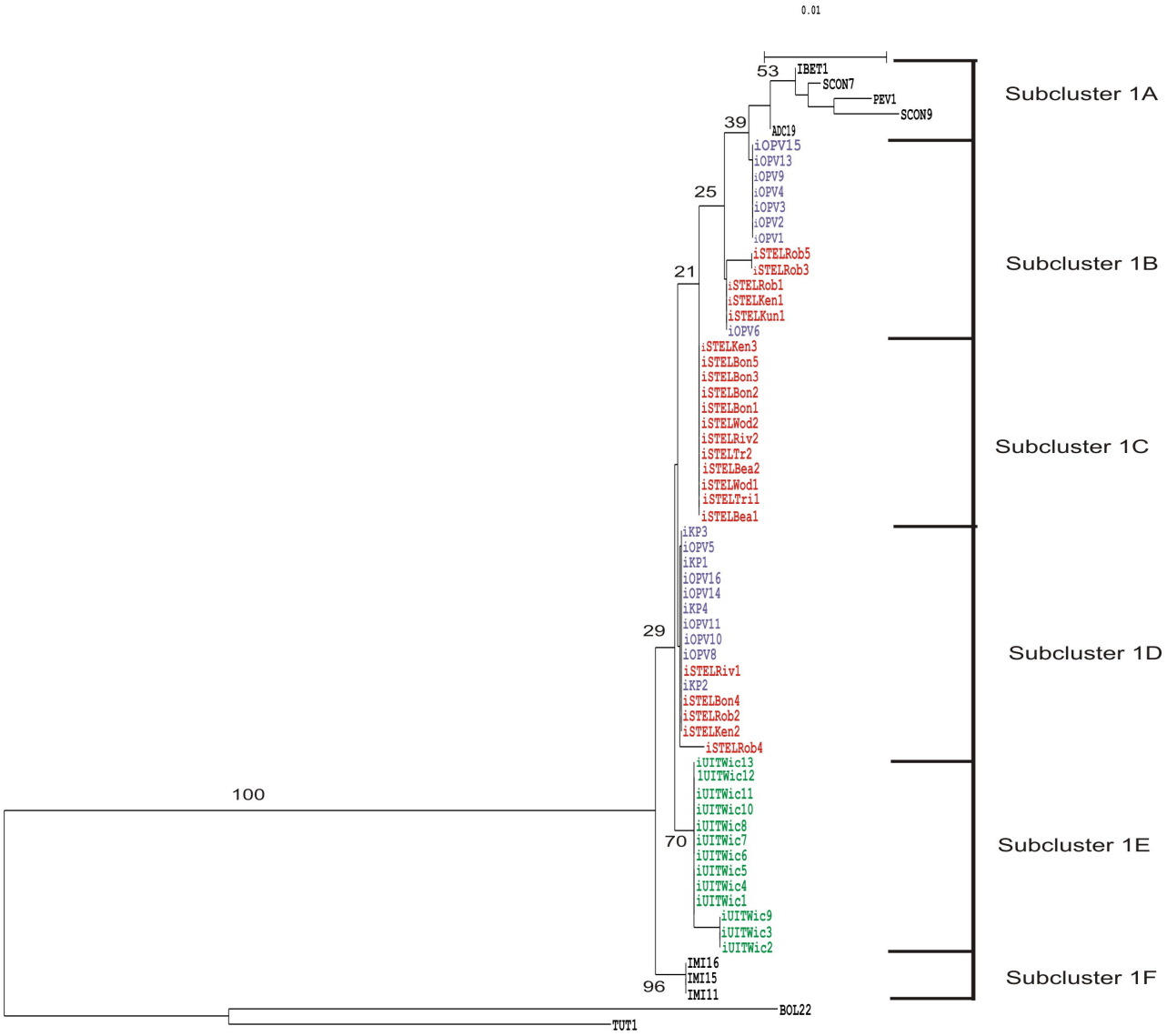


Figure 4.4: Phylogenetic relationships determined using partial nucleotide sequences of the *C. imicola* mitochondrial DNA cytochrome oxidase I gene fragments of individual midges. A Neighbor-joining tree was constructed from alignments of 472 bp of single *C. imicola* species from the three different geographical areas with bootstrap proportion of 1 000 replicates. Homologous sequences from *C. bolitinos* (BOL22) and *C. tuttifrutti* (TUT1) were used to root the tree. The nucleotide substitution per site is indicated by the scale bar. Populations are described by different colours: Black - GenBank, Purple - Onderstepoort, Red – Stellenbosch, Green – Kirkwood.

Table 4.6 The 11 COI haplotypes for *C. bolitinos* populations from different geographic areas

Population	Site code	Number of specimens	Number of haplotypes	BOLCOI haplotype
Clarens	Cla	13	3	01, 02,03
George	Kid Rid Oute	4 8 1	4	04, 05,06,07
Stellenbosch	Far Rob Ros	2 3 7	2	01,08
Port Elizabeth	Asc	12	4	01,09,10,11

The BOL22 sequence was used as a reference sequence to determine haplotype structures of *C. bolitinos* individuals.

Table 4.7 The COI diversity of *C. bolitinos* from different geographic areas

Population	Nucleotide Diversity (*Pi*)	Standard Deviation (SD)	Haplotype Diversity (Hd)	No. of Polymorphic Sites (S)
Clarens	0.01195	0.094	0.455	14
George	0.02102	0.060	0.714	27
Stellenbosch	0.01220	0.074	0.443	13
Port Elizabeth	0.01961	0.078	0.665	24
Overall results	0.02151	0.042	0.734	32

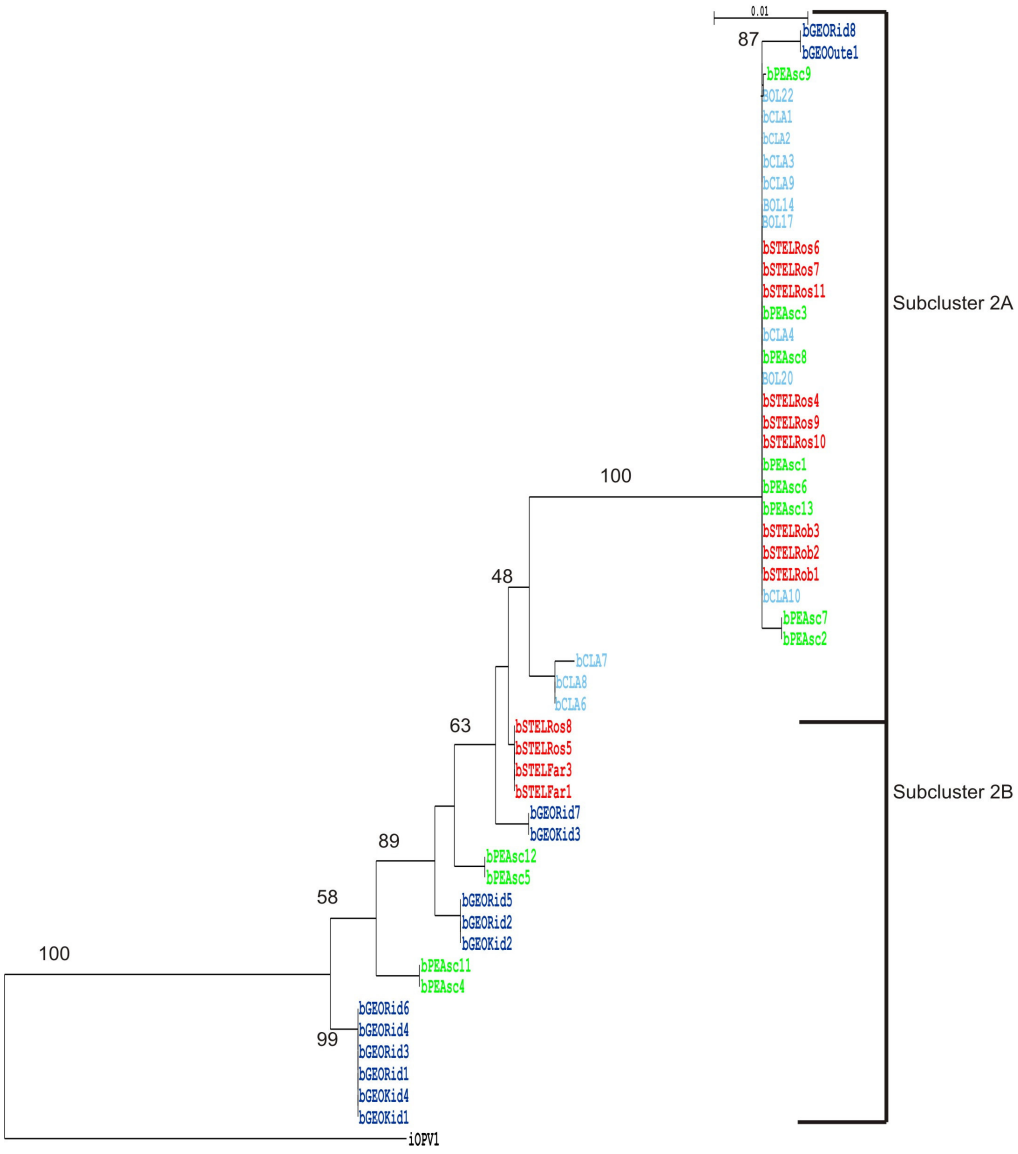


Figure 4.5: Phylogenetic relationships among *C. bolitinos* determined using partial nucleotide sequences of the mitochondrial DNA cytochrome oxidase I gene fragments of individual midges. The nucleotide alignments of 472 bp were used to construct the phylogenetic tree using neighbor-joining method. Numbers at the nodes indicate bootstrap confidence values. The iOPV1 sequence was used as an out group. Colours of different populations are: Black - GenBank, blue - George, red – Stellenbosch, bright green – Port Elizabeth, ice blue – Clarens.

4.3 DISCUSSION

The purpose of this chapter was to amplify the extracted midge DNA using a set of COI primer (Table 4.1) previously described by Dallas *et al.* (2003), verify the expected fragment size by loading the PCR product on an agarose gel (Figs 4.1 & 4.2), sequence the product and use phylogenetic trees to clarify the genetic relationship between individuals and groups of midges collected at different sites in South Africa.

In this study, the phylogenetic relationship of the *C. imicola* and *C. bolitinos* species were established. Comparison of the multiple sequence alignments of the two *Culicoides* species display variations within the COI region of the mitochondria. The number of nucleotide substitutions or differences between sequences of the two species occurred at 56 positions along a total length of 472 bases (Appendix 1). The most frequent substitutions were A-T tranversions which occurred at 26 positions, followed by A-G transitions at 14 positions, T-C transitions at 11 positions, T-G transitions at three positions and A-C transitions at two positions. No G-C tranversions were observed and on average, most transitions/transversions occurred sequentially every 10 bases. The nucleotide base composition of the sequenced mitochondrial COI fragment has shown that the *Culicoides* mitochondrial genome has a high A+T content. It was between 66 and 71% for all the species included in this study. These A+T percentages are comparable to most insect species including *Lybella cyanea* with 66%, *Gryllus ovisopis* 69%, *Locusta migratoria* and *Chrothippus parrallelus* both 70% (Herbeck & Novembre 2003). In Othorpterathan fishes, the A+T content ranged between 54 and 57% (Persis *et al.* 2009). The differences between these two *Culicoides* species were further shown by their location on two separate branches of the tree with high bootstrap values indicating confidence in the tree topology (Fig. 4.4). The results of the phylogenetic analysis has shown that although *C. imicola* and *C. bolitinos* sequences are clearly distinguishable from each other, these two species appear to be genetically closer to each other than to the other members within the Imicola complex (Linton *et al.* 2002).

4.3.1 *Culicoides imicola*

The genetic relationship of the *C. imicola* collected at Onderstepoort, Stellenbosch and Kirkwood was determined. GenBank sequences of samples from three European countries were included in the analysis for comparison and verification. Comparison of the COI multiple sequence alignment has shown that there are variations between sequences of individuals, these are, however, not directly linked to geographic origin. Due to this, individual midges from Onderstepoort and Stellenbosch could not be distinguished from each other and are grouped together in subcluster 1D (Fig. 4.4). Sequences of midges from the latter two areas are identical and also share haplotypes 03 and 04 (Table 4.4) that are dominant in both areas. However, subclusters 1B and 1C suggest the opposite since other members of these two populations clustered separately on different clades and could be clearly identified by the possession of haplotypes unique to their areas of origin. The results suggest that although there are "genetic identities" of midges from the two areas, some members of these two groups could be separated according to their geographical origin, low bootstrap values, however, indicate low confidence in the branching pattern.

The most important feature observed in Fig. 4.4 is the differences shown by the Onderstepoort *C. imicola* sequences generated in the present study and the Onderstepoort sequences obtained from the GenBank (i.e. IMI11, IMI15 and IMI16) which occur on separate clades. This difference was mainly due to the substitutions of A by T at position 370 and G by A at position 424 of the nucleotide sequence from the GenBank (Appendix 2). The same two substitutions were the same as in a *C. bolitinos* (Bol 22) sequence included in the alignment as an out group. This is a clear indication that these two species are indeed closely related.

DnaSP 4.0 (Rozas *et al.* 2003) was used to measure the degree of genetic variation within and between each group of midges. The low standard deviation of a particular midge population serves as an indication of high genetic diversity

within that group of midges. For example, Table 4.5 and 4.7 clearly indicate that in all populations, nucleotide diversity is inversely proportional to its standard deviation. The results have shown that the Onderstepoort midges are the most diverse and highly variable of the three as indicated by the highest nucleotide diversity, Pi=0.00407 and the lowest standard deviation, SD=0.044 (Table 4.5), hence they are wide spread on the phylogenetic tree (Fig. 4.4). These values are higher than the combined total average for all study areas. Also the genetic diversity of the Kirkwood population is the lowest of the three as indicated by the highest SD and the lowest Pi.

Although large number of Onderstepoort and Stellenbosch samples in this study are genetically identical, all the Kirkwood samples have clearly shown to be distinct from the rest as they are grouped separately from others within subcluster 1E (Fig. 4.4), and this is supported by a high bootstrap value of 70%. The presence of the two unique haplotypes (i.e. haplotype 08 and 09) found in this population (Table 4.4) and the substitution of C by A at position 1 of their nucleotide sequences which is common in all members of this population (Appendix 2) indicates the existence of a *C. imicola* subpopulation in the Kirkwood area. However, further studies would be required to confirm this. The results of the DnaSP have shown that sequences of individuals from the Kirkwood area are less diverse compared to sequences of midges from the other two areas as shown by the lowest nucleotide diversity, Pi=0.00078 and highest standard deviation, SD=0.091 (Table 4.5). These results indicate that this population is stable and that breeding with midges from other areas might not have occurred in the recent past. This could mean that an outbreak of AHS disease in Kirkwood might not spread via infected midges to other areas such as Stellenbosch or Onderstepoort. This does, however, not preclude the spread of the virus via infected hosts.

Samples of *C. imicola* from Europe included are easily distinguishable from South African samples due to the possession of unique haplotypes (Table 4.4)

and different nucleotide sequences (Appendix 2). This has also been shown by the location of these midges on a separate clade in subcluster 1A (Fig. 4.4). This indicates that although there are small differences between midges from different geographic areas in South Africa, South African populations can be clearly distinguished from samples of European countries. These genetic differences could be influenced by differences in environmental conditions that exist in those parts of Europe (i.e. Israel, Portugal and Spain) and South Africa, and also by the greater distances between the concerned areas.

The present results of the COI phylogenetic analysis for *C. imicola* from various localities in South Africa have shown that in general, there is a very low level of genetic divergence between local populations compared to midges from well separated countries of the Mediterranean basin (Dallas *et al.* 2003). This low genetic diversity is likely to be influenced by similar climatic conditions and the closer geographic distances between the study areas situated within the same country as opposed to distances between study areas that are situated in different countries with different climates.

4.3.2 *Culicoides bolitinos*

Currently there is no published work on a detailed genetic study of *C. bolitinos.* In this study an attempt was made to determine the genetic relationship between different geographical populations of *C. bolitinos* midges. The sequence and phylogenetic analysis of the four different South African populations (Port Elizabeth, George, Stellenbosch and Clarens) of *C. bolitinos* were performed using partial sequence of the COI gene of the mitochondrial genome. A multiple sequence alignment was generated and a total of 17 nucleotide substitutions were observed among individuals of different *C. bolitinos* populations along a total length of 472 bp. The same multiple sequence alignment was used to construct Fig. 4.5 which shows that there is a clear genetic diversity within this species, however, this is not linked to the geographic origin. Therefore these sequences do not provide any information about genetic differences of various

midge populations. However, variations were detected using DnaSP between individual midges of each group from different locations. The results of the DnaSP analysis have shown that midges from George are the most diverse of the four as shown by the highest nucleotide diversity, Pi=0.02102, highest haplotype diversity, HD=0.714 and the lowest standard deviation, SD=0.060 and all these values are above the combined average of the four populations (Table 4.7). The genetic diversity of midges from this area is further shown by the highest bootstrap values and the location of its individuals on four different branches of the tree (Fig. 4.5). This George population has the highest number of unique haplotypes that vary among its individuals (Table 4.6). These results may indicate that breeding between midges from George and other areas does take place, to a certain extent, and that if *C. bolitinos* is proven to be a vector, disease might spread via infected vectors during an outbreak.

The tree has also shown that some members of *C. bolitinos* from Clarens, Port Elizabeth and Stellenbosch areas are grouped together on a single branch due to identical sequences as shown in subcluster 2A (Fig. 4.5), as well as the possession of haplotype 01 that is dominant in midges from the three localities. The individual midges which share similar haplotypes are closely related. The mitochondrial COI gene could not provide any indication that geographical population of this species are genetically separated. However, the branching pattern between subcluster 2A and 2B (Fig. 4.5) suggests the existence of *C. bolitinos* individuals of distinct genotypes.

4.4 CONCLUSION

The COI marker seems to reflect the geographical separation of the different *C. imicola* populations, and this could be useful for phylogeographic and population genetics studies. However, a further study will be required to confirm this.

There is a clear genetic divergence or separation within the species classified as *C. bolitinos* according to morphological markers. These genetic subgroupings are

not artifacts as can be seen from their repeated occurrence within these samples. It can be speculated that this might be a case of the so called “cryptic species” and further investigations will be required for confirmation.

CHAPTER 5

PHYLOGENETIC ANALYSIS OF *CULICOIDES* POPULATIONS BASED ON MITOCHONDRIAL 16S rRNA GENE FRAGMENT

5.1 INTRODUCTION

This chapter describes the amplification of a specific fragment of the mitochondrial 16S rRNA gene for the species of *C. imicola* and *C. bolitinos* using PCR. The DNA used in Chapter 4 were also used for analysis in this study. The PCR products were used for sequencing and phylogenetic analysis as in the previous chapter. The results of the two species were compared and discussed.

5.2 RESULTS

5.2.1 Primer selection

Culicoides imicola 16S rRNA partial gene sequence obtainable from the GenBank (accession: AF083045) was used to design a pair of primers shown in Table 5.1. These primers were used for PCR as well as DNA sequencing of the two *Culicoides* species.

Table 5.1 16S rRNA primers, binding sites, sizes and their nucleotide sequences

Primer Name	Orientation of primer	Primer binding site	Amplicon size (bp)	Primer Sequence
16S rRNA **1A**	Forward	71-90	468	5'- CCGCAGTATACTGACTGTGC -3'
16S rRNA **1B**	Reverse	515-538	468	5'-ATCATGTAAGAATTCAAAAGTCG-3'

5.2.2 PCR and nucleotide sequencing

A volume of 5 µl genomic DNA (0.5-1 µg) was amplified using the 16S rRNA primers described in Table 5.1. Both *C. imicola* and *C. bolitinos* DNA were successfully amplified using this set of primers. The size of the PCR products was approximately 460 bp (Fig. 4.1). Cycle sequencing was done using approximately 5-20 ng of purified PCR product with the same primers that were

used for PCR. The nucleotide sequencing produced sequences ranging from 445-475 bp.

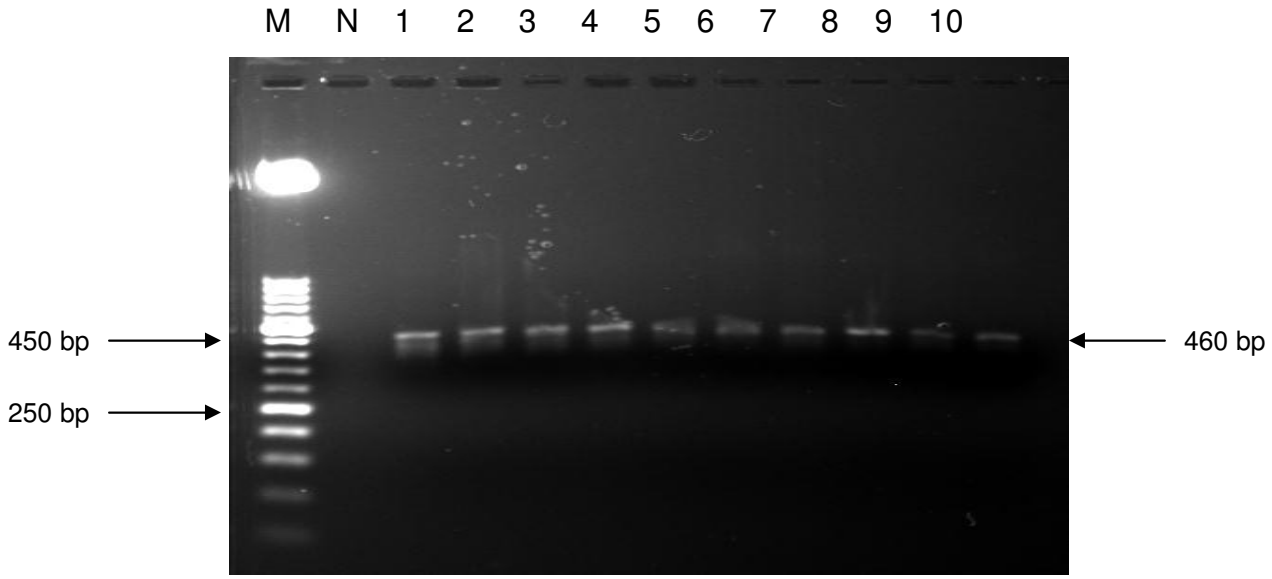


Figure 5.1: A 1.2% TBE agarose gel showing 16S rRNA-specific PCR products from the individual *C. imicola* and *C. bolitinos* from various regions of South Africa. Lanes **M** = Molecular Weight Marker (50 bp), Lane **N** = negative control with no DNA, Lane **1-5** = *C. imicola*, Lane **6-10** = *C. bolitinos*. The gel shows a uniform banding pattern for both species with an approximate size of 460 bp

5.2.3 Phylogenetic analysis

Phylogenetic analysis was done as explained in section 4.2.4. Sequences of 445 bp were generated after trimming the 16S rRNA multiple sequence alignments. The *C. imicola* and C. *bolitinos* phylogenetic trees were constructed based on 57 and 53 nucleotide sequences respectively. Fig. 5.2 was used to determine the relationship of the two species. Different colours were used in Figs 5.3 & 5.4 to

differentiate individuals from different localities. GenBank sequences (Table 5.2) were included to compare with the sequences generated in this study. Multiple sequence alignments shown in Appendix 4, 5 and 6 were used to construct the phylogenetic trees in Figs 5.2, 5.3 & 5.4 respectively using Neighbor joining method (Saitou & Nei 1987).

Table 5.2 *Culicoides* GenBank sequences included in this investigation

Species and code	Isolation year	Place of origin	Genbank Accession no.
C. tuttifrutti NHMT15	May 2004	Kimberly, S.A	AY294137
C. imicola RSA 1	July 1999	Onderstepoort, S.A	AF083045
C. imicola RSA 2	July 1999	Onderstepoort, S.A	AF083048
C. imicola RSA 6	July 1999	Onderstepoort, S.A	AF083046
C. imicola RSA 10	July 1999	Onderstepoort, S.A	AF083047
C. imicola NHM161	May 2004	Onderstepoort, S.A	AF294138
C. imicola NHM171	May 2004	Onderstepoort, S.A	AF294139
C. imicola NHM181	May 2004	Onderstepoort, S.A	AF294140
C. imicola NHM191	May 2004	Onderstepoort, S.A	AF294141
C. imicola AOND 1	January 2001	Onderstepoort, S.A	AF281314
C. imicola AOND 2	January 2001	Onderstepoort, S.A	AF281317
C. imicola AOND 6	January 2001	Onderstepoort, S.A	AF281315
C. imicola AOND 10	January 2001	Onderstepoort, S.A	AF281316
C. imicola IBET 15	July 1999	Israel: Bet Dagan	AF083054
C. imicola IBET 18	July 1999	Israel: Bet Dagan	AF083055
C. imicola IBET 31	July 1999	Israel: Bet Dagan	AF083057
C. imicola IBET 35	July 1999	Israel: Bet Dagan	AF083058
C. imicola IBET 45	July 1999	Israel: Bet Dagan	AF083059

KEY: RSA = Republic of South Africa, SA = South Africa, NHM = Onderstepoort Isolate name, AOND = Onderstepoort isolate name, IBET = Israel Bet isolate name.

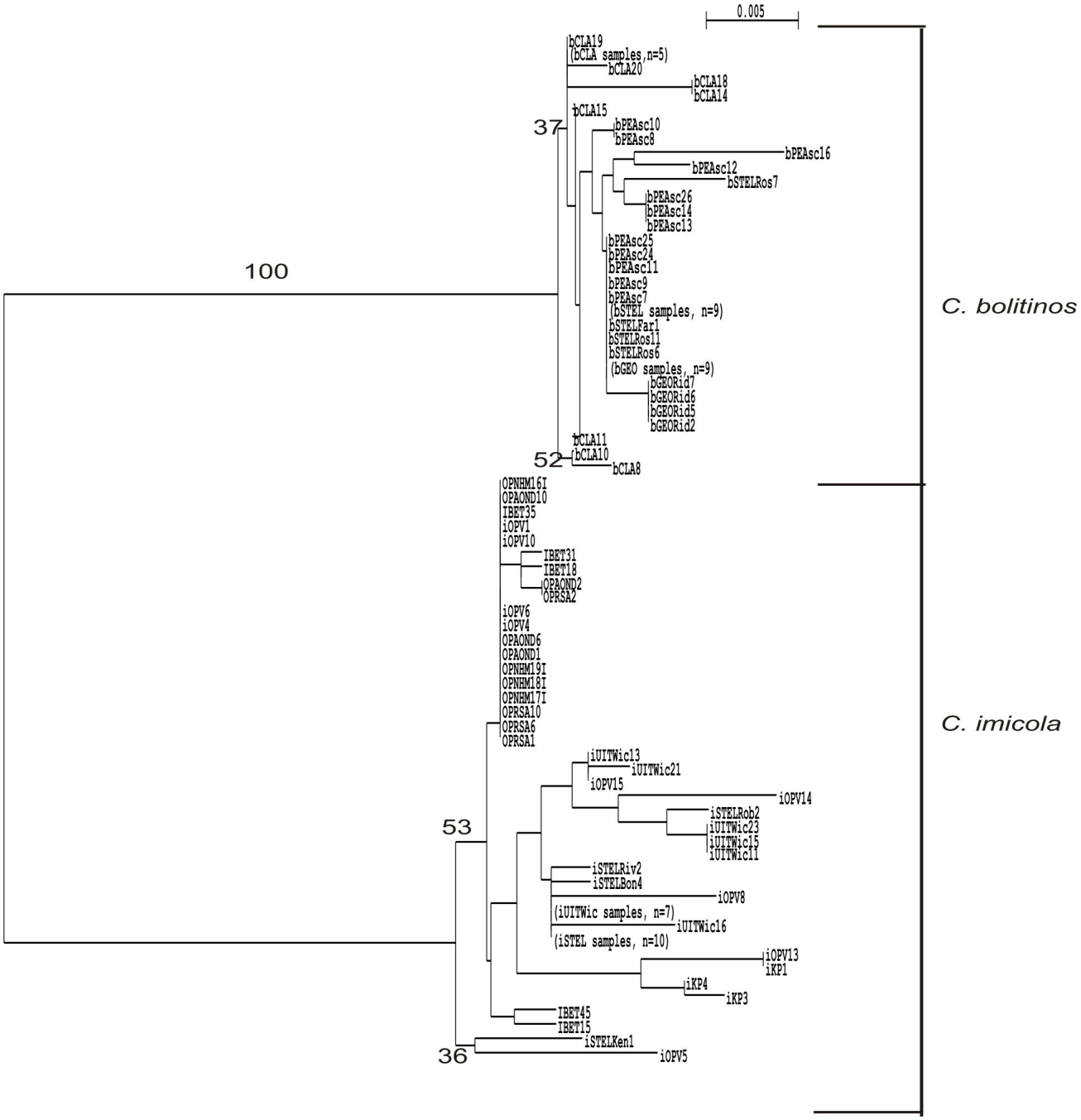


Figure 5.2: Phylogenetic relationships among the two members of the Imicola Complex analysed using mitochondrial 16S rRNA gene fragments. Neighbor-joining tree constructed from alignments of 445 bp partial nucleotide sequences of the mitochondrial DNA.

Table 5.3 Comparison of twenty one 16S rRNA haplotypes for *C. imicola* populations from different geographical areas generated using DnaSP (Rozas *et al*. 2003)

Population	**Site Code**	**Number of specimens**	**Number of haplotypes**	**IMI16S haplotype**
Onderstepoort	OVI KP	20 4	9	01, 02,07,08,09, 10,11,12,13
Stellenbosch	Bea Bon Ken Kun Riv Rob Tri	1 2 2 2 3 3 1	4	14,15,16,17
Kirkwood	Wic	13	4	14,19,20,21
Bet Dagan (Israel)	IBET	5	5	01, 03,04,05,06

Table 5.4 Statistical values used to estimate the 16S rRNA diversity of *C. imicola* from different geographic areas in South Africa (Rozas *et al*. 2003)

Population	**Nucleotide Diversity (*Pi*)**	**Standard Deviation (SD)**	**Haplotype Diversity (Hd)**	**No. of Polymorphic Sites (S)**
Onderstepoort	0.00814	0.075	0.649	22
Stellenbosch	0.00309	0.111	0.487	10
Kirkwood	0.00492	0.081	0.665	8
Overall results	0.00787	0.025	0.832	31

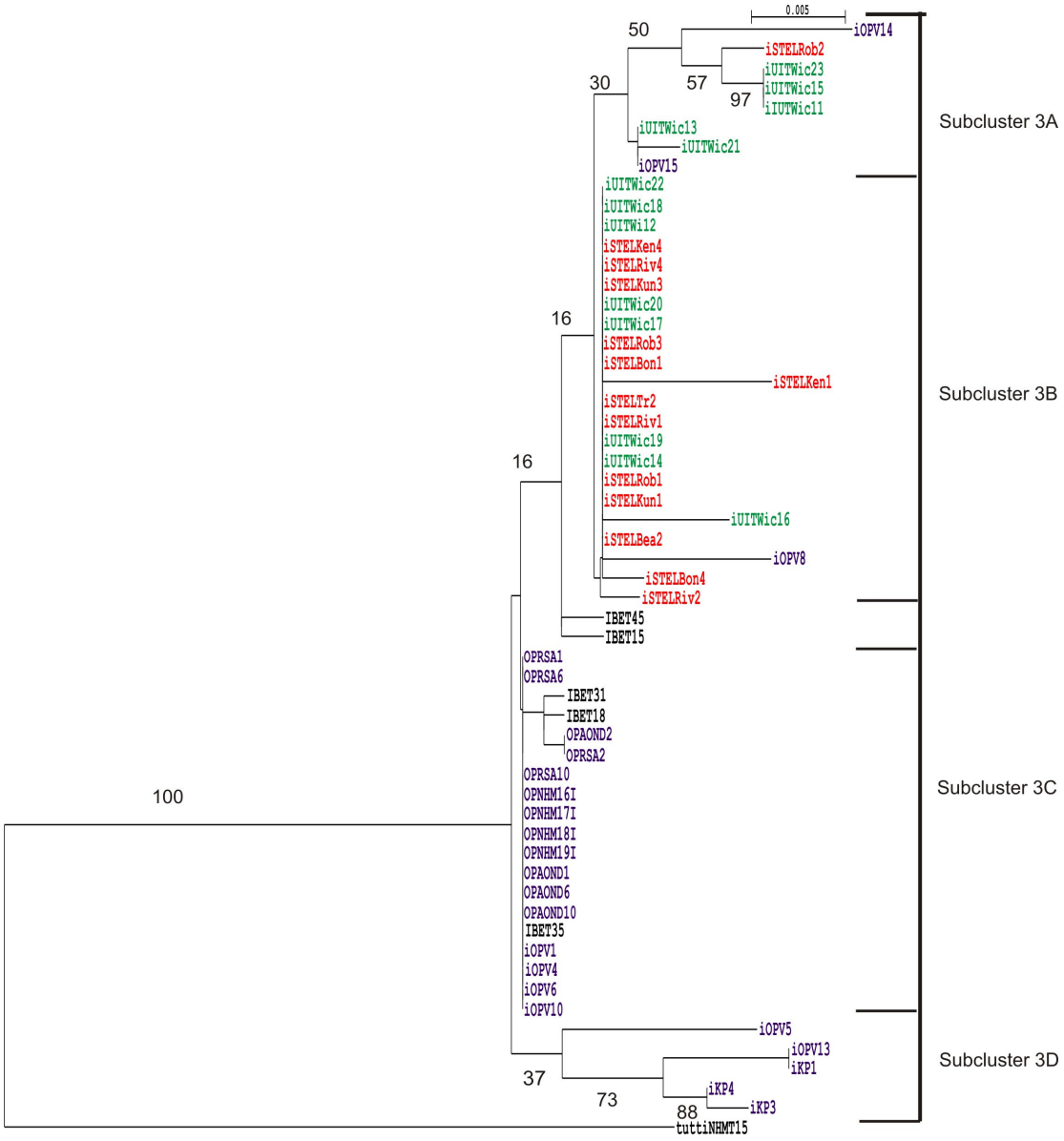


Figure 5.3: Phylogenetic relationships of the *Culicoides imicola* based on mitochondrial 16S rRNA gene fragments of individual midges from different localities. The DNA nucleotide alignment of 445 bp was used to construct the phylogenetic tree using Neighbor-joining method. *Culicoides tuttifrutti* (tuttiNHMT15) was used as an out group to root the tree. Populations are described by different colours; Black - GenBank, Purple - Onderstepoort, Red – Stellenbosch, Green - Kirkwood

Table 5.5 Comparison of the nineteen 16S rRNA haplotypes for *C. bolitinos* populations from different geographical areas generated using DnaSP (Rozas *et al*. 2003)

Population	Site Code	No. of specimens	No. of haplotypes	BOL16S haplotype
Clarens	Cla	13	6	01, 02,03,04,05,06
George	Geo	13	6	07, 08,09,10,11,12
Stellenbosch	Far Ros	2 11	5	07, 09,13,14,15
Port Elizabeth	Wic	13	5	07,16,17,18,19

Table 5.6 Statistical values used to estimate 16S rRNA diversity of *C. bolitinos* from different geographic areas in South Africa (Rozas *et al*. 2003)

Population	Nucleotide Diversity (*Pi*)	Standard Deviation (SD)	Haplotype Diversity (Hd)	No. of Polymorphic Sites (S)
Clarens	0.16813	0.071	0.751	171
George	0.04143	0.037	0.837	42
Stellenbosch	0.03045	0.114	0.551	41
Port Elizabeth	0.00435	0.051	0.810	6
Overall results	0.0832	0.042	0.870	196

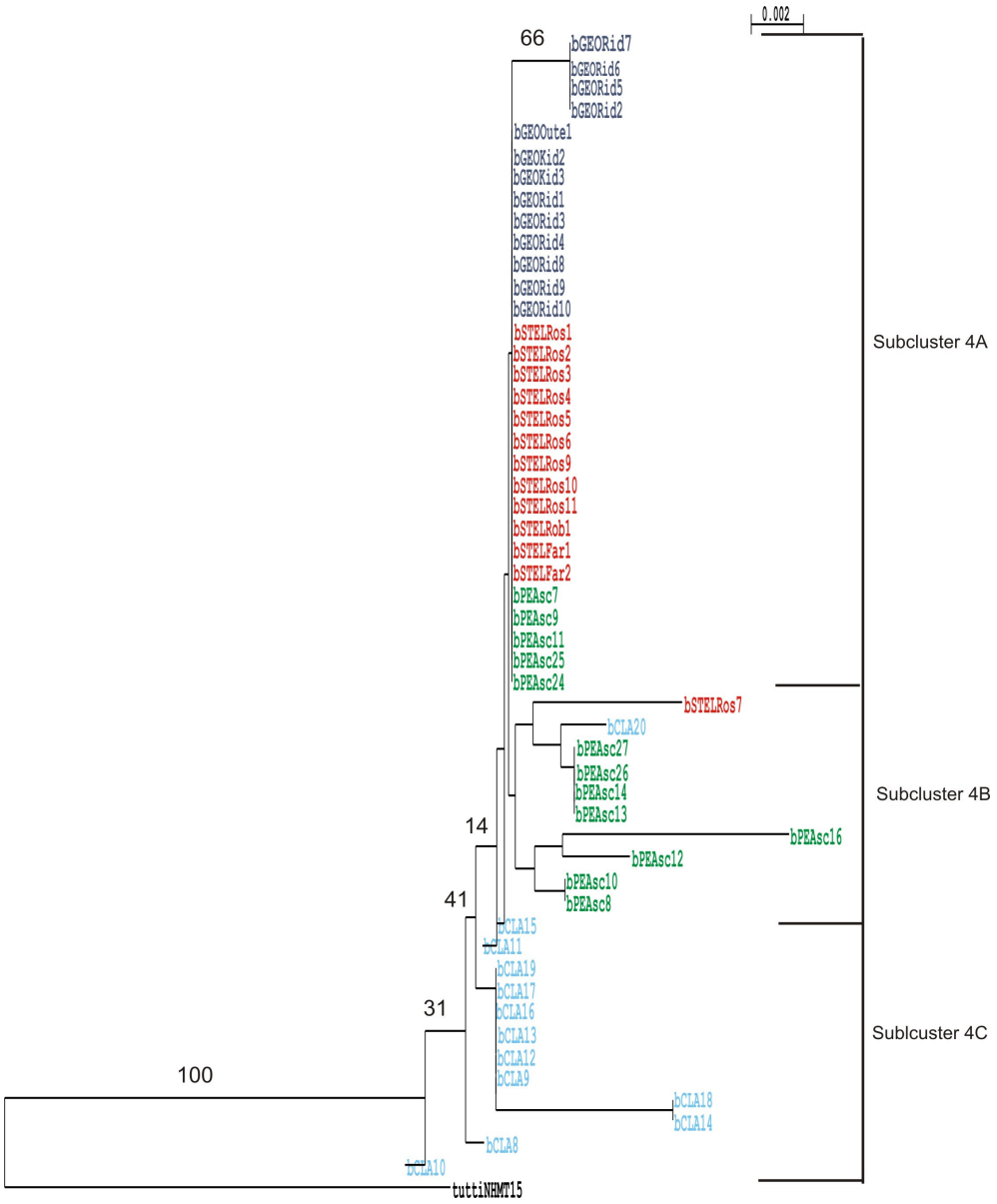


Figure 5.4: Phylogenetic relationships among *Culicoides bolitinos* from different regions of South Africa. The DNA nucleotide alignment of 446 bp was used to construct the phylogenetic tree using Neighbor-joining method. TuttiNHMT15 was used as an out group to root the tree. Populations are described by different colours: Black - GenBank, dark blue: George, Ice blue: Clarens, Red: Stellenbosch, Green: Port Elizabeth

5.3 DISCUSSION

In this chapter, the 16S rRNA gene was used for the characterisation of *C. imicola* and *C. bolitinos* samples used in Chapter 4. Amplified PCR products of 460 bp (Fig. 5.1) for the two species were sequenced. Sequences of the 16S rRNA gene fragment for the two *Culicoides* species have shown that their nucleotide base composition has high A+T content like most other insect mitochondrial rRNA sequences (Xiang & Kochar 1991; Shouche & Patole 2000). It was on average 80% and 82% for *C. bolitinos* and *C. imicola* sequences respectively. Fig. 5.2 shows that *C. imicola* and *C. bolitinos* from South Africa belonged to different phylogenetic clades of 16S rRNA, and are phylogenetically distinct from each other. This is as expected from two distinct species and confirmed the classification of the specimens on morphological basis. The high bootstrap value of 99% observed between main branches of the two *Culicoides* species indicates high confidence in the tree topology.

5.3.1 *Culicoides imicola*

The relationship between *C. imicola* midges collected in all study areas was determined using the mitochondrial 16S rRNA gene sequences. A total of 56 *C. imicola* sequences were included in the analysis. Comparison of sequences through multiple sequence alignment has shown that the total number of substitutions within individuals of *C. imicola* was 16 over a total length of 445 bases. These differences were observed in 21 individual midges of which 12 are from Onderstepoort. The most common substitution was the transition type which accounted for approximately 74% of the total substitutions in *C. imicola* (Appendix 5). These substitutions were not linked to midge geographic origin. *Culicoides imicola* collected in Kirkwood and Stellenbosch could be easily distinguished from those collected in Onderstepoort due to the substitution of A by T at position 442 and T by C at 444th base in all sequences and also share haplotype 14 (Table 5.3) which is dominant within the two populations. This has also been shown by the clustering together of midges from the two areas (Fig 5.3), however, the low bootstrap values indicates low confidence in the tree

topology. These results do not correlate with the findings made using COI gene analysis where Kirkwood midges appeared as a distinct genotype. This shows a limited ability of this gene to separate midges from this area according to geography.

Similarly, as in the previous chapter, Table 5.4 and 5.6 show that the nucleotide diversity which reflects genetic diversity is always inversely proportional to the standard deviation (i.e. the lower the standard deviation the higher the genetic diversity and vice-versa). The results from DnaSP have shown that *C. imicola* from Onderstepoort are more diverse and highly variable than midges from the other two study areas as indicated by the highest nucleotide diversity, Pi=0.0814 and the lowest standard deviation, SD=0.075 (Table 5.4). This diversity has also been shown by the wide spread of individuals from Onderstepoort on the phylogenetic tree (Fig. 5.3). This results correlate with the findings made using COI in Chapter 4 and support the notion that Onderstepoort midges do interbreed with midges from other areas in this study and that an outbreak of a disease in this region is likely to spread via infected vectors to other locations. High genetic diversity of the *C. imicola* from this region could also be influenced by the abundance of this species in the region (i.e. high numbers increases chances of breeding with individuals of different genotypes which may increase variations).

Midges from Bet Degan (Israel) and Onderstepoort (South Africa) are clustered together in subcluster 3C and 3D (Fig. 5.3) due to identical sequences, which indicates that the 16S rRNA partial gene fragment fails to discriminate individuals from two different countries on separate continents. The two groups of midges also share haplotype 01 which is not found in both Kirkwood and Stellenbosch midges (Table 5.3). This implies that if the 16S rRNA cannot distinguish between midges originating from different hemispheres, it is highly unlikely that it will be able to distinguish between midges from different areas within a single country. This partial gene fragment has low discriminating power in *C. imicola* compared

to COI as it could not separate midges from any of the three areas according to geography. These results do not provide enough information that can be used to predict disease spread and/or help with the implementation of control strategies.

5.3.2 *Culicoides bolitinos*

Sequence analysis of the *C. bolitinos* 16S rRNA gene has shown that the transition and transversion substitutions accounted for 50% each. A multiple sequence alignment of this species has shown a very low number of substitutions observed at 14 different positions in 13 individuals along a total length of 445 bp (Appendix 6). Most of these nucleotide substitutions occurred in sequences of individuals caught in Clarens. The substitution of T by A at position 305 is the only unique character state that distinguishes Clarens population of *C. bolitinos* from the other three. Midges from Clarens contain six unique haplotypes with no shared haplotype and have been grouped separately from others as shown in subcluster 4C (Fig. 5.4). However, the low bootstrap values indicate low confidence in tree topology.

Phylogenetic analysis indicated that *C. bolitinos* midges from Stellenbosch, George and Port Elizabeth are closely related due to clustering together in subcluster 4A as a result of their identical sequences (Fig 5.4; Appendix 6). Haplotype analysis has also indicated that haplotype 07 is dominant in populations from Stellenbosch, George and Port Elizabeth whereas haplotype 09 is only dominant in Stellenbosch and George (Table 5.5). This is partly expected since the latter two areas are geographically closer to each other than to Port Elizabeth, hence two dominant haplotypes are shared by the two groups. It could be that the closer the distance between the two study areas the more related their midges will be. It is therefore assumed that low bootstrap values and high sequence homology within this species is an indication that the 16S rRNA partial gene has a very low discriminating power between populations of *C. bolitinos* compared to that of the COI gene. Haplotype analysis, however, has shown that

midges from George are more diverse (HD=0.837) than midges from other areas (Table 5.6).

5.4 Conclusion

The genetic analysis of midges using 16S rRNA marker has shown that there are clear genetic diversities within *C. imicola* and *C. bolitinos* species. However, this marker does not provide any meaningful information that reflects the geographic origin of individual midges within the two species. Therefore this target region is not appropriate for phylogeographic and population genetic studies. It could be more likely due to the nature of this gene and the short area that was sequenced. However, this partial gene could still be useful in determining the diversity of a single *Culicoides* species within one area.

CHAPTER 6

6 GENERAL DISCUSSIONS AND CONCLUSIONS

In South Africa, the geographic distribution of the *Culicoides* species, in particular that of *C. imicola,* has been well-described (Meiswinkel 1989; Baylis *et al* 1998; Meiswinkel 2004b). Although most livestock associated *Culicoides* species, and especially *C. imicola,* are found wide spread in the country, it is not clear to what extent populations from different localities are genetically isolated from each other. The dispersal of vectors in the country might be linked to the risk of virus spread from one geographic area to another. It is known that the dispersal of *Culicoides* can be influenced by environmental and geophysical factors acting as natural barriers (Bishop *et al*. 2004). Since morphological characteristics are usually inadequate to determine relationships among individuals and populations of the midge vector species, genetic characterization was investigated for this purpose.

The present work was intended to identify a suitable genetic marker that can subsequently be applied to reveal subpopulations of *C. imicola* and *C. bolitinos* with the objective of doing a phylogeographic and population genetic studies. This study was partly motivated by oral susceptibility results that indicate that the *C. imicola* population from Stellenbosch was significantly more susceptible to AHSV serotype 8 (AHSV serotype 8 88/99 Pietermaritzburg) than the Onderstepoort population of *C. imicola* (Venter *et al.* 2009). Although oral susceptibility and vector competence are influenced by external environmental factors, mainly are dependent on the genetic makeup of the vector midge (Tabachnick 1991; Wellby *et al.* 1996; Mellor *et al.* 1998; Wittmann *et al.* 2001). Differences in oral susceptibility in *C. imicola* from Onderstepoort and from Stellenbosch suggested that these two populations could differ genetically.

Due to their rapid rate of evolution (Brown *et al*, 1979; 1982), mitochondrial genes are believed to give better resolutions in population genetic studies than

nuclear DNA genes and hence were chosen for genetic analysis in this study. Examples include the use of the ITS2 ribosomal nuclear DNA gene to determine the phylogenetic relationship of *C. obsoletus* populations in Italy (Ludivik *et al.* 2005). The results indicated that there was no significant difference between and within populations of this species, which suggested that ITS2 could not detect any population substructures (Ludivik *et al.* 2005). The ITS1 nuclear ribosomal DNA gene located between 18S and 5.8S was chosen, due to its well known structural and evolutionary properties (Elder & Turner 1995), as a molecular tool to study populations of *Culicoides* in France (Perrin *et al.* 2006). However, the results from the French study indicated that populations of *C. obsoletus* and *C. imicola* displayed 99.2% and 99.8% sequence identities respectively (Elder & Turner 1995). This clearly indicated that these targeted regions of nuclear DNA origin are not appropriate for genetic discrimination among individuals of the same species.

Several authors have shown that mitochondrial COI has the ability to discriminate various geographic populations of *C. imicola* in the Mediterranean basin (Dallas *et al*. 2003; Nolan *et al.* 2008; Calvo *et al.* 2009). Similarly, macro-geographic population structure was detected in populations of tsetse fly *Glossina pallidipes* from six countries in East and southern Africa at the mitochondrial level using 16S ribosomal RNA (r16SII), COI, COII, fragment between cytochrome oxidase II and transfer RNA leucine (COIITLII) and cytochrome B1 (cyB1) through SSCP (Ouma *et al.* 2005). This clearly illustrates the ability of mitochondrial markers to distinguish among populations of the same species in various insects species. Based on that, COI and 16S rRNA were preferred markers in this study for the analysis of populations of *C. imicola* and *C. bolitinos* in South Africa. The selection of study areas were based on the association of the region with the history of AHS outbreaks. It was hoped that the characterisation of these populations would assist with the implementation of effective disease control strategies based on the risk of the viral spread from endemic areas.

6.1 Intrapopulation variation

The molecular markers used have shown different results when used to detect differences among midges within each sampling area for each of the two target species. Both markers have indicated that individuals of *C. imicola* from Onderstepoort were more variable compared to individuals from other regions as reflected by high nucleotide diversities, high haplotype diversities, low standard deviations, number of polymorphic sites (Tables 4.5 & 5.4) and the branching patterns of the phylogenetic trees (Figs 4.5 & 5.3). This high genetic variation in individuals of the Onderstepoort population could be the result of interaction of this population with midges from other locations. Such interaction will support the accepted idea that large number of *C. imicola* found in the north, due to warmer temperatures, may eventually spread to the southern part of the country when environmental conditions become suitable during the warmer summer months. It is known that AHS can occur throughout the year in the north of the country (Barnard 1993) and then spread towards the south. This southwards spread of the virus could be attributed to midge movement.

The *C. imicola* population from Kirkwood is more uniform and has high sequence identity. These identical sequences resulted in the clustering of midges from this area on a single branch (Fig. 4.5) and have high standard deviation, low nucleotide diversity and low number of haplotypes as well as few polymorphic sites within the COI gene of individual midges (Table 5.4). This could indicate that natural geographic barriers have isolated midges in this region from the other study areas. The implication will be that AHS from other areas will not spread via infected *C. imicola* into the area and visa versa. Based on the results of the 16S rRNA gene, sequences of *C. imicola* from Kirkwood are not identical as shown by COI gene. This part of the gene has shown that it could not distinguish midges according to geography. Clear genetic diversities were observed among *C. bolitinos* individuals within each study area when using COI, however, the George population was the most diverse as shown by nucleotide

diversities, haplotype diversities, standard deviations as well as high bootstrap values between branches.

6.2 Interpopulation variation

Generally, not many differences were observed between populations of each of the two target *Culicoides* species. The exception being the clear genetic differences, within the COI region of the mitochondrial DNA, that distinguish *C. imicola* from Kirkwood from that of other areas. These differences were detected through the analysis of the multiple sequence alignment (Appendix 2), haplotype analysis (Table 4.4) as well as the branching pattern of the phylogenetic tree (Fig. 4.5). Twelve of 22 individual *C. imicola* from Stellenbosch possess a distinct genotype that could be used to separate them from midges of other areas as shown by subcluster 1C (Fig. 4.5). However, the results of the COI gene of *C. imicola* from Onderstepoort did not provide meaningful information that could be used to classify them according to geography. Individual midges from the latter area were always clustered together with midges from other regions.

The use of mitochondrial COI partial gene to characterise *C. bolitinos* based on their geographic origin did not yield any significant results. The *C. bolitinos* collected at different locations did not display any genetic differences according to geography, but differences were clear between certain individuals of this species regardless of their origin.

Genetic analysis of the *C. imicola* and *C. bolitinos* using 16S rRNA yielded very limited information regarding the geographic origin of the two species. No significant sequence differences were observed between *C. imicola* midges from all study areas. The overall 16S rRNA results have shown that these midges are genetically similar, however, minor sequence differences that were not linked to geographical location, were observed within few individuals. Although. *C. bolitinos* midges from Clarens have shown to possess unique haplotypes as well as clustering together in subcluster 4C (Fig. 5.4), the low bootstrap values

indicate low confidence in tree topology. Therefore, these results did not provide sufficient information that can be used to separate midges based on their geography. Overall, the results obtained in the present study support the findings made using RAPD markers whereby high level of genetic variation within South African *C. imicola* populations were found, but this variation did not correlate with geographic origin (Sebastiani *et al*. 2001).

6.3 Conclusion

Both markers have shown that the Onderstepoort population of *C. imicola* shares some genetic similarities with midges from all other study areas. This could indicate that Onderstepoort midges do interbreed with midges from other areas thus suggesting that an outbreak of AHSV in this area could spread to other localities in the vector. It does seem unlikely though, that this could happen naturally between Onderstepoort and Stellenbosch populations. Another explanation could be that midges might have been transported in one way or another between the areas.

The COI partial gene could not distinguish any of the *C. bolitinos* midges according to geography. It has, however, shown that there are two distinct genotypes within this species in all sampled areas in South Africa because of the clear genetic separation that was observed. This could indicate that some taxa do not interbreed even though they are of the same species occurring within the same location. As a result, phylogenetic analysis would not group these individuals of *C. bolitinos* according to geography but based on their genetic makeup and this could be an indication of a cryptic species. Genetic characterization using different markers will be important in confirming this finding. Overall, COI has the potential to resolve populations of *C. imicola* and has shown to be a better marker than the 16S rRNA.

6.4 Recommendations

1. More genetic markers will be needed to clarify South African midge population structures, especially microsatellites markers which are known to exhibit high discriminatory power and are more polymorphic.
2. Increasing the sample size will improve the current knowledge of the midge genetic structure in the country.
3. It is recommended that additional areas (i.e. other South African Provinces and neighbouring countries of the South African Development Community) be sampled to improve the current understanding of the midge movement and dispersal.
4. These genetic studies need to be extended to include other equally wide spread but the less abundant *Culicoides* species. E.g. *Culicoides* species with more restricted breeding site preferences.
5. Results generated by genetic analyses need to be supported by taxonomical and biological studies.

CHAPTER 7

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Appendix 1

Multiple sequence alignment of the *C. imicola* and *C. bolitinos* nucleotide sequences of the COI partial gene fragment for all individual midges used in this study. *Culicoides tuttifrutti* (TUT1) sequence was used as a reference to generate the alignments. All the nucleotide sites that are similar to TUT1 are represented by the dots. The alignment was used to compare the two species of *Culicoides*. All the names of the sequences that were obtained from the GenBank start with capital letters. The alignment was carried out using the Clustal X program. The prefix i represents *C. imicola* and b represents *C. bolitinos*. The symbol (-) indicates deletions.

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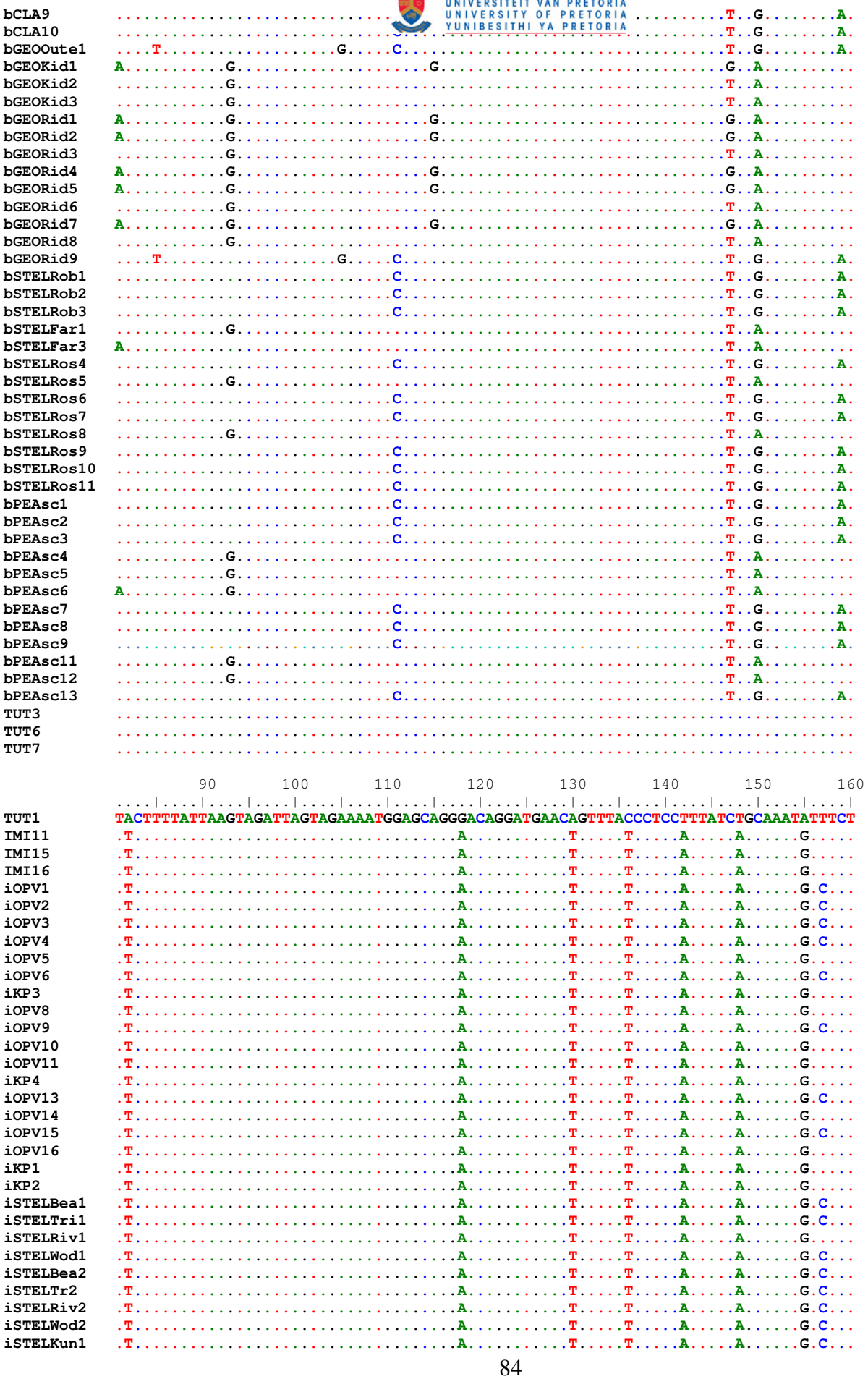

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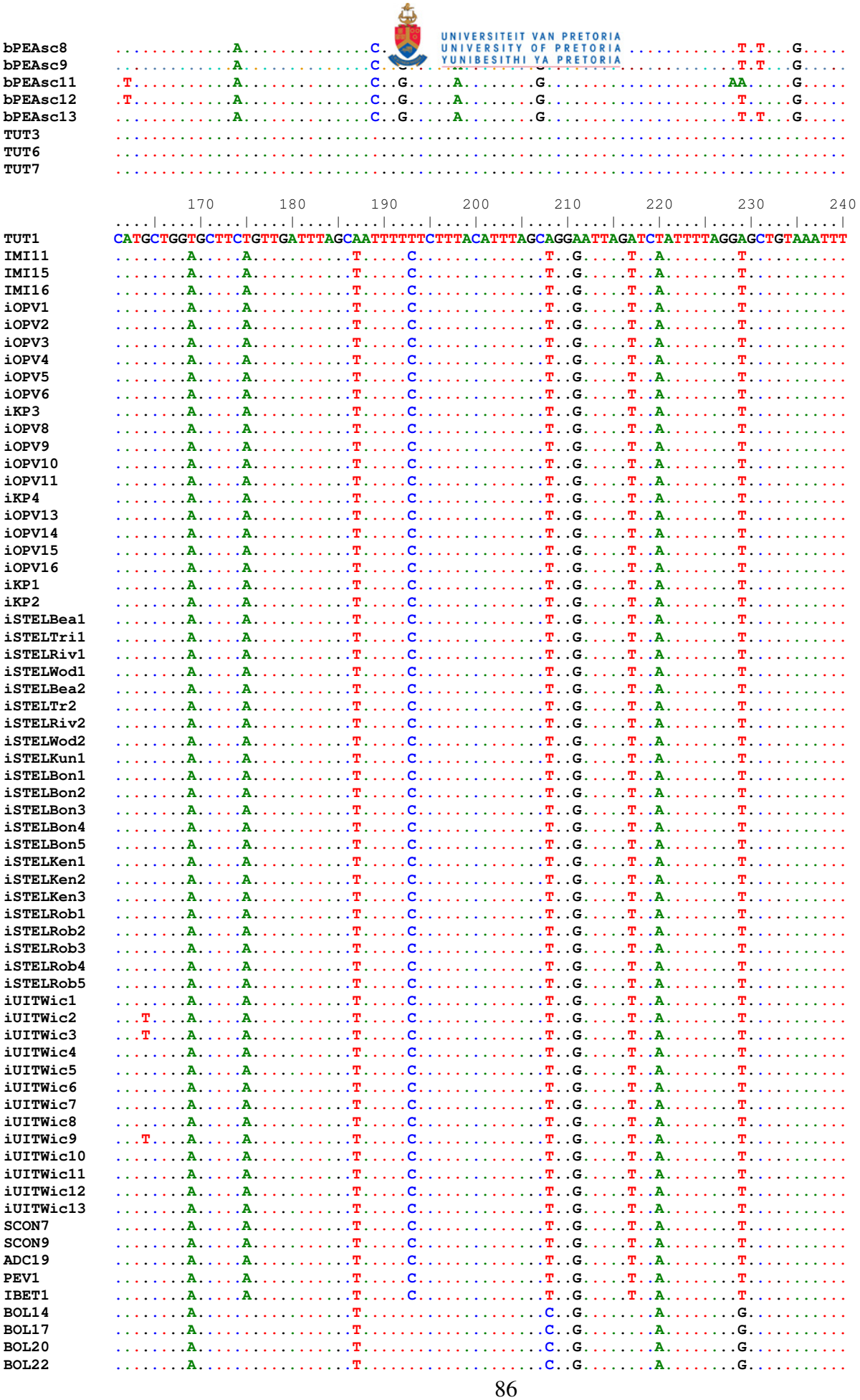

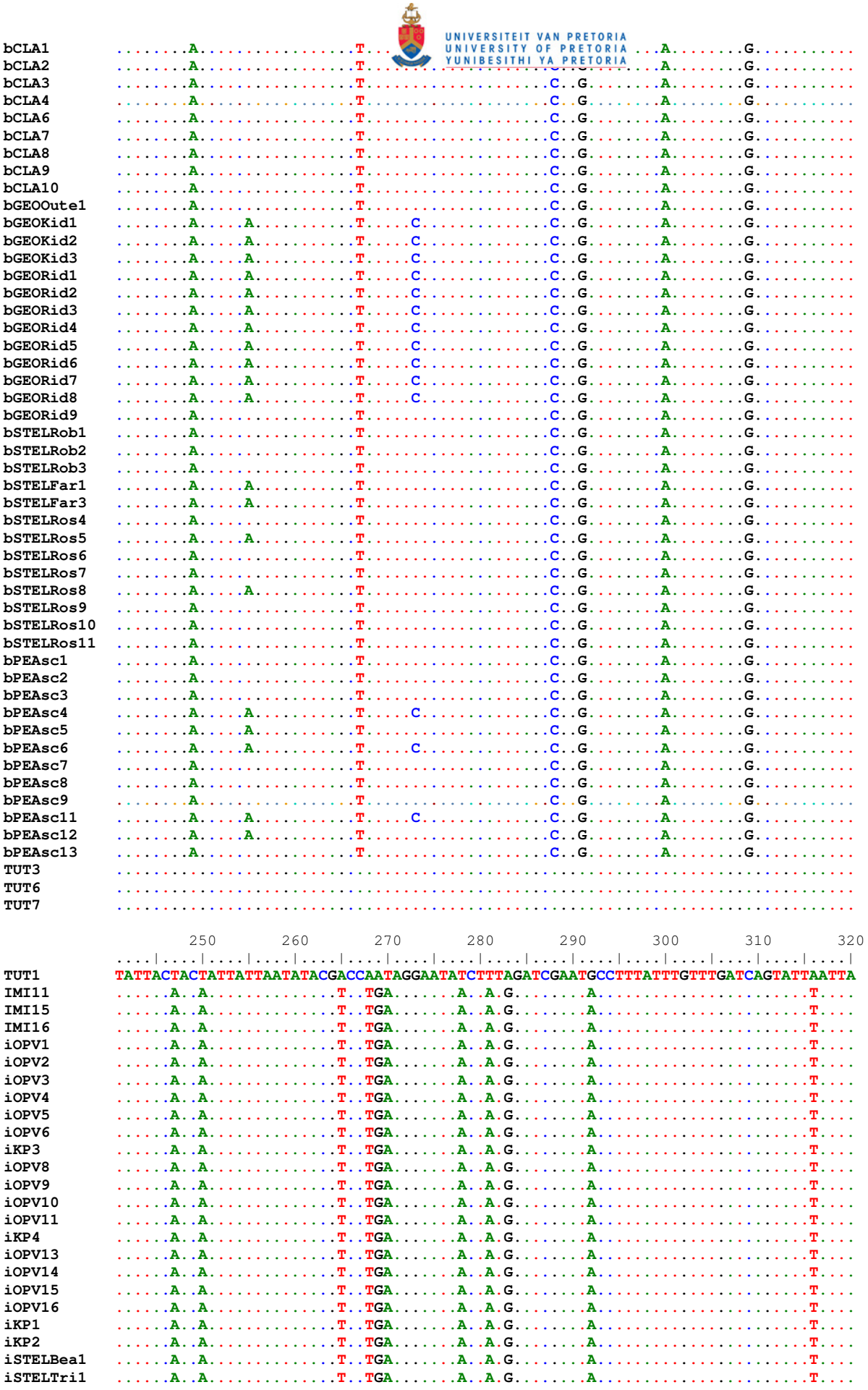

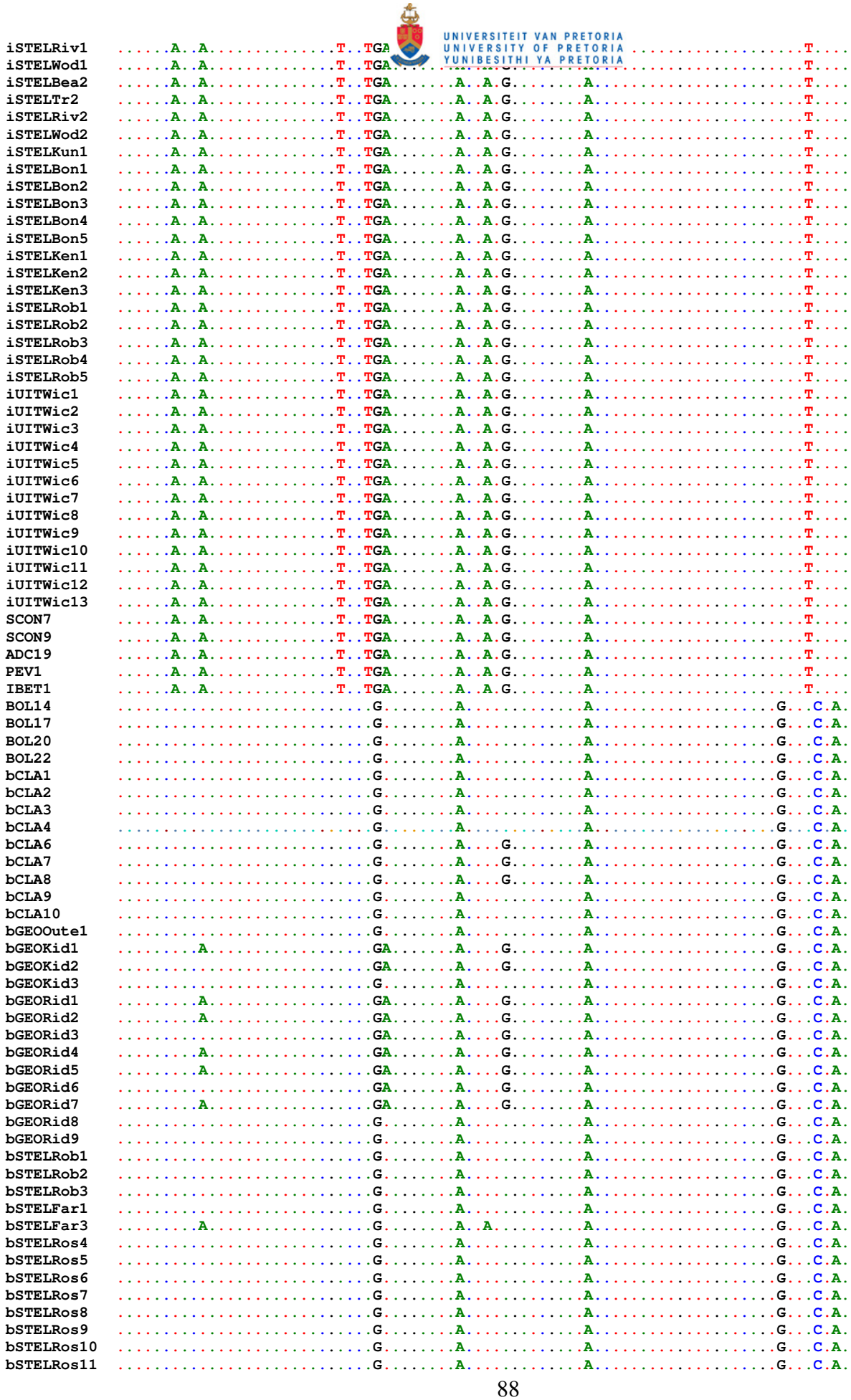

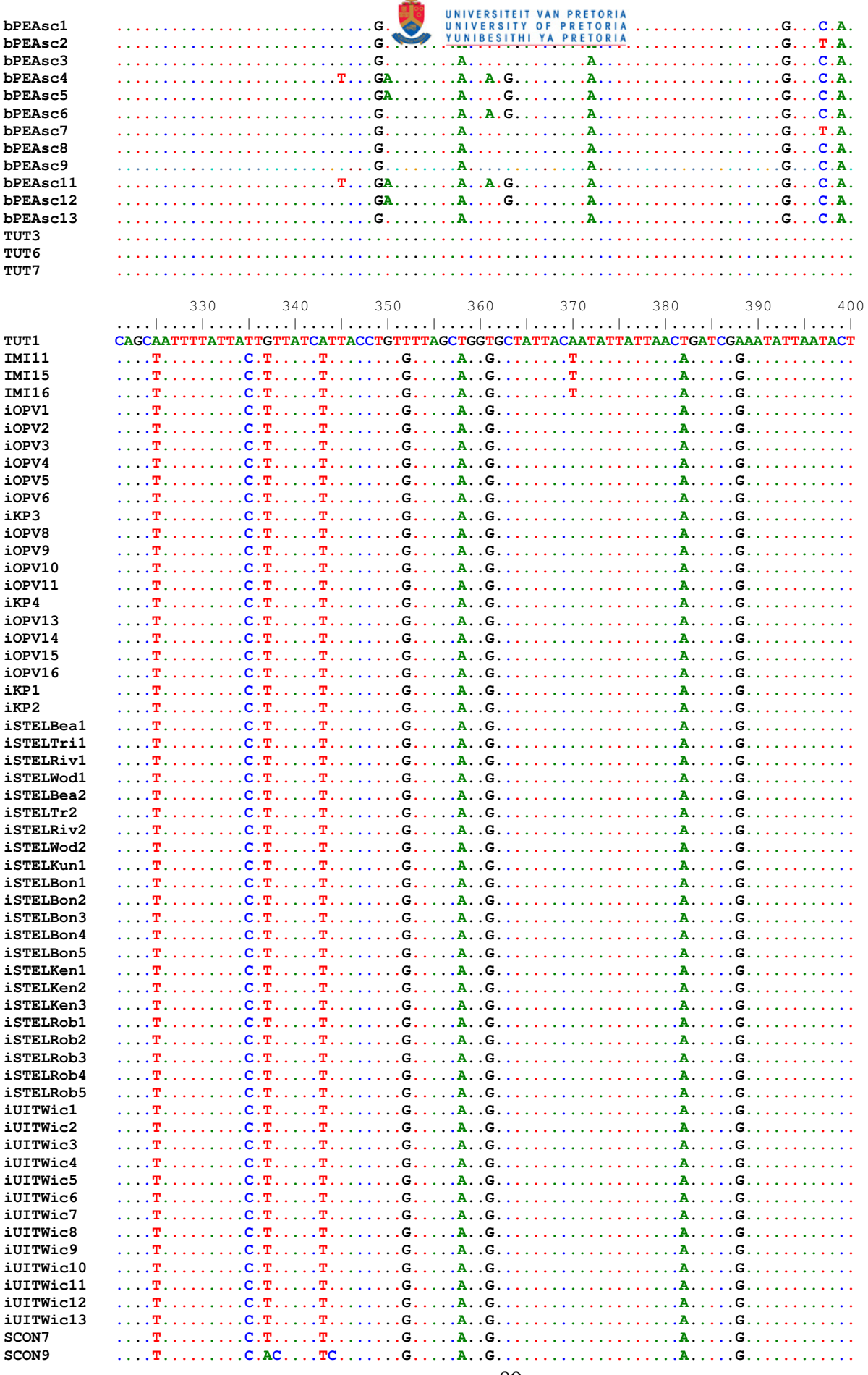

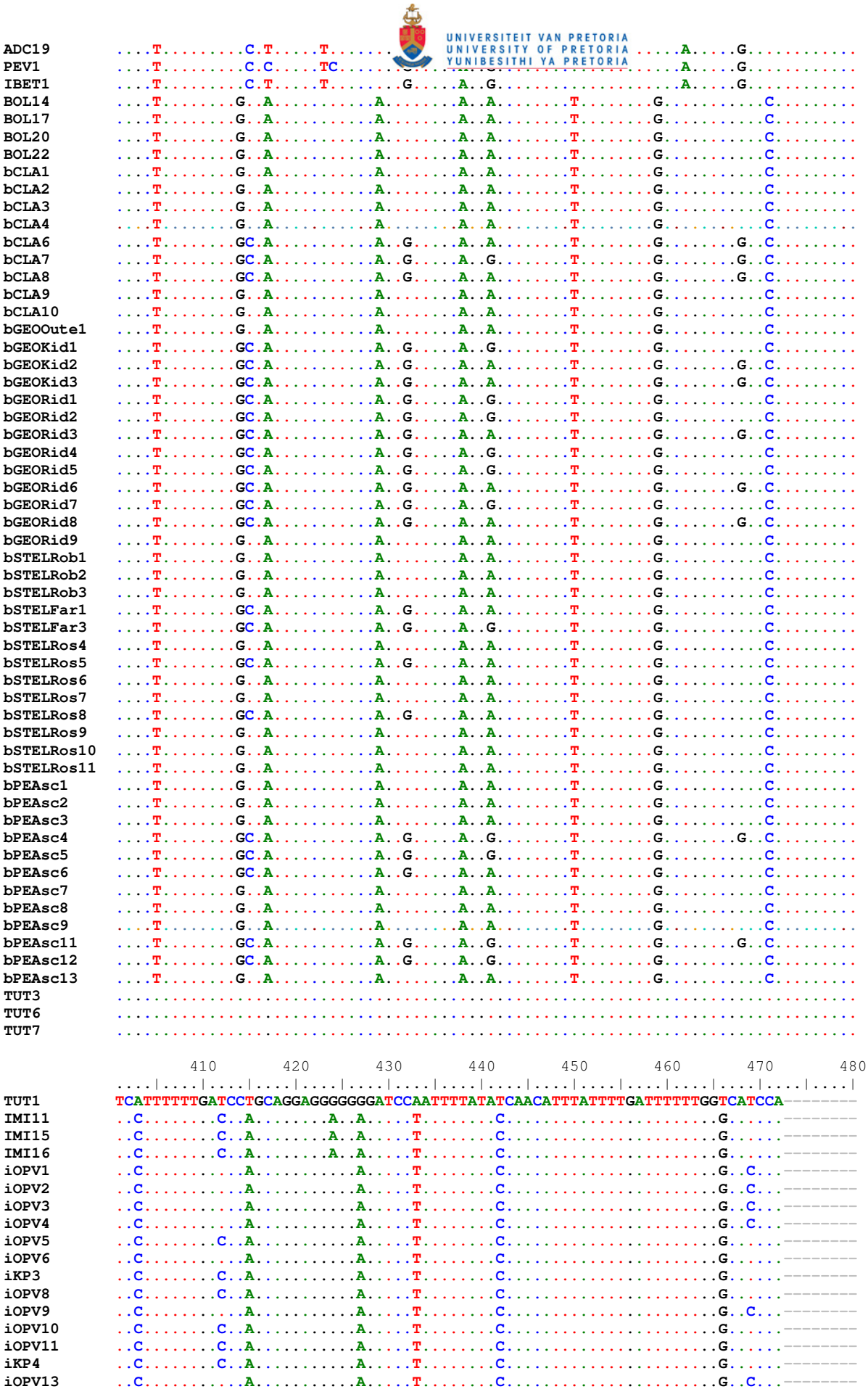

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bPEAsc11      ..C.......C.........A.....C...............G..C...--------
bPEAsc12      ..C.......C.........A.....C...............G..C...--------
bPEAsc13      ..........C.........A.....................G.....--------
TUT3          ..........................................--------
TUT6          ..........................A.....G.....C.A.....--------
TUT7          ..........................................--------
```

Appendix 2

Multiple alignment of the *C. imicola* nucleotide sequences of the COI partial gene fragment for all 61 individuals from different geographic areas. *Culicoides tuttifrutti* (TUT1) and *C. bolitinos* (BOL22) sequences were used as reference species to generate the alignments. All the nucleotide sites that are similar to TUT1 are represented by the dots. The alignment was used to compare the *C. imicola* individuals of all the populations. The alignment was carried out using the Clustal X program.

```
                10        20        30        40        50        60        70        80
                |         |         |         |         |         |         |         |
TUT1            TTTAATATTAGGAGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAGATTTTGAATATTACCCCCTTCAATTACTT
BOL22           ..........................C.....................................T..G.......A.
IMI11           A.........G.....................................T.............G..A..T......C
IMI15           A.........G.....................................T.............G..A..T......C
IMI16           A.........G.....................................T.............G..A..T......C
iOPV1           A.........G.....................................T.............G..A..T......C
iOPV2           A.........G.....................................T.............G..A..T......C
iOPV3           A.........G.....................................T.............G..A..T......C
iOPV4           A.........G.....................................T.............G..A..T......C
iOPV5           A.........G.....................................T.............G..A..T......C
iOPV6           A.........G.....................................T.............G..A..T......C
iKP3            A.........G.....................................T.............G..A..T......C
iOPV8           A.........G.....................................T.............G..A..T......C
iOPV9           A.........G.....................................T.............G..A..T......C
iOPV10          A.........G.....................................T.............G..A..T......C
iOPV11          A.........G.....................................T.............G..A..T......C
iKP4            A.........G.....................................T.............G..A..T......C
iOPV13          A.........G.....................................T.............G..A..T......C
iOPV14          A.........G.....................................T.............G..A..T......C
iOPV15          A.........G.....................................T.............G..A..T......C
iOPV16          A.........G.....................................T.............G..A..T......C
iKP1            A.........G.....................................T.............G..A..T......C
iKP2            A.........G.....................................T.............G..A..T......C
iSTELBea1       A.........G.....................................T.............G..A..T......C
iSTELTri1       A.........G.....................................T.............G..A..T......C
iSTELRiv1       A.........G.....................................T.............G..A..T......C
iSTELWod1       A.........G.....................................T.............G..A..T......C
iSTELBea2       A.........G.....................................T.............G..A..T......C
iSTELTr2        A.........G.....................................T.............G..A..T......C
iSTELRiv2       A.........G.....................................T.............G..A..T......C
iSTELWod2       A.........G.....................................T.............G..A..T......C
iSTELKun1       A.........G.....................................T.............G..A..T......C
iSTELBon1       A.........G.....................................T.............G..A..T......C
iSTELBon2       A.........G.....................................T.............G..A..T......C
iSTELBon3       A.........G.....................................T.............G..A..T......C
iSTELBon4       A.........G.....................................T.............G..A..T......C
iSTELBon5       A.........G.....................................T.............G..A..T......C
iSTELKen1       A.........G.....................................T.............G..A..T......C
iSTELKen2       A.........G.....................................T.............G..A..T......C
iSTELKen3       A.........G.....................................T.............G..A..T......C
iSTELRob1       A.........G.....................................T.............G..A..T......C
iSTELRob2       A.........G.....................................T.............G..A..T......C
iSTELRob3       A.........G.........G...........................T.............G..A..T......C
iSTELRob4       A.........G.........G...........................T.............G..A..T......C
iSTELRob5       A.........G.........G...........................T.............G..A..T......C
iUITWic1        C.........G.....................................T.............G..A..T......C
iUITWic2        C.........G.....................................T.............G..A..T......C
iUITWic3        C.........G.....................................T.............G..A..T......C
iUITWic4        C.........G.....................................T.............G..A..T......C
iUITWic5        C.........G.....................................T.............G..A..T......C
iUITWic6        C.........G.....................................T.............G..A..T......C
iUITWic7        C.........G.....................................T.............G..A..T......C
iUITWic8        C.........G.....................................T.............G..A..T......C
iUITWic9        C.........G.....................................T.............G..A..T......C
iUITWic10       C.........G.....................................T.............G..A..T......C
iUITWic11       C.........G.....................................T.............G..A..T......C
iUITWic12       C.........G.....................................T.............G..A..T......C
iUITWic13       C.........G.....................................T.............G..A..T......C
SCON7           ..........G.....................................T...............A..T.......
SCON9           ..........G.....................................T...............A..T......C
ADC19           ..........G.....................................T.............G..A..T......C
PEV1            ..........G.....................................T...............A..T.......
IBET1           ..........G.....................................T...............A..T......C

                90        100       110       120       130       140       150       160
                |         |         |         |         |         |         |         |
TUT1            TACTTTTATTAAGTAGATTAGTAGAAAATGGAGCAGGGACAGGATGAACAGTTTACCCTCCTTTATCTGCAAATATTTCT
BOL22           ..........A.............C..G.....A........G.....................T.T...G.....
IMI11           .T.....................A.........T.....T.....A.....A......G.....
IMI15           .T.....................A.........T.....T.....A.....A......G.....
IMI16           .T.....................A.........T.....T.....A.....A......G.....
iOPV1           .T.....................A.........T.....T.....A.....A......G.C...
iOPV2           .T.....................A.........T.....T.....A.....A......G.C...
iOPV3           .T.....................A.........T.....T.....A.....A......G.C...
iOPV4           .T.....................A.........T.....T.....A.....A......G.C...
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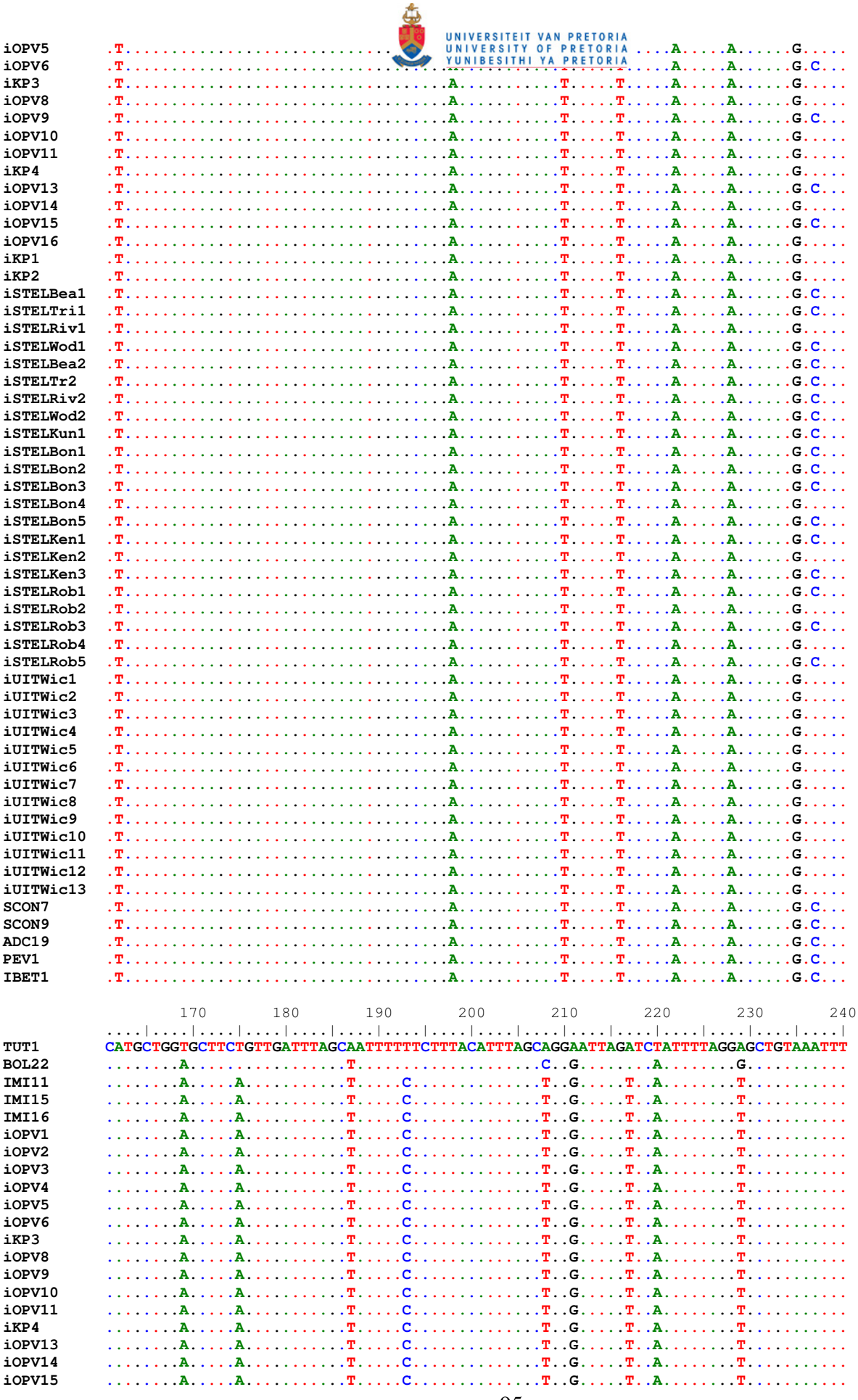

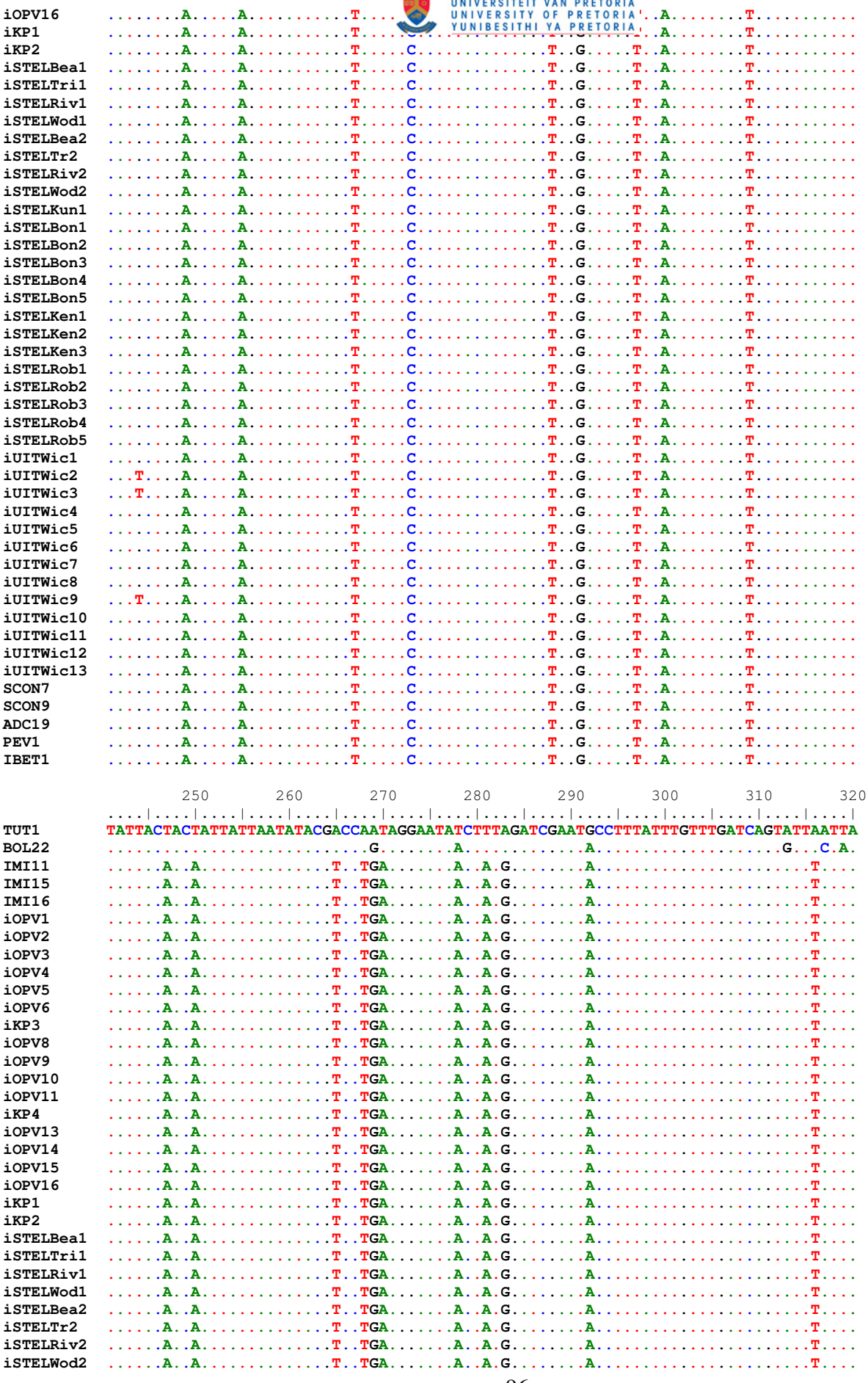

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                330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TUT1            CAGCAATTTTATTATTGTTATCATTACCTGTTTTAGCTGGTGCTATTACAATATTATTAACTGATCGAAATATTAATACT
BOL22           ....T.........G..A...........A........A..A.........T.........G...........C.........
IMI11           ....T.........C.T.....T........G.....A..G.........T...........A.....G.............
IMI15           ....T.........C.T.....T........G.....A..G.........T...........A.....G.............
IMI16           ....T.........C.T.....T........G.....A..G.........T...........A.....G.............
iOPV1           ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV2           ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV3           ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV4           ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV5           ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV6           ....T.........C.T.....T........G.....A..G.....................A.....G.............
iKP3            ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV8           ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV9           ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV10          ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV11          ....T.........C.T.....T........G.....A..G.....................A.....G.............
iKP4            ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV13          ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV14          ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV15          ....T.........C.T.....T........G.....A..G.....................A.....G.............
iOPV16          ....T.........C.T.....T........G.....A..G.....................A.....G.............
iKP1            ....T.........C.T.....T........G.....A..G.....................A.....G.............
iKP2            ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELBea1       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELTri1       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELRiv1       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELWod1       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELBea2       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELTr2        ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELRiv2       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELWod2       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELKun1       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELBon1       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELBon2       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELBon3       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELBon4       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELBon5       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELKen1       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELKen2       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELKen3       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELRob1       ....T.........C.T.....T........G.....A..G.....................A.....G.............
iSTELRob2       ....T.........C.T.....T........G.....A..G.....................A.....G.............
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iSTELRob3  ....T.........C.T.....T.........G.....A..G.....A.....G...........
iSTELRob4  ....T.........C.T.....T.........G.....A..G.....A.....G...........
iSTELRob5  ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic1   ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic2   ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic3   ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic4   ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic5   ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic6   ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic7   ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic8   ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic9   ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic10  ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic11  ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic12  ....T.........C.T.....T.........G.....A..G.....A.....G...........
iUITWic13  ....T.........C.T.....T.........G.....A..G.....A.....G...........
SCON7      ....T.........C.T.....T.........G.....A..G.....A.....G...........
SCON9      ....T.........C.AC....TC........G.....A..G.....A.....G...........
ADC19      ....T.........C.T.....T.........G.....A..G.....A.....G...........
PEV1       ....T.........C.C.....TC........G.....A..G.....A.....G...........
IBET1      ....T.........C.T.....T.........G.....A..G.....A.....G...........

                 410        420        430        440        450        460        470
           ....|....|....|....|....|....|....|....|....|....|....|....|....|..
TUT1       TCATTTTTTGATCCTGCAGGAGGGGGGGATCCAATTTTATATCAACATTTATTTTGATTTTTTGGTCATCCA
BOL22      ..........C..........A..................G.........................
IMI11      ..C........C..A........A..A.....T........C.....................G......
IMI15      ..C........C..A........A..A.....T........C.....................G......
IMI16      ..C........C..A........A..A.....T........C.....................G......
iOPV1      ..C..........A..........A.....T........C.....................G..C...
iOPV2      ..C..........A..........A.....T........C.....................G..C...
iOPV3      ..C..........A..........A.....T........C.....................G..C...
iOPV4      ..C..........A..........A.....T........C.....................G..C...
iOPV5      ..C........C..A..........A.....T........C.....................G......
iOPV6      ..C..........A..........A.....T........C.....................G......
iKP3       ..C........C..A..........A.....T........C.....................G......
iOPV8      ..C........C..A..........A.....T........C.....................G......
iOPV9      ..C..........A..........A.....T........C.....................G..C...
iOPV10     ..C........C..A..........A.....T........C.....................G......
iOPV11     ..C........C..A..........A.....T........C.....................G......
iKP4       ..C........C..A..........A.....T........C.....................G......
iOPV13     ..C..........A..........A.....T........C.....................G..C...
iOPV14     ..C........C..A..........A.....T........C.....................G......
iOPV15     ..C..........A..........A.....T........C.....................G..C...
iOPV16     ..C........C..A..........A.....T........C.....................G......
iKP1       ..C........C..A..........A.....T........C.....................G......
iKP2       ..C........C..A..........A.....T........C.....................G......
iSTELBea1  ..C........C..A..........A.....T........C.....................G......
iSTELTri1  ..C........C..A..........A.....T........C.....................G......
iSTELRiv1  ..C........C..A..........A.....T........C.....................G......
iSTELWod1  ..C........C..A..........A.....T........C.....................G......
iSTELBea2  ..C........C..A..........A.....T........C.....................G......
iSTELTr2   ..C........C..A..........A.....T........C.....................G......
iSTELRiv2  ..C........C..A..........A.....T........C.....................G......
iSTELWod2  ..C........C..A..........A.....T........C.....................G......
iSTELKun1  ..C..........A..........A.....T........C.....................G......
iSTELBon1  ..C........C..A..........A.....T........C.....................G......
iSTELBon2  ..C........C..A..........A.....T........C.....................G......
iSTELBon3  ..C........C..A..........A.....T........C.....................G......
iSTELBon4  ..C........C..A..........A.....T........C.....................G......
iSTELBon5  ..C........C..A..........A.....T........C.....................G......
iSTELKen1  ..C..........A..........A.....T........C.....................G......
iSTELKen2  ..C........C..A..........A.....T........C.....................G......
iSTELKen3  ..C........C..A..........A.....T........C.....................G......
iSTELRob1  ..C..........A..........A.....T........C.....................G......
iSTELRob2  ..C........C..A..........A.....T........C.....................G......
iSTELRob3  ..C..........A..........A.....T........C.....................G......
iSTELRob4  ..C........C..A..........A.....T........C.....................G......
iSTELRob5  ..C..........A..........A.....T........C.....................G......
iUITWic1   ..C........C..A..........A.....T........C.....................G......
iUITWic2   ..C........C..A..........A.....T........C.....................G......
iUITWic3   ..C........C..A..........A.....T........C.....................G......
iUITWic4   ..C........C..A..........A.....T........C.....................G......
iUITWic5   ..C........C..A..........A.....T........C.....................G......
iUITWic6   ..C........C..A..........A.....T........C.....................G......
iUITWic7   ..C........C..A..........A.....T........C.....................G......
iUITWic8   ..C........C..A..........A.....T........C.....................G......
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iUITWic9    ..C........C..A..........A.....T.........C.....................G......
iUITWic10   ..C........C..A..........A.....T.........C.....................G......
iUITWic11   ..C........C..A..........A.....T.........C.....................G......
iUITWic12   ..C........C..A..........A.....T.........C.....................G......
iUITWic13   ..C........C..A..........A.....T.........C.....................G......
SCON7       ..C..........A..........A.....T.........C.....................G..C...
SCON9       ..C..........A..........A.....T...C.....C.....................G..C...
ADC19       ..C..........A..........A.....T.........C.....................G..C...
PEV1        ..C..........A..........A.....T.........C.....................G..C...
IBET1       ..C..........A..........A.....T.........C.....................G..C...
```

Appendix 3

Multiple alignment of the *C. bolitinos* nucleotide sequences of the COI partial gene fragment for all 51 individuals from different geographic areas. *Culicoides imicola* (iOPV1) sequence was used as a reference species to generate the alignment. Only the nucleotide sites that differ from the iOPV1 are shown. The dots represent identical nucleotide sites and the symbol (-) represent deletions. The alignment was used to compare the *C. bolitinos* individuals of all sampled populations. The alignment was generated using the Clustal X program.

```
                  10        20        30        40        50        60        70        80
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
iOPV1             ATTAATATTAGGGGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTTTGAATATTACCGCCATCTATTACTC
BOL14             T..........A..................C.....................A..............T..G..A.....AT
BOL17             T..........A..................C.....................A..............T..G..A.....AT
BOL20             T..........A..................C.....................A..............T..G..A.....AT
BOL22             T..........A..................C.....................A..............T..G..A.....AT
bCLA1             T..........A..................C.....................A..............T..G..A.....AT
bCLA2             T..........A..................C.....................A..............T..G..A.....AT
bCLA3             T..........A..................C.....................A..............T..G..A.....AT
bCLA4             T..........A..................C.....................A..............T..G..A.....AT
bCLA6             T.....................................................A..............T.....A......T
bCLA7             T.....................................................A..............T.....A......T
bCLA8             T.....................................................A..............T.....A......T
bCLA9             T..........A..................C.....................A..............T..G..A.....AT
bCLA10            T..........A..................C.....................A..............T..G..A.....AT
bGEOOute1         T...T.......A...........G.....C.....................A..............T..G..A.....AT
bGEOKid1          ..............................G.....................A..................A......T
bGEOKid2          T.....................................................A..............T.....A......T
bGEOKid3          T.....................................................A..............T.....A......T
bGEOKid4          ..............................G.....................A..................A......T
bGEORid1          ..............................G.....................A..................A......T
bGEORid2          T.....................................................A..............T.....A......T
bGEORid3          ..............................G.....................A..................A......T
bGEORid4          ..............................G.....................A..................A......T
bGEORid5          T.....................................................A..............T.....A......T
bGEORid6          ..............................G.....................A..................A......T
bGEORid7          T.....................................................A..............T.....A......T
bGEORid8          T...T.......A...........G.....C.....................A..............T..G..A.....AT
bSTELRob1         T..........A..................C.....................A..............T..G..A.....AT
bSTELRob2         T..........A..................C.....................A..............T..G..A.....AT
bSTELRob3         T..........A..................C.....................A..............T..G..A.....AT
bSTELFar1         T.....................................................A..............T.....A......T
bSTELFar3         T.....................................................A..............T.....A......T
bSTELRos4         T..........A..................C.....................A..............T..G..A.....AT
bSTELRos5         T.....................................................A..............T.....A......T
bSTELRos6         T..........A..................C.....................A..............T..G..A.....AT
bSTELRos7         T..........A..................C.....................A..............T..G..A.....AT
bSTELRos8         T.....................................................A..............T.....A......T
bSTELRos9         T..........A..................C.....................A..............T..G..A.....AT
bSTELRos10        T..........A..................C.....................A..............T..G..A.....AT
bSTELRos11        T..........A..................C.....................A..............T..G..A.....AT
bPEAsc1           T..........A..................C.....................A..............T..G..A.....AT
bPEAsc2           T..........A..................C.....................A..............T..G..A.....AT
bPEAsc3           T..........A..................C.....................A..............T..G..A.....AT
bPEAsc4           T.....................................................A..............T.....A......T
bPEAsc5           T.....................................................A..............T.....A......T
bPEAsc6           T..........A..................C.....................A..............T..G..A.....AT
bPEAsc7           T..........A..................C.....................A..............T..G..A.....AT
bPEAsc8           T..........A..................C.....................A..............T..G..A.....AT
bPEAsc9           T..........A..................C.....................A..............T..G..A.....AT
bPEAsc11          T.....................................................A..............T.....A......T
bPEAsc12          T.....................................................A..............T.....A......T
bPEAsc13          T..........A..................C.....................A..............T..G..A.....AT

                  90        100       110       120       130       140       150       160
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
iOPV1             TTCTTTTATTAAGTAGATTAGTAGAAAATGGAGCAGGAACAGGATGAACTGTTTATCCTCCATTATCAGCAAATGTCTCT
BOL14             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
BOL17             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
BOL20             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
BOL22             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
bCLA1             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
bCLA2             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
bCLA3             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
bCLA4             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
bCLA6             .A..........A..............C..G..............G..A.....C.....T.....TT.......T...
bCLA7             .A..........A..............C..G..............G..A.....C.....T.....TT.......T...
bCLA8             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
bCLA9             .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
bCLA10            .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
bGEOOute1         .A..........A..............C..G..............G..A.....C.....T.....TT.T.....T...
bGEOKid1          ............A..............C..G..............G..A...........T......T.......T...
bGEOKid2          ..T.........A..............C..G..............G..A.....C.....T.....TT.......T...
bGEOKid3          .C..........A..............C..G..............G..A.....C.....T.....TT.......T...
bGEOKid4          ............A..............C..G..............G..A...........T......T.......T...
bGEORid1          ............A..............C..G..............G..A...........T......T.......T...
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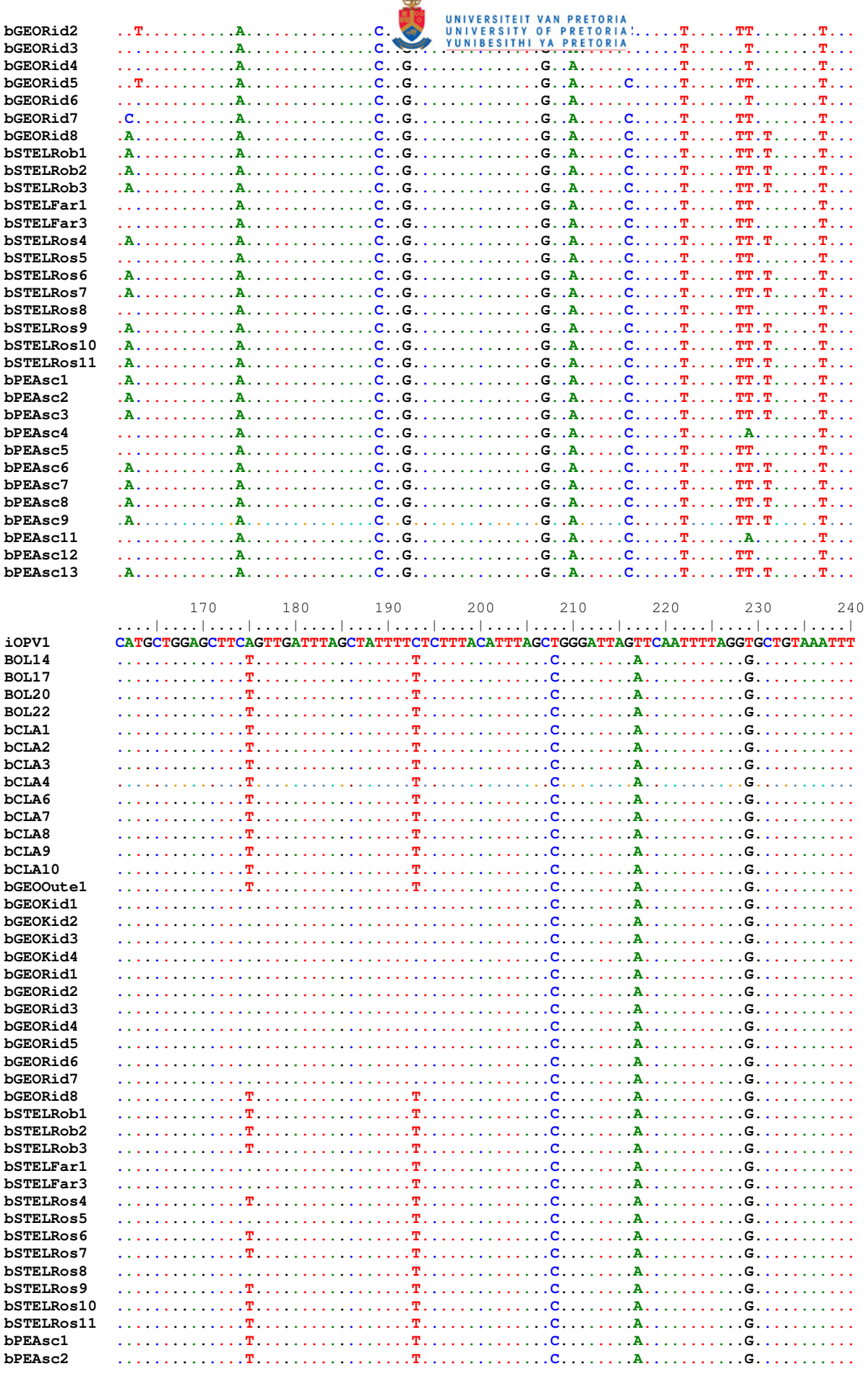

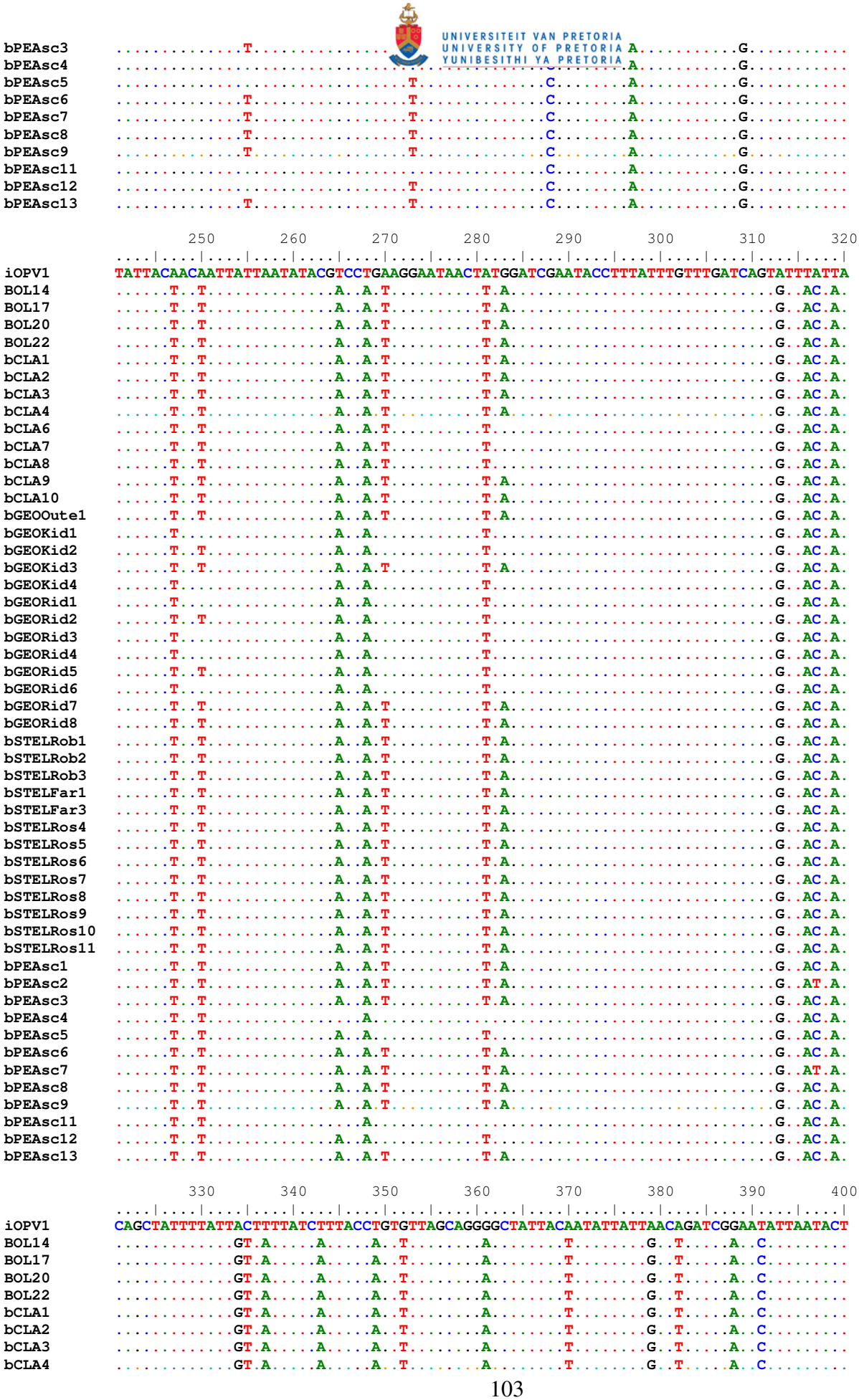

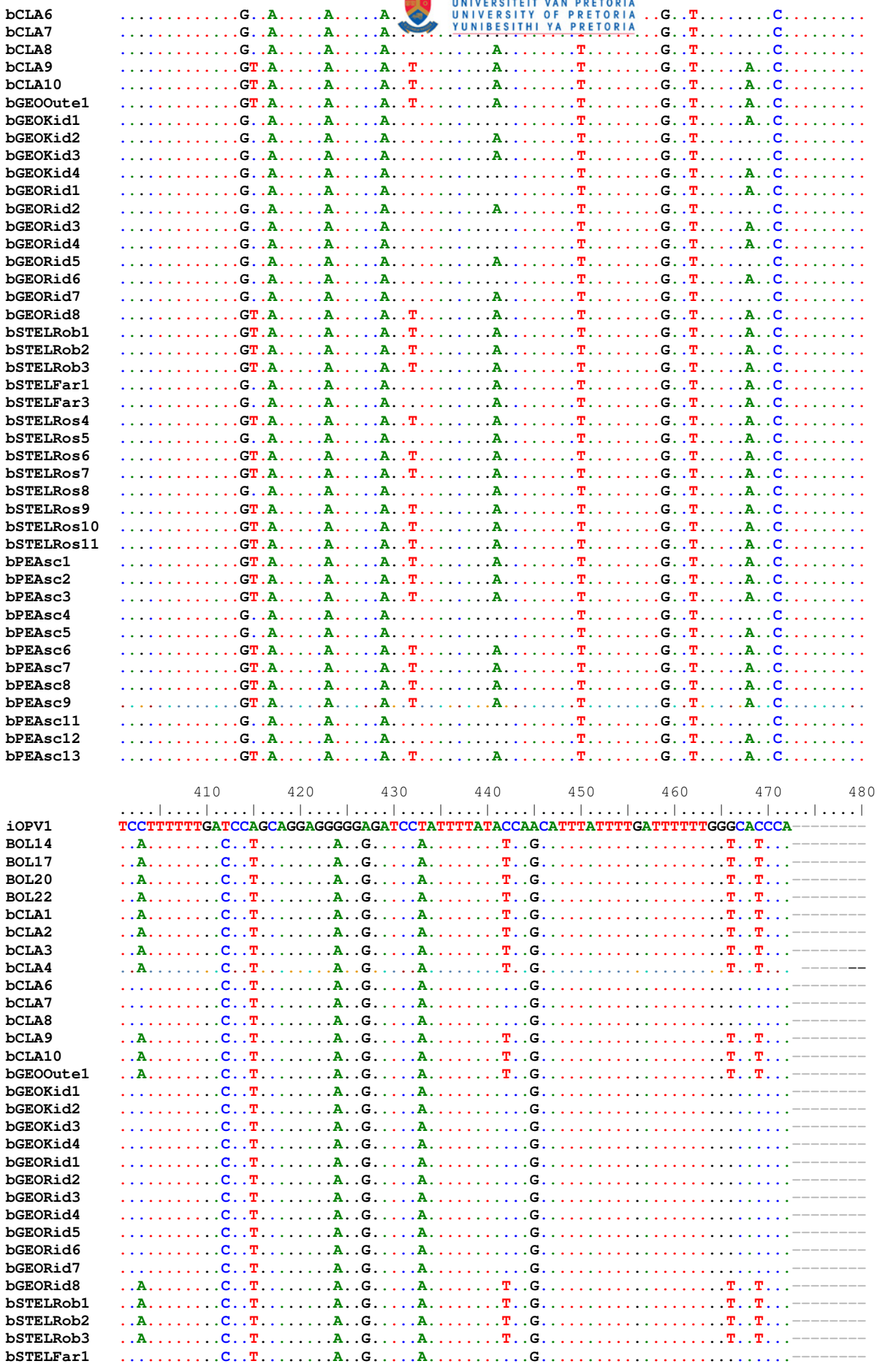

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bSTELRos6     ..A.......C..T........A..G.....A.....T..G..........T..T...--------
bSTELRos7     ..A.......C..T........A..G.....A.....T..G..........T..T...--------
bSTELRos8     ..........C..T........A..G.....A..........G..........T..T...--------
bSTELRos9     ..A.......C..T........A..G.....A.....T..G..........T..T...--------
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bSTELRos11    ..A.......C..T........A..G.....A.....T..G..........T..T...--------
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bPEAsc13      ..A.......C..T........A..G.....A.....T..G..........T..T...--------
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Appendix 4

Multiple sequences alignment of the *C. imicola* and *C. bolitinos* nucleotide sequences of the 16S rRNA partial gene fragment for all individuals midges from all sampled populations. *Culicoides tuttifrutti* (tuttiNHMT15) sequence was used to align both *C. imicola* and *C. bolitinos*. All the nucleotide sites that are similar to TUT1 are represented by the dots. The alignment was used to compare the two species of *Culicoides*. All the names of the sequences that were obtained from the GenBank start with capital letters. The alignment was carried out using the Clustal X program. The prefix i represents *C. imicola* and b represents *C. bolitinos*. The symbol (-) indicates deletions.

```
                       10        20                                     60        70        80
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OPRSA2                    .....................T.....................T.............A.........T
OPRSA6                    .....................T.....................T.............A.........T
OPRSA10                   .....................T.....................T.............A.........T
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OPNHM17I                  .....................T.....................T.............A.........T
OPNHM18I                  .....................T.....................T.............A.........T
OPNHM19I                  .....................T.....................T.............A.........T
OPAOND1                   .....................T.....................T.............A.........T
OPAOND2                   .....................T.....................T.............A.........T
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IBET31                    .....................T.....................T.............A.........T
IBET35                    .....................T.....................T.............A.........T
IBET45                    .....................T.....................T.............A.........T
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iOPV12                    .....................T.....................T.............A.........T
iKP1                      .CTG.................T.....................T.............A.........T
iOPV13                    .CTG.................T.....................T.............A.........T
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OPRSA2          ................................................................................
OPRSA6          ................................................................................
OPRSA10         ................................................................................
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OPNHM17I        ................................................................................
OPNHM18I        ................................................................................
OPNHM19I        ................................................................................
OPAOND1        ................................................................................
OPAOND2        ................................................................................
OPAOND6        ................................................................................
OPAOND10       ................................................................................
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IBET31          ................................................................................
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iOPV6           ................................................................................
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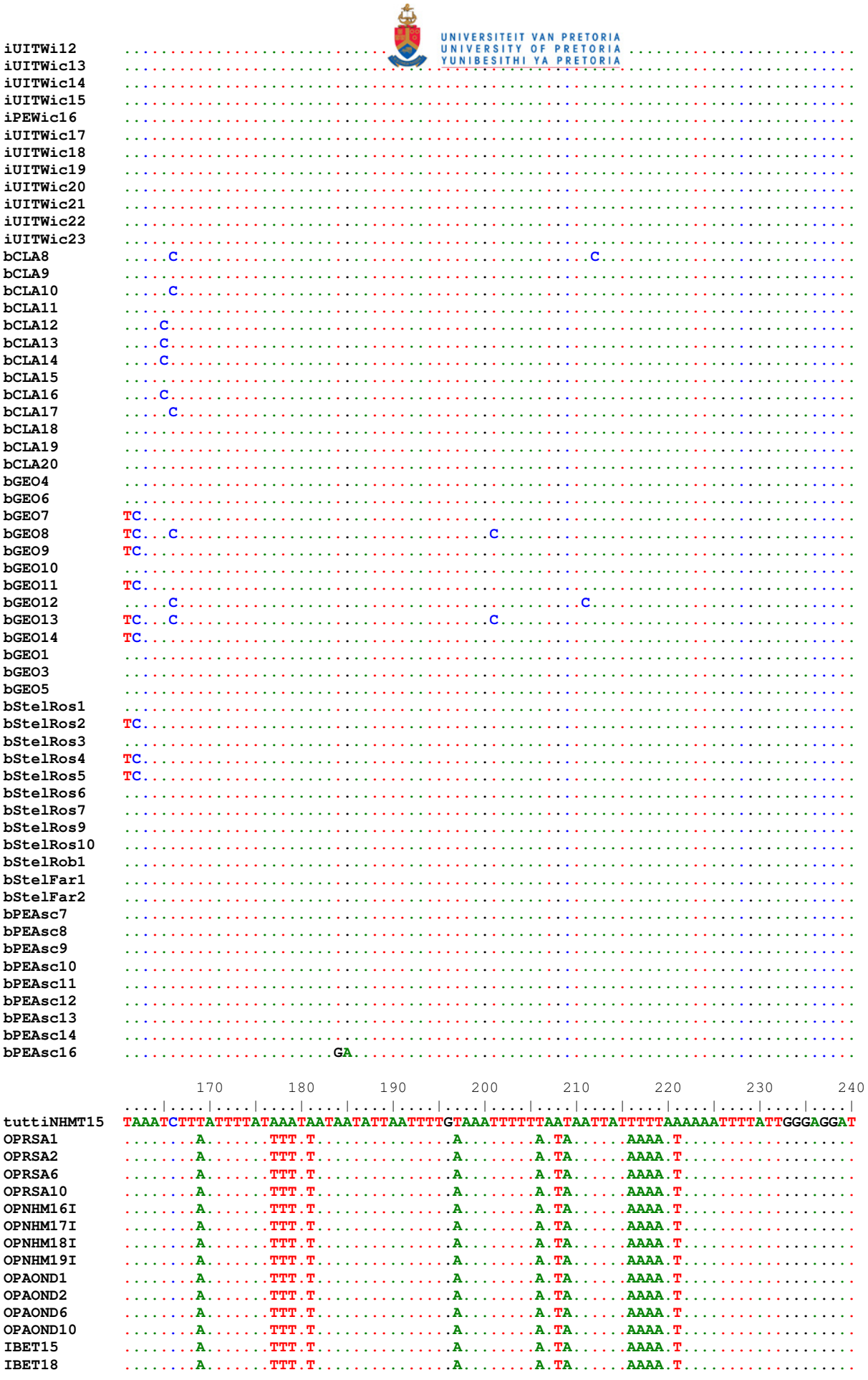
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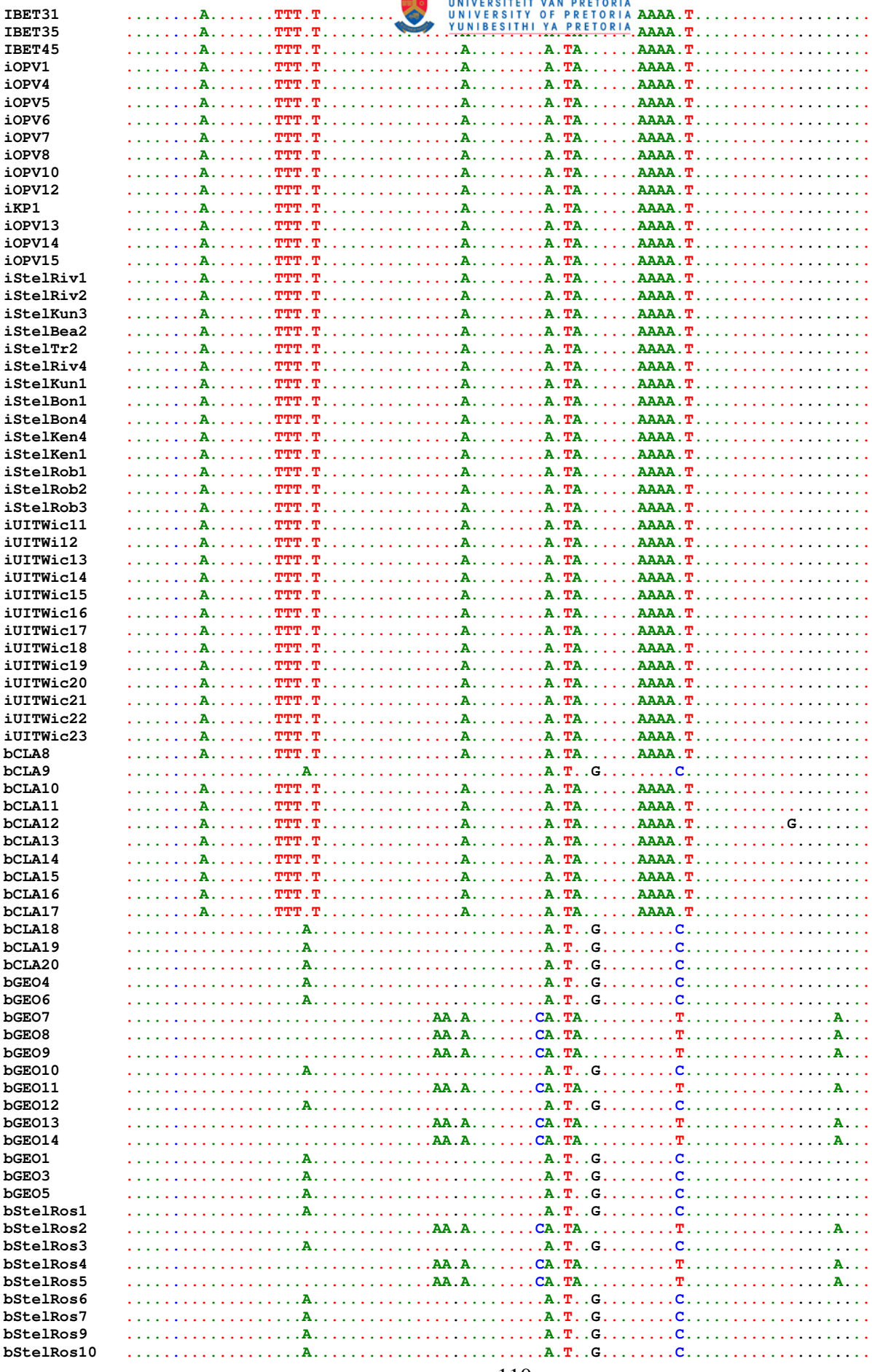

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OPRSA2        ........................T.TT...-..................T.........A.........A........
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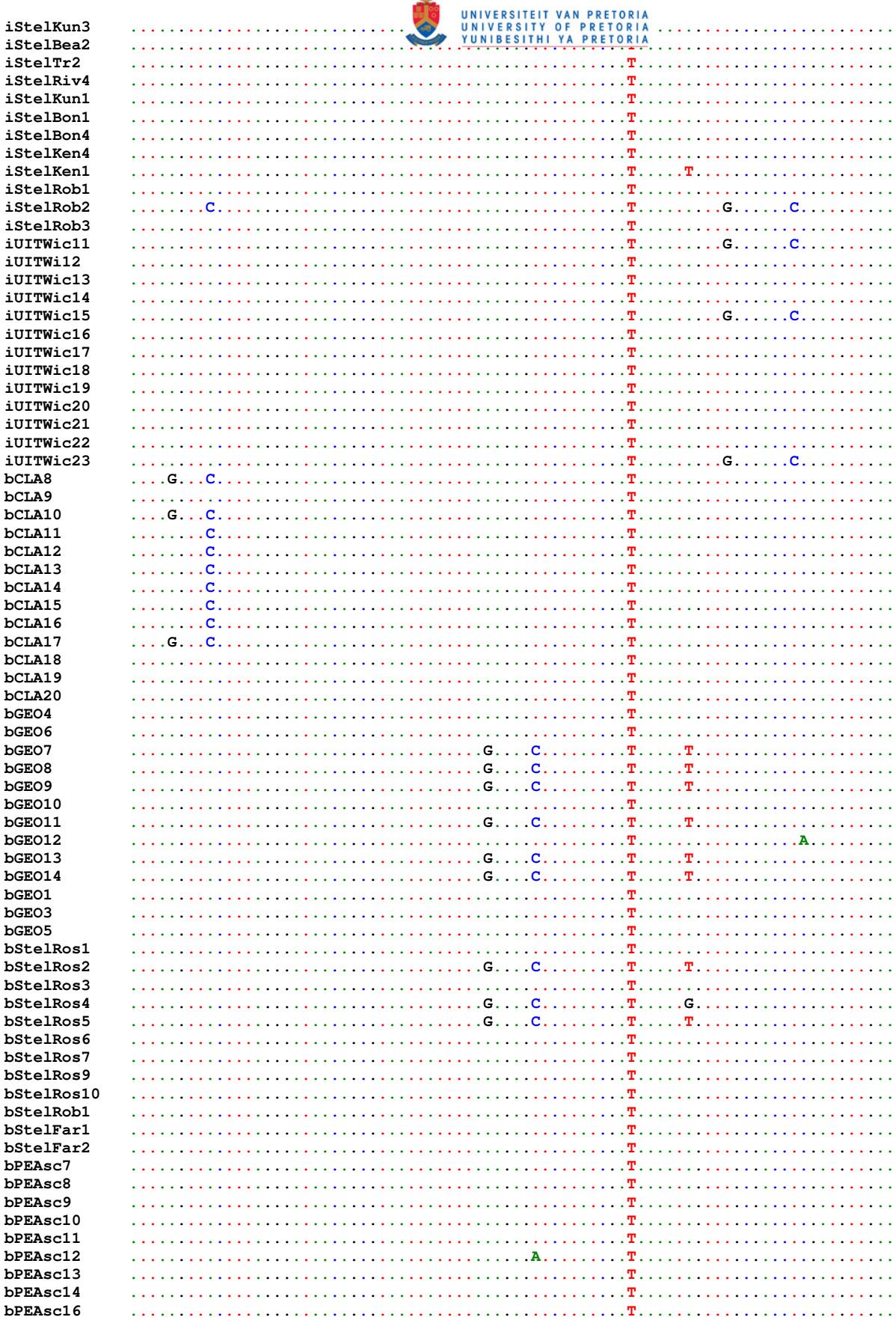

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OPRSA2              ..................T.C..G.........................G.....
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iOPV4               ..................T....G.........................G.....
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bCLA12              ......G.A.........T....G...................
bCLA13              ......G.A.........T....G...................
bCLA14              ......G.A.........T....G...................
bCLA15              ......G.A.........T....G...................
bCLA16              ......G.A.........T....G...................
bCLA17              ......G.A.........T....G...................
bCLA18              ......G.A.........T....G...................
bCLA19              ......G.A.........T....G...................
bCLA20              ......G.A.........T....G...................
bGEO4               ......G.A.........T....G...................
bGEO6               ......G.A.........A....G...................
bGEO7               ..............AA....A..G...................
bGEO8               ............A.A.CAA.A..G...................
bGEO9               ..............AA....A..G...................
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bGEO10          ......G.A..........A....G.....................
bGEO11          ..............AA....A..G.....................
bGEO12          ......G..............A..G.....................
bGEO13          ............A.A.CAA.A..G.....................
bGEO14          ..............AA....A..G.....................
bGEO1           ......G.A..........T....G.....................
bGEO3           ......G.A..........T....G.....................
bGEO5           ......G.A..........T....G.....................
bStelRos1       ......G.A..........T....G.....................
bStelRos2       ..............AA....A..G.....G...............
bStelRos3       ......G.A..........T....G.....................
bStelRos4       ............A.A.CA.CA..G.....................
bStelRos5       ..............AA....A..G.....................
bStelRos6       ......G.A..........T....G.....................
bStelRos7       ......G.A..........T....G..............C.....
bStelRos9       ......G.A..........T....G.....................
bStelRos10      ......G.A..........T....G.....................
bStelRob1       ......G.A..........T....G.....................
bStelFar1       ......G.A..........T....G.....................
bStelFar2       ......G.A..........T....G.....................
bPEAsc7         ......G.A..........T....G.....................
bPEAsc8         ......G.A..........T....G.....................
bPEAsc9         ......G.A..........T....G.....................
bPEAsc10        ......G.A..........T....G.....................
bPEAsc11        ......G.A..........T....G.....................
bPEAsc12        ......G.A..........T....G.....................
bPEAsc13        ......G.A..........T....G.....................
bPEAsc14        ......G.A..........T....G.....................
bPEAsc16        ......G.A..........T....G.....................
```
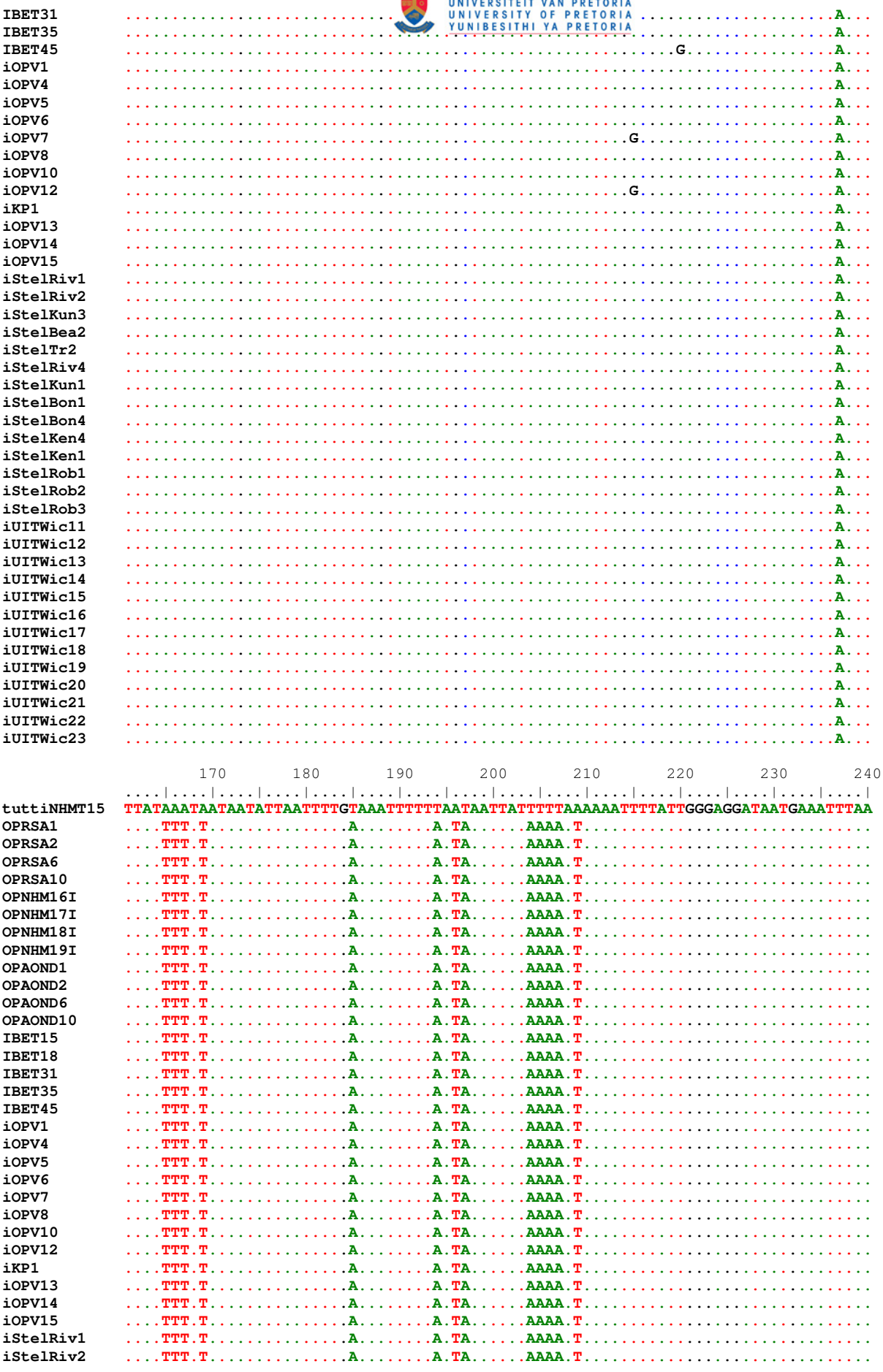

Appendix 5

Multiple alignment of the *C. imicola* nucleotide sequences of the 16S rRNA partial gene fragment for all 56 individuals from different geographic areas. *Culicoides tuttifrutti* (tuttiNHMT15) sequence was used as reference species to generate the alignments. All the nucleotide sites that are similar to tuttiNHMT15 are represented by the dots. The alignment was used to compare the *C. imicola* individuals of all the sampled populations. The alignment was carried out using the Clustal X program.

```
                    10        20        30        40        50        60        70        80
                    |....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tuttiNHMT15         CTGTGCAAAGGTAGCATAATCCATTGTCTTTTAATTAAAGGCTAGTATGAATGGTTGGATGAGATATCAACTATTTTTAT
OPRSA1              ....................T....................T............A.........T...........
OPRSA2              ....................T....................T............A.........T...........
OPRSA6              ....................T....................T............A.........T...........
OPRSA10             ....................T....................T............A.........T...........
OPNHM16I            ....................T....................T............A.........T...........
OPNHM17I            ....................T....................T............A.........T...........
OPNHM18I            ....................T....................T............A.........T...........
OPNHM19I            ....................T....................T............A.........T...........
OPAOND1             ....................T....................T............A.........T...........
OPAOND2             ....................T....................T............A.........T...........
OPAOND6             ....................T....................T............A.........T...........
OPAOND10            ....................T....................T............A.........T...........
IBET15              ....................T....................T............A.........T...........
IBET18              ....................T....................T............A.........T...........
IBET31              ....................T....................T............A.........T...........
IBET35              ....................T....................T............A.........T...........
IBET45              ....................T....................T............A.........T...........
iOPV1               ....................T....................T............A.........T...........
iOPV4               ....................T....................T............A.........T...........
iOPV5               ....................T....................T............A.........T...........
iOPV6               ....................T....................T............A.........T...........
iOPV7               ..............C.....T....................T............A.........T...........
iOPV8               ..............C.....T....................T............A.........T....TCC.....
iOPV10              ....................T....................T............A.........T...........
iOPV12              ....................T....................T............A.........T...........
iKP1                .CTG................T....................T............A.........T...........
iOPV13              .CTG................T....................T............A.........T...........
iOPV14              ..TG................T....................T............A.........T...........
iOPV15              ....................T....................T............A.........T...........
iStelRiv1           ....................T....................T............A.........T...........
iStelRiv2           ....................T....................T............A.........T...........
iStelKun3           ....................T....................T............A.........T...........
iStelBea2           ....................T....................T............A.........T...........
iStelTr2            ....................T....................T............A.........T...........
iStelRiv4           ....................T....................T............A.........T...........
iStelKun1           ....................T....................T............A.........T...........
iStelBon1           ....................T....................T............A.........T...........
iStelBon4           ....................T....................T............A.........T...........
iStelKen4           ....................T....................T............A.........T...........
iStelKen1           ....................T....................T............A.........T...........
iStelRob1           ....................T....................T............A.........T...........
iStelRob2           ....................T....................T............A.........T...........
iStelRob3           ....................T....................T............A.........T...........
iUITWic11           ....................T....................T............A.........T...........
iUITWic12           ....................T....................T............A.........T...........
iUITWic13           ....................T....................T............A.........T...........
iUITWic14           ....................T....................T............A.........T...........
iUITWic15           ....................T....................T............A.........T...........
iUITWic16           ....................T....................T............A.........T...........
iUITWic17           ....................T....................T............A.........T...........
iUITWic18           ....................T....................T............A.........T...........
iUITWic19           ....................T....................T............A.........T...........
iUITWic20           ....................T....................T............A.........T...........
iUITWic21           ..............C.....T....................T............A.........T...........
iUITWic22           ....................T....................T............A.........T...........
iUITWic23           ....................T....................T............A.........T...........

                    90        100       110       120       130       140       150       160
                    |....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tuttiNHMT15         TTTAATAAAAATGAATTTTAATTTTTAGTAAAAATGCTAAAATAAAAAAATTAGACGAGAAGACCCTATAAATCTTTATT
OPRSA1              ...............................................................................A...
OPRSA2              ...............................................................................A...
OPRSA6              ...............................................................................A...
OPRSA10             ...............................................................................A...
OPNHM16I            ...............................................................................A...
OPNHM17I            ...............................................................................A...
OPNHM18I            ...............................................................................A...
OPNHM19I            ...............................................................................A...
OPAOND1             ...............................................................................A...
OPAOND2             ...............................................................................A...
OPAOND6             ...............................................................................A...
OPAOND10            ...............................................................................A...
IBET15              ...............................................................................A...
IBET18              ...............................................................................A...
```



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iStelKun3     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelBea2     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelTr2      ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelRiv4     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelKun1     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelBon1     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelBon4     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelKen4     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelKen1     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelRob1     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelRob2     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iStelRob3     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic11     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic12     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic13     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic14     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic15     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic16     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic17     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic18     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic19     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic20     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic21     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic22     ....TTT.T.............A.....A.TA.....AAAA.T.....................
iUITWic23     ....TTT.T.............A.....A.TA.....AAAA.T.....................

                       250       260       270       280       290       300       310       320
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tuttiNHMT15   TAAACTTTTATTCTAATTTATAAATATAATTAAATTAATGAATATATTATTATAAAATGTTTAAAAAATTAAGTTACTTT
OPRSA1        ...........T.TT.....................T.........A.........A.....................
OPRSA2        ...........T.TT.....................T.........A.........A.....................
OPRSA6        ...........T.TT.....................T.........A.........A.....................
OPRSA10       ...........T.TT.....................T.........A.........A.....................
OPNHM16I      ...........T.TT.....................T.........A.........A.....................
OPNHM17I      ...........T.TT.....................T.........A.........A.....................
OPNHM18I      ...........T.TT.....................T.........A.........A.....................
OPNHM19I      ...........T.TT.....................T.........A.........A.....................
OPAOND1       ...........T.TT.....................T.........A.........A.....................
OPAOND2       ...........T.TT.....................T.........A.........A.....................
OPAOND6       ...........T.TT.....................T.........A.........A.....................
OPAOND10      ...........T.TT.....................T.........A.........A.....................
IBET15        ...........T.TT.....................T.........A.........A.....................
IBET18        ...........T.TT.....................T.........A.........A.....................
IBET31        ...........T.TT.....................T.........A.........A.....................
IBET35        ...........T.TT.....................T.........A.........A.....................
IBET45        ...........T.TT.....................T.........A.........A.....................
iOPV1         ...........T.TT.....................T.........A.........A.....................
iOPV4         ...........T.TT.....................T.........A.........A.....................
iOPV5         ...........T.TT.....................T.........A.........A.....................
iOPV6         ...........T.TT.....................T.........A.........A.....................
iOPV7         ...........T.TT.....................T.........A.........A.....................
iOPV8         ...........T.TT.....................T.........A.........A.....................
iOPV10        ...........T.TT.....................T.........A.........A.....................
iOPV12        ...........T.TT.....................T.........A.........A.....................
iKP1          ...........T.TT.....................T.........A.........A.....................
iOPV13        ...........T.TT.....................T.........A.........A.....................
iOPV14        ...........T.TT.....................T.........A.........A..............C....
iOPV15        ...........T.TT.....................T.........A.........A.....................
iStelRiv1     ...........T.TT.....................T.........A.........A.....................
iStelRiv2     ...........T.TT.....................T.........A.........A.....................
iStelKun3     ...........T.TT.....................T.........A.........A.....................
iStelBea2     ...........T.TT.....................T.........A.........A.....................
iStelTr2      ...........T.TT.....................T.........A.........A.....................
iStelRiv4     ...........T.TT.....................T.........A.........A.....................
iStelKun1     ...........T.TT.....................T.........A.........A.....................
iStelBon1     ...........T.TT.....................T.........A.........A.....................
iStelBon4     ...........T.TT.....................T.........A.........A.....................
iStelKen4     ...........T.TT.....................T.........A.........A.....................
iStelKen1     ...........T.TT.....................T.........A.........A.....................
iStelRob1     ...........T.TT.....................T.........A.........A.....................
iStelRob2     ...........T.TT.....................T.........A.........A..............C....
iStelRob3     ...........T.TT.....................T.........A.........A.....................
iUITWic11     ...........T.TT.....................T.........A.........A.....................
iUITWic12     ...........T.TT.....................T.........A.........A.....................
iUITWic13     ...........T.TT.....................T.........A.........A.....................
iUITWic14     ...........T.TT.....................T.........A.........A.....................
iUITWic15     ...........T.TT.....................T.........A.........A.....................
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iUITWic16  ...........T.TT.....................................A.....................
iUITWic17  ...........T.TT.....................................A.....................
iUITWic18  ...........T.TT.....................T.........A.....A.....................
iUITWic19  ...........T.TT.....................T.........A.....A.....................
iUITWic20  ...........T.TT.....................T.........A.....A.....................
iUITWic21  ...........T.TT.....................T.........A.....A.....................
iUITWic22  ...........T.TT.....................T.........A.....A.....................
iUITWic23  ...........T.TT.....................T.........A.....A.....................

                    330       340       350       360       370       380       390       400
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tuttiNHMT15  AGGGATAACAGCGTAATTATTTTAAAGAGTTCTTATCGACAAAATAGTTTGCGACCTCGATGTTGAATTAAGAATTAATT
OPRSA1       ...............................................T.....................................
OPRSA2       ...............................................T.....................................
OPRSA6       ...............................................T.....................................
OPRSA10      ...............................................T.....................................
OPNHM16I     ...............................................T.....................................
OPNHM17I     ...............................................T.....................................
OPNHM18I     ...............................................T.....................................
OPNHM19I     ...............................................T.....................................
OPAOND1      ...............................................T.....................................
OPAOND2      ...............................................T.....................................
OPAOND6      ...............................................T.....................................
OPAOND10     ...............................................T.....................................
IBET15       ...............................................T.................C.....................
IBET18       ...............................................T.....................................
IBET31       ...............................................T.....................................
IBET35       ...............................................T.....................................
IBET45       ...............................................T.....................................
iOPV1        ...............................................T.....................................
iOPV4        ...............................................T.....................................
iOPV5        ...............................................T.....................................
iOPV6        ...............................................T.....................................
iOPV7        ...............................................T.....................................
iOPV8        ...............................................T.....................................
iOPV10       ...............................................T.....................................
iOPV12       ...............................................T.....................................
iKP1         ...............................................T.....................................
iOPV13       ...............................................T.....................................
iOPV14       ...............................................T.............C..C.....................
iOPV15       ...............................................T.....................................
iStelRiv1    ...............................................T.....................................
iStelRiv2    ...............................................T.....................................
iStelKun3    ...............................................T.....................................
iStelBea2    ...............................................T.....................................
iStelTr2     ...............................................T.....................................
iStelRiv4    ...............................................T.....................................
iStelKun1    ...............................................T.....................................
iStelBon1    ...............................................T.....................................
iStelBon4    ...............................................T.....................................
iStelKen4    ...............................................T.....................................
iStelKen1    ...............................................T.....T.....................................
iStelRob1    ...............................................T.....................................
iStelRob2    ...............................................T.........G......C.....................
iStelRob3    ...............................................T.....................................
iUITWic11    ...............................................T.........G......C.....................
iUITWic12    ...............................................T.....................................
iUITWic13    ...............................................T.....................................
iUITWic14    ...............................................T.....................................
iUITWic15    ...............................................T.........G......C.....................
iUITWic16    ...............................................T.....................................
iUITWic17    ...............................................T.....................................
iUITWic18    ...............................................T.....................................
iUITWic19    ...............................................T.....................................
iUITWic20    ...............................................T.....................................
iUITWic21    ...............................................T.....................................
iUITWic22    ...............................................T.....................................
iUITWic23    ...............................................T.........G......C.....................

                    410       420       430       440
                    ....|....|....|....|....|....|....|....|
tuttiNHMT15  TAGGTGCAGAAATTTAAATTTTAAGTCTGTTCGACTTTTAAATTC
OPRSA1       ......T....G.........................G.....
OPRSA2       ......T.C..G.........................G.....
OPRSA6       ......T....G.........................G.....
OPRSA10      ......T....G.........................G.....
OPNHM16I     ......T....G.........................G.....
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OPNHM17I      ......T....G.....................G.....
OPNHM18I      ......T....G.....................G.....
OPNHM19I      ......T....G.....................G.....
OPAOND1       ......T....G.....................G.....
OPAOND2       ......T.C..G.....................G.....
OPAOND6       ......T....G.....................G.....
OPAOND10      ......T....G.....................G.....
IBET15        ......T....G.....................G...C.
IBET18        ......T.A..G.....................G.....
IBET31        ......T.T..G.....................G.....
IBET35        ......T....G.....................G.....
IBET45        ......T....G.....................G...C.
iOPV1         ......T....G.....................G.....
iOPV4         ......T....G.....................G.....
iOPV5         ......T....G.............C.C.....C.GAC.....
iOPV6         ......T....G.....................G.....
iOPV7         ....G.T....G.............C.G..C.....G.....
iOPV8         ......T....G.....................G.T.C.
iOPV10        ......T....G.....................G.....
iOPV12        ....G.T....G.............C.G..C.....G.....
iKP1          ....G.T....G.............C.G..C.....G.....
iOPV13        ....G.T....G.............C.G..C.....G.....
iOPV14        ....G.T....G.....................G.T...
iOPV15        ....G.T....G.....................G.T.C.
iStelRiv1     ......T....G.....................G.T.C.
iStelRiv2     ......T....G.............C.......G.T.C.
iStelKun3     ......T....G.....................G.T.C.
iStelBea2     ......T....G.....................G.T.C.
iStelTr2      ......T....G.....................G.T.C.
iStelRiv4     ......T....G.....................G.T.C.
iStelKun1     ......T....G.....................G.T.C.
iStelBon1     ......T....G.....................G.T.C.
iStelBon4     ......T....G.................C.....G.T.C.
iStelKen4     ......T....G.....................G.T.C.
iStelKen1     ......T....G.................GAC.....G.T.C.
iStelRob1     ......T....G.....................G.T.C.
iStelRob2     ....G.T....G.....................G.T.C.
iStelRob3     ......T....G.....................G.T.C.
iUITWic11     ....G.T....G.............G.......G.T.C.
iUITWic12     ......T....G.....................G.T.C.
iUITWic13     ....G.T....G.....................G.T.C.
iUITWic14     ......T....G.....................G.T.C.
iUITWic15     ....G.T....G.............G.......G.T.C.
iUITWic16     ......T...CGGC..................G.T.C.
iUITWic17     ......T....G.....................G.T.C.
iUITWic18     ......T....G.....................G.T.C.
iUITWic19     ......T....G.....................G.T.C.
iUITWic20     ......T....G.....................G.T.C.
iUITWic21     ....G.T....G.....................G.T.C.
iUITWic22     ......T....G.....................G.T.C.
iUITWic23     ....G.T....G.............G.......G.T.C.
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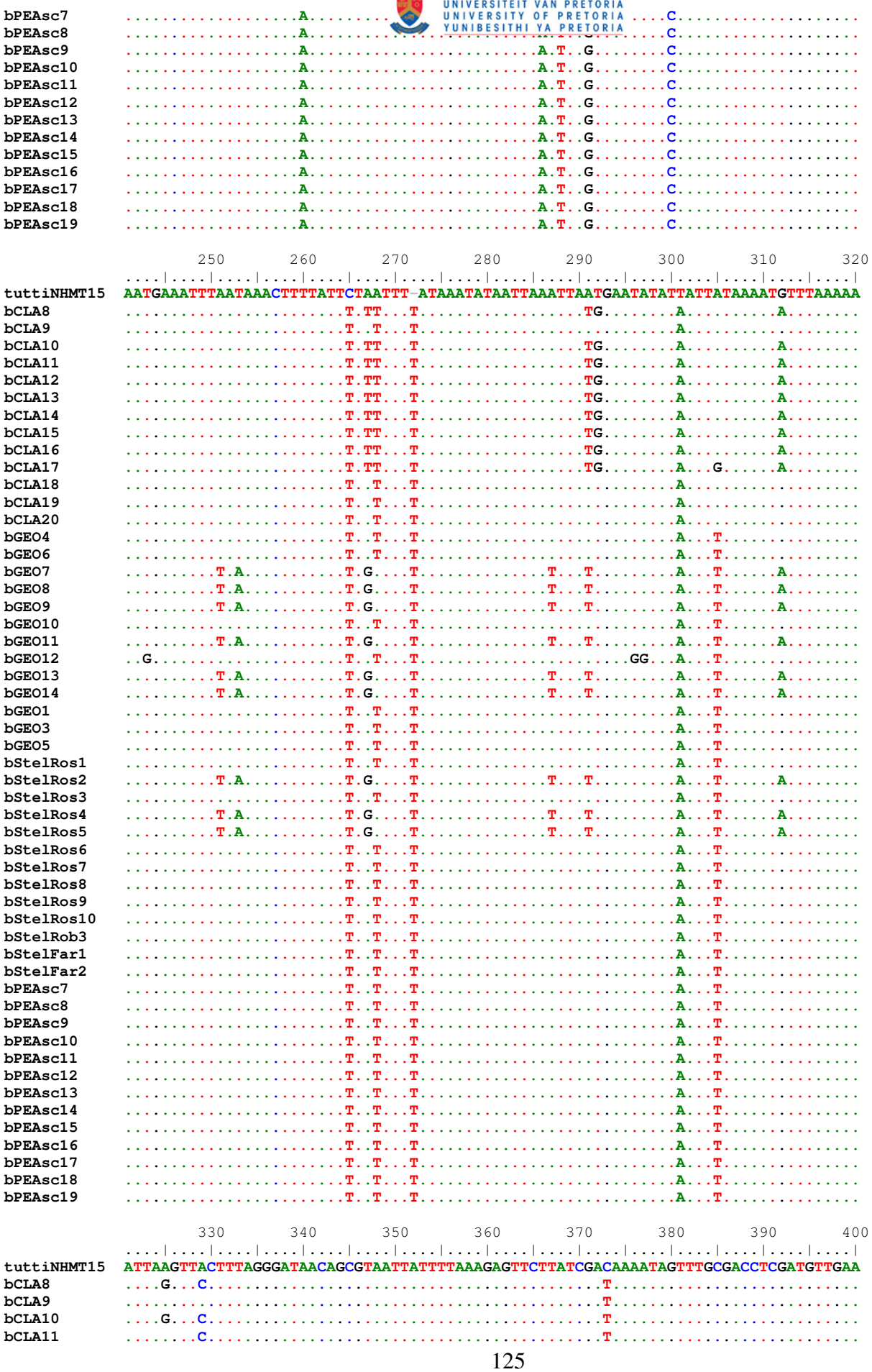
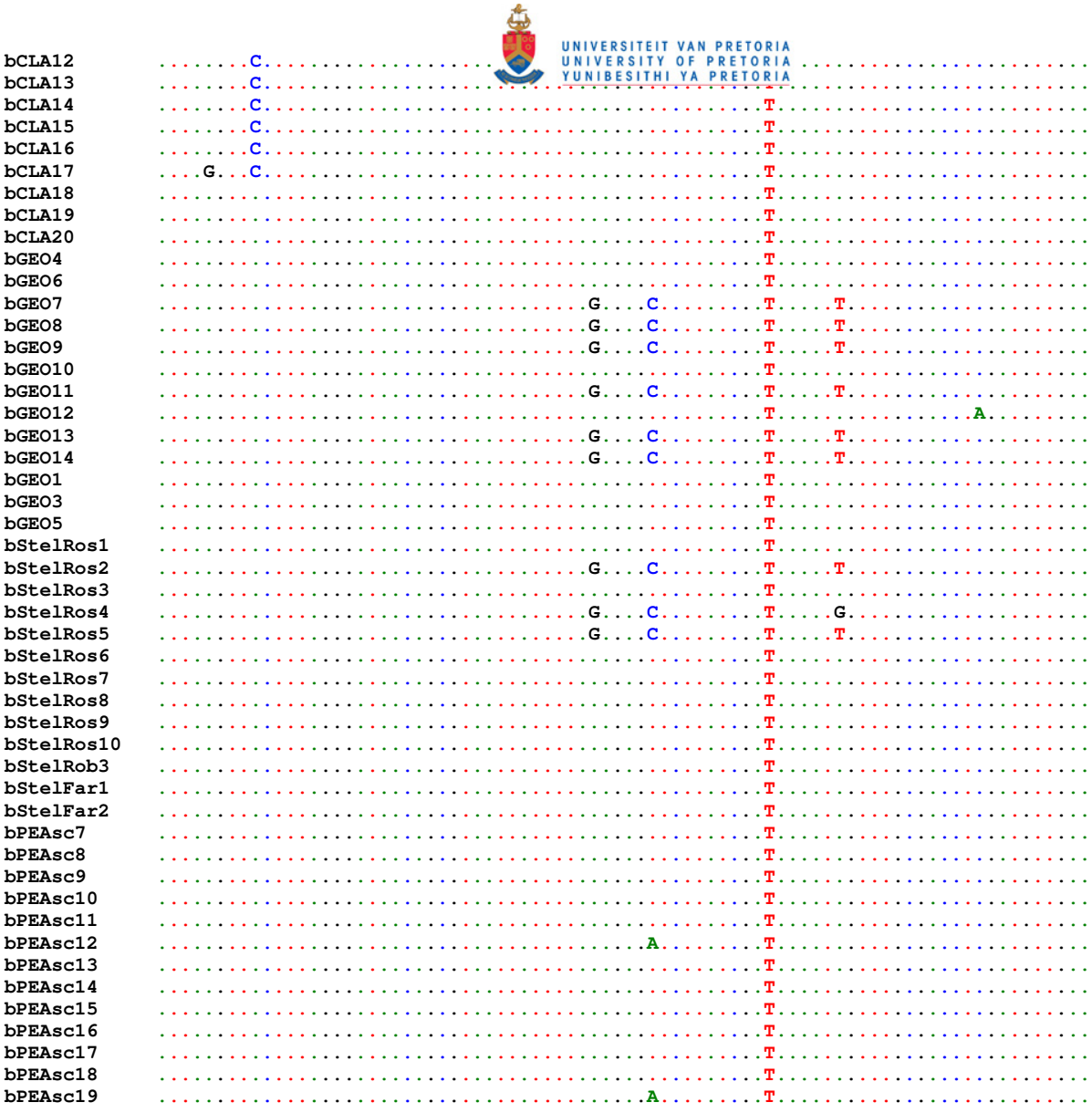
Appendix 6

Multiple alignment of the *C. bolitinos* nucleotide sequences of the 16S rRNA partial gene fragment for all 52 individuals from different geographic areas. *Culicoides tuttifrutti* (tuttiNHMT15) sequence was used as a reference species to generate the alignment. Only the nucleotide sites that differ from the tuttiNHMT15 are shown. The dots represent identical nucleotide sites and the symbol (-) represent deletions. The alignment was used to compare the *C. bolitinos* individuals of all sampled populations. The alignment was generated using the Clustal X program.

```
                    10        20                                                  60        70        80
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tuttiNHMT15                 CTGTGCAAAGGTAGCATAATCCATTGTCTTTTAATTAAAGGCTAGTATGAATGGTTGGATGAGATATC
bCLA8       CAGTATACCCGA..............GCAATC.CTGACA..T.........T.............A.........T
bCLA9       CAGTATACCCGA.....................................A.................G....
bCLA10      CAGTATACCCGA..............GCGATC.CTGACA..T.........T.............A.........T
bCLA11      CAGTATACCCGA................ATC.CTGA.A..T.........T.............A.........T
bCLA12      CAGTATACCCGA................ATC.CTGA.A...........T.............A.........T
bCLA13      CAGTATACCCGA...........T.........A.TGACA..T.........T.............A.........T
bCLA14      CAGTATACCCGA...........T......ATCA.TGA.A..T.........T.............A.........T
bCLA15      CAGTATACCCGA...........T......ATCC.C.GAA..T.........T.............A.........T
bCLA16      CAGTATACCCGA...........T.............AA..T.........T.............A.........T
bCLA17      CAGTATACCCGA...........T.............AA............T.....C......A.........T
bCLA18      CAGTATACCCGA..............GCA.....................A.................G....
bCLA19      CAGTATACCCGA.....................................A.................G....
bCLA20      CAGTATACCCGA.....................C...............A.................G....
bGEO4       CAGTATACCCGA.....................................A.................G....
bGEO6       CAGTATACCCGA.....................................A.................G....
bGEO7       CAGTATACCCGA.....................................T.....A.....A.........T
bGEO8       CAGTATACCCGA.....................................C.....A.....A.........T
bGEO9       CAGTATACCCGA.....................................A.....A.....A.........T
bGEO10      CAGTATACCCGA.....................................A.................G....
bGEO11      CAGTATACCCGA.....................................T.....A.....A.........T
bGEO12      CAGTATACCCGA.....................................C.....A.................G....
bGEO13      CAGTATACCCGA.....................................C.....A.....A.........T
bGEO14      CAGTATACCCGA.....................................A.....A.....A.........T
bGEO1       CAGTATACCCGA.....................................A.................G....
bGEO3       CAGTATACCCGA.....................................A.................G....
bGEO5       CAGTATACCCGA.....................................A.................G....
bStelRos1   CAGTATACCCGA.....................................A.................G....
bStelRos2   CAGTATACCCGA.....................................T.....A.....A.........T
bStelRos3   CAGTATACCCGA.....................................A.................G....
bStelRos4   CAGTATACCCGA.....................A.A.............A.....A.....A.........T
bStelRos5   CAGTATACCCGA.....................................T.....A.....A.........T
bStelRos6   CAGTATACCCGA.....................................A.................G....
bStelRos7   CAGTATACCCGA.....................................A.................G....
bStelRos8   CAGTATACCCGA.....................CA..............A.................G....
bStelRos9   CAGTATACCCGA.....................................A.................G....
bStelRos10  CAGTATACCCGA.....................................A.................G....
bStelRob3   CAGTATACCCGA.....................................A.................G....
bStelFar1   CAGTATACCCGA.....................................A.................G....
bStelFar2   CAGTATACCCGA.....................................A.................G....
bPEAsc7     CAGTATACCCGA.....................................A.................G....
bPEAsc8     CAGTATACCTGA.....................................A.................G....
bPEAsc9     CAGTATACCCGA.....................................A.................G....
bPEAsc10    CAGTATACCTGA.....................................A.................G....
bPEAsc11    CAGTATACCCGA.....................................A.................G....
bPEAsc12    CAGTATACCCGA.....................................A.........G.......G....
bPEAsc13    CAGTATACCCGA.....................................A.................G....
bPEAsc14    CAGTATACCCGA.....................C...............A.................G....
bPEAsc15    CAGTATACCCGA.....................C...............A.................G....
bPEAsc16    CAGTATACCTGA.....................C...............A.........G.......G....
bPEAsc17    CAGTATACCCGA.....................................A.................G....
bPEAsc18    CAGTATACCCGA.....................C...............A.................G....
bPEAsc19    CAGTATACCCGA.....................................A.........G.......G....

                          90        100       110       120       130       140       150       160
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tuttiNHMT15 AACTATTTTTATTTTAATAAAAATGAATTTTAATTTTTAGTAAAAATGCTAAAATAAAAAAATTAGACGAGAAGACCCTA
bCLA8       .....C.....................................C.....................................
bCLA9       ......................................................................................
bCLA10      .....C.....................................C.....................................
bCLA11      ......................................................................................
bCLA12      ....C.....................................C.....................................
bCLA13      ....C.....................................C.....................................
bCLA14      ....C.....................................C.....................................
bCLA15      ......................................................................................
bCLA16      ....C.....................................C.....................................
bCLA17      .....C.....................................C.....................................
bCLA18      ......................................................................................
bCLA19      ......................................................................................
bCLA20      ......................................................................................
bGEO4       ......................................................................................
bGEO6       ......................................................................................
bGEO7       TC....................................................................................
bGEO8       TC...C.....................................C.....................................
bGEO9       TC....................................................................................
```





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bGEO5          ......G.A..........T....G.....
bStelRos1      ......G.A..........T....G.....
bStelRos2      ..............AA....A..G.....G.....
bStelRos3      ......G.A..........T....G.....
bStelRos4      ............A.A.CA.CA..G.....
bStelRos5      ..............AA....A..G.....
bStelRos6      ......G.A..........T....G.....
bStelRos7      ......G.A..........T....G.....
bStelRos8      ......G.A..........T....G.............C.....
bStelRos9      ......G.A..........T....G.....
bStelRos10     ......G.A..........T....G.....
bStelRob3      ......G.A..........T....G.....
bStelFar1      ......G.A..........T....G.....
bStelFar2      ......G.A..........T....G.....
bPEWic7        ......G.A..........T....G.....
bPEAsc8        ......G.A..........T....G.....
bPEAsc9        ......G.A..........T....G.....
bPEAsc10       ......G.A..........T....G.....
bPEAsc11       ......G.A..........T....G.....
bPEAsc12       ......G.A..........T....G.....
bPEAsc13       ......G.A..........T....G.....
bPEAsc14       ......G.A..........T....G.....
bPEAsc15       ......G.A..........T....G.....
bPEAsc16       ......G.A..........T....G.....
bPEAsc17       ......G.A..........T....G.....
bPEAsc18       ......G.A..........T....G.....
bPEAsc19       ......G.A..........T....G.....
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