Plasmodium sp. INFECTIONS IN EX-CAPTIVE BORNEAN ORANGUTANS (*Pongo pygmaeus*) HOUSED AT THE ORANGUTAN CARE CENTER AND QUARANTINE, PASIR PANJANG, KALIMANTAN TENGAH, INDONESIA

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ABSTRACT

This thesis reports infections of *Plasmodium* sp. in wild-born, ex-captive orangutans housed at the Orangutan Care Center and Quarantine (OCC&Q) in Kalimantan, Indonesia. We microscopically examined blood from 1) OCC&Q residents (n=69); 2) newly confiscated orangutans (n=14); and 3) previously released ex-captives (n=2). We observed *Plasmodium* sp. parasites in blood smears collected from 24 individuals. Blood from these individuals was collected and preserved for species determination using Polymerase Chain Reaction and sequence alignment tools.

We amplified, cloned, and sequenced a ~1500 bp region of the 18S sRNA from 13 of 24 *Plasmodium* sp. infected animals. Our sequences formed four distinct groupings at the nucleotide level which may represent four *Plasmodium* sp. infecting orangutans at OCC&Q. Our data suggest cross species infection of orangutans with macaque (*Macaca* sp.) and human plasmodia, which may have serious implications for conservation and rehabilitation efforts of endangered species.

DEDICATION

To Intan, Oro and the other orphans who have lost the battle. May your losses not be in vain.

To Yuda, Ucok, Yayat and the other orangutans I have gotten to know. Thank you for introducing me to your world and showing me how we can make a difference.

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GLOSSARY

1. NHP=non human primate

2. MYA=million years ago

3. mm=millimetres

4. km^2=square kilometres

5. a.s.l.=above sea level

6. SORC= SEPILOK Orangutan Rehabilitation Center

7. GAHMU=Great Ape Health Monitoring Unit

8. PCR=polymerase chain reaction

9. OWM=Old Wolrd Monkey

10. NWM=New World Monkey

Chapter 1
EVOLUTION OF THE ORANGUTAN (*Pongo* sp.): BIOGEOGRAPHY OF THE PAST

1.1 Introduction

Currently orangutans are classified into two species; the Bornean orangutan (*Pongo pygmaeus*) and the Sumatran orangutan (*Pongo abelii*). Where do these modern orangutan species originate? Where and when did they evolve and what is/are their ancestor species and/or subspecies? Fortunately, orangutans are unlike African apes in that a fossil record exists which allows us to answer some of these questions. This chapter will discuss the "pre-*Pongo*" origins of the orangutan. This will be followed by a discussion of the South East Asian sites where *Pongo* fossils have been found. This chapter concludes with a look at the genetics of the modern orangutan and a discussion as to whether they are in fact one species or two.

1.2 Pre-*Pongo* Relatives of the Modern Orangutan (*Pongo pygmaeus*)

Orangutans have been said to belong to a sister clade to the clade that represents the African apes and humans (Andrews and Cronin, 1985). This clade has been referred to as the "Orangutan Clade" and said to include *Lufengpithecus*, *Sivapithecus*, *Ouranopithecus* and *Bodvapithcus* (Schwartz, 1988). It is believed that this "Orangutan Clade" can actually be subdivided further into to two additional clades. The first clade is called the Lufengpithecini and is a sister clade to the orangutan clade

which is now called the Sivapithecini and includes *Sivapithecus*, *Gigantopithecus* and *Pongo*. It is now believed that the evolutionary line leading to orangutans probably separated from the line leading to the other great apes over 13 million years ago (MYA) (Stewart and Disotell, 1998). This inference is supported by past studies which found that the nucleic acid and serum protein relationships between orangutans and the African apes indicated a separation at approximately 10±3 MYA (Andrews and Cronin, 1985). During the Late Miocene at approximately 12.5 MYA the most easily recognizable orangutan ancestors belong to the Genus *Sivapithecus* and evolved in India and Pakistan (Kelley, 2002). This was then followed by the rise of a younger cousin, represented by Chinese fossils which have been ascribed to the Genus *Lufengpithecus* (Harrison *et al.*, 2002 and Liu *et al.*, 2001).

1.2.1 *Lufengpithecus* sp.

China has a unique and diverse hominoid fauna. It has been hypothesized that the uplift of the Tibetan Plateau that occurred between ten and eight million years ago may have played an important role in hominoid survival in the region (Harrison *et al.*, 2002). This geological occurrence is believed to have isolated the Yunnan hominoids into a region where the subtropical climate remained fairly stable, while the climate in the rest of South and East Asia was becoming increasingly seasonal (Harrison *et al.*, 2002).

Since the late 1950's, China's Yunnan Province provided several fossil hominoid sites from which *Lufengpithecus* fossils have been recovered (Harrison *et al.*, 2002). A total of four Yunnan Province site localities are discussed and reanalyzed by Harrison *et al.* (2002). These sites include Xiaolangtan (a coal mine), Shihuiba (found in a lignite horizon), the Yuanmou Basin (Xiaohe Formation Layer) and Yangyi on the eastern slopes of Gaoligangshan Mountain.

Originally the hominoid fossils at the site of Xiaolangtan were given the name *Dryopithecus keiyuanensis*. This was followed by a reclassification based on large and small size variants found at the site into one of two genera: a) *Ramapithecus* or b) *Sivapithecus*. The Xiaolangtan hominoids have been dated to 8.30 +/- 0.10 MYA (Harrison *et al.*, 2002). Recently, Harrison *et al.* (2002) reclassified the fossils from Xiaolangtan and the four Yuanmou localities as belonging to the species *Lufengpithecus keiyuanensis*. Interestingly, just one year prior, the Yuanmou fossils had been found to differ very little from the Siwaliks *Sivapithecus* fauna (Liu *et al.*, 2001), supporting the original belief that *Lufengpithecus* was a descendent of *Sivapithecus*. These authors (Liu *et al.*, 2001) believed that the Yuanmou fossils belonged to a species that was related to *Lufengpithecus lufengensis* as either a geographical or temporal variant, but do not attempt to classify them. It appears that they were right in their assumed relatedness of these fossils to *L. lufengensis.*

The fossil hominoids found at the site of Shihuiba have recently been reclassified by Harrison *et al.* (2002). The Shihuiba fossils date to approximately 7-9 MYA and include both cranial and post-cranial remains. Since their discovery in 1975, they have been classified as belonging to a number of different genera and species. It is these fossils that Harrison *et al.* (2002), believe represent the species *Lufengpithecus lufengensis*.

There are no firm dates on the recently discovered fossil material from Yangyi, but it is speculated to date to between three and five million years ago (Harrison *et al.*, 2002). If these data prove true the fossils would represent the youngest extinct Eurasian fossil hominoid other than *Gigantopithecus* (Harrison *et al.*, 2002). At this time these fossils have not been ascribed to either a genus or species.

1.2.2 *Khoratpithecus* sp.

Khoratpithecus sp. from Northern Thailand is a recently discovered fossil ape genus believed by the authors to be an ancestor to orangutans (Chaimanee *et al.*, 2004). This new ape genus is represented by two species; *Khoratpithecus (Lufengpithecus) chiangmuanensis* and *Khoratpithecus piriyai* (Chaimanee *et al.*, 2003, 2004). These fossils date to the Middle/Late Miocene and it has been proposed that they represent the closest known orangutan ancestor and may, in fact, be a direct ancestor to Pleistocene orangutans (Chaimanee *et al.*, 2004). One of the strongest indicators of this fossil ape's relatedness to extant and fossil orangutans is the lack of muscle insertion impression corresponding to the anterior digastric muscles on the mandible (Chaimanee *et al.*, 2004). This absence is seen solely in extant orangutans as a result of the development of the laryngeal air sac (Chaimanee *et al.*, 2004).

1.2.3 *Sivapithecus sp.*

One of the major characteristics that orangutans share with *Sivapithecus* is their thick enamel which is seen only in the members of *Pongo*, *Lufengpithecus*, *Ramapithecus* and *Sivapithecus* (Andrews and Cronin, 1985). Other indicators of shared ancestry between *Sivapithecus* and *Pongo* include characters related to the separate functional complexes of their face and dentition, eight shared measurements of the palate and lower face and distinctive facial characteristics such as the size and conformation of the zygomatic region (Andrews and Cronin, 1985). Early researchers believed that *Sivapithecus* may have ranged as far West as Turkey, Greece and the former Czechoslovakia. Included in this assumption was a fossil found in the late Miocene deposits of Turkey which was ascribed to the species *Sivapithecus meteai*. This European fossil hominoid was said to be similar in morphology to modern

orangutans (Andrews and Cronin, 1985). Today, there is some uncertainty as to whether all of these fossils should continue to be ascribed to the *Sivapithecus* Genus.

Indo-Pakistan is the area from which *Sivapithecus* fossils are best known (Harrison *et al.*, 2002). In particular these fossils are very well represented in the Siwalik deposits of northern Pakistan. The Siwalik deposits provide a sample of the region's terrestrial fauna from the early Miocene until the Pleistocene (Scott *et al.*, 1999). There are a number of species of *Sivapithecus* represented in the Nagri formation of the Siwalik deposits, of which one of the largest is *Sivapithecus parvada* which was larger than the earlier Sivapithecoid (all members of the *Sivapithecus* Genus) forms (Scott *et al.*, 1999). The Siwalik deposits have produced many postcranial remains including both forelimb and hindlimb elements (Madar *et al.*, 2002). *Sivapithecus* lived in this region during the late Miocene around ten MYA (Scott *et al.*, 1999). Sivapithecoids are estimated to have been a relatively large bodied ape weighing in the region of 68 kg (Nelson and Jurmain, 1988). These arboreal apes travelled quadrupedally through the forest on the tops of medium to large sized tree branches (Madar *et al.*, 2002). This is partly supported by the palaeoenvironmental evidence of the region which suggests that this region was comprised of dense closed canopy forests at ten MYA(Scott *et al.*, 1999) indicating that this region could be home to an arboreal ape. Although this ape was arboreal, it interestingly was not as specialized in its locomotory pattern in the same way as hylobatids and humans (Madar *et al.*, 2002). Sivapithecoids probably had a degree of mobility that fell somewhere between that of extant African apes and extant *Pongo* (Madar *et al.*, 2002). Further, these apes were probably capable of a number of suspensory positions including uni- or bi-manual and perhaps even quadrepedal (Madar *et al.*, 2002). If this species was an arboreal primate what caused its extinction?

Siwaliks Palaeoclimate and *Sivapithecus* Habitats

Evidence from the paleoclimatological record of the Siwalik indicates that there was a cooling event about 9.4-8.37 MYA and that this was followed by a shift in the flora of the region from C3 plants to C4 grasslands (Scott *et al.*, 1999) occurring at around eight MYA. C3 and C4 plants refer to the type of photosynthetic pathways used by the plants for their metabolic processes. A C3 photosynthetic pathway refers to plants such as trees, most shrubs, herbs and cool-season grasses, while C4 plants generally refer to tropical or warm-season grasses (Pearsall, 2000). This vegetational shift was believed to indicate a shift in the region from the dense closed canopy forests characteristic in the Siwaliks at ten MYA to a more open, perhaps even savannah type habitat by six MYA (Scott *et al.*, 1999). The most recent evidence suggests that as early as 9.3 MYA the habitat of *Sivapithecus* was not closed canopy forest but a vegetation mosaic made up of forest, woodlands and open habitats (Nelson, 2003). As the monsoons became drier and these open habitats spread into the region (Nelson, 2003), Sivapithcoids would have been forced to migrate elsewhere or go extinct.

Perhaps more interesting for those studying extant apes is the evidence indicating that *Sivapithecus* exploited an extremely seasonal environment with five to six dry months per year much like the monsoon forests of South China (Nelson, 2003). It is possible that *Sivapithecus* adapted to life in this extremely seasonal environment by being capable of fat storage much like modern orangutans (see Chapter 2), or by inhabiting gallery forests or riverine forest environments (Nelson, 2003). It is possible that the Siwalik flora was also specialized allowing *Sivapithecus* to take advantage of an otherwise less than desirable habitat. For example, it has been postulated that the Siwalik Miocene forests may have contained a huge diversity of fruiting species allowing *Sivapithecus* greater access to its preferred food (Nelson, 2003). We do know that

despite these potential adaptations to deal with their unusually seasonal environment the Sivapithecoids were unable to survive in this habitat and by seven MYA, all Sivapithecoids were extinct in the Indo-Pakistan region (Harrison et al., 2002).

1.3 Evidence of Past Orangutan (*Pongo*) Distributions and Their Habitats:

Fossils of the genus *Pongo* have been found in much of South East Asia. Based on the literature, it now seems likely that orangutans migrated from China to the South East Asian regions of Vietnam, Thailand, Laos, until they eventually crossed onto the islands of Sumatra, Borneo and Java. This migration route is called the Sino-Malayan route (Vu *et al.*, 1996). It is not clear whether there was only one migration or many migrations followed by extinctions and new orangutan migratory incursions. Evidence from the fossil record seems to indicate that the latter is more plausible. The *Pongo* migrations into the Greater Sunda Islands during the Pliocene or early Pleistocene probably consisted of a number of members of the *Pongo* genera. It is believed that during this migration the orangutan began a gradual decrease in dental size, with an overall 16% decrease in tooth size from the time of the Pleistocene orangutan to the appearance of the Holocene orangutan (Hooijer, 1948, 1949). This size decrease culminates in appearance of the smallest orangutan ever known, the Javan orangutan (Hooijer, 1949). This hypothesis has led to the speculation that had this orangutan species survived it would have been smaller than the extant Sumatran form (Hooijer, 1948). As with many classifications of species which are now extinct or no longer found in that region and only described from fossil remains, there is the problem of lumping and splitting species. The splitters generally believe that there is little harm done in making geographic or temporal subpopulations into distinct species. This may,

in fact have a deleterious effect on orangutan palaeontology by obscuring several aspects of species biology (Drawhorn, 1994).

Caves have been the localities from which most of the fossil orangutan faunal assemblages have been recovered. This is probably related to the excellent environmental conditions that are found in caves for preservation and fossilization. Most of the recovered fossil *Pongo* material has been in the form of teeth. These are believed to have been brought into the caves by the porcupine *Hystrix brachyurus*, who collects and gnaws on defleshed and defatted bones (Drawhorn, 1994). Perhaps some of the caves containing fossil orangutan material may be the result of faunal assemblages at kill or scavenging sites. For example, there is a cave mentioned by Drawhorn (1994) that he believes represents a scavenging site because of its unusual male to female orangutan sex ratio. The Sumatran tiger (*Panthera tigris sumatrae*) may also contribute to these fossil assemblages as it tends to eat a kill in safe densely covered place (usually flora) (Borner, 1978).

1.3.1 China

The *Pongo* fauna of China seems to be very extensive and has been fairly well studied (e.g., Ho *et al.* 1995, Hooijer 1948, Kahlke 1972, Liu *et al.* 2001, and von Koengswald 1982). There are still very little published data on these fossil sites such as their palaeoecology. The East Asian monsoon was probably the controlling force for palaeoenvironmental change in East Asia for the time period dating from 130,000 years ago to 20,000 years ago (An, 2000). The East Asian monsoon is formed by the interacting thermal differences between the Asian landmass and the Pacific Ocean. Some researchers believe that its intensity could be further influenced by the occurrence of the Tibetan Plateau (An, 2000). Evidence from loess-palaeosol-red clay

indicates that the East Asian monsoon has been occurring for the past 7.2million years in which winter and summer monsoons have alternated dominance regimes with major climatic oscillations occurring every ten thousand years and less major oscillations every 1,000 years (An, 2000).

The first estimate of the number of fossil *Pongo* localities in China was done by Kahlke in 1972. At this time he recognized six sites containing *Pongo* fossil material (Kahlke, 1972). By 1982, eight sites were recognized in China within the five Provinces of Kwangsi, Kwantung, Szechwan, Kiangsu and Yunnan (von Koengswald 1982). The most recent estimate of the number of Chinese *Pongo* sites was thirteen made by Ho *et al.* (1995). These 13 fossil orangutan sites range in date from the early Pleistocene (Liucheng *Gigantopithecus* cave) to the Middle Pleistocene (Guanyindon, Shuanyandong, Niushuishan and Xioshandong caves) and the Late Pleistocene (Hexiandong, Xing-An, Tangshancun, Ganqionyan, Tobo, Liujiang *Homo sapiens*, Luokeng and Qixinyan caves) (Ho *et al.* 1995).

The Chinese sites containing fossil orangutans tend to contain a very diverse faunal assemblage including animals such as the macaque (*Macaca* sp.), the panda (*Ailuropoda melanoleuca fovealis)*, and the leopard (*Panthera pardus)* (Vu *et al.*, 1996). There has even been speculation that one of the extinct orangutan ancestors may have in fact been a mountain orangutan (von Koengswald 1982). This speculation is based on the range of higher altitude (over 600 m a.s.l.) adapted faunal material that has been discovered in association with orangutan fossils (von Koengswald 1982).

The earliest documentation of a novel fossil orangutan sub-species in China comes from Hoshangtung Cave in Southern China and consists of the lower canine from a female animal. This became the type specimen for *Pongo pygmaeus weidenreichi* dating to the lower or middle Pleistocene (Hoojier, 1948). Currently, it is believed that

the Chinese *Pongo* fossil assemblages represent two subspecies, *Pongo pygmaeus pygmaeus* and Hoojier's *Pongo pygmaeus weidenreichi* (Ho *et al.*, 1995). As for the relationships between these Chinese orangutan species, it has been speculated that the Chinese orangutan fauna are the ancestral forms of the Pleistocene Sumatran orangutan (*Pongo pygmaeus paleosumatrensis*) or another orangutan "race" with a unique specialization (Hooijer, 1949). This orangutan seems to have been larger than extant orangutans supporting its relation to this the early Sumatran subspecies of *Pongo*. This provides further support for the hypothesis that orangutans diminished in size as they migrated from Southern China, through mainland South-East Asia and into the Sunda Shelf Islands.

1.3.2 Vietnam

The orangutan fossils of Vietnam may be the most important to the palaeoprimatology community. This is not a function of their numbers or because of the amount of detail in which they have been studied, but because the Binh Province of Vietnam has produced the first and only complete fossil skeleton of an orangutan (Bacon and Vu, 2001). Previously, the orangutan fossils of Vietnam have been discussed in general by Kahlke (1972) and by Schwartz *et al.* (1995). Two separate cave systems containing fossil orangutans, such as the Lang Trang caves (Vu *et al.*, 1996) and Tham Khuyen cave (Schwartz *et al.* 1994) have also been reported.

The most important of these finds is, as mentioned above, the complete fossil orangutan skeleton found in the Binh Province of Vietnam. Interestingly, the upper and lower molars of this fossil orangutan seem to be similar to both extant subspecies; whereas at other Vietnamese orangutan fossil sites the fossil orangutan faunas seem to have tremendous variation in tooth size (Schwartz *et al.*, 1995). This skeleton is

something of an ambiguity because it contains traits belonging to both extant Sumatran orangutans (*P. abelii*) and Bornean orangutans (*P. pygmaeus*) (Bacon and Vu, 2001). For example, it has a long face with a weakly prominent interorbital region and a long nuchal surface, all characteristics of modern *P. abelii*. In addition, however, it demonstrates a marked prognathism, a straight orientation of the lower teeth, a concave facial profile and a long brain case, all of which are characteristics of modern Bornean orangutans (Bacon and Vu, 2001). The confusion over this fossil orangutan does not end here, as the skull of this animal is as large as that found on an extant male, while the long bones are equivalent to a small female (Bacon and Vu, 2001). The limb bone proportions are also confusing because the upper limbs are longer than those found in either extant form (Bacon and Vu, 2001).

In 1972, only four caves had been identified as containing fossil orangutans. The Hang-Hum cave, the Tan-Van cave, the Thung-Long cave and the Hang-Quit cave were identified as containing fossil orangutan material (Kahlke, 1972). By the mid 1990's Tham Khuyen cave in North Eastern Vietnam (Schwartz *et al.*, 1994), the Lang Trang caves (Vu *et al.*, 1996) and the sites of Tham Hai, Keo Leng, Nguom rockshelter, Diea Rockshelter and Tham On cave had been identified and added to this list (Schwartz *et al.*, 1995). A total of 201 fossil orangutan specimens dating to around 500,000 years BP have been recovered from the Lang Trang caves (Vu *et al.*, 1996). It has been speculated that these specimens represent a distinct orangutan subspecies *Pongo pygmaeus ciiochoni* (Schwartz *et al.*, 1995).

Originally, it was believed that the Tham Khuyen breccia cave produced two types of fossil orangutan. One was assigned to the species *Pongo pygmaeus* while the other has was assigned to the *Pongo* genus and not given a species name (Schwartz *et al.*, 1994). Further study indicated that the 300,000-250,000 year old cave actually

contained three *Pongo* morphs (Schwartz *et al.*, 1995). One of these morphs was a completely new genus with no apparent orangutan affinities. It was eventually named with both a new genus and a new species name being called *Langsonia liquidens* (Schwartz *et al.*, 1995). More recent studies have indicated that this orangutan is not a new Genus or Species of orangutan, but that it belongs to the orangutan subspecies *Pongo pygmaeus weidenreichi* that was described by Hooijer, (1948) (Harrison *et al.*, 2002). The second orangutan morph found at the site was the new orangutan species *Pongo hooijeri* which is larger than most fossil and extant orangutan subspecies (Schwartz *et al.*, 1995). The final fossil orangutan morph located in these caves was identified as a new orangutan subspecies and given the name *Pongo pygmaeus kahlkei.* This orangutan subspecies is unique in that it has the largest molars of any orangutan subspecies (Schwartz *et al.*, 1995).

The other Vietnamese cave sites have not been as well studied for various reasons such as small samples. These sites include Tham Hai cave where one orangutan tooth was recovered, the Nguom Rockshelter where one individual orangutan dating to about 23,000 years BP was identified and Diea Rockshelter (Schwartz *et al.*, 1995). A brief discussion of the orangutan fossil fauna from some of these less well known orangutan sites follows below.

The orangutan fossils in Hang Hum cave date to between 140,000 and 80,000 years BP and have been identified as belonging to a subspecies described by Hooijer (1948), *Pongo pygmaeus weidenreichi* (See Chinese orangutan fossils above) (Schwartz *et al.*, 1995), while the Tham On cave orangutans date to between 250,000 and 140,000 years BP. This earlier orangutan morph appears to be a different subspecies based on the observation that it is a markedly sexually dimorphic orangutan (Schwartz *et al.*, 1995). The male dentition from this orangutan tends to be slightly

smaller than the dentition of extant males, while the female dentition is markedly smaller than that of extant females (Schwartz *et al.*, 1995). As a result of this size differentiation this fossil orangutan has been assigned to the subspecies of *P.p. fromageti* (Schwartz *et al.*, 1995). It has been concluded that all of the fossil orangutans from Vietnam are probably larger on average than extant forms (Schwartz *et al.*, 1995), providing further evidence for a reduction in orangutan size between the forms that lived in Vietnam and those that migrated to the Sunda Islands.

1.3.3 Thailand/Laos

Again, like much of the available fossil orangutan assemblages the fossil orangutan faunas of these two neighbouring countries are not well known. There appears to be a large void in the literature concerning the orangutan fossil assemblages of these countries yet these sites may represent important points along the migration route of orangutans from China to the islands of Sumatra and Borneo. Three fossil orangutan sites have been identified in Laos. They are Tham-P'a-Loi, Tham-Hang and Houei-Hoc caves (Drawhorn 1994 and Kahlke 1972). Interestingly, there have also been three fossil orangutan sites identified in Thailand. Nam Phnom Dam and Kao Pa Nam are briefly discussed in Drawhorn (1994) while Thum Wiman Nakin cave has the remains of Pongo pygmaeus represented by 16 fossil teeth dating to the later mid-Pleistocene is discussed by Tougaard and Ducrocq (1999). It has been speculated that these orangutans were terrestrial and moving southward, forced by the cooling that occurred in South East Asia at this time (Tougaard and Ducrocq, 1999). This is supported by the findings of Jablonski et al. (2000) who found that shrinking habitats and the increased severity of seasonal climates pushed many South East Asian primates southward.

1.3.4 Sumatra

The *Pongo* faunas of Sumatra are very important because it is the one place where there are both fossil and extant forms of orangutans. This presents a partial reason as to why the fossil orangutans of Sumatra have been fairly well studied, described and re-described over the years by researchers such as Hooijer (1948 and 1949) and Drawhorn (1994).

The most important fossil orangutan locality in Sumatra is the Padang Highlands with a total of 3,170 recovered fossil teeth (Table 1.1) (Hooijer, 1948). The site encompasses three very important cave sites: a) Simbrambang cave dating to the end of the Pleistocene or early Holocene having the largest collection of teeth with approximately half the total collected in the Padang Highlands, b) Lida Ajer cave dating to 80,000 BP with about 1/3 of the total tooth assemblage and c) Djamboe cave dating between 85,000 and 56,000 BP and contributes 1/9 of the fossil tooth assemblage for the region (Hooijer, 1948). A fourth unknown cave called Cave "?" was identified by Drawhorn (1994) in his reanalysis of the Sumatran fossil orangutan material. It is unclear as to where the orangutan fossils from this cave were discussed by Hooijer (1948).

The type specimen (left M_3) for the novel orangutan subspecies *Pongo pygmaeus paleosumatrensis* was discovered at the Simbrambang cave site (Hooijer, 1948). It has been stated that this site was not conducive to creating a type specimen because some of the Padang Highland localities were not suitably identified by Eugene Dubois (who originally excavated these sites) and confounded by Hooijer who during his analysis of this material pooled all of the Padang Highlands localities into a single collection for description (Drawhorn, 1994) confounding the earlier problem caused by Dubois collection techniques.

Of the fossil teeth attributed to *P.p. paleosumatrensis* found in the Padang highlands a total of 312 incisors were identified (see Table 1.1) (Hooijer, 1948). The upper incisors seem to be less hypsodont than those found in extant orangutans while the lower incisors appear more hypsodont (Hooijer, 1948). A total 82 upper and 131 lower canines were also identified in this assemblage. The upper male canines are less hypsodont than those of extant orangutans while the female upper canines are larger than those of extant orangutans. The lower male canines are also larger than those of extant orangutans while the lower female canines are less hypsodont than those of extant orangutans (Hooijer, 1948). A total of 476 upper and 538 lower premolars were identified in this assemblage. Molars were very common at the Padang Highland localities. A total of 924 upper molars and 664 lower molars were recovered (Hooijer, 1948). These molars seemed to have the same basic pattern as those found in *Sivapithecus* and other Anthropoid apes, while showing a trend towards a gradual suppression of the third molar (Hooijer, 1948). A total of 43 deciduous teeth were also identified in the assemblages of these caves (Hooijer, 1948).

Table 1.1: Fossil *Pongo pygmaeus paleosumatrensis* teeth recovered at Padang Highland localities (from Hoojier, 1948).

Tooth Description	Total Recovered
I^1	44
I^2	55
I_1	56
I_2	154
Upper Canines	47♂ and 35♀
Lower Canines	81♂ and 50♀
P^3	199
P^4	277
P_3	270
P_4	268
M^1	331
M^2	353
M^3 and M^4	240
M_1	209
M_2	230
M_3 and M_4	225
Deciduous Teeth	43
Totals	3,170

There is some disagreement with the conclusions of Hooijer (1948). Drawhorn (1994) believes that the material from the Mid-Pleistocene level at Lida Ajer is probably sufficiently different to be used as a new type specimen and that it should be renamed *Pongo duboisi lidaajarensis* which he believes survived at least until the late Pleistocene at 80,000 years BP. A total of 84 individuals [Minimum Number of Individuals (MNI)] of this highly sexually dimorphic and large orangutan have been identified by Drawhorn (1994). Using these MNI numbers Drawhorn (1994) attempts to estimate orangutan mortality at Lida Ajer by dividing the population into three age-classes. Drawhorn (1994) divides the sites orangutans as being adolescents, prime adults and mature adults. Based on this subdivision Drawhorn (1994) estimates male orangutan mortality at 23.8%

for adolescents, 47.6% for prime adults, and 28.6% for mature adults for this site and female mortality at 11.1% for adolescents, 55.6% for prime adults, and 33.3% for mature adults (Drawhorn, 1994). While these figures may represent the distribution of mortality based on the fossil teeth found in this locality it seems unlikely that these represent true mortality figures. Losing so many adults at the prime of their lives would quickly lead a population to extinction if over a short period of time.

This disagreement between the findings of Hooijer (1948) and Drawhorn (1994) continues into the interpretation of the Djamboe cave site. At Djamboe, Drawhorn (1994) believes that there is sufficient evidence to create another new subspecies of orangutan named *Pongo duboisi djamboensis* dating to between 85,000 and 55,000 years BP. He believes that this orangutan morph is a chronological subspecies of *Pongo duboisi lidaajarensis*. The older *Pongo duboisi djamboensis* assemblages at 70-85,000 years BP are believed to be contemporary with the orangutans at Lida Ajer until about 56,000 years BP when the Djamboe fossils become almost indistinguishable from *Pongo pygmaeus* (Drawhorn, 1994).

Based on his observations, Drawhorn (1994) develops two possible models for orangutan evolution in Sumatra's Padang Highlands. The first sees the sequence being; the Lida Ajer population → the Djamboe population → the Simbrambang population → the extant population. While the second model sees Djamboe as a temporally mixed composite of *P. duboisi* and *P.p. paleosumatrensis*, it would seem likely that the first of these models is the more accurate. Either way it can be concluded that the disappearance of *P. duboisi* and the appearance of *P. pygmaeus* coincide with the beginning of the terminal Pleistocene glaciation at the eruption of the Toba volcano and that the reduction in orangutan dentition seems to coincide with this cooler and drier period of geological time (Drawhorn, 1994).

1.3.5 Java

Trinil is probably the most well known of the Javan fossil orangutan sites and has been discussed by several researchers (i.e. Drawhorn 1994, Hooijer 1948 and de Vos 1983). For once Drawhorn (1994) and Hooijer (1948) agree that this site contains a novel orangutan. It has been referred to as *Pongo brevirostris* by Drawhorn (1994) but he believes that this fossil represents a Pleistocene orangutan subspecies which he names *P.p. javenensis*. Hooijer (1948) also believed that this fossil probably represents an orangutan subspecies, but that there is too little material for it to be named. Drawhorn's (1994) suggested name of *P.p. javenensis* for the Javan orangutan of the late Pleistocene is rooted in minor differences such as an increased cingular development and increased molar crown flare. Fossil orangutans have also been discovered at the Punung Fissures (Drawhorn 1994 and de Vos 1982) and Sangiran (Drawhorn, 1994). It has been proposed that the Punung Fissures orangutan assemblage may represent an orangutan shifting towards a dryer period with a more open woodland type habitat (de Vos, 1982).

1.3.6 Borneo

The fossil Bornean orangutan assemblages are another poorly researched group of fossils. Niah cave in Malaysian Borneo is often referred to but is not much discussed in much of the literature. The cave dates to approximately 50,000 years BP and was probably tropical rainforest that was attached to mainland Southeast Asia (Long *et al.* 1994). This site is currently in a region of limestone forest (MacKinnon *et al.*, 1996).

1.4 Biogeographical Changes to the Malay Archipelago

The islands of Sumatra, Borneo and the Malayan Peninsula occupy an ever-wet climate (Marley and Henley, 1987). Ever-wet conditions create rainforest when there is a minimum of 60 mm of rain in every month (MacKinnon *et al.*, 1996). The Malay Archipelago was created during the Mid-Miocene when the South-East Asian and Australian plates collided near Sulawesi (George, 1987). Three main types of environmental change have been identified in the region. These changes have been identified as: sea level changes, changes in the degree of seasonality as defined by precipitation, and temperature changes (Marley and Henley, 1987). The consequences of these environmental changes would have potentially provided the Malay Archipelago with land linkages from mainland South-East Asia. These land linkages might have been available to organisms that require seasonal climates and may have lowered the montane zone boundaries making the lower hills available to lower temperature adapted organisms (Marley and Henley, 1987). At the same time these Pleistocene climatic changes caused the distribution of Catarrhines to be contracted and pushed southward (Jablonski *et al.*, 2000).

Seasonality is an important aspect of some mammalian lives. Thus, we might assume that the changing seasonality of the past may have had a tremendous influence on mammal evolution, dispersal and ranging patterns in the past thus explaining their current distribution. For example, Cercopithecoids were able to flourish during the Pleistocene as a result of their greater adaptability to the more seasonal environments that arose at this time (Jablonski *et al.*, 2000). The climate of the Malay Archipelago during the late Tertiary and early Quaternary was much more seasonal than today. Today's ever-wet forests were pushed much further south during the Mid-Pleistocene drying (Marley and Henley, 1987). These dryer conditions (George, 1987) along with the

lowering of the sea levels, allowed for the creation of a savannah corridor during the Pleistocene through which animals may have been able to migrate from mainland South East Asia (namely Thailand) to Java (Marley and Henley, 1987; George, 1987). Perhaps there was even the potential for a Philippine-Sulawesi-Sunda Island migration route (George, 1987). Although this is unlikely as the flora and fauna of the island of Borneo are more like those of the Asian mainland and the other Sunda Islands than those of Sulawesi (MacKinnon *et al.*, 1996). In fact these two islands have been separated for about 10 million years ago and are the boundary of Wallace's Line (MacKinnon *et al.*, 1996). Orangutans may have been able to survive and perhaps even exploit this savanna habitat as many researchers have argued that the orangutan ancestor may have been in fact more terrestrial (Delgado and van Schaik, 2001). Peat pollen assemblages have allowed scientists to infer that this savanna corridor did exist during the Pleistocene (Marley and Henley, 1987). Other evidence for a dryer Pleistocene climate in the Sunda region comes in the form of Kunkar Nodules which only form in arid conditions and have been identified on the Sunda Shelf. This provides even greater evidence to support the idea that the Pleistocene climate of the region was drier and much more strongly seasonal (Marley and Henley, 1987). South Kalimantan has been said to have been especially strongly seasonal during the early Holocene, while Sumatra has many landforms that only form under semi-arid conditions (Marley and Henley, 1987).

The thermal changes in the Malay Peninsula region have been extremely variable with approximately 21 sea temperature variations over the last 1.6 million years (Marley and Henley, 1987). At this time sea temperature changed by approximately 2° Celsius (Marley and Henley, 1987) which probably translated into a drop in temperature of about 2-3° Celsius in the lowlands of Sumatra and about 6° Celsius in the Sumatran

highlands (Whitten *et al.*, 1997). Part of the reason for this significant cooling was the eruption of Mount Toba in Sumatra at 75,000 BP which may have been the trigger for the final dramatic global cooling of the late Pleistocene (Drawhorn, 1994). Mount Toba may have ejected as much as 1,500-2,000 km^3 of molten rock and debris during its eruption and created a caldera 100 km long. The extant of this volcanic explosion may have triggered local orangutan extinctions and allowed for their replacement from mainland South East Asia (Drawhorn, 1994). These changes in temperature were enough that the mountains of *Gunung Leuser* (today a rich orangutan habitat up to about 2,000 meters) probably did not deglaciate until around 14,000 to 9,000 BP, while *Gunung Kemiri* did not deglaciate until 7,590 +/- 40 BP (Marley and Henley, 1987). Evidence from glacial till on *Gunung Leuser* indicates that at 15 thousand years ago there was still a region of glaciation in excess of 100 km^2 and that the temperature was still 6° Celsius colder than present (Drawhorn, 1994). The region began to warm in the early Holocene around 8,300 years BP when the subalpine/montane forest boundaries climbed by 350 meters (Drawhorn, 1994).

One might not have expected such a great change in environmental conditions in a region of such low latitudes, yet the Malay Archipelago has been greatly influenced by environmental changes especially during the last glaciation of the Pleistocene. In realizing this one can gain a better appreciation for some of the climatic conditions that this region's biota may have had to deal with during their evolution and adaptation to their present habitats. We will now look at the ecology of the habitats used by modern orangutans.

1.5 Current Ecology of Orangutan Habitats in Borneo

As stated earlier much of the flora and fauna of the island of Borneo are more closely related to those of the South East Asian mainland rather than its eastern neighbour the island of Sulawesi (MacKinnon *et al.*, 1996). The Island of Borneo is made up of three political units. Much of the northern part of the island (about the top 1/3) is made up of the Malaysian provinces of Sabah and Sarawak along with the Sultanate of Brunei. The rest of the island is made up of the four (East, Central, West, South) provinces of the Indonesian region of Kalimantan. The most complete source on the ecology of this region is MacKinnon *et al.* (1996). For this reason this section will summarize those data and information that have been provided by these authors that are relevant to orangutans and their habitats. Unless otherwise referenced all information in this section comes from this source because of its broad scope and incredible synthesis of this island's ecology. While many of the orangutan studies do discuss the ecology of their study sites that data will be included in Chapter 2-Orangutan Ecology. This section is meant only as an overview of the ecology of Borneo and how orangutans fit into this environment.

Seven distinct biounits have been identified on the island of Borneo. These biounits are: 1) the Meratus Mountains of South Kalimantan; 2) Northeast Borneo including Northeastern Kalimantan and Sabah; 3) the North Coast including Brunei and Sarawak; 4) Northwest Borneo north of the Kapus River and including western Sarawak; 5) Southern lowland plains, this is an area of vast peat and fresh water swamp forests; 6) The East coast of Borneo and finally, 7) the hilly and mountainous central part of the island. For some unknown reason the Southeastern part of the island of Borneo has no natural orangutan populations. Based on current orangutan distribution (Singleton et al.,

2004) orangutans appear to be present at least in small remnant populations in six of the seven biounits with only biounit 1, the Meratus Mountains not being home to wild orangutans.

Swamp forests are the most important biounit for our study as it takes place in the peat and fresh water swamp forests of Central Kalimantan (*Kalimantan Tengah*) Indonesia. There are three distinct types of swamps and their corresponding forests. The first of these types is mangrove swamp which has no orangutans. The second swamp type is rain-fed peat swamp forest and the third is fresh water swamp. The latter two habitat types seem to be important for the orangutan (See Chapter 2- Orangutan Ecology). Most of those tree families found in lowland evergreen Dipterocarp forests are also found in these swamp forests. Freshwater swamp forests cover about 7% of Kalimantan and are one of the more productive forest types as they are regenerated by the mineral rich floodwaters that reinvigorate them with nutrients (e.g. at *Gunung Palung* an important orangutan habitat). This type of forest contains everything from low scrub forest less than ten meters in height to mixed lowland Dipterocarp-type forest.

The important tree types in swamp include *Campnosperma sp., Alstonia sp., Eugenia sp., Canarium sp., Koompassia sp., Culophyllum sp.* and *Melanorrhea sp.* (MacKinnon *et al.*, 1996). Unfortunately, much of this forest has been cleared for agriculture because of its high productivity.

The lowland forests of Borneo are the most important forest type in terms of both ecology and commercial value in the Sundaland region. This forest tends to have a canopy between 45 meters and 60 meters in height in which ten percent of all trees and 80% of all emergents are Dipterocarps. The importance of this forest type to primates and orangutans can be seen in the diversity of the primate species that can be found in

lowland forests. In Borneo, as many as 13 different species of primates can be found in these forests.

A second type of lowland-type forest is ironwood forest or *ulin* which is dominated by the ironwood tree (*Eusideroxylon zwagari*). The ironwood tree is partially characterized by its extremely large seed weighing as much as 230 g. This forest type once encompassed 1440 km^2 of the island of Borneo but now less than 40% of it remains intact. Important orangutan habitats such as Kutai National Park (See Chapter 2-Orangutan Ecology and Rodman, 1973, 1977, 1979, and 1988) are comprised of this forest type. Ironwood forest has two canopies; a higher open canopy at 30-35 meters and a lower closed sub-canopy at 20-25 meters. Other important tree species in this forest type include *Intsia palembonica*, *Eugenia* sp. and *Palaquium* sp.

Heath forests (*kerangas*) are another type of orangutan habitat yet, generally, orangutans are found here in low densities. Heath forests are found on soils that are created from siliceous parent materials which create acidic, coarse, and free-draining soils that are often referred to white sand soils. These forests create low single layered canopies formed by the crowns of large saplings. Under the best conditions heath forest may attain canopy heights of 27-31 meters, but are more commonly characterized by small densely packed trees with small buttresses, stilt type root systems, thin climbers and epiphytes. Some of the characteristic species of heath forest include; *Casuarina sumatrana*, *Cratoxylum glaucum*, *Dacrydium elatum*, *Baectia frutescans*, and *Tristaniopsis obovata*.

Limestone forests form on the remains of what was once an ancient sea floor where the remains of calcium carbonate organisms such as corals and brachiopods have accumulated. Among these limestone forest regions is Niah Cave an important orangutan fossil site (See Chapter 1-Orangutan Origins). Five types of limestone forests

have been identified. These are: 1) lowland scree characterized by widely spaced trees dominated by massive emergents five meters in girth and 50 meters high; 2) lowland limestone cliff communities that do not contain much vegetation; 3) lowland limestone forest characterized by large Dipterocarp emergents which may reach 40 meters or more; 4) lower montane limestone forest characterized by a large deep mat of peat-like humus and a canopy under 25 meters and 5) upper montane (over 1,200 meters) characterized by small trees.

The final habitat on the island of Borneo that contains wild orangutans is the mountain habitats. Here, orangutans and other primates are limited to the lower mountain slopes and lower montane regions. There are few mountains on the island of Borneo above 2,000 meters in height. The lower mountain slopes contain habitat similar to that found in the lowland rainforests of the region, while the lower montane habitat is dominated by chestnut (*Castanopsis*) and oak (*Lithocarpus* and *Quercus*) trees. The upper montane forests generally tend to contain small and stunted trees making them less than prime habitat for primates. Thus, these forests tend not to contain any primates. For every 1,000 meters in altitude the temperature in these forests tends to drop five degrees Celsius and the daily variations in temperature become more drastic.

The climate of Borneo tends to be warm with annual average temperatures between 25 and 35° Celsius. There are two main monsoon seasons in Indonesia influencing the rainfall on the island of Borneo. The "Dry" monsoon runs from May to October while the "Wet" monsoon runs from November to April. Borneo has very few months with less than 200 mm of rain. Much of the hilly interior of the island receives between 2,000 and 4,000 mm of rain annually. Kalimantan has been divided into five broad agroclimate zones. In these zones wet months are defined as those with more than 200 mm of rain and dry months are those with less than 100 mm of rain. Zone A

covers 43% of Kalimantan and has nine or more wet months and two or less dry months. Zones B (32% of Kalimantan), C (15% of Kalimantan), D (4% of Kalimantan) and E (6% of Kalimantan) are sub-divided into zones 1 and 2. Zone B1 has between seven and nine wet months and less than two dry months; while B2 has seven to nine wet months and 2 to three dry months. Zone C1 has five to six wet months and less than two dry months; while C2 has five to six wet months and two to three dry months. Zone D1 has three to four wet months and less than two dry months; while D2 has three to four wet months and two to three dry months. Finally, zone E1 has less than three wet months and less than two dry months and zone E2 has less than three wet months and two to three dry months.

1.6 Current Ecology of Orangutan Habitats in Sumatra

Data regarding the ecology of Sumatra has recently been synthesized by Whitten *et al.*, (1997). Interestingly orangutans are currently only found north of Lake Toba on the island of Sumatra. The island of Sumatra is made up of ten vegetation types. Many of the vegetational types were discussed above in the section on the ecology of Borneo and are sufficiently similar that to re-discuss them here would be redundant. These vegetation types include the three swamp forest types: mangrove, freshwater and peat, montane rainforest, lowland evergreen rainforest, semi-evergreen rainforest, heath forest, tropical pine forest, ironwood forest and forest on limestone. Of these only lowland semi-evergreen rainforest and tropical pine forest are not discussed in the ecology of Borneo above. The tropical pine forests are probably not important to orangutans as they are generally found in sub-alpine zones 2,000-2,500 meters above

sea level, and never occur under 1,000 meters, while semi-evergreen forests are not discussed as a separate habitat type.

The island of Sumatra is also in the ever-wet zone and may experience rainfall of more than 6,000 mm west of the Barisan mountain range to less than 1,500 mm in other areas. The main rainy season is between the months of May to September. As discussed above this differs from the island of Borneo which is experiencing its dry monsoon at this time. Much like the island of Borneo, the island of Sumatra has 5 agroclimatic zones (A-E). Zone A has nine or more consecutive wet months and two or less consecutive dry months. Zone B has seven to nine consecutive wet months and three or less consecutive dry months. Zone C has five to six consecutive wet months and three or less consecutive dry months. Zone D has three or four consecutive wet months and two to six dry months. Finally, Zone E has up to 3 consecutive wet months and up to 6 consecutive dry months. It is important to note that 71% of Sumatra has seven or more consecutive wet months and up to three consecutive dry months. This section is only meant as a brief overview and for a more in depth understanding of the ecology of Sumatra one should read Whitten *et al.* (1997).

1.7 Conclusion

The orangutan has gone through a transition and evolution from its prehistoric predecessors *Sivapithecus*, *Lufengpithecus* and the various *Pongo* species and subspecies to become the only modern great ape other than humans to live outside of Africa. Why has this ape survived until now in Borneo and Sumatra when all of the other apes outside of Africa went extinct? It has been speculated that once *Pongo* sp. evolved and had migrated to Borneo and Sumatra this ape had advantages over mainland apes

such as decreased body size and increased arboreality making these animals harder to hunt than their mainland cousins (Harrison *et al.*, 2002).

It is clear that there is a great deal of knowledge regarding fossil orangutans that has already been discovered in many areas such as Sumatra, China and Vietnam. Yet, at the same time there is still a great deal of work to be done in more complete descriptions of the orangutan fossil faunas of places such as Thailand and Laos. The more complete our fossil descriptions are the better we will be able to interpret the migratory movements and evolution of the orangutan.

Chapter 2
ECOLOGY OF MODERN ORANGUTANS (*Pongo* sp.) FROM THE ISLANDS OF BORNEO (*P. pygmaeus*) AND SUMATRA (*P. abelii*).

2.1 Introduction

The orangutan is an especially interesting great ape with many characteristics that make it unique from all other great apes. It is the only great ape to live a generally solitary and unsocial lifestyle and it uses vocalizations as spacing mechanisms rather than physical confrontations to protect females and territories (Galdikas, 1979). The orangutan is also the only great ape other than humans to live outside of Africa, and apparently has the longest interbirth interval of any primate species (See 2.3 Life History below). As a result of these factors the orangutan has become a popular scientific study subject and one of the most recognizable great ape and primate species in popular culture (Rijksen, 1995). The orangutan has been said to be the most endangered of the great apes (The Editors in the Preface to *The Neglected Ape*). Orangutans are in grave danger of extinction due to a dramatic decrease in numbers which was first noted in the late 1980's (Rijksen, 1995). The world's conservation community has been slow to fight to save the orangutan (Rijksen, 1995).

Orangutans once ranged over a vast area including much of South East Asia from southern China through Vietnam, Thailand, Laos, and into the Sunda Islands (see Chapter 1). Orangutans have already gone extinct over much of this area and today only exist on the islands of Sumatra and Borneo. Two possible explanations for the

collapse of the orangutan range have been proposed. The first of these is the ecological explanation which proposes that orangutan distributions shifted southward as a result of the shifting tropical and subtropical zones of South East Asia (Delgado and van Schaik, 2001). The increase in temperature since the last glaciation has raised sea levels and perhaps restricted the amount of habitat available for large mammals such as orangutans (Delgado and van Schaik, 2001). The second hypothesis is that humans are to blame for the decline in orangutan distribution because of the fact that orangutans are fairly slow, arboreal animals making them easy to exploit while their slow reproduction makes them susceptible to extinction (Delgado and van Schaik, 2001). It seems likely that these two hypotheses should not be thought of as mutually exclusive. Both ecological and human factors have probably contributed to the elimination of orangutans from its former habitat.

Many orangutan rehabilitation centres have been established to deal with the modern threats caused by humans that are facing orangutans. These sites may at one time combine the study of wild orangutans with the conservation goal of returning wild-born ex-captive orangutans that had been confiscated by various government agencies back to the wild (see Yeager, 1997). In 1995, it was suggested that ex-captive orangutans only be released into former habitats that today contain no wild orangutans (Soemarna *et al.*, 1995). This and other recommendation made by environmental organizations eventually became part of the *Indonesian Orangutan Action Plan* (Soemarna *et al.*, 1995). These guidelines were adopted in 1995 and as a result, the Indonesian government no longer allows the release of wild-born ex-captives into areas with viable wild orangutan populations (Rijksen and Meijaard, 1999). There are some key advantages to the rehabilitation and reintroduction process. It may provide demographic and genetic reservoirs from which new populations can be established and

genetic diversity can be increased. It also reduces the threat of extinction of wild populations by increasing their numbers (Yeager, 1997).

Currently there are five active wild orangutan research sites (Delgado and van Schaik, 2001). The two current research sites in Sumatra are Ketambe and Suaq Balimbing which are both in the Gunung Leuser ecological area (Delgado and van Schaik, 2001). There are also three current research sites in Indonesian Borneo. The current Bornean research sites are Tanjung Puting National Park and Cabang Panti in Gunung Palung National Park (Delgado and van Schaik, 2001) and Danau Sentarum Wildlife Reserve (Russon *et al.*, 2001). Previously, four other orangutan research sites also existed in Borneo. These sites were Lokan, Ulu Segama, Mentoko and Renun (Delgado and van Schaik, 2001) but for whatever reason these sites are no longer active centers of wild orangutan field research. One site in Sarawak (Lanjak Entimau Wildlife Sanctuary) has looked at estimates of orangutan abundance inside the wildlife sanctuary (Blouch, 1997). Yet, this project was not specific to orangutans and looked at the density of all primates in the area.

From these orangutan studies three types of ranging patterns have been postulated. These may explain why there is such variation in home range estimates at the sites discussed below. Rijksen and Meijaard, 1999) identified these three ranging patterns as: (a) residents who are those orangutans that live in an area for many years and are present in that area for much of the year; (b) commuters who are those orangutans that each year for many years visit an area and are seen regularly in that area for many weeks or months and (c) wanderers who are seen very infrequently for a period of three years and may in fact never return to the area. Males often tend to fall into the latter category and thus have been called the wandering sex (Te Boekhorst *et al.*, 1990). Both the female attraction hypothesis and the food attraction hypothesis have

been forwarded as explanations for male wandering (Te Boekhorst *et al.*, 1990). The female attraction hypothesis was originally developed by MacKinnon (1974) and was later revised by Te Boekhorst *et al.*, (1990). This hypothesis states that males move into an area when females in the area are ready to conceive. The food attraction hypothesis postulates that non-resident orangutans arrive at periods of high fruit production (Te Boekhorst *et al.*, 1990). It has been pointed out that these two hypotheses are probably not mutually exclusive (Te Boekhorst *et al.*, 1990).

2.2 Orangutan Home and Day Ranges, Density and Population Estimates

2.2.1 Bornean Orangutan Population Estimates

In 1995, it was estimated that between 10,282 and 15,546 orangutans lived on the island of Borneo in the remaining 22,360 km^2 of suitable habitat (Rijksen *et al.*, 1995). A more recent estimate of the Bornean orangutan population released in the summer of 2004 based on the creation of a new PHVA (Population and Habitat Viability Analysis) suggests that the Bornean orangutan population is actually greater than it was believed to be in 1995. This new estimate indicates that more than 40,000 orangutans survive on the island of Borneo (Singleton *et al.*, 2004). This estimate is based on the largest ever cooperative study by orangutan researchers to survey intensively all potential orangutan habitats on the islands of Borneo and Sumatra. This population estimate includes very large population concentrations in Tanjung Puting National Park (6,000 individuals), Sebangau (6,900 individuals) and Arut-Belantikan (Minimum 6,000 individuals). Nine other areas in Borneo are believed to house orangutan populations numbering between 1,000 and 3,350 individuals (Singleton *et al.*, 2004). This indicates that orangutan populations are faring better than was believed prior to this PHVA.

Bornean orangutans probably numbered around 230,000 animals at the turn of

the 20th century based on available habitat (Rijksen and Meijaard, 1999). The decline in Bornean orangutan populations has been attributed to the Indonesian government's recent decision to open up Kalimantan to economic development in 1986 (Eudey, 1995). For example as many as 3,000, and at least 1,000 orangutans, were smuggled out of Kalimantan between 1988 and 1990 (Eudey, 1995).

Tanjung Puting

Tanjung Puting National Park (TPNP) in the Province of Kalimantan Tengah, Indonesia is probably the best known orangutan research site in the world because it encompasses the research site of Dr. Birute Galdikas at Camp Leakey. The most recent estimate of the population of orangutans in the park is 6,000 individuals making it one of the last true strongholds for orangutans (Singleton *et al.*, 2004). The Park itself is relatively flat and low lying (Galdikas, 1978a, 1979), with most of the park being lower than 30 meters a.s.l. (Galdikas, 1988). The park is made up of 63% mixed dipterocarp forest and 27% peat swamp forest (Galdikas, 1988). There are two rainfall peaks in the park, the major one occurring between the months of December and May, followed by a second peak in the month of September (Galdikas, 1978a and 1988). The dry season tends to fall between June and August (Galdikas, 1988). The temperature in the Park varies between a maximum temperature of 37.5°C to 34°C with the lows in the park varying between 18° and 21° C (Galdikas, 1978a, 1988). Temperatures tend to vary by about 10° C during a single 24 hour period (Galdikas, 1978a).

The orangutans at TPNP tend to live in one of three group types; either as a solitary male, an adult female with one or two dependant offspring or immature animals who are in a transitory phase between living with their mothers and having their own separate independent life (Galdikas, 1979). Female and male orangutans in the

park tend to have different sized home ranges, but these home ranges themselves tend to overlap (Galdikas, 1978a). A home range can be defined as "the area a group or individual uses" (Strier, 2000: 376). There is uncertainty at TPNP as to the true size of male home ranges other than to say they are very large and in some instances may be larger than the study area of Camp Leakey (Galdikas and Teleki, 1981). Female orangutans within the park tend to have home ranges that vary based on their age (see Table 2.1) (Galdikas, 1978a and 1988).

Orangutan day ranges tend to vary greatly between males and females at TPNPk. The average day range for an orangutan is between 90 and 3,050 meters with a mean value of 790 meters (Galdikas, 1988). Males tend to travel farther and faster than females during the day with a mean day range of 850 meters with a maximum of four kilometers (Galdikas, 1988). Females tend to travel slower than males and thus tend to have smaller day ranges than males averaging about 710 meters with a maximum recorded value of 2.5 kilometers (Galdikas, 1988). Orangutan densities in the Park have been observed to fluctuate as orangutans seek seasonally available food sources (Galdikas, 1979). This corresponds to the predictions made by Te Beokhorst *et al.*'s (1990) food attraction hypothesis.

Gunung Palung: Cabang Panti

Gunung Palung National Park and the research site of Cabang Panti is currently the only other active site of long term wild orangutan research on the island of Borneo. The research being conducted at this park has just begun to be published over the past few years and doesn't have the time depth of sites like Tanjung Puting can provide. The site is made up of peat swamp forest, freshwater swamp forest, alluvial terrace forest, lowland sandstone forest, lowland and upland granite forest (Knott, 1997). Mast (or mass) fruitings have occurred at the site in 1987, 1991 and 1995 (Knott, 1997).

Gunung Palung has both prime orangutan habitat and a less desirable orangutan habitat. For the prime orangutan habitat available in the park orangutan populations have been estimated as high as 2,100 orangutans (Rijksen et al., 1995). The less desirable orangutan habitat in the park and along its boundaries had an estimated population of 1,650 animals (Rijksen et al., 1995). More recent estimates indicate that the orangutan populations in this park may have dropped to a total of 2,500 individuals (Singleton et al., 2004). To help save wildlife in this park an experimental type of forest buffering was established at this site in the early 1990's. These forest buffers generally take one of two forms. They are either forest extension buffers which increase the forest habitat available for the local flora and fauna, or they are sociobuffering zones that allow the local peoples living around the park to use them for goods and services (Salafsky, 1993). Unfortunately, there are no published accounts of orangutan home range or day range size for this park.

Kutai: Mentoko

The Kutai research site (Mentoko) was at one time a well studied site focusing on wild orangutans (e.g. Rodman, 1973, 1977, 1979 and 1988). Unfortunately, according to Delgado and van Schaik (2001) this site is no longer active in terms of wild orangutan research. The Kutai Nature Reserve (Now Kutai National Park) is located in East Kalimantan and is made-up of primary dipterocarp forest (Rodman, 1979). The monthly maximum for rainfall in the park is 355.12 mm while the monthly minimum is 124.54 mm (Rodman, 1973). The site is often flooded by the nearby river (Rodman, 1973) increasing the amount of river silts that provide excellent conditions for dipterocarp growth (MacKinnon et al., 1996).

A current estimate indicates that more than 600 orangutans live in the Kutai region (Singleton *et al.*, 2004). Estimates of orangutan densities in the park range from

3/km^2 (Rodman, 1973) to 1/km^2 (Rijksen *et al.*, 1995). Unlike Tanjung Puting, the male orangutans at Kutai tend to have small discrete home ranges (See Table 2.1) (Rodman, 1973). The female home range at Kutai may also be smaller than those at Tanjung Puting and are estimated at between 0.4km^2 and 0.6 km^2 (Rodman, 1971) to 1.5km^2 (Rodman and Mitani,1987 as discussed by Rodman, 1988). The average day range at Kutai is an average of 305 meters per day (Rodman, 1977 and 1988). This is also very small when compared to those data for Tanjung Puting National Park.

Danau Sentarum

This site has only recently been studied and the authors caution that estimates of orangutan density based on nests can be inaccurate. For nest surveys to be representative of the actual population a valid estimate needs to be established for the proportion of nest builders who are mothers with dependent offspring. A proper estimate of nest decay rates must be also be established though it should be noted that these estimates can't be used to extrapolate populations over a wide area (Russon *et al.*, 2001). The site contains four forest types: 1) swamp and peat swamp forest; 2) lowland hill forest; 3) farmland, clearing or secondary forest and 4) unusable forest (Ibid). There are three proposed protected areas at the study site with a total population of 3771 orangutans divided as follows: DSWR-206 orangutans; DSWR-M-1024 orangutans and SSWR-G-2741 orangutans. The best habitat at the site is swamp or peat forest and lowland forest (Russon *et al.*, 2001). The estimated orangutan densities range between 4.09 orangutans per km^2 in best habitat comprising of low swamp forest to a low of 0.43/km^2 in high swamp forest (Ibid). The most up to date population estimates for Denau Sentarum indicate that this population has also seen a decline over the past few years with only 1,500 individuals remaining in this region (Singleton *et al.*, 2004). Unfortunately, there is little other information available for this site.

Ulu Segama

The research site in the Ulu Segama Reserve, Sabah, Malaysia was used between 1968 and 1970 by John MacKinnon (MacKinnon, 1974a and 1974b). In 1995 the total number of orangutans living in all of Sabah was inaccurately estimated at between 456 to 760 orangutans (Rijksen et al., 1995). More recently, an estimate of approximately 13,615 orangutans living in Sabah was provided by Singleton et al., (2004) indicates that the orangutan population in Sabah is much healthier than was previously believed. The Ulu Segama Reserve is at an elevation of between 400 and 500+ meters above sea level, and is made up of mixed dipterocarp forest. January and December are the wettest months, experiencing approximately 700mm and 600mm of rain respectively, but the region receives a large amount of rainfall throughout the year (MacKinnon, 1974a). Ulu Segama was divided into three regions by MacKinnon (1974). Segama A has an estimated orangutan density of 1.5/km^2, Segama B an estimated orangutan density of between 1 and 2/km^2 and Segama C an estimated orangutan density of less than 1/km^2 (MacKinnon, 1974a).

Lanjak Entimau Wildlife Sanctuary, Sarawak Malaysia

The Lanjak Entimau Wildlife Sanctuary contains the only viable population of orangutans living in the Malaysian Province of Sarawak (Blouch, 1997). The sanctuary is located at an elevation ranging from 100 to 1,284 meters above sea level (Blouch, 1997). The forest at the site is a mixture of lowland and submontane evergreen rainforest dominated by dipterocarps (Blouch, 1997). There is a total annual rainfall here of about 3,500mm with the driest months receiving about 200mm of rain (Blouch, 1997). The site is estimated to have approximately 1,000 (1,024-1,181) orangutans but their densities are controlled by food availability and hunting pressure (Blouch, 1997, Singleton et al., 2004). The overall orangutan density at the site is about 0.5/km^2, but this number increases to 1.73/km^2 in the south region of the sanctuary

where the greatest densities of fruit trees are found (Blouch, 1997). There is no published literature pertaining to this site. This region offers the best site for orangutan research in Sarawak simply because it contains that Province's only viable orangutan population.

Table 2.1: Ecological Variables from Bornean Orangutan Research Sites

Site	Park Size	Orangutan Density /km^2	Orangutan HR km^2	Annual Temp.	Annual Rainfall
Tanjung Puting	415,040 ha.	1-2	♂ 20+? ♀ 3.5-6	37.5°C-18°C	Up to 3,819 mm
Gunung Palung	90,000 ha.	3	n/a	n/a	4,300 mm
Kutai	198,629 ha.	1-3	♂ 0.8-1.6 ♀ 0.4-1.5	30.55°C-18.88°C	2,364.74 mm
Danau Sentarum	109,000-190,000 ha.	0.43-4.09	n/a	n/a	Na
Ulu Segama	n/a	1-2	♂2+ ♀much smaller	n/a	Up to 700 mm per month
Lanjak Entimau	168,768 ha.	0.5/km^2-1.73/km^2	n/a	n/a	3,500 mm

2.2.2 Sumatran Orangutan Population Estimates

The Sumatran orangutan is currently restricted to the northern part of the island. Five distinct populations were identified by van Schaik et al., (1995). They include 1) The Singkil Population; 2) the Sembabala-Dolok Sembelin Population; 3) The Greater Gunung Leuser West Population; 4) The Greater Gunung Leuser East Population and 5) A small population west of Takegnon may exist but cannot be confirmed because of conflicting reports (van Schaik et al., 1995). The overall population of the Sumatran orangutan has decreased on the island by about 86% in the last 100 years (Rijksen and Meijaard, 1999). The number of Sumatran orangutans at the beginning of the Twentieth century has been estimated at 85,000 animals (Rijksen and Meijaard, 1999). There are some discrepancies as to how many orangutans are left in Sumatra. A generous estimate of about 12,000 animals is given by Rijksen and Meijaard (1999), while the total

population estimate in 1995 was 9,200 orangutans according to van Schaik et al., (1995). Today a total of approximately 7,501 orangutans are believed to survive on the island of Sumatra (Singleton et al., 2004). By 1999 the estimated number of orangutan living in the Leuser Ecosystem had decreased to less than 6,500 (van Schaik et al., 2001). Logging is the most serious threat to orangutans in Sumatra (Robertson and van Schaik, 2001) as it is to orangutans in Borneo. The forests in which Sumatran orangutans live are being lost, degraded and fragmented pushing them towards rapid extinction (Robertson and van Schaik, 2001). In 1999 it was predicted that the Sumatra orangutan would be extinct within a decade because as many as 1,000 animals were being lost each year (van Schaik et al., 2001).

Both Ketambe and Suaq Balimbing research sites are located inside the Gunung Leuser Ecosystem which encompasses about 25,000 km^2 (van Schaik et al., 2001). The Gunung Leuser National Park is currently insufficiently protected (Robertson and van Schaik, 2001). This has led to a proposal to create a private management system to protect the entire 25,000 km^2 habitat. This project is aimed at conserving the Leuser Ecosystem and securing a basis for sustainable economic development in the surrounding regions (Robertson and van Schaik, 2001). The project hopes to accomplish these goals by establishing and protecting a new nature conservation area in which there are intact ecologically sustainable boundaries keeping the area as untouched as possible. Likewise, the project will need to facilitate the creation of sustainable buffer zones inside the ecosystem and the excellent production forests that surround it (Robertson and van Schaik, 2001).

Population estimates within the Leuser ecosystem have been made based on habitat type along with the corresponding orangutan densities for each habitat type. The West Leuser region has an estimated 7,639 orangutans with a corrected value of 5,700

orangutans (van Schaik et al., 1995), while the East Leuser region has an estimated population of 4,636 orangutans. These data are summarized in Tables 2.2 and 2.3.

Table 2.2: Ecological Variables from Bornean Orangutan Research Sites

Habitat Type	Population Size (#of Ind.)	Population Density /km^2
Secondary Forest	6	0.5
Swamp Forest	255	5
Below 500m a.s.l.	2,079	2.5
500-1,000m a.s.l.	4,161	1.8
1,000-1,800m a.s.l.	1,138	0.4

Table 2.3: Orangutan Densities in the East Leuser Region of Sumatra

Habitat Type	Population Size (# of Ind.)	Population Density /km^2
Secondary Forest	43	0.5
Under 500 m a.s.l.	592	2.5
Logged Lowland	177	1.0
Under 1,000m a.s.l.	3,015	1.8
1,000-1,800m a.s.l.	809	0.4

If the Gunung Leuser National Park in Sumatra was lost, the orangutan population in the Leuser Ecosystem would probably quickly drop to 3,325 animals (Faust *et al.*, 1995).

Ketambe

Ketambe is perhaps the second best studied orangutan research site in terms of site ecology and the wild orangutans that live in it (see Table 2.4). At this time it is the best studied of the Sumatran orangutan research sites. It owes this to the fact that research at the site has been conducted since June, 1971 (Rijksen, 1978). The site consists of pristine rainforest at an altitude varying between 250 and 2,000 meters above sea level (Buij *et al.*, 2002) making part of the region mixed hill or upland rainforest (Rijksen, 1978). The forest ranges from swamp and lowland dipterocarp forest to hill

and mountain forests (Rijksen, 1978). The temperature at Ketambe has a number of similarities with Tanjung Puting in that the yearly average is 29.2°C (Rijksen, 1978). Two dry seasons exist at the site, one from January to February, and the other from July to August (Rijksen, 1978). The annual rainfall at the site is around 3000mm with 2827mm of precipitation in a very dry year and 3744mm of precipitation in a very wet year (Wich and van Schaik, 2000). Ketambe has experienced mastfruiting episodes at a rate of 0.29 for the years spanning 1971-1998 (Wich and van Schaik, 2000). This includes episodes in 1981, 1984, 1988, 1992, 1995 and 1998 (Wich and van Schaik, 2000).

Orangutan populations within the park have been estimated at 3,450 for the western extent of the park and 2,400 for the eastern extent of the park (van Schaik *et al.*, 1995). Orangutan densities in the park range between 7/km^2 in the prime habitat type of coastal swamps to 1/km^2 in good habitats (van Schaik *et al.*, 2001). Orangutan densities correlate with the number of fruit sources at each elevation (Buij *et al.*, 2002). This may indicate that the Ketambe orangutans follow a seasonal migration following the shifting phases of fruit ripening from the lowland to the intermediate elevation zone and finally into the highlands as the fruit in each of these zones ripen (Buij *et al.*, 2002). Observations at Ketambe indicate that in the lowland regions "strange" orangutans do tend to enter the study area when fruit is abundant and resident animals tend to leave the area only when fruit is very scarce (Buij *et al.*, 2002). Some researchers believe that this fruiting cycle controls the density of orangutans at Ketambe with it being a major contributor to the site's carrying capacity during non-fruiting seasons, while becoming an available resource to visitors during fruiting season (Te Boekhorst *et al.*, 1990).

Orangutans at Ketambe have overlapping home ranges estimated at 1-2km^2 for adult females and 2km^2 for adult males (Rijksen, 1978 and reviewed in Rodman, 1988).

It is very interesting that these numbers differ from the maximum home ranges observed at Suaq Balimbing (see next section below) considering how close they are in geographical proximity. The day ranges for individual orangutans at Ketambe averaged 480 meters for an adult male, 550 meters for a female and 890 meters for a subadult male (Rijksen, 1978 and reviewed in Rodman, 1988).

Suaq Balimbing

The site of Suaq Balimbing is a fairly young center of wild orangutan research. It was established in 1992 and was expanded between 1992 and 1996 (Singleton and van Schaik, 2001). As a result there is very little published data on the site, but it is part of the Leuser Ecosystem and thus may be considered similar to Ketambe. Suaq Balimbing has two rainy and two dry seasons with a total annual rainfall in the region of 3,500mm (Fox *et al.*, 1999) The site contains four main habitat types; tall riverine forest; regularly flooded backswamps; closed canopy peat swamp forest and mixed dipterocarp forest (Singleton and van Schaik, 2001). Both male and female orangutans have overlapping home ranges, but these vary widely depending on the method used to estimate home range size (Singleton and van Schaik, 2001). The home ranges for the orangutan population at Suaq Balimbing is quite large when they are compared to the home range estimates from other sites (see Table 2.4) (Singleton and van Schaik, 2001). The typical home ranges at the site are in the range of 8.5km^2 for adult females and 25 km^2 for adult males (Singleton and van Schaik, 2001). Orangutan density at the site is believed to be between 6 and 6.5/km^2 (Singleton and van Schaik, 2001). These estimates of home range size are much higher than those obtained by researchers at other sites and may be attributed to either the swamp habitats patchiness or the definition of home range (Singleton and van Schaik, 2001). Food abundance does not explain the extreme variation that is seen in orangutan home range size at this

site (Singleton and van Schaik, 2001). The poor swamp habitats and their lowered species richness may force orangutans to use larger areas to obtain food (Singleton and van Schaik, 2001). Two reasons are postulated: 1) the trees in the swamp which represent a limited number of species may fruit during different periods due to regional variations in cloudiness or the availability of suitable water and nutrients; and 2) the fact that trees are clumped in distribution throughout the swamp may force orangutans to range widely because of the time variations that may be seen in the fruiting of the important tree species (Singleton and van Schaik, 2001). These large home ranges incorporate both areas of hill and swamp forest (Singleton and van Schaik, 2001).

The Other Sumatran Sites: West Langkat, Ranun S. and N.

The "other" Sumatran sites used for the study of wild orangutans are very limited in the information that they provide because of the relatively short period of study conducted there and the fact that only one researcher visited these sites between April and November 1971 (Mackinnon, 1974a). The elevation at the sites was as high as 2,000 meters at West Langkat and as low as 300 meters at Ranun (Mackinnon, 1974a). The only information that is important for this section is the estimates of orangutan densities. At West Langkat orangutans have a density of between 1 and 2/km^2, at Ranun South the estimate was 1/km^2 and at Ranun north it was <1/km^2 (Mackinnon, 1974a). It is clear that these sites were not well studied during MacKinnon's (1974a) time. These sites were the scene of "quick and dirty" studies providing comparisons between Sumatran orangutans and the orangutans observed in his longer study in Borneo.

Table 2.4: Ecological Variables from Sumatran Orangutan Research Sites

Site	Altitude	Annual Temp	Humidity	Annual Rainfall	Orangutan Densities /km^2	Home Ranges
Ketembe	250-2,000 m a.s.l.	34.2°-17°C	62-100% Avg. = 86.9%	2,827 mm-3,744 mm	1-7	♂2km^2 ♀1-2km^2
Suaq Balimbing	See Ketembe	See Ketembe	See Ketembe	3,500 mm	6.-6.5	♂0.59-39.12 km^2 ♀0.65-26.92 km^2
Others	300-2,000m a.s.l.	n/a	n/a	n/a	Less than 1-2	n/a

2.2.3 Conclusions on Populations, Densities, Home and Day Ranges

Orangutans live in a fairly diverse type of habitat. This habitat tends to be very wet rainforest where precipitation is in the range of 3,000mm or more per year in a normal non-drought year. Based on the current orangutan populations and densities it appears that orangutans are making the most of the little pristine habitat that still exists, while not thriving in disturbed habitats (Felton *et al.*, 2003; van Schaik *et al.*, 2001). Orangutans are most abundant in lowland forests, probably reflecting that this is the preferred type of "good" habitat left. Of these habitats lowland and good swamp forests support the highest orangutan population densities while orangutan population densities decline as the site's elevation increases. Orangutan densities vary greatly and no one explanation can be used as a reason for this variation. Yet habitat type, fruiting tree densities and availability probably do have some impact on these home range sizes. The average orangutan home range is probably in the region of 5-7 km^2 for adult females living in swamp forests, and 25+km^2 for male orangutans living in swamp forest. These home ranges may decline significantly in prime lowland forest but an estimate of about 2km^2 for adult females and 5km^2 for adult males is probably fairly accurate. Orangutan home ranges overlap for both adult males and adult females and those sites where this has not been found to be the case, occur where only short term and limited

research studies have been conducted. Orangutans probably do not choose to live on mountains or mountain slopes above 1,800 meters above sea level, this may also be related to fruit trees and other environmental factors at these elevations. Orangutan day ranges also seem to vary greatly, but an average of 0.5-1.0 kilometers per day is probably fairly accurate.

There do not appear to be a great number of differences between Sumatran and Bornean orangutans in terms of the discussion provided here. Densities of orangutans are significantly higher in Sumatra. So far no definitive explanation has been provided to explain this difference, though hypotheses based on quality of the habitat in Sumatra as compared to Borneo have been presented in the past. It is unclear as to how one might quantify a study to examine such a question. Is the orangutan habitat of Sumatra that much better than any place where orangutans are currently being, or have been, in the past studied on the island of Borneo? It seems highly unlikely to me, but perhaps a difference in certain important plant species could potentially cause such interesting differences in densities between the islands. Otherwise much of the data summarized here seems to indicate that orangutans on both islands generally share similar day ranging and home ranging patterns.

2.2 Feeding Ecology of the Orangutan

The orangutan is frugivorous. This implies that the orangutan must have some dietary flexibility because fruit lacks some of the required essential amino acids (Chivers, 1998). Orangutans are believed to have three categories of food preference. Esteemed foods attract temporary associations of two or more orangutans, while preferred food sources are those visited by more than three orangutans during a fruiting season. The final food preference type consists of all those foods that do not fit into the

above two groups (Rijksen, 1978). The most common method used to estimate diet is to study the time spent feeding on different food types (Chivers, 1998). The orangutan along with other primates should be selective about their diet in terms of the intake of essential nutrients, the reduced consumption of indigestible plant components and in the intake of plant secondary compounds such as alkaloids and tannins (Hamilton and Galdikas, 1994). This last point is especially important as orangutans lack the enzyme uricase which means that they have more difficulty in dealing with tannins than some other primates (Chivers, 1998). Rijksen (1978) originally divided orangutan food types into five broad categories: 1) fruit; 2) leaf material; 3) bark; 4) insects and 5) miscellaneous. These categories along with the sixth category of flowers and their parts will be used to help organize the discussion of orangutan feeding ecology.

2.2.1 Orangutan Energy Requirements

Before discussing the common diet of an orangutan it is first necessary to review the energetic requirements of the species. To get an accurate estimate of orangutan metabolism one needs to examine a number of variables. These estimates should use the variables of: the time spent feeding on each item, the food type, the weight of each food item, the rate of intake and the composition of each food in terms of sugars, proteins, fats, minerals, vitamins, and secondary compounds such as alkaloids and tannins (Chivers, 1998). The first attempt to estimate orangutan dietary requirements was done by Rodman (1979). He estimated these requirements based on the formula:

$$M_{tot} = 70\ W^{0.75} + 34\ DW^{0.75} + 2.3\ DW$$

Where M= metabolic requirements, W= weight, D= day Journey in km, and 2.3 DW is the cost of vertical changes in the canopy. So for example, Rodman (1979) estimated the average weight of an adult male orangutan at 90kg and average daily

travel distance of 0.4 km to estimate adult male orangutan daily caloric needs. Based on this Rodman (1979) calculated the following:

$$M_{tot} = 70\ (90)^{0.75} + 34\ [(0.4)(90)]^{0.75} + 2.3\ [(0.4)(90)]$$

With this calculation Rodman (1979) estimated that an adult male orangutan has a daily caloric requirement of 2,530 Kcal. He repeated this equation using a 50 kg estimate for the average mass of an adult female orangutan and 30 kg for the average mass of a juvenile female orangutan. These resulted in daily caloric estimates of 1620 Kcal for an adult female and 1100 Kcal for a juvenile female (Rodman, 1979). These values do not include any correction for thermoregulation, growth, pregnancy, lactation (Rodman, 1979) and disease.

A seemingly more accurate measurement of orangutan metabolic needs is provided by Knott (1997). This method used a human model based on weight and activity level and came up with figures of 3,344 Kcal for adult males and 1,512 Kcal for females (Knott, 1997). As with Rodman's metabolic estimates these figures do not take into consideration the energetic requirements of pregnancy, lactation and disease (Knott, 1997). The estimation by Knott is probably a little low as ketones, otherwise known as the products of metabolized fat stores, were recovered from animals eating diets estimated at 3,824 Kcal per day for adult males and 1,793 Kcal per day for adult females (Knott, 1997). One conclusion that could be made from this study is that males ate more than females by a significant margin and were undernourished (Knott, 1997) (See section 2.2.4 below).

2.2.2 Fruit

As stated above the highest proportion of the orangutan diet in all studies is made up of fruit. Orangutans normally will eat several fruits at once, processing them

with their lips and teeth (Rodman, 1988). Generally, this is done very sloppily, resulting in a great deal of waste (MacKinnon, 1974a). Orangutans also tend to be very selective in the type of fruits that they prefer eating those fruits with ripe mature mesocarps (i.e. fleshy fruits) (Rodman, 1988). While frugivory is a commonality for all orangutans, slight differences among various orangutan research sites have been observed in terms of the time spent eating fruit and the species of fruits eaten. At Ketambe a total of 92 different fruit types are eaten making up a total of 58% of the total feeding time and thus constituting the staple dietary component for orangutans at Ketembe (Rijksen, 1978). At Tanjung Puting orangutans eat a great deal more fruit species at 169 with, fruit accounting for 50% of their diet (Galdikas, 1988). In 1977 (Rodman, 1977) the orangutans at Kutai were reported to spend 53.8% of their feeding time eating fruit, but in 1979 (Rodman, 1979) this figure increased slightly to 61.2% of their diet. The orangutans at Kutai rely on two very important fruiting tree species; *Draconium mangiferum* and *Koordersiodendron pinnatum* (Rodman, 1977). The total percentage of fruit in the diet at Ulu Segama based on data from MacKinnon (1974a) was 62% (Rodman, 1988). A similar estimation was done by Rodman (1988) based on data from MacKinnon (1974a) for Ranun and an estimate of 84.7% of the orangutan diet at this site was fruit. The fruit component of the orangutan diets at these sites seems to be high and may reflect the shorter length of time spent by researchers at these study sites.

Different orangutan research sites do have different floral compositions and thus these sites differ in the type of fruits eaten by each respective site's orangutans. This might be expected because these research sites are on two separate islands. Apparently, there is also a great deal of variation on the islands as only a total of 32 plant species used by orangutans for food (not just fruit species) were eaten at both sites (Rodman, 1988). This raises a huge question as to why the diet is so different between

the research sites. As yet no study has attempted to deal with this question. Figs are a very important aspect of the orangutan diet at Ketambe representing a total of 54% of the fruit eaten (Rijksen, 1978). There are five types of fig plants at Ketambe: free standing trees; terrestrial shrubs; epiphytes; climbers and stranglers (Rijksen, 1978). Tanjung Puting differs in that it has a complete absence of large figs for the orangutans to feed on (Galdikas, 1988). Orangutans only spend 0.4% of their feeding time eating figs at that site (Galdikas, 1988). Lanjak Entimau is similar in that strangling figs are rare and thus not a major part of the orangutan diet (Blouch, 1997). Figs are also an important part of the orangutan diet at Ulu Segama but no exact figures are provided (MacKinnon, 1974a). At Kutai figs tend to be an important food source when other fruit sources were not in season (Rodman, 1979). *Ficus sp.* may be especially important to the orangutan in part because they may fruit as often as once every four to five months (Rodman, 1978). Orangutans may even form temporary groups when large fig trees are fruiting and the size of these aggregations appear to be correlated to the quality of the fruit patch (Utami *et al.*, 1997).

2.2.4 Seeds

Another important aspect of the orangutan fruit diet is what happens to the seeds. Are they eaten and digested or do they pass through the system unharmed or perhaps even enhanced as was suggested for the seeds of *Koordersiodendron pinnatum* (Rodman, 1977). The answer to both questions is yes. As part of their fruit diet orangutans do consume fruit seeds. Some of these seeds such as those of the *Neesia sp.* fruit are very high in fat (approximately 46%) (Knott, 1997). Orangutans are also seen as the most important non-flying seed dispersers at the site of Tanjung Putting because of their arboreality, large size, long ranging distances and their ability to move between habitat types taking seeds from the fruits that they eat with them (Galdikas,

1982). In 29% of seed consumption the seeds were chewed and presumably consumed (Galdikas, 1982). Three types of orangutan seed dispersion have been identified by Galdikas (1982). Orangutans may eat fruits with intact seeds, swallow them, and then pass them unharmed in their faeces. They may carry fruit flesh and fibers for long distances before spitting out a wad of seeds. They may carry a completely intact fruit in their mouth a considerable distance and their sloppy eating habits may result in the droppage and wastage of fruits that are eaten by other animals on the ground who in turn deposit those seeds in their feces (Galdikas, 1982). Seeds at Kutai were commonly observed to be swallowed and dispersed in the faeces (Rodman, 1977). A total of 14% of orangutan faeces at Ketambe had seeds in them (Rijksen, 1978). The three most common seeds seen in the Ketambe orangutan faeces were *Aglaia speciosa*, *Mallotus sphaerocarpus* and *Xerospermum sp.* (Rijksen, 1978). Galdikas (1982) reported a total of 94% of faecal samples containing at least one seed and 78% had the intact seeds of two or more plant species, supporting the hypothesis that orangutans are important seed dispersers.

2.2.5 Mass Fruiting and Flowering

The tropical forests of South East Asia are unique in that the plants living here tend to have phenological (i.e. flowering and fruiting) cycles that are much more seasonal than in the tropical zones of Africa and South America (Appanah, 1993). These variable phenological cycles are not seen in other parts of the world, especially not to the same intensity (Appanah, 1993). Two types of flowering trees can be identified in South East Asia; those that flower mainly during mass flowering events and those that flower outside mass flowering events (Appanah, 1993). The trees that flower outside the mass flowering events generally flower annually but may skip a year or two, while those that flower at irregular intervals may flower every two or three years

(Appanah, 1993). Mass flowering events occur at varying intervals of between two and ten years (Appanah, 1993). These mass events have been postulated to relate to irradiation (i.e. more hours of direct sunlight per day), drier than normal weather conditions and sharp drops in the night-time temperature (Appanah, 1993; Wich and van Schaik, 2000). These mass flowerings generally lead to mass fruitings, but is not always the case as observed at Ketambe in 1997 (Wich and van Schaik, 2000). Mass fruiting episodes have been suggested as a strong selection force for orangutans, determining when they might maximize their caloric intake to sustain them during lean times (Knott, 1997). Mass fruitings in eastern and western Borneo and peninsular Malaysia are more likely to occur following an El Nino-Southern Oscillation event but this is not true of Sumatran mass fruiting events (Wich and van Schaik, 2000). Foods that are available during mass fruiting events tend to be higher in caloric intake than those foods available during non-mass events (Knott, 1997). Adult orangutan males have been estimated to increase their caloric intake during a mass fruiting event by almost 5,000 Kcal per day, and adult females may increase their caloric intake to almost 6,000 Kcal per day (Knott, 1997). This may translate into a weight gain of 0.66 kg per day per orangutan (Knott, 1997). Even if a mass event only lasted one month an orangutan could conceivably gain 19.8 kilograms.

2.2.6 Leaves

Leaf material is generally the second most common type of food consumed by orangutans. At Kutai, leaves account for 29% of the time spent feeding (Rodman, 1977). At Ketambe this figure was slightly lower taking up to 25% of the total feeding time and including the consumption of leaf galls, epiphytic fungi, aerial roots and 22 other vegetative materials (Rijksen, 1978). Tanjung Puting had a much lower rate of leaf consumption with leaves representing merely 15% of the total feeding time

(Galdikas, 1988). 43% of the leaf-eating bouts by orangutans at Tanjung Puting consisted of *Gironniera nervosa* and *Xanthophylum rufum* or *G. nervosa* and *Melanorrhea wallichii* (Galdikas, 1988). Young leaves are the preferred choice among orangutans (Galdikas, 1988). *Xanthophyllum sp.* account of as much as 50% of the foraging time spent eating young leaves (Hamilton and Galdikas, 1994). This may be correlated to the fact that the young leaves of this plant have the most available crude protein and the general young leaf qualities of high water content and high crude protein to fiber ratios (Hamilton and Galdikas, 1994).

2.2.7 Bark

Bark consumption in orangutans may be the most interesting part of their feeding ecology because of a common trend seen at many sites. This trend is the observation that orangutans will often consume bark by chewing it into a wadge, sucking on it for a while and then spitting out (Description from Galdikas, 1988). This behaviour has been reported at many of major orangutan research centers including Tanjung Puting (Galdikas, 1988), Ketambe (Rijksen, 1978), Ulu Segama (MacKinnon, 1974a) and Kutai (Rodman, 1977). This behaviour could possibly be like bitter pith chewing reported in chimpanzees and has been hypothesized to be used by chimpanzees as a control measure against parasitic nematodes (Huffman, 1997). Secondary compounds possibly available in the bark and cambium wadges eaten by orangutans may have an anti-parasitic or a similar function. If this were true, bark as a proportion of the orangutan diet would certainly decrease because it is not being consumed for nutritional value. However, bark as a portion of the orangutan diet has been reported to be the primary source of dietary sustenance at certain times of the year (Knott, 1997). At Ketambe, bark was only observed to make up 3% of the total time spent feeding and this included chewing on wadges (Rijksen, 1978). Bark as a part of the orangutan diet is much more

common at Kutai and Tanjung Puting. At Kutai bark accounts for 14.2% of orangutan feeding time (Rodman, 1977) and at Tanjung Puting a total of 55 species of bark were consumed accounting for 11% of the total foraging time (Galdikas, 1988). Rodman (1979) identified a total of three tree species from which orangutans seemed to chew the tree's cambium then spit it out. He identified these trees as *Uncario sp.*, *Cratoxylon sp.* and *Shorea sp.* (Rodman, 1979).

2.2.8 Flowers

The part of the orangutan diet that includes flowers is often clumped in with "other" foods because orangutans do not eat much in the way of flowers. Flowers though may be at times an important aspect of orangutan diet that is often glossed over. For example flowers such as those from *Dillenia* sp. and *Xanthophyllum* sp. with available crude protein values of 16.6% and 18.6% respectively had the highest available crude protein values of any of the orangutan food types tested by Hamilton and Galdikas (1994), Because they often make up a very small proportion of the diet, however, they have in the past been undervalued in their importance to orangutans. At Kutai, flowers only account for 2.2% of orangutan feeding time (Rodman, 1978), and at Tanjung Puting flowers account for only 4% of the time spent feeding (Galdikas, 1988). When a food type makes up such a small proportion of the overall diet such as is seen in the case of flowers it is easy to understand how their importance to the overall diet may be overlooked.

2.2.9 Insects

Insects are an interesting aspect of the orangutan diet as there is great variation in the amount and types of insects eaten at some of the major research sites. At Kutai orangutans were seen to eat ants and termites, spending a total of 0.8% of their total

feeding time in this pursuit (Rodman, 1977). At Ulu Segama honey bees, Meliponid bees, termites, ants and wasp galls were all observed to be eaten (MacKinnon, 1974a). The Ketambe orangutans were estimated to spend 14.4% of their total feeding time searching for and eating insects with a total of 17 insect species being included in their diet (Rijksen, 1978). Ants of the genera *Camponotus* and *Polyrhachis* were identified along with two species of termites; *Nasutitermes matagensis* and *Captotermes curvingnatus* as being part of the orangutan diet here, along with lesser amounts of caterpillars, crickets, moths and other insect eggs (Rijksen, 1978). At Tanjung Puting termites are eaten on the ground, though this behaviour is generally found only among males (Galdikas, 1988). These termite feeding bouts account for about 4% of the total orangutan feeding time at Tanjung Puting (Galdikas, 1988). Wasps are also eaten by orangutans unintentionally. The remains of fig wasps (*Blastophaga*) are eaten incidentally along with the rest of the fig by orangutans (Rijksen, 1978).

2.2.10 "Other" Foods

The final category of orangutan food types has been termed "other" and includes all those foods that are not encompassed in the above four categories. Other foods include a potentially long list of items such as vertebrate meat (Utami and van Hooff, 1997), bird eggs (MacKinnon, 1974a and Rijksen, 1978), cob webs (Rodman, 1978), and soil (MacKinnon, 1974a). Meat eating has only been observed in Sumatra and is probably an idiosyncratic and opportunistic occurrence rather than representative of true "hunting" behaviour (Utami and van Hooff, 1997). In all the reported incidents of orangutan meat-eating, the choice of prey was another primate; the slow loris (*Nycticebus coucang*). It would seem that all of these "other" food types could probably be classified as rare and opportunistic food types. They are probably not overly important to overall nutrition of the orangutan. Soil eating, also referred to as geophagy,

could be an exception to this rule, however. The soils eaten by orangutans in Ulu Segama and in Sumatra as observed by MacKinnon (1974a) were soils that were very rich in minerals, especially alkaline metals such as sodium, potassium and calcium. These metals could be important to the orangutan in more than one way. They may provide the orangutan with some essential minerals that are lacking or rare in their diet. Geophagy is seen in many primates such as rhesus macaques (*Macaca mulatta*) and mountain gorillas (*Gorilla gorilla beringei*) (Huffman, 1997). This dietary behaviour may help ease symptoms of stomach upset. This would be especially true if the soils that orangutans eat were mineral rich clay soils (Huffman, 1997).

2.2.11 Tool Use to Get at Prized Food Items

Orangutans have occasionally been observed to make and use tools as an aid in acquiring or processing foods. It has been observed that Sumatran orangutans will use sticks for help in extracting insects or honey from tree holes and for prying the seeds out of hard husked fruits (Fox *et al.*, 1999). Orangutans also use leaves in feeding on *Erythrina* trees (Fox and bin'Muhammad, 2002). Tool use in orangutans has only been reported in Sumatra even though the same foods which the Sumatran orangutans use tools to process or acquire may be eaten in Borneo (van Schaik and Knott, 2001). It is interesting that this behaviour is only seen on the island of Sumatra and it is most likely a local, learned tradition.

2.2.12 Drinking

Young leaves are high in water content and may determine the amount of water an orangutan takes in, but in addition, orangutans have been observed to drink about once a day from the water that accumulated in natural tree bowls (Rijksen, 1978). This

may increase when feeding on *Neesia sp.* fruit as was observed at Suaq Balimbing (van Schaik and Knott, 2001).

2.3 Orangutan Life History

2.3.1 General Life History

The life stages of the orangutan are probably best represented by the divisions established by Rijksen (1978). He believes that there are four major life stages for females and five for males. There are probably five life stages for both sexes but detecting the difference between subadult and fully adult female is more difficult if the age of the animal is not known. A female subadult could be classified as any female between 8-13 years which would roughly end at the time when wild females are most likely to have their first offspring. The currently accepted life stages include infants (0-2½ years of age), juveniles (2½-5 years), adolescents (5-8 years), adult females (8+ years), subadult males (8 to between 13 and 15 years), and adult males (between 13 and 15 years +). The first permanent teeth of the orangutan appear between the ages of 5 and 7 years with the canines and the third molars taking much longer to develop fully (Beynon *et al.*, 1991). Orangutan canines develop differently between the sexes though. Female canine crowns are completed around the age of 5.47+/- 0.73 years, while male canine crowns are completed around the age of 8.73+/-0.86 years (Schwartz and Dean, 2001). Orangutans are considered to be adults only after their adult dentition has fully erupted (Rijksen, 1978). This will occur only after the age of 9+ years when the third molar erupts (Beynon *et al.*, 1991)

The life history of the orangutan was studied in detail and summarized by Leighton *et al.* (1995) as part of the Habitat Viability Analysis Workshop (HVAW) for orangutans. Much of the information was compiled from the study sites of Ketembe (see

Rijksen, 1978) and Tanjung Puting (see Galdikas, e.g. 1978). More recently, the accumulated data from two Sumatran sites [Ketembe and Suaq Balimbing (See Section 2.1.2)] collected over the length of each study (32 and 5½ years respectively) were presented by Wich *et al.* (2004). Based on the accumulated data the HVA workshop established ten aspects of orangutan life history that help define how orangutans live, important life milestones, and population characteristics. As a result of the new data provided by Wich *et al.* (2004) we can get a more detailed estimate of life history for Sumatran orangutans (*Pongo abelii*). Orangutans birth occur at an approximate 1:1 sex ratio, and that during the first year infant mortality rates for orangutans aged 0-1 was under 10% (6.9% at Ketembe) (Leighton *et al.*, 1995; Wich *et al.*, 2004). This is considerably higher than the infant mortality rates found at Wamba for the gracile chimpanzee (or Bonobo) which averaged only 4% (Coxe *et al.*, 2000). Orangutan infant mortality may rise to 30% during periods of extreme food shortage which tend to happen one to ten times per century. Between 56 and 67% of all infants survive until at least 11 years of age (Wich *et al.*, 2004). Females first reproduce around the age of 15 with a range of 13-17 years, while males don't reproduce until around the age of twenty (Leighton *et al.*, 1995; Wich *et al.*, 2004). Female orangutans have one of the longest inter-birth intervals (IBI) of any mammal at between eight years for Bornean orangutans (Galdikas and Teleki, 1981) and 9.3 years for Sumatran orangutans (Wich *et al.*, 2004). It is estimated that each year 87.5% of all adult female orangutans will produce no offspring (Leighton *et al.*, 1995). This being said, females may continue to give birth to healthy offspring between until the ages of 40 and 50 (Wich *et al.*, 2004). The maximum age for both male and female orangutans was estimated at 45 years in the wild by Leighton *et al.*, (1995), but the long-term data from Ketembe indicates that orangutans (at least the Sumatran species) may live well into their 50s and perhaps to almost 60 years (Wich *et al.*, 2004).

2.3.2 Two Adult Male Morphs

The male orangutan may go through two stages of adulthood. The first is a fully mature male cheekpadder which is the adult male morph that the general public is most likely to recognize. These males display very well-developed secondary sexual characteristics such as well-developed throat pouches, wide cheek pads, and long dense hair (Delgado and van Schaik, 2000). The second type of adult male orangutan is the unflanged adult male. These are the adult males who have not yet developed secondary sexual characteristics. They are also much smaller having not yet attained the size of the cheekpadder males, thus weighing about the same as an adult female (Delgado and van Schaik, 2000).

Why do these two male morphs exist? It has been speculated that the developmentally arrested male may avoid the metabolic and stress related costs of the secondary sexual characteristics of the cheekpadder males (Maggioncalda *et al.*, 2002). Somewhat different is the hypothesis set forward by Utami *et al.*, (1996a) who take the metabolic and stress related costs of the secondary sexual characteristics into consideration while relating the occurrence of the two male morphs to different reproductive strategies. At Ketembe, Utami *et al.*, (1996a) found that six out of ten offspring tested were fathered by unflanged males while only 4 were fathered by flanged males. They felt that this evidence supports the "frequency-dependent two-strategies" hypothesis (Utami *et al.*, 1996b) where flanged males trade the high cost contest competition between flanged males for access to females with the attractive nature of their secondary sexual characteristics to females. While unflanged males do not have the high cost contest competition for females they must expend more energy to find and convince females to mate with them.

2.3.3 Diseases and Health of the Wild Orangutan

Orangutans suffer from a number of diseases including protozoans such as malaria, intestinal parasites and ectoparasites, viruses and bacteria. While there is some limited knowledge concerning the diseases affecting orangutans, very little of this knowledge has been obtained through the study of wild orangutans. One reason for this is that wild orangutans will not allow a human to poke and prod them meaning that animals must be anesthetized before examination. There is an inherent risk in the anesthetization of any animal and orangutans are no exception to this rule. A recent study exploring which drugs were the "best" drugs for orangutan anaesthesia experienced two fatalities, one was an infant shot with a dart meant for its mother and the other was an animal that fell asleep in direct sunshine and died from overheating (Hiang *et al.*, 1995). Thus, people have been very apprehensive and selective about capturing wild orangutans for medical diagnosis unless the situation is critical or the animal needs to be captured for some other reason such as translocation.

Intestinal Parasites and Ectoparasites

The intestinal parasitic fauna of the orangutan represents one of the more studied medical/disease aspects of wild orangutans. The most complete published study was by Collet *et al.* (1985), but intestinal parasitic data were also reported by MacKinnon (1974a) and by Rijksen (1978). Recently, intestinal parasitic data has been accumulated by Skinner (unpublished raw data). In a study of 89 orangutan faecal samples, Collet et al. (1985) identified four types of gastro-intestinal parasite (GIP). This is low in comparison with Rijksen (1978) who identified eight species of GIP. MacKinnon (1974a) does not report how many types of GIP were found in the orangutans he studied but he reports that only seven of the twenty-four orangutan faecal samples collected contained any kind of GIP. Orangutans seem to commonly suffer from infections of

Strongyloides sp. (Collet *et al.* 1985, Rijksen 1978 and Uemura *et al.* 1979). These infections may affect a large number of animals at a particular site such as the 78.7% of animals that tested positive for this parasite at Tanjung Puting (Collet *et al.*, 1985). This parasite is supposed to be fairly benign (Uemura et al., 1979) but has been also known to cause deaths in young orangutans (Uemura *et al.*, 1979 and Dr. Kay Mehren-Toronto Zoo, Chief of Veterinary Medicine-Pers. Comm.). *Trichuris trichuria* is probably the second most common intestinal parasite to infect wild orangutans. At Tanjung Puting 15.7% of the orangutans tested were infected with this parasite (Collet et al., 1985),while at Ketambe 22% of orangutans tested were infected with this parasite (Rijksen, 1978). Orangutans are also infected from a number of other parasites such as: *Enterobius buckleyi* and *Abbreviata caucasica*, *Pithocostrongylus sp.*, *Trichostrongylus sp.* and *Gastrocoides sp.* at Ketambe (Rijksen, 1978), *Oesophagostomum sp.* at Ketambe and Gunung Palung (Rijksen, 1978 and M. Skinner unpublished data), and various other strongylids, oxyurids, (Collet *et al,* 1985) trichostrongylids (Rijksen 1978 and Skinner Data), *Necator americanus*, *Enterobius vermicularis*, *Giardia lamblia* and *Cyclospora* (M. Skinner unpublished data). With regards to these parasites, there is a lack of epidemiological data in terms of how these parasites influence the lives of the primates that they infect. Unfortunately, while there has been a fair amount of work done on the parasites of these animals nobody as of yet has attempted to correlate these parasite infections to behaviour changes such as increased lethargy, decreased appetite (measured by studying feeding time) and possible zoopharmacognosy (animals using plant medicines to cure themselves or relieve symptoms) in orangutans such as the bark and pith chewing discussed above. Other GIP's have been identified in orangutans that have had contact with humans such as *Platynosomum fastosum* (Warren *et al.*, 1998) and *Balamuthia mandrillaris* (Canfield *et al.*, 1997).

Orangutans may also suffer from ectoparasites. Although, MacKinnon (1974a) found that all the orangutans that he tested were ectoparasite free Rijksen (1978) reports that ex-captive orangutans under his care were found to suffer from red chiggers (*Eutrombicala wickmanni*) and ticks (*Haemaphysalis cornigera*).

Viruses

Orangutans carry a great number of viral diseases such as a Simian T-Lymphotrophic Viruses (STLV) (Ibuki *et al.*, 1997 and Verschoor *et al.*, 1998), Hepadnaviruses (Warren *et al.*, 1999 and Verschoor et al., 2001), simian retroviruses (probably a cross infection from a macaque) (Warren *et al.*, 1998), and herpes virus (Warren *et al.*, 1998). While it is great that scientists have been able to determine the existence of these viruses in orangutans there is really no literature base on which diseases if any are important in the everyday lives of wild orangutans. Questions regarding how these viruses contribute to wild orangutan morbidity and mortality have not yet been answered. This is a common occurrence for primates in general.

Falls

While not a disease, another important factor in orangutan health is that of falling from trees. Orangutans tend to spend most of their time in the trees and it can be expected they would fall from these trees on occasion. Adult male orangutans tend to use the lower parts of a tree probably in part because of their size, while females and younger orangutans use the higher parts of a tree (Sugardjito, 1988). It has been estimated that adult male Sumatran orangutans will fall on average two times per day (Delgado and van Schaik, 2000). This is reflected in the high number of healed fractures that have been found in orangutan long bones (Lovell, 1990). The bones that can be studied have healed fractures because these animals were collected live and so we do not see the remains of those wild orangutans that fall to their deaths. Nonetheless, this

phenomenon may be more prevalent than we would like to believe. At Ketambe one adult female was observed to fall, become a paraplegic and eventually die, while another young ex-captive orangutan fell and died from a broken neck (Rijksen, 1978). While we would like to believe that orangutans are quick, agile and beautifully coordinated forest travellers, it is clear that on occasion they fall whether it is from a branch breaking or misjudgement of the distance between trees. Furthermore, these falls can be very serious and even deadly.

2.3.4 Other Aspects of Orangutan Ecology

There are two major aspects of orangutan ecology that have yet to be discussed. The first is sympatric primates and other mammals, while the second is predation. These two aspects of orangutan ecology are sometimes under-studied by researchers not only in orangutan studies but in the studies of many primates.

Sympatric Primates and Other Animals

There are 14 species of primates on Borneo that may or may not always be sympatric with orangutans. These include prosimians such as the slow loris (*Nyceticebus coucang*) and the western tarsier (*Tarsius bancanus*); monkeys such as the maroon langur (*Presbytis rubicunda*), Hose's langur (*Presbytis hosei*-3 subspecies), the white-fronted langur (*Presbytis frontata*), the silvered langur (*Presbytis cristata*), the banded langur (*Presbytis melalophos*), the proboscis monkey (*Nasalus larvatus*), the crab eating macaque (*Macaca fascicularis*) and the pig-tailed macaque (*Macaca nemestrina*); and lesser apes such as the agile gibbon (*Hylobates agilis*) and the Bornean gibbon (*Hylobates muelleri*) (Payne and Francis, 1998). Rodman (1973) discusses the presence of eight other primate species at Kutai, while at Ketambe both species of macaques, the Thomas' langur (*Presbytis thomasi*), the white-handed gibbon

(*Hylobates lar*) and the siamang (*Symphangus* (*Hylobates*) *syndactylus*) have been reported (Rijksen, 1978). It is well known that gibbons and siamangs are also frugivorous primates. These are the primates that are most likely to compete with orangutans for fruit foods, although macaques may occasionally also feed on fruits (Rijksen, 1978). Other animals such as hornbills, fruit bats, Malayan sun bears (*Helarctos malayanus*) and the binterong (*Arctitis binturong*) may also compete with orangutans for fruit, especially for figs (Rijksen, 1978). The leaf monkeys (genus *Presbytis*) and the proboscis monkey (*Nasalis larvatus*) are mainly leaf eaters and probably don't usually compete directly with orangutans for food, but as we have seen leaves, though not eating in great quantity may be an important component of the orangutan diet.

Predation

Since the observation indicating that Sumatran orangutans are less terrestrial than Bornean orangutans was first made it has been speculated that Sumatran orangutans may avoid ground travel because of the existence of the Sumatran tiger (*Panthera tigris sumatrae*). Even adult male orangutans have been known to be killed and eaten by Sumatran tigers (Borner, 1978). Sumatran tigers have a large home range of about 100km^2 (Borner, 1978). The tiger's home range would encompass the home ranges of many orangutans at a density like those seen at Ketambe of 5/km^2. Other animals have also been known to eat orangutans such as the Bornean bearded pig (*Sus barbatus*) (Galdikas, 1978b). Whether this was a case of opportunistic predation on a past prime male is unknown, but it has been speculated that wild pigs probably do play an important role in at least the scavenging of orangutan remains (Galdikas, 1978b). There has been speculation and rare observations of other animals preying on orangutans. MacKinnon (1974a) has speculated that the eagles

such as *Ictinaetus malayensis*, the reticulated python (*Python reticulatus*), the panther (*Panthera pardus*)[1] (only in Sumatra), the golden cat (*Felis temminchi*) (only in Sumatra), the dhole (*Cuon javanica*-a type of wild hunting dog found only in Sumatra) and the clouded leopard (*Neofelis nebulosa*) may also be capable of taking orangutans. All of these animals may not be capable of taking on adult orangutans, but they may play a role in the predation mortality of infants and juveniles. Rijksen (1978) witnessed a number of juvenile ex-captive orangutans being hunted and eaten by a clouded leopard and speculated that these may be orangutan predators until the point where juvenile orangutans reach about ten kilograms. Some if not all of these animals would probably take an appropriately sized orangutan if the opportunity presented itself.

2.4 Comparison of Sumatran and Bornean Orangutans

There are a large number of differences between Sumatran orangutans (*P. abelii*) and Bornean orangutans (*P. pygmaeus*) (see Table 2.5). There are enough differences both physical and genetic that these orangutans have now been elevated to separate species rather than their previously accepted taxonomic division at the sub specific level (See Groves, 2001 and Singleton *et al.*, 2004). There are two excellent sources that examine the variations between Bornean and Sumatran orangutans (Delgado and van Schaik, 2000 and Courtenay *et al.*, 1988). Most of the information provided here comes from one or both of these sources unless otherwise specified.

Adult male orangutans from the two islands differ in face shape, cheek pad shape, beard length and beard appearance, long call tone and intensity, and throat sacs. There are also some behaviours that only the males of one species participate in such

[1] There is some confusion as to the existence of the panther in Sumatra. Variouspeople mention it such as Rijksen (1978) and McKinnon (1974a) yet its existence in Sumatra has never been confirmed but it is highly speculated that it does exist here.

as the Sumatran male orangutan's courtship display or the long-term consort pairs that they form with females. Orangutan females also show some differences such as the appearance of a beard on Sumatran females. Overall, there are also a number of differences that are characteristic of both sexes of orangutan, such as terrestrial behaviour. Sumatran orangutans are much less terrestrial than Bornean orangutans which may reflect the presence of tigers. Bornean orangutans also seem to spend more time eating the inner cambium of tree bark than Sumatran orangutans, though Sumatran orangutans seem to eat more insects than the Bornean subspecies. Sumatran and Bornean orangutans also have a great deal of differences in their fur, such as colour (Bornean is darker), texture (Sumatran is fleecier) and cross section. Some of these differences such as the fleecier texture of Sumatran orangutans may be related to the higher altitude range at which they may be found. Sumatran orangutans also tend to be more social than Bornean orangutans. This may be reflected in the densities at which orangutans live on the different islands. There are also a number of differences in the genetics of Sumatran and Bornean orangutans. The most prevalent of these is the difference in the second and fourteenth chromosomes. Other genetic differences are discussed in the previous chapter. A difference in orangutan foot structure was noticed by MacKinnon (1974a) who saw the Sumatran orangutan as having a flatter foot, yet this observation does not appear elsewhere. While it is apparent that there are a fair number of differences between Sumatran and Bornean orangutans, the fact remains that in captivity they have produced viable hybrids that have produced viable offspring themselves.

It is worth noting that there is speculation as to another potential orangutan or great ape species living in Northern Sumatra. Local traditions refer to this animal as *orang pandek* and it is believed to inhabit the Angkola region of Sumatra (van Schaik et al., 2001). Whether this is truly a new species of orangutan is debatable as this may

simply be a local legend or myth.

2.5 Conclusion

Clearly, orangutans have a very diverse ecology. This brief overview is based on thousands of pages of literature which researchers have produced as part of a devoted study of orangutans. There are very few generalizations that can be made about orangutan ecology because of the variation not only between islands but also between research sites on the islands. Orangutans are frugivorous, but as described here they eat a more diverse diet than one might expect and even have different ways of procuring these food resources. Surprisingly, there is also a great deal of information that remains to be discovered such as on the diseases of wild orangutans. One would think that of the thousands of pages dedicated to the explanation of the ecology of wild orangutans that it would be possible to locate more disease epidemiology. Much of this lack of knowledge can probably be partially blamed on the orangutan's arboreal and unsocial lifestyle. It is more difficult to study arboreal animals than it is to study terrestrial animals. The other broad sweeping generalization that can be made about the orangutan is their threat of extinction. Orangutan numbers have dropped badly over the past Century and if we do not take serious conservation action now we may never be able to fill those knowledge gaps that remain in the study of orangutan ecology.

Table 2.5: The Major Differences Between Bornean and Sumatran Orangutans

	Pongo pygmaeus	*Pongo abelii*
Adult Male Face Shape	Concave in Profile-Clearly Expressed Suborbital Fossa	Straight in Profile-No Suborbital Fossa
Cheek Pads	Curve Forward	Flat Against The Face
Adult Male Beard	Short and Scruffy	Long, Lush, May Have Moustache
Female Beards		Long Beard
Sociality	More Aggressive-Males	More Social
Foot Structure		More Plantigrade (Flat Feet)
Genetics: Chromosome 2	Homozygous Type 1	Homozygous Type 2
Genetics: Chromosome 14		Has Rare Variant
Genetics: Other	Various Differences-See Chapter 2-Genetics of Modern Orangutans	Various Differences-See Chapter 2- Genetics of Modern Orangutans
Terrestrial Behaviour	More Terrestrial	Less Terrestrial
Fur Colour	Red, Deep Maroon or Blackish Brown	Rusty Red or Light Cinnamon
Fur Texture	Coarse	Fleecier
Fur Cross Section	Flatter, Thick Black Pigment Column	Thin, Round, Dark Central Pigment Column
Throat Sac-Adult Male	Large and Pendulous	Less Large and Less Pendulous
Maximum Altitude	Under 2,000 Meters	Have Been Found Over 2,000 Meters
Feeding Differences	Eat More Cambium	More Insect Feeding Time
Densities	Lower 1-3/km^2	Higher 5-7/km^2
Mating Behaviour	Short Consortships	Longer Consortships-Several Days to Several Months
Courtship		Males Have Courtship Display
Long Call	Long and Drawn Out	Short, Fast Tempo
Tool Use	No Tool Use	Use Tools to Procure Food

Chapter 3
PLASMODIUM SP. INFECTIVE TO PRIMATES

3.1 Introduction

Malaria is a disease that has been known to humanity for ages. Many of the earliest civilizations documented its presence (Schmidt and Roberts 2000). Malaria is a disease caused by a protozoan parasite of vertebrate blood belonging to the Phylum Apicomplexa, Order Haemosporidia and the Genus *Plasmodium* (Cox, 1993; Escalante and Ayala, 1994). Malaria is often referred to as being a cosmopolitan disease of the tropical and sub-tropical zones (Marquardt et al., 2000) also having species infective to rodents, birds and lizards (Escalante and Ayala, 1994). In 1955 the Eighth World Health Organization Assembly began laying the groundwork to completely eradicate malaria and its causative agent through the Malaria Eradication Program (Coatney, 1971). At that time malaria had an estimated prevalence of 350 million cases worldwide (Schmidt and Roberts, 2000). Today, we now know that this attempt to eradicate Plasmodium has been a complete failure. Current estimates put the current worldwide malaria prevalence at 439 million people with almost 1.5 billion people living in malaria endemic zones (Marquardt et al., 2000). Today, malaria remains one of the major causes of death and illness in Developing Countries (Waters et al., 1993). In these regions malaria may cause as many as three million deaths annually (Escalante and Ayala, 1994; Marquardt et al., 2000).

Plasmodium sp. requires two types of host to complete its lifecycle: an invertebrate vector and a vertebrate host. Some mammal plasmodia are transmitted by

female *Anopheles* mosquitoes (Cox, 1993; Marquardt *et al.*, 2000; Schmidt and Roberts, 2000; WHO, 1987). Female *Anopheles* mosquitoes do not obtain the required nutrients from taking this blood meal; rather the blood meal is used in the formation of eggs (Lowenberger Pers. Comm., 2003). Once infected female mosquitoes remain infective for life and can potentially infect any susceptible organism on which it feeds (Schmidt and Roberts, 2000). For the plasmodia, mosquitoes are considered to be the definitive hosts because sexual reproduction of *Plasmodium* sp. occurs in the mosquito while vertebrates are considered intermediate hosts because in them asexual reproduction occurs (Marquardt *et al.*, 2000). The development and lifecycle of the *Plasmodium* parasite is one that is typical to the Apicomplexan group of organisms (Marquardt *et al.*, 2000).

This section will begin with a brief discussion of the *Plasmodium* lifecycle. I will then discuss the non-human primate (NHP) malarias. Finally, this chapter will conclude with a brief discussion of the evolutionary relationships of the malarias with a special focus on the human and non-human primate malarias.

3.2 Life Cycle of the *Plasmodium* Parasite

The lifecycle of *Plasmodium* has been well documented because of its relevance to human morbidity and mortality. There are an almost unlimited number of sources discussing this Protozoa's lifecycle. The sources of this *Plasmodium* lifecycle information contained in this section are from: Coatney *et al.*, (1971); Cox (1993); Magraith (1984); Marquardt *et al.*, (2000); Schmidt and Roberts (2000); and the WHO (1987) unless otherwise referenced. Members of the genus *Plasmodium* have a complicated lifecycle. Their lifecycle can be divided into three major stages: The

invertebrate stage which occurs in the mosquito; the **exoerythrocytic stage** and the **erythrocytic stage**, both of which occur in the vertebrate host.

3.2.1 The Invertebrate Stage

The lifecycle of the *Plasmodium* Protozoan begins when a female mosquito lands on an infected vertebrate host and begins to ingest blood containing gametocytes. Once the ingested gametocytes reach the stomach of the mosquito, they form male and female gametes. It is at this stage that the *Plasmodium* organism's lifecycle involves sexual reproduction as the male and female gametes fuse to form **ookinetes**.

The ookinetes eventually pierce the interior gut wall of the mosquito and encyst on the gut's epithelial cells. This is referred to as the oocyst stage. During the oocyst stage the ookinetes undergo meiotic division to form the infective stage called the sporozoites. After a maturation period ranging from seven to fifteen days, the cyst ruptures and the sporozoites invade the invertebrate host's body. Sporozoites that have migrated into the mosquito's salivary glands are then injected with the salivary juices of the mosquito during its next feeding.

3.2.2 The Exoerythrocytic Stage

This stage begins once the sporozoites have been injected into the vertebrate (intermediate) host's bloodstream. Sporozoites then enter the parenchymatous cells of the liver. This is facilitated by the production of the circumsporozoite protein by the sporozoites, which inhibits them from entering other cell types (Schmidt and Roberts, 2000). Once in the liver the sporozoites form **trophozoites** and begin **schizogony**. The nucleus then divides repeatedly forming daughter nuclei which form schizonts. The schizonts then proceed to mature over the next six to sixteen days when the mature schizont divides to produce **merozoites**, which are single-celled and nucleated. The

liver cells then rupture allowing merozoites to escape into the bloodstream beginning the Erythrocytic Stage.

3.2.3 Erythrocytic Stage

The Erythrocytic Stage begins when the **merozoites** invade the erythrocytes (or red blood cells). The merozoites then again form into **trophozoites** which uses the host cell's cytoplasm to form a large vacuole. This is often referred to as the "Ring Stage" because the young *Plasmodium* sp. parasite appears as cytoplasm with a nucleus on one edge. Pigment granules made of hemozoin then appear in the vacuoles as a result of the parasite's digestion of the host haemoglobin. These trophozoites grow and become less amoeboid in shape. The nucleus then begins to divide forming the meront. Merozoites in the meront are released when the erythrocyte ruptures. These escaped merozoites then enter other erythrocytes repeating this cycle. Some merozoites may enter erythrocytes and form gametocytes (either in the form of microgametocytes or macrogametocytes). It is these gametocytes which may be ingested by the definitive mosquito host to facilitate further transmission.

In the human *Plasmodium* species there are two time cycles on which the Erythrocytic Stage occurs. *Plasmodium malariae* is the only human plasmodia having a 72-hour Erythrocytic Stage length, while the other three species of human *Plasmodium* have 48-hour cycle Erythrocytic stages. It is for this reason that *P. malariae* is often referred to as quartan malaria, while *P. vivax* is referred to as benign tertian malaria and *P. falciparum* is referred to as malignant tertian malaria, while *P. ovale* malaria is referred to simply as tertian malaria (Marquardt *et al.*, 2000).

3.3 *Plasmodium* Species of the Non-Human Primates

There are a total of 29 plasmodia that are naturally infective to non-human primates (NHPs) (Gysin, 1998). These include *Plasmodium* spp. infecting prosimians, New World monkeys (NWM), the Old World monkeys (OWM), the gibbons (Hylobatidae), the African great apes (*Pan sp.*, and *Gorilla sp.* and the Asian great ape or the Orangutan (*Pongo sp.*). The two species of orangutan malaria (*P. silvaticum* and *P. pitheci*) will be discussed in greater detail than the other primate malarias because it is this thesis' focus of study.

Primate plasmodia have been of special interest to malariologists because they represent excellent animal models for human malaria (Waters *et al.*, 1993). Animal models for human malaria are seen as key components in an attempt to better understand the malaria parasite and in the development of a vaccine (Waters *et al.*, 1993). Non-human primate malarias have been hypothesized as a potential cause of **Zoonotic** disease. A zoonosis is defined as diseases and infections which naturally occur in animals and can be transmitted to humans (Coatney, 1971). Later, Robert Coatney (1971) realized that there existed a possibility that human diseases such as human *Plasmodium* sp could infect animals. He thus coined the term **Anthroponosis**. He defined an Anthroponosis as diseases and infections naturally infective to humans which may be transmitted to vertebrate animals (Coatney, 1971).

Previously, much of the research that has been done involving primates and their plasmodia has focused on using NHP hosts to study the four human *Plasmodium* sp. For example, tamarins (*Saguinus mystax* and *Saguinus fuscicolis*) have often been used as experimental hosts for *P. vivax* because of their small size and relative availability (Collins and Skinner, 1982). Use of the squirrel monkey (*Saimiri sciureus*) has also led to important findings regarding human falciparum malaria (*P. falciparum*) which is the

most severe form of malaria in humans (Gysin *et al.*, 1980). For example, experiments with *P. falciparum* malaria in the squirrel monkey allowed researchers to create a model for cerebral malaria and to ascertain its exact cause (Gysin *et al.*, 1992). Symptoms of cerebral malaria may include retinal blindness, impaired coordination, hypothermia and respiratory distress, which can lead to pulmonary edema (Gysin *et al.*, 1992).

3.3.1 *Plasmodium* Species of the Prosimians

Interestingly, all known prosimian plasmodia use the lemurs of Madagascar as their vertebrate hosts. A total of seven lemur plasmodia have been identified from three lemur species (*Lemur fulvus fulvus, Lemur fulvus rufus.* and *Lemur macaco macaco*) (Gysin, 1998; Landau *et al.*, 1989). The first lemur *Plasmodium* sp. was identified in 1951 when *Plasmodium girardi* was isolated from the blood of a lemur (*Lemur fulvus rufus*) (Coatney *et al.*, 1971). This lemur taxonomy is no longer used but we can assume that this lemur is a type of Brown Lemur (*Eulemur fulvus*). In 1964, Dunn (1964) confirmed the lack of plasmodia in lorises. That study concluded that no lorisoids were hosts for malaria parasites. For our purposes here, we must assume this to be true, but we must remain open to the fact that there very well could be *Plasmodium* spp. infective to lorisoids that have not yet been found because of the scale of study that is often necessary to find new malaria parasites in previously unidentified vertebrate hosts. African Pottos and Bush Babies have also never been identified as being infected with *Plasmodium* sp. parasites.

Recently, a number of new lemur *Plasmodium* spp. have been proposed. These include *P. uilenbergi*, *P. bucki*, *P. foleyi*, *P. percygarnhami*, and *P. coulangesi* (Landau *et al.*, 1989 and Lepers *et al.*, 1989). The diversification and classification of the *Plasmodium* sp. infective to lemurs was completely overhauled by Landau *et al.*, (1989).

This reclassification was based on a "phenomenon of vicariance" that is said to be similar to that which is found in African rodent malarias (Landau *et al.*, 1989). This refers to an interesting occurrence where *Plasmodium* sp. in one species of mammal is only slightly different than the *Plasmodium* sp. that is infecting a closely related species of mammal leading to debates on whether these species of *Plasmodium* are truly distinct. The original scientific name, *P. girardi*, now only refers to this parasite if it develops in *L. f. rufus*. The nomenclature changes to *Plasmodium* sp. if it develops in the blood of *L. f. fulvus* and changes again to *P. percygarnhami* if it develops in the blood of *L. m. macaco* (Landau *et al.*, 1989). This study also concludes that *P. lemuris* is probably not a *Plasmodium* sp. parasite but that it is probably a Haemoproteid (Landau et al., 1989).

3.3.2 *Plasmodium* Species of the New World Monkeys

It has long been hypothesized that the parasite causing malaria is not native to the New World, but that it was brought to the New World either by the Spanish Conquistadors or their African Slaves (Desowitz, 1991). There are currently two recognized species of NWM *Plasmodium* parasites. These are *P. simium* and *P. brasilianum* (Coatney *et al.*, 1971; Gysin, 1998; WHO 1987). *Plasmodium brasilianum* was first identified in 1908 by two German scientists in blood taken from a Cacajao (*Cacajao colvus*), while *P. simium* was first identified in 1951 by Fonseca in a Howler monkey (*Alouatta fusca*) (Coatney *et al.*, 1971). The New World monkey plasmodia are unique in that each has a direct human *Plasmodium* sp. counterpart (Coatney *et al.*, 1971). *Plasmodium brasilianum* was at one time believed to be closely related to the human malaria parasite *P. falciparum*, while *P. simium* is believed to be closely related to the human malaria, *P. vivax* (Coatney, 1971), while more recent genetic evidence indicates that *P. brasilianum* may in fact be more closely related to *P. malariae* (Leclerc *et al.*, 2004; White, 2004). *Plasmodium simium* and *P. brasilianum* may both represent

potential zoonoses in part because of their close evolutionary relationship with the human *Plasmodium* sp. (Coatney, 1971).

Interestingly, *P. simium* has reportedly only been found in regions of the New World where the mosquito vector *Anopheles cruzi* is present (Collins *et al.*, 1973). This parasite is known to naturally infect two primate species from genera that are known to live sympatrically, *Alouatta fusca* and *Brachyteles arachnoides* (WHO, 1987). The vector for *P. brasilianum* is yet unknown. *P. brasilianum* is known to infect at least twenty species of NWM representing at least ten genera including: *Alouatta*, *Aotus*, *Ateles*, *Brachyteles*, *Callicebus*, *Cebus*, *Chiroptes*, *Lagothrix*, *Saguinus* and *Saimiri* (Baerg, 1971 and WHO, 1987). Both of these parasites have been found to naturally infect humans with the greatest number of recorded cases coming from Brazil (Coatney, 1971).

3.3.3 *Plasmodium* Species of the Old World Monkeys

The malaria parasites of the Old World monkeys (OWM) are some of the most studied of the non-human primate (NHP) plasmodia. This is probably due to the shared characteristics of some human and OWM plasmodia. For example, *P. fragile* has often been used as a model for human *P. falciparum* because of their shared traits such as tertian periodicity and small ring shaped trophozoites (Coatney *et al.*, 1971 and Collins *et al.*, 1974). In addition to *P. fragile*, the macaque malarias *P. fieldi, P. cynomolgi* and *P. semiovale* have been used as animal models for human malaria because they share a relapse mechanism that is similar to those found in two of the human malarias *P. vivax* and *P. ovale* which is caused by the persistence of the liver stages of this parasite and potentially continuing for a number of years (Coatney *et al.*, 1971, Collins *et al.*, 1974 and Sullivan *et al.*, 1998).

There are eleven currently recognized *Plasmodium* sp. found in OWM (Gysin, 1998). These include: *P. cynomologi*, *P. knowlesi*, *P. fieldi*, *P. fragile*, *P. gonderi*, *P. semiovale*, *P. inui*, *P. coatneyi*, *P. shortii*, *P. georgesi* and *P. petersi* (Coatney et al., 1971; Gysin, 1998; Poirriez *et al.*, 1995; and WHO, 1987). Only three of the eleven known OWM *Plasmodium* species (*P. gonderi, P. petersi* and *P. georgesi*) have been identified as being naturally infective to African OWM. These parasites have been found to naturally infect Drills (*Mandrillus leucophaeus*), Vervet monkeys (*Chlorocebus aethiops*), and the Mangabeys [e.g. the Sooty Mangabey (*Cercocebus torquatus atys*); the Tana River Mangabey (*Cercocebus galeritus*); and the Gray-Cheeked Mangabey (*Lophocebus* (*Cercocebus*) *albigena*)] (Gysin, 1998; Poirriez *et al.*, 1995; and WHO, 1987).

The other OWM malaria parasites all use Asian OWM as their vertebrate hosts. The most common OWM host are the macaques (Genus *Macaca*). *M. arctoides*, *M. cyclopsis*, *M. fascicularis*, *M. mulatta*, *M. nemestrina*, *M. radiata*, and *M. sinica* have all been identified as natural vertebrate hosts for at least one species of *Plasmodium* (WHO, 1987 and Coatney *et al.*, 1971). At least five species of Langur (Genus *Presbytis*) are also known to be natural vertebrate hosts for OWM malaria. Some OWM plasmodia such as *P. cynomolgi* could potentially be shared by OWM in areas where macaques and langurs live sympatrically.

Of the Asian OWM plasmodia only *Plasmodium knowlesi* has been found to infect humans under completely natural conditions. In 1965, a surveyor for the United States Army was infected with this parasite while surveying in the Jungles of Peninsular Malaysia (Coatney *et al.*, 1971), but it seems likely that the transmission of non-human primate plasmodia to humans has been occurring for millennia.

3.3.4 *Plasmodium* Species of the Hylobatidae

The *Plasmodium* sp. that infect gibbons are probably the least researched of the *Plasmodium* sp. infective to NHPs. To date four distinct *Plasmodium* species have been identified from gibbons. These are *P. hylobati*, *P. eyelsi*, *P. jeffreyi* and *P. youngi* (Coatney *et al.*, 1971 and Gysin, 1998). It is probably not a coincidence that three of the four gibbon malarias have been found in the white handed or lar gibbon (*Hylobates lar*) in that the lar gibbon is by far the most studied and best known member of the Hylobatidae. The only other gibbon with a known species of malaria parasite is the Silvery Javan Gibbon (*Hylobates moloch*) which is one of the least known and most endangered of the gibbons. It is currently listed as critically endangered by the IUCN (Harcourt and Parks, 2003 and Rowe, 1996). It is probable that the researchers who identified *P. hylobati* in *Hylobates moloch* from North Borneo were mistaken as *Hylobates moloch* or as it is more commonly know, the Silvery Javan Gibbon, is found only on the island of Java while *Hylobates muelleri* and *Hylobates agilis* are the two species of gibbon known from the island of Borneo (Payne *et al.*, 1998).

Plasmodium hylobati was first identified in 1939 by the eminent malariologist Dr. Jerome Rodhain but remained lost to science until it was re-discovered and re-described during the 1960s and 1970s (Coatney *et al.*, 1971). The controversy over this malaria's gibbon hosts is interesting because when its detailed description was published there were three criteria that were suggested for use in its identification (Collins *et al.*, 1972). One of these diagnostic criteria was this parasite's occurrence in the same species of gibbon as the type host, a Silvery Javan Gibbon identified as belonging to the species *Hylobates lensciseus* (Coatney et al., 1971) which is later identified to be a synonymous name for *Hylobates moloch* (Collins *et al.*, 1972). Yet, this *Plasmodium* sp.'s detailed description was published based on its identification from an adult male *Hylobates*

moloch caught in Sarawak, Malaysian North Borneo (Collins *et al.*, 1972) and as mentioned above this species of gibbon is not found in that region. This detailed description used three major criteria as a guide for researchers to distinguish this gibbon *Plasmodium* sp. from the other gibbon *Plasmodium* sp. Among these criteria was this parasite's occurrence in the same species of gibbon as the type specimen (Collins *et al.*, 1972). These criteria for identification are now problematic in that we have here established that there are at least two and possibly more species of gibbon that are susceptible to this particular *Plasmodium* parasite.

A second recently identified *Plasmodium* sp. in the gibbon is *P. jeffreyi*. *P. jeffreyi* was first identified in the blood of a Lar or White Handed gibbon (*Hylobates lar*) from Kedah, a province in Peninsular Malaysia (Warren *et al.*, 1966). This parasite was identified as being found in disturbed areas where primary and secondary forests merged with treed crops (Warren *et al.*, 1966); perhaps because these type of environments are more suitable to the mosquito vector.

3.3.5 *Plasmodium* Species of the African Large Apes

Three *Plasmodium* sp. (*P. schwetzi*, *P. rodhaini*, and *P. reichenowi*) have been identified from the blood of the African large apes (Coatney *et al.*, 1971; Gysin, 1998; Ollomo *et al.*, 1997). These plasmodia are known to be infective to the common chimpanzee (*Pan troglodytes*) and the lowland gorillas (*Gorilla gorilla gorilla* and *Gorilla gorilla graueri*) (Coatney *et al.*, 1971; Dunn, 1964 and Gysin, 1998). In addition, there have been references to malaria parasites being found in the blood of the bonobo (*Pan paniscus*) (Bray, 1963). In the case of the bonobo no novel or distinct malaria parasite species have been identified. No *Plasmodium* sp. parasites have been identified in the blood of mountain gorillas (*Gorilla gorilla beringei*). It has been suggested that the

habitat used by the mountain gorilla may prevent exposure to malaria parasites because of the altitude at which they live (over 2000 meters a.s.l) which has been identified as being beyond the altitudinal limits for *Plasmodium* sp. because their mosquito vectors do not range into higher altitudes (Dunn, 1964).

Research examining potential relatedness of the African ape and human *Plasmodium* sp. during the late 1890s concluded that each of the ape *Plasmodium* sp. had a close relative within the human *Plasmodium* sp. It was believed that *P. reichenowi* was closely related to *P. falciparum*, *P. rodhaini* was closely related to *P. vivax* and *P. schwetzi* was closely related to *P. malariae* (Gysin, 1998). It has been suggested that *P. rodhaini* was not in fact a distinct species of primate *Plasmodium* sp., as it was hypothesized that this malaria was in fact the human *Plasmodium* sp., *P. malariae* (Coatney, 1971; Coatney et al., 1971 and Dunn, 1964). This hypothesis indicated this parasite's uniqueness in that if this was the case it would be both a natural zoonosis and a natural anthroponosis (Coatney, 1971). These relationships are discussed further in the section 3.4 below.

A more recent study examined the potential transmission of *Plasmodium* parasites between humans and apes in Gabon. The study was based at the International Centre for Medical Research, which houses fifty-nine gorillas and nine chimpanzees (or 59 chimpanzees and nine gorillas-both combinations are mentioned in this article) (Ollomo *et al.*, 1997). This site was seen to be unique because it housed captive great apes near a human neighbourhood and there were wild chimpanzees and gorillas living in the forest/savannah areas surrounding the city (Ollomo *et al.*, 1997). Only indirect evidence of *Plasmodium* infections were found in 13 apes through a measurement of titres against *P. falciparum* sporozoites. Interestingly, this study still concludes that the natural infection of apes by plasmodia is limited despite an implied

infection of 13/68 animals tested (Ollomo *et al.*, 1997). This assumption seems premature without further study as almost 20% of the gorillas and chimpanzees tested for *P. falciparum* titres show exposure to this human malaria causing parasite, or could this be explained by these apes being previously infected with *P. reichenowi* which as mentioned above is very closely related to *P. falciparum*. These data have been seen by some as proof that African apes cannot act as reservoirs for human plasmodia species (Gysin, 1998). It has also been concluded that because this study did not find any development of the blood stages into the gametocytic stages that *Plasmodium* transmission in this region was nearly impossible (Gysin, 1998). This is contrary to earlier findings that *P. schweitzi* and *P. rodhaini* are infective to human hosts in a laboratory setting (Contacos *et al.*, 1970 and Coatney *et al.*, 1971). The former interpretation may be supported the fact that *P. schweitzi* was not found to be infective to African-American volunteers (Contacos *et al.*, 1970).

3.3.6 *Plasmodium* Species of the Asian Large Ape (The Orangutan)

There are two recognized species of *Plasmodium* sp. that are naturally infective to orangutans. Orangutan malarias have long been considered to be a benign parasitic infection causing little morbidity (Peters *et al.*, 1976), although there are questions as to how truly benign these parasites are. The death of an orangutan believed caused by malaria occurred at the Sydney Zoological Gardens was reported by Dodd (1913-as cited in Peters *et al.*, 1976). There have never been any reports of malaria in the Sumatran subspecies (*P. abelii*). Yet, researchers working with rehabilitant orangutans in Sumatra have reported animals with symptoms consistent with malarial infections whose health recovered after treatment with antimalarial drugs (Carel van Schaik Pers. Comm. 2002).

The first *Plasmodium* sp. parasite known to be infective to the orangutan was *P. pitheci*. It was first identified and described in 1907 by two German scientists Halberstaedter and van Prowazek (Coatney *et al.*, 1971). They isolated this malaria parasite from the blood of a Bornean orangutan that had been sent to the Berlin Zoo (Coatney et al., 1971). During the late 1960's and early 1970's two major expeditions were mounted to "rediscover" Halberstaedter and von Prowazek's orangutan malaria (Wolfe *et al.*, 2002). Both of these studies took place at the Sepilok Orangutan Rehabilitation Centre (SORC) in the Malaysian Province of Sabah. At the time, these studies were undertaken because of the fear that orangutans (and thus their malaria parasites) would soon be extinct (Peters *et al.*, 1976). These expeditions eventually led to the discovery of a new orangutan *Plasmodium* sp. named *Plasmodium silvaticum* (Garnham *et al.*, 1972; Peters *et al.*, 1976).

Both *P. pitheci* and *P. silvaticum* have tertian periodicities (Coatney et al., 1971, and Peters et al., 1976). This periodicity refers to the length of time between the rupture of the schizonts. The rupturing schizonts are what generally cause illness in human malarias, as high malarial fevers are often associated with the mass rupture of schizonts. *Plasmodium pitheci* and *P. silvaticum* are distinguishable in a number of ways including size, shape, enlargement of the host cell and characteristic stippling effects (Peters et al., 1976) (See Table 3.1).

Table 3.1: Differentiating Characteristics of *P. pitheci* and *P. silvaticum* (From Peters *et al.*, 1976)

	Plasmodium pitheci	*Plasmodium silvaticum*
Size	Smaller	Larger
Shape	Less Amoeboid	More Amoeboid
Effects on the Host Cell	Only Slight Enlargement	More Significant Enlargement
Stippling	Characteristic	Schuffner's dots
Schizony in Peripheral Blood	Profuse	Rare
Sexual Forms	Unique	Unique

The relatively high prevalence of *Plasmodium* sp. in orangutans has been noted by many researchers (i.e., Peters *et al.*, 1976 and Wolfe *et al.*, 2002). These studies questioned why orangutans show such high rates of malaria infection when they tend to be solitary animals living in low population densities (Peters *et al.*, 1976 and Wolfe *et al.*, 2002). Of the three major studies of orangutan malaria (Coatney *et al.*, 1971; Peters *et al.*, 1976; Wolfe *et al.*, 2002) all have found infection rates over 50% for the animals housed at SORC (SEPILOK Orangutan Rehabilitation Centre). The unusually high population density of orangutans at SORC which has been estimated at 100/km^2 (Wolfe *et al.*, 2002) may contribute to these high rates. The first major expedition to study orangutan malaria was conducted in the late 1960s. This study identified a parasite prevalence of 55.6% in the SORC orangutans (10 of 18) based on the microscopic examination of blood films (Coatney *et al.*, 1971). Peters *et al.* (1976) found a *Plasmodium* sp. prevalence of 84.6% (11 of 13) in this same orangutan population during the expedition in which they discovered *P. silvaticum*. The most recent study by Wolfe and colleagues (2002) revealed the highest *Plasmodium* sp. prevalence of all

orangutan studies at 93.5% (29 of 31). The fact that this study recorded the highest prevalences may be in part due to the use of a relatively new genetic technique, namely Polymerase Chain Reaction (PCR). These researchers were also the first to obtain *Plasmodium* sp. prevalences for completely wild orangutans. They reported a remarkable difference between wild and semi-captive orangutan *Plasmodium* sp. infection rates with only 11.6% (5 of 43) of the wild orangutans testing positive.

A number of factors possibly contributing to the high prevalence of *Plasmodium* sp. in semi-captive orangutans were discussed by Wolfe *et al.* (2002). They identified six potential causes including increased orangutan densities, orangutans living close to humans and human settlements, a decrease in the use of trees, smaller day ranges, unnatural social structures, abnormal stress levels and the change in diet (Wolfe *et al.*, 2002). To date no conclusions have yet been reached as to why orangutans at SEPILOK suffer from such high rates of infection with the *Plasmodium* parasite.

3.4 Evolutionary Relationships of the *Plasmodium* Parasite

3.4.1 Origins of Primate *Plasmodium*

Scientists debate the origins of the *Plasmodium* sp. infective to primates, including humans, to this day. In the 1971 book The Primate Malarias, Coatney and colleagues were the first to offer the hypothesis that the primate malarias arose in Southeast Asia as a result of a coevolutionary event between the parasites and their hosts. The parasites then moved into Africa with migrating hominids and into the Americas with European colonization (See discussion of this subject in Escalante *et al.*, 1998; Also thoroughly discussed in Waters *et al.*, 1993). Three factors were seen by Waters et al, (1993) to support this theory. First, almost all of the *Plasmodium* species infective to monkeys are found in Asia. Secondly, all four of the human malarias can be

found in Asia, Africa and the Americas. Thirdly, the primate malarias have favoured but not absolute host/parasite relationships. In 1993 only *P. falciparum* was believed to be an exception to this evolutionary hypothesis. At that time *P. falciparum* was believed to be of recent evolutionary origins arising from the chicken malaria *P. gallinaceum* (Waters *et al.*, 1993). It was believed that *P. falciparum* had arisen with the commencement of intensive agriculture as recently as 5,000-10,000 years ago (Escalante *et al.*, 1995; Waters *et al.*, 1993). Agriculture was seen as providing a suitable climatic effect so as to create favourable conditions for the transmission of highly pathogenic ailments yet not threaten entire human populations (Waters *et al.*, 1993).

There have been two major changes in the Primate *Plasmodium* sp. origins paradigm over the past few years. The first and most important in terms of human morbidity and mortality is that *P. falciparum* is not a parasite that recently jumped from bird to human hosts. The most recent genetic evidence based on analysis of (small sub-unit) SSU rRNA, the circumsporozoite protein (CSP) gene, and cytochrome *b* from (Mitochondrial DNA) mtDNA indicates that *P. falciparum* is most closely related to the chimpanzee plasmodia, *P. reichenowi* with an estimated divergence date for the two of between six and ten million years ago (Escalante and Ayala, 1994; Escalante *et al.*, 1995, 1998; Ayala *et al.*, 1998). The second major change in this paradigm is the movement away from an Asian origin for the primate plasmodia to an African origin for these parasites. Two evidentiary lines support this hypothesis; 1) the close genetic relationship of all primate malarias from Southeast Asia and 2) the positioning of the African primate malarias in the primate malaria phylogeny (Escalante *et al.*, 1998). This new paradigm is further supported by phylogenetic evidence based on CSP and cytochrome *b* genes in orangutan and gibbon plasmodia which indicates that the orangutan malarias align more closely with the Asian primate malaria clade than with the

African ape malaria clade (Wolfe *et al.*, 1999b). Evidence provided by Escalante *et al*, (1998) further indicates that all the primate plasmodia with the exception of *P. falciparum* and *P. reichenowi* form a monophyletic group, while the African monkey plasmodia seem to form a clade with the primate plasmodia of Southeast Asia and *P. vivax*.

3.5 Conclusion

Since the beginning of the academic study on the *Plasmodium* sp. of humans, NHPs and their *Plasmodium* sp. parasites have been used as models for human malaria. In fact, primates and their malarias are so important to the study of the human disease that Coatney *et al.* (1971:14) were led to state that: "No discussion of the history of man's conflict with malaria would be complete without consideration of the plasmodia of other animals." They continue (p. 14): "The evolution of an understanding of the plasmodia of non-human primates parallels, in many ways, and complements the story of human malaria."

Although studies using NHP and NHP *Plasmodium* sp. as models for human malaria continue, especially in the search for the ever-elusive vaccine, the study of NHP diseases such as malaria has begun to move from its human focus towards helping the primates that may be afflicted by these malarias. There is a great deal of basic information which is lacking regarding natural infections with NHP plasmodia and their primate hosts such as whether NHP malarias also make their host sick. These studies were ignored in the past because they seemingly had no importance to the study of human malaria. This view is beginning to change. Recent observations of plants used by chimpanzees very irregularly, or when they appeared ill has lead to the discovery of two new chemical compounds called limonoids (Trichirubines A and B) which have very potent anti-malarial properties (Kreif *et al.*, 2004). At the recent conference at which the

Great Ape Health Monitoring Unit (GAHMU) was established, malaria was discussed as potentially being one of the diseases which may pose a threat to the survival of great apes in the wild at least until more is known on how apes react to *Plasmodium* sp. infections. The study of malaria in NHPs has become important in terms of conservation biology and medicine as well as public health in many regions where humans and primates live in close proximity.

Chapter 4
METHODOLOGY

4.1 Introduction

The methodology section of this thesis is divided into two major sections. The first section is entitled "Field Methodology". This section discusses all methodologies, including laboratory and microscopic procedures, used at the Orangutan Care Centre and Quarantine (OCC&Q), in Kalimantan Tengah, Indonesia. The second section is entitled "Laboratory Methodology". This section discusses all methodologies involved in the DNA analysis of blood samples exported to Canada. These analyses were completed in the laboratory of Dr. Carl Lowenberger in the Department of Biological Sciences at Simon Fraser University.

4.2 Field Methodology

4.2.1 Study Site and Population

The Orangutan Care Center and Quarantine (OCC&Q) is located in the Province of Kalimantan Tengah, Indonesian Borneo. The field portion of this study took place at the OCC&Q between July 20th 2003 and August 23rd, 2003 which corresponded to the dry season in Kalimantan Tengah. Approximately 200 orangutans were housed at the OCC&Q at that time. Sampled individuals were chosen by the OCC&Q's head veterinarian, Dr. Rosa Garriga, from two groups of orangutans based on which orangutans were in need of routine blood checks. The first group included all orangutans arriving at the OCC&Q as part of their initial medical assessment, while the second group of blood samples were taken from resident orangutans during their semi-

annual health checks. We also took blood samples from any resident orangutans undergoing medical procedures requiring anaesthesia.

4.2.2 Sample Collection

Blood samples were collected by an OCCQ Veterinarian using a 25G 5/8 gauge PrecisionGlide™ needle and 3-ml syringe, between eight and nine AM. All blood samples were placed into plastic vials containing EDTA (an anti-coagulant and preservative for blood), then gently mixed by hand. The animal's name, size (Small-15 kilograms or less, Medium-15-30 kilograms and Large-over 30 kilograms) and gender were recorded on data sheets during the blood collection process. Blood vials were labelled with the animal's name and date.

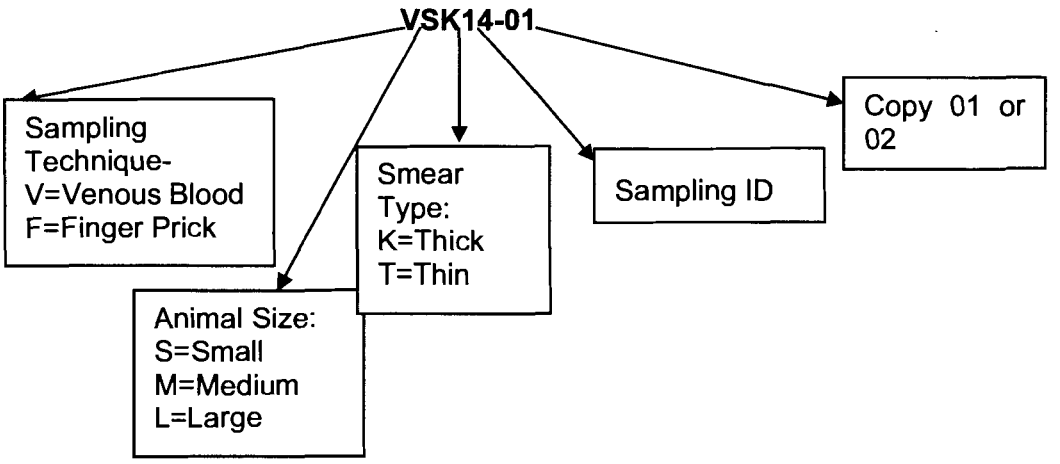
4.2.3 FTA® Card Preparation

In the OCC&Q laboratory an aliquot of blood (5-10 μl) was pipetted onto each of four circular areas on Whatman® FTA® Classic Cards. A sampling number based on the information collected during the blood collection process was transcribed on to the FTA® cards. For example, DNA sample VS42 would indicate that this was a blood sample collected from the vein of a small animal and that it was the 42nd animal sampled. FTA® cards containing blood were allowed to dry completely overnight and placed in individual plastic bags with corresponding identification numbers for storage and transport back to Canada. Bags containing blood stained cards were placed in a cardboard box for protection and transport. The box containing Whatman® FTA® Classic Cards was stored in Indonesia from September 2003 to October 2004 when permission was obtained to send the samples to Canada. PCR analysis of the blood stored on the Whatman® FTA® cards began in November 2004.

4.2.4 Blood Smear Preparation

Four blood smears were prepared from each blood sample; two thick and two thin smears for each orangutan. To prepare thick smears we placed a small amount of blood (approximately 3-4μl) onto the middle of a pre-cleaned slide using a 20 μl pipette. The blood was spread into a rectangular pattern on the slide using a pipette tip (Cheesbrough, 1998). In preparing thin smears we placed a small drop (approximately 1-2μl) of blood at the top end of the pre-cleaned, dry slide. A clean microscope slide or cover slip approximately held at a 45° angle was placed at the bottom end of the slide and drawn up into the area of the slide containing the blood. The blood spread along the rim of the spreading slide which was then slid quickly along the smear slide creating a thin blood film encompassing approximately 2/3 of the microscope slide (Cheersbrough, 1998). All thick and thin smears were labelled with a detailed coding system that allowed subsequent identification, date and sample type (See Figure 4.1).

Figure 4.1: Microscope Slide Numbering and Its Meaning



All thick and thin smears were dried using a small fan. Once dry the thin smears were fixed with absolute methanol. Slides were placed facedown on toothpicks in a

plastic tray and immersed in Giemsa stain (10X dilution in physiological saline) for 30 minutes. We washed the slides under cold running water until all excess staining fluid was removed and the stained area was light pink. Thick smears were washed carefully to remove all excess stain from around the smear and slide back while not washing away stained blood. The slides were allowed to dry completely using an incubator or small battery operated fan.

We covered the stained area with cover slips to protect the prepared slides using *Micromount*™ microscope slide glue. Completely prepared microscope slides were allowed to dry in an incubator.

4.2.5 Finger Prick Methodology

One of the original goals of this study was to test the "Finger-Prick" method of blood collection on the orangutans housed at OCC&Q. We used Microtainer® Brand Safety Flow Lancets to prick a small hole in the finger, and collected blood on a clean microscope slide to make a thick smear. Because the orangutans bled very little from a finger prick and the bleeding ceased almost immediately (See Results, Chapter 5), we decided to abandon this sampling style and chose to take small amounts of blood from animals undergoing routine blood checks.

4.2.6 Microscope Analysis

We analyzed blood smears microscopically under 1000 X magnification. For all samples deemed positive for *Plasmodium* sp. we estimated the total parasitemia with standard protocols used in the OCC&Q laboratory. This technique estimates the number of parasites per volume of blood (μl) by counting the number of *Plasmodium* infected red blood cells (RBC), comparing this number with the number of white blood

cells (WBC) then multiplying this by the number of WBC's per volume (μl) as determined with a haemocytometer (Cheesbrough, 1998). For example, when looking at the thick smear under microscopy we would count the number of *Plasmodium* infected RBC seen as we counted 200 WBC. Thus, the formula (Cheesbrough, 1998) used by the OCC&Q is:

$$\frac{\text{WBC Count/}\mu\text{l}}{\text{Counted WBCs up to 200}} \times \text{Red Blood Cells Infected with Parasites}$$

So for example, if we found a total of 65 RBCs infected for every 200 WBCs and use an average number of WBCs/μl of blood for an orangutan, say 12,000 (the normal range of WBCs for an orangutan is between 8,000 and 15,000/μl) the formula would work as follows:

$$\frac{\text{WBC Count/}\mu\text{l}\ (12{,}000)}{200} \times 65 \text{ Infected RBCs} = 3900 \text{ parasites/}\mu\text{l}$$

All positive infections were reported to a staff veterinarian and diagnoses were confirmed by staff technicians. We examined the thin smears of individuals whose thick smears contained >30 parasites per 200 WBC. This allows a more precise *Plasmodium* sp. diagnosis and is more useful in species identification (Dr. Garriga, Pers. comm.). We took photographs of infected red blood cells in the thin smear.

4.2.7 Mosquito Collection

Every Wednesday evening between the hours of 5 and 8 p.m. we collected mosquitoes from areas around the orangutan sleeping cages. We waved an insect collection net in the direction of any mosquito that was seen. Mosquitoes were sealed in glass vials and then placed in a freezer overnight. The next morning all collected mosquitoes were placed into a glass vial containing 95% ethanol.

4.2.8 Division into Subpopulations

To facilitate more accurate analyses we further subdivided our samples into four categories. 1) OCC&Q residents were individuals untreated for malaria for at least one year and had been living at OCC&Q longer a minimum of three months (n=69), 2) newly confiscated arrivals, living at OCC&Q for less than one month and with no previous treatment history for malaria (n=14), 3) newly confiscated arrivals to OCC&Q with previous history of treatment for malaria (n=1), 4) newly arrived animals that had at one time been ex-captives and previously released back into the forest (n=2) (See Figures 5.1-5.4). Both of the animals in the final category came to the OCC&Q with severe amoebic dysentery from Pondok Tanggui. The *Plasmodium* sp. infection was discovered during the routine blood work done on all new arrivals, and both animals had significant infections requiring treatment.

4.3 Laboratory Methodology

4.3.1 Overview

This section discusses the procedures used in the molecular analysis of the orangutan blood samples. This section discusses DNA extraction; PCR Amplification of *Plasmodium* specific DNA; cloning of PCR products; sequencing; and data analysis.

4.3.2 DNA Extraction Protocols

Subsequent laboratory techniques required the extraction of the DNA preserved on the FTA® Cards. We extracted DNA from the Whatman® FTA® Classic Cards following the manufacturer's instructions (Whatman, 2000 and 2004), using either of two protocols. The first involved washing punched disks for direct use in PCR reactions. The second method involved the elution of DNA from punched disks into of 100 µl TE buffer.

We punched a 1.2 mm disk from a blood stained card using a Harris Micro-Punch. We placed individual disks into 0.6 ml PCR tubes and treated them with 2.5 µl Proteinase-K (10mg/ml) in 247.5µl Whatman® FTA® Purification Reagent (FTA-PR), and placed them in a 65°C water bath for 30 minutes. This process removes protein and allows DNA to be accessible. We then washed each punched disk three times in 200µl of FTA-PR, allowing the disk to soak for five minutes per wash. We then washed the disk twice in 200µl TE Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) incubating five minutes per wash at room temperature. The disks were allowed to dry (air dry or in 37°C incubator) before use in a PCR reaction.

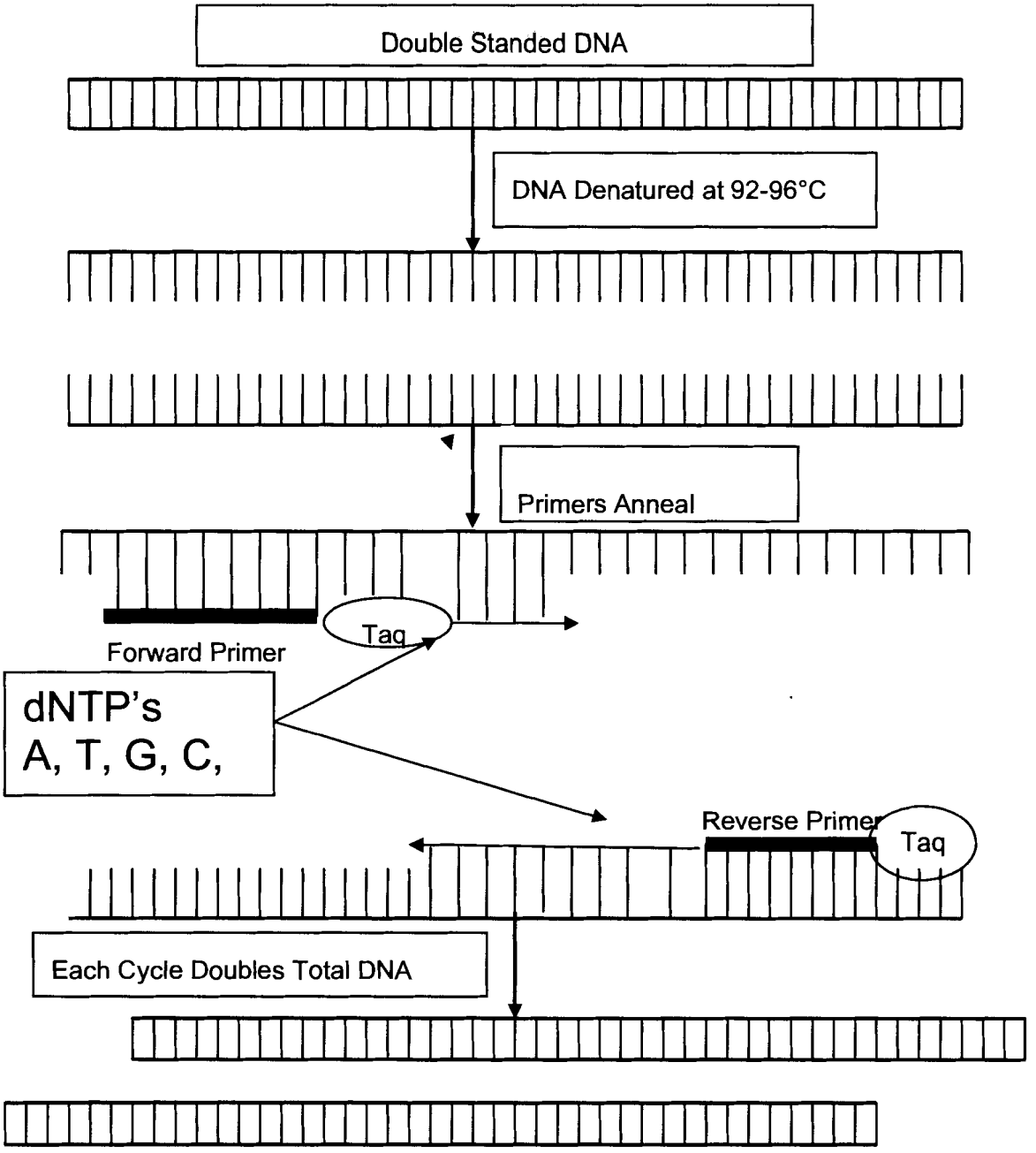
Alternatively, we eluted DNA from three to five disks per blood sample. Disks were punched and washed as described above. Once the disks had dried we added 35µl of Elution Solution 1 (0.1 N NaOH, 0.3 mM EDTA, pH13) to the tube and let disks soak for five minutes at room temperature. We then added 65µl of Elution Solution 2 (0.1M Tris-HCl, pH7.0) and vortexed five times quickly to mix and then incubated tubes for ten minutes at room temperature. The elutant was then vortexed ten times and the disks were removed. 2µl of elutant was used as template in the initial PCR.

What is Polymerase Chain Reaction (PCR)?

Polymerase Chain Reaction (or PCR) is a process in which selected regions of DNA, as defined by specifically designed primers, are amplified creating >1,000,000 copies of the target DNA sequence. There are three main stages in a PCR reaction (Dieffenbach and Dveksler, 1995). In PCR double stranded DNA is denatured by heating the reaction to 92°-96°C which separates and linearizes the DNA into two strands. Subsequently, two oligonucleotides (primers) designed and purchased specifically for this purpose, anneal to their complimentary sequence on either DNA

strand. An enzyme (*Taq* polymerase) then binds to each primer and moves down the single strand enzymatically adding the complimentary nucleotides and creating two copies of the original DNA sequence. Over a number of cycles (generally 20-35) this process produces millions of copies of the sequence. Most reagents are common to all PCR reactions with the specific design of the primers allowing a specific region of DNA to be amplified (See Figure 4.2).

Figure 4.2: PCR in Action: dNTPs refer to Deoxyribonucleotide triphosphate.



4.3.3 Primer Design

Four of the primers used during this study were designed by Singh *et al.* (1999) for human plasmodia and used by Wolfe *et al.* (2002) in a study of *Plasmodium* in

orangutans. These primers were designed against the 18s small sub-unit ribosomal RNA gene, and were designated rPLU1, rPLU3, rPLU4, and rPLU5 (See Table 4.1). We designed two additional reverse nested PCR primers (rPLUCal and rPLU Cal 2) using *Plasmodium* sp. 18S sRNA sequences available in GenBank (an NIH database). To date no species specific primers exist for *P. silvaticum* or *P. pitheci*, the *Plasmodium* sp. believed to infect orangutans. Nested PCR is a technique which increases the sensitivity of PCR reactions by using a set of internal primers in second PCR reaction with an aliquot from the initial PCR acting as template (Roux, 1995).

Table 4.1: *Plasmodium* Genus Specific PCR Primers

Primer Designation	Forward Primers	Tm
rPLU1	5′-TCA AAG ATT AAG CCA TGC AAG TGA-3′	66°C
rPLU3-Nested	5′-TTT TTA TAA GGA TAA CTA CGG AAA AGC TGT-3′	64°C
	Reverse Primers	
rPLU4-Nested	5′-TAC CCG TCA TAG CCA TGT TAG GCC AAT ACC-3′	74°C
rPLU5	5′ -CCT GTT GTT GCC TTA AAC TCC-3′	62°C
rPLUCal-Nested	5′-ACA CAW RGT KCC TCT AAG AAG C-3′	59°C
RPLU Cal 2-Nested	5′-CGC TAT TGG AGC TGG AAT TAC C-3′	65°C

Figure 4.3: Primers and their placement on the Desired Sequence of the *Plasmodium*18S sRNA Gene

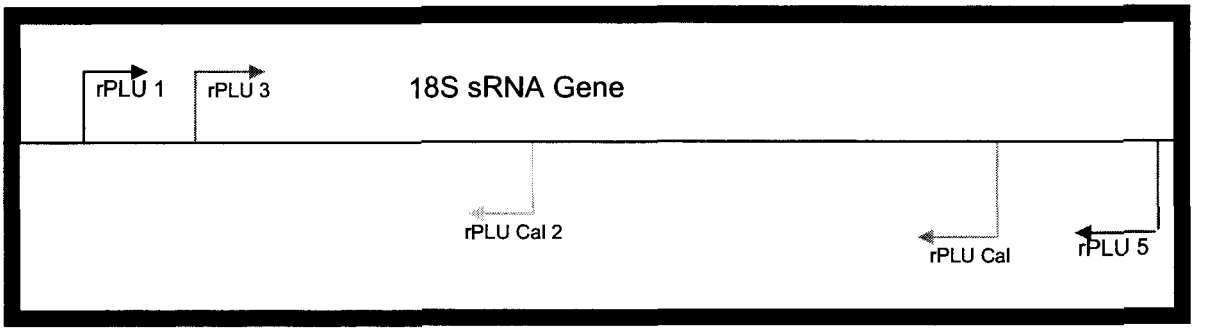


Table 4.2: Primer Combinations and Their Expected Amplicon Lengths

Forward and Reverse Primer Combinations	Expected Amplicon Length
rPLU 1 and rPLU 5	~1640 (basepair) bp
rPLU 3 and rPLU Cal 2	~500 bp
rPLU 3 and rPLU Cal	~1500bp

4.3.4 Polymerase Chain Reaction Protocols (PCR)

Overview

Because of the intricacies and difficulties of this study we used a three step PCR process.

Step 1: PCR of DNA on the disks or from elutant with primers rPLU1 and rPLU5. This should amplify a ~1600 (basepair) bp DNA fragment.

Step 2: Because of the low amplification of large DNA segments, we tested the product obtained in Step 1 with a nested PCR reaction. Using 2µl of the product obtained in Step 1 as DNA template we performed a reaction containing primers rPLU 3 and rPLU Cal 2, which produced a 500 bp band. We used this to confirm a positive PCR reaction in Step 1.

Step 3: Using PCR samples from Step 1 that were confirmed to contain amplified DNA in Step 2, we did a third reaction using primers rPLU 3 and rPLU Cal. These nested primers amplified a ~1500 bp fragment that contains three to four variable regions that can be used for species determination. These products were used for cloning and sequencing.

Initial PCR Reaction

During the initial PCR reaction a prepared 1.2 mm disk (or 2 μl of elutant) from each animal was used as template in a 50μl PCR reaction containing 34.8 μl H_2O, 5 μl 10X PCR Buffer, 2μl MgCl, 2μl dNTPs, 2μl Primer rPLU1, 2μl Primer rPLU 5 and 0.1 μl *Taq* DNA Polymerase. This reaction was run on a PTC-2000 Thermocycler (MJ Research; Waltham MA) under the conditions of: Step 1) 94°C for 4 min, Step 2) 35 cycles of denaturation at 94°C for 30 sec; annealing at 55°C for 1 min; extension at 72°C for 1 min; Step 3) additional extension at 72°C for 4 min; Step 4) hold PCR reaction at 4°C.

Nested PCR Reactions

We used 2μl of the initial PCR reaction as template in a nested PCR reaction as described by Singh et al., (1999). The PCR reaction comprised 16.4μl H_20, 2.5μl 10X Buffer, 1μl MgCl, 1μl dNTPs, 1μl primer rPLU 3, 1μl primer rPLU Cal 2, and 0.1μl *Taq* DNA polymerase (25 μl total volume). This reaction was run on a PTC-2000 Thermocycler (MJ Research; Waltham MA) under the conditions of: Step 1) 94°C for 4 min, Step 2) 35 cycles of denaturation at 94°C for 10 sec; annealing at 60°C for 10 sec; extension at 72°C for 45 sec; Step 3) additional extension at 72°C for 4 min; Step 4) hold PCR reaction at 4°C.

PCR products were size fractionated on a 1% Agarose gel containing Ethidium Bromide, visualized on a BioDoc gel documentation System (UVP, California) and the size of the band was compared to a molecular marker of known size. If we found the Nested 2 reaction to be positive for the presence of *Plasmodium* sp. DNA we performed an additional reaction on the original PCR product using primers rPLU 3 and rPLU CAL to amplify a ~1500 bp section of DNA in order to obtain a longer DNA fragment for phylogenetic analysis and multiple alignment after sequencing. We used the same PCR reagents and conditions as in the initial PCR reaction but changed the primers to rPLU3 and rPLU Cal and used an annealing temperature of 55°C.

We also used a commercially available PCR kit specifically designed to amplify long DNA fragments (BD Strips, BD Biosciences Clontech, Palo Alto, Ca) We ran this 25μl reaction with 1μl rPLU 3, 1 μl rPLU CAL and 1.5-2μl template from the initial PCR reaction. The PCR conditions for these samples were 1) 95° for 1 min, 2) 35 cycles of 95° for 30, 58° for 3 min; 3) 58° for 3 min, 4) hold at 4°.

Analysis of PCR Products

We used two types of agarose gels, 1.2% low melting temperature (LMT) agarose, or 1% regular agarose, both containing ethidium bromide. Bands of ~1500 bp were excised from the gel with a sterile scalpel. Bands excised from 1% Agarose gels were purified using a Qiagen Gel Purification Kit (Qiagen, Valencia Ca) following manufacturers instructions while bands excised from LMT gels were liquefied at 65°C then vortexed. The DNA fragments from Gel purified PCR products and LMTs were cloned directly into P-GEM-T-Easy vector (Promega, Madison WI, USA) following manufacturers protocols. Potential transformants were identified using blue-white screening of XL1-Blue cells (Strategene, USA). Transformed colonies were grown overnight in 5ml LB medium with 5μl Ampicillin (100μg/μl) and purified using Wizard Plus

Miniprep DNA Purification System (Promega, Madison WI, USA). Bi-directional sequencing of PCR products (Lowenberger *et al.*, 1999) was done using Big Dye Chemistry (v3.1) (Applied Biosystems, Foster City, Ca) using plasmid vector primers SP6 and T7.

4.3.5 Bioinformatics

The nucleotide (nt) sequences were compared to those *Plasmodium* sp. sequences available in public databases using BLAST N (Nucleotide-Nucleotide Blast), T-BLAST X (Translated Query-Translated Database) and BLAST X (Translated query – Protein database) public databases. BLAST N compares nucleotide sequences entered by the user to all nucleotide sequences in the databases. BLAST X translates nucleotide sequences entered by the user into all six reading frames and compares these to all proteins in the databases. T-BLAST-X translates entered nucleotide sequences into all six reading frames and compares them to translated nucleotide sequences available in the databases. This process produces a report on both DNA and protein sequences and indicates with which species our submitted sequences share the greatest homology. This usually indicates the genus and species from which the DNA originates.

All available sequences of the *Plasmodium* sp. 18S sRNA gene which contained complete sequences of our target region were downloaded. These included; *P. gallinaceum* (Chicken), *P. berghei* (Rodent), and the primate plasmodia; *P. falciparum*, *P. ovale*, *P. malariae*, *P. vivax*, *P. cynomolgi*, *P. fragile*, *P. knowlesi*, *P. reichenowi*, *P. simium* and *P. inui* (See Chapter 3 for discussion of primate plasmodia). These sequences were edited to begin and end with our primers (rPLU3 and rPLU Cal), using EditSeq (DNA Star, Madison, WI) allowing for easier comparisons between sequences. These reference sequences were also translated into proteins, using our forward primer

as a guide, with EditSeq (DNA Star, Madison, WI). Reference nucleotide sequences (Appendix 2) and their protein translations (Appendix 3) were aligned with sequences amplified from our samples and their protein translations using MegAlign (DNA Star, Madison WI). This allowed for the comparison of our DNA and protein sequences with known DNA/protein sequences.

Chapter 5
RESULTS

5.1 Introduction

As with the methodology section above, the results section is divided into two parts. The first part of this chapter is entitled "Field Results" and reports the findings of the microscopic analysis performed at the OCC&Q. The second part is entitled "Laboratory Results" and reports on the findings of the DNA analysis of the Whatman® FTA® Card preserved samples analyzed via PCR in the Laboratory of Dr. Carl Lowenberger at Simon Fraser University.

5.2 Field Results

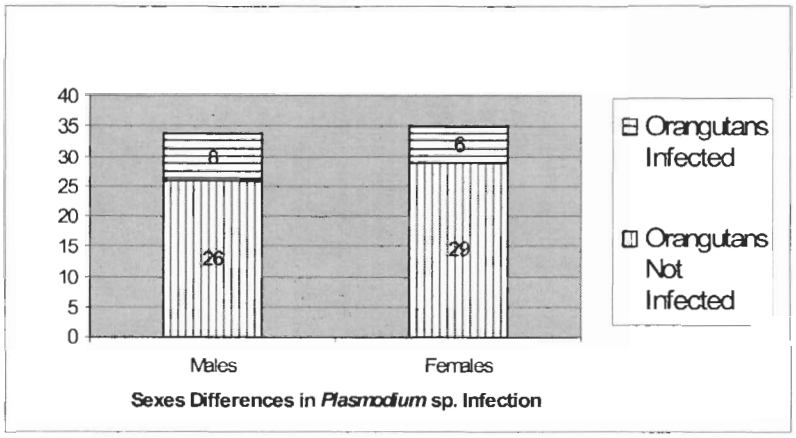
5.2.1 Orangutan Blood Collection

We collected a total of 97 blood samples from 87 orangutans. Of these, 21 blood samples were from small orangutans (under 15 kg), 62 blood samples were collected from medium orangutans (15-30 kg) and the final four samples were from large orangutans (over 30 kg). Large orangutans were sampled rarely because of the difficulty in handling them. Blood samples were divided almost equally among males and females with 43 males, and 44 females sampled.

Of the 69 OCC&Q residents tested (34 males, 35 females), only 14 had *Plasmodium* sp infections (20.3%) (8 males: 23.5%, 6 females: 17.1%) (Figure 5.1). Of these 14 animals, only 3 had infections requiring medical treatment (as determined by

OCC&Q staff veterinarians) and none of the 14 animals showed any outward sign of illness as a result of the *Plasmodium* infection.

Figure 5.1: Results of Testing OCC&Q Resident Orangutans for *Plasmodium* sp. Infections



We tested 14 newly arrived orangutans for *Plasmodium* sp. infections. This represented all orangutans arriving at the OCC&Q during the study period and three females who had arrived three months previously with low level *Plasmodium* sp. infections and were being re-evaluated to determine if these infections had progressed. All of these orangutans had no known history of treatment for *Plasmodium* sp. infections during their time in captivity. Eight of these 14 animals tested positive for *Plasmodium* sp. (57%) (Figures 5.2 and 5.3). In this sample subset, variables such as sample size, individual size class and sex were uncontrollable because of the nature of the population being sampled.

Of the eight animals with *Plasmodium* infections, four infections were deemed severe enough to require treatment by OCC&Q staff veterinarians. One of these animals (Oro-VM28) showed symptoms of lethargy, anorexia, and dehydration. We monitored this animal's activities using one 20-minute focal animal sample (Altmann, 1974) per hour during the daytime hours when orangutans tend to be most active (~between 8 AM and 4:30 PM) over two and a half days during his treatment for

Plasmodium sp. infection. After two and a half days the chemotherapy did not reduce the symptoms and we concluded the symptoms were being caused by some other infection.[2]

Figure 5.2: Sex Differences in *Plasmodium* sp. Infections Among Newly Confiscated Orangutans

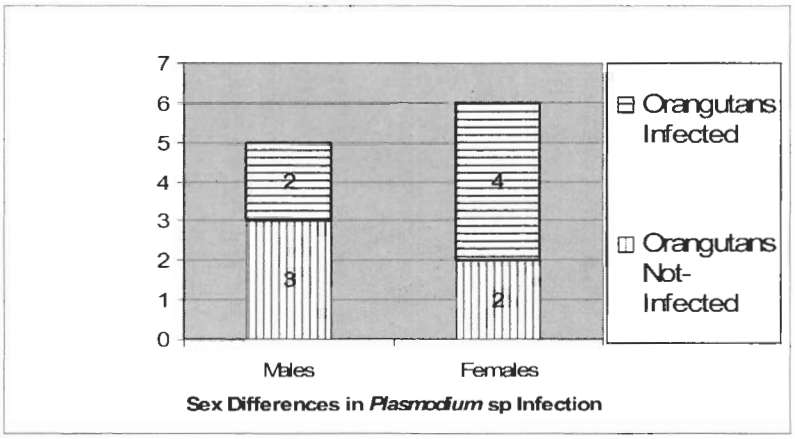
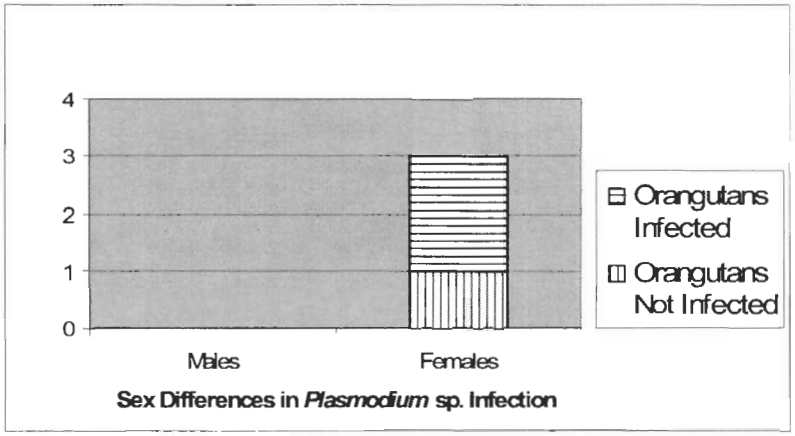


Figure 5.3: *Plasmodium* sp. Testing of Newly Arrived Orangutans/Who Arrived with *Plasmodium* sp. Infections 3 Months Prior that Did Not Require Treatment



Two previously released ex-captive orangutans were brought to the OCC&Q from Pondok Tanggui for treatment of amoebic dysentery, which was causing severe and bloody diarrhoea. We collected blood from these animals as part of the routine

[2] Oro eventually died as a result of severe malnutrition prior to his arrival at OCC&Q

testing done to all new arrivals at the clinic. Both animals were found to have levels of *Plasmodium* sp. for which staff veterinarians administered treatment.

All blood smears positive for the presence of *Plasmodium* were reported to one of the OCC&Q staff veterinarians who then checked the animal's condition and decided whether the level of parasitemia was sufficient to warrant chemotherapeutic treatment or further monitoring for potential increases in parasitemia.

All orangutans treated for *Plasmodium* infections were treated with Artecef©. A loading dose of 4.8 mg/kg was given at hour 0 of treatment and subsequent doses of 1.6 mg/kg at hour 6, 24, 48 and 72 hours. All doses are given via intra-muscular injection usually in the quadriceps. At hour 96 all orangutans treated for *Plasmodium* were re-checked to ensure the effectiveness of the drug in clearing the infection.

One orangutan named "Frankie" (VM40) had the highest level of parasitemia at the OCC&Q during the field season with his maximum parasitemia estimated between ~28-30,000/µl of blood (calculated as per formula in Chapter 4 page 91). Blood samples for both smears and DNA diagnosis were collected from Frankie at each treatment interval to follow the effectiveness of chemotherapeutic treatment more closely. By hour-72 Frankie was microscopically free of *Plasmodium* and diagnosed negative. The final dose was given to ensure the clearance of the infection and Frankie remained negative during his post-treatment re-check.

5.2.2 Finger-Prick

It quickly became apparent that for the purposes of this study, this technique was unsuitable because of the small quantity of blood acquired made accurate diagnoses and later DNA work difficult. This technique would require repeated

sampling to obtain suitable blood volumes, which would have caused needless pain and stress so this aspect of the project was abandoned.

5.2.3 Mosquitoes

We collected a number of mosquitoes in and around OCC&Q during my time there. Mosquitoes were collected from dwellings, near orangutan cages, swamp ponds, orangutan Pondoks and at the Lamandau release site. Because of the small sample size and time constraints this aspect of the research was not pursued.

5.3 Laboratory Results

5.3.1 *Plasmodium* DNA Amplification

We amplified, cloned and sequenced a ~1500 bp segment of the *Plasmodium* sp. 18S sRNA gene from 13 of the 24 orangutans who were microscopically positive for *Plasmodium* sp. (Appendix 1). We were not able to amplify *Plasmodium* sp. specific DNA from some animals with very low parasitemias.

5.3.2 Results of Bioinformatic Analyses

We downloaded the sequences of various plasmodia available in the databases. We aligned the sequences (See Appendix 2) and generated phylogenetic trees based on a nearest neighbour association at the nucleotide level (Figure 5.4) and at the amino acid level (Figure 5.5).

We then aligned the sequences we obtained from our 13 *Plasmodium* sp. infected orangutans at the nucleotide level (Appendix 3). Phylogenetic trees showing the nearest neighbour relationships of our sequences were created from these alignments (Figure 5.6).

Figure 5.4: Phylogenetic Tree of Reference Nucleotide Sequences

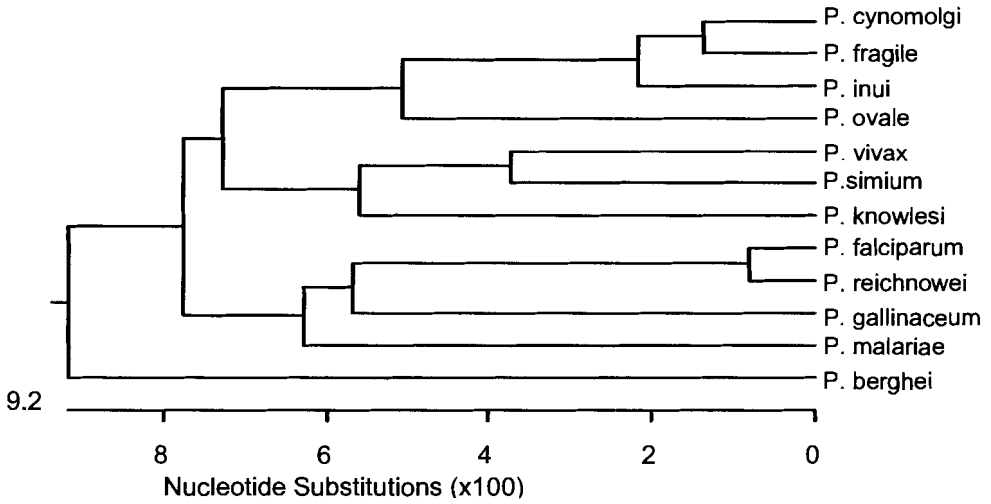
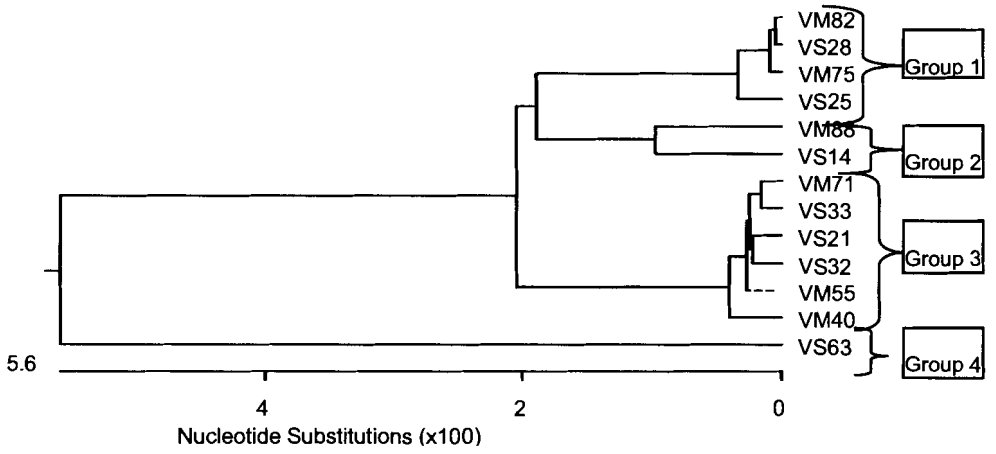


Figure 5.5: Phylogenetic Tree of Reference Sequences Translated into Putative Proteins

Our samples form four distinct groupings at the nucleotide level. There is some variation in how our sequences align at the protein level that may be due to natural shifts in reading frame, sequencing errors that may result in premature stop codons.

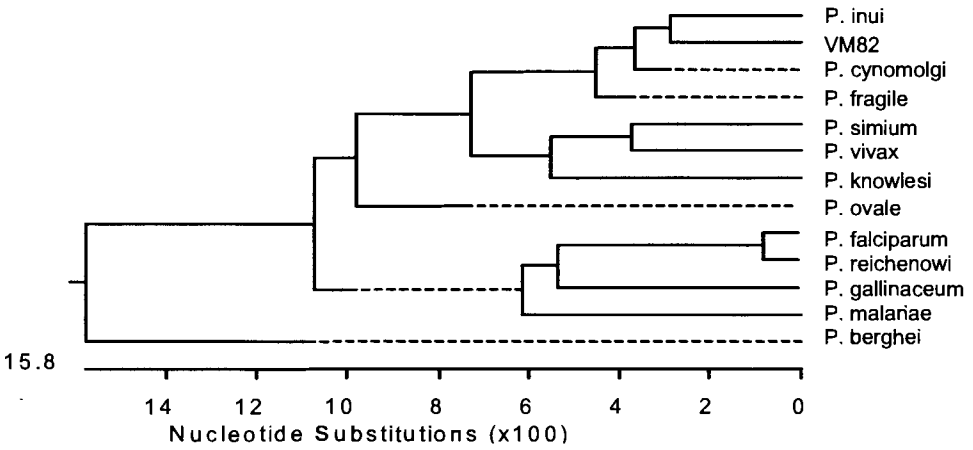
Figure 5.6: Phylogenetic Tree of Obtained Sequences at the Nucleotide Level: Dotted lines indicate weaker relationships



Based on these natural groupings we designated samples VS25, VS28, VM75 and VM82 as Group 1; samples VS14 and VM88 as Group 2; VS21, VS32, VS33, VM40, VM55 and VM71 as Group 3 ; and VS63 as Group 4.

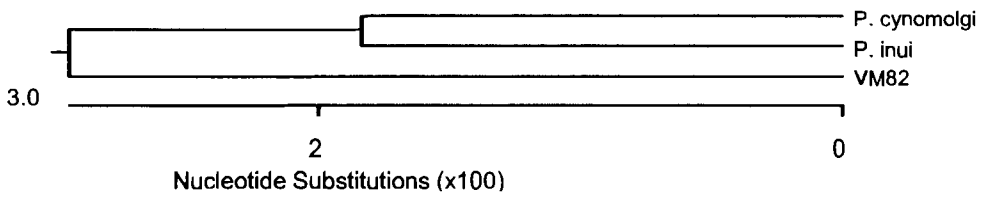
For Group 1, we randomly designated sequence VM82 as our representative sample. Sequence VM82 consists of 1519 bp at the nucleotide level and translates to a putative protein with a length of 506 amino acids. VM82 shares significant sequence identity with *P. cynomolgi* (94%) and *P. inui* (95%) in the region of bases 160-1509 (Appendix 3). A phylogenetic tree based on alignments of VM82 with our reference sequences shows closest association with *P. inui* and *P. cynomolgi* (Figure 5.7).

Figure 5.7: Sequence VM82 Aligned with Reference Sequences-Nucleotide Level



To aid in the determination of the *Plasmodium* sp. represented by this group of sequences VM82 was aligned solely with its two closest complete homologues *P. inui* and *P. cynomolgi* (Appendix 4). A phylogenetic tree showing the nearest neighbour association between these three sequences shows VM82 to be distinct from both *P. inui* and *P. cynomolgi* (Figure 5.8).

Figure 5.8: Sequence VM82 Aligned with Reference Sequences of *P. inui* and *P. cynomolgi*-Nucleotide Level

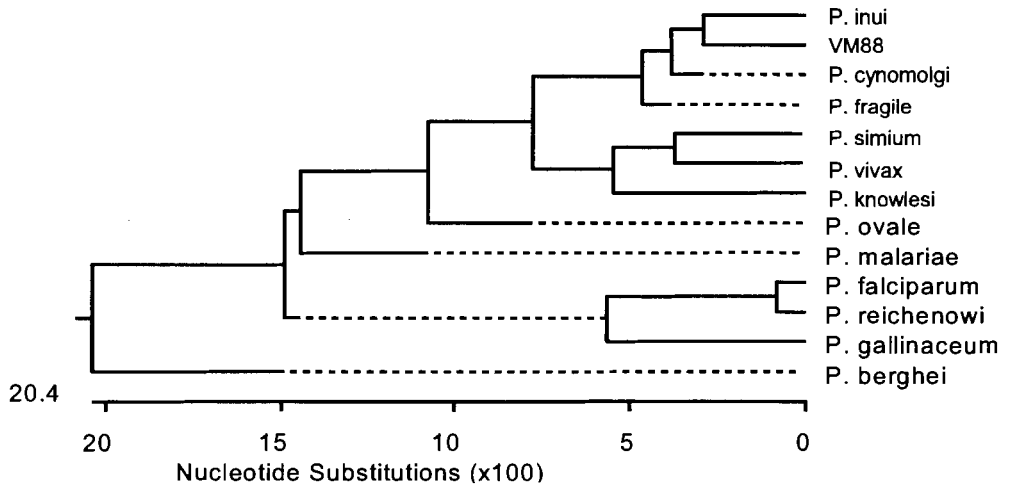


These three sequences are highly conserved showing variable areas in the region of bases 106-155, 633-672, 720-730 and 1018-1050 which indicates a closer

homology to *P. cynomolgi*. Despite sharing the greatest homology with *P. cynomolgi*, differences between VM82 and *P. cynomolgi* in the region of bases 143-155, 646-673 and 1023-1043 indicate that this is only a *P. cynomolgi*-like parasite. At the protein level, sample VM82 shares the greatest homology with *P. cynomolgi* at 85% in the region of bases 376-543, 92% in the region of bases 730-768, 92% in the region of bases 814-1023 and 96% in the region of bases 1040-1414.

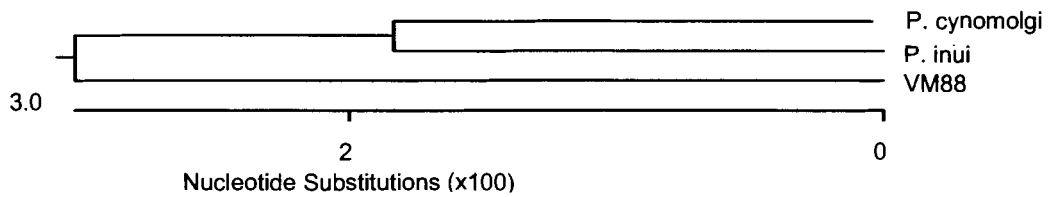
For Group 2, we designated sequence VM88 as our representative sample. Sample VM88 is 1544 bp in length and translates to a putative protein 514 amino acids in length. It shares significant similarity with *P. hylobati*, *P .inui*, *P. fieldi*, and *P. cynomolgi*. Sample VM88 shows 96% identity to *P. hylobati* in the region of bases 837-1400, 89% similarity in the region of bases 523-783 and 84% similarity in the region of bases 1436-1487. Similarity with *P. inui* is 97% in the region of bases 16-95, 96% in the region of bases 837-1408, 92% in the region of bases 1436-1487 and 90% in the region of bases 120-783. VM88 also shares 95% identity with *P. fieldi* in the region of bases 837-1408. 89% in the region of bases 525-700 and 92% in the region of bases 730-783. Finally, VM88 shares 95% identity to *P. cynomolgi* in the region of bases 837-1408, 91% identity in the region of bases 16-700, 92% in the region of bases 730-783 and 87% in the region of bases 1440-1487 (Appendix 3). A phylogenetic tree based on a nearest neighbour association of VM88 and downloaded reference sequences confirms a close association between VM88, *P. inui* and *P. cynomolgi* (See Figure 5.9).

Figure 5.9: Sequence VM88 Aligned with Reference Sequences-Nucleotide Level



To aid in the determination of which *Plasmodium* sp. is represented by this group, sample VM88 was aligned for comparison at the nucleotide level with its two most complete homologous matches (*P. inui* and *P. cynomolgi*) (Appendix 5) and a nearest neighbour phylogenetic tree was created (Figure 5.10).

Figure 5.10: Sequence VM88 Aligned with Reference Sequences of *P. inui* and *P. cynomolgi*-Nucleotide Level

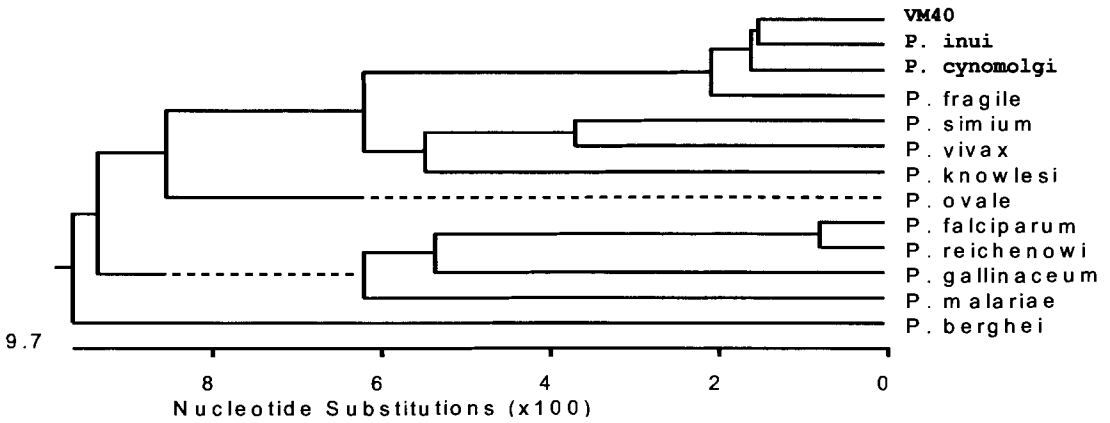


The sequences show high conservation but analysis of variable regions in these three sequences, especially two large areas in the region of bases 113-175 and 630-670, led to the identification of this *Plasmodium* sp. as being a *P. inui*-like parasite. This species is most homologous with *P. inui*, but varies from *P. inui* in the region of bases 110-175, 712-735, 789-887 and 1495-1551. At the protein level VM88 shares the greatest homology to *P. cynomolgi* at 88% in the region of bases 143-361, 44% in the

region of bases 476-610, 92% in the region of bases 914-1036, 94% in the region of bases 1053-1382.

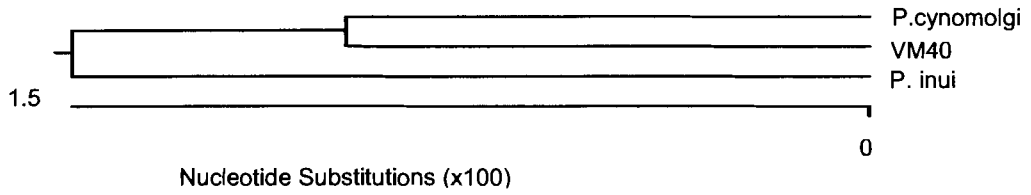
For Group 3, we designated sequence VM40 as our representative sample. VM40 is 1515 bp in length with a putative protein length of 505 amino acids. VM40 shares significant identity with three *Plasmodium* sp. at the nucleotide level: *P. hylobati*, *P. inui* and *P. cynomolgi*. Sample VM40 shares 96% and 97% identities with *P. hylobati* in the region of bases 522- 641 and 675-1510 respectively. It shares 95% identity to *P. inui* between regions 10-635, and 97% identity between regions 671-1510. VM40 also shares a close identity to *P. cynomolgi* sharing 97% identity in the region of bases 4-641 and 96% identity in the region of bases 671-1510 (Appendix 3). A nearest neighbour phylogenetic tree based on the alignment of VM40 with our reference sequences showed a close association between VM40, *P. inui* and *P. cynomologi* (Figure 5.11).

Figure 5.11: Sequence VM40 Aligned with Reference Nucleotide Sequences



To aid in the species identification of this sample it was aligned with its two closest complete homologues in (*P. inui* and *P. cynomologi*) (Appendix 6) and a nearest neighbour phylogenetic tree was created showing that VM40 associates more closely with *P. cynomolgi* (Figure 5.12).

Figure 5.12: Sequence VM40 Aligned with Reference Sequences of *P. inui* and *P. cynomolgi* -Nucleotide Level

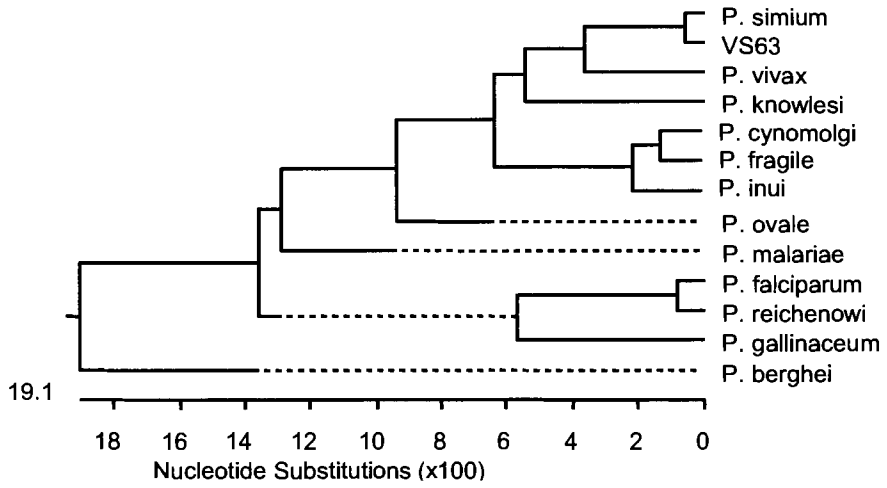


These sequences show large conserved areas but variable areas in the region of bases 143-173 and 637-671 led to the identification of this *Plasmodium* sp. as being homologous to *P. cynomolgi.*

At the protein level, T-BLAST X queries of this sequence returned 95% identity to *P. cynomolgi* in the region of bases 141-356, 100% identity in the region of bases 364-456, 97% identity in the region of bases 757-1026, and 96% identity in the region of bases 1042-1416.

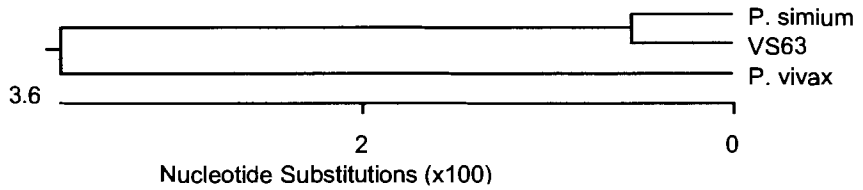
Group 4 is represented by one sequence that does not closely match any other sequences obtained in this study. This sequence (VS63) is 1582 bp in length and translates into a putative protein consisting of 526 amino acids. At the nucleotide level VS63 shows significant similarity to *P. simium* sharing 97% identity in the region of bases 10-786 and 98% identity in the region of bases 802-1582 and to *P. vivax* at 93% in the region of bases 5-786 and 98% in the region of bases 802-1358 (Appendix 3). A nearest neighbour phylogenetic tree shows the close association of VS63 with *P. simium* and *P. vivax* (See Figure 5.13).

Figure 5.13: Sequence VS63 Aligned with Reference Nucleotide Sequences



Sample VS63 was aligned with its two closest nucleotide homologues *P. simium* and *P. vivax* (Appendix 7) and a nearest neighbour phylogenetic tree was also created (Figure 5.14).

Figure 5.14: Sequence VS63 Aligned with Reference Sequences of *P. simium* and *P. vivax*



Alignments of *P. vivax*, *P. simium* and VS63 at the nucleotide are highly conserved but regions of variation in the region of bases 54-74, 147-167, 569-599, 1365-1447 and 1483-1520 indicate a closer homology to *P. simium*.

This relationship is strongly supported by the results of protein searches in T-BLAST X which indicate that VS63 shares 94% identity to *P. simium* in the region of

bases 10-273, 100% identity in the region of bases 284-460 and 96% identity in the region of bases 505-603 and 659-1447.

Chapter 6
DISCUSSION AND CONCLUSIONS

6.1 Discussion of Field Results

6.1.1 The Usefulness of the "Finger Prick" Method

One of the initial purposes of this research was, in part, to test for *Plasmodium* sp. infections without taking blood from the veins of orangutans through a method called the "Finger Prick" method. The success of this method has been well documented on humans in many countries. Unfortunately, orangutans are not ideal candidates for this method to collect blood samples. While it was possible to diagnose *Plasmodium* sp. infections from the "Finger Prick" it was not possible to get enough blood from an orangutan finger for more than one thick smear. At times even getting enough blood to make one thick smear was a challenge. Orangutans seem to have excellent clotting factors in their blood and combined with their thick skin and long bent fingers made it almost impossible at times to get any blood from their fingers. This technique was dropped because we decided that taking blood from the veins of an orangutan was easier and less traumatic on the animal.

6.1.2 Resident Orangutans vs. Newly Arrived Orangutans

Our results indicate that newly arrived orangutans to the OCC&Q are more likely to be infected with *Plasmodium* sp. than resident individuals (X^2=7.39, df=1, p=>0.01) but there are no differences between the sexes. There may be a number of reasons for higher *Plasmodium* sp. infection rates among newly confiscated orangutans such as

their increased proximity to humans, lack of a nutritious and normal diet, and stress (Wolfe *et al.*, 2002). Our data support a hypothesis that increased proximity to humans may increase the risk of *Plasmodium* sp. infections for newly confiscated individuals. If human proximity played a critical role in the transmission of *Plasmodium* sp. to orangutans we would expect that these individuals would be more likely to be infected with human *Plasmodium* sp. There are a number of possible explanations for higher *Plasmodium* sp. prevalences among newly arrived orphaned orangutans versus resident individuals, such as increased stress, insufficient diet, and increased exposure to vectors.

Unfortunately, there are no data available on the level of morbidity and/or mortality suffered by orangutans due to *Plasmodium* sp. nor is there data on the potential role of the immune response in clearing these infections. Observational data collected during this study found that a sick orangutan that was positive for infection with *Plasmodium* sp. showed no improvement upon treatment with antimalarial drugs that cleared the infection. This observational data was only collected from one individual that is far too small a sample size to make any generalizations.

Our resident orangutans are most comparable to the semi-captive orangutans tested by Wolfe *et al.* (2002) whose population showed an extremely high prevalence of *Plasmodium* sp. at 93.5%. This contrasts greatly from the prevalence of *Plasmodium* sp. we found in the semi-captive orangutans housed at the OCC&Q (20.3%). Originally, because of the location of the OCC&Q in a peatswamp forest, we had expected higher rates of *Plasmodium* sp. infection similar to those found at SORC by Wolfe *et al.* (2002). Wolfe *et al.* (2002) suggested that a number of behavioural and ecological factors may contribute to higher rates of *Plasmodium* sp. infection among semi-captive orangutans such as decreased arboreality, decreased day ranges, changes in social structure, increased population density, dietary changes and stress. Each of these factors is also

present to some degree in the orangutans housed at OCC&Q. Wolfe *et al.* (2002) also discussed the potential role of human activities in the high prevalence of *Plasmodium* sp. at SORC, including proximity to humans and human settlements, and the effects of facilities such as drainage ditches which increase the availability of standing water and vector mosquito population. However there is no data on what mosquito species might transmit non-human plasmodia to orangutans. Our much lower prevalence of *Plasmodium* sp. infections in the orangutans housed at the OCC&Q may indicate the presence of some factor limiting *Plasmodium* sp. infection rates at OCC&Q which is not present at SORC.

6.2 Discussion of DNA Laboratory Results

6.2.1 Difficulty Amplifying *Plasmodium* sp. DNA from Microscopically Positive Individuals

There were a number of factors that need to be weighed in choosing how to collect and preserve samples. We chose Whatman® FTA® Classic Cards as our storage medium mainly because of the ease in preparing them with samples in the field. Samples were prepared and allowed to dry on the cards preserving them in a non-biohazardous format. This meant that we did not need Health Canada permits to import biohazardous materials, or Agriculture Canada permits for the importation of primate "products". This also meant that while we waited 15 months to obtain the CITES permits necessary to export the blood samples to Canada, our samples were safely preserved.

We found difficulties in amplifying or eluting *Plasmodium* sp. DNA from individuals with low level infections. Singh *et al.* (1999) found that they were able to detect lower parasitemia levels through PCR more reliably than via microscopy. Our results are unable to support this finding as we had difficulty in amplifying *Plasmodium* sp DNA from samples estimated to have infections under 200 parasites per microlitre.

6.2.2 Sequencing Results

As stated at the end of Chapter 5 the results of our laboratory DNA analyses indicate the presence of four distinct groups of *Plasmodium* sp. present in the orangutans housed at the OCC&Q. This is based on sequencing a ~1500 bp segment of the 18S sRNA considered an "ideal target" for *Plasmodium* sp. identification (Li *et al.*, 1995). The isolation of four plasmodia from the blood of orangutans may be a remarkable finding. Previous *Plasmodium* sp. identification in orangutans had been based solely on parasite morphology and had identified only two plasmodia species in orangutans; *P. pitheci* and *P. silvaticum*. There are no DNA sequences for these two species with which we could compare our data. There are unpublished reports that claim that the macaque *P. inui* has also been found in orangutans housed at SORC in Sabah Malaysia (Wolfe *et al.*, 2002), but these data have yet to be published.

The finding of four species of *Plasmodium* sp. parasites in the blood of orangutans sampled at OCC&Q may be also important to the veterinary staff at OCC&Q. Until now, they believed that the OCC&Q orangutans were experiencing only infections with *P. pitheci*. This assumption was supported by the data collected at SORC by Wolfe *et al.* (2002) who also found only evidence of *P. pitheci* infections in their orangutans despite it being the only site from which samples morphologically identified as *P. silvaticum* had been found. Because we do not have access to *P. silvaticum* or *P. pitheci* sequences, it is possible that two of our four sequences may represent *P. pitheci* or *P. silvaticum*. These are *P. vivax* like plasmodia as are the macaque plasmodia *P. cynomolgi*, *P. inui, P. fragile* and *P. knowlesi* (Escalante and Ayala, 1995; Escalante *et al.*, 2005; Leclerc *et al.*, 2004; Mu *et al.*, 2005; Rathore *et al.*, 2001 and Waters *et al.*, 1993).

Based on our analyses we believe that the sequences described above as Group 1 represent a *P. cynomolgi*-like parasite, while the sequences described above as Group 2 represent a *P. inui*-like parasite. These two *Plasmodium* species may represent the two orangutan specific members of the *P. vivax*/Southeast Asian primate *Plasmodium* sp. group of parasites, *P. pitheci* and *P. silvaticum* which had previously been solely identified via morphological characteristics (Peters *et al.*, 1976 and Wolfe *et al.*, 2002). Unfortunately, the confirmation of, or exclusion against, these two *Plasmodium* sp. as being *P. pitheci* and/or *P. silvaticum* is not possible. There are no known reference samples of *P. pitheci* or *P. silvaticum* which have been previously identified based on morphological characteristics that we could use as genetic type specimens for comparison against our two unknown *Plasmodium* sp. sequences.

The group 3 sequences align best with *P. cynomolgi* which is another macaque specific *Plasmodium* sp. that can infect humans. *Plasmodium cynomolgi* was originally identified by Martin Mayer in 1907 in the blood of a crab-eating macaque (*Macaca fascicularis*) from Java (Coatney *et al.*, 1971) and is also a member of the *P. vivax*/Southeast Asian primate plasmodia group (Escalante and Ayala, 1995; Escalante *et al.*, 2005; Leclerc *et al.*, 2004; Mu *et al.*, 2005; Rathore *et al.*, 2001 and Waters *et al.*, 1993). Interestingly, this species has been studied in great detail because it shares a number of traits with *P. vivax*, such as similar trophozoites and gametocytes (Coatney *et al.*, 1971), and as a result has been studied as the primate counterpart to human *P. vivax* (Waters *et al.*, 1993).

If our interpretation is correct, and our Group 3 *Plasmodium* sp. is *P. cynomolgi* this would represent strong evidence of the cross-species transfer of *Plasmodium* sp between macaques and orangutans. Macaques are quite common throughout Kalimantan including the area surrounding the OCC&Q. They can often been seen crossing the road both into and out of the nursery forest used by orphaned orangutans

housed at OCC&Q as part of their rehabilitation process. Thus, finding a macaque *Plasmodium* sp. in orangutans sharing these forests should come as no surprise.

Until now there have never been any confirmed reports of orangutans being infected with *Plasmodium* sp. specific to other species of primate. There is one report of orangutans being infected by the macaque specific parasite *P. inui* (Wolfe *et al.*, 2002) but the data supporting this claim have never been published. Humans have been infected naturally with at least two other species of macaque plasmodia so it should not be surprising that we might find one of these species in orangutans housed at OCC&Q.

Another macaque parasite *P. knowlesi*, also may infect other primates. For over 70 years it has been known to be infective to humans and was employed as a treatment for neurosyphilis (Coatney *et al.*, 1971). Since 1965, *P. knowlesi* has been known to naturally infect humans in Malaysia (Coatney *et al.*, 1971) and it continues to be an important zoonotic infection in Southeast Asia (Jongwutiwes *et al.*, 2004 and Singh *et al.*, 2004).

The *Plasmodium* sp. represented by the sequence referred to above as Group 4 is the most surprising. As stated in Chapter 5, this sequence aligns most closely with *P. simium* and *P. vivax*. Because *P. simium* is a *Plasmodium* sp. of New World monkeys, we can be fairly certain that this infection is not *P. simium*. If we only include plasmodia known to be in Southeast Asia in our analysis of this sequence then it becomes apparent that what we truly have here is the first report of an orangutan being infected with human *P. vivax*. Our interpretation of these results is supported by the fact that many types of *P. vivax* are almost identical to *P. simium* (Escalante et al., 1998 and 2005) and the fact that this individual was a recent arrival at OCC&Q, that had had extensive interactions with humans, and who arrived with this infection.

Plasmodium vivax is referred to as the benign tertian malaria that refers to its 48 hour asexual reproductive phase (Marquardt *et al.*, 2000) and its lower levels of mortality

than *P. falciparum*. This is one of the most widespread of the human plasmodia infecting 70-80 million humans in the low-lying, coastal or marshy areas of the temperate zones of Latin America, Africa, the Middle East and Asia (Coatney *et al.*, 1971 and Mu *et al.*, 2005). This geography and climate of Central Kalimantan in the region around the OCC&Q has the potential to harbour *P. vivax* and its vector(s) as it is warm throughout the year and is swampy. The *P. vivax* parasite(s) of Central Kalimantan have not yet been studied but we can assume they are present in the region based on the results of our sequence and reports of chloroquine resistant *P. vivax* in West Kalimantan (the next province to the west of Central Kalimantan) (Fryauff, 1998).

It should not come as a big surprise that *P. vivax* is infective to orangutans but it does have major implications for orangutan conservation initiatives. Current evidence suggests a zoonotic origin for *P. vivax* arising 10,000-81,607 years ago from a macaque *Plasmodium* sp (Escalante *et al.*, 2005; Leclerc *et al.*, 2004 and Mu *et al.*, 2005). With humans being genetically closer to orangutans than to macaques, it only seems logical that if *P. vivax* arose as a result of a recent host switch then orangutans could also be infected with *P. vivax*.

The finding that *P. vivax* may be infective to orangutans could have major implications in terms of orangutan rehabilitation and reintroduction efforts in Kalimantan and perhaps even in Sumatra. In humans, *P. vivax* can cause true relapse for 2 to 30 years (Coatney *et al.*, 1971). If this also occurs in orangutans, then individuals released back to the wild have the potential (if suitable vectors are present in the release area) to spread this parasite to other orangutans in the area. For this reason it is important that we learn how this parasite affects orangutans. We need to understand whether the human plasmodia cause morbidity or mortality in orangutans to determine whether it is a serious threat to orangutan health and conservation efforts. It may require that all orangutans coming from human captivity need to be treated with antimalarial drugs

effective at killing *P. vivax* but to date no antimalarial drugs are capable of preventing true relapse. These data may provide the impetus for studying Sumatran orangutans for the presence of *Plasmodium* sp. because even if *P. pitheci* and *P. silvaticum* are not naturally found on Sumatra, *P. vivax* is present and may infect Sumatran orangutans.

6.3 Recommendations for Future Research

This section gives recommendations for future research along with suggested changes to the research methodologies used in this study. We hope that others can learn from our methods and use our recommendations to make future research on this topic easier, giving us a broader picture of how *Plasmodium* sp. infections may impact orangutan conservation efforts in Indonesian and Malaysian Borneo. This section is divided into three parts: 1) Recommendations for Future Field Research; 2) Methodology for Field Laboratory Analysis; and 3) DNA Laboratory Methodology.

6.3.1 Recommendations for Future Field Research

We have a number of recommendations for future field research and data collection techniques on this topic. The first and probably most important is a longer-term study of *Plasmodium* sp. prevalence and species abundance at OCC&Q especially, in light of our discovery of potential human *P. vivax* in orangutans. The potential role of *P. vivax* infections in orangutan rehabilitation and reintroduction needs to be further explored. This study should be focused on collecting data during the wet season as our current study provides data from the height of the dry season. This would allow for seasonal comparisons of *Plasmodium* sp. prevalence and species abundance.

Part of a longer-term field study should be a greater attempt to determine how *Plasmodium* sp. infections affect orangutans through behavioural observations and then collection of more detailed medical data. We are still uncertain as to the impact of

Plasmodium sp. infections in orangutans and without these data it is difficult to estimate its threat to orangutan conservation efforts. If *Plasmodium* sp. infections are found to cause morbidity and mortality in semi-captive orangutans, but not in wild orangutans then wild orangutans should be monitored and samples of any plants eaten should be collected for the analysis of any secondary compounds which may be present, such as those recently isolated by Krief et al., (2004). Such data may help explain the discrepancy between the *Plasmodium* sp. prevalence in newly confiscated orangutans and OCC&Q residents.

Another key aspect of any future research projects of *Plasmodium* sp. in orangutans should be to expand the geographical areas covered. This project is one of the first studies of *Plasmodium* sp. in Indonesian Borneo. Research needs to be expanded to cover more orangutan research and rehabilitation sites in other parts of Kalimantan. *Plasmodium* sp. research also needs to be undertaken in Sumatra. Officially, *Plasmodium* sp. does not exist in Sumatran orangutans, but there are unconfirmed reports of malaria-like symptoms and their response to antimalarial drugs, which is indicative of possible *Plasmodium* sp. infections.

Finally, it is important for any future research on this subject to include two research goals not tied directly to orangutans. The first goal should be directed at attempting to identify potential mosquito vectors in the region. This can be done both relatively easily and cheaply using baited mosquito traps hung near orangutan sleeping cages. We have no information on the potential vectors in this region, which is a key to understanding how the plasmodia in this region are transmitted. It is believed that all mammalian plasmodia are transmitted by *Anopheles* sp. mosquitoes (Cox, 1993), but there is no evidence to suggest or contradict this speculation. The second goal should study the *Plasmodium* sp. prevalence and species abundance in the human population

in the region. This would help confirm whether primate and human plasmodia are being passed between the human and nonhuman primate populations in the region.

6.3.2 Methodology for Field Laboratory Analyses

There are two methodological changes to our field data collection methods that we would make to any future study of *Plasmodium* sp. in orangutans. First, we would find a better way to estimate *Plasmodium* sp parasitemias. The current OCC&Q protocols for estimating *Plasmodium* sp. parasitemias is a modification of the method described in Cheesbrough (1999) (See page 91). This method may not be very accurate and may over or underestimate the total parasitemias because it is based on the number of WBCs present. WBC levels may be artificially inflated in these orangutans due to illness and infections thus using this technique may artificially over-estimate parasitemia levels.

The other methodological change has to do with the preparation and storage of the Whatman®FTA® Cards. For any future research we would suggest collecting the maximum amount of blood (500µl) recommended by the manufacturer for each card. Also, to maximize the number of red blood cells collected we would recommend centrifuging the blood prior to loading onto the FTA® Cards in order to concentrate the blood cells and separate the blood plasma. This may allow for easier detection of low parasitemias via PCR. Genomic DNA is said to be stable for up to 11 years if preserved on Whatman® FTA® Cards (Whatman, 2002). To help ensure the best preservation of blood samples on the cards we recommend that they be stored with silica gel in order to ensure their protection from humidity and moisture (Karesh Pers. comm.).

6.3.3 DNA Laboratory Methodology

We only have a couple of minor recommendations for future DNA research into the plasmodia infective to orangutans. Our first recommendation is to elute DNA from the FTA® Cards. The sensitivity of our PCR reactions seemed to increase if the DNA was previously eluted from the cards prior to use in PCR reactions rather than using a cleaned disc in the PCR reaction.

6.4 Conclusions

This project used a combination of traditional microscopic diagnosis and molecular biological analysis via PCR to identify the *Plasmodium* sp. found in semi-captive orangutans housed at the Orangutan Care Center and Quarantine in Central Kalimantan, Indonesian Borneo. We microscopically examined the blood of 87 orangutans that were divided into three residence categories. We tested 69 OCC&Q resident orangutans that had been living at OCC&Q for at least three months or, if they had been resident for a year or more they could not have been treated for *Plasmodium* sp. infections during the past year. Of these 69 orangutans 14 were microscopically diagnosed with *Plasmodium* sp. We also tested 14 newly confiscated orangutans who had been living at OCC&Q for anywhere from a few hours to a maximum of three months. Of these 14 newly arrived orangutans, eight were microscopically positive for *Plasmodium* sp. Finally, we tested two previously released ex-captive orangutans from *Pondok Tanggui* who were brought to the OCC&Q to get treatment for severe amoebic dysentery infections, both of which had *Plasmodium* sp infections.

Blood samples collected and preserved on Whatman® FTA® Cards from the 24 orangutans that were microscopically positive for *Plasmodium* sp. were used in species identification via PCR. Using specially designed primers we were successfully able to

amplify, clone and sequence a ~1500 bp region of the *Plasmodium* sp. 18S sRNA gene from 13 of the 24 microscopically positive orangutans. We experienced difficulty in amplifying *Plasmodium* sp. DNA from the blood of orangutans whose parasitemia had been estimated at less than 200/μl.

Bioinformatic analysis of these 13 sequences indicates the presence of four *Plasmodium* sp. in the orangutans housed at the OCC&Q. Each of these *Plasmodium* species appears to be members of the *P. vivax*/Southeast Asian primate *Plasmodium* sp. group. Two of the species we detected, a *P. inui*-like parasite and a *P. cynomolgi*-like parasite, may represent the two orangutan *Plasmodium* spp. (*P. pitheci* and *P. silvaticum*) which had been previously described based on morphological characteristics. Unfortunately, no reference materials of these previously identified orangutan *Plasmodium* sp. are available for comparison. A third group of sequences appears to be a cross-species infection with the macaque *Plasmodium* sp., *P. cynomolgi*. This is the first confirmed report of a macaque *Plasmodium* sp. infecting orangutans. Our final group of genetically identified *Plasmodium* species is the human *Plasmodium* sp., *P. vivax*. This may be our most important finding for researchers involved in orangutan research and conservation efforts because it is the first scientific evidence of a human *Plasmodium* sp. infecting orangutans.

APPENDICES

Appendix 1: Sequences of Amplified *Plasmodium* sp. DNA from Orangutans.

Sample ID	Sex	Resident/New Arrival/Free Ranging	Sequence Length (bp)	Group	Greatest Homology
VS14	Male	New Arrival	1518	Group 2	*P. inui*-like
VS21	Female	New Arrival	1515	Group 3	*P. cynomolgi*
VS25	Female	New Arrival	1523	Group 1	*P. cynomolgi*-like
VS28	Male	New Arrival	1520	Group 1	*P. cynomolgi*-like
VS32	Female	New Arrival	1524	Group 3	*P. cynomolgi*
VS33	Female	New Arrival	1516	Group 3	*P. cynomolgi*
VM40	Male	Resident	1515	Group 3	*P. cynomolgi*
VM55	Male	Resident	1514	Group 3	*P. cynomolgi*
VS63	Female	New Arrival	1582	Group 4	*P. vivax*
VM71	Male	Free Ranging	1518	Group 3	*P. cynomolgi*
VM75	Male	Free Ranging	1528	Group 1	*P. cynomolgi*-like
VM82	Female	Resident	1519	Group 1	*P. cynomolgi*-like
VM88	Male	Resident	1544	Group 2	*P. inui*-like

Appendix 2: Aligned Sequences of the 18S sRNA *Plasmodium* sp. Gene Available in GenBank

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                10        20        30        40        50        60        70        80
P.berghei       TTTATTATAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTG-TTA-AG TACTTTTA-- --CTCCCCGG AGTAA----T
P.cynomolg      -TTCTTATAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AG CACTCTTG-- ---ATTAAGT TCTTG-----
P.falcipar      TTTATTATAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AT TATCCTTT-- -GATTTT-TA TCTTT----G
P.fragile       -TCTATATAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AG CACTCTTG-- ---ATTTATT TCTTG-----
P.gallinac      -TTTTTATAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGACCG-AT TGTTCCTCAT CGCCTTTATA GGTAATGAAG
P.inui          ---------- GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTTCAG CACTCTTG-- ---ATTAAGT TCTTG-----
P.knowlesi      -TCTATATAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AG CACTCTTGAT --TT---ATT TCTTG-----
P.malariae      -TTTTTATAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AA TACTCTTA-- --ATTCTTTA TGTTTTTT-G
P.ovale         -TACTTACAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AA TACGTTTGGT -TCATTTTTG TCTCTT---A
P.reicheno      -TTATTATAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AT TATCCTTT-- -GATTTTATA TCTTA----G
P.simium        ---------- GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AA TGCTCCCGAC --GTGTTACG TCTTGT---G
P.vivax         -TTTCTATAA GGATAACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AA TGCTCTCGAC --GA---ATG TCTTG-----
Clustal Co                **********  **********  **********  ***     *                       *

                  90        100       110       120       130       140       150       160
P.berghei       TGTATGTATT TGTTAAGACC C-CTAAGAAA AAA-T-GAT- -ATTAAAGGA ATTATAACAA AGAAGCAACA CAT--AATAT
P.cynomolg      AGTGTGTACT TGTTAAGCCT T-TTAAGAAA AAAGT-TATT AACTTAAGGA ATTATAACAA AGAAGTAACA CGT--AATGG
P.falcipar      GATAAGTATT TGTTAGGCCT T-ATAAGAAA AAAGT-TATT AACTTAAGGA ATTATAACAA AGAAGTAACA CGT--AATAA
P.fragile       AGTGTGTACT TGTTAAGCCT T-ATAAGAAA GAAGT-TATT AACTTAAGGA ATTATAACAA AGAAGTAACA CGTGAAATGG
P.gallinac      AACACGTATT TGTTAAGCCT T-ATAAGAAA AAAGT-TATT AATTTAAGGA ATTATAACAA AGAAGCAACA CAT--AATAA
P.inui          AGTGTGTACT TGTTAAGCCT T-TTAAGAAA AAAGT-TATT AACTTAAGAA GTTATAACAA AGAAGCAACA CGT--AATAG
P.knowlesi      AGTGTGTACT TGTTAAGCCT T-ATAAGAAA AGAGT-TATT AACTTAAGGA ATTATAACAA AGAAGTAACA CGT--AATGG
P.malariae      AGTATGTATT TGTTAAGCCT T-ATAAGAGA AAAGTATATT AACTTAAGGA AT-ATAACAA AGAAGTAACA CAT--AATAA
P.ovale         CGTATGTACT TGTTAAGCCT T--TAAGAGA AAAGTTTAC- AACTTAAGGA ATTATAACAA AGAAGTAACA CAT--AATAA
P.reicheno      GATAAGTACT TGTTAGGCCT T-ATAAGAAA AAAGT-TATT AACTTAAGGA ATTATAACAA AGAAGTAACA CGT--AATAA
P.simium        AGCATGTACT TGTTAAGCCT TTATAAGAAA AAAGT-TAAT AACTTAAGGA ATGATAACAA AGAAGTGACA CATAAAAAGG
P.vivax         GGCATGTACT TGTTAAGCCT TTATAAGAAA AAAGT-TAAT AACTTAAGGA ATGATAACAA AGAAGTGACA CATAGAA-GG
Clustal Co        *** * ***** * *     ***** *   * *  *    * * *** *  * ******* *****  *** * *  **
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                    170        180        190        200        210        220        230        240
P.berghei           A--------- ATTATTCAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TA-GGGTATT GACCT-AACA
P.cynomolg          ATCCG-TCCA TTTTT--AGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TA-GGGTATT GGCCT-AACA
P.falcipar          A----TTTAT TTTATTTAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TA-GGGTATT GGCCT-AACA
P.fragile           ATTAACTCCA TTTTTTTAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TACGGGTATT GGCCT-AACA
P.gallinac          AA--CTTTGT TTTATTTAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TA-GGGTATT GGCCT-AACA
P.inui              GTATTTTCCA TTTTT--AGT GTGTTACTAA CGAGTTTCTG ACCTATCAGC TTTTGA-TGT A--GGGTATT GGCCT-AACA
P.knowlesi          ATTC-TTCCA TTT-TTTAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TA-GGGTATT GGCCTTAACA
P.malariae          A---TTTCGA TTTATTTAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TA-GGGTATT GGCCT-AACA
P.ovale             G----TTTAC CTTATTTAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TA-GGGTATT GGCCT-AACA
P.reicheno          A----TTTAT TTTATTTAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TA-GGGTATT GGCCT-AACA
P.simium            ACTCGTCCCA TTT-TCTAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGAATGT TA-GGGTAT- GGCCT-AACA
P.vivax             ACCTGCGTCC ATT-TATAGT GTGTATCAAT CGAGTTTCTG ACCTATCAGC TTTTGA-TGT TA-GGGTATT GGCCT-AACA
Clustal Co                      ** *  *** ****  * *  ********** ********** ****** ***    ******  * *** ****

                    250        260        270        280        290        300        310        320
P.berghei           TGGCTTTGAC GGGTAACGGG GAATTAGAGT TCGATT--CC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.cynomolg          TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGATT--CC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.falcipar          TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGATT--CC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.fragile           TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGATT--CC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.gallinac          TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGATT--CC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.inui              TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGATC--CG GAGAGGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.knowlesi          TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGACT--TC CGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.malariae          TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGATT--CC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.ovale             TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGATT--CC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.reicheno          TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGATT--CC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.simium            TGGCTATGAC GGGTAACGGG GAATTAGAGT TCGATTCTCC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
P.vivax             TTGCTATGAC GGGTAACGGG GAATTAGAGT TCGATT--CC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG
Clustal Co          * *** **** ********** ********** ****           ***** ********** ********** **********
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                330        340        350        360        370        380        390        400
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P.cynomolg      GCAGCAGGCG CGTAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TACAAGGCCA A--TCTGGCT
P.falcipar      GCAGCAGGCG CGTAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TGCAAGGCCA ATTTTTGGTT
P.fragile       GCAGCAGGCG CGTAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TACAAGGCCA A--TCTGGCT
P.gallinac      GCAGCAGGCG CGTAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TGCAAGGCCA AATTTTGGTT
P.inui          GCAGCAGGCG CGTAAATTAC CCAATTCTAA ATGAAGAGAG GTAGTGACAA GAAATAACAA TACAAGGCCA A--TCTGGCT
P.knowlesi      GCAGCAGGC- --TAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TACAAGGCCA AT--CTGGCT
P.malariae      GCAGCAGGCG CGTAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TGCAAGGCCA AATTTTGGTT
P.ovale         GCAGCAGGCG CGTAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TACAAGGCCA TTTCATGGTT
P.reicheno      GCAGCAGGCG CGTAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TGCAAGGCCA ATTTTTGGTT
P.simium        GCAGCAGGCG CGTAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACGA TACAAGACCA AA-ACTGGTT
P.vivax         GCAGCAGGCG CGTAAATTAC CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TACAAGACCA AA-ACTGGTT
Clustal Co      *********    ******** ********** *  ******* ********** ******** * *  *** ***      *** *

                410        420        430        440        450        460        470        480
P.berghei       TTATAATTGG AATGATGGGA ATTTAAAACC TTCCCAAAA- ATCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.cynomolg      TTGTAATTGG AATGATGGGA ATTTAAAACC TTCCCAAAA- CTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.falcipar      TTGTAATTGG AATGGTGGGA ATTTAAAACC TTCCCAGAGT AACAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.fragile       TTGTAATTGG AATGATGGGA ATTTAAAACC TTCCCAAAA- TTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.gallinac      TTGCAATTGG AATGATAGGA ATTTAAAAAC TTCCTAAAGT AACAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.inui          TTGTAATTGG AATGATGGGA ATTTAAAACC TTCCCAAAA- CTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.knowlesi      TTGTAATTGG AATGATGGGA ATTTAAAACC TTCCCAAAA- TTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.malariae      TTGCAATTGG AATGATGGGA ATTTAAAACC TTCCCAGAA- GGCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.ovale         TTGTAATTGG AATGATGGGA ATTTAAAACC TTCCCAAAA- TTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.reicheno      TTGTAATTGG AATGGTGGGA ATTTAAAACC TTCCCAGAGT AACAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
P.simium        TTGTAATTGG AATGATGGGA ATTTAAATCC TTCCCATAA- TACAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCG-TAA
P.vivax         TTGTAATTGG AATGATGGGA ATTTAAATCC TTCCCATAA- TACAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA
Clustal Co      **  ****** **** * *** *******  * **** * *    ******** ********** ********** ****** ***
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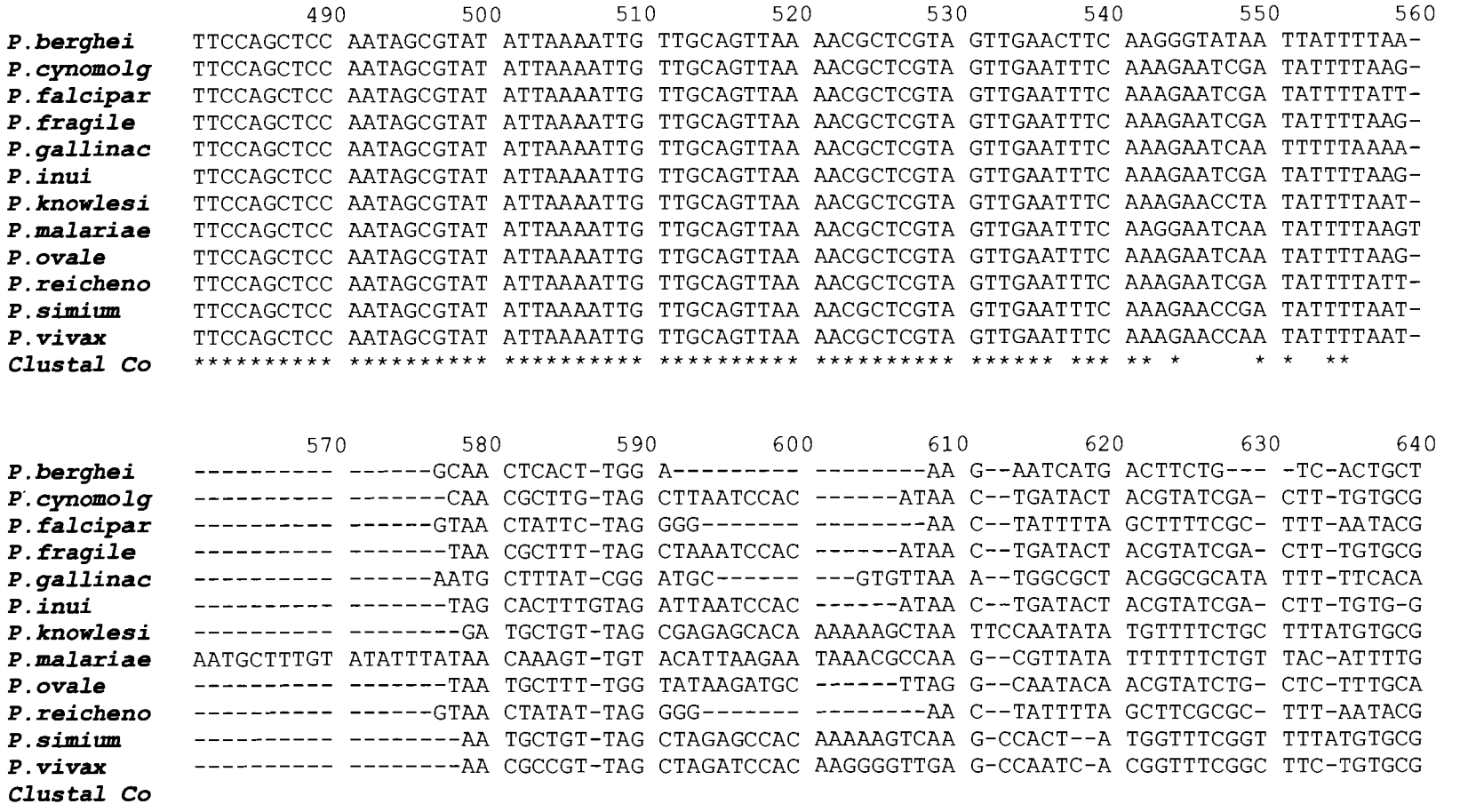
490 500 510 520 530 540 550 560
P.berghei TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAACTTC AAGGGTATAA TTATTTTAA-
P.cynomolg TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTAAG-
P.falcipar TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTATT-
P.fragile TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTAAG-
P.gallinac TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCAA TTTTTAAAA-
P.inui TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTAAG-
P.knowlesi TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAACCTA TATTTTAAT-
P.malariae TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAGGAATCAA TATTTTAAGT
P.ovale TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCAA TATTTTAAG-
P.reicheno TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTATT-
P.simium TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAACCGA TATTTTAAT-
P.vivax TTCCAGCTCC AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAACCAA TATTTTAAT-
Clustal Co ********** ********** ********** ********** ********** ****** *** ** * * * ** *
570 580 590 600 610 620 630 640
P.berghei ---------- ------GCAA CTCACT-TGG A--------- --------AA G--AATCATG ACTTCTG--- -TC-ACTGCT
P.cynomolg ---------- -------CAA CGCTTG-TAG CTTAATCCAC ------ATAA C--TGATACT ACGTATCGA- CTT-TGTGCG
P.falcipar ---------- ------GTAA CTATTC-TAG GGG------- --------AA C--TATTTTA GCTTTTCGC- TTT-AATACG
P.fragile ---------- -------TAA CGCTTT-TAG CTAAATCCAC ------ATAA C--TGATACT ACGTATCGA- CTT-TGTGCG
P.gallinac ---------- ------AATG CTTTAT-CGG ATGC------ ---GTGTTAA A--TGGCGCT ACGGCGCATA TTT-TTCACA
P.inui ---------- -------TAG CACTTTGTAG ATTAATCCAC ------ATAA C--TGATACT ACGTATCGA- CTT-TGTG-G
P.knowlesi ---------- --------GA TGCTGT-TAG CGAGAGCACA AAAAAGCTAA TTCCAATATA TGTTTTCTGC TTTATGTGCG
P.malariae AATGCTTTGT ATATTTATAA CAAAGT-TGT ACATTAAGAA TAAACGCCAA G--CGTTATA TTTTTTCTGT TAC-ATTTTG
P.ovale ---------- -------TAA TGCTTT-TGG TATAAGATGC ------TTAG G--CAATACA ACGTATCTG- CTC-TTTGCA
P.reicheno ---------- ------GTAA CTATAT-TAG GGG------- --------AA C--TATTTTA GCTTCGCGC- TTT-AATACG
P.simium ---------- --------AA TGCTGT-TAG CTAGAGCCAC AAAAAGTCAA G-CCACT--A TGGTTTCGGT TTTATGTGCG
P.vivax ---------- --------AA CGCCGT-TAG CTAGATCCAC AAGGGGTTGA G-CCAATC-A CGGTTTCGGC TTC-TGTGCG
Clustal Co

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                650        660        670        680        690        700        710        720
P.berghei       TTT-ATCCTT GT-TGCA--- GTTCTTT--- TAATACAGGG CCCTTTGAGA GCCCATTAAT T--------- TATGACTGGG
P.cynomolg      CATTTTGCTA -T-TATGT-- GTTCTTTTAA TTAAAATGAT TCTCTTTTAA GGTCTTTCTT TTGC--TTCG GCATTTGAAG
P.falcipar      CTT-CCTCTA TTATTAT--- GTTCTTTAAA TAACAAAGAT TCTTTTTAAA ATCCCCACTT TTGCTTTTGC TTTTTTTGGG
P.fragile       CATTTTGCTA -T-TATGT-- GTTCTTTTAA TTAAAATGAT TCT-TTTTAA GGTCTTTCTT TGGC--TCCG GC-TATGAAG
P.gallinac      AAT-CTGATA ATATGCG--- TTTTTTAAAA TAAAATAGAT TCTTTTTAAA AATTCTTCGT T-GCTTTTTA AGTGATGAGA
P.inui          CATTTTTCTA CT-TATGT-- GTTCTTTTAA TTAAAATGAT TCTTTTTAAG ACTTTCTTTT TTGC--TTCG GCATTTTAGG
P.knowlesi      CATCCTCTAC CTATTAAGT- GTTAATTAAA TTAAAGGGTT TCTTTTTAAA ATCTTCTATA ACTAATAAAA ATATATGGAA
P.malariae      TTTTATTAAT ATATATATGC GTTCTTATTA TAAAAATGAT TCTTTTTAAA ATTCTTTTGT GTAATTTTT- TATGCATGGG
P.ovale         TTC-CTTATC CAAAATGT-- GTTCTTATTA TAAAAAGGAT TCTTTTTAAA ATCTCCTTTA C------TTT TTGTACTGGA
P.reicheno      CTT-CCTCTA TTATTAT--- GTTCTTTAAA TAACAAAGAT TCTTTTTAAA ATCCCCACTT T-GCTTTTAT GCTTTTTGGG
P.simium        CATCT-CTAC CTATCAAGTT GTTTTTTTAA TTAAAGTGTT TCTTTTTAAA ATCTTCTTTA GCTTA---AA ACATATGGAA
P.vivax         CATC--CTAC CTATCAAGC- GTTTTTTTAA TTAAAGTGTT TCTTTTTAAA ATCTTCTTTA CCTTA---AC -CATATGGAA
Clustal Co                            **  *     * * *  *    *  **

                730        740        750        760        770        780        790        800
P.berghei       TTTCTCGTTA CTTTGAGTAA ATTAGAGTGT TTAAAGCAAA CAGA-TAAAG CGTATTTTAC TGTGTTT-GA ATACTATAGC
P.cynomolg      AT-CTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGA-TATAG C----ATT-G CGCGTTT-GA ATACTACAGC
P.falcipar      GATTTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGT-TAAAG C--ATTT-AC TGTGTTT-GA ATACTATAGC
P.fragile       AT-CTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGA-TATAA C----CTTTG TGTGTTT-GA ATACTACAGC
P.gallinac      ATTTTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGTGTATAA C--AGGCAAC TGTGTTT-GA ATACTACAGC
P.inui          AGACTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGA-TATAG C----ATT-A TGCGTTT-GA ATACTACAGC
P.knowlesi      GATTTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGA-TATAT A--GCACTTG CGCGTTT-GA ATACTACAGC
P.malariae      AATTTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGT-TAAAA C---AGTTTC TGTGTTT-GA ATACTACAGC
P.ovale         GATTTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGT-TAAAG C--ATTTTAC TGCGTTT-GA ATACTACAGC
P.reicheno      GATTTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGT-TAAAG C--ATTTTAC TGTGTTT-GA ATACTATAGC
P.simium        GGTTTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA CAGA-TATA- ---GCA-TAA TGCGTTT-GA ATACTACAGC
P.vivax         GATTTTGTTA CTTTGAGTAA ATTAAAGTGT TCATAGCAAA CAGA-TACA- ---GCA-TTG CGCGTTTTGA ATACTACAGC
Clustal Co        * **** ********** **** ***** * * ****** ***  ** *             * **** ** ****** ***
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                  810        820        830        840        850        860        870        880
P.berghei         ATGGAATAAC AACATTGAAT AGGTCAAAAG TTTTTGAAAA ATTTTT--CT TATTTTGGCT TAGATACAGT TAATAGGAGT
P.cynomolg        ATGGAATAAC GAAATTGAAC AAGTCAGAA- -TTTT-GTTC TTTTTT--CT TATTTTGGCT TAGTTACGAT TAATAGGAGT
P.falcipar        ATGGAATAAC AAAATTGAAC AAGCTAAAA- TTTTTTGTTC TTTTTT--CT TATTTTGGCT TAGTTACGAT TAATAGGAGT
P.fragile         ATGGAATAAC AAAATTGAAC AAGTCAGAA- -TTTTTGTTC TTTTTT--CT TATTTTGGCT TAGTTACGAT TAATAGGAGT
P.gallinac        ATGGAATAAC AAAATTGAAC AAGCTAAAA- TTTTT-GTTC TTTTTT--CT TATTTTGGCT TAGTTACGAT TAATAGGAGT
P.inui            ATGGAATAAC AAAATTGAAC AAGT-AGAA- -TTTT-GTTC TTATTTTACT TATTTTGGCT TAGTTACGAT TAATAGGAGT
P.knowlesi        ATGGAATAAC AAAATTGAAC AGGTCAAAA- --CTATGTTT CTTTTTTTTT ATTTTTGGCT TAGTTACGAT TAATAGGAGT
P.malariae        ATGGAATAAC AAAATTGAAC AAGTCAGAA- --TTTTGTTC TTTTTT--CT TATTTTGGCT TAGTTACGAT TAATAGGAGT
P.ovale           ATGGAATAAC AAAATTGAAC AAGTCAAAA- --CTCTGTTC CTTTTT--CT TATTTTGGCT TAGTTACGAT TAATAGGAGT
P.reicheno        ATGGAATAAC AAAATTGAAC AGGCTGAAA- TTTTTTGTTC TTTTTT--CT TATTTTGGCT TAGTTACGAT TAGTAGGAGT
P.simium          ATGGAATAAC AAAATTGAAC AAGTCAAAA- --CTATGTTT CTTTTTTTTT ATTTTTGGCT TAGTTACGAT TAATAGGAGT
P.vivax           ATGGAATAAC AAAATTGAAC AAGTCAAAA- --CTATGTTT CTTTTTTTT- ATTTTTGGCT TAGTTACGAT TAATAGGAGT
Clustal Co        **********  * ******  * *    **     *        * ***       ******** *** ***  * ** *******

                  890        900        910        920        930        940        950        960
P.berghei         AGCTTGGGGG CATTTGTATT CAGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACAA ACAACTGCGA AA--GCATTT
P.cynomolg        AGCTTGGGGG CGTTTGTATT CAGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACAA ACAACTGCGA AA--GCATTT
P.falcipar        AGCTTGGGGA CATTCGTATT CAGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACGA ACAACTGCGA AA--GCATTT
P.fragile         AGCTTGGGGG CATTTGTATT CAGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACAA ACAACTGCGA AA--GCATTT
P.gallinac        AGTTTGGGGG CATTCGTATT CAGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACGA ACAACTGCGA AACGGCATTT
P.inui            AGCTTGGGGG CATTTGTATT CAGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACAA ACAACTGCGA AA--GCATTT
P.knowlesi        AGTTTGGGGA CATTTGTATT CAGATGTCAG AGGTGAAATT CTAAGATTTT CTGGAGACAA ACAACTGCGA AA-GGCATTT
P.malariae        AGCTTGGGGG CATTTGTATT CAGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACAA GCAACTGCGA AA--GCATTT
P.ovale           AGCTTGGGGG CATTTGTATT CAGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACAA ACAACTGCGA AA--GCATTT
P.reicheno        AGCTTGGGGA CATTCGTATT CGGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACGA ACAACTGCGA AA--GCATTT
P.simium          AGTTTGGGGA CATTTGTATT CAGATGTCAG AGGTGAAATT CTTAGATTTT CTGGAGACAA ACAACTGCGA AA-G--ATTT
P.vivax           AGTTTGGGGA CATTTGTATT CAGATGTCAG AGGTGAAATT CTAAGATTTT CTGGAGACAA ACAACTGCGA AG-GC-ATTT
Clustal Co        ** ******  * ** ***** * ******** ********** ** ******* ******** *  ********* *     ****
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                970         980         990         1000        1010        1020        1030        1040
P.berghei       GCCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.cynomolg      GCCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.falcipar      GTCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.fragile       GCCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.gallinac      GCCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.inui          GCCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAAGATAC CGTCGTAATC TTAACCATAA
P.knowlesi      GTCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.malariae      GCCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.ovale         GCCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.reicheno      GTCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.simium        GCCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA
P.vivax         GTCTAAAATA CTTCCATTAA TCAAGAACGA AAGTTAAGGG AGTGAAAACG ATCA-GATAC CGTCGTAATC TTAACCATAA
Clustal Co      * ******** ********** ********** ********** ****** *** **** ***** ********** **********

                1050        1060        1070        1080        1090        1100        1110        1120
P.berghei       ACTATGCCGA CTAAG-TGTT GGATGAAAAT -TTATAAATA AAACTATCTT CTTT------ AAAGGAGTAG TTTTTTAGAT
P.cynomolg      ACTATGCCGA CTAGG-CTTT GGATGAAAGA TTTTAAAATA AGAGATTTT- CTCTTCG--G AGT---TAAT CTCTTAGATT
P.falcipar      ACTATGCCGA CTAGG-TGTT GGATGAAAGT GTTAAAAATA AAAGTCATCT T--------- -TCGAGGTGA CTTTTAGATT
P.fragile       ACTATGCCGA CTAGG-CTTT GGATGAAAGA TTTTAAAATA AGAGCTTTTT CTCTTCG--G AGA---AGAA GTCTTAGATT
P.gallinac      ACTATGCCGA CTAGG-TGTT GGATGAAAGT GTTAAAAATA AAAGACGATC TGATGTAACA ATCGGATTGT CTTTTAGCTT
P.inui          ACTATGCCGA CTAGG-CTTT GGATGAAAGA TTTTAAAATA AGAGTTTTT- CTCTGAA--G AGAGTTAAAA CTCTTAGATT
P.knowlesi      ACTATACCGA CTAGGCTTTT GGATGAAAGT AAAACAAATG AGGATAGTCT CTT------- -CGGGGATAG TTCTTAGATT
P.malariae      ACTATGCCGA CTAGG-TGTT GGATGATAGA GTAAAAAATA AAAGAGACAT TCATATA--T ATGAGTGTTT CTTTTAGATA
P.ovale         ACTATGCCGA CTAGG-TTTT GGATGAAAGA TTTTTAAATA AGAAAATTCC T--------- -TTTGGAAAT TTCTTAGATT
P.reicheno      ACTATGCCGA CTAGG-TGTT GGATGAAAGT GTTAAAAATA AAAGTCATCT T--------- -TCGAGGTGA CTTTTAGATT
P.simium        ACTATACCGA CTAGG-TTTT GGATGAAAGT AAAACAAATA AGGATAGTCT CTT------- -CGGGGATAG TCCTTAGATT
P.vivax         ACTATACCGA CTAGG-TTTT GGATGAAAGT TAAACAAATA AGGATAGTCT CTT------- -CGGGGATAG TCCTTAGATT
Clustal Co      ***** **** *** *   ** ****** *       ****  *                                 **
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                1130       1140       1150       1160       1170       1180       1190       1200
P.berghei       GCTTCCTTCA GTACCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
P.cynomolg      GCTTCCTTCA GTGCCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
P.falcipar      GCTTCCTTCA GTACCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
P.fragile       GCTTCCTTCA GTGCCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
P.gallinac      GCTTCCTTCA GTACCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC ATAGCGAGAA AGTTAAAAGA
P.inui          GCTTCCTTCA GTGCCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AG-GAGAA AGTTAAAAGA
P.knowlesi      TCTTCCTTCA GTACCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
P.malariae      GCTTCCTTCA GTACCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
P.ovale         GCTTCCTTCA GTACCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
P.reicheno      GCTTCCTTCA GTACCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
P.simium        TCTTCCTTCA GTACCCTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
P.vivax         TCTTCCTTCA GTACCCTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC A-AGCGAGAA AGTTAAAAGA
Clustal Co       ********* ** ** **** ********** ********** ********** ********** * ** ***** **********

                1210       1220       1230       1240       1250       1260       1270       1280
P.berghei       ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGGA AACTCACTAG TTTAAGACAA
P.cynomolg      ATTGA-GGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGAA AACTCACTAG TTTAAGACAA
P.falcipar      ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGGA AACTCACTAG TTTAAGACAA
P.fragile       ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGAA AACTCACTAG TTTAAGACAA
P.gallinac      ATT-ACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGAA AACTCACTAG TTTAAGACAA
P.inui          ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGAA AACTCACTAG TTTAAGACAA
P.knowlesi      ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGAA AACTCACTAG TTTAAGACAT
P.malariae      ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGGA AACTCACTAG TTTAAGACAA
P.ovale         ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGGA AACTCACTAG TTTAAGACAA
P.reicheno      ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGGA AACTCACTAG TTTAAGACAA
P.simium        ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGAA AACTCACTAG TTTAAGACAA
P.vivax         ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGAA AACTCACTAG TTTAAGACAA
Clustal Co      *** * **** ********** ********** ********** ********** ******** * ********** *********
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                1290       1300       1310       1320       1330       1340       1350       1360
P.berghei       GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
P.cynomolg      GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
P.falcipar      GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
P.fragile       GAGTAGGATT GACAG-TTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
P.gallinac      GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
P.inui          GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
P.knowlesi      CCGTAGGATT GACAGATTAC TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG CGAATATGAT
P.malariae      GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
P.ovale         GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
P.reicheno      GAGTAGGATT GACAGGTTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTTG TGAATATGAT
P.simium        GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
P.vivax         GAGTAGGATT GACAGATTGA GAGCTCTTTC TTGATTTCTT GGATGGTGAT GCATGGCCGT TTTTAGTTCG TGAATATGAT
Clustal Co        ******** ***** **    ********* ********** ********** ********** ******** *  *********

                1370       1380       1390       1400       1410       1420       1430       1440
P.berghei       TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GTGGATATGT GATATTCTTC G--AAGGT-G
P.cynomolg      TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GCAAATACGA TATATTCTTA TGTGGGAT-T
P.falcipar      TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GCGAGTACAC TATATTCTTA TTTGAAAT-T
P.fragile       TTGTCTGGTT AATTCTGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GCAAATACGA TATATTCTTA TGTGGAAT-T
P.gallinac      TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GTAAATACAA CATATTCTTA AACAAAGA-A
P.inui          TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GCAAATACGA TATATTCTTA TGTGGAAT-T
P.knowlesi      TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GCAAATACAA CATATTTCTA TGCTGAATTT
P.malariae      TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GTAAATACAC TATATTCTTA AGTGAAATTA
P.ovale         TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GCGAATACGT TATATTCCTA CTTGAAAT-T
P.reicheno      TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GTAAGTACGC TATATTCTTA ATTGAAAT-T
P.simium        TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GCAAATACGA TATATTCTTA CGTGGAATT-
P.vivax         TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG GTAAGTACGA CATATTTTTA TGTCGGATT-
Clustal Co      ********** ***** **** ********** ********** ********** *    **     *****  *
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                1450       1460       1470       1480       1490       1500       1510       1520
P.berghei       GACTAACTAT AGCGTT---T TCGAAGGTAT GTTGCATAAT CA-------- ---------- ---------- ----------
P.cynomolg      GAATACGGTT GA--TT---T GCTTATTTTG AAGAAAATAT TG-------- ---------- ---------- ----------
P.falcipar      GAACATAGGT AACTAT---A CATTTATTCA GTAATCAAAT TA-------- ---------- ---------- ----------
P.fragile       GAATATGGTT GA--TT---T GCTTATTTTG CAGAAAATAT TG-------- ---------- ---------- ----------
P.gallinac      GAATATAGAT AA--AA---A TTACAAATAA GTGAAAATAT TA-------- ---------- ---------- ----------
P.inui          GAATATAGTT GA--TT---T GCTTATTTTG AAGAAAATAT TG-------- ---------- ---------- ----------
P.knowlesi      GTATTTGGAT AATTTAAAAG GTTTTTTTGC CGTGCA-TAT AATGAGTACG GAAAAAATGC ATTTTGCTAC CTTGTACCT-
P.malariae      GAATATAGAT AAATTG---T GCTAATTTTG ATTAAAATAT TA-------- ---------- ---------- ----------
P.ovale         GAATATAGCT GA-ATT---T GCTTATTTTG AAGAATATAT TA-------- ---------- ---------- ----------
P.reicheno      GAATATAGGT AATTAT---A CATTTATTCA GTGATCAAAT TA-------- ---------- ---------- ----------
P.simium        GCATACTTAT GGTTT----G TCTCTATTGA GCTGCATAAT AA-TGAAGGA TCGTGATTGC TTTTCGTGTC AATCTTTCTT
P.vivax         GGATCTGGAT GATTT----G CTTATATTGA GGTGCAATCT AAATAGGGGA TTGCAATTAT ACTTCGTGTC GGTGTTTCTT
Clustal Co      *        *                  *            *

                1530       1540       1550       1560       1570       1580       1590       1600
P.berghei       ----AATTGG TTT------- -------ACC CTTTGTT-TT TTTGTAGC-- ---------A TATTCTTTTA TTT-CGTTGG
P.cynomolg      ----GGATGC GTAA------ ----AGTGTC CCTTTCC-CT TTTCTACT-- ---------- TAATTTGCTT ATCATACTGT
P.falcipar      ----GGATAT TTTTATTA-- ---AAATATC CTTTTCC-CT GTTCTACTA- ---------A TAATTTGTTT TTTACTCTAT
P.fragile       ----GAATGC GTAAC----- ---AAGTGTT CCTTTCC-CT TTTCTACT-- ---------- AAATTTGCTT ATCATACTAT
P.gallinac      ----GGATAT TTTT------ ---TAATATC CTTTTCC-CT TTTCAACTT- ---------A TTTTGTTTTT TTTACTCTAT
P.inui          ----GGATAC GTAA------ ---ATGTATC CCTTTCC-CT TTTCTACT-- ---------- TAATTTGCTT ATCATACTAT
P.knowlesi      AAT--AATAC TTG-TGCGCC TATGCATATT TCCAACC-CT CTATTCCCTG TAC-CGCATA AAACTGGATT ATGATGCCAT
P.malariae      ----GAATGT TTTTTTTAAT A-AAAACGTT CTTTTCC-CT TTTTTTCTT- ---------A ATTATGCATA TTTATTCTTT
P.ovale         ----GGATAC ATTA------ ---TAGTGTC CTTTTCC-CT TTTCTACT-- ---------- TAATTCGCTA TTCATGCTGT
P.reicheno      ----GGATAT TTTTATTA-- ---AAATATC CTTTTCC-CT GTTCTACTA- ---------A TAATTTGTTT TTTACTCTAT
P.simium        AATCGAATCG TTGATGTGTT T-TGTATATC TCGTTC--TT TCATTACCT- TGCAATCACT CATTTGCCTT ATCATACTGT
P.vivax         AATCGAATAG CTGATGCGTT T-GGTATATT GCTTTCCTTT TTTTTATTTC TGCGCTTCTT TACTTGGCTT ATCGTACCGT
Clustal Co            *    *                            *                                  *   *
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                1610       1620       1630       1640       1650       1660
P.berghei       GTTTTTTCCC TAGTAAGGAT GTATCTGCTT TATT-TAATG CTTCTTAGAG GAACGATGTG T
P.cynomolg      TTCTTTTTC- GCGTAAGAAT GTATTTGCTT GATTGTAAAG CTTCTTAGAG GAACGATGTG T
P.falcipar      TTCTCTCTTC TTTTAAGAAT GTACTTGCTT GATTGAAAAG CTTCTTAGAG GAACATTGTG T
P.fragile       TTCTTTTCT- GCGTATGAAT GTATTTGCTT GATTGTAAAG CTTCTTAGAG GAACGATGTG T
P.gallinac      TTCTTTTTTT GTATAAGAAT GTATTTACTT GATTGTAAAG CTTCTTAGAG GGACATTGTG T
P.inui          TTCTTTTTT- GGGTAAGAAT GTATTTGCTT GATTGTAAAG CTTCTTAGAG GAACGATGTG T
P.knowlesi      TTCACTTTT- ATGTAGAAAT GTATTTGCTT TTACATAAAG CTTCTTAGAG GAACGATGTG T
P.malariae      TTCTTTTTTC GCATAAGAAT GTATTTGCTT AATTGTAAAG CTTCTTAGAG GAACGATGTG T
P.ovale         TTCTTTTTT- GTGTAGGAAT GTATTCGTTT GATTGTAAAG CTTCTTAGAG GAACGATGTG T
P.reicheno      TTCTCTCTTC TTTTAAGAAT GTACTTGCTT GATTGTAAAG CTTCTTAGAG GAACATTGTG T
P.simium        TTCTCTTTT- GCGTAAGAAT GTATTTGCTT GATTGTAACG CTTCTTAGAG GAACGATGTG -
P.vivax         TTCCTTTTT- GTGTAGAAAT GTATTTGCAT TATATTAAAG CTTCTTAGAG GAACGATGTG T
Clustal Co       *   *        **   ** ***      *       ** * ********** * **  ****
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Appendix 3: Alignment of Representative Sequences with *P. inui, P. cynomolgi* and *P. vivax*

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                    10         20         30         40         50         60         70         80
P.inui      ---------G GATAA--CTA CGGAAAAGCT GTAGCTAATA CTTGCTTTCA GCACTCTTGA TTAAGT---T CTTG--AGTG
P.cynomolg  TTCTTATAAG GATAA--CTA CGGAAAAGCT GTAGCTAATA CTTGCTTT-A GCACTCTTGA TTAAGT---T CTTG--AGTG
P.vivax     TTTCTATAAG GATAA--CTA CGGAAAAGCT GTAGCTAATA CTTGCTTT-A ATGCTCTCGA CGAAT---GT CTTG--GGCA
VM40        TTTTTATAAG GATAA--CTA CGGAAAAGCT GTAGCTAATA CTTGCTTT-A GCACTCTTGA TTAAGT---T CTTT--NNTG
VS63        TTTTTATAAG GATAA--CTA CGGAAAAGCT GTAGCTAATA CTTGCTTT-A ATGCTCCAGA CGTGTTACGT CTTGTGAGCA
VM82        TTTTTATAAG GATAA--CTA CGGAAAAGCT GTAGCTAATA TTTGCTAT-A GTACTCTTGA TTAACT---T CTTG--GGTG
VM88        -TTCACTAGT GATTATACTA CGGAAAAGCT GTAGCTAATA CTTGCTTT-A GCACTCTTGA TTAAGT---T CTTG--AGTG
Clustal Co             *** *  *** ********** **********  ***** * *    ***  **          * ***

                   90        100        110        120        130        140        150        160
P.inui      TGTACTTGTT AAGCCTTT-T AAGAAAAAAG TTAT--TAAC TTAAGA-AGT TATAACAAAG AAGCA--ACA CGTAATAGGT
P.cynomolg  TGTACTTGTT AAGCCTTT-T AAGAAAAAAG TTAT--TAAC TTAAGG-AAT TATAACAAAG AAGTA--ACA CGTAA-TGGA
P.vivax     TGTACTTGTT AAGCCTTTAT AAGAAAAAAG TTAA--TAAC TTAAGG-AAT GATAACAAAG AAGTGACACA TAGAA-GGAC
VM40        TGTACTTGTT AAGCCTTT-T AAGAAAANAG TNAT--TAAC TTAAGG-AAT TATAACAAAG AAGTA--ACA CGTAAATGGA
VS63        TGTACTTGTT AAGCCTTTAT AAGAAAAAAG TTAA--TAAC TTAAGG-AAT GATAACAAAG AAGTGACACA TAAAAAGGAC
VM82        CGTATTTGTC AAGTCTTT-T AAGAAGAAAG TTTTAATAGC TTTATG-AAT AATAACAACG AAGTGTGAGA CATAAACGAA
VM88        TGTNCTTGTT AAGCCTTT-T AAGAAAAAAN TTAT--TANC TGGGGGGAAT TATAACAAAG AAGTA--ACA CGTAAATGGA
Clustal Co   **  ****  *** **** * ***** * *  *     ** * *      * *  ******* * ***    * *    **  *

                  170        180        190        200        210        220        230        240
P.inui      ATTTTCCATT TTT-AGTGTG TTACTAACGA GTTTCTGACC TATCAGCTTT TGATGT-AGG GTATTGGCCT AACATGGCTA
P.cynomolg  TCCGTCCATT TTT-AGTGTG TATCAATCGA GTTTCTGACC TATCAGCTTT TGATGTTAGG GTATTGGCCT AACATGGCTA
P.vivax     CTGCGTCCAT TTATAGTGTG TATCAATCGA GTTTCTGACC TATCAGCTTT TGATGTTAGG GTATTGGCCT AACATTGCTA
VM40        TTT-TCCATT TTTTAGTGTG TATCAATCGA GTTTCTGACC TATCAGCTTT TGATGTTAGG GTATTGGCCT AACATGGCTA
VS63        TCGTCCCATT TTCTAGTGTG TATCAATCGA GTTTCTGACC TATCAGCTTT TGATGTTAGG GTATTGGCCT AACATGGCTA
VM82        TTCGTCCATT TTTTAGTGTG TATCAATCGA GTTTCTGACC TATCAGCTTT TGATGTTAGG GTATTGGCTT AACATGGCTA
VM88        TTT-TCCATT TTTTAGTGTG TATCAATCGA GTTTCTGACC TATCAGCTTT TGATGTTAGG GTATTGGCCT AACATGGCTA
Clustal Co        *  * **  ****** *  * * *** ********** ********** ****** *** ******** * ***** ****
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                  250        260        270        280        290        300        310        320
P.inui            TGACGGGTAA CGGGGAATTA GAGTTCGATC CGGAGAGGGG AGCCTGAGAA ATAGCTACCA CATCTAAGGA AGGCAGCAGG
P.cynomolg        TGACGGGTAA CGGGGAATTA GAGTTCGATT CCGGAGAGGG AGCCTGAGAA ATAGCTACCA CATCTAAGGA AGGCAGCAGG
P.vivax           TGACGGGTAA CGGGGAATTA GAGTTCGATT CCGGAGAGGG AGCCTGAGAA ATAGCTACCA CATCTAAGGA AGGCAGCAGG
VM40              TGACGGGTAA CGGGGAATTA GAGTTCGATT CCGGAGAGGG AGCCTGAGAA ATAGCTACCA CATCTAAGGA AGGCAGCAGG
VS63              TGACGGGTAA CGGGGAATTA GAGTTCGATT CCGGAGAGGG AGCCTGAGAA ATAGCTACCA CATCTAAGGA AGGCAGCAGG
VM82              TAACGGGTAA CCTGGAATTA GAGTTCGAAA CGGGAGAGGG AGCCTGATAA ATAGTTATCA CATCTAAGGA AGGCAGCAGA
VM88              TGACGGGTAA CGGGGAATTA GAGTTCGATT CCGGAGAGGG AGCCTGATAA ATAGTTATCA CATCTAAGGA AGGCAGCAGA
Clustal Co        * ******** *  ******* ********   * *    *** ******* ** **** ** ** ********** *********

                  330        340        350        360        370        380        390        400
P.inui            CGCGTAAATT ACCCAATTCT AAATGAAGAG AGGTAGTGAC AAGAAATAAC AATACAAGGC CAAT-CTGGC TTTGTAATTG
P.cynomolg        CGCGTAAATT ACCCAATTCT AAAG-AAGAG AGGTAGTGAC AAGAAATAAC AATACAAGGC CAAT-CTGGC TTTGTAATTG
P.vivax           CGCGTAAATT ACCCAATTCT AAAG-AAGAG AGGTAGTGAC AAGAAATAAC AATACAAGAC CAAAACTGGT TTTGTAATTG
VM40              CGCGTAAATT ACCCAATTCT AAAG-AAGAG AGGTAGTGAC AAGAAATAAC AATACAAGGC CAAT-CTGGC TTTGTAATTG
VS63              CGCGTAAATT ACCCAATTCT AAAG-AAGAG AGGTAGTGAC AAGAAATAAC AATACAAGAC CAAAACTGGT TTTGTAATTG
VM82              CGCGTAAATT ACCCAATTGT AAAC-AAGAG AGCTAGTGAC AAGAAATAAC AATACTAGGC CAAT-CTG-C TTTGTAATTA
VM88              CGCGTAAATT ACCCAATTGT AAAC-AAGAG AGCTAGTGAC AAGAAATAAC AATACTAGGC CAAT-CTG-C TTTGTAATTA
Clustal Co        ********** ******** * ***  ***** ** ******* ********** ***** ** * ***  ***   *********

                  410        420        430        440        450        460        470        480
P.inui            GAATGATGGG AATTTAAAAC CTTCCCAAAA CTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA TTCCAGCTCC
P.cynomolg        GAATGATGGG AATTTAAAAC CTTCCCAAAA CTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA TTCCAGCTCC
P.vivax           GAATGATGGG AATTTAAATC CTTCCCATAA TACAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA TTCCAGCTCC
VM40              GAATGATGGG AATTTAAAAC CTTCCCAAAA CTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGNTAA TTCCAGCTCC
VS63              GAATGATGGG AATTTAAATC CTTCCCATAA TACAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA TTCCAGCTCC
VM82              GAATGCTGGG AATTTAAAAC CTTCCCAAAA TTCAATTGGA GGGCAAGTCT GGTGCCAGCA GTCTCGGTAA TTCGAGCTCC
VM88              GAATGCTGGG AATTTAAAAC CTTCCCAAAA TTCAATTGGA GGGCAAGTCT GGTGCCAGCA GTCTCGGTAA TTCGAGCTCC
Clustal Co        ***** **** ******** * ******* **   ******** ********** ********** * * ** *** *** ******

                  490        500        510        520        530        540        550        560
P.inui            AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTAAGT AGCACTTTGT
P.cynomolg        AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTAAGC AACGCTT-GT
P.vivax           AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAACCAA TATTTTAA-T AACGCCGT-T
VM40              AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA NTTGAATTTC AAAGAATCGA TATTTTAAGT AACGCTTTGT
VS63              AATAGCGTAT ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAACCGA TATTTTAA-T AATGCTGT-T
VM82              ACTAGCGTAW GTTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTAAGT AACGCTTTGT
VM88              ACTAGCGTAT GTTAAAATTG TTGCAGNTAA AACGCTCGTA NTTGAATTTC AAAGAATCGA TATTTTAAGT AACTCTTTGT
Clustal Co        * *******   ********* ****** *** **********  ********* ****** * * ********   *   *    *
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                570        580        590        600        610        620        630        640
P.inui          AGATTAATCC AC-ATAACTG ATACTA---- -CGTATCGAC TTT--GTG-G CATT-TTTCT ACTTATGTGT TCTTTTAATT
P.cynomolg      AGCTTAATCC AC-ATAACTG ATACTA---- -CGTATCGAC TTT--GTGCG CATT-TTGCT A-TTATGTGT TCTTTTAATT
P.vivax         AGCTAGATCC ACAAGGGGTT GAGCCAATCA CGGTTTCGGC TTC-TGTGCG CATC-CTACC TATCAAGCGT TTTTTTAATT
VM40            AGCTTAATCC ACCATAACTG ATACTA---- -CGTATCGAC TTTT-GTGCG CATT-TTGCT ACTTATGTGT TCTTTTAATT
VS63            AGCTAGAGCC ACAAAAAGTC MAGCCA-CTA TGGTTTCGGT TTTATGTGCG CATCTCTACC TATCAAGCGT TTTTTTAAWT
VM82            AGCTTAATCC AC-ATAACTG ATACTA---- -CGTATCGAA TTT--GTGCG CATT-TTGCT ACTTATGTGT TCTTTTAATT
VM88            AGCTTAATCC AC-ANAACTG ATACTA---- -CGTATCGAA TTT--GTGCG CATT-TTGCT ACTTATGTGT TCTTTTAATT
Clustal Co      ** *  * ** ** *    *     * *       ** ***   **   *** * ***   * *    * * * ** * ****** *

                650        660        670        680        690        700        710        720
P.inui          AAAATGATTC T-TTTTAAGA -CTTTCTTTT TT-GCTTCGG CATTTTA-GG AGA-CTT-GT TACTTTG-AG TAAATTAGAG
P.cynomolg      AAAATGATTC TCTTTTAAGG TCTTTCTTTT ---GCTTCGG CATTTGA--- AGATCTT-GT TACTTTG-AG TAAATTAGAG
P.vivax         AAAGTGTTTC T-TTTTAAAA TCTTCTTTAC CT---TAAC- CATATGG--A AGATTTT-GT TACTTTG-AG TAAATTAAAG
VM40            AAAATGATTC T-TTTTAAGA TCTTTCTTTT TTTGCTTCNG NCATTTA-GG AGATCTTTGT TACTTTG-AG TAAATTAGAG
VS63            AAAGTGTTTC T-TTTTAAAA TCTTCTTTAG CT---TAAAA CATATGG--A AGATTTT-GK TACTTTG-AG TAAATTAAAG
VM82            AAAATGATTC T-TTTTAAGA TCTTTCTGTT TT-GTTTCGG CATCTTA-GG AGATCTT-GT TACTTTG-AG TAAATTAGAG
VM88            AAAATGATTC T-TTTNAAGA TCTTTCTGTT TT-GTTNCGG CATCTTAAGG AGATCTT-GT TACTTTGGAG TAAATTAGAG
Clustal Co      *** ** *** * *** **    ***  *         *         *      ***  ** *  ******* ** ******* **

                730        740        750        760        770        780        790        800
P.inui          TGTTCAAA-G CAAACAGAT- ATAGCAT--- -TATGCGTTT -GAATACTAC -AGCATGGAA TAACAAAATT G-AACAAGT-
P.cynomolg      TGTTCAAA-G CAAACAGAT- ATAGCAT--- -TGCGCGTTT -GAATACTAC -AGCATGGAA TAACGAAATT G-AACAAGTC
P.vivax         TGTTCATA-G CAAACAGAT- ACAGCAT--- -TGCGCGTTT TGAATACTAC -AGCATGGAA TAACAAAATT G-AACAAGTC
VM40            TGTTCAAA-G MAAACAGATT ATAGCATCTT TTATGCGTTT -GAATACTAC -AGCATGGAA TAACAAAAAT TGAACAAGTC
VS63            TGTTCAAA-G CAAACAGAT- ATAGCAT--- -AATGCGTTT -GAATACTAC -AGCATGGAA TAACAAAATT G-AACAAGTC
VM82            TGTTCAAA-G CAAACAAATT ATAGCATCTT -TATGTGTTT -GAATACTAC -AGCATGGAA TAACAAAATT G-AACAAGTC
VM88            TGTTCAAAAG CAAACAAATT ATGGCATCTT -TATGTGTTT TGAATACTAC CAGCATGGAA TAACAAANTT GGAACANGTC
Clustal Co      ****** * *  ***** **  *  ****       * ****  *********  ********* **** **  *   **** **
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                  810        820        830        840        850        860        870        880
P.inui            AGAATTTTGT TCTTATTTTA CTTATTTTGG CTTAG-TTAC GATTAATA-G GAGTAGCTT- --GGGGGCAT TTGTATTCAG
P.cynomolg        AGAATTTTGT TCTTTTTT-- CTTATTTTGG CTTAG-TTAC GATTAATA-G GAGTAGCTT- --GGGGGCGT TTGTATTCAG
P.vivax           AAAACTATGT TTCTTTTTTT T-ATTTTTGG CTTAG-TTAC GATTAATA-G GAGTAGTTT- --GGGGACAT TTGTATTCAG
VM40              AGAATTTTGT TCTTTTTT-- CNTATTTTGG CTTAG-TTAC GATTAATA-G GAGTAGCTT- --GGGGGCAT TTGTATTCAG
VS63              AAAACTATGT TTCTTTTTTT TTATTTTTGG CTTAG-TTAC GATTAATA-G GAGTAGTTT- --GGGGACAT TTGTATTCAG
VM82              AGAATTTTGT TCTTTTTT-- CTTATTTTGG CTTAG-TTAC GATTAATA-G GAGTAGCTT- --GGGGGCAT TTTTATTCAG
VM88              AGAATTTTGT TCTTTTTT-- CTWWTTTTGG CTTAGGTTNC GATTAATNAG GAGTAGGCTT GGGGGGGCAT TTGTATTCAG
Clustal Co        * ** * *** *  * ***       ****** ***** ** * *******  * ******  *    **** * * ** *******

                  890        900        910        920        930        940        950        960
P.inui            ATGTCAGA-- GGTGAAATT- CTTAGATTTT C-TGGAGACA AACAACTGCG AAAGCATTTG CCTAAAA-TA CTTCCATTAA
P.cynomolg        ATGTCAGA-- GGTGAAATT- CTTAGATTTT C-TGGAGACA AACAACTGCG AAAGCATTTG CCTAAAA-TA CTTCCATTAA
P.vivax           ATGTCAGA-- GGTGAAATT- CTAAGATTTT C-TGGAGACA AACAACTGCG AAGGCATTTG TCTAAAA-TA CTTCCATTAA
VM40              ATGTCAGA-- GGTGAAATT- CTTAGATTTT C-TGGAGACA AACAACTGCG AAAGCATTTG CCTAAAA-TA CTTCCATTAA
VS63              ATGTCAGA-- RGTGAAATT- CTTAGATTTT C-TGGAGACA AACAACTGCG AAGGCATTTG TCTAAAA-TA CTTCCATTAA
VM82              ATGTCAGA-- GGTGAAATT- CTTAGATTTT C-TGGAGACA AACAGCTGCG AAAGCATTTG CCTAAAA-TA CTTCCATTAA
VM88              ATGTCNAGAG GGTGAAATTT CTTAGATTTT NCTGGAGACA AACAACTGNG AAAGCNTTTG CCTAAAAATA CTTCCATTAA
Clustal Co        *****       ********  ** *******   ******** **** *** * ** ** ****  ****** ** **********
                  970        980        990        1000       1010       1020       1030       1040
P.inui            TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAAGATAC CGTCGTAATC TTAACCATAA ACTATGCCGA CTAGGCTTTG
P.cynomolg        TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA ACTATGCCGA CTAGGCTTTG
P.vivax           TCAAGAACGA AAGTTAAGGG AGTGAAAACG ATCA-GATAC CGTCGTAATC TTAACCATAA ACTATACCGA CTAGGTTTTG
VM40              TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA ACTATGCCGA CTAGGCTTTG
VS63              TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTAACCATAA ACTATACCGA CTAGGTTTTG
VM82              TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCG-GATAC CGTCGTAATC TTAACCATAA ACTATGCCGA CTAGGCTTTG
VM88              TCAAGANCGA AAGTTAAGGG AGTGAAGACG ATCA-GATAC CGTCGTAATC TTANCCATAA ACTATGCCGA CTAGGCTTTG
Clustal Co        ****** *** ********** ****** *** ***  ***** ********** *** ****** ***** **** ***** ****

                  1050       1060       1070       1080       1090       1100       1110       1120
P.inui            GATGAAAGAT TTTAAAATAA GAGTTTTTCT CTGAAGAGAG TTAAAACTCT TAGATTGCTT CCTTCAGTGC CTTATGAGAA
P.cynomolg        GATGAAAGAT TTTAAAATAA GAGATTTTCT CTTCGG--AG TTAAT-CTCT TAGATTGCTT CCTTCAGTGC CTTATGAGAA
P.vivax           GATGAAAGTT AAACAAATAA GGATAGTCTC TTCGGG---- -GATAGTCCT TAGATTTCTT CCTTCAGTAC CCTATGAGAA
VM40              GATGAAAGAT TTTAAAATAA GAGTTTTTTC TCTGAG-GAG TTAAAACTCT TAGATTGCTT CCTTCAGTGC CTTATGAGAA
VS63              GATGAAAGTA AAACAAATAA GGATAGTCTC TTCGGG---- -GATAGTCCT TAGATTTCTT CCTTCAGTAC CCTATGAGAA
VM82              GATGAAAGAT TTTAAAGTAA GAGCTTTTTC TCTGAG-GAG TTAAAAATCT TAGATTGCTT CCTTCAGTGC CTTATGAGAA
VM88              GATGAAAGAT TTTAAAATAA GAGTTTTTTC TCTGAG-GAG TTAAAACTCT TAGATTGCTT CCTTCAGTGC CTTATGAGAA
Clustal Co        ********       ** *** *     *         *       *     ** ****** *** ******** * * ********
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                1130       1140       1150       1160       1170       1180       1190       1200
P.inui          ATCAAAGTCT TTGGGTTCTG GGGCGAGTAT TCGCGCAAG- GAGAAAGTTA AAAGAATTGA CGGAAGGGCA CCACCAGGCG
P.cynomolg      ATCAAAGTCT TTGGGTTCTG GGGCGAGTAT TCGCGCAAGC GAGAAAGTTA AAAGAATTGA -GGAAGGGCA CCACCAGGCG
P.vivax         ATCAAAGTCT TTGGGTTCTG GGGCGAGTAT TCGCGCAAGC GAGAAAGTTA AAAGAATTGA CGGAAGGGCA CCACCAGGCG
VM40            ATCAAAGTCT TTGGGTTCTG GGGCGAGTAT TCGCGCAAGC GAGAAAGTTA AAAGAATTGA CGGAAGGGCA CCACCAGGCG
VS63            ATCAAAGTCT TTGGGTTCTG GGGCGAGTAT TCGCGCAAGC GAGAAAGTTA AAAGAATTGA CGGAAGGGCA CCACCAGGCG
VM82            ATCAAAGTCT TTGGGTTCTG GGGCGAGTAT TCGCGCAAGC GAGAAAGTTA AAAGAATTGA CGGAAGGGCA CCACCAGGCG
VM88            ATCAAAGTCT TTGGGTTCTG GGGCGAGTAT TCGCGCAAGC GAGAAAGTTA AAAGAATTGA CGGAAGGGCA CCACCAGGCG
Clustal Co      ********** ********** ********** *********  **********  **********  *********  **********

                1210       1220       1230       1240       1250       1260       1270       1280
P.inui          TGGAGCTTGC GGCTTAATTT GACTCAACAC GGGAAAACTC ACTAGTTTAA GACAAGAGTA GGATTGACAG ATTAATAGCT
P.cynomolg      TGGAGCTTGC GGCTTAATTT GACTCAACAC GGGAAAACTC ACTAGTTTAA GACAAGAGTA GGATTGACAG ATTAATAGCT
P.vivax         TGGAGCTTGC GGCTTAATTT GACTCAACAC GGGAAAACTC ACTAGTTTAA GACAAGAGTA GGATTGACAG ATTGAGAGCT
VM40            TGGAGCTTGC GGCTTAATTT GACTCAACAC GGGAAAACTC ACTAGTTTAA GACAAGAGTA GGATTGACAG ATTAATAGCT
VS63            TGGAGCTTGC GGCTTAATTT GACTCAACAC GGGAAAACTC ACTAGTTTAA GACAAGAGTA GGATTGACAG ATTGAGAGCT
VM82            TGGAGCTTGC GGCTTAATTT GACTCAACAC GGGAAAACTC ACTAGTTTAA GACAAGAGTA GGATTGACAG ATTAATAGCT
VM88            TGGAGCNTGC GGCTTAATTT GACTCAACAC GGGAAAACTC NCTAGTTTAA GACAAGAGTA GGATTGACAG ATTAATAGCT
Clustal Co      ****** *** ********** ********** **********  *********  **********  **********  *** * ****

                1290       1300       1310       1320       1330       1340       1350       1360
P.inui          CTTTCTTGAT TTCTTGGATG GTGATGCATG GCCGTTTTTA GTTCGTGAAT ATGATTTGTC TGGTTAATTC CGATAACGAA
P.cynomolg      CTTTCTTGAT TTCTTGGATG GTGATGCATG GCCGTTTTTA GTTCGTGAAT ATGATTTGTC TGGTTAATTC CGATAACGAA
P.vivax         CTTTCTTGAT TTCTTGGATG GTGATGCATG GCCGTTTTTA GTTCGTGAAT ATGATTTGTC TGGTTAATTC CGATAACGAA
VM40            CTTTCTTGAT TTCTTGGATG GTGATGCATG GCCGTTTTTA GTTCGTGAAT ATGATTTGTC TGGTTAATTC CGATAACGAA
VS63            CTTTCTTGAT TTCTTGGATG GTGATGCATG GCCGTTTTTA GTTCGTGAAT ATGATTTGTC TGGTTAATTC CGATAACGAA
VM82            CTTTCTTGAT TTCTTGGATG GTGATGCATG GCCGTTTTTA GTTCGTGAAT ATGATTTGTC TGGTTAATTC CGATAACGAA
VM88            CTTTCTTGAT TTCTTGGATG GTGATGCATG NCCGTTTTTA GTTCGNGAAT ATGATTTGTN TGGTTAATTC CGATAACGAA
Clustal Co      ********** ********** **********  ********* ***** **** *********  **********  **********

                1370       1380       1390       1400       1410       1420       1430       1440
P.inui          CGAGATCTTA ACCTGCTAAT TAGCGGCAAA TACGATATAT TCTTATGTGG AA-TTGAATA TAGTTGATTT GCTTATTTTG
P.cynomolg      CGAGATCTTA ACCTGCTAAT TAGCGGCAAA TACGATATAT TCTTATGTGG GA-TTGAATA CGGTTGATTT GCTTATTTTG
P.vivax         CGAGATCTTA ACCTGCTAAT TAGCGGTAAG TACGACATAT TTTTATGTCG GA-TTGGATC TGGATGATTT GCTTATATTG
VM40            CGAGATCTTA ACCTGCTAAT TAGCGGCAAA TACGATATAT TCTTATGTGG AA-TTGAATA TAGTTGATTT GCTTATTTTG
VS63            CGAGATCTTA ACCTGCTAAT TAGCGGCAAA TACGATATAT TCTTACGTGG AA-TTGCATA CTTATGGTTT GTCTCTATTG
VM82            CGAGATCTTA ACCTGCTAAT TAGCGGCAAA TACGATATAT TCTTATGTGG AA-TTGAATA TAGTTGATTT GCTCATTTTG
VM88            CGAGATCTTA ACCTGCTAAT TAGCGGCAAA TACGATATAT TCTTATGTGG AACTTGAATA TAGTTGATTT GCTTATTTTG
Clustal Co      ********** ********** ****** **  ***** **** * *** ** *  * *** **      ** *** *    * ***
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                1450       1460       1470       1480       1490       1500       1510       1520
P.inui          AA-------- ---------- ---GAAAATA T--------- ---------T -----GGGAT ACGTAAA--- -----TGTAT
P.cynomolg      AA-------- ---------- ---GAAAATA T--------- ---------T -----GGGAT GCGTAAA--- ------GTGT
P.vivax         AGGTGCAATC TAAATAGGGG ATTGCAATTA TACTTCGTGT CGGTGTTT-C TTAATCGAAT AGCTGATGCG TTTGGTATAT
VM40            AA-------- ---------- ---GAAAATA T--------- ---------T -----GGGAT ATGTAAA--- -----TGTAT
VS63            AGCTGCA-TG ATAGTGAAGG GTCGTGATTG CTTCTCTTGT CAATCTTTTC TTAATCGAAT CGTTGATGTG TTTTGTATAT
VM82            AA-------- ---------- ---GAAAATA T--------- ---------T -----GGGAT ACGTAAA--- -----TGTAT
VM88            ANT------- ---------- ---GAAAACN CCC------- ---------N -----NGGAT AAANTAAAC- -----TGTAT
Clustal Co      *                       *  *                              * **      *           * *

                1530       1540       1550       1560       1570       1580       1590       1600
P.inui          CCCTTTCC-C TTTTCTACT- ---------- -TAATTTGCT TATCATACTA TTTCTTTTTT --GGGTAA-G AATGTATTTG
P.cynomolg      CCCTTTCC-C TTTTCTACT- ---------- -TAATTTGCT TATCATACTG TTTCTTTTTC --GCGTAA-G AATGTATTTG
P.vivax         TGCTTTCCTT TTTTTTATTT CTGCGCTTCT TTACTTGGCT TATCGTACCG TTTCCTTTTT --GTGTAG-A AATGTATTTG
VM40            CCCTTTCC-C NAATCTACT- ---------- -TAATTTGCT TATCATACTA TTTCTTTTTC --GCGTAA-G AATGTATTTG
VS63            CTCGTTC--T TTCATTACCT -TGCAATCAC TCATTTGCCT TATCATACTG TTTCTCTTTT --GCGTAA-G AATGTATTTG
VM82            CCCTTTCC-C TTTTCTACT- ---------- -TAATTTGCT TATCATACTA TTTCTTTTTC --GCGTAA-G AATGTATTTG
VM88            CCCTTTCC-C TTTTCNANAA ---------- -TAATTTGCT TATCATACTA TTTCTTTTTA CGNTGTAACG AATGTATTTN
Clustal Co        * ***          *                 * **  ** **** ***   ****  ***      ***    *********

                1610       1620       1630
P.inui          CTTGATT-GT AAAGCTT-CT TAGAGGAACG ATGTGT
P.cynomolg      CTTGATT-GT AAAGCTT-CT TAGAGGAACG ATGTGT
P.vivax         CATTATA-TT AAAGCTT-CT TAGAGGAACG ATGTGT
VM40            CTTGATT-GT AAAGCTT-CT TAGAGNCANG ------
VS63            CTTGATT-GT AAAGCTT-CT TAGAGGCACC ATGTG-
VM82            CTTGATT-GT AAAGCTT-CT TAGAGGCACC TTGTGT
VM88            CNNCCCCCGN AAANNTTACN TAGAGGCNCC TGGAAT
Clustal Co      *          ***  ** *  *****
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Appendix 4: Alignment of VM82 with *P. inui* and *P. cynomolgi*

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                    10         20         30         40         50         60         70         80
P.cynomolg  TTCTTATAAG GATAACTACG GAAAAGCTGT AGCTAATACT TGCTTT-AGC ACTCTTGATT AAGTTCTTGA GTGTGTACTT
P.inui      ---------G GATAACTACG GAAAAGCTGT AGCTAATACT TGCTTTCAGC ACTCTTGATT AAGTTCTTGA GTGTGTACTT
VM82        TTTTTATAAG GATAACTACG GAAAAGCTGT AGCTAATATT TGCTAT-AGT ACTCTTGATT AACTTCTTGG GTGCGTATTT
Clustal Co           * ********** ********** ******** * **** * **  ********** ** ******  *** *** **

                     90        100        110        120        130        140        150        160
P.cynomolg  GTTAAGCCTT TTAAGAAAAA AGTTAT--TA ACTTAAGGAA TTATAACAAA GAAGTA--AC ACGTAATGGA T-CCGTCCAT
P.inui      GTTAAGCCTT TTAAGAAAAA AGTTAT--TA ACTTAAGAAG TTATAACAAA GAAGCA--AC ACGTAATAGG TATTTTCCAT
VM82        GTCAAGTCTT TTAAGAAGAA AGTTTTAATA GCTTTATGAA TAATAACAAC GAAGTGTGAG ACATAAACGA ATTCGTCCAT
Clustal Co  ** *** *** ******* ** **** *  **  *** *  *  * *******  ****    *  ** ***  *       *****

                    170        180        190        200        210        220        230        240
P.cynomolg  TTTT-AGTGT GTATCAATCG AGTTTCTGAC CTATCAGCTT TTGATGTTAG GGTATTGGCC TAACATGGCT ATGACGGGTA
P.inui      TTTT-AGTGT GTTACTAACG AGTTTCTGAC CTATCAGCTT TTGATGT-AG GGTATTGGCC TAACATGGCT ATGACGGGTA
VM82        TTTTTAGTGT GTATCAATCG AGTTTCTGAC CTATCAGCTT TTGATGTTAG GGTATTGGCT TAACATGGCT ATAACGGGTA
Clustal Co  **** ***** **  * * ** ********** ********** ******* ** *********  ********** ** *******

                    250        260        270        280        290        300        310        320
P.cynomolg  ACGGGGAATT AGAGTTCGAT TCCGGAGAGG GAGCCTGAGA AATAGCTACC ACATCTAAGG AAGGCAGCAG GCGCGTAAAT
P.inui      ACGGGGAATT AGAGTTCGAT CCGGAGAGGG GAGCCTGAGA AATAGCTACC ACATCTAAGG AAGGCAGCAG GCGCGTAAAT
VM82        ACCTGGAATT AGAGTTCGAA ACGGGAGAGG GAGCCTGATA AATAGTTATC ACATCTAAGG AAGGCAGCAG ACGCGTAAAT
Clustal Co  **  ****** *********   * *    ** ******** * ***** ** * ********** **********  *********

                    330        340        350        360        370        380        390        400
P.cynomolg  TACCCAATTC TAAA-GAAGA GAGGTAGTGA CAAGAAATAA CAATACAAGG CCAATCTGGC TTTGTAATTG GAATGATGGG
P.inui      TACCCAATTC TAAATGAAGA GAGGTAGTGA CAAGAAATAA CAATACAAGG CCAATCTGGC TTTGTAATTG GAATGATGGG
VM82        TACCCAATTG TAAA-CAAGA GAGCTAGTGA CAAGAAATAA CAATACTAGG CCAATCTG-C TTTGTAATTA GAATGCTGGG
Clustal Co  *********  ****  **** *** ****** ********** ****** *** ******** * *********  ***** ****
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                410        420        430        440        450        460        470        480
P.cynomolg      AATTTAAAAC CTTCCCAAAA CTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA TTCCAGCTCC AATAGCGTAT
P.inui          AATTTAAAAC CTTCCCAAAA CTCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA TTCCAGCTCC AATAGCGTAT
VM82            AATTTAAAAC CTTCCCAAAA TTCAATTGGA GGGCAAGTCT GGTGCCAGCA GTCTCGGTAA TTCGAGCTCC ACTAGCGTAW
Clustal Co      ********** **********  ********* ********** ********** * * ****** *** ****** * *******

                490        500        510        520        530        540        550        560
P.cynomolg      ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTAAGC AACGCTT-GT AGCTTAATCC
P.inui          ATTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTAAGT AGCACTTTGT AGATTAATCC
VM82            GTTAAAATTG TTGCAGTTAA AACGCTCGTA GTTGAATTTC AAAGAATCGA TATTTTAAGT AACGCTTTGT AGCTTAATCC
Clustal Co       ********* ********** ********** ********** ********** *********  * * *** ** ** *******

                570        580        590        600        610        620        630        640
P.cynomolg      ACATAACTGA TACTACGTAT CGACTTTGTG CGCATTTTGC TA-TTATGTG TTCTTTTAAT TAAAATGATT CTCTTTTAAG
P.inui          ACATAACTGA TACTACGTAT CGACTTTGTG -GCATTTTTC TACTTATGTG TTCTTTTAAT TAAAATGATT CTTTTTAAGA
VM82            ACATAACTGA TACTACGTAT CGAATTTGTG CGCATTTTGC TACTTATGTG TTCTTTTAAT TAAAATGATT CTTTTTAAGA
Clustal Co      ********** ********** *** ******  ******* * ** ******* ********** ********** ** *** *

                650        660        670        680        690        700        710        720
P.cynomolg      -GTCTTTCTT TTGCTTCGGC ATTTGAAGAT --CTTGTTAC TTTGAGTAAA TTAGAGTGTT CAAAGCAAAC AGAT-ATAGC
P.inui          -CTTTCTTTT TTGCTTCGGC ATTTTAGGAG A-CTTGTTAC TTTGAGTAAA TTAGAGTGTT CAAAGCAAAC AGAT-ATAGC
VM82            TCTTTCTGTT TTGTTTCGGC ATCTTAGGAG ATCTTGTTAC TTTGAGTAAA TTAGAGTGTT CAAAGCAAAC AAATTATAGC
Clustal Co        * * * ** *** ****** ** * * **    ******** ********** ********** ********** * ** *****

                730        740        750        760        770        780        790        800
P.cynomolg      AT---TGCGC GTTTGAATAC TACAGCATGG AATAACGAAA TTGAACAAGT CAGAATTTTG TTCTTTTTT- -CTTATTTTG
P.inui          AT---TATGC GTTTGAATAC TACAGCATGG AATAACAAAA TTGAACAAGT -AGAATTTTG TTCTTATTTT ACTTATTTTG
VM82            ATCTTTATGT GTTTGAATAC TACAGCATGG AATAACAAAA TTGAACAAGT CAGAATTTTG TTCTTTTTT- -CTTATTTTG
Clustal Co      **   *  *  ********** ********** ****** *** **********  ********* ***** ***   *********

                810        820        830        840        850        860        870        880
P.cynomolg      GCTTAGTTAC GATTAATAGG AGTAGCTTGG GGGCGTTTGT ATTCAGATGT CAGAGGTGAA ATTCTTAGAT TTTCTGGAGA
P.inui          GCTTAGTTAC GATTAATAGG AGTAGCTTGG GGGCATTTGT ATTCAGATGT CAGAGGTGAA ATTCTTAGAT TTTCTGGAGA
VM82            GCTTAGTTAC GATTAATAGG AGTAGCTTGG GGGCATTTTT ATTCAGATGT CAGAGGTGAA ATTCTTAGAT TTTCTGGAGA
Clustal Co      ********** ********** ********** **** *** * ********** ********** ********** **********
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                890        900        910        920        930        940        950        960
P.cynomolg      CAAACAACTG CGAAAGCATT TGCCTAAAAT ACTTCCATTA ATCAAGAACG AAAGTTAAGG GAGTGAAGAC GATCA-GATA
P.inui          CAAACAACTG CGAAAGCATT TGCCTAAAAT ACTTCCATTA ATCAAGAACG AAAGTTAAGG GAGTGAAGAC GATCAAGATA
VM82            CAAACAGCTG CGAAAGCATT TGCCTAAAAT ACTTCCATTA ATCAAGAACG AAAGTTAAGG GAGTGAAGAC GATCG-GATA
Clustal Co      ****** *** ********** ********** ********** ********** ********** ********** ****  ****

                970        980        990        1000       1010       1020       1030       1040
P.cynomolg      CCGTCGTAAT CTTAACCATA AACTATGCCG ACTAGGCTTT GGATGAAAGA TTTTAAAATA AGAG-ATTTT CTCTTCGGAG
P.inui          CCGTCGTAAT CTTAACCATA AACTATGCCG ACTAGGCTTT GGATGAAAGA TTTTAAAATA AGAG-TTTTT CTCTGAAGAG
VM82            CCGTCGTAAT CTTAACCATA AACTATGCCG ACTAGGCTTT GGATGAAAGA TTTTAAAGTA AGAGCTTTTT CTCTGAGGAG
Clustal Co      ********** ********** ********** ********** ********** ******* ** ****  **** ****   ***

                1050       1060       1070       1080       1090       1100       1110       1120
P.cynomolg      ---TTAATCT CTTAGATTGC TTCCTTCAGT GCCTTATGAG AAATCAAAGT CTTTGGGTTC TGGGGCGAGT ATTCGCGCAA
P.inui          AGTTAAAACT CTTAGATTGC TTCCTTCAGT GCCTTATGAG AAATCAAAGT CTTTGGGTTC TGGGGCGAGT ATTCGCGCAA
VM82            --TTAAAAAT CTTAGATTGC TTCCTTCAGT GCCTTATGAG AAATCAAAGT CTTTGGGTTC TGGGGCGAGT ATTCGCGCAA
Clustal Co        * **  * ********** ********** ********** ********** ********** ********** **********

                1130       1140       1150       1160       1170       1180       1190       1200
P.cynomolg      GCGAGAAAGT TAAAAGAATT GA-GGAAGGG CACCACCAGG CGTGGAGCTT GCGGCTTAAT TTGACTCAAC ACGGGAAAAC
P.inui          G-GAGAAAGT TAAAAGAATT GACGGAAGGG CACCACCAGG CGTGGAGCTT GCGGCTTAAT TTGACTCAAC ACGGGAAAAC
VM82            GCGAGAAAGT TAAAAGAATT GACGGAAGGG CACCACCAGG CGTGGAGCTT GCGGCTTAAT TTGACTCAAC ACGGGAAAAC
Clustal Co      * ******** ********** ** ******* ********** ********** ********** ********** **********

                1210       1220       1230       1240       1250       1260       1270       1280
P.cynomolg      TCACTAGTTT AAGACAAGAG TAGGATTGAC AGATTAATAG CTCTTTCTTG ATTTCTTGGA TGGTGATGCA TGGCCGTTTT
P.inui          TCACTAGTTT AAGACAAGAG TAGGATTGAC AGATTAATAG CTCTTTCTTG ATTTCTTGGA TGGTGATGCA TGGCCGTTTT
VM82            TCACTAGTTT AAGACAAGAG TAGGATTGAC AGATTAATAG CTCTTTCTTG ATTTCTTGGA TGGTGATGCA TGGCCGTTTT
Clustal Co      ********** ********** ********** ********** ********** ********** ********** **********

                1290       1300       1310       1320       1330       1340       1350       1360
P.cynomolg      TAGTTCGTGA ATATGATTTG TCTGGTTAAT TCCGATAACG AACGAGATCT TAACCTGCTA ATTAGCGGCA AATACGATAT
P.inui          TAGTTCGTGA ATATGATTTG TCTGGTTAAT TCCGATAACG AACGAGATCT TAACCTGCTA ATTAGCGGCA AATACGATAT
VM82            TAGTTCGTGA ATATGATTTG TCTGGTTAAT TCCGATAACG AACGAGATCT TAACCTGCTA ATTAGCGGCA AATACGATAT
Clustal Co      ********** ********** ********** ********** ********** ********** ********** **********
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                    1370       1380       1390       1400       1410       1420       1430       1440
P.cynomolg  ATTCTTATGT GGGATTGAAT ACGGTTGATT TGCTTATTTT GAAGAAAATA TTGGGATGCG TAAA-GTGTC CCTTTCCCTT
P.inui      ATTCTTATGT GGAATTGAAT ATAGTTGATT TGCTTATTTT GAAGAAAATA TTGGGATACG TAAATGTATC CCTTTCCCTT
VM82        ATTCTTATGT GGAATTGAAT ATAGTTGATT TGCTCATTTT GAAGAAAATA TTGGGATACG TAAATGTATC CCTTTCCCTT
Clustal Co  ********** ** ******* *  ******* **** ***** ********** ******* ** **** ** ** **********

                    1450       1460       1470       1480       1490       1500       1510       1520
P.cynomolg  TTCTACTTAA TTTGCTTATC ATACTGTTTC TTTTTCGCGT AAGAATGTAT TTGCTTGATT GTAAAGCTTC TTAGAGGAAC
P.inui      TTCTACTTAA TTTGCTTATC ATACTATTTC TTTTTTGGGT AAGAATGTAT TTGCTTGATT GTAAAGCTTC TTAGAGGAAC
VM82        TTCTACTTAA TTTGCTTATC ATACTATTTC TTTTTCGCGT AAGAATGTAT TTGCTTGATT GTAAAGCTTC TTAGAGGCAC
Clustal Co  ********** ********** ***** **** ***** * ** ********** ********** ********** ******* **

P.cynomolg  GATGTGT
P.inui      GATGTGT
VM82        CTTGTGT
Clustal Co    *****
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Appendix 5: Alignment of VM88 with *P. inui* and *P. cynomolgi*

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                    10         20         30         40         50         60         70         80
P.cynomolg  TTCTTATAAG GATA-ACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AG CACTCTTGAT TAAGTTCTTG AGTGTGTACT
P.inui      ---------G GATA-ACTAC GGAAAAGCTG TAGCTAATAC TTGCTTTCAG CACTCTTGAT TAAGTTCTTG AGTGTGTACT
VM88        TTCACTAGTG ATTATACTAC GGAAAAGCTG TAGCTAATAC TTGCTTT-AG CACTCTTGAT TAAGTTCTTG AGTGTGTNCT
Clustal Co           *   ** ***** ********** ********** ******* ** ********** ********** ******* **

                   90        100        110        120        130        140        150        160
P.cynomolg  TGTTAAGCCT TTTAAGAAAA AAGTTATTAA CTTAAGG-AA TTATAACAAA GAAGTAACAC GTAATGGAT- CCGTCCATTT
P.inui      TGTTAAGCCT TTTAAGAAAA AAGTTATTAA CTTAAGA-AG TTATAACAAA GAAGCAACAC GTAATAGGTA TTTTCCATTT
VM88        TGTTAAGCCT TTTAAGAAAA AANTTATTAN CTGGGGGGAA TTATAACAAA GAAGTAACAC GTAAATGGAT TTTCCATTTT
Clustal Co  ********** ********** ** ******  **   *  *  ********** **** ***** ****  *        *  ***

                  170        180        190        200        210        220        230        240
P.cynomolg  TTAGTGTGTA TCAATCGAGT TTCTGACCTA TCAGCTTTTG ATGTTAGGGT ATTGGCCTAA CATGGCTATG ACGGGTAACG
P.inui      TTAGTGTGTT ACTAACGAGT TTCTGACCTA TCAGCTTTTG ATGT-AGGGT ATTGGCCTAA CATGGCTATG ACGGGTAACG
VM88        TTAGTGTGTA TCAATCGAGT TTCTGACCTA TCAGCTTTTG ATGTTAGGGT ATTGGCCTAA CATGGCTATG ACGGGTAACG
Clustal Co  *********   * * ***** ********** ********** **** ***** ********** ********** **********

                  250        260        270        280        290        300        310        320
P.cynomolg  GGGAATTAGA GTTCGATTCC GGAGAGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG GCAGCAGGCG CGTAAATTAC
P.inui      GGGAATTAGA GTTCGATCCG GAGAGGGGAG CCTGAGAAAT AGCTACCACA TCTAAGGAAG GCAGCAGGCG CGTAAATTAC
VM88        GGGAATTAGA GTTCGATTCC GGAGAGGGAG CCTGATAAAT AGTTATCACA TCTAAGGAAG GCAGCAGACG CGTAAATTAC
Clustal Co  ********** ******* *  *    ***** ***** **** ** ** **** ********** ******* ** **********

                  330        340        350        360        370        380        390        400
P.cynomolg  CCAATTCTAA A-GAAGAGAG GTAGTGACAA GAAATAACAA TACAAGGCCA ATCTGGCTTT GTAATTGGAA TGATGGGAAT
P.inui      CCAATTCTAA ATGAAGAGAG GTAGTGACAA GAAATAACAA TACAAGGCCA ATCTGGCTTT GTAATTGGAA TGATGGGAAT
VM88        CCAATTGTAA A-CAAGAGAG CTAGTGACAA GAAATAACAA TACTAGGCCA ATCTG-CTTT GTAATTAGAA TGCTGGGAAT
Clustal Co  ****** *** *  *******  ********* ********** *** ****** ***** **** ****** *** ** *******

                  410        420        430        440        450        460        470        480
P.cynomolg  TTAAAACCTT CCCAAAACTC AATTGGAGGG CAAGTCTGGT GCCAGCAGCC GCGGTAATTC CAGCTCCAAT AGCGTATATT
P.inui      TTAAAACCTT CCCAAAACTC AATTGGAGGG CAAGTCTGGT GCCAGCAGCC GCGGTAATTC CAGCTCCAAT AGCGTATATT
VM88        TTAAAACCTT CCCAAAATTC AATTGGAGGG CAAGTCTGGT GCCAGCAGTC TCGGTAATTC GAGCTCCACT AGCGTATGTT
Clustal Co  ********** ******* ** ********** ********** ******** *  *********  ******* * ******* **
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                490        500        510        520        530        540        550        560
P.cynomolg      AAAATTGTTG CAGTTAAAAC GCTCGTAGTT GAATTTCAAA GAATCGATAT TTTAAGCAAC GCTT-GTAGC TTAATCCACA
P.inui          AAAATTGTTG CAGTTAAAAC GCTCGTAGTT GAATTTCAAA GAATCGATAT TTTAAGTAGC ACTTTGTAGA TTAATCCACA
VM88            AAAATTGTTG CAGNTAAAAC GCTCGTANTT GAATTTCAAA GAATCGATAT TTTAAGTAAC TCTTTGTAGC TTAATCCACA
Clustal Co      ********** *** ****** ******* ** ********** ********** ****** * *  *** ****  **********

                570        580        590        600        610        620        630        640
P.cynomolg      TAACTGATAC TACGTATCGA CTTTGTGCGC ATTTTGCTA- TTATGTGTTC TTTTAATTAA AATGATTCTC TTTTAAG-GT
P.inui          TAACTGATAC TACGTATCGA CTTTGTG-GC ATTTTTCTAC TTATGTGTTC TTTTAATTAA AATGATTCTT TTTAAGA-CT
VM88            NAACTGATAC TACGTATCGA ATTTGTGCGC ATTTTGCTAC TTATGTGTTC TTTTAATTAA AATGATTCTT TTNAAGATCT
Clustal Co       ********* **********  ****** ** ***** ***  ********** ********** *********  **  *    *

                650        660        670        680        690        700        710        720
P.cynomolg      CTTTCTTTTG CTTCGGCATT TGAAGAT--- CTTGTTACTT TG-AGTAAAT TAGAGTGTTC AAA-GCAAAC AGAT-ATAGC
P.inui          TTCTTTTTTG CTTCGGCATT TTAGGAGA-- CTTGTTACTT TG-AGTAAAT TAGAGTGTTC AAA-GCAAAC AGAT-ATAGC
VM88            TTCTGTTTTG TTNCGGCATC TTAAGGAGAT CTTGTTACTT TGGAGTAAAT TAGAGTGTTC AAAAGCAAAC AAATTATGGC
Clustal Co       * * *****  * ******  * * *      ********** ** ******* ********** *** ****** * ** ** **

                730        740        750        760        770        780        790        800
P.cynomolg      AT---TGCGC GTTT-GAATA CTAC-AGCAT GGAATAACGA AATTG-AACA AGTCAGAATT TTGTTCTTTT TT--CTTATT
P.inui          AT---TATGC GTTT-GAATA CTAC-AGCAT GGAATAACAA AATTG-AACA AGT-AGAATT TTGTTCTTAT TTTACTTATT
VM88            ATCTTTATGT GTTTTGAATA CTACCAGCAT GGAATAACAA ANTTGGAACA NGTCAGAATT TTGTTCTTTT TT--CTWWTT
Clustal Co      **   *  *  **** ***** **** ***** ******** * * *** ****  ** ****** ******** * **  **  **

                810        820        830        840        850        860        870        880
P.cynomolg      TTGGCTTAG- TTACGATTAA TA-GGAGTAG CTT---GGGG GCGTTTGTAT TCAGATGTCA GA--GGTGAA ATT-CTTAGA
P.inui          TTGGCTTAG- TTACGATTAA TA-GGAGTAG CTT---GGGG GCATTTGTAT TCAGATGTCA GA--GGTGAA ATT-CTTAGA
VM88            TTGGCTTAGG TTNCGATTAA TNAGGAGTAG GCTTGGGGGG GCATTTGTAT TCAGATGTCN AGAGGGTGAA ATTTCTTAGA
Clustal Co      *********  ** ******* *  *******   *   **** ** ******* *********      ****** *** ******

                890        900        910        920        930        940        950        960
P.cynomolg      TTTTC-TGGA GACAAACAAC TGCGAAAGCA TTTGCCTAAA A-TACTTCCA TTAATCAAGA ACGAAAGTTA AGGGAGTGAA
P.inui          TTTTC-TGGA GACAAACAAC TGCGAAAGCA TTTGCCTAAA A-TACTTCCA TTAATCAAGA ACGAAAGTTA AGGGAGTGAA
VM88            TTTTNCTGGA GACAAACAAC TGNGAAAGCN TTTGCCTAAA AATACTTCCA TTAATCAAGA NCGAAAGTTA AGGGAGTGAA
Clustal Co      ****  **** ********** ** ******  ********** * ******** **********  ********* **********
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                970        980        990        1000       1010       1020       1030       1040
P.cynomolg      GACGATCA-G ATACCGTCGT AATCTTAACC ATAAACTATG CCGACTAGGC TTTGGATGAA AGATTTTAAA ATAAGAGATT
P.inui          GACGATCAAG ATACCGTCGT AATCTTAACC ATAAACTATG CCGACTAGGC TTTGGATGAA AGATTTTAAA ATAAGAGTTT
VM88            GACGATCA-G ATACCGTCGT AATCTTANCC ATAAACTATG CCGACTAGGC TTTGGATGAA AGATTTTAAA ATAAGAGTTT
Clustal Co      ******** * ********** ******* ** ********** ********** ********** ********** ******* **

                1050       1060       1070       1080       1090       1100       1110       1120
P.cynomolg      TT-CTCTTCG GAG---TTAA TCTCTTAGAT TGCTTCCTTC AGTGCCTTAT GAGAAATCAA AGTCTTTGGG TTCTGGGGCG
P.inui          TT-CTCTGAA GAGAGTTAAA ACTCTTAGAT TGCTTCCTTC AGTGCCTTAT GAGAAATCAA AGTCTTTGGG TTCTGGGGCG
VM88            TTTCTCTGAG GAG--TTAAA ACTCTTAGAT TGCTTCCTTC AGTGCCTTAT GAGAAATCAA AGTCTTTGGG TTCTGGGGCG
Clustal Co      ** ****    ***   * **  ********* ********** ********** ********** ********** **********

                1130       1140       1150       1160       1170       1180       1190       1200
P.cynomolg      AGTATTCGCG CAAGCGAGAA AGTTAAAAGA ATTGA-GGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC
P.inui          AGTATTCGCG CAAG-GAGAA AGTTAAAAGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC
VM88            AGTATTCGCG CAAGCGAGAA AGTTAAAAGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CNTGCGGCTT AATTTGACTC
Clustal Co      ********** **** ***** ********** ***** **** ********** ********** * ******** **********

                1210       1220       1230       1240       1250       1260       1270       1280
P.cynomolg      AACACGGGAA AACTCACTAG TTTAAGACAA GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT
P.inui          AACACGGGAA AACTCACTAG TTTAAGACAA GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT
VM88            AACACGGGAA AACTCNCTAG TTTAAGACAA GAGTAGGATT GACAGATTAA TAGCTCTTTC TTGATTTCTT GGATGGTGAT
Clustal Co      ********** ***** **** ********** ********** ********** ********** ********** **********

                1290       1300       1310       1320       1330       1340       1350       1360
P.cynomolg      GCATGGCCGT TTTTAGTTCG TGAATATGAT TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG
P.inui          GCATGGCCGT TTTTAGTTCG TGAATATGAT TTGTCTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG
VM88            GCATGNCCGT TTTTAGTTCG NGAATATGAT TTGTNTGGTT AATTCCGATA ACGAACGAGA TCTTAACCTG CTAATTAGCG
Clustal Co      ***** **** **********  ********* **** ***** ********** ********** ********** **********

                1370       1380       1390       1400       1410       1420       1430       1440
P.cynomolg      GCAAATACGA TATATTCTTA TGTGGGA-TT GAATACGGTT GATTTGCTTA TTTTGAA-GA AAATAT--TG GGATGC-GTA
P.inui          GCAAATACGA TATATTCTTA TGTGGAA-TT GAATATAGTT GATTTGCTTA TTTTGAA-GA AAATAT--TG GGATAC-GTA
VM88            GCAAATACGA TATATTCTTA TGTGGAACTT GAATATAGTT GATTTGCTTA TTTTGANTGA AAACNCCCNN GGATAAANTA
Clustal Co      ********** ********** ***** * ** *****  *** ********** ******  ** ***        ****    **
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                1450       1460       1470       1480       1490       1500       1510       1520
P.cynomolg      AA--GTGTCC CTTTCCCTTT TCTACT-TAA TTTGCTTATC ATACTGTTTC TTTTTC--GC GTAA-GAATG TATTTGCTTG
P.inui          AAT-GTATCC CTTTCCCTTT TCTACT-TAA TTTGCTTATC ATACTATTTC TTTTTT--GG GTAA-GAATG TATTTGCTTG
VM88            AACTGTATCC CTTTCCCTTT TCNANAATAA TTTGCTTATC ATACTATTTC TTTTTACGNT GTAACGAATG TATTTNCNNC
Clustal Co      **  ** *** ********** ** *   *** ********** ***** **** *****      **** ***** ***** *

                1530       1540       1550
P.cynomolg      ATT-GTAAAG CTT-CTTAGA GGAACGATGT GT
P.inui          ATT-GTAAAG CTT-CTTAGA GGAACGATGT GT
VM88            CCCCGNAAAN NTTACNTAGA GGCNCCTGGA AT
Clustal Co          * ***   ** * **** **  *   *   *
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Appendix 6: Alignment of VM40 with *P. inui* and *P. cynomolgi*

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P.cynomolg   TTCTTATAAG GATAACTACG GAAAAGCTGT AGCTAATACT TGCTTT-AGC ACTCTTGATT AAGTTCTTGA GTGTGTACTT
P.inui       ---------G GATAACTACG GAAAAGCTGT AGCTAATACT TGCTTTCAGC ACTCTTGATT AAGTTCTTGA GTGTGTACTT
VM40         TTTTTATAAG GATAACTACG GAAAAGCTGT AGCTAATACT TGCTTT-AGC ACTCTTGATT AAGTTCTTTN NTGTGTACTT
Clustal Co            * ********** ********** ********** ****** *** ********** ********    *********

                    90         100        110        120        130        140        150        160
P.cynomolg   GTTAAGCCTT TTAAGAAAAA AGTTATTAAC TTAAGGAATT ATAACAAAGA AGTAACACGT AA-TGGATCC GTCCATTTTT
P.inui       GTTAAGCCTT TTAAGAAAAA AGTTATTAAC TTAAGAAGTT ATAACAAAGA AGCAACACGT AATAGGTATT TTCCATTTTT
VM40         GTTAAGCCTT TTAAGAAAAN AGTNATTAAC TTAAGGAATT ATAACAAAGA AGTAACACGT AAATGGATTT TCCATTTTTT
Clustal Co   ********** *********  *** ****** ***** * ** ********** ** ******* **  **       *  *****

                   170        180        190        200        210        220        230        240
P.cynomolg   AGTGTGTATC AATCGAGTTT CTGACCTATC AGCTTTTGAT GTTAGGGTAT TGGCCTAACA TGGCTATGAC GGGTAACGGG
P.inui       AGTGTGTTAC TAACGAGTTT CTGACCTATC AGCTTTTGAT GT-AGGGTAT TGGCCTAACA TGGCTATGAC GGGTAACGGG
VM40         AGTGTGTATC AATCGAGTTT CTGACCTATC AGCTTTTGAT GTTAGGGTAT TGGCCTAACA TGGCTATGAC GGGTAACGGG
Clustal Co   *******  *  * ******* ********** ********** ** ******* ********** ********** **********
                   250        260        270        280        290        300        310        320
P.cynomolg   GAATTAGAGT TCGATTCCGG AGAGGGAGCC TGAGAAATAG CTACCACATC TAAGGAAGGC AGCAGGCGCG TAAATTACCC
P.inui       GAATTAGAGT TCGATCCGGA GAGGGGAGCC TGAGAAATAG CTACCACATC TAAGGAAGGC AGCAGGCGCG TAAATTACCC
VM40         GAATTAGAGT TCGATTCCGG AGAGGGAGCC TGAGAAATAG CTACCACATC TAAGGAAGGC AGCAGGCGCG TAAATTACCC
Clustal Co   ********** ***** * *     ******* ********** ********** ********** ********** **********

                   330        340        350        360        370        380        390        400
P.cynomolg   AATTCTAAA- GAAGAGAGGT AGTGACAAGA AATAACAATA CAAGGCCAAT CTGGCTTTGT AATTGGAATG ATGGGAATTT
P.inui       AATTCTAAAT GAAGAGAGGT AGTGACAAGA AATAACAATA CAAGGCCAAT CTGGCTTTGT AATTGGAATG ATGGGAATTT
VM40         AATTCTAAA- GAAGAGAGGT AGTGACAAGA AATAACAATA CAAGGCCAAT CTGGCTTTGT AATTGGAATG ATGGGAATTT
Clustal Co   *********  ********** ********** ********** ********** ********** ********** **********

                   410        420        430        440        450        460        470        480
P.cynomolg   AAAACCTTCC CAAAACTCAA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC GGTAATTCCA GCTCCAATAG CGTATATTAA
P.inui       AAAACCTTCC CAAAACTCAA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC GGTAATTCCA GCTCCAATAG CGTATATTAA
VM40         AAAACCTTCC CAAAACTCAA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC GNTAATTCCA GCTCCAATAG CGTATATTAA
Clustal Co   ********** ********** ********** ********** ********** * ******** ********** **********
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490 500 510 520 530 540 550 560
P.cynomolg AATTGTTGCA GTTAAAACGC TCGTAGTTGA ATTTCAAAGA ATCGATATTT TAAGCAACGC TT-GTAGCTT AATCCAC-AT
P.inui AATTGTTGCA GTTAAAACGC TCGTAGTTGA ATTTCAAAGA ATCGATATTT TAAGTAGCAC TTTGTAGATT AATCCAC-AT
VM40 AATTGTTGCA GTTAAAACGC TCGTANTTGA ATTTCAAAGA ATCGATATTT TAAGTAACGC TTTGTAGCTT AATCCACCAT
Clustal Co ********** ********** ***** **** ********** ********** **** * * * ** **** ** ******* **
570 580 590 600 610 620 630 640
P.cynomolg AACTGATACT ACGTATCGAC TTT-GTGCGC ATTTTGCTA- TTATGTGTTC TTTTAATTAA AATGATTCTC TTTTAAGGTC
P.inui AACTGATACT ACGTATCGAC TTT-GTG-GC ATTTTTCTAC TTATGTGTTC TTTTAATTAA AATGATTCT- TTTTAAGACT
VM40 AACTGATACT ACGTATCGAC TTTTGTGCGC ATTTTGCTAC TTATGTGTTC TTTTAATTAA AATGATTCT- TTTTAAGATC
Clustal Co ********** ********** *** *** ** ***** *** ********** ********** ********* *******
650 660 670 680 690 700 710 720
P.cynomolg TTTCTTTT-- -GCTTCGGC- ATTT-GAAGA TCTT-GTTAC TTTGAGTAAA TTAGAGTGTT CAAAGCAAAC AGAT-ATAGC
P.inui TTCTTTTTT- -GCTTCGGCA TTTTAGGAGA CTT--GTTAC TTTGAGTAAA TTAGAGTGTT CAAAGCAAAC AGAT-ATAGC
VM40 TTTCTTTTTT TGCTTCNGNC ATTTAGGAGA TCTTTGTTAC TTTGAGTAAA TTAGAGTGTT CAAAGMAAAC AGATTATAGC
Clustal Co ** **** ***** * *** * *** * ***** ********** ********** ***** **** **** *****
730 740 750 760 770 780 790 800
P.cynomolg AT----TGCG CGTTTGAATA CTACAGCATG GAATAACGAA A-TTGAACAA GTCAGAATTT TGTTCTTTTT T--CTTATTT
P.inui AT----TATG CGTTTGAATA CTACAGCATG GAATAACAAA A-TTGAACAA GT-AGAATTT TGTTCTTATT TTACTTATTT
VM40 ATCTTTTATG CGTTTGAATA CTACAGCATG GAATAACAAA AATTGAACAA GTCAGAATTT TGTTCTTTTT T--CNTATTT
Clustal Co ** * * ********** ********** ******* ** * ******** ** ******* ******* ** * * *****
810 820 830 840 850 860 870 880
P.cynomolg TGGCTTAGTT ACGATTAATA GGAGTAGCTT GGGGGCGTTT GTATTCAGAT GTCAGAGGTG AAATTCTTAG ATTTTCTGGA
P.inui TGGCTTAGTT ACGATTAATA GGAGTAGCTT GGGGGCATTT GTATTCAGAT GTCAGAGGTG AAATTCTTAG ATTTTCTGGA
VM40 TGGCTTAGTT ACGATTAATA GGAGTAGCTT GGGGGCATTT GTATTCAGAT GTCAGAGGTG AAATTCTTAG ATTTTCTGGA
Clustal Co ********** ********** ********** ****** *** ********** ********** ********** **********
890 900 910 920 930 940 950 960
P.cynomolg GACAAACAAC TGCGAAAGCA TTTGCCTAAA ATACTTCCAT TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCA-GA
P.inui GACAAACAAC TGCGAAAGCA TTTGCCTAAA ATACTTCCAT TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAAGA
VM40 GACAAACAAC TGCGAAAGCA TTTGCCTAAA ATACTTCCAT TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCA-GA
Clustal Co ********** ********** ********** ********** ********** ********** ********** ******* **

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                970        980        990        1000       1010       1020       1030       1040
P.cynomolg      TACCGTCGTA ATCTTAACCA TAAACTATGC CGACTAGGCT TTGGATGAAA GATTTTAAAA TAAGAGATTT T-CTCTTCGG
P.inui          TACCGTCGTA ATCTTAACCA TAAACTATGC CGACTAGGCT TTGGATGAAA GATTTTAAAA TAAGAGTTTT T-CTCTGAAG
VM40            TACCGTCGTA ATCTTAACCA TAAACTATGC CGACTAGGCT TTGGATGAAA GATTTTAAAA TAAGAGTTTT TTCTCTGAGG
Clustal Co      ********** ********** ********** ********** ********** ********** ****** *** * ****   *

                1050       1060       1070       1080       1090       1100       1110       1120
P.cynomolg      AG--TTAAT- CTCTTAGATT GCTTCCTTCA GTGCCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC
P.inui          AGAGTTAAAA CTCTTAGATT GCTTCCTTCA GTGCCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC
VM40            AG--TTAAAA CTCTTAGATT GCTTCCTTCA GTGCCTTATG AGAAATCAAA GTCTTTGGGT TCTGGGGCGA GTATTCGCGC
Clustal Co      **  ****   ********** ********** ********** ********** ********** ********** **********

                1130       1140       1150       1160       1170       1180       1190       1200
P.cynomolg      AAGCGAGAAA GTTAAAAGAA TTGA-GGAAG GGCACCACCA GGCGTGGAGC TTGCGGCTTA ATTTGACTCA ACACGGGAAA
P.inui          AAG-GAGAAA GTTAAAAGAA TTGACGGAAG GGCACCACCA GGCGTGGAGC TTGCGGCTTA ATTTGACTCA ACACGGGAAA
VM40            AAGCGAGAAA GTTAAAAGAA TTGACGGAAG GGCACCACCA GGCGTGGAGC TTGCGGCTTA ATTTGACTCA ACACGGGAAA
Clustal Co      *** ****** ********** **** ***** ********** ********** ********** ********** **********

                1210       1220       1230       1240       1250       1260       1270       1280
P.cynomolg      ACTCACTAGT TTAAGACAAG AGTAGGATTG ACAGATTAAT AGCTCTTTCT TGATTTCTTG GATGGTGATG CATGGCCGTT
P.inui          ACTCACTAGT TTAAGACAAG AGTAGGATTG ACAGATTAAT AGCTCTTTCT TGATTTCTTG GATGGTGATG CATGGCCGTT
VM40            ACTCACTAGT TTAAGACAAG AGTAGGATTG ACAGATTAAT AGCTCTTTCT TGATTTCTTG GATGGTGATG CATGGCCGTT
Clustal Co      ********** ********** ********** ********** ********** ********** ********** **********

                1290       1300       1310       1320       1330       1340       1350       1360
P.cynomolg      TTTAGTTCGT GAATATGATT TGTCTGGTTA ATTCCGATAA CGAACGAGAT CTTAACCTGC TAATTAGCGG CAAATACGAT
P.inui          TTTAGTTCGT GAATATGATT TGTCTGGTTA ATTCCGATAA CGAACGAGAT CTTAACCTGC TAATTAGCGG CAAATACGAT
VM40            TTTAGTTCGT GAATATGATT TGTCTGGTTA ATTCCGATAA CGAACGAGAT CTTAACCTGC TAATTAGCGG CAAATACGAT
Clustal Co      ********** ********** ********** ********** ********** ********** ********** **********

                1370       1380       1390       1400       1410       1420       1430       1440
P.cynomolg      ATATTCTTAT GTGGGATTGA ATACGGTTGA TTTGCTTATT TTGAAGAAAA TATTGGGATG CGTAAA-GTG TCCCTTTCCC
P.inui          ATATTCTTAT GTGGAATTGA ATATAGTTGA TTTGCTTATT TTGAAGAAAA TATTGGGATA CGTAAATGTA TCCCTTTCCC
VM40            ATATTCTTAT GTGGAATTGA ATATAGTTGA TTTGCTTATT TTGAAGAAAA TATTGGGATA TGTAAATGTA TCCCTTTCCC
Clustal Co      ********** **** ***** ***  ***** ********** ********** *********   ***** **  **********
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                1450       1460       1470       1480       1490       1500       1510       1520
P.cynomolg      TTTTCTACTT AATTTGCTTA TCATACTGTT TCTTTTTCGC GTAAGAATGT ATTTGCTTGA TTGTAAAGCT TCTTAGAGGA
P.inui          TTTTCTACTT AATTTGCTTA TCATACTATT TCTTTTTTGG GTAAGAATGT ATTTGCTTGA TTGTAAAGCT TCTTAGAGGA
VM40            NAATCTACTT AATTTGCTTA TCATACTATT TCTTTTTCGC GTAAGAATGT ATTTGCTTGA TTGTAAAGCT TCTTAGAGNC
Clustal Co         ******* ********** ******* ** ******* *  ********** ********** ********** ********

P.cynomolg      ACGATGTGT
P.inui          ACGATGTGT
VM40            ANG------
Clustal Co      * *
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Appendix 7: Alignment of VS63 with *P. simium* and *P. vivax*

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                    10         20         30         40         50         60         70         80
P.simium    ---------G GATAACTACG GAAAAGCTGT AGCTAATACT TGCTTTAATG CTCCCGACGT GTTACGTCTT GTGAGCATGT
VS63        TTTTTATAAG GATAACTACG GAAAAGCTGT AGCTAATACT TGCTTTAATG CTCCAGACGT GTTACGTCTT GTGAGCATGT
P.vivax     TTTCTATAAG GATAACTACG GAAAAGCTGT AGCTAATACT TGCTTTAATG CTCTCGACGA AT---GTCTT --GGGCATGT
Clustal Co           * ********** ********** ********** ********** ***  ****   *   *****   * ******

                    90        100        110        120        130        140        150        160
P.simium    ACTTGTTAAG CCTTTATAAG AAAAAAGTTA ATAACTTAAG GAATGATAAC AAAGAAGTGA CACATAAAAA GGACTCGTCC
VS63        ACTTGTTAAG CCTTTATAAG AAAAAAGTTA ATAACTTAAG GAATGATAAC AAAGAAGTGA CACATAAAAA GGACTCGTCC
P.vivax     ACTTGTTAAG CCTTTATAAG AAAAAAGTTA ATAACTTAAG GAATGATAAC AAAGAAGTGA CACATAGAA- GGACCTGCGT
Clustal Co  ********** ********** ********** ********** ********** ********** ****** **  ****  *

                   170        180        190        200        210        220        230        240
P.simium    CATTTTCTAG TGTGTATCAA TCGAGTTTCT GACCTATCAG CTTTTGAATG TTAGGGTAT- GGCCTAACAT GGCTATGACG
VS63        CATTTTCTAG TGTGTATCAA TCGAGTTTCT GACCTATCAG CTTTTGA-TG TTAGGGTATT GGCCTAACAT GGCTATGACG
P.vivax     CCATTTATAG TGTGTATCAA TCGAGTTTCT GACCTATCAG CTTTTGA-TG TTAGGGTATT GGCCTAACAT TGCTATGACG
Clustal Co  *  *** *** ********** ********** ********** ******* ** *********  **********  *********

                   250        260        270        280        290        300        310        320
P.simium    GGTAACGGGG AATTAGAGTT CGATTCTCCG GAGAGGGAGC CTGAGAAATA GCTACCACAT CTAAGGAAGG CAGCAGGCGC
VS63        GGTAACGGGG AATTAGAGTT CGATT--CCG GAGAGGGAGC CTGAGAAATA GCTACCACAT CTAAGGAAGG CAGCAGGCGC
P.vivax     GGTAACGGGG AATTAGAGTT CGATT--CCG GAGAGGGAGC CTGAGAAATA GCTACCACAT CTAAGGAAGG CAGCAGGCGC
Clustal Co  ********** ********** *****  *** ********** ********** ********** ********** **********

                   330        340        350        360        370        380        390        400
P.simium    GTAAATTACC CAATTCTAAA GAAGAGAGGT AGTGACAAGA AATAACGATA CAAGACCAAA ACTGGTTTTG TAATTGGAAT
VS63        GTAAATTACC CAATTCTAAA GAAGAGAGGT AGTGACAAGA AATAACAATA CAAGACCAAA ACTGGTTTTG TAATTGGAAT
P.vivax     GTAAATTACC CAATTCTAAA GAAGAGAGGT AGTGACAAGA AATAACAATA CAAGACCAAA ACTGGTTTTG TAATTGGAAT
Clustal Co  ********** ********** ********** ********** ****** *** ********** ********** **********

                   410        420        430        440        450        460        470        480
P.simium    GATGGGAATT TAAATCCTTC CCATAATACA ATTGGAGGGC AAGTCTGGTG CCAGCAGCCG CG-TAATTCC AGCTCCAATA
VS63        GATGGGAATT TAAATCCTTC CCATAATACA ATTGGAGGGC AAGTCTGGTG CCAGCAGCCG CGGTAATTCC AGCTCCAATA
P.vivax     GATGGGAATT TAAATCCTTC CCATAATACA ATTGGAGGGC AAGTCTGGTG CCAGCAGCCG CGGTAATTCC AGCTCCAATA
Clustal Co  ********** ********** ********** ********** ********** ********** ** ******* **********
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                490        500        510        520        530        540        550        560
P.simium        GCGTATATTA AAATTGTTGC AGTTAAAACG CTCGTAGTTG AATTTCAAAG AACCGATATT TTAATAATGC TGTTAGCTAG
VS63            GCGTATATTA AAATTGTTGC AGTTAAAACG CTCGTAGTTG AATTTCAAAG AACCGATATT TTAATAATGC TGTTAGCTAG
P.vivax         GCGTATATTA AAATTGTTGC AGTTAAAACG CTCGTAGTTG AATTTCAAAG AACCAATATT TTAATAACGC CGTTAGCTAG
Clustal Co      ********** ********** ********** ********** ********** **** ***** ******* **  *********

                570        580        590        600        610        620        630        640
P.simium        AGCCACAAAA AGTCAAGCCA CT-ATGGTTT CGGTTTTATG TGCGCATCTC TACCTATCAA GTTGTTTTTT TAATTAAAGT
VS63            AGCCACAAAA AGTCMAGCCA CT-ATGGTTT CGGTTTTATG TGCGCATCTC TACCTATCAA GC-GTTTTTT TAAWTAAAGT
P.vivax         ATCCACAAGG GGTTGAGCCA ATCACGGTTT CGGCTTC-TG TGCGCATC-C TACCTATCAA GC-GTTTTTT TAATTAAAGT
Clustal Co      * *******    **  *****  * * ***** *** **  ** ******** * ********** *  ******* *** ******

                650        660        670        680        690        700        710        720
P.simium        GTTTCTTTTT AAAATCTTCT TTAGCTTAAA ACATATGGAA GGTTTTGTTA CTTTGAGTAA ATTAGAGTGT TCAAAGCAAA
VS63            GTTTCTTTTT AAAATCTTCT TTAGCTTAAA ACATATGGAA GATTTTGKTA CTTTGAGTAA ATTAAAGTGT TCAAAGCAAA
P.vivax         GTTTCTTTTT AAAATCTTCT TTACCTTAAC -CATATGGAA GATTTTGTTA CTTTGAGTAA ATTAAAGTGT TCATAGCAAA
Clustal Co      ********** ********** *** *****   ********* * ***** ** ********** **** ***** *** ******
                730        740        750        760        770        780        790        800
P.simium        CAGATATAGC ATAATGCGTT T-GAATACTA CAGCATGGAA TAACAAAATT GAACAAGTCA AAACTATGTT TCTTTTTTTT
VS63            CAGATATAGC ATAATGCGTT T-GAATACTA CAGCATGGAA TAACAAAATT GAACAAGTCA AAACTATGTT TCTTTTTTTT
P.vivax         CAGATACAGC ATTGCGCGTT TTGAATACTA CAGCATGGAA TAACAAAATT GAACAAGTCA AAACTATGTT TCTTTTTTTT
Clustal Co      ****** *** **   ***** * ******** ********** ********** ********** ********** **********

                810        820        830        840        850        860        870        880
P.simium        TATTTTTGGC TTAGTTACGA TTAATAGGAG TAGTTTGGGG ACATTTGTAT TCAGATGTCA GAGGTGAAAT TCTTAGATTT
VS63            TATTTTTGGC TTAGTTACGA TTAATAGGAG TAGTTTGGGG ACATTTGTAT TCAGATGTCA GARGTGAAAT TCTTAGATTT
P.vivax         -ATTTTTGGC TTAGTTACGA TTAATAGGAG TAGTTTGGGG ACATTTGTAT TCAGATGTCA GAGGTGAAAT TCTAAGATTT
Clustal Co       ********* ********** ********** ********** ********** ********** ** ******* *** ******

                890        900        910        920        930        940        950        960
P.simium        TCTGGAGACA AACAACTGCG AAAG-ATTTG CCTAAAATAC TTCCATTAAT CAAGAACGAA AGTTAAGGGA GTGAAGACGA
VS63            TCTGGAGACA AACAACTGCG AAGGCATTTG TCTAAAATAC TTCCATTAAT CAAGAACGAA AGTTAAGGGA GTGAAGACGA
P.vivax         TCTGGAGACA AACAACTGCG AAGGCATTTG TCTAAAATAC TTCCATTAAT CAAGAACGAA AGTTAAGGGA GTGAAAACGA
Clustal Co      ********** ********** ** * *****  ********* ********** ********** ********** ***** ****
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                970        980        990        1000       1010       1020       1030       1040
P.simium        TCAGATACCG TCGTAATCTT AACCATAAAC TATACCGACT AGGTTTTGGA TGAAAGTAAA ACAAATAAGG ATAGTCTCTT
VS63            TCAGATACCG TCGTAATCTT AACCATAAAC TATACCGACT AGGTTTTGGA TGAAAGTAAA ACAAATAAGG ATAGTCTCTT
P.vivax         TCAGATACCG TCGTAATCTT AACCATAAAC TATACCGACT AGGTTTTGGA TGAAAGTTAA ACAAATAAGG ATAGTCTCTT
Clustal Co      ********** ********** ********** ********** ********** ******* ** ********** **********

                1050       1060       1070       1080       1090       1100       1110       1120
P.simium        CGGGGATAGT CCTTAGATTT CTTCCTTCAG TACCCTATGA GAAATCAAAG TCTTTGGGTT CTGGGGCGAG TATTCGCGCA
VS63            CGGGGATAGT CCTTAGATTT CTTCCTTCAG TACCCTATGA GAAATCAAAG TCTTTGGGTT CTGGGGCGAG TATTCGCGCA
P.vivax         CGGGGATAGT CCTTAGATTT CTTCCTTCAG TACCCTATGA GAAATCAAAG TCTTTGGGTT CTGGGGCGAG TATTCGCGCA
Clustal Co      ********** ********** ********** ********** ********** ********** ********** **********

                1130       1140       1150       1160       1170       1180       1190       1200
P.simium        AGCGAGAAAG TTAAAAGAAT TGACGGAAGG GCACCACCAG GCGTGGAGCT TGCGGCTTAA TTTGACTCAA CACGGGAAAA
VS63            AGCGAGAAAG TTAAAAGAAT TGACGGAAGG GCACCACCAG GCGTGGAGCT TGCGGCTTAA TTTGACTCAA CACGGGAAAA
P.vivax         AGCGAGAAAG TTAAAAGAAT TGACGGAAGG GCACCACCAG GCGTGGAGCT TGCGGCTTAA TTTGACTCAA CACGGGAAAA
Clustal Co      ********** ********** ********** ********** ********** ********** ********** **********

                1210       1220       1230       1240       1250       1260       1270       1280
P.simium        CTCACTAGTT TAAGACAAGA GTAGGATTGA CAGATTAATA GCTCTTTCTT GATTTCTTGG ATGGTGATGC ATGGCCGTTT
VS63            CTCACTAGTT TAAGACAAGA GTAGGATTGA CAGATTGAGA GCTCTTTCTT GATTTCTTGG ATGGTGATGC ATGGCCGTTT
P.vivax         CTCACTAGTT TAAGACAAGA GTAGGATTGA CAGATTGAGA GCTCTTTCTT GATTTCTTGG ATGGTGATGC ATGGCCGTTT
Clustal Co      ********** ********** ********** ****** * * ********** ********** ********** **********

                1290       1300       1310       1320       1330       1340       1350       1360
P.simium        TTAGTTCGTG AATATGATTT GTCTGGTTAA TTCCGATAAC GAACGAGATC TTAACCTGCT AATTAGCGGC AAATACGATA
VS63            TTAGTTCGTG AATATGATTT GTCTGGTTAA TTCCGATAAC GAACGAGATC TTAACCTGCT AATTAGCGGC AAATACGATA
P.vivax         TTAGTTCGTG AATATGATTT GTCTGGTTAA TTCCGATAAC GAACGAGATC TTAACCTGCT AATTAGCGGT AAGTACGACA
Clustal Co      ********** ********** ********** ********** ********** ********** *********  ** ***** *

                1370       1380       1390       1400       1410       1420       1430       1440
P.simium        TATTCTTACG TGGAATTGCA TACTTATGGT TTGTCTCTAT TGAGCTGCAT AATAA-TGAA GGATCGTGAT TGCTTTTCGT
VS63            TATTCTTACG TGGAATTGCA TACTTATGGT TTGTCTCTAT TGAGCTGCAT GATAG-TGAA GGGTCGTGAT TGCTTCTCTT
P.vivax         TATTTTTATG TCGGATTGGA TCTGGATGAT TTGCTTATAT TGAGGTGCAA TCTAAATAGG GGATTGCAAT TATACTTCGT
Clustal Co      **** *** * * * **** * *    *** * ***  * *** **** ****    **  *    ** * *  ** *     ** *
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                1450       1460       1470       1480       1490       1500       1510       1520
P.simium        GTCAATCTTT -CTTAATCGA ATCGTTGATG TGTTTTGTAT ATCTCGTTC- -TTTCATTAC CT-TGCAATC ACTCATTTGC
VS63            GTCAATCTTT TCTTAATCGA ATCGTTGATG TGTTTTGTAT ATCTCGTTC- -TTTCATTAC CT-TGCAATC ACTCATTTGC
P.vivax         GTCGGTGTTT -CTTAATCGA ATAGCTGATG CGTTTGGTAT ATTGCTTTCC TTTTTTTTAT TTCTGCGCTT CTTTACTTGG
Clustal Co      ***  * ***  ********* ** * *****  **** **** **  * ***   ***  ***   * ***  *    * * ***

                1530       1540       1550       1560       1570       1580       1590
P.simium        CTTATCATAC TGTTTCTCTT TTGCGTAAGA ATGTATTTGC TTGATTGTAA CGCTTCTTAG AGGAACGATG TG-
VS63            CTTATCATAC TGTTTCTCTT TTGCGTAAGA ATGTATTTGC TTGATTGTAA AGCTTCTTAG AGGCACCATG TG-
P.vivax         CTTATCGTAC CGTTTCCTTT TTGTGTAGAA ATGTATTTGC ATTATATTAA AGCTTCTTAG AGGAACGATG TGT
Clustal Co      ****** ***  *****  ** *** ***  * **********  * **  ***  ********* *** ** *** **
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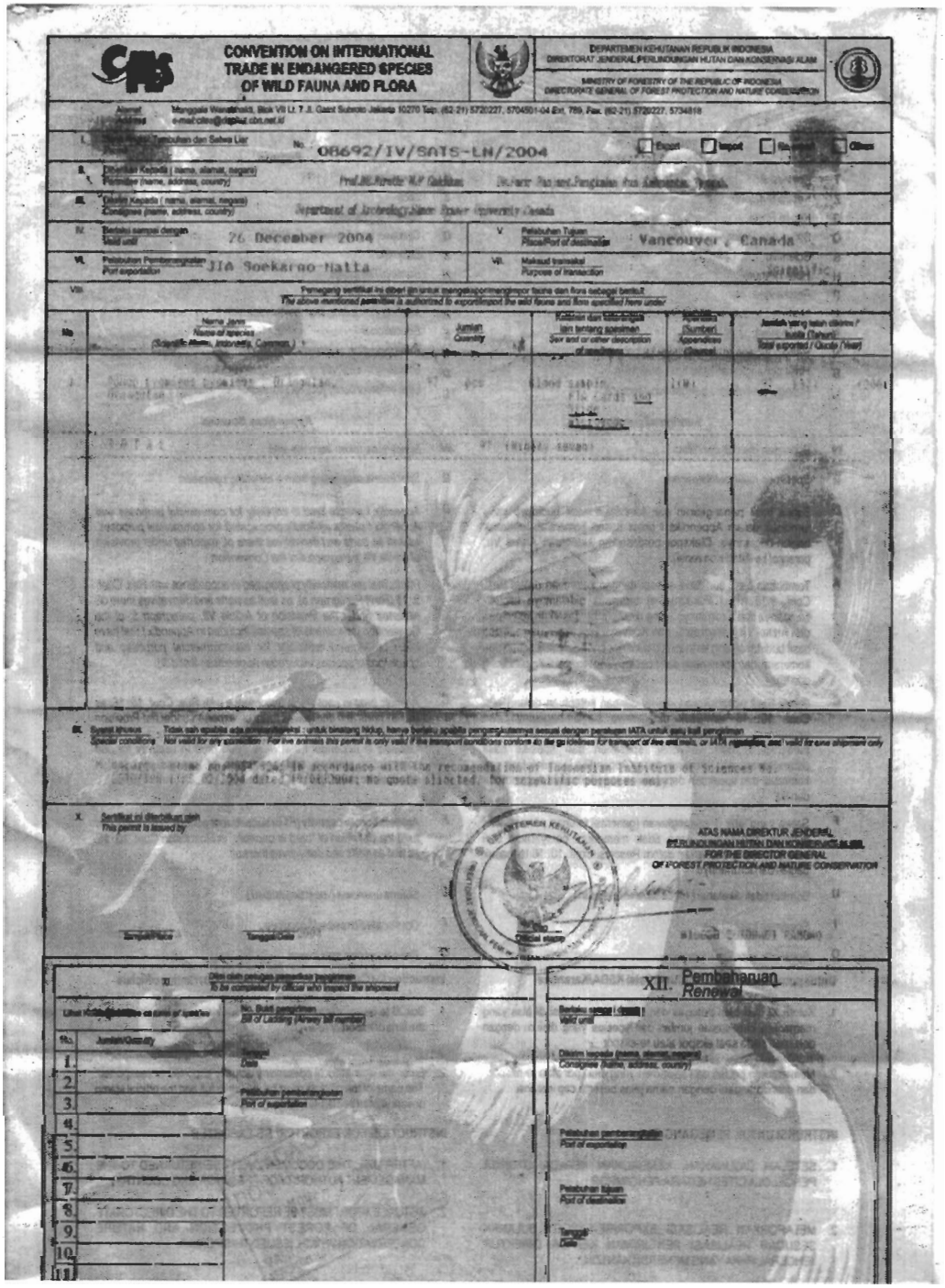
Appendix 8: Copy of CITES Permit



CONVENTION ON INTERNATIONAL TRADE IN ENDANGERED SPECIES OF WILD FAUNA AND FLORA

DEPARTEMEN KEHUTANAN REPUBLIK INDONESIA
DIREKTORAT JENDERAL PERLINDUNGAN HUTAN DAN KONSERVASI ALAM

MINISTRY OF FORESTRY OF THE REPUBLIC OF INDONESIA
DIRECTORATE GENERAL OF FOREST PROTECTION AND NATURE CONSERVATION

No. 08692/IV/SATS-LN/2004

Export

Vancouver , Canada

26 December 2004

Jl. Soekarno Hatta

ATAS NAMA DIREKTUR JENDERAL
PERLINDUNGAN HUTAN DAN KONSERVASI ALAM
FOR THE DIRECTOR GENERAL
OF FOREST PROTECTION AND NATURE CONSERVATION

XII. Pembaharuan
Renewal

Appendix 9: SFU Ethics Approval for Research Involving Animals

TO: **Dr C. Lowenberger**
Biology

FROM: **M. Weeks**
Chair, UACC

SUBJECT: **Project # 675B-03**
DATE: **June 9, 2003**

Your Project # 675B-03 "Identification of malaria parasites in Orang Utans" is approved.

Grant Title: N/A
Grant No.: NA
Funding Source: private funding- (student fellowship +/- or supervisor is funding)
Account number: N/A

Michael Weeks

cc. Madeleine Stephens (Animal Care Facility)
Chris Kennedy (Department Representative)
Nancy McNiel (ORS)

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