MOLECULAR PHYLOGENETICS AND THE EVOLUTION OF HIGH-FREQUENCY ECHOLOCATION IN HORSESHOE BATS (GENUS *RHINOLOPHUS*)

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DECLARATION

I, Samantha Stoffberg, hereby declare that the work contained in this thesis is the result of my own research and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. The text does not exceed 80,000 words and no part has been submitted in the past, or is being submitted, to any other university in fulfilment of a degree.

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ABSTRACT

Horseshoe bats (genus *Rhinolophus*) belong to the Old World family Rhinolophidae. They are high-duty cycle bats and many species use echolocation calls dominated by high frequencies (above 60 kHz). Much is known about how they use their echolocation calls, but very little is known about why these bats use echolocation calls of such high frequencies, or what has caused the divergence in echolocation call frequency between rhinolophid species. I test five hypotheses that may explain the evolution and divergence of high frequencies in the horseshoe bats: (1) The Allotonic Frequency Hypothesis – echolocation frequencies outside of moth hearing range (allotonic frequencies) have evolved in response to moth hearing; (2) The Allometry Hypothesis – high-frequency echolocation calls are simply a function of body size; (3) The Acoustic Adaptation Hypothesis – selection pressures linked to habitat structure have shaped the evolution of high-frequency echolocation calls; (4) The Foraging Habitat Hypothesis – foraging style and habitat of a bat should correspond to echolocation call frequency and wing design; and (5) The Acoustic Communication Hypothesis – echolocation frequencies evolved under selection pressure which eliminated overlap among sympatric species of rhinolophids, within the context of effective communication.

To explore the evolution and divergence of high frequencies, a robust phylogeny is required. To date no robust phylogenetic hypothesis has been erected for the family, and in many instances South African species have been excluded from partial phylogenies. To develop a robust phylogeny for the majority of the rhinolophids and in particular for the South African *Rhinolophus* species, I used a molecular supermatrix approach which included mitochondrial cytochrome *b* and three nuclear introns (TG, THY and PRKC1). The resultant robust phylogenetic hypothesis allowed me to investigate the geographical centre of origin for the Rhinolophidae and to estimate dates of divergence using a relaxed Bayesian

clock. The phylogeny for the family, multivariate analyses on echolocation and morphological data, together with the inclusion of habitat data, were used to test the predictions made by the above hypotheses.

Acceptance of the Allotonic Frequency Hypothesis requires two independent predictions to be validated. Numerous studies support the first prediction that the proportion of tympanate insects will be highest in the diets of bats whose echolocation calls are dominated by frequencies outside the hearing range of moths. Here I tested the second prediction of the Allotonic frequency Hypothesis, that within any family of bats, species using allotonic frequencies should be phylogenetically more derived. I found no support for the second prediction of the Allotonic Frequency Hypothesis, *viz* that bats using allotonic frequencies should be derived rather than ancestral if high frequencies in this genus have evolved in response to the selection pressure imposed by moth hearing. Rather, high frequencies are the ancestral condition. Rhinolophids evolved in forests, probably within Asia, and the high frequencies that characterize this genus are an adaptation to the cluttered habitats in which they arose and still occur today.

An allometric relationship exists between body size and echolocation frequency within the Rhinolophidae: however, body size alone cannot explain the evolution of high frequencies in this family. There is a complete overlap in body sizes between the Rhinolophidae and other families, such as the Vespertilionidae, which use lower echolocation frequencies. Among the rhinolophids echolocation frequency has stronger allometric relationships with morphological characters directly associated with sound production, emission, and reception. This contrast strongly suggests that selection has acted directly on echolocation rather than on body size. Furthermore, many species echolocate at frequencies much higher than predicted by body size allometry.

These deviations towards higher frequencies cannot be explained by the Acoustic Adaptation Hypothesis. For present-day species, differences in habitat

structure and climatic variables cannot explain differences in echolocation call frequency, nor can they explain why some species use echolocation calls of much higher frequency than would be predicted by the allometric relationship between call frequency and body size. Both low-frequency and high-frequency species occupy syntopic habitats (the same habitat within the same geographical range) and, because of this, habitat alone cannot explain the higher frequencies used by this family of bats. Limited support was found for the Foraging Habitat hypothesis, with some species that deviated from the body size/echolocation frequency relationship also deviating from the body size/wing design relationship. However no relationship exists between wing loading and peak echolocation frequency for the South African rhinolophids, nor for the global set of horseshoe bats. Thus, the evolution of frequencies higher than predicted by allometry are not due to selection acting simultaneously on wing morphology and call frequency.

Echolocation call frequencies of South African rhinolophids do, however, support predictions of the Acoustic Communication Hypothesis and also show support for the hypothesis that the high frequencies used by many rhinolophid species have evolved to enable efficient conspecific communication. Thus, evolutionary divergence of echolocation frequency among the Rhinolophidae was driven by the partitioning of sonar frequency bands for effective communication.

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CHAPTER 1

THE EVOLUTION OF HIGH-FREQUENCY ECHOLOCATION IN HORSESHOE BATS: AN INTRODUCTION TO THE HYPOTHESES

ECHOLOCATION IN BATS

Bats (Order Chiroptera) are unique among mammals because they are capable of true powered flight. Among mammals, bats are second in species richness only to rodents, with over 1000 species occurring worldwide (Simmons 2005). Excluding the fossil taxa, extant bats are grouped into 19 families: Craseonycteridae, Emballonuridae, Furipteridae, Hipposideridae, Megadermatidae, Miniopteridae, Molossidae, Mormoopidae, Mystacinidae, Myzopodidae, Natalidae, Noctilionidae, Nycteridae, Phyllostomidae, Pteropodidae, Rhinolophidae, Rhinopomatidae, Thyropteridae and Vespertilionidae (Simmons 2005). Bats fill almost every niche available to a nocturnal flying mammal and their ecological diversity is clearly evident in their diet. Some bats are frugivorous or nectarivorous, whilst others are piscivorous, carnivorous and even sanguivorous (Fenton 2001; Schnitzler & Kalko 2001). However, despite this wide range of diets, the majority of bats are insectivorous.

The successful capture of insect prey in the dark requires effective sensory adaptations for locating food. Although bats are not blind, sight alone is not sufficient to hunt moving objects in darkness. The primary method by which bats locate food can be used to divide the extant bat families into two broad categories - the echolocating and non-echolocating bats.

The Old World fruit bats (family Pteropodidae) do not echolocate, but locate their fruit using sight and olfaction (Fenton 2001). The exception in this family is the genus *Rousettus* which roosts in caves. *Rousettus* species use a very different form of echolocation than that used by insectivorous bats: they echolocate using tongue clicks produced by flicking the tongue against the roof of the mouth (Sales & Pye 1974). The clicks are used for orientation only and it has been proposed that this rudimentary form of echolocation evolved for orientating in their cave roosts in a manner similar to that used by oilbirds (*Steatornis caripensis*) and cave-dwelling swiftlets (*Aerodramus* spp. and *Collocalia* spp.) (Griffin 1953; Medway 1959; Novick 1959; Griffin & Suthers 1970; Konishi & Knudsen 1979; Henson & Schnitzler 1980; Griffin & Thompson 1982; Suthers & Hector 1982; Fenton 2001; Price *et al.* 2004). All other bat species, particularly the insectivores, use sophisticated laryngeal echolocation to a lesser or greater extent for orientation and hunting.

Echolocation is the location of objects by their echoes (Griffin 1960) and involves the broadcasting of pulses of sound using the larynx and vocal chords. These sounds are reflected back to the bat as echoes from objects. The bat's ears detect these echoes and the brain analyzes the spectral differences between the outgoing pulse and the returning echo to form a three-dimensional image of its environment. Bats that use echolocation can be divided into two broad categories depending on the amount of time they are "calling" (Fenton *et al.* 1995). The majority of bats use low duty-cycle (LDC) echolocation in which the duration of each call is much less than the interval between successive calls (Fenton *et al.* 1995). The second group of bats is the high duty-cycle (HDC) bats which use calls that are of longer duration than the inter-call interval (Fenton *et al.* 1995). High duty-cycle bats operate with search phase cycles greater than 30% (50-70% in rhinolophids) and are tolerant of pulse-echo overlap (Jones 1999). Calls of HDC bats are dominated by a long, constant-frequency component. With the exception of one New World species *Pteronotus parnellii* (Mormoopidae, Schnitzler & Henson 1980; Henson *et al.* 1987; Keating *et al.* 1994), bats that fall

into the HDC category are restricted to the Old World families Rhinolophidae and Hipposideridae.

The echolocation calls of HDC bats are characterized by a strong constant frequency (CF) component of relatively long duration (8–120 ms; Neuweiler 1989) and high frequencies (37–212 kHz, Black 1979; Jones 1992; Jacobs 2000; Taylor 2000), usually in combination with a short frequency modulated (FM) component at the beginning and/or end of the call (Neuweiler 1989). Numerous species use harmonics to provide greater resolution of their targets. The majority of HDC bats put most of their energy into the second harmonic (Sales & Pye 1974).

Bats that use HDC calls detect and classify prey in cluttered habitats by evaluating Doppler shifts that encode information about relative movement and allow the location of insect prey by detecting fluttering wings (Neuweiler 1989). Rhinolophid and hipposiderid bats possess an "auditory fovea" which can be compared to the fovea in the visual system of some animals. Within the basilar membrane of HDC bats, there is an area of thickening and lengthening that corresponds to the particular frequency component of the echolocation call (Schuller & Pollak 1979). Furthermore, in the auditory cortex of the brain, neurons are excited by a particular frequency and amplitude. In HDC bats, there are proportionally more neurons that are sensitive to the narrow range of frequencies within the range of the peak frequency of the bat's echolocation call (Schuller & Pollak 1979). These bats compensate for the shift in the frequency of the echoes of emitted signals, as a result of the relative motion of the bat to the target, by adjusting the frequency of subsequent calls to ensure that the returning echo is within this narrow frequency range. This behaviour is referred to as Doppler shift Compensation (DSC, Schnitzler 1968). It allows these bats to separate the emitted pulse from the returning echo in frequency, thus avoiding self-deafening (Fenton *et al.* 1995; Fenton 2001).

By using DSC, the bat effectively keeps echoes from the background constant. Against this constant acoustic background the movement of insect wings is detectable as amplitude modulations resulting from the emitted calls being reflected by the fluttering wings of insects at different angles of incidence to the impinging call (Neumann & Schuller 1991; Behrend *et al.* 1999). When an insect's wing is perpendicular to the emitted echolocation call, the returning echo will be more intense than if the wings were at an angle that is greater or less than 90º to the call (Bell & Fenton 1984; Schnitzler 1987; Altringham 1996). Thus, the movement of insect wings causes acoustic glints (brisk changes in intensity and spectral content, Neuweiler 1989) in returning echoes, which are easily detected against the acoustically constant background created by DSC calls (Bell & Fenton 1984; Schnitzler 1987). These echolocation calls are well adapted for the detection of fluttering targets in clutter (Bell & Fenton 1984; Schnitzler 1987), but are incapable of detecting insects whose wings are stationary (Bell & Fenton 1984; Link *et al.* 1986) because of the absence of acoustic glints. Furthermore, rhinolophid calls are dominated by high frequencies which are more directional than lower frequencies and provide greater resolution in cluttered habitats (Neuweiler 1989; Rydell *et al.* 1995). Although much is known about how rhinolophids and hipposiderids use their echolocation calls, very little is known about a) why these bats use echolocation calls of such high frequencies, or b) what has caused the divergence in echolocation frequency between rhinolophid species. Rhinolophids (23–121 kHz; Novick 1977, Zhao *et al.* 2003) and hipposiderids (60-212 kHz; Fenton & Bell 1981, Robinson 1996) echolocate on average at higher frequencies than most other families, including the Molossidae, Vespertilionidae, and Emballonuridae (Jones 1996), despite many species from families such as the Vespertilionidae also hunting in clutter (Norberg & Rayner 1987; Schnitzler & Kalko 2001; Ratcliffe *et al.* 2006).

HIGH-FREQUENCY ECHOLOCATION IN THE FAMILY RHINOLOPHIDAE

The horseshoe bats (genus *Rhinolophus* Lacépède 1799) are the second most speciose genus of bats, the most speciose being the genus *Myotis*, and is the only recognized extant genus in the family Rhinolophidae (Bell 1836). The 77 species of *Rhinolophus* (Simmons 2005) are restricted to the Old World and occur in both temperate and tropical areas throughout the Afrotropical, Australian, Indo-Malayan, Oceanian and Palearctic regions (Csorba *et al.* 2003). They are characterized by a solid pectoral ring formed by the fusion of the manubrium (top bone of the three bones forming the sternum), the first two ribs and the last cervical and first two thoracic vertebrae (Vaughan 1970; Csorba *et al.* 2003). The pectoral ring, together with the flattened and extended ribs, make the rib cage more rigid so that its contraction during the expulsion of the echolocation pulse is more efficient and energetically less costly, allowing these bats to echolocate while stationary (Speakman *et al.* 1989; Speakman & Racey 1991; Speakman *et al.* 2004). This is an adaptation for the 'perch-and-sally' hunting or 'flycatching' foraging strategy used by rhinolophids which involves scanning the habitat for insect prey from a perch (Schnitzler *et al.* 1985; Neuweiler *et al.* 1987; Jones & Rayner 1989; Csorba *et al.* 2003; Jones & Rydell 2003; Pavey & Burwell 2004). Another distinguishing characteristic of horseshoe bats is the intricate folds of skin in the nasal region. These "noseleaves" generally consist of three parts (lancet, sella and noseleaf) which may vary in arrangement, size and shape between species. The characteristic horseshoe-shaped anterior noseleaf gives these bats their common name.

All members of the genus *Rhinolophus* are HDC echolocators; the duration of their call is long relative to the time between consecutive calls. This is possible because these bats separate their emitted signal from the returning echo in frequency and not time (Fenton *et al.* 1995). Low duty-cycle bats separate call and echo in time so they have to wait for the returning echo. As a result, they are "quiet" for most of the time between two consecutive calls (Fenton *et al.* 1995).

The range of frequencies used by rhinolophids is from 23.7 kHz (*R. rex* from Guizhou, China; Liang 1993, Zhao *et al.* 2003) to 121 kHz (*R. landeri landeri* from Nigeria; Pye & Roberts 1970, Novick 1977). Although some species echolocate below 60 kHz (e.g. *R. philippinensis*, *R. hildebrandti*, *R. luctus* and *R. trifoliatus*), 35 of the 44 species for which echolocation data are available use frequencies above 60 kHz (e.g. *R. blasii*, *R. clivosus*, *R. capensis*, *R. denti* and *R. swinnyi*).

Several hypotheses that are not mutually exclusive could be advanced to explain the use of high-frequency echolocation in bats. I will restrict my discussion of these hypotheses to the Rhinolophidae because some aspects of the hypotheses may not apply to LDC bats or to other HDC bats.

Allotonic Frequency Hypothesis

The evolution of echolocation represented an innovation that increased the effectiveness of bats in catching nocturnal insects and exerted a strong selection pressure on these insects to evolve effective defences against foraging bats. Echolocation signals make bats conspicuous and may alert potential prey to a bat's approach. Certain nocturnal insects, including species of beetles (Coleoptera), praying mantids (Mantodea), lacewings (Neuroptera) and moths (Lepidoptera), have evolved tympanic organs that allow them to hear the echolocation calls of an approaching bat and to take evasive action (Roeder 1967; Fullard 1982, 1987, 1990; Surlykke 1988). Some moths, for example, have evolved ears apparently in direct response to bat echolocation (Fullard 1987; Surlykke 1988; Miller & Surlykke 2001) and the interaction between echolocating bats and tympanate moths is one of the most frequently cited examples of predator/prey coevolution (Fullard 1988; Rydell *et al.* 1995; Brewer 1998; Rydell *et al.* 2000; Fullard 2001; Hoagland *et al.* 2001). Evidence for such coevolution includes the fact that the ears of moths are highly sensitive to frequencies between 20 and 60 kHz which coincide with the peak-frequency range of most echolocating bats (Fullard 1987).

The ability to detect the echolocation calls of a foraging bat provides the moth with an opportunity to avoid capture by rapidly altering its flight path or dropping towards the ground (Roeder 1967). It has been estimated that tympanate moths have a 40% greater chance of evading foraging bats than non-tympanate moths (Roeder 1967; Rydell 1992; Acharya & Fenton 1999). However, there is a decrease in the sensitivity of moth ears to frequencies above 65 kHz and below 20 kHz (Fenton & Fullard 1979; Fullard 1987; Surlykke 1988; Fullard *et al.* 1997). For bats echolocating above 65 kHz, the distance over which a moth can detect the bat is reduced (Fenton & Fullard 1979). A possible counter-adaptation by bats would therefore be to exploit the limited hearing capabilities of moths by using frequencies above and below the greatest sensitivities of moth ears, so-called "allotonic frequencies" (Novick 1977; Fullard 1987). Allotonic frequencies are thus frequencies below 20 kHz or above 60 kHz (after Fullard 1982). Syntonic frequencies are frequencies within the hearing range of moths (20–60 kHz; Fullard 1982). Examples of bats using allotonic frequencies and whose diet is dominated by moths include *Euderma maculatum* (12 kHz; Woodsworth *et al.* 1981), *Tadarida teniotis* (11-12 kHz; Rydell & Arlettaz 1994) and *Cloeotis percivali* (212 kHz; Whitaker & Black 1976, Jacobs 2000).

The evolution of tympanate organs in moths, and their subsequent ability to avoid bat predation, may have resulted in selection pressure on bats to evolve echolocation calls that are dominated by allotonic frequencies. As many rhinolophids echolocate above 60 kHz, moth hearing has been hypothesized to be the ultimate cause driving the evolution of high frequencies in these bats (Novick 1977; Fenton & Fullard 1979).

The Allotonic Frequency Hypothesis proposes that echolocation frequencies outside of moth hearing range have evolved to circumvent moth hearing. It makes at least two predictions: 1) that the proportion of tympanate insects will be highest in the diets of bats whose echolocation calls are dominated by

frequencies outside the hearing range of moths, and 2) that within any family of bats, species using allotonic frequencies should be phylogenetically more derived. The first prediction has been supported by numerous studies that have shown a positive relationship between the frequency of bat echolocation calls and the incidence of moths in their diets at the phylogenetic level of families (Jones 1992; Bogdanowicz *et al.* 1999), including the Rhinolophidae (Schoeman 2006) and the ecological level of community structure (Jacobs 2000; Schoeman & Jacobs 2003; Pavey *et al.* 2006).

If moth ears evolved in response to bat predation, and high echolocation frequencies in some bats, which made them less audible to moths, evolved in response to moth hearing, high-frequency echolocation should have evolved more recently than echolocation frequencies within the range of moth hearing. This prediction of the allotonic frequency hypothesis has not yet been tested and the reason for the high-frequency echolocation calls used by bats, for example, in the families Rhinolophidae and Hipposideridae remains unresolved (Fenton & Fullard 1979; Fullard 1987; Schnitzler 1987; Heller & von Helversen 1989; Neuweiler 1989; Jones 1996).

The rhinolophids are ideal for testing the allotonic frequency hypothesis because the genus contains species that echolocate at frequencies both within and outside moth hearing range. Furthermore, Jones & Waters (2000) suggest that the potential for coevolution between bats and moths may be greater in bats that use calls dominated by a narrow constant frequency than in bats using broadband calls. Although LDC bats using broadband calls may place most of the energy into an allotonic frequency, part of the call may still be syntonic with moth hearing because each call comprises a range of frequencies. If high-frequency echolocation in the Rhinolophidae is the result of selection pressure from moth hearing, species that echolocate within the range of moth hearing (20-60 kHz) should be ancestral whereas species that use allotonic frequencies (>60 kHz) should be more derived. Bats using frequencies at the lower end of the

spectrum (<20 kHz) are also using allotonic frequencies and should therefore also be derived, but as no rhinolophid uses frequencies below 20 kHz, this prediction was not tested.

Alternative explanations for the evolution of allotonic frequencies

The Prey Detection Hypothesis

A positive relationship exists between predator and prey body sizes (Peters 1983) with predator body size influencing the size of prey that can be handled effectively. Among bats, an inverse relationship also exists between call frequency and the size of the prey that can be detected (Pye 1983). Bats using higher frequencies are able to detect smaller targets than bats using lower frequencies. This suggests that small bats require echolocation calls of high frequencies that will enable them to detect prey items that are small enough for them to handle. This could explain the absence of larger prey items that require a longer handling time, (e.g. beetles) in the diets of small bats (because they are well able to assess prey size before attacking), but does not however explain why the diets of larger bats contain so few moths (which should be both detectable and rewarding, Barclay & Brigham 1991; Jones 1992). Because call frequency is related to body size, these studies suggest that the high frequencies used by most of the rhinolophid and hipposiderid species have evolved to detect small prey items rather than as a means of overcoming prey defences. If this is the case, then bats with higher frequencies should take smaller prey and have broader diets than bats with lower frequencies (Prey Detection Hypothesis; Jacobs *et al.* 2007). However, this may not always be the case. For example, *R. clivosus* emits a higher frequency call than the sympatric *R. capensis*, but there is considerable overlap in the range of prey sizes taken (Jacobs *et. al.* 2007). Furthermore, contrary to the prediction made by the Prey Detection Hypothesis, *R. clivosus* (with the higher frequency echolocation calls) takes, on average, larger insects than *R. capensis* (Jacobs *et al.* 2007). These results may be due to

the high echolocation frequencies used by both *R. clivosus* (92 kHz, Jacobs *et al.* 2007) and *R. capensis* (84 kHz, Jacobs *et al.* 2007).

The minimal detectable prey size is influenced by the wavelength of a bat's echolocation call. At 20 kHz, the size of a detectable insect is 17.2 mm, at 25 kHz it is 13.8 mm, at 40 kHz it is 8.6 mm and at 50 kHz it is 6.9 mm. At the higher frequencies, differences in wavelengths are much smaller than differences at lower frequencies for the same absolute difference in frequency. At 70 kHz the wavelength of sound is 4.9 mm, at 80 kHz it is 4.3 mm, and at 90 it is 3.8 mm. Thus the wavelength difference associated with a frequency difference of 5 kHz between 20 and 25 kHz is 3.4 mm, but is only 0.6 mm for a frequency difference of 10 kHz between 70 and 80 kHz. This suggests that differences in prey size that are detectable by a bat are greater at lower frequencies than at higher frequencies. Thus, differences in echolocation frequencies are more likely to influence differences in prey size at the lower frequency ranges (Kingston *et al.* 2001; Houston *et al.* 2004; Kingston & Rossiter 2004), but at higher frequencies the differences between wavelengths are not likely to be functionally significant. Thus at the high frequencies at which most rhinolophids operate, prey size is unlikely to be the reason why one bat would echolocate at 70 kHz and another at 90 kHz. The Prey Detection Hypothesis is therefore unlikely to explain the evolution of high frequencies within the Rhinolophidae (Jacobs *et al.* 2007).

Because echolocation calls are used for both orientation and for hunting, they are probably under a variety of selection pressures. It is possible, therefore, that the call structure and relatively high frequencies used by the rhinolophids and hipposiderids have evolved for reasons other than to make their calls less audible to moths, or to facilitate resource partitioning based on prey size. The high-frequency calls could: 1) be associated with body size (Jones 1996, 1999), 2) have evolved to provide greater resolution in drier climates where the lower humidity means less atmospheric attenuation (Heller & von Helversen 1989; Guillén *et al.* 2000), or 3) have evolved in response to more densely vegetated habitats (Neuweiler 1989; Rydell *et al.* 1995) which have many obstacles that a

bat has to detect and avoid by echolocation, i.e. they are more cluttered. Finally, high frequencies may have evolved to permit effective communication amongst conspecifics through the partitioning of echolocation frequency bands.

Allometry Hypothesis

In the Rhinolophidae, body size (indexed by forearm length and body mass) and call frequency are inversely correlated (Heller & von Helversen 1989; Jones 1996, 1999): it is possible that the high-frequency calls used by these bats are simply a function of body size or, more precisely, a function of the size of the organs producing the echolocation sounds. In other words, the high frequencies are a reflection of the allometric relationship between size and echolocation calls within the rhinolophids, a prediction termed the Allometry Hypothesis. This relationship can be explained in terms of the resonating characteristics of the structures needed for sound production and reception. Longer vocal chords and larger cavities produce sounds of lower frequencies (Guillén *et al.* 2000). This implies that the resting frequency of the CF component should remain relatively constant within individual bats (Heller & von Helversen 1989) and that a closer relationship should exist between echolocation frequency and the sound-producing and -processing apparatus, than with overall body size (Francis & Habersetzer 1998).

The elaborate noseleaves of rhinolophids are associated with the emission of echolocation calls. A significant relationship exists between call frequency, frequency wavelength and the breadth of the noseleaf (Bogdanowicz 1992, Robinson 1996). In *R. ferrumequinum* and *R. hipposideros*, the distance between the nostrils is equal to half the wavelength of the bat's echolocation call (Pye 1972). Low-frequency calls are characterized by longer wavelengths than high-frequency calls. If internarial spacing is equal to half the call wavelength, and lower frequencies are characterized by long wavelengths, then lower frequency calls should correlate with wider noseleaves. If noseleaf width always increases with body size, this would be another explanation of the inverse relationship

between body sizes and call frequency. Furthermore, in some bats, call frequency is correlated with the height of the pinnae, such that longer pinnae are associated with lower call frequencies (Gannon *et al.* 2001; Zhao *et al.* 2003). This can also be related back to body size, with larger bats possessing longer pinnae. However the longer pinnae may also be an adaptation for detecting prey-generated sounds (i.e. calls and/or movements) in species that glean, for example *Megaderma lyra* (Marimuthu & Neuweiler 1987), *Myotis myotis* (Arelettaz *et al.* 2001), *Antrozous pallidus* (Furzessery *et al.* 1993) and *Nycteris grandis* (Fenton *et al.* 1983, 1990). Although *R. megaphyllus* (single incident - Pavey & Burwell 2004) and *R. blasii* (Siemers & Ivanova 2004) have been shown to glean, *R. blasii* and *R. megaphyllus* are not characterized by longer-than-average ears as found in other gleaning bats. In *R. blasii* this may be because it does not rely on prey-generated sounds for gleaning (Siemers & Ivanova 2004). If differences in the frequency of echolocation calls are the result of variations in body size, I predict that irrespective of phylogenetic affinities, larger bats will have echolocation calls characterized by lower frequencies and smaller bats will have echolocation calls characterized by higher frequencies. Furthermore a relationship should exist between noseleaf width and body size (forearm length) and between pinnae length, skull parameters and call frequency.

The Acoustic Adaptation Hypothesis

The Acoustic Adaptation Hypothesis (Morton 1975; Hansen 1979) proposes that selection pressures linked to habitat structure have shaped the evolution of the acoustic properties of bird songs. This hypothesis assumes that songs used by birds have been shaped by habitat-driven selection to enhance sound propagation (Boncoraglio & Saino 2007). Bat echolocation calls also may have evolved in response to habitat structure and environmental factors associated with species' distributions that influence the propagation of sound. Various environmental variables, (e.g. humidity) may therefore influence echolocation call frequency.

Species' distributions are not random. For example, the distributions of three hipposiderids have been shown to be associated with annual rainfall, rainfall seasonality, distance to the coast, and habitat complexity (Milne *et al.* 2006). If selection is acting to enhance the sound propagation of echolocation calls, the distribution of species using either high- or low-frequency calls may coincide with unique combinations of environmental variables.

Humidity influences the degree to which atmospheric attenuation affects echolocation calls. The rate at which the absorption of sound energy increases is directly related to the humidity of the air (Lawrence & Simmons 1982). Lower frequency sounds have longer wavelengths than sounds of higher frequency and are therefore less sensitive to atmospheric attenuation. High frequencies are inherently subject to rapid atmospheric attenuation, and attenuate even more rapidly under humid conditions. This suggests that echolocation frequency and humidity interact to influence the distance at which prey and obstacles can be detected. For a bat occupying cluttered habitat in a humid area (for example, tropical rainforests) call frequency may be a response to the trade-off between selection for lower frequencies, which provide a longer detection range at high humidity, and higher frequencies, which provide greater resolution for prey identification within clutter.

It has been proposed that the rhinolophids originated in humid areas such as the tropical rainforests of south-east Asia or Africa (Bogdanowicz 1992). Bogdanowicz (1992) suggests that the relatively wide noseleaves in tropical rhinolophids, and their associated lower call frequencies, may be an adaptation to hunting in humid areas. Bats occupying drier habitats have calls of higher frequency relative to body size than bats occupying wetter environments (Heller & von Helversen 1989). This is expected, because higher frequencies attenuate more rapidly under humid conditions thus reducing the bat's detection range. These studies suggest that the high frequencies used by rhinolophids and hipposiderids may be an adaptation to occupying dry habitats. If rhinolophids

originated in humid areas where low-frequency calls are more suitable, then low-frequency calls could be predicted to have arisen relatively early in the rhinolophid lineage, a prediction shared with the Allotonic Frequency Hypothesis. However, if humidity is driving the evolution of call frequency, bats possessing low-frequency calls should be restricted to humid areas, and bats using high frequencies should be characteristic of more arid regions (Heller & von Helversen 1989). Relatively derived rhinolophid species that moved to more arid habitats may have been released from the constraint imposed on their calls by atmospheric attenuation and humidity, possibly enabling them to make use of calls of higher frequencies (and thus benefiting from the increased resolution). If echolocation calls have evolved in response to environmental variables that can influence the propagation of echolocation call frequency (e.g. humidity), then irrespective of phylogenetic affinities, the distributions of rhinolophids using lower frequencies should be different to those using high frequencies. I would also expect these predictions to be supported intraspecifically within widely distributed species, such that populations of the same species (e.g. *R. clivosus*) occupying different habitats would have different peak echolocation frequencies, e.g. higher frequencies in drier habitats.

Foraging Habitat Hypothesis

Foraging habitat and behaviour coincide with particular adaptations in both echolocation calls and wing morphology (Aldridge & Rautenbach 1987; Norberg & Rayner 1987; Fenton 1990; Schnitzler & Kalko 2001; Schnitzler *et al.* 2003; Stoffberg & Jacobs 2004) and may be independent of phylogeny (Ruedi & Mayer 2001). Due to the joint constraints of flight and detection of food in different habitats, echolocation and wing design have been assumed, to form an adaptive complex (Aldridge & Rautenbach 1987).

Rhinolophids have low wing loadings, low aspect ratios and rounded wingtips - all adaptations for slow, manœuvrable flight (Norberg & Rayner 1987). This is necessary to avoid collisions with obstacles when foraging in cluttered habitats.

Cluttered habitats are characterized by dense vegetation which increases the amount of acoustic clutter ("echoes from objects other than the target of interest", Fenton *et al.* 1995 p235) when hunting for prey or during orientation. Horseshoe bats therefore require echolocation calls (and hence echoes) that allow the bat to overcome the key perceptual problem associated with cluttered habitats, i.e. being able to distinguish prey from background echoes (Fenton 1990).

Rhinolophid echolocation calls are uniquely adapted to foraging in cluttered habitats because they can reject background clutter at the same time as detecting fluttering prey (Schuller 1984; Neuweiler 1989). This is due to the use of Doppler Shift Compensation. Furthermore, high-frequency calls are more directional and allow for the detection of small prey items in cluttered habitats by providing greater resolution (Neuweiler 1989; Rydell *et al.* 1995). Thus the high-frequency calls used by the Rhinolophidae may represent an adaptation for foraging in cluttered habitats, the so called Foraging Habitat Hypothesis (Jones & Barlow 2004, Jacobs *et al.* 2007). If so, then given that rhinolophids reportedly originated in tropical rainforests (Bogdanowicz 1992), high-frequency echolocation should appear relatively early in the lineage of the Rhinolophidae, whilst lower frequencies should appear later.

The Foraging Habitat Hypothesis (Jones & Barlow 2004) proposes that the foraging style and habitat of a bat should correspond to the echolocation call frequency and the wing design of that species, and that both should be influenced by the degree of clutter such that peak frequency and wing loading are inversely related (Jacobs *et al.* 2007). This hypothesis differs from the Acoustic Communication Hypothesis in that the FHH deals with how bats through their flight and echolocation deal with clutter while the ACH deals with limitations imposed by the propagation of sound in different habitats. The reason for this is that as the habitat becomes more cluttered, the species foraging there would require increased manœuvrability (lower wing loading) and high-resolution echolocation (i.e. higher frequencies). Furthermore, if selection pressures are

favouring wing and call combinations for certain habitats, those species with call frequencies higher than predicted by the relationship between body size and echolocation frequency should have a wing design that also deviates from the family norm (Jacobs *et al.* 2007).

The previous three hypotheses assume that selection pressures acting over a gradient of increasing body size or habitat variable (e.g. clutter) have resulted in an increase in echolocation frequency. This effect would have had to be more pronounced for the Rhinolophidae than for other families to explain the higher frequencies used by rhinolophids.

The Acoustic Communication Hypothesis

The Acoustic Communication Hypothesis (Jacobs *et al.* 2007, also referred to as Acoustic Resource Partitioning by Duellman & Pyles 1983) proposes that the evolution of different echolocation frequencies among sympatric species may be a means of partitioning sonar bands to permit efficient communication between conspecifics. This hypothesis may also explain why some species deviate from the allometric relationship between call frequency and body size by using higher or lower echolocation frequencies (Jacobs *et al.* 2007). Deviations from allometry may serve to partition the frequencies used by sympatric *Rhinolophus* species (e.g. Heller & von Helversen 1989; Jacobs *et al.* 2007). Similarly divergence in echolocation frequency between morphologically cryptic species may have evolved to facilitate communication among conspecifics (Jones & Barlow 2004). If high frequencies have evolved for partitioning communication channels, then I would predict that the range of frequencies within species should be small, and within a bat community there should be no overlap in the peak echolocation frequency used by sympatric species.

A MOLECULAR PHYLOGENETIC APPROACH

I use a molecular phylogenetic approach to test the above predictions. Both mitochondrial and nuclear markers are used to generate a phylogeny for the majority of the rhinolophids and in particular for the South African *Rhinolophus* species.

An accurate knowledge of the phylogeny of a group of species is needed to identify common processes underlying the evolution of diversification among the component species (Sanderson & Donoghue 1996). A phylogeny is also necessary to determine whether the calls used by some bats are the legacy of an ancestral condition, or whether they have evolved independently.

Previous phylogenies for the rhinolophids have been based on morphological and phenetic characters (Bogdanowicz 1992; Bogdanowicz & Owen 1992). However, depending on which characters were used, or the different weighting methods applied, the resultant phylogenies were very different. Allozyme studies of southern African rhinolophids (Maree & Grant 1997) recovered trees that are in conflict with those based on morphology, and, more recently, a supertree approach failed to elucidate the relationships among the majority of rhinolophids (Jones *et al.* 2002). Furthermore, Guillén *et al.* (2003) attempted to use the mitochondrial cytochrome *b* gene to generate a phylogeny. However, this gene failed to provide adequate resolution at deeper nodes. Some of these problems encountered in the generation of a phylogeny for the rhinolophids may have resulted from the fact that in many cases only one data set or one gene was used. Numerous studies have demonstrated that it is important to use multiple and diverse sources of phylogenetic information such as data sets comprising sequences from several markers (nuclear and mitochondrial) because studies using several data sets consistently demonstrate the limited ability of single data sets to reconstruct phylogenies with any accuracy (Cao *et al.* 1994; Cummings *et al.* 1995).

A robust phylogeny for the genus *Rhinolophus* is not yet available and part of this study will attempt to resolve the evolutionary relationships among certain *Rhinolophus* species by making use of both nuclear introns and the mitochondrial cytochrome *b* gene. Nuclear introns provide sufficient resolution at deeper nodes in bats (Eick et al. 2005) and other mammalian groups at both the specific and deeper taxonomic levels (Matthee & Davis 2001; Matthee *et al.* 2001; Willows-Munro 2003; Willows-Munro *et al.* 2005; Matthee *et al.* 2007). By contrast, cytochrome *b* does not provide good resolution at deeper nodes (Guillén *et al.* 2003), but it is useful for resolving terminal nodes. A robust phylogeny will allow me to map echolocation frequency to test not only the allotonic frequency hypothesis but also several alternative hypotheses for the evolution of high-frequency calls.

RESEARCH AIMS

The primary aims of this study are to use a molecular phylogenetic approach to:

- Construct a robust molecular phylogeny for the genus *Rhinolophus*;
- Using the above, test the predictions of the Allotonic Frequency Hypothesis; and
- Investigate the influence of body size and habitat on the evolution of rhinolophid echolocation call frequency, testing the Allometry, Acoustic Adaptation, Foraging Habitat and Acoustic Communication hypotheses.

CHAPTER 2

A MOLECULAR PHYLOGENY OF THE RHINOLOPHIDAE

INTRODUCTION

Bats are one of the most diverse mammalian groups, yet the relationships amongst extant taxa have remained poorly understood until recently. Classifications based on morphology suggested two suborders: the Megachiroptera, or Old World fruit bats, and the Microchiroptera, or echolocating bats (Simmons 1995; Simmons & Geisler 1998).

Much controversy has surrounded the monophyletic status of bats (*e.g.* Smith & Madkour 1980; Pettigrew *et al.* 1989; Goodman 1991; reviewed in Simmons 1994). Some authors claim that the morphology of the two suborders of bats suggest separate origins, with the Megachiroptera being more closely related to primates than to the Microchiroptera (Smith & Madkour 1980; Pettigrew 1986, Pettigrew *et al.* 1989; Pettigrew & Kirsch 1995). In contrast, more recent molecular analyses strongly support the original notion of bat monophyly (Miyamoto *et al.* 2000; Teeling *et al.* 2000; Jones *et al.* 2002; Eick *et al.* 2005). Controversy has also surrounded the monophyly of the groups making up the suborder Microchiroptera. Analyses based on morphology suggest that all Microchiroptera have a common ancestor with all extant taxa possessing a complex laryngeal echolocation system (Simmons 1998; Simmons & Geisler 1998), and that the Megachiroptera comprise all other non-echolocating bats making up the other suborder. However, molecular studies demonstrate that the microbats previously grouped as the Rhinolophoidea (Rhinolophidae, Hipposideridae, Nycteridae, and Megadermatidae) are in fact paraphyletic

(Teeling *et al.* 2002; Springer *et al.* 2004; Van Den Bussche & Hoofer 2004; Eick *et al.* 2005). These molecular studies suggest that the nycterids are distinct from other Rhinolophoidea and that the remaining members of the Rhinolophoidea (Rhinolophidae, Hipposideridae, and Megadermatidae), which now also include the Rhinopomatidae and the Craseonycteridae (Teeling *et al.* 2005; Miller-Butterworth *et al.* 2007), are more closely related to the non-echolocating megachiropteran family, Pteropodidae (Teeling *et al.* 2002; Eick *et al.* 2005).

Previous classifications also regard the Family Rhinolophidae as comprising two subfamilies, the Hipposiderinae and the Rhinolophinae (Koopman 1993, 1994; McKenna & Bell 1997; Simmons & Geisler 1998; Teeling *et al.* 2002). However, in this study I consider the Family Rhinolophidae to comprise only the rhinolophids and exclude the hipposiderids from this family following Corbet & Hill (1992), Bates & Harrison (1997) and Simmons (2005). This treatment is congruent with morphological studies (Bogdanowicz & Owen 1998; Hand & Kirsch 1998) and recent molecular studies which treat the rhinolophids and hipposiderids as separate families (Eick *et al.* 2005). Furthermore, rhinolophids and hipposiderids are immunologically as distinct as other taxa placed in separate families (Pierson 1986).

THE HORSESHOE BATS: GENUS *RHINOLOPHUS*

The family Rhinolophidae Gray 1825 consists of a single genus *Rhinolophus* Lacépéde 1799. The taxon is exclusively Old World, with its 77 species (Simmons 2005) occurring in both temperate and tropical areas throughout the Afrotropical, Australian, Indomalayan, Oceanian and Palearctic regions (Csorba *et al.* 2003).

Horseshoe bats are characterized by intricate folds of skin in the nasal region. These "noseleaves" generally consist of three parts (lancet, sella and noseleaf) which vary in arrangement, size and shape between species (Csorba *et al.*

2003). The characteristic horseshoe-shaped anterior noseleaf gives these bats their common name. In addition to nasal skin folds, members of this genus possess a synapomorphic solid pectoral ring formed by the fusion of the manubrium (top bone of the three bones forming the sternum), the first two ribs, and the last cervical and first two thoracic vertebrae (Vaughan 1970, Csorba *et al.* 2003).

RHINOLOPHID RELATIONSHIPS

Early studies on the evolutionary relationships within the genus *Rhinolophus* were based on body size, shape of the noseleaf and position of the third upper premolar. Early classifications of the rhinolophids (Anderson 1905a; 1905b; 1918) remained relatively unchanged until the advent of modern taxonomic techniques (chromosomal studies, electrophoresis, and molecular analyses) and the advancement of analytical techniques. Over the past few decades the relationships among the rhinolophids have been reassessed (e.g. Harada *et al.* 1985; Qumsiyeh *et al.* 1988; Guillén *et al.* 2003).

Cladistic and phenetic studies based on 16 external characters and 19 cranial characters from 62 species of *Rhinolophus* provide some information on interrelationships, however the analysis of morphological data under different sets of assumptions recovered conflicting trees (Bogdanowicz 1992; Bogdanowicz & Owen 1992). In addition, the more recent molecular analyses indicate a large amount of convergence in morphological characters, particularly those associated with echolocation (Eick *et al.* 2005).

In an attempt to provide a conservative phylogeny for the Chiroptera, Jones *et al.* (2002) made use of a supertree approach. However, the supertree constructed for the Rhinolophidae, using the technique of Matrix Representation with Parsimony, provided poor resolution of the evolutionary relationships among the 64 *Rhinolophus* species included in the analysis. The only relationships that had

support were those at the tips of the tree between sister taxa. Source data for the construction of the supertree were obtained from diverse studies which applied informal character analyses, discrete character clustering methods, and distance data clustering methods to morphological and/or molecular data (Jones *et al.* 2002). Due to the potential bias towards older, less robust analyses, phylogenetic information was collated from studies published between 1970 and 2000. Source data for the Rhinolophidae were obtained from Pierson (1986), Qumsiyeh *et al.* (1988), Bogdanowicz & Owen (1992), Koopman (1994), and Maree & Grant (1997). The data from these studies were based on different characters and methodologies and recovered conflicting relationships (discussed later in this chapter), and because the level of conflict among source trees has not been assessed, these data may therefore have been less than ideal for constructing a supertree of the rhinolophids.

The most recent molecular study of the interrelationships among rhinolophids was published as part of a description of the taxonomic background of the genus (Guillén *et al.* 2003), using the complete mitochondrial cytochrome *b* gene from a total of forty-three species (or putative species). Five main clades were identified: 1) the *R. trifoliatus* clade, 2) the *R. hipposideros* clade, 3) the African clade, 4) the *R. megaphyllus* clade, and 5) the clade comprising the *R. rouxii* and *R. euryotis* groups. Bootstrap support for these clades was low, with limited intra-clade resolution. However, the *R. megaphyllus* - *R. pusillus* clade did have some support in terms of the criterion of Hillis & Bull (1993) who suggest that nodes with less than 70% bootstrap support are not well supported. The low resolution of the cytochrome *b* gene tree, especially close to the base of the topology, may be due to a rapid radiation within the family and/or homoplasy in the cytochrome *b* data set (see also Matthee & Davis 2001). In addition to the poor resolution among species, three South African taxa (*R. capensis*, *R. denti* and *R. swinnyi*) were not included in Guillén *et al.*'s (2003) study.

SOUTHERN AFRICAN RHINOLOPHIDS

Of the 25 rhinolophid species that occur in Africa (Csorba *et al.* 2003; Simmons 2005) ten (*R. blasii*, *R. capensis*, *R. clivosus*, *R. darlingi*, *R. denti*, *R. fumigatus*, *R. hildebrandti*, *R. landeri*, *R. simulator* and *R. swinnyi*) occur in southern Africa, where they are widely distributed. These species are usually distinguished on the basis of body size (in particular forearm length), position of the anterior upper premolar, and differences in the shape and size of noseleaves around the nostrils.

There is much disagreement over phylogenetic relationships among southern African rhinolophids. Morphological analyses by Bogdanowicz & Owen (1992) recover conflicting clades. In both the size-removed and common-part-removed analyses the southern African rhinolophids either group with other African rhinolophids or Asian species, and the composition of the various clades differ according to the analysis. Phenetic analysis of the same data set proposes that the southern African rhinolophids can be grouped into three clades (Bogdanowicz 1992). The first clade grouped *R. blasii*, *R. capensis*, *R. denti, R. simulator, R. landeri* and *R. swinnyi* together. *Rhinolophus darlingi* and *R. clivosus* were placed in a separate clade and the third clade comprised *R. hildebrandti* and *R. fumigatus (*Bogdanowicz *op. cit.*). Bogdanowicz & Owen (1992) further suggest that *R. hildebrandti* is the most plesiomorphic species of the extant African rhinolophids. Phylogenies produced by Erasmus & Rautenbach (1984), which were based on cranial features, suggest that *R. darlingi* groups more closely with *R. clivosus* and *R. capensis.* In the same classification, *R. fumigatus* and *R. hildebrandti* group together, with the rest of the southern African rhinolophids grouping together based on skull size. These results are more similar to the phenetic analyses of Bogdanowicz (1992), but differ from results of the allozyme study of Maree & Grant (1997). Furthermore, the phenetic analyses of Bogdanowicz (1992) and Bogdanowicz & Owen (1992) also found details of the systematic relationships between the southern African rhinolophids to be in

disagreement with a study by Qumsiyeh *et al.* (1988) based on chromosomal and allozyme data. However the studies did agree on the possibility of a monophyletic African assemblage.

Analyses by Maree & Grant (1997), based on 34 allozyme encoding loci, grouped *R. fumigatus,* and *R. hildebrandti* with *R. darlingi* and *R. clivosus*. *Rhinolophus capensis, R. denti, R. simulator* and *R. swinnyi* were grouped together and *R. blasii* was placed in a separate clade based on its genetic distinctiveness. *Rhinolophus clivosus* and *R. landeri* also appear to be the most divergent among the ten southern African species. Because only one specimen was sampled, the phylogenetic affinities of *R. landeri* could not be resolved by allozymes. Furthermore, the use of isozyme characters did not permit systematic inference of *Rhinolophus* in southern Africa and no differences in gene expression were observed between the two distinct species, *R. clivosus* and *R. landeri* (Maree & Grant 1996).

On the basis of cytochrome *b* analysis, Guillén *et al.* (2003) concluded that *R. clivosus* was more closely related to *R. ferrumequinum* than any other African rhinolophid. However, because *R. capensis*, *R. denti*, and *R. swinnyi* were not included in this analysis, the authors adopted the clade described in Maree & Grant (1997). *Rhinolophus blasii* is more closely affiliated with *R. euryale* and *R. mehelyi* (from Europe) than other African rhinolophids, supporting its placement in a separate clade by Maree & Grant (1997), rather than with the *R. capensis* clade described by Bogdanowicz (1992). Relationships among the remaining southern African rhinolophids (*R. fumigatus*, *R. hildebrandti*, and *R. darlingi*) have poor bootstrap support (Guillén *et al.* 2003).

From the above it is evident that relationships among the species of rhinolophids, and in particular those occurring in southern Africa are poorly understood. Current phylogenies are based on single data sets and do not yield well-resolved trees (e.g. Maree & Grant 1997). Supertree analyses incorporating most of the

species in this genus are also poorly resolved, with many of the source trees being based on morphology. To date there is little agreement between traditional taxonomies and cladistic analyses (Jones *et al.* 2002). One possible explanation for these discrepancies may be the types of data sets, or analyses, used.

Supertrees do not make use of raw character data, but use nodes in topologies as phylogenetic structure. Source trees from the published literature are recorded as a series of binary characters describing each node which are then incorporated into a matrix that is analysed using the matrix representation with parsimony method (Gatesy *et al.* 2002; Jones *et al.* 2002; Bininda-Emonds 2004; de Queiroz & Gatesy 2007). One shortfall of this method is that support for the various nodes in the source topologies is not taken into account. Inaccurate source topologies can also result in the supertree recovering incorrect relationships. Furthermore, even with recent advances in supertree analyses, hidden support for nodes within conflicting trees cannot be detected (Pisani & Wilkinson 2002) unless the primary data on which the topologies are based are available. Similarly, the absence of primary character data in supertree analyses can lead to pseudoreplication, thus violating the assumption of data independence (Bininda-Emonds 2004). This suggests that using character data directly (e.g. a supermatrix approach) would be a better approach. Furthermore, the use of more than one gene fragment to construct phylogenies may provide better resolution between taxa (de Queiroz 1993). If different gene partitions recover similar phylogenies, it is more likely that the phylogenetic hypothesis is correct.

MOLECULAR PHYLOGENY OF THE GENUS *RHINOLOPHUS*: A SUPERMATRIX APPROACH

The supermatrix approach may be more reliable than using a supertree approach because raw character data (and not tree topologies - see above), are combined to create a single matrix in which all the characters are analysed simultaneously (de Queiroz & Gatesy 2007). The benefit of the supermatrix over the supertree

analysis is that the characters themselves are used to their full extent when constructing phylogenies and no data are lost when individual character sets are summarized into trees (de Queiroz & Gatesy 2007). The supermatrix approach has the benefit of providing greater resolution and accuracy, because different data sets comprising genes which may evolve at different rates are used (Gatesy *et al.* 2002). Furthermore, hidden support for various nodes in separate data sets can be revealed in supermatrix analyses because the combined data set may reveal phylogenetic signals (synapomorphies) that could otherwise be hidden by phylogenetic 'noise' (homoplasies) in the analysis of separate data sets (de Queiroz 1993; de Queiroz & Gatesy 2007).

Problems can arise when analyzing single-gene data sets because gene trees and species trees may be conflicting. Species trees reflect the evolutionary history of a group, whereas gene trees reflect the diversification of a group of sequences derived from different gene fragments. Unlike species trees, gene trees are sensitive to the effects of gene duplication, hybridization and lineage sorting (Moritz & Hillis 1996; Lyons-Weiler & Milinkovitch 1997), but the congruence of multiple independently segregating markers may provide a more reliable estimate of the species tree (Pamilo & Nei 1988). Combining different data sets into a supermatrix may result in a dominant tree being recovered which, based on available knowledge, best represents the species tree (de Queiroz & Gatesy 2007). Recently, the supermatrix approach has been successful in elucidating evolutionary relationships at various taxonomic levels using matrices based on mitochondrial and/or nuclear intron genes (Matthee & Davis 2001; Montgelard *et al.* 2003; Matthee *et al.* 2004; Reyes *et al.* 2004; Eick *et al.* 2005; Willows-Munro *et al.* 2005; Moyle & Marks 2006; Piaggio & Perkins 2006; Matthee *et al.* 2007).

Genetic markers: mitochondrial and nuclear DNA

The high mutation rate of mtDNA makes it a useful tool for recovering phylogenies at low taxonomic levels (between genera and species within the

same family; Brown *et al.* 1982). In addition, the rapid rate of sequence divergence in animal mtDNA makes this molecule appropriate for discriminating recently diverged lineages (Harrison 1989).

Cytochrome *b* is a mtDNA protein in the electron transport chain and, unlike the other mitochondrial proteins, it is not a subunit of a large enzyme complex. As the only protein product of the mitochondrial genome that is a functional monomer (Hillis *et al.* 1996), this gene has been used successfully in numerous studies to address systematic questions at the level of both genus and species (Van Den Bussche *et al.* 1998; Avise & Walker 1999). The phylogenetic utility of this gene for resolving species-level relationships in bat genera has been well documented (Sudman *et al.* 1994; Wilkinson *et al.* 1997; Cooper *et al.* 1998; Wright *et al.* 1999; Bastian *et al.* 2001; Lewis-Oritt *et al.* 2001; Kawai *et al.* 2003; Stadelmann *et al.* 2004; Hoofer *et al.* 2006; Li *et al.* 2006; Pulvers & Colgan 2007; Russell *et al.* 2007; Stadelmann *et al.* 2007). Furthermore, the use of this mitochondrial region permits the comparison of my results with those from published data (e.g. Guillén *et al.* 2003).

The mammalian nuclear genome is larger than the mitochondrial genome and nuclear DNA can provide markers that segregate independently (Matthee & Davis 2001; Matthee *et al.* 2001; Matthee *et al.* 2007). Multiple nuclear markers may thus prove more useful in reflecting the species tree than are multiple mtDNA markers, which are all linked (Springer *et al.* 2001). Furthermore, non-coding introns and coding exons are under different evolutionary constraints and may provide different phylogenetic information, thus providing resolution at different taxonomic levels. As introns are non-coding, sequences may contain unique insertions and deletions (idels) which can also be phylogenetically informative (Matthee *et al.* 2004).

Due to the different evolutionary rates of nuclear and mtDNA genes, patterns of variation for mitochondrial and nuclear markers are usually not congruent

(Harrison 1989). Systematic studies based solely on maternally inherited genes such as cytochrome *b* have been criticized because they are susceptible to introgressive hybridization (i.e. the retention of ancestral polymorphisms). They are also susceptible to independent lineage sorting which can result in increased homoplasy (Avise 1994). Thus, phylogenies incorporating for example cytochrome *b* and nuclear DNA could provide valuable insight into the evolutionary relationships among the rhinolophid species, specifically by providing phylogenetic signal at different levels within the tree (see also Halanych & Robinson 1999). Three nuclear DNA regions, protein-kinase (PRKC1), thyroglobulin (TG), and thyrotropin (THY) have been used to resolve relationships at different taxonomic levels (Matthee & Davis 2001; Matthee *et al.* 2004; Eick *et al.* 2005; Willows-Munro *et al.* 2005).

Dating *Rhinolophus* divergence: a molecular clock

Increasingly, molecular divergence data are being used to predict times of divergence from a common ancestor (the Molecular Clock Hypothesis, Zuckerkandl & Pauling 1965). The molecular clock hypothesis proposes that the constant rate of molecular change (Kimura 1968, 1983; but see Ohta 1992) may be useful for predicting times of divergence. Although the rate of the molecular clock varies between evolutionary lineages and taxon generation times (Ohta 1993), related species should share the attributes which affect substitution rates, and reliable estimates of divergence times may be increased with the inclusion of more than one gene (Kumar & Hedges 1998), and several calibration points (Drummond *et al.* 2006, Pulquério & Nichols 2007).

Molecular clocks have been the centre of some controversy, especially regarding their reliability and the confidence that can be placed in the dates, as well as the uncertainty associated with dating calibration points (reviewed in Pulquério & Nichols 2007). Furthermore, studies (e.g. Britten 1986) have shown that it is naïve to assume a constant rate of evolution throughout the tree, because

substitution rates can vary among lineages and also in a punctuated fashion (Drummond *et al.* 2006).

Recent advances in the field allow for statistical methods which do not assume a constant rate of substitution and these can be applied to estimate divergence times (e.g. Yoder & Yang 2000). Relaxed Bayesian clock methods do not require the assumption of a strict molecular clock and take the variability in substitution rate into account (Drummond *et al.* 2006). Bayesian methods model the molecular rates among lineages by *a priori* selecting a branch rate from a parametric distribution, where the mean parametric distribution is a function of the molecular rate on the parent branch (Drummond *et al.* 2006). The relaxed Bayesian clock method also permits the inclusion of more than one gene fragment, multiple calibration points, and the rate for each gene fragment and branch on the tree is allowed to vary.

The main aims of this chapter are to use a molecular supermatrix comprising mitochondrial cytochrome *b* and three nuclear introns to:

1) Produce a robust molecular phylogeny for the genus *Rhinolophus*;

2) Test the monophyly of the rhinolophids and within them also the monophyly of the African rhinolophids; and

3) Provide a timescale for the radiation of extant *Rhinolophus* species.

METHODS

TAXON SAMPLING

Data were collected from individuals of the ten southern African rhinolophids throughout South Africa. Study areas included the following sites: Algeria Forestry station (AFS – GPS 32°22'S, 19°03'E); De Hel in the Groot Wintershoek Nature Reserve (DHL - 33°05'S, 19°05'E); De Hoop Nature Reserve (DHP - 34°26'S, 20°25'E); Goodhouse (GDH - 28°56'S, 18°07'E); Kokstad (KSD - 30° 48.3'S, 29°16.6'E); Koegelbeen Cave, Griekwastad (KGB - 28°40'S, 23°22'E); Mkuze (MKZ - 27°36'S, 32°10'E); Pirie Forest, King William's Town (PFT - 31°38'S, 29°33'E); Knysna – (KYS - 33°57'S, 23°10'E); Pafuri, Kruger National Park (PAF - 22°25'S, 31°12'E) and Sudwala (SUD - 25°23'S, 30°41'E) (Fig. 2.1). The South African distributions of the ten southern African *Rhinolophus* species are shown in Fig. 2.2, with more detailed maps provided in Chapter 5 (Fig. 5.2). Bats were caught either by using mist nets and/or harp traps (Tuttle 1974) placed in flight paths, outside caves and over rivers; or with hand-nets from their roost sites during the day. Mist nets were checked regularly throughout trap nights to ensure that bats were not injured while caught in the nets. Sub-adults were distinguished from adults by the presence of cartilaginous epiphyseal plates in their finger bones (Anthony 1988) and only adult bats were used in all subsequent analyses.

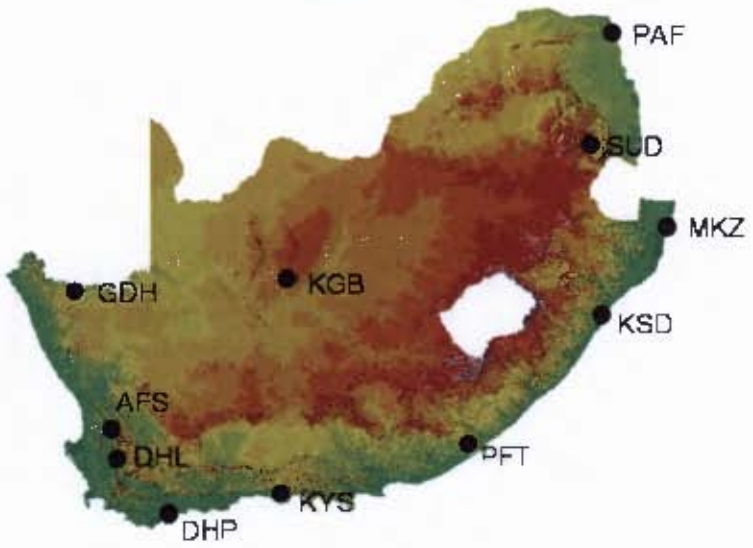


Fig. 2.1 A topographical map of South Africa showing the distribution of sampling sites where rhinolophids were collected. AFS = Algeria Forestry station; DHL = De Hel; DHP = De Hoop Nature Reserve; GDH = Goodhouse; KSD = Kokstad; KGB = Koegelbeen; MKZ = Mkuze; PFT = Pirie Forest; KYS = Knysna; PAF = Pafuri; and SUD = Sudwala. Localities correspond to those described for South African rhinolophids in Tables 2.1 and 2.2. Colours correspond to elevation where green indicates the lowest areas and red the highest areas.

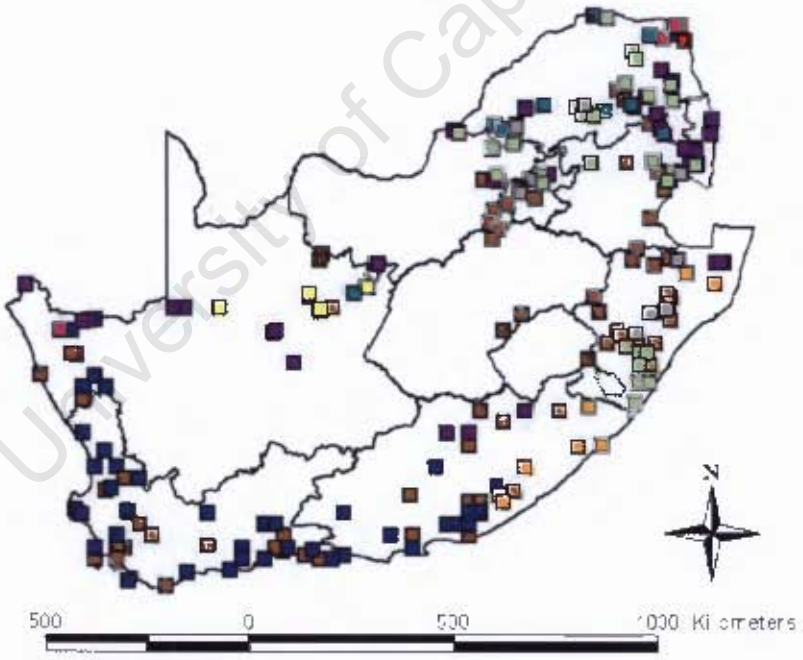


Fig. 2.2 Distribution of the ten *Rhinolophus* species within South Africa. Species include: R. *blasii* (■), *R. capensis* (■), *R. clivosus* (■), *R. darlingi* (■), *R. denti* (■), *R. fumigatus* (■), *R. hildebrandti* (■), *R. landeri* (■), *R. simulator* (■) *and R. swinnyi* (■). Distribution data was taken from Keith (2004).

TISSUE SAMPLE COLLECTION

Two biopsies of skin were taken from the tail or wing membrane of each specimen using a sterile 3 mm-diameter medical biopsy punch (Wilkinson *et al.* 1997). Biopsies were taken from areas with no visible large blood vessels. For transportation to the laboratory, skin samples were placed in a 2 ml Eppendorf tube containing 90% ethanol or 20% Dimethylsulphoxide (DMSO) in saturated NaCl solution. In addition to the *Rhinolophus* spp., tissue samples were also taken from sister taxa (Family Hipposideridae). *Hipposideros caffer* and *Cloeotis percivali* caught at Sudwala (SUD) were used to root the trees.

Rhinolophus specimens from localities outside South Africa

Tissue samples (liver, wing punches or in the case of some museum samples, pieces of skin) for *Rhinolophus* species from outside of South Africa were received from various museums and individuals. To increase taxonomic representation, GenBank sequences were obtained for 17 individuals representing four additional *Rhinolophus* species and two additional outgroup taxa (*Rhinopoma hardwickei* and *Rousettus aegyptiacus*). I sampled 31 *Rhinolophus* species for the supermatrix analyses (Table 2.1) and 38 *Rhinolophus* species for the cytochrome *b* analyses (Table 2.2).

Table 2.1 *Rhinolophus* species and outgroup taxa used in the supermatrix data set. Abbreviations used: FMNH - Field Museum of Natural History, Chicago; HNHM – Hungarian Natural History Museum, Budapest; SA - South Africa. * indicates missing data.

Genus	Species	Source	Collection locality	Accession Number	Sequence Codes			
					CYT *b*	THY	TG	PRKC1
Cloeotis	*percivali*	Schoeman & Stoffberg	Sudwala, South Africa	17.12.02CIP2(SDC)	cmr c1	cmr c1	*	cmr c1
Hipposideros	*caffer*	Schoeman & Stoffberg	Sudwala, South Africa	17.12.02HC2(SDC)	cmr h2	cmr h2	cmr h2	cmr h2
Hipposideros	*ruber*	FMNH	Morogoro Region, Tanzania	WTS 2030	cmr 79	cmr 79	cmr 79	cmr 79
Rhinolophus	*acuminatus*	Iain Mackie	Myanmar	TM 4	cmr 93	cmr 93	cmr 93	cmr 93
Rhinolophus	*affinis*	MNHN	Kapoe, Thailand	99.512	cmr 31	cmr 31	cmr 31	cmr 31
Rhinolophus	*affinis*	Iain Mackie	Myanmar	IL 6	cmr 87	cmr 87	cmr 87	cmr 87
Rhinolophus	*blasii*	Schoeman & Stoffberg	Sudwala, South Africa	18.12.02RB2(SC)	cmr 30	cmr 30	cmr 30	cmr 30
Rhinolophus	*blasii*	M. Ruedi	Peloponnese, Greece	MHNG 1807.41	cmr 49	cmr 49	cmr 49	cmr 49
Rhinolophus	*borneensis*	HNHM	Cambodia	CSOCA 17	cmr 129	cmr 129	cmr 129	cmr 129
Rhinolophus	*capensis*	University of Cape Town	De Hoop, South Africa	30.01.03RC1(HH)	cmr 10	cmr 10	cmr 10	cmr 10
Rhinolophus	*clivosus*	Schoeman & Stoffberg	Sudwala, South Africa	18.12.02RCL1(SM)	cmr 1	cmr 1	cmr 1	cmr 1
Rhinolophus	*darlingi*	David S. Jacobs	Mkuze, South Africa	26.07.02RD(MC)	cmr 76	cmr 76	cmr 76	cmr 76
Rhinolophus	*denti*	David S. Jacobs	Koggelbeen, South Africa	20.07.04RDNT4(KGB)	cmr 56	cmr 56	cmr 56	cmr 56
Rhinolophus	*ferrumequinum*	M. Ruedi	Lesvos, Greece	MHNG 1807.97	cmr 51	*	cmr 51	*
Rhinolophus	*formosae*	HNHM	Taiwan	2005.65.40	cmr 119	cmr 119	cmr 119	cmr 119
Rhinolophus	*fumigatus*	David S. Jacobs	Pafuri, South Africa	4.08.02RF1(LG)	cmr 12	cmr 12	cmr 12	cmr 12
Rhinolophus	*fumigatus*	FMNH	Tanga Region, Tanzania	WTS 1533	cmr 80	cmr 80	cmr 80	cmr 80
Rhinolophus	*hildebrandti*	Schoeman & Stoffberg	Sudwala, South Africa	18.12.02RH1(SM)	cmr 4	cmr 4	cmr 4	cmr 4
Rhinolophus	*hildebrandti*	FMNH	Morogoro Region, Tanzania	WTS 2562	cmr 83	cmr 83	cmr 83	cmr 83
Rhinolophus	*hipposideros*	P. Benda	Kombatados, Greece	PB 875	cmr 52	cmr 52	*	cmr 52
Rhinolophus	*landeri*	David S. Jacobs	Pafuri, South Africa	2.08.02RL1(TK)	cmr 13	cmr 13	cmr 13	cmr 13
Rhinolophus	*lepidus*	Iain Mackie	Myanmar	MW 2	cmr 91	cmr 91	cmr 91	*
Rhinolophus	*maclaudi*	FMNH	Uganda	WTS 889	cmr 78	cmr 78	cmr 78	cmr 78
Rhinolophus	*macrotis*	Iain Mackie	Myanmar	KL 1	cmr 89	cmr 89	cmr 89	cmr 89
Rhinolophus	*malayanus*	Iain Mackie	Myanmar	IL 1	cmr 86	cmr 86	*	cmr 86
Rhinolophus	*marshalli*	Iain Mackie	Myanmar	TA 3	cmr 90	cmr 90	*	cmr 90
Rhinolophus	*megaphyllus*	Australian Museum	Buladelah Forest, Australia	EBU 12131	cmr 36	cmr 36	cmr 36	cmr 36
Rhinolophus	*mehelyi*	M. Ruedi	Lesvos, Greece	MHNG 1808.01	cmr 50	cmr 50	cmr 50	cmr 50
Rhinolophus	*monoceros*	HNHM	Taiwan	2005.65.57	cmr 121	cmr 121	cmr 121	cmr 121
Rhinolophus	*nippon*	HNHM	South Korea	2003.37.28	*	cmr 118	cmr 118	cmr 118
Rhinolophus	*pearsonii*	Iain Mackie	Myanmar	SH 8	*	cmr 95	cmr 95	*
Rhinolophus	*pusillus*	Iain Mackie	Myanmar	SH 15	*	cmr 96	cmr 96	cmr 96
Rhinolophus	*shameli*	Iain Mackie	Myanmar	MDI 8	cmr 92	*	cmr 92	cmr 92
Rhinolophus	*simulator*	David S. Jacobs	Sudwala, South Africa	12.08.03 Runk1(SM2)	cmr 27	cmr 27	cmr 27	cmr 27
Rhinolophus	*simulator*	FMNH	Morogoro Region, Tanzania	WTS 2561	cmr 82	cmr 82	cmr 82	cmr 82
Rhinolophus	*sinicus*	HNHM	Nepal	98.5.6	AF 109651	cmr 126	cmr 126	cmr 126
Rhinolophus	*stheno*	Iain Mackie	Myanmar	MN 14	cmr 97	*	*	*
Rhinolophus	*swinnyi*	David S. Jacobs	Kokstad, South Africa	23.07.04RSW4(KSM)	cmr 60	cmr 60	cmr 60	cmr 60
Rhinolophus	*thomasi*	Iain Mackie	Myanmar	SH 13	cmr 94	cmr 94	cmr 94	cmr 94
Rhinopoma	*hardwickei*	GenBank		GenBank	AY 056462	AJ 865680	*	AJ 866328
Rousettus	*aegyptiacus*	GenBank		GenBank	DQ 445714	AJ 865671	*	AJ 866320
Triaenops	*persicus*	Schoeman & Stoffberg	Mozambique	27.08.06TP3(GCMZ)	cmr 153	cmr 153	cmr 153	*

Table 2.2 *Rhinolophus* species and outgroup taxa used in the cytochrome *b* data set. Abbreviations used: FMNH - Field Museum of Natural History, Chicago; HNHM – Hungarian Natural History Museum, Budapest; MNHN – Muséum National d'Histoire Naturelle, Paris; SA - South Africa. Seq. Code refers to sequences generated in this study.

Genus	Species	Source	Collection locality	Accession Number	Seq. Code
Cloeotis	*percivali*	Schoeman & Stoffberg	Sudwala, South Africa	17.12.02CIP2(SDC)	cmr c1
Hipposideros	*caffer*	Schoeman & Stoffberg	Sudwala, South Africa	17.12.02HC2(SDC)	cmr h2
Hipposideros	*cyclops*	FMNH	Tanga Region, Tanzania	WTS 1554	cmr 81
Hipposideros	*ruber*	FMNH	Morogoro Region, Tanzania	WTS 2030	cmr 79
Rhinolophus	*acuminatus*	Iain Mackie	Myanmar	TM 4	cmr 93
Rhinolophus	*acuminatus*	GenBank		DQ 178988	
Rhinolophus	*affinis*	MNHN	Kapoe, Thailand	99.512	cmr 31
Rhinolophus	*affinis*	Iain Mackie	Myanmar	IL 6	cmr 87
Rhinolophus	*affinis*	GenBank		AF 460975	
Rhinolophus	*arcuatus*	Australian Museum	Waratem Cave, Papua New Guinea	EBU 23054	cmr 37
Rhinolophus	*blasii*	M. Ruedi	Lesvos, Greece	MHNG 1807.98	cmr 41
Rhinolophus	*blasii*	M. Ruedi	Peloponnese, Greece	MHNG 1807.41	cmr 49
Rhinolophus	*blasii*	Schoeman & Stoffberg	Sudwala, South Africa	18.12.02RB1(SC)	cmr 8
Rhinolophus	*blasii*	Schoeman & Stoffberg	Sudwala, South Africa	18.12.02RB2(SC)	cmr 30
Rhinolophus	*borneensis*	HNHM	Cambodia	CSOCA 17	cmr 129
Rhinolophus	*borneensis*	GenBank		DQ 178985	
Rhinolophus	*capensis*	David S. Jacobs	De Hel,South Africa	8.04.02RCL4(CDH)	cmr 9
Rhinolophus	*capensis*	David S. Jacobs	De Hoop, South Africa	30.01.03RC1(HH)	cmr 10
Rhinolophus	*capensis*	David S. Jacobs	Knysna, South Africa	30.09.03RCL1(BMK)	cmr 34
Rhinolophus	*capensis*	David S. Jacobs	De Hoop, South Africa	26.01.04RD1(HH)	cmr 42
Rhinolophus	*capensis*	David S. Jacobs	Knysna, South Africa	1.10.03RD2(HFK)	cmr 44
Rhinolophus	*capensis*	David S. Jacobs	Knysna, South Africa	3.10.03RD1(HFK)	cmr 45
Rhinolophus	*capensis*	David S. Jacobs	Knysna, South Africa	30.09.03RCL2(BMK)	cmr 46
Rhinolophus	*capensis*	David S. Jacobs	De Hoop, South Africa	2.02.04RC4(HH)	cmr 48
Rhinolophus	*capensis*	M. Ruedi			cmr 85
Rhinolophus	*clivosus*	Schoeman & Stoffberg	Sudwala, South Africa	18.12.02RCL1(SM)	cmr 1
Rhinolophus	*clivosus*	David S. Jacobs	Sudwala, South Africa	DSJ 17	cmr 18
Rhinolophus	*clivosus*	FMNH	Morogoro Region, Tanzania	WTS 3010	cmr 84
Rhinolophus	*cornutus*	GenBank		AB 085720	
Rhinolophus	*creaghi*	GenBank		DQ 178986	
Rhinolophus	*darlingi*	Schoeman & Stoffberg	Sudwala, South Africa	18.12.02RD1(SM)	cmr 3
Rhinolophus	*darlingi*	Schoeman & Stoffberg	Sudwala, South Africa	18.12.02RD2(SM)	cmr 7
Rhinolophus	*darlingi*	David S. Jacobs	Mkuze, South Africa	26.07.02RD1(MC)	cmr 11
Rhinolophus	*darlingi*	David S. Jacobs	Sudwala, South Africa	DSJ 11	cmr 17
Rhinolophus	*darlingi*	M. Ruedi	Goodhouse, South Africa	14.11.02M975(RD)	cmr 47
Rhinolophus	*darlingi*	David S. Jacobs	Koggelbeen, South Africa	20.07.04RD1(KGB)	cmr 57
Rhinolophus	*darlingi*	David S. Jacobs	Mkuze, South Africa	26.07.02RD(MC)	cmr 76
Rhinolophus	*darlingi*	E. Seamarck	Swaziland	ECJS 09 2/2/2006	cmr 148
Rhinolophus	*darlingi*	E. Seamarck	Swaziland	ECJS 01 2/2/2006	cmr 149
Rhinolophus	*darlingi*	E. Seamarck	Swaziland	ECJS 02 2/2/2006	cmr 150
Rhinolophus	*denti*	David S. Jacobs	Koggelbeen, South Africa	20.07.04RDNT4(KGB)	cmr 56
Rhinolophus	*denti*	David S. Jacobs	Koggelbeen, South Africa	20.07.04RDNT12(KGB)	cmr 59
Rhinolophus	*euryotis*	Australian Museum	Fulleborn, Papua New Guinea	EBU 25314	cmr 38
Rhinolophus	*ferrumequinum*	M. Ruedi	Lesvos, Greece	MHNG 1807.97	cmr 51
Rhinolophus	*formosae*	HNHM	Taiwan	2005.65.40	cmr 119
Rhinolophus	*fumigatus*	David S. Jacobs	Pafuri, South Africa	4.08.02RF1(LG)	cmr 12
Rhinolophus	*fumigatus*	David S. Jacobs	Pafuri, South Africa	31.07.02RF(TK)	cmr 14
Rhinolophus	*fumigatus*	FMNH	Tanga Region, Tanzania	WTS 1533	cmr 80

Table 2.2 continued. *Rhinolophus* species and outgroup taxa used in the cytochrome *b* data set. Abbreviations used: FMNH - Field Museum of Natural History, Chicago; HNHM – Hungarian Natural History Museum, Budapest; MNHN – Muséum National d'Histoire Naturelle, Paris; SA- South Africa. Seq. Code refers to sequences generated in this study.

Genus	Species	Source	Collection locality	Accession Number	Seq. Code
Rhinolophus	*hildebrandti*	David S. Jacobs	Sudwala, South Africa	18.12.02RH1(SM)	cmr 4
Rhinolophus	*hildebrandti*	David S. Jacobs	Pafuri, South Africa	4.08.02RH1(LG)	cmr 15
Rhinolophus	*hildebrandti*	FMNH	Morogoro Region, Tanzania	WTS 2562	cmr 83
Rhinolophus	*hildebrandti*	Schoeman & Stoffberg	Mozambique	27.08.06RH1(GCMZ)	cmr 152
Rhinolophus	*hipposideros*	P. Benda	Kombatados, Greece	PB 875	cmr 52
Rhinolophus	*hipposideros*	GenBank		AY 141040	
Rhinolophus	*landeri*	David S. Jacobs	Pafuri, South Africa	2.08.02RL1(TK)	cmr 13
Rhinolophus	*landeri*	David S. Jacobs	Pafuri, South Africa	2.08.02RL2(TK)	cmr 16
Rhinolophus	*lepidus*	Iain Mackie	Myanmar	MW 2	cmr 91
Rhinolophus	*luctus*	GenBank		DQ 178987	
Rhinolophus	*maclaudi*	FMNH	Uganda	WTS 889	cmr 78
Rhinolophus	*macrotis*	Iain Mackie	Myanmar	KL 1	cmr 89
Rhinolophus	*macrotis*	GenBank		AF 460976	
Rhinolophus	*malayanus*	Iain Mackie	Myanmar	KTI 1	cmr 101
Rhinolophus	*marshalli*	Iain Mackie	Myanmar	TA 3	cmr 90
Rhinolophus	*megaphyllus*	Australian Museum	Buladelah Forest, Australia	EBU 12131	cmr 36
Rhinolophus	*mehelyi*	M. Ruedi		MHNG 1808.01	cmr 50
Rhinolophus	*monoceros*	HNHM	Taiwan	2005.65.57	cmr 121
Rhinolophus	*monoceros*	Genbank		NC 005433	
Rhinolophus	*paradoxolophus*	HNHM	Vietnam	98.3.7	cmr 122
Rhinolophus	*pearsonii*	GenBank		AF 451340	
Rhinolophus	*perditus*	GenBank		AY 141039	
Rhinolophus	*philippinensis*	GenBank		AY 057945	
Rhinolophus	*pumilus*	GenBank		NC 005434	
Rhinolophus	*pusillus*	GenBank		DQ 178984	
Rhinolophus	*rex*	GenBank		AF 451339	
Rhinolophus	*shameli*	Iain Mackie	Myanmar	MDI 8	cmr 92
Rhinolophus	*simulator*	Schoeman & Stoffberg	Sudwala, South Africa	16.12.02RS5(SDC)	cmr 2
Rhinolophus	*simulator*	Schoeman & Stoffberg	Sudwala, South Africa	19.12.02RS2(SDC)	cmr 6
Rhinolophus	*simulator*	David S. Jacobs	Sudwala, South Africa	12.08.03 Runk1(SM2)	cmr 27
Rhinolophus	*simulator*	David S. Jacobs	Sudwala, South Africa	12.08.03 Runk2(SM2)	cmr 28
Rhinolophus	*simulator*	David S. Jacobs	Sudwala, South Africa	12.08.03 Runk3(SM2)	cmr 29
Rhinolophus	*simulator*	Schoeman & Stoffberg	Mozambique	28.08.06RSpp?(GCMZ)	cmr 154
Rhinolophus	*simulator*	FMNH	Morogoro Region, Tanzania	WTS 2561	cmr 82
Rhinolophus	*stheno*	Iain Mackie	Myanmar	MN 14	cmr 97
Rhinolophus	*swinnyi*	David S. Jacobs	Kokstad mine	23.07.04RSW4(KSM)	cmr 60
Rhinolophus	*swinnyi*	David S. Jacobs	Pirie forest, South Africa	26.07.04RSW1(SPF)	cmr 64
Rhinolophus	*swinnyi*	David S. Jacobs	Pirie forest, South Africa	26.07.04RSW8(SPF)	cmr 65
Rhinolophus	*swinnyi*	David S. Jacobs	Pirie forest, South Africa	26.07.04RSW2(SPF)	cmr 66
Rhinolophus	*thomasi*	Iain Mackie	Myanmar	SH 13	cmr 94
Rhinolophus	*thomasi*	HNHM			cmr 112
Rhinolophus	*unknown*	Iain Mackie	Myanmar	MDI 3	cmr 88
Rousettus	*aegyptiacus*	GenBank		DQ 445714	
Triaenops	*persicus*	Schoeman & Stoffberg	Mozambique	27.08.06TP3(GCMZ)	cmr 153

Complete taxon sampling was problematic due to the difficulty in obtaining tissue samples from rhinolophid species outside of South Africa. However, taxon sampling in this study was well represented as at least one species from each species-group as described by Csorba *et al.* (2003) was sequenced. Furthermore, recent molecular studies show that missing data do not have a strong effect on supermatrix analyses provided a sufficient number of informative characters are used (e.g. Fulton & Strobeck 2006). Because nuclear intron sequences were difficult to amplify for very small or old pieces of tissue and skin, the number of taxa for which sequences were obtained differed between the molecular supermatrix and cytochrome *b* data sets (Table 2.1, Table 2.2).

LABORATORY PROCEDURES

The molecular work was performed in the Evolutionary Genomics Group in the Department of Botany and Zoology, University of Stellenbosch, under the supervision of Associate Professor C. A. Matthee.

DNA EXTRACTION AND NUCLEOTIDE SEQUENCING

To ensure that proteinaceous material was digested before DNA extraction, skin samples were incubated overnight at 55°C in the presence of 20 µl proteinase-K (concentration of 10 mg/ml) in 500 µl extraction buffer. Total genomic DNA from the skin samples was extracted using the standard phenol-chloroform-iso-amyl alcohol extraction method as described in Sambrook *et al.* (1989). Extracted DNA was re-suspended in 100 µl buffer comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (pH 8), and stored at -20°C.

Standard polymerase chain reaction (PCR) procedures were employed, where the PCR volume of 25 µl contained 0.2 µM of each primer, 0.2 mM dNTP's, 2.5 µl of 10x buffer (50 mM KCl, 10 mM Tris-HCl), 1.5 – 2.5 mM $MgCl_2$ sterile double-distilled water and 1 unit of either Super-therm (Southern Cross Biotechnology) or BIOTAQ (Bioline) thermostable polymerase. PCR amplifications were conducted under the following conditions:

- one cycle of 94°C for 3 min denaturing;
- 35 cycles: 94°C for 30 s;
  Annealing temperature* for 30 s;
  72°C for 45 s;
- one cycle at 72°C for 5 min for final extension

* Annealing temperature for cytochrome *b* 48-50°C and for nuclear introns 53-56° C.

Each time PCR was performed, one additional sample was included to act as a negative control for contamination (all reagents, but template-free). The PCR products were visualized on 1.0% agarose gel containing ethidium bromide (Ausubel *et al.* 1994) and purified using commercially available kits (Wizard® SV Gel and PCR Clean-Up System, Promega Corporation; GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences) following the manufacturers' instructions. The cycle sequencing reactions were done using the BigDye Terminator Ready-Reaction Kit (version 3, Applied Biosystems, Perkin Elmer) where cycle parameters were the following:

- 25 cycles: 94°C for 30 s;
  Annealing temperature* for 5 s;
  60°C for 4 min;
  Final ramping temperature at 4°C

* Annealing temperature for cytochrome *b* 50° C and for nuclear introns 55° C.

Sequencing products were analysed on an ABI 3100 (Applied Biosystems, Perkin Elmer) automated sequencer at the Core Sequencing Facility, University of Stellenbosch. Where possible, PCR products from two to five individuals from different locations for each species were sequenced to assess the level of intraspecific polymorphism and to confirm the monophyly of species.

Mitochondrial DNA marker

I generated mtDNA sequence data from the mitochondrial cytochrome *b* region. The following universal mammalian primers were used to amplify sections of the cytochrome *b*

gene: L14841, L14990, L15162, H15494 and H15915 (Kocher *et al.* 1989; Irwin *et al.* 1991). A 1157 bp size fragment was sequenced for cytochrome *b*.

One potential problem associated with mtDNA is the possible amplification of non-functional nuclear copies (*numts*) of mtDNA genes. In an attempt to avoid the amplification of *numts*, I took the following precautions: 1) In the majority of cases, the cytochrome *b* gene region was sequenced for more than one individual of a species to allow for comparison and the identification of deviations from the 'normal' base composition; 2) Both the 3' and 5' strands were sequenced to improve accuracy of base identification; 3) All sequences were aligned using Clustal X (Thompson *et al.* 1997) and MacClade (Maddison & Maddison 2005) and checked for the presence of any insertions or deletions characteristic of *numts*; 4) Sequences were translated into their amino acids and checked for termination codons. Finally, the high guanine biased base composition, the absence of insertions and/or deletions, and the absence of termination codons, allowed moderate confidence that *numts* were avoided.

Nuclear markers

Primers designed for bovids (Matthee *et al.* 2001; Matthee & Davis 2001) and bats (Eick *et al.* 2005; Table 2.3) were used to amplify and sequence PCR products from the following nuclear introns:

- PRKC1 (Protein kinase C, iota 1);
- TG (Thyroglobulin); and
- THY (Thyrotropin beta chain precursor).

For the above nuclear introns the following size fragments were sequenced: PRKC1 – 419 bp; TG – 721 bp; and THY – 521 bp.

Table 2.3 Bat-specific primers used for PCR amplification and sequencing (from Eick *et. al.* 2005).

BAT	PRKCI	A	5'	CTT GTC AAT GAT GAT GAG G	3'
		B	5'	CTT ATT TTA AAA TAT GAA AGA AAT C	3'
BAT	THY	A	5'	GGG TAT GAT GTT CAT CTT ACT TC	3'
		B	5'	GGC ATC CTG GTA TTT CTA CAG TCT TG	3'
BAT	TG	A	5'	GTT AAT TTA AAA ATT GCC CAG A	3'
		B	5'	TCC ACT GCC CGC TGG CAC TGC A	3'

ANALYTICAL METHODS

OUTGROUPS

It has been suggested that outgroups should ideally include the nearest sister taxa to the ingroup because the probability of homoplasy ("independent evolutionary origin or loss of one or more traits in different organisms", Givnish & Sytsma 1997, p55) increases with time since divergence from a common ancestor (Lewis-Oritt *et al.* 2001). In addition, if the outgroup is distantly related it is also possible that long branches can be attracted to each other, causing spurious results (e.g. Sanderson *et al.* 2000; Wiens & Hollingsworth 2000). Smith (1994) proposes that the effects of long-branch attraction in parsimony analyses can be reduced by including multiple outgroups with successive relatedness to the ingroup. Furthermore, the assumption of ingroup monophyly can be tested when multiple outgroups are included (Baverstock & Moritz 1996).

Previous systematic studies have placed *Hipposideros* as sister to *Rhinolophus* (Maree & Grant 1997; Teeling *et al.* 2002; Van Den Bussche & Hoofer 2004; Eick *et al.* 2005). In this study, species of different degrees of relatedness to the rhinolophids such as *Hipposideros caffer*, *Triaenops persicus* and *Cloeotis percivali* (Family Hipposideridae) were used as outgroup taxa. *Rhinopoma hardwickei* (Rhinopomatidae) and *Rousettus aegyptiacus*

(Pteropodiade) were used as distant outgroup taxa. These specimens were included because they belong to families included with the Rhinolophidae and Hipposideridae in the suborder Pteropodiformes (Eick *et al.* 2005). Species of *Rhinolophus* which occur in Asia, Australia and Europe were also used to test whether the African rhinolophids form a monophyletic clade, as suggested both by morphological analyses (Bogdanowicz 1992, Bogdanowicz & Owen 1992) and by molecular analyses based on cytochrome *b* (Guillén *et al.* 2003).

ALIGNMENT

Sequence data were edited and assembled using Sequence Navigator 1.01 (Applied Biosystems) or 4Peaks version 1.6 (Griekspoor & Groothuis 2005). Sequences for the nuclear DNA and mtDNA data partitions were aligned using the default parameters in Clustal X (Thompson *et al.* 1997). Cytochrome *b* does not contain insertions or deletions (indels), and was easily aligned. The authenticity of the gene and the alignments were confirmed by amino acid translation. Large indels, predominantly between outgroup and ingroup taxa, made some alignments difficult for the nuclear introns, and in this case BlastAlign was used (Belshaw & Katzourakis 2005). Nuclear intron alignments are provided in Appendix 2, pages 213-234). The initial alignment was then visually edited using MacClade 4 version 4.07 (Maddison & Maddison 2005) to minimize the number of gaps resulting from indels. For the THY data set, an approximately 19bp section of guanine-thymine repeats was deleted as they were of unequal lengths across taxa. Furthermore, 4bp of thymine repeats in the THY data set were deleted as they varied among taxa and were difficult to align. Aligned sequences were converted to NEXUS file format (Maddison *et al.* 1997) for subsequent phylogenetic analysis using PAUP* v 4.0b10 (Swofford 2002) and MRBAYES 3.1.2 (Huelsenbeck 2000). Indels can be useful in defining clades (Keeling & Palmer 2000; Venkatesh *et al.* 2001) and including them as characters may be more preferable than coding them as "missing data" (Ogden & Rosenberg 2006). Gaps were treated as additional characters by coding them as presence and absence data using Gap Recoder software (http://lamar.colostate.edu/~psimmons/phylogenetics.htm). The gap matrix was analysed with the nucleotide data sets (Matthee *et al.* 2007).

PHYLOGENETIC ANALYSES

Congruence among different analytical methods can provide additional support for certain hypotheses. The present study includes parsimony (e.g. Wahlberg *et al.* 2005) and model-based methods (e.g. Reyes *et al.* 2004; Wiens *et al.* 2005; Moyle & Marks 2006). Parsimony analysis estimates a tree (or trees) that requires the minimum number of evolutionary changes to explain the particular data set and assumes that shared derived characters are due to inheritance from a common ancestor (Swofford *et al.* 1996). Unlike parsimony, which does not require explicit models of evolutionary change (Swofford *et al.* 1996; Steel & Penny 2000), model-based analyses such as maximum likelihood and Bayesian inference require a model of DNA evolution that takes into account the uneven nucleotide composition of the gene pool, the bias in transition mutations relative to transversions, and the rate heterogeneity of DNA evolution within and among sequences (Yang 1993; Huelsenbeck & Crandall 1997). The use of model-based phylogenetic methods can lead to more accurate estimates of phylogenies by better accounting for the different properties of character evolution and by incorporating information regarding branch lengths (Swofford *et al.* 2001). Although weighting schemes used in parsimony analyses may account for differences in character evolution and branch lengths, equally weighted parsimony analyses may perform better than those implementing both simple and complex weighting schemes (Kjer *et al.* 2007).

Although likelihood analyses (as implemented in PAUP*) are very time-consuming, Bayesian analysis, through the use of Markov chain Monte Carlo (MCMC) searching, is generally faster and permits the use of different models for different data sets or gene fragments (Ronquist & Huelsenbeck 2003; Nylander *et al.* 2004).

Maximum parsimony (MP) and maximum likelihood (ML) were implemented in PAUP* 4.0b10 (Swofford 2002) and Bayesian Inference (BI) was conducted using MRBAYES 3.1.2 (Huelsenbeck 2000). Minimum Evolution was not used because of missing data in the gene data sets which can heavily influence distance-based methods. Data sets (cytochrome *b*, TG, THY, PRKC1) were analysed separately and combined using

parsimony and Bayesian inference, and the supermatrix data set was also analysed using maximum likelihood.

The sequence data were subjected to a parsimony analysis using the heuristic search option in PAUP* 4.0b10. Parsimony analyses were run with all sites weighted equally (Kjer *et al.* 2007) and 100 sequential heuristic searches were performed with the starting trees obtained via 100 random stepwise additions followed by tree-bisection-reconnection (TBR) branch swapping. Multistate characters were treated as polymorphic. Tree statistics such as tree length, consistency index (CI; Kluge & Farris 1969), and retention index (RI; Farris 1989) were obtained from PAUP. Both the RI and the CI measure the amount of homoplasy present in the data. However, the RI is considered to be a more reliable indicator of saturation because it excludes autapomorphies which all have a consistency value of 1 (Nei & Kumar 2000).

For each data set (gene fragment) and for the supermatrix data set, an optimal model of evolution was chosen before conducting likelihood and Bayesian analyses. MODELTEST v 3.06 (Posada & Crandall 1998) was used to determine the DNA substitution model that best describes the different datasets. MODELTEST determines the best-fit model of nucleotide substitution by using likelihood ratio tests to compare models sequentially. When the addition of a parameter no longer significantly increases the fit between the model and the data, the result is assumed to be the best-fit model (Posada & Crandall 1998). Statistical tests such as the Akaike information criterion (AIC) and the hierarchical likelihood ratio test (hLRT) are used by MODELTEST to select the appropriate model of evolution. AIC provides an indication of the expected distance between any model and the evolutionary processes that produced the data. The smaller the AIC the better the fit of the model to the data because the AIC penalizes an increase in the number of parameters. The hLRT compares the maximum likelihood value of a topology under a simple model to the optimal topology under a more complex model. Although the AIC is considered to be preferable to the hLRT for estimating the optimal model (Johnson & Omland 2004), if the models selected by AIC and hLRT differed, then the data partition was analysed for both

models. The same optimal model parameters were also applied in the distance analyses to calculate maximum likelihood pairwise sequence divergence values between taxa.

Maximum likelihood recovered two trees and a strict consensus tree was enforced. Due to time constraints, bootstrapping was not performed. For the Bayesian analyses, random starting trees were used and eight Markov chains (two cold and six heated) were run for one, two and five million generations with trees being sampled every 50 generations. The supermatrix data set was analysed in a partitioned manner to allow the selection of different optimal models for each fragment (Ronquist & Huelsenbeck 2003). For the Bayesian analysis of the cytochrome *b* data set, first-, second- and third-position codons were unlinked and analysed in a partitioned manner. The fixed burn-in (number of trees that need to be discarded as they were sampled before equilibrium was reached) was based on inspection of the number of generations required to reach stationarity in all posterior outputs. All MCMC analyses were repeated three times.

Evaluating character support

To assess the reliability of the clades, bootstrap support (Felsenstein 1985) was calculated in PAUP* 4.0b10 using 1000 replicates. Furthermore posterior probability values, generated using Bayesian Inference, were also used to assess statistical significance of nodes. Nodes with ≥ 95% Bayesian posterior probability were considered significantly supported.

Data partitions

Congruence between numerous independent data partitions is one way of providing an indication of the reliability of the evolutionary relationships recovered by the analyses (Miyamoto & Fitch 1995). In this regard, each gene fragment was analysed separately and then combined to evaluate visually potential conflict in gene trees among genes. De Queiroz's (1993) recommendations for evaluating incongruence between data sets, as used by Teeling *et al.* (2003) and Eick *et al.* (2005), were followed. This was done by determining whether there were any strongly supported nodes based on one gene fragment that were in conflict with a strongly supported node based on another gene

fragment. Nodes were considered well supported if there was ≥70% bootstrap support with Bayesian posterior probabilities of ≥0.95. Because there were no conflicting clades that were well-supported between individual data sets, cytochrome *b*, TG, THY and PRKC1 were combined to form a supermatrix.

MOLECULAR CLOCK

To date the evolution of the rhinolophid lineages I used the relaxed Bayesian clock method (Thorne *et al.* 1998; Thorne & Kishino 2002) following the methodology of Matthee *et al.* (2004). The supermatrix Bayesian inference tree (Fig 2.5) was used as the reference. As priors I used 41 million years (Myr) between the root and the tip with a standard deviation of 41 Myr. This is believed to be a reasonable maximum age for the divergence of the Rhinolophidae from the Hipposideridae (Eick *et al.* 2005). The rate at the root node was set to 0.003 (SD=0.003) substitutions per site per million years as was used for the Chiropteran families (Eick *et al.* 2005). The values were obtained by averaging the number of substitutions for sites from the root to the tips and then dividing by the 41 Myr, the putative age of rhinolophid divergence. In the present study, these values were variable and ranged from 0.0004 to 0.001. Varying the root rate, even by large changes has a negligible effect on the estimates (Matthee *et al.* 2004; Eick *et al.* 2005). The Markov chain Monte Carlo was sampled 10 000 times every 100th cycle and an initial burn-in of 10 000 was used. I used the default values for the Brownian rate parameter. The highest possible divergence time for the ingroup was set to 65 Myr, because Chiroptera are believed to have diversified in the early Tertiary (Simmons & Geisler 1998; Simmons & Conway 2003; Teeling *et al.* 2005). Because no fossil rhinolophids are currently recognized as extant species, the incorporation of the hipposiderids is needed to establish a time axis for the extant rhinolophids. Two time constraints were incorporated from the fossil record and from the literature. The constraints were a minimum of 37 million years ago (MYA) and a maximum of 55 MYA for the split between the rhinolophids and hipposiderids (Maree & Grant 1997; McKenna & Bell 1997; Teeling *et al.* 2003).

RESULTS

SEQUENCE DATA

Cytochrome *b* showed a bias against guanine (Table 2.4), which is typical of mammalian mitochondrial genes (Irwin *et al.* 1991). PRKC1 showed a bias against cytosine and guanine (Table 2.4). The adenine-thymine (A-T) bias common to both rhinolophids and megachiropterans (Pettigrew 1995) was evident in the nuclear genes and the supermatrix data set, but not in cytochrome *b* data. This nuclear intron bias towards A and T has been found in other mammalian groups (Matthee *et al.* 2007). Similar topologies were recovered for cytochrome *b* and PRKC1 when either model was used as input. Although different models were selected for each gene fragment, both the LRT and AIC criteria selected the TVM + I + G for the supermatrix data set (Table 2.4).

The combined supermatrix data set comprised 2818 characters and two most parsimonious trees were recovered. A 50% majority rule consensus tree was constructed for each of the six runs in the Bayesian analyses. Similar posterior probabilities were obtained from each run per data set and the topologies for the 95% posterior intervals ranged from: 8706 to 18 408 for individual data sets, but was 2134 for the supermatrix data set. This is due to the increased resolution obtained by combining each data set into a supermatrix (Buckley *et al.* 2002). Similarly the number of trees sampled in the supermatrix data set was much lower (2230 trees) than for each individual data set (9182 to 19 384 trees).

Table 2.4 Optimal models and model parameters selected by MODELTEST for each data partition and the supermatrix. Included are the frequencies of nucleotide bases and the rates of base change. Where applicable, values for the proportion of invariable sites and gamma shape distribution are provided.

		DATA SETS						
		CYT *b*		TG	THY	PRKC1		SUPERMATRIX
MODEL		(hLRT)	(AIC)	(hLRT & AIC)	(hLRT & AIC)	(hLRT)	(AIC)	(hLRT & AIC)
	MODEL	TVM+I+G	GTR+I+G	K80+G	K81uf+G	HKY	TrN+I	TVM+I+G
	AIC		30267 54	8193 25	5560 69		3482 16	38973 91
	-lnL	15127 49	15123 77	4094 62	2774 35	1740 54	1736 08	19477 95
BASE FREQUENCIES	% A	32 3	31 3	equal frequencies	27 4	32 4	31 4	27 6
	% C	33 1	34 2		20 0	11 3	12 4	24 4
	% G	12 8	11 5		20 6	18 1	16 7	18 2
	% T	21 9	23 0		32 0	38 2	39 5	29 8
RATES NUCLEOTIDE CHANGE	A-C	0 4023	0 3719		1 0000		1 0000	1 3104
	A-G	2 9869	3 4351		2 8636		5 2195	4 0819
	A-T	0 4731	0 4223		0 5533		1 0000	0 7592
	C-G	0 3536	0 3704		0 5533		1 0000	0 6943
	C-T	2 9869	2 4763		2 8636		2 9739	4 0819
	G-T	1 0000	1 0000		1 0000		1 0000	1 0000
	Invariable Sites (I)	0 2958	0 2948	0	0	0	0 1756	0 2010
	Gamma Shape Distribution (G)	0 7801	0 7641	0 9259	1 9228	equal rates all sites	equal rates all sites	0 5465
	Ti/Tv ratio			1 3828		1 6502		

PHYLOGENETIC ANALYSES

The phylogenetic signal contained within each of the data sets (each gene fragment) was variable when compared using either the consistency (CI) or retention indices (RI), which are measures of homoplasy. Parsimony analysis of the mitochondrial cytochrome *b* gene (including all taxa) recovered 3100 equally most parsimonious trees, with a tree length of 3070 (CI=0.361, RI=0.609). Although not directly comparable due to differences in the number of taxa, the reduced nuclear intron data sets, PRKC1 (tree length = 110, CI=0.755, RI=0.897), TG (tree length = 363, CI=0.667, RI=0.798), and THY (tree length = 213, CI=0.681, RI=0.84) indicated lower levels of homoplasy as inferred from the RI and CI values. Parsimony analysis of the supermatrix data set recovered two most parsimonious trees with a length of 2619 (CI=0.467, RI=0.529). Trees for the individual

data sets (only cytochrome *b* shown in Fig 2.3 & 2.4) were not as well resolved or supported when compared with the supermatrix tree. This was true for analyses conducted using both parsimony and Bayesian Inference. For cytochrome *b*, Bayesian analysis resolved the polytomy comprising the majority of ingroup taxa obtained in parsimony analysis, although deeper nodes were generally not supported (Figs 2.3, 2.4).

Results from the supermatrix data set indicated that there are at least two well-supported rhinolophid clades. The first main clade comprises *Rhinolophus* species predominantly from the Afrotropical biogeographic region, and the second from the Oriental region (Fig. 2.5). These clades were also evident in the maximum likelihood consensus topology (Fig. 2.6) and the strict parsimony topology (Fig. 2.7). With a few exceptions, such as the placement of *R. fumigatus*, the lower level associations within both the African and Oriental clade were recovered by both Bayesian and maximum likelihood analyses. Parsimony analysis recovered support for the two clades, and found good support for *R. landeri* and *R. blasii* being basal within the African clade (Fig. 2.7). Although Bayesian analysis of the cytochrome *b* gene lacked support for the deeper nodes, the *Rhinolophus* species comprising the African and Oriental clades are once again evident (Fig. 2.3).

Within the Oriental clade, the placement of *R. hipposideros* and *R. formosae* were unresolved. Similarly the placement of *R. pearsonii* within the Rhinolophidae was not resolved, possibly due to the absence of cytochrome *b* and PRKC1 sequences for that specimen.

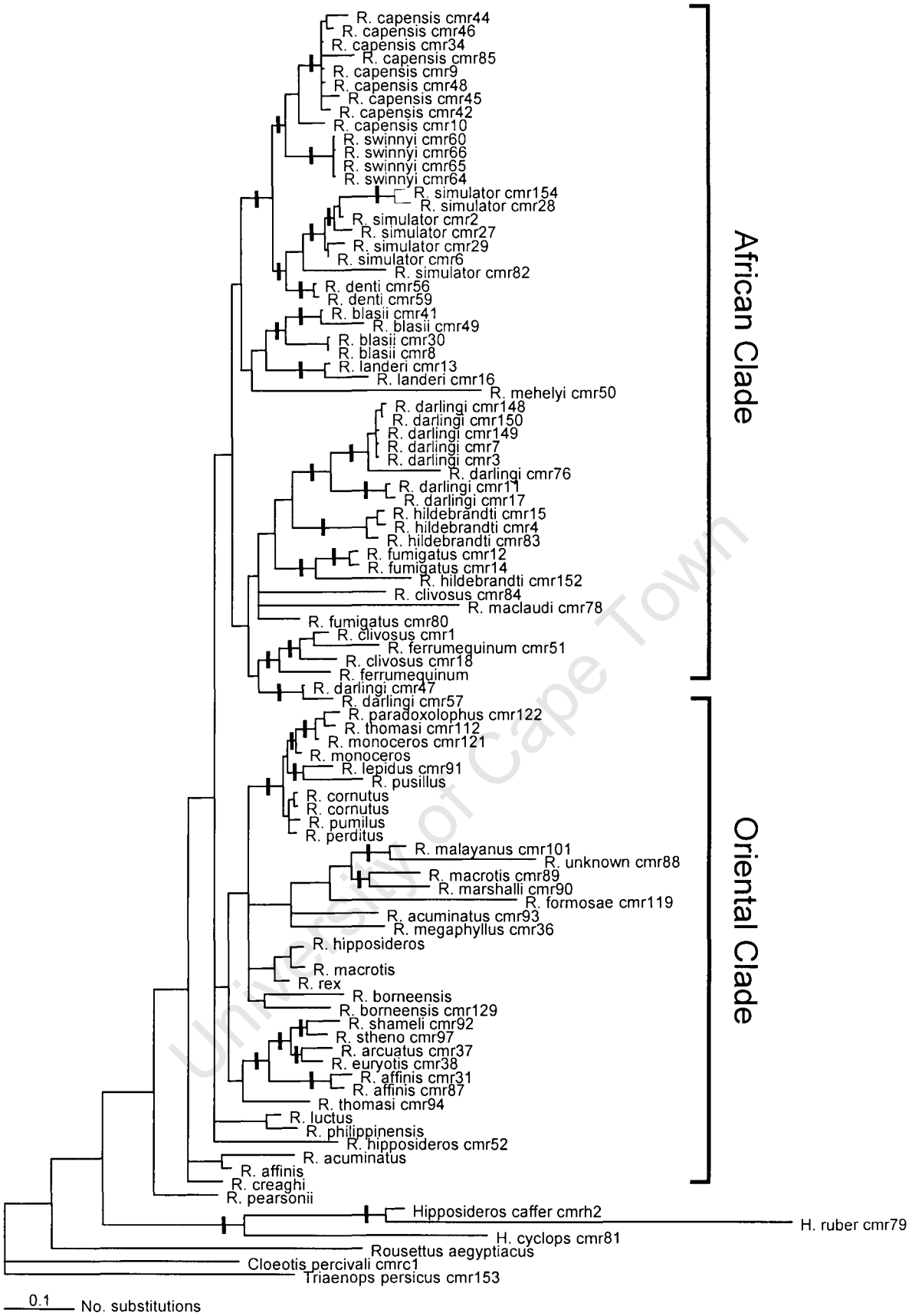


Fig. 2.3 Consensus topology of the posterior distribution (excluding burn-in) of trees sampled in a 2 million generation BI run of the cytochrome *b* data set. Black vertical lines indicate nodes with posterior probabilities of ≥0.95

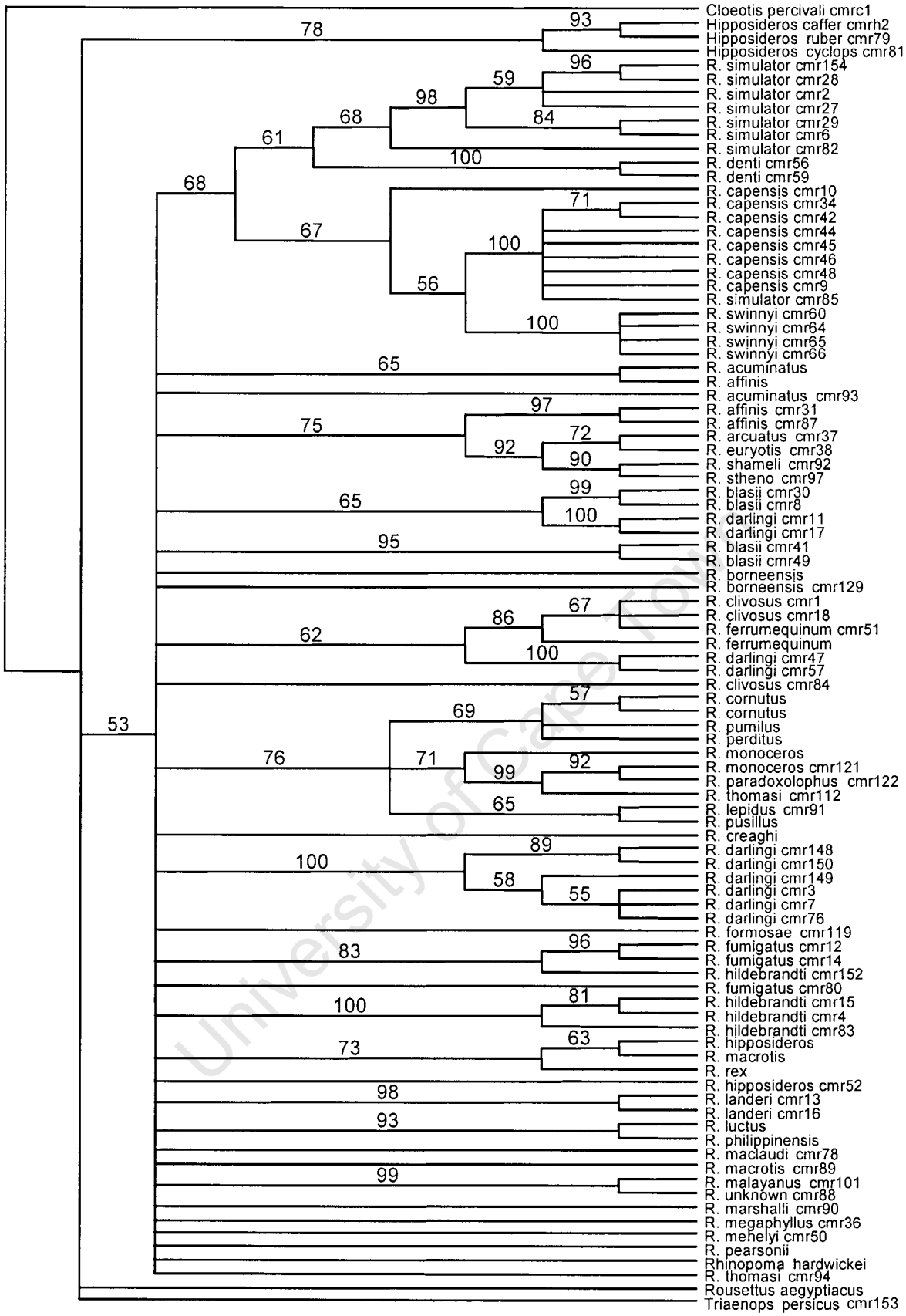


Fig. 2.4 Parsimony topology based on the cytochrome *b* data set. Values above nodes refer to bootstrap support percentages generated from 1000 replicates.

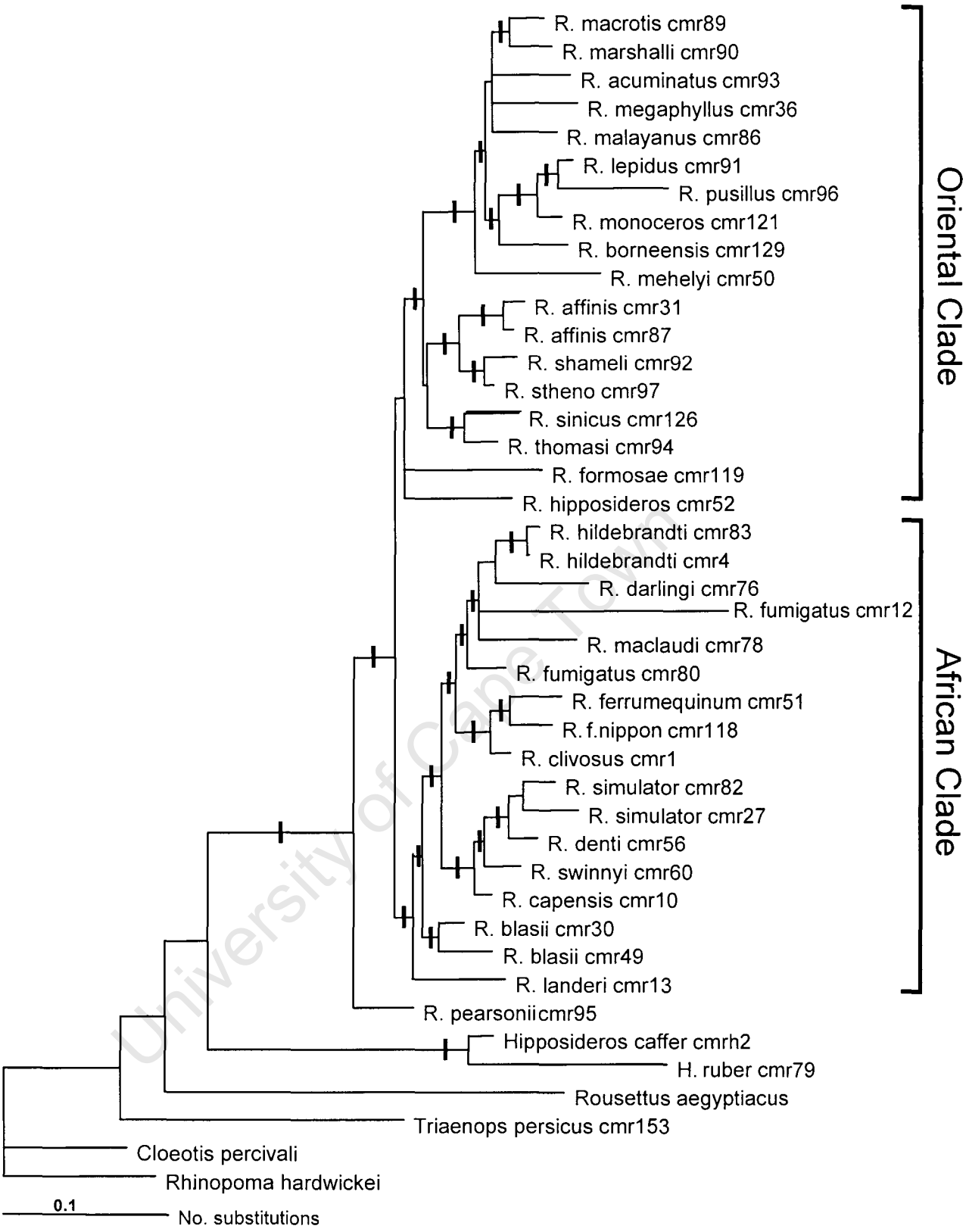


Fig. 2.5 Consensus topology of the posterior distribution (excluding burn-in) of trees sampled in a 5 million generation BI run of the supermatrix data set. Black vertical lines indicate nodes with posterior probabilities of ≥ 0.95.

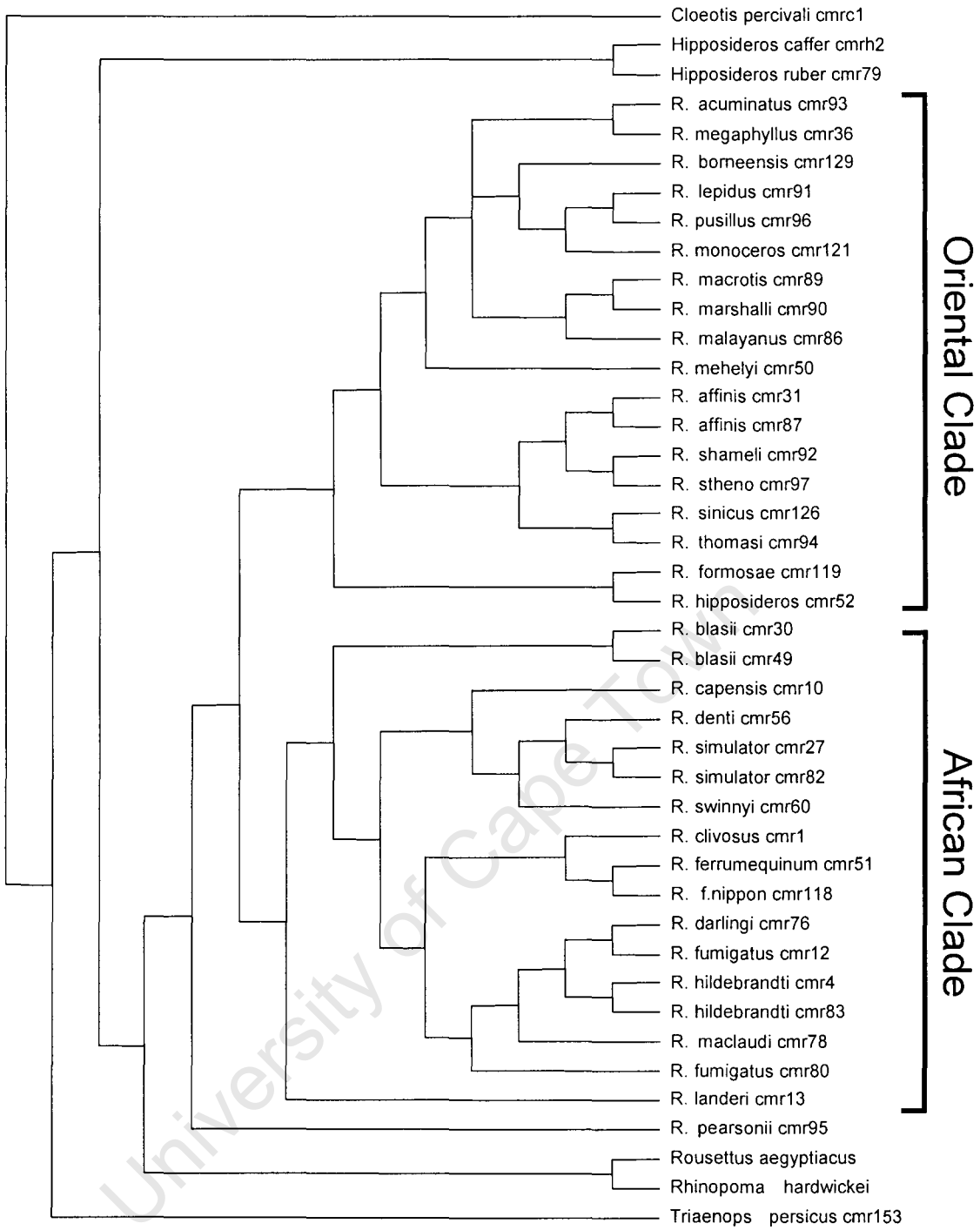


Fig. 2.6 Maximum likelihood strict consensus topology (-ln = 18,185.07) obtained from the supermatrix data set with the TVM + I +G model of evolution.

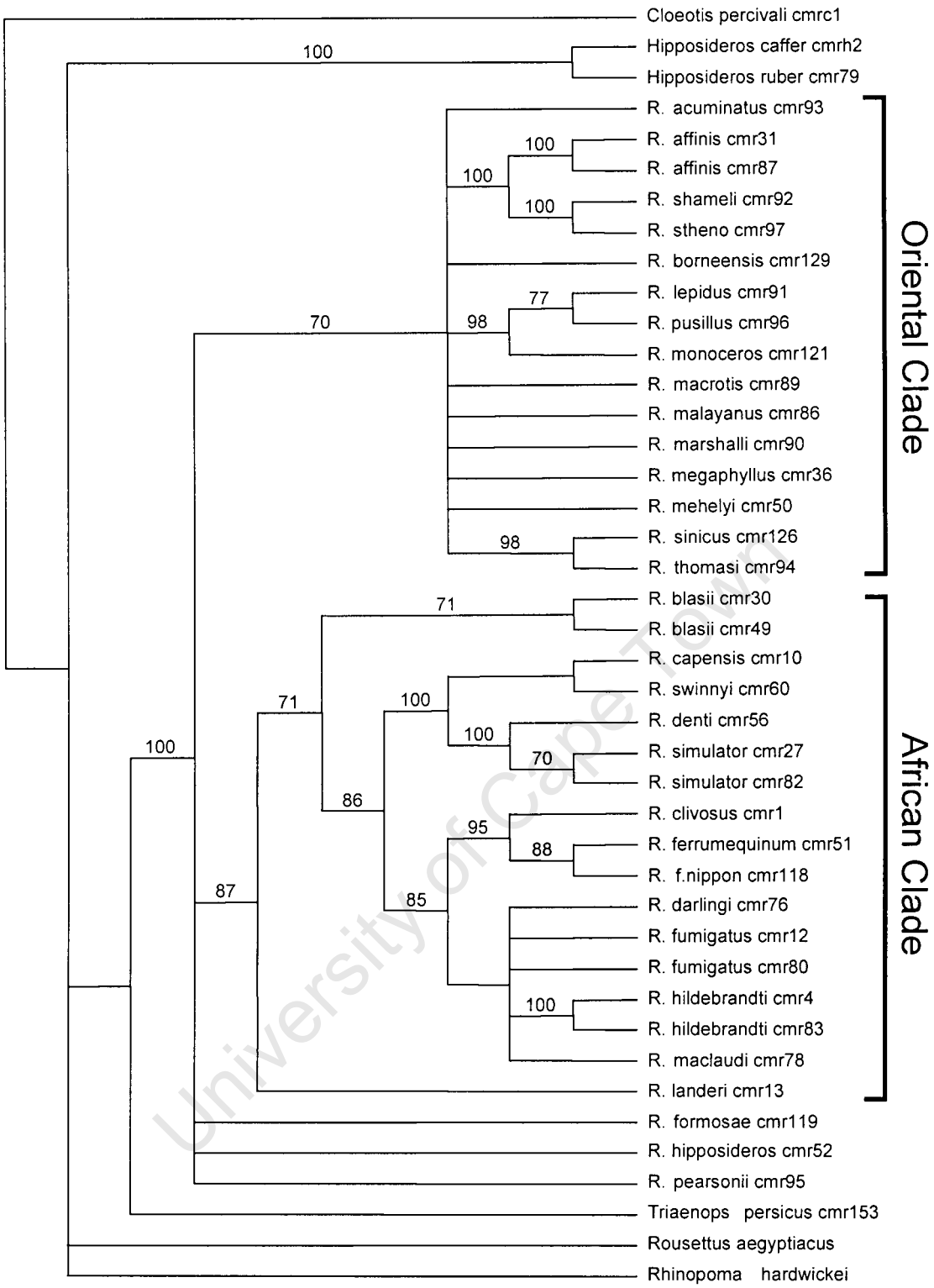
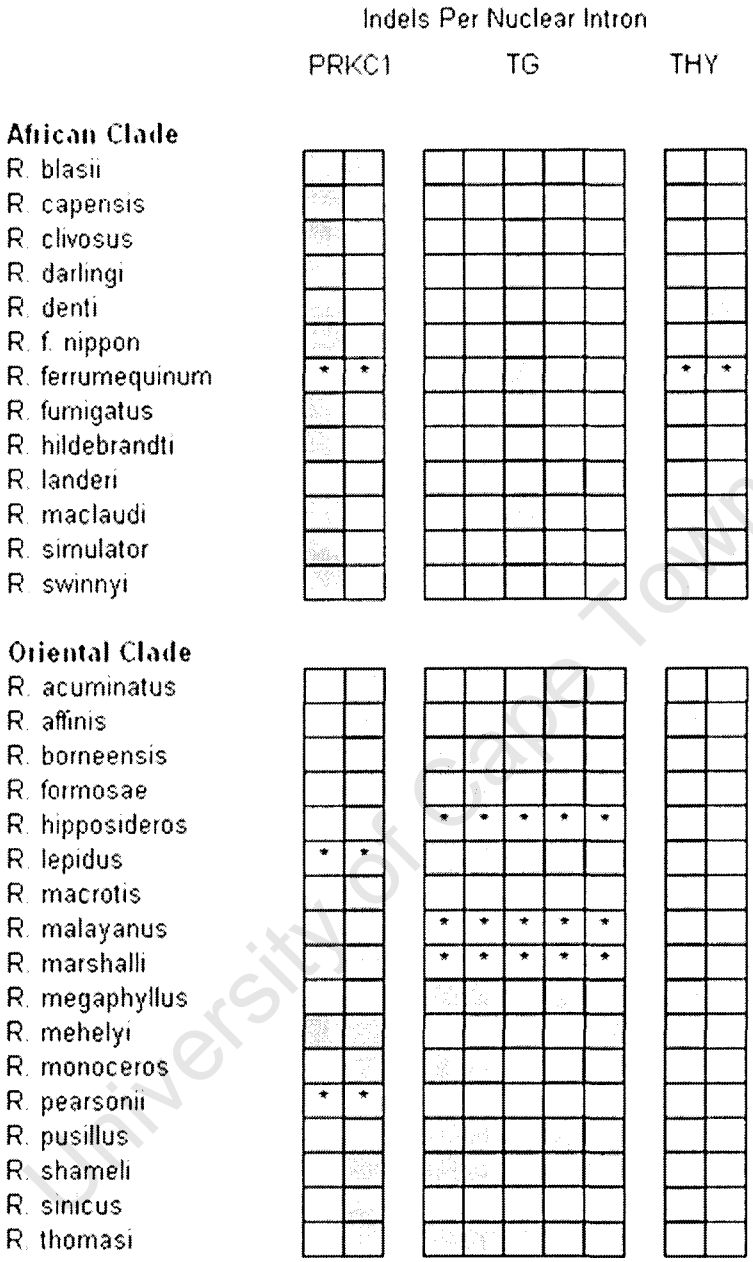


Fig. 2.7 Supermatrix parsimony topology. Values above nodes refer to bootstrap support percentages generated from 1000 replicates. Only bootstrap values of ≥ 70% are shown (Hillis & Bull 1993).

An area of conflict between the cytochrome *b* tree and the tree constructed using the supermatrix data set surrounded the placement of *R. mehelyi*. In the cytochrome *b* tree (Fig. 2.3), *R. mehelyi* grouped with the African rhinolophid clade, although this relationship is not well supported. In the supermatrix topology, the same species grouped with the Oriental rhinolophid clade with nodal support of 70% and posterior probability values ≥0.95 (Figs 2.5, 2.7). *Rhinolophus mehelyi* also groups with the Oriental clade in the likelihood consensus topology (Fig. 2.6). In comparison to the supermatrix data set, which identified *R. blasii* and *R. landeri* as basal within the African clade, in the cytochrome *b* analyses these species grouped more closely with the clade comprising *R. capensis*. However, this placement was not supported by either bootstrap or posterior probability values (Figs 2.3, 2.4).

Cytochrome *b* analyses placed *R. capensis* and *R. swinnyi* as well as *R. denti* and *R. simulator* as sister taxa respectively, both with posterior probabilities of ≥ 0.95 (Fig 2.3). Furthermore, within the cytochrome *b* data set, *R. darlingi* appeared to be paraphyletic (Fig. 2.3) as did *R. ferrumequinum* and *R. clivosus*. However, analyses of the nuclear introns and the supermatrix data set showed that the species are monophyletic. Where taxa were paraphyletic, representatives which had the complete cytochrome *b* strand were used in the supermatrix data set. The discrepancies in the cytochrome *b* data set may be due to the short fragment of cytochrome *b* used, saturation, or introgression. For *R. darlingi*, the individual chosen for the supermatrix data set was a voucher specimen for which skull parameters were checked. Similarly the *R. clivosus* specimen chosen was well within the expected range of forearm length and echolocation call frequency for this species, and was collected within South Africa. By contrast, the *R. ferrumequinum* specimen was collected in Greece, where *R. clivosus* does not occur.

Table 2.5 Insertions and deletions present in nuclear introns used in the supermatrix data set. Only indels ≥ 3bp were used and autapomorphies were excluded. Grey squares indicate the presence of an indel, * indicates that no data were available for that specimen.



No indels were unique to either the African or Oriental clade (Table 2.5). A unique insertion in THY was present in both *R. denti* and *R. simulator*, but absent from the other rhinolophid species. Unlike other species in the Oriental clade, *R. mehelyi* shared a deletion in the PRKC1 gene with the species in the African clade. The reason no indel events were unique to either the African or the

Oriental clade is that indel events which characterize the majority of *Rhinolophus* species within the Oriental clade were often shared by the basal African clade species *R. blasii* and *R. landeri*. These characters are thus probably symplesiomorphic.

Relationships among the southern African rhinolophids based on the supermatrix data set were well resolved, with good posterior probability and bootstrap support (Figs 2.5, 2.7). The cytochrome *b* data set recovered the *capensis-simulator-denti-swinnyi* clade with posterior probability support of ≥ 0.95 and 68% bootstrap support. The clade comprising *R. clivosus* and *R. ferrumequinum* has ≥ 0.95 posterior probability and 86% bootstrap support. Similarly, some *R. fumigatus* and *R. hildebrandti* specimens grouped to form a clade with 83% bootstrap and ≥ 0.95 posterior probability support (Figs 2.3, 2.4). My results differ with those of previous morphological and allozyme analyses. Unlike Bogdanowicz & Owen (1992) in which *R. fumigatus* and *R. hildebrandti* group with Oriental species such as *R. macrotis* and *R. marshalli*, in this study *R. fumigatus* and *R. hildebrandti* were identified as more closely related to other African rhinolophids than to the Oriental species. Furthermore, *R. landeri* did not comprise a clade with *R. mehelyi*, which was in the Oriental clade (Fig. 2.5), and both *R. blasii* and *R. landeri* were more basal than the other southern African rhinolophids. Similarities between this study and the phenetic analyses of Bogdanowicz & Owen (1992) include the grouping together of *R. capensis, R. denti* and *R. simulator*, as well as a relationship between *R. darlingi* and the *R. clivosus-R. ferrumequinum* clade.

Relationships among the South African rhinolophids based on the supermatrix data set were similar to those recovered from the allozyme study (Maree & Grant 1997). My results differed only slightly in that based on molecular data *R. clivosus* was placed within a separate group from that comprising *R. darlingi*, *R. fumigatus*, and *R. hildebrandti* (Fig. 2.5), and was not part of the same group as suggested by Maree & Grant (1997). However, the *R. clivosus* - *R. ferrumequinum* group was sister to that comprising *R. darlingi*, *R. hildebrandti*, and *R. fumigatus*, with ≥0.95 posterior probability support.

SEQUENCE DIVERGENCES

Pairwise maximum likelihood sequence divergences values for the *Rhinolophus* species supermatrix ranged from 1.5% (*R. thomasi* and *R. sinicus*) to 22.9% (*R. marshalli* and *R. ferrumequinum*). General time reversible (GTR) corrected sequences for the same taxa ranged from 2% between *R. thomasi* and *R. sinicus* to 37% between *R. marshalli* and *R. ferrumequinum* (Table 2.6). Maximum likelihood sequence divergence comparisons between ingroup *Rhinolophus* taxa and outgroup taxa ranged from 23.2% between *R. swinnyi* and *Triaenops persicus*, to 47.4% between *R. pearsonii* and *Cloeotis percivali* (Table 2.6). Among the individual data sets, cytochrome *b* showed higher GTR-corrected sequence divergence values than the nuclear intron data sets (Table 2.7).

The intraspecific GTR-corrected sequence divergence distances for South African rhinolophids from localities within and outside South Africa showed variable degrees of divergence for the cytochrome *b* gene (Table 2.8). *Rhinolophus blasii* from two localities in Greece differed by 3.6%, and two specimens from the same location in South Africa (Sudwala) showed 0.1% sequence divergence. Within *R. hildebrandti*, individuals from two localities within South Africa (Pafuri and Sudwala) had sequence divergence values of 1%. Sequence divergence values for an individual *R. hildebrandti* specimen from Mozambique ranged from 13% when compared with South African samples (n=2) and 12.7% when compared with a single specimen from Tanzania (Table 2.8).

Table 2.6 Average sequence divergence values based on the supermatrix data set. Values above the diagonal line represent maximum likelihood sequence divergence values. Values below the diagonal line represent GTR corrected sequence divergence values.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
(1)	*C.percivali*		0.32	0.62	0.28	0.50	0.25	0.30	0.26	0.31	0.33	0.34	0.33	0.36	0.29	0.28	0.31	0.50	0.28	0.33	0.39	0.65	0.61	0.56	0.27	0.38	0.29	0.16	1.06	2.12	0.30	0.31	0.32	2.12	0.38	0.35	0.27	0.33
(2)	*H.caffer*	0.22		0.08	0.18	0.28	0.18	0.18	0.18	0.20	0.20	0.21	0.21	0.35	0.19	0.29	0.21	0.16	0.22	0.21	0.23	0.17	0.19	0.17	0.21	0.21	0.18	0.18	0.14	0.13	0.17	0.23	0.19	0.11	0.28	0.21	0.19	0.35
(3)	*H.ruber*	0.31	0.07		0.19	0.43	0.19	0.29	0.19	0.31	0.20	0.22	0.32	0.39	0.21	0.30	0.27	0.18	0.32	0.23	0.27	0.30	0.31	0.31	0.25	0.32	0.20	0.19	0.15	0.13	0.17	0.39	0.20	0.12	0.75	0.32	0.28	0.57
(4)	*R.acuminatus*	0.20	0.14	0.14		0.30	0.05	0.06	0.06	0.07	0.09	0.08	0.08	0.13	0.09	0.14	0.07	0.11	0.08	0.08	0.08	0.05	0.08	0.05	0.05	0.08	0.06	0.05	0.10	0.18	0.08	0.06	0.10	0.03	0.18	0.10	0.05	0.34
(5)	*Ro.aegyptiacus*	0.29	0.19	0.25	0.20		0.30	0.31	0.29	0.31	0.30	0.32	0.32	0.49	0.32	0.39	0.32	0.30	0.32	0.33	0.39	0.35	0.37	0.37	0.30	0.39	0.31	0.25	0.26	0.27	0.30	0.37	0.31	0.21	0.43	0.34	0.32	0.54
(6)	*R.affinis*	0.18	0.14	0.14	0.05	0.20		0.06	0.05	0.07	0.06	0.08	0.08	0.11	0.07	0.13	0.06	0.08	0.08	0.07	0.07	0.06	0.07	0.06	0.06	0.08	0.06	0.05	0.05	0.13	0.05	0.05	0.08	0.04	0.09	0.08	0.06	0.3
(7)	*R.blasii*	0.20	0.14	0.19	0.05	0.21	0.06		0.06	0.05	0.06	0.06	0.06	0.10	0.06	0.09	0.05	0.07	0.05	0.07	0.07	0.13	0.13	0.13	0.08	0.09	0.06	0.03	0.07	0.13	0.05	0.08	0.05	0.03	0.12	0.06	0.07	0.36
(8)	*R.borneensis*	0.18	0.13	0.14	0.06	0.20	0.05	0.05		0.07	0.08	0.08	0.07	0.10	0.09	0.13	0.06	0.10	0.08	0.07	0.08	0.06	0.08	0.07	0.05	0.08	0.05	0.06	0.09	0.18	0.07	0.06	0.08	0.04	0.14	0.08	0.05	0.31
(9)	*R.capensis*	0.21	0.14	0.19	0.06	0.21	0.06	0.04	0.06		0.05	0.06	0.03	0.09	0.07	0.09	0.05	0.07	0.07	0.09	0.06	0.11	0.13	0.12	0.08	0.09	0.07	0.03	0.08	0.14	0.06	0.08	0.03	0.04	0.12	0.03	0.07	0.38
(10)	*R.clivosus*	0.22	0.15	0.18	0.08	0.20	0.06	0.05	0.07	0.04		0.05	0.06	0.05	0.09	0.08	0.04	0.09	0.08	0.10	0.07	0.09	0.11	0.09	0.08	0.07	0.08	0.02	0.09	0.16	0.08	0.08	0.06	0.04	0.18	0.06	0.07	0.35
(11)	*R.darlingi*	0.22	0.15	0.16	0.07	0.21	0.07	0.06	0.07	0.06	0.05		0.08	0.09	0.08	0.09	0.04	0.09	0.09	0.10	0.05	0.11	0.12	0.11	0.10	0.09	0.08	0.02	0.07	0.15	0.06	0.11	0.06	0.04	0.24	0.06	0.08	0.36
(12)	*R.denti*	0.21	0.15	0.20	0.07	0.21	0.07	0.05	0.06	0.03	0.06	0.07		0.11	0.08	0.11	0.06	0.08	0.08	0.09	0.09	0.14	0.14	0.14	0.10	0.11	0.08	0.04	0.09	0.15	0.08	0.10	0.03	0.05	0.13	0.04	0.09	0.41
(13)	*R.ferrumequinum*	0.23	0.22	0.23	0.11	0.27	0.10	0.08	0.09	0.08	0.04	0.08	0.09		0.14	0.08	0.08	0.14	0.13	0.14	0.11	0.33	0.28	0.37	0.13	0.12	0.12	0.02	0.21	0.29	0.17	0.13	0.13	2.12	0.22	0.12	0.12	0.36
(14)	*R.formosae*	0.20	0.14	0.15	0.08	0.21	0.06	0.06	0.08	0.06	0.08	0.07	0.07	0.12		0.13	0.07	0.10	0.08	0.11	0.10	0.08	0.11	0.09	0.07	0.08	0.08	0.06	0.12	0.20	0.10	0.08	0.11	0.04	0.22	0.10	0.07	0.35
(15)	*R.fumigatus*	0.19	0.18	0.19	0.10	0.23	0.10	0.07	0.10	0.07	0.06	0.07	0.09	0.07	0.10		0.08	0.16	0.13	0.18	0.09	0.15	0.16	0.15	0.14	0.10	0.13	0.07	0.25	0.25	0.14	0.10	0.12	0.13	0.18	0.10	0.13	0.36
(16)	*R.hildebrandti*	0.21	0.15	0.18	0.06	0.21	0.06	0.04	0.05	0.04	0.04	0.04	0.05	0.07	0.07	0.07		0.07	0.07	0.07	0.05	0.08	0.09	0.09	0.08	0.06	0.07	0.02	0.05	0.14	0.05	0.08	0.05	0.04	0.14	0.05	0.07	0.35
(17)	*R.hipposideros*	0.28	0.13	0.14	0.09	0.20	0.07	0.06	0.08	0.06	0.08	0.08	0.07	0.12	0.09	0.11	0.07		0.07	0.12	0.11	0.08	0.11	0.09	0.08	0.11	0.07	0.05	0.10	0.19	0.09	0.09	0.11	0.04	0.18	0.10	0.08	0.36
(18)	*R.landeri*	0.19	0.16	0.20	0.07	0.22	0.07	0.05	0.07	0.06	0.07	0.07	0.07	0.11	0.07	0.10	0.07	0.07		0.10	0.09	0.14	0.15	0.13	0.10	0.10	0.08	0.06	0.06	0.15	0.08	0.10	0.08	0.05	0.12	0.08	0.09	0.38
(19)	*R.lepidus*	0.22	0.15	0.16	0.07	0.22	0.07	0.06	0.06	0.07	0.09	0.08	0.08	0.12	0.10	0.13	0.06	0.10	0.08		0.11	0.05	0.09	0.07	0.07	0.11	0.02	0.08	0.09	0.18	0.05	0.11	0.11	0.02	0.16	0.10	0.07	0.35
(20)	*R.maclaudi*	0.24	0.16	0.18	0.07	0.24	0.07	0.07	0.07	0.06	0.06	0.05	0.08	0.10	0.08	0.07	0.05	0.09	0.08	0.09		0.11	0.13	0.12	0.10	0.09	0.09	0.02	0.12	0.15	0.07	0.12	0.07	0.04	0.26	0.08	0.08	0.42
(21)	*R.macrotis*	0.32	0.13	0.19	0.05	0.22	0.06	0.10	0.05	0.09	0.08	0.09	0.11	0.22	0.08	0.11	0.07	0.07	0.11	0.05	0.10		0.06	0.04	0.06	0.11	0.05	0.04	0.08	0.12	0.02	0.18	0.08	0.02	0.27	0.14	0.10	0.68
(22)	*R.malayanus*	0.31	0.15	0.20	0.07	0.23	0.07	0.10	0.07	0.11	0.10	0.10	0.11	0.20	0.09	0.11	0.08	0.09	0.12	0.07	0.11	0.05		0.07	0.07	0.12	0.07	0.05	0.12	0.20	0.07	0.15	0.12	0.03	0.16	0.15	0.11	0.55
(23)	*R.marshalli*	0.29	0.13	0.19	0.05	0.23	0.06	0.10	0.06	0.10	0.08	0.09	0.11	0.23	0.08	0.11	0.08	0.07	0.11	0.06	0.10	0.04	0.06		0.05	0.11	0.05	0.04	0.07	0.13	0.01	0.18	0.09	0.02	0.28	0.15	0.10	0.66
(24)	*R.megaphyllus*	0.19	0.15	0.17	0.05	0.21	0.06	0.07	0.05	0.07	0.07	0.08	0.08	0.11	0.06	0.11	0.07	0.07	0.09	0.06	0.08	0.06	0.06	0.05		0.09	0.05	0.06	0.05	0.13	0.05	0.09	0.09	0.03	0.16	0.09	0.07	0.35
(25)	*R.mehelyi*	0.24	0.15	0.20	0.07	0.24	0.07	0.08	0.07	0.07	0.07	0.07	0.09	0.10	0.07	0.08	0.06	0.09	0.09	0.09	0.08	0.10	0.10	0.09	0.08		0.09	0.04	0.16	0.15	0.07	0.13	0.08	0.06	0.29	0.10	0.11	0.42
(26)	*R.monoceros*	0.21	0.14	0.15	0.06	0.20	0.05	0.05	0.05	0.06	0.07	0.07	0.07	0.11	0.07	0.10	0.06	0.08	0.07	0.02	0.08	0.05	0.07	0.05	0.05	0.07		0.06	0.08	0.18	0.05	0.08	0.09	0.03	0.15	0.08	0.05	0.34
(27)	*R.f.nippon*	0.14	0.13	0.14	0.04	0.19	0.04	0.02	0.05	0.03	0.01	0.02	0.04	0.02	0.05	0.05	0.02	0.05	0.05	0.07	0.02	0.04	0.05	0.04	0.05	0.04	0.06		0.06	0.18	0.08	0.05	0.04	0.05	2.12	0.03	0.05	0.28
(28)	*R.pearsonii*	0.47	0.11	0.12	0.09	0.18	0.05	0.06	0.08	0.06	0.08	0.06	0.07	0.16	0.10	0.12	0.05	0.09	0.06	0.08	0.10	0.07	0.10	0.06	0.05	0.12	0.07	0.06		0.17	0.11	0.18	0.12	0.03	0.23	0.10	0.05	0.39
(29)	*Rp.hardwickei*	0.95	0.10	0.11	0.14	0.19	0.11	0.11	0.14	0.11	0.13	0.12	0.12	0.20	0.15	0.15	0.11	0.14	0.12	0.13	0.12	0.10	0.14	0.10	0.11	0.12	0.14	0.14	0.14		0.19	0.16	0.19	0.14	0.38	0.17	0.13	0.35
(30)	*R.pusillus*	0.20	0.13	0.13	0.07	0.20	0.04	0.04	0.06	0.05	0.07	0.06	0.06	0.13	0.08	0.10	0.04	0.08	0.07	0.04	0.06	0.02	0.07	0.01	0.05	0.06	0.04	0.06	0.09	0.14		0.07	0.10	0.02	0.30	0.08	0.05	0.38
(31)	*R.shameli*	0.21	0.16	0.22	0.06	0.24	0.04	0.07	0.05	0.07	0.08	0.09	0.08	0.11	0.07	0.08	0.07	0.09	0.08	0.10	0.10	0.13	0.12	0.14	0.08	0.11	0.07	0.05	0.15	0.14	0.08		0.09	0.03	0.05	0.10	0.07	0.38
(32)	*R.simulator*	0.21	0.14	0.15	0.09	0.21	0.07	0.05	0.07	0.03	0.06	0.06	0.03	0.11	0.09	0.09	0.05	0.10	0.07	0.09	0.06	0.08	0.10	0.08	0.08	0.07	0.08	0.03	0.10	0.14	0.08	0.08		0.05	0.19	0.04	0.08	0.33
(33)	*R.sinicus*	0.95	0.09	0.10	0.03	0.16	0.04	0.03	0.03	0.04	0.04	0.04	0.05	0.95	0.04	0.08	0.04	0.04	0.05	0.02	0.04	0.02	0.03	0.02	0.03	0.06	0.03	0.05	0.03	0.11	0.02	0.03	0.04		2.12	0.04	0.02	2.12
(34)	*R.stheno*	0.23	0.19	0.32	0.15	0.26	0.09	0.11	0.12	0.11	0.15	0.18	0.11	0.17	0.17	0.15	0.12	0.14	0.11	0.13	0.19	0.19	0.13	0.20	0.14	0.21	0.12	0.95	0.18	0.24	0.20	0.05	0.16	0.95		0.18	0.11	0.44
(35)	*R.swinnyi*	0.23	0.15	0.20	0.08	0.22	0.07	0.05	0.07	0.03	0.06	0.06	0.04	0.10	0.09	0.08	0.05	0.08	0.07	0.09	0.07	0.11	0.12	0.11	0.08	0.09	0.07	0.03	0.08	0.13	0.07	0.08	0.04	0.04	0.15		0.08	0.39
(36)	*R.thomasi*	0.19	0.14	0.18	0.05	0.21	0.05	0.06	0.05	0.06	0.06	0.07	0.07	0.10	0.06	0.10	0.06	0.07	0.08	0.06	0.07	0.09	0.09	0.09	0.06	0.09	0.05	0.05	0.05	0.11	0.05	0.07	0.07	0.01	0.10	0.07		0.39
(37)	*T.persicus*	0.22	0.22	0.29	0.21	0.29	0.19	0.21	0.19	0.22	0.22	0.21	0.24	0.22	0.21	0.21	0.21	0.22	0.23	0.21	0.24	0.32	0.28	0.30	0.21	0.24	0.21	0.18	0.23	0.23	0.22	0.22	0.20	0.95	0.26	0.23	0.23	

Table 2.7 Average GTR-corrected sequence divergence values based on the cytochrome *b* data set.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
(1) *C.percivali*																			
(2) *H.caffer*	0.24																		
(3) *H.ruber*	0.35	0.19																	
(4) *R.acuminatus*	0.26	0.21	0.22																
(5) *Ro.aegyptiacus*	0.29	0.24	0.36	0.26															
(6) *R.affinis*	0.23	0.18	0.19	0.12	0.25														
(7) *R.arcuatus*	0.22	0.20	0.33	0.12	0.26	0.10													
(8) *R.blasii*	0.25	0.18	0.33	0.15	0.27	0.15	0.14												
(9) *R.borneensis*	0.26	0.18	0.20	0.13	0.24	0.12	0.10	0.14											
(10) *R.capensis*	0.25	0.20	0.35	0.18	0.26	0.13	0.12	0.10	0.13										
(11) *R.clivosus*	0.27	0.20	0.29	0.18	0.24	0.11	0.13	0.14	0.13	0.11									
(12) *R.darlingi*	0.29	0.22	0.22	0.21	0.28	0.17	0.19	0.17	0.18	0.17	0.16								
(13) *R.denti*	0.26	0.20	0.34	0.16	0.26	0.13	0.12	0.11	0.11	0.07	0.12	0.18							
(14) *R.ferrumequinum*	0.34	0.27	0.26	0.17	0.27	0.17	0.18	0.17	0.13	0.17	0.06	0.18	0.16						
(15) *R.formosae*	0.29	0.23	0.25	0.18	0.26	0.17	0.16	0.15	0.17	0.15	0.18	0.20	0.15	0.18					
(16) *R.fumigatus*	0.27	0.24	0.24	0.21	0.29	0.17	0.16	0.11	0.15	0.13	0.12	0.12	0.15	0.13	0.17				
(17) *R.hildebrandti*	0.26	0.22	0.30	0.20	0.27	0.14	0.13	0.12	0.13	0.11	0.13	0.14	0.13	0.19	0.19	0.12			
(18) *R.hipposideros*	0.29	0.21	0.23	0.18	0.25	0.16	0.13	0.13	0.14	0.13	0.14	0.18	0.13	0.12	0.16	0.17	0.16		
(19) *R.landeri*	0.23	0.20	0.31	0.15	0.27	0.13	0.12	0.07	0.11	0.10	0.12	0.16	0.10	0.16	0.14	0.12	0.13	0.12	
(20) *R.lepidus*	0.27	0.19	0.23	0.12	0.28	0.14	0.11	0.15	0.10	0.15	0.16	0.18	0.12	0.16	0.18	0.18	0.17	0.17	0.14
(21) *R.maclaudi*	0.30	0.24	0.29	0.23	0.32	0.14	0.16	0.18	0.19	0.15	0.18	0.16	0.18	0.24	0.25	0.14	0.14	0.24	0.15
(22) *R.macrotis*	0.32	0.20	0.35	0.14	0.31	0.12	0.18	0.21	0.13	0.19	0.15	0.21	0.21	0.22	0.19	0.19	0.14	0.17	0.21
(23) *R.malayanus*	0.31	0.21	0.33	0.13	0.28	0.12	0.19	0.20	0.13	0.20	0.15	0.22	0.19	0.20	0.16	0.19	0.15	0.17	0.21
(24) *R.marshalli*	0.29	0.20	0.35	0.14	0.32	0.12	0.19	0.22	0.16	0.20	0.15	0.22	0.21	0.23	0.21	0.19	0.16	0.18	0.20
(25) *R.megaphyllus*	0.23	0.20	0.27	0.11	0.26	0.14	0.14	0.17	0.12	0.15	0.15	0.21	0.16	0.19	0.17	0.19	0.16	0.16	0.17
(26) *R.mehelyi*	0.31	0.20	0.33	0.20	0.31	0.14	0.21	0.20	0.15	0.17	0.14	0.17	0.20	0.20	0.22	0.13	0.12	0.19	0.19
(27) *R.monoceros*	0.28	0.21	0.23	0.12	0.26	0.13	0.12	0.15	0.09	0.16	0.15	0.18	0.14	0.15	0.16	0.16	0.17	0.15	0.14
(28) *R.pearsonii*	0.47	0.22	0.26	0.16	0.20	0.15	0.16	0.18	0.13	0.23	0.13	0.19	0.18	0.16	0.17	0.12	0.15	0.16	0.15
(29) *Rp.hardwickei*	0.95	0.95	0.95	0.22	0.26	0.95	0.95	0.95	0.19	0.95	0.17	0.95	0.95	0.20	0.26	0.95	0.95	0.23	0.95
(30) *R.pumilus*	0.24	0.19	0.32	0.12	0.26	0.12	0.12	0.14	0.11	0.14	0.12	0.19	0.13	0.20	0.16	0.19	0.14	0.15	0.14
(31) *R.pusillus*	0.95	0.95	0.95	0.19	0.30	0.95	0.95	0.95	0.14	0.95	0.17	0.95	0.00	0.19	0.25	0.95	0.95	0.21	0.95
(32) *R.shameli*	0.25	0.19	0.33	0.13	0.27	0.10	0.06	0.13	0.12	0.12	0.13	0.20	0.13	0.20	0.18	0.16	0.12	0.14	0.11
(33) *R.simulator*	0.29	0.21	0.23	0.19	0.26	0.17	0.14	0.16	0.14	0.13	0.14	0.19	0.09	0.16	0.18	0.20	0.18	0.17	0.14
(34) *R.stheno*	0.23	0.19	0.32	0.15	0.26	0.09	0.06	0.11	0.12	0.11	0.15	0.18	0.12	0.17	0.17	0.15	0.12	0.14	0.11
(35) *R.swinnyi*	0.29	0.21	0.36	0.20	0.27	0.16	0.16	0.13	0.15	0.07	0.12	0.17	0.09	0.16	0.19	0.17	0.13	0.15	0.13
(36) *R.thomasi*	0.22	0.19	0.32	0.15	0.27	0.10	0.11	0.13	0.12	0.12	0.12	0.18	0.12	0.18	0.18	0.16	0.12	0.16	0.13
(37) *T.persicus*	0.30	0.25	0.35	0.24	0.30	0.22	0.27	0.25	0.22	0.27	0.25	0.27	0.27	0.25	0.27	0.25	0.26	0.23	0.26

	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
(19) *R.landeri*																			
(20) *R.lepidus*	0.14																		
(21) *R.maclaudi*	0.15	0.23																	
(22) *R.macrotis*	0.21	0.12	0.19																
(23) *R.malayanus*	0.21	0.12	0.21	0.10															
(24) *R.marshalli*	0.20	0.15	0.20	0.09	0.11														
(25) *R.megaphyllus*	0.17	0.12	0.19	0.11	0.12	0.10													
(26) *R.mehelyi*	0.19	0.20	0.19	0.15	0.15	0.14	0.17												
(27) *R.monoceros*	0.14	0.04	0.23	0.13	0.12	0.14	0.10	0.20											
(28) *R.pearsonii*	0.15	0.15	0.38	0.30	0.18	0.30	0.19	0.36	0.13										
(29) *Rp.hardwickei*	0.95	0.23	0.95	0.95	0.23	0.95	0.95	0.95	0.21	0.19									
(30) *R.pumilus*	0.14	0.06	0.16	0.09	0.10	0.09	0.11	0.14	0.05	0.07	0.95								
(31) *R.pusillus*	0.95	0.08	0.95	0.95	0.18	0.95	0.95	0.95	0.09	0.18	0.22	0.95							
(32) *R.shameli*	0.11	0.15	0.20	0.20	0.17	0.20	0.15	0.21	0.14	0.15	0.95	0.13	0.11						
(33) *R.simulator*	0.14	0.16	0.20	0.18	0.18	0.19	0.20	0.19	0.16	0.16	0.23	0.16	0.18	0.16					
(34) *R.stheno*	0.11	0.13	0.19	0.19	0.13	0.19	0.13	0.20	0.12	0.18	0.24	0.11	0.20	0.04	0.15				
(35) *R.swinnyi*	0.13	0.16	0.20	0.23	0.20	0.24	0.18	0.22	0.15	0.14	0.19	0.15	0.15	0.15	0.11	0.15			
(36) *R.thomasi*	0.13	0.13	0.15	0.18	0.18	0.18	0.14	0.19	0.13	0.15	0.95	0.11	0.95	0.11	0.18	0.10	0.16		
(37) *T.persicus*	0.26	0.22	0.30	0.32	0.28	0.31	0.23	0.32	0.23	0.24	0.22	0.25	0.25	0.26	0.22	0.25	0.26	0.27	

For specimens of South African rhinolophids that were only obtained from localities within South Africa, pairwise maximum likelihood sequence divergence values ranged from 0.5% between *R. denti* specimens from Koegelbeen; 3.9% between *R. landeri* from Pafuri; 0.1% between *R. swinnyi* specimens from Pirie Forest, and 0.13% between *R. swinnyi* specimens from Kokstad and Pirie forest. Although *R. capensis* is endemic to the Cape floristic region and is ostensibly monotypic, sequence divergence values ranged from 0.02-9.1% (Table 2.8).

Rhinolophus darlingi showed interesting sequence divergence patterns. Specimens from the Northern Cape had large divergence values when compared with specimens from the eastern side of South Africa. Individuals from Goodhouse and Koegelbeen in the Northern Cape had a sequence divergence value of 2.5%. These bats had divergence values that ranged from 10% to 14.9% when compared with bats from localities in eastern South Africa and Swaziland (Table 2.8). In the cytochrome *b* topology, the Northern Cape (Goodhouse, Koggelbeen) *R. darlingi* did not group with the *R. darlingi* specimens from further east in Swaziland, Mkuze, and Sudwala (Fig. 2.3). Instead, they grouped with the *R. clivosus – R. ferrumequinum* clade, although the posterior probability for that node is less than 0.95 (Fig. 2.3). It is unlikely that the Northern Cape specimens were misidentified, because echolocation calls were taken, and the skulls of voucher specimens were checked. Due to these large sequence divergence values, only *R. darlingi* from the eastern side of South Africa was used in the supermatrix data set.

Table 2.8 Intraspecific sequence divergence values for *Rhinolophus* species from localities within South Africa and Africa. Values are GTR-corrected sequence divergence values based on the cytochrome *b* gene.

Code	Location	Sequence Divergence Values								
R. blasii		cmr 8	cmr 30	cmr 41	cmr 49					
cmr 8	Sudwala, RSA									
cmr 30	Sudwala, RSA	0.001								
cmr 41	Greece	0.057	0.065							
cmr 49	Greece	0.059	0.087	0.036						
R. capensis		cmr 9	cmr 10	cmr 34	cmr 42	cmr 44	cmr 45	cmr 46	cmr 48	
cmr 9	De Hel, RSA									
cmr 10	De Hoop, RSA	0.038								
cmr 34	Knysna, RSA	0.002	0.091							
cmr 42	De Hoop, RSA	0.010	0.074	0.005						
cmr 44	Knysna, RSA	0.023	0.053	0.020	0.026					
cmr 45	Knysna, RSA	0.021	0.038	0.022	0.031	0.034				
cmr 46	Knysna, RSA	0.008	0.039	0.012	0.020	0.014	0.007			
cmr 48	De Hoop, RSA	0.005	0.039	0.003	0.010	0.024	0.020	0.010		
R. clivosus		cmr 1	cmr 18	cmr 84						
cmr 1	Sudwala, RSA									
cmr 18	Sudwala, RSA	0.049								
cmr 84	Tanzania	0.124	0.137							
R. darlingi		cmr 3	cmr 7	cmr 11	cmr 17	cmr 47	cmr 57	cmr 76	cmr 148	cmr 149	cmr 150
cmr 3	Sudwala, RSA										
cmr 7	Sudwala, RSA	0.001									
cmr 11	Mkuze, RSA	0.074	0.074								
cmr 17	Sudwala, RSA	0.080	0.081	0.009							
cmr 47	Goodhouse, RSA	0.100	0.100	0.115	0.115						
cmr 57	Koggelbeen, RSA	0.100	0.115	0.140	0.12	0.025					
cmr 76	Mkuze, RSA	0.043	0.043	0.091	0.098	0.149	0.144				
cmr 148	Swaziland	0.002	0.002	0.082	0.080	0.112	0.118	0.053			
cmr 149	Swaziland	0.002	0.002	0.067	0.075	0.103	0.110	0.049	0.007		
cmr 150	Swaziland	0.002	0.002	0.079	0.077	0.109	0.114	0.051	0.003	0.004	
R. denti		cmr 56	cmr 59								
cmr 56	Koggelbeen, RSA										
cmr 59	Koggelbeen, RSA	0.005									

Table 2.8 continued.

Code	Location	Sequence Divergence Values						
R. fumigatus		cmr 12	cmr 14	cmr 80				
cmr 12	Pafuri, RSA							
cmr 14	Pafuri, RSA	0.011						
cmr 80	Tanzania	0.108	0.101					
R. hildebrandti		cmr 4	cmr 15	cmr 83	cmr 152			
cmr 4	Sudwala, RSA							
cmr 15	Pafuri, RSA	0.010						
cmr 83	Tanzania	0.016	0.018					
cmr 152	Mozambique	0.130	0.129	0.127				
R. landeri		cmr 13	cmr 16					
cmr 13	Pafuri, RSA							
cmr 16	Pafuri, RSA	0.039						
R. simulator		cmr 2	cmr 6	cmr 27	cmr 28	cmr 29	cmr 82	cmr 154
cmr 2	Sudwala, RSA							
cmr 6	Sudwala, RSA	0.010						
cmr 27	Sudwala, RSA	0.019	0.020					
cmr 28	Sudwala, RSA	0.038	0.047	0.051				
cmr 29	Sudwala, RSA	0.021	0.015	0.055	0.046			
cmr 82	Tanzania	0.079	0.085	0.080	0.081	0.084		
cmr 154	Mozambique	0.026	0.022	0.037	0.017	0.074	0.085	
R. swinnyi		cmr 60	cmr 64	cmr 65	cmr 66			
cmr 60	Kokstad, RSA							
cmr 64	Pirie Forest, RSA	0.001						
cmr 65	Pirie Forest, RSA	0.001	0.001					
cmr 66	Pirie Forest, RSA	0.001	0.002	0.001				

MOLECULAR CLOCK

The estimated divergence of the rhinolophids from their sister taxa, the hipposiderids, occurred during the Eocene approximately 40.5 (sd 5.9) MYA. The estimated divergence time of the African clade from the Oriental clade using the relaxed Bayesian clock method was 36.3 ± 6.2 MYA (Fig. 2.8). A discussion on the biogeography of the rhinolophids together with possible causes for their radiation is provided in Chapter 5.

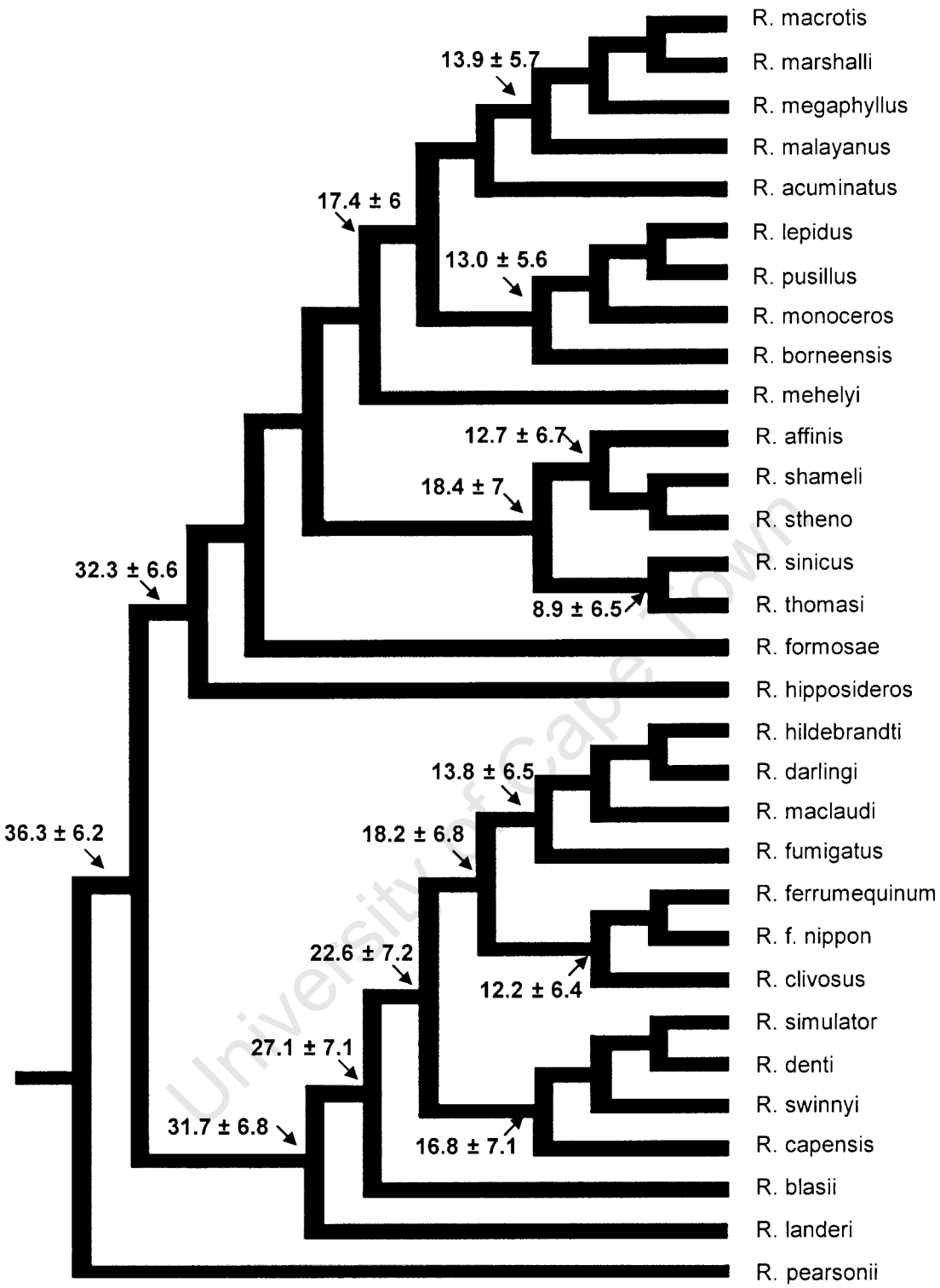


Fig. 2.8 Estimated divergence times for the genus *Rhinolophus*. Values at nodes refer to ages ± SD (MYA) estimated using a relaxed Bayesian clock on the supermatrix data set in which the supermatrix topology (Fig. 2.5) was used as reference.

DISCUSSION

The mitochondrial cytochrome *b* gene failed to recover well-supported relationships at deeper nodes in the topology for *Rhinolophus* species used in this study. Relationships among closely related species received good nodal support and many intraspecific associations were well supported. This marker is clearly suitable at the population level, but does not provide sufficient resolution at higher levels between clades with deeper nodes (also see Guillén *et al.* 2003). Nuclear introns evolve at a much slower rate than cytochrome *b*, and as a result can provide resolving power at deeper nodes within the phylogeny. However, nuclear intron data sets alone do not recover well-supported topologies for the rhinolophids, terminal nodes often being unresolved. Thus, analysis of the evolutionary relationships among the rhinolophids provides a good example of the limitations inherent in using only one data set. Through combining mtDNA (cytochrome *b*) and nuclear introns, the supermatrix data set recovered a well-resolved phylogeny for the rhinolophids, with good bootstrap and posterior probability support for most associations.

The Rhinolophidae form a monophyletic group and split from their sister family, the Hipposideridae, during the Eocene (*ca* 40.5 MYA). These results are similar to those reported by Maree & Grant (1997), McKenna & Bell (1997), Teeling *et al.* (2003) and Eick *et al.* (2005). Furthermore, this estimate of divergence is congruent with the fossil data, with fossils of extinct *Rhinolophus* and *Hipposideros* species first occurring in Middle Eocene deposits (*ca* 49 to 37 MYA; Simmons & Geisler 1998).

The rhinolophids can be divided into at least two major clades – the predominantly African and the predominantly Oriental clades - based on the current biogeographical distributions of the majority of species within each clade. Using the zoogeographical regions in Csorba *et al.* (2003) these clades would translate into an 'Afrotropical' and an 'Indomalayan' clade. The split between

these two major clades occurred within the late Eocene, approximately 36 MYA, with the ancestors of the extant African and Oriental species being present during the Oligocene (*ca* 34 to 20 MYA). Within each of these clades, radiation into smaller clades (as identified by Guillén *et al.* 2003) occurred during the Miocene (Fig. 2.8), a time when radiation also occurred in other groups of bats, e.g. *Myotis* (Stadelmann *et al.* 2007). The rhinolophid radiation occurred quite rapidly, as indicated by the short internal branches at the base of the African and Oriental clades (Figs 2.3, 2.5). Based on the cytochrome *b* gene, Guillén *et al.* (2003) proposed that a rapid radiation of the rhinolophids started approximately 17 – 15 MYA. These dates are congruent with the divergence dates recovered in this study for other rhinolophid subclades within the African and Oriental clades (Fig. 2.8). Further discussion of the historical biogeography of the rhinolophids, with a discussion on possible factors leading to speciation are provided in Chapter 5.

Morphological (Bogdanowicz 1992) and molecular (Guillén *et al.* 2003) analyses suggest that the African rhinolophids form a monophyletic clade. However, included in this clade are *R. ferrumequinum* and *R. f. nippon*, which has a very large distribution (Csorba *et al.* 2003). The distribution of *R. ferrumequinum* within Africa is restricted to localities north of the Sahara Desert (in the Palearctic biogeographic zone). It is likely that *R. ferrumequinum* may have originated in Africa and dispersed into Europe and Asia. All other rhinolophids included in the African clade in my analyses have distributions within the Afrotropical biogeographic zone.

In revising rhinolophid relationships, Guillén *et al.* (2003) proposed, based on phylogenetic affinities, that the genus *Rhinolophus* should be divided into six subgenera each comprising different groups. Results from this study support the above proposal. The radiation of the African rhinolophids began approximately 31.7± 6.8 MYA (Fig. 2.8). The African clade comprises species assigned (by Guillén *et al.* 2003) to the 'subgenus' *Rhinolophus* which includes several smaller

subclades. My results are in accord with those of Guillén *et al.* (2003) except for *R. mehelyi*. The distribution of *R. mehelyi* extends from Europe into the Middle East, and into North Africa (Csorba *et al.* 2003). Guillén *et al.* (2003) placed *R. mehelyi* within the African clade as closely related to *R. blasii* and within the *R. euryale*-group. However, support for this node was low (50 - 72%). In this study, the supermatrix approach places *R. mehelyi* firmly within the Oriental clade with posterior probability values of ≥0.95 and bootstrap support of 70% (Figs 2.5, 2.7).

The radiation of species within the Oriental clade occurred approximately 32.3 ± 6.6 MYA, with the diversification into many smaller clades occurring around 17 to 18 MYA (Fig. 2.8). The relationships of *R. formosae* and *R. hipposideros* to the other rhinolophids are not resolved within the Oriental clade. The Oriental clade can be divided into at least three subclades each comprising representatives of the three proposed 'subgenera' of Guillén *et al.* (2003). *Rhinolophus sinicus* and *R. thomasi* are part of Guillén *et al.*'s (*op. cit.)* putative 'subgenus' *Indorhinolophus*. The putative subgenus *Coellophyllus* comprises *R. affinis R. stheno* and *R. shameli,* and the putative *Rhinolophyllotis* subgenus comprises *R. acuminatus, R. malayanus, R. borneensis, R. megaphyllus, R. macrotis, R. marshalli, R. monoceros*, *R. lepidus*, and *R. pusillus*.

Species whose phylogenetic affinities were marked as uncertain by Guillén *et al.* (2003), but which have been confirmed in this study, include *R. capensis, R. swinnyi*, *R. denti* (in Guillen *et al.*'s *op.cit R. capensis*-group in the *Rhinolophus* subgenus); and *R. monoceros* and *R. lepidus* (in Guillen *et al.*'s *op. cit. R. pusillus*-group in the *Rhinophyllotis* subgenus). Although *R. f. nippon* is still referred to as a subspecies of *R. ferrumequinum* in Csorba *et al.* (2003), the GTR-corrected sequence divergence value between *R. ferrumequinum* and *R. f. nippon* is 2%. This is the same as the sequence divergence value between *R. clivosus* and *R. f. nippon*, and between sister taxa *R. thomasi* and *R. sinicus*. However, the specific status in the latter two pairs of species have been questioned. A comparison of genetic variability across the species' range for *R.*

ferrumequinum suggests that *R. f. nippon* may not warrant specific status (Rossiter *et al.* in press). Furthermore, *R. thomasi* and *R. sinicus* (at least in China) may be conspecific (Gareth Jones, personal communication).

Southern African rhinolophids

Among the southern African rhinolophids, evolutionary relationships are well resolved. Results obtained from the supermatrix analyses differ from those described earlier by Bogdanowicz (1992), but differ only slightly from the conclusions of Maree & Grant (1997). The discrepancies among the different studies may be due to the different data sets analysed. The groups identified by Bogdanowicz (1992) and Bogdanowicz & Owen (1992) appear to place species that are similar in size together. Similarly, *R. fumigatus* and *R. hildebrandti* (two low-frequency echolocators) may be grouped together on the basis of noseleaf shape. Maree & Grant (1997) failed to identify the relationship of *R. landeri* with the other rhinolophids. This may be due to the fact that *R. landeri* appears to occupy a more basal position among the African rhinolophids, and is more distantly related to the southern African rhinolophids. In agreement with Maree & Grant (1997), *Rhinolophus clivosus*, together with *R. ferrumequinum* and *R. f. nippon*, form the sister clade to the clade comprising *R. darlingi, R. hildebrandti* and *R. fumigatus* (Fig. 2.5). Bogdanowicz & Owen (1992) proposed that *R. hildebrandti* possessed the most plesiomorphic morphological characters among the African rhinolophids, and, based on this, suggested that it was one of the most basal of the rhinolophids. However, the molecular analyses in this study show that *R. landeri* and *R. blasii* are more likely to be the most basal of the African rhinolophids (Fig. 2.5).

At a qualitative level, patterns of intraspecific sequence divergence are positively related to geographic distance. For example, sequence divergence in *R. blasii* from the same locality in South Africa is < 1% (Table 2.8) but between South Africa and Greece is 6%. This sequence divergence (Bradley & Baker 2001)

between European and African specimens, combined with divergence in echolocation call frequency (respectively 94 kHz, Heller & von Helversen 1989; and 86.6 kHz, this study) indicates that they may be distinct at the species level and warrants further investigation.

Rhinolophus clivosus from the same locality within South Africa diverges by 5% but, on average, differs from *R. clivosus* from Tanzania by 12%. Similarly, populations of *R. darlingi* from Northern Cape, South Africa (Goodhouse, Koggelbeen) differ from those in eastern South Africa (10-14.9%) as do populations of *R. fumigatus* and *R. hildebrandti* from within and outside South Africa. However, geographic distance may only partly explain patterns of sequence divergence because divergence between individuals from the same species from the same locality can be greater than that between individuals from different localities. For example, sequence divergence between *R. capensis* from De Hoop can be as high as 10% but averages only 4% between individuals from De Hoop and Knysna (± 240 km to the east). These patterns, together with sequence divergence values of the same magnitude or higher for recognized *Rhinolophus* species (e.g. *R. landeri* and *R. capensis*, and *R. denti* and *R. capensis*; Table 2.7) suggests that there may be several species complexes (as in *R. maclaudi*; Fahr *et al.* 2002) that need to be investigated using genes that evolve more rapidly than cytochrome *b*, and with samples from populations throughout the species' distribution ranges.

To conclude, nuclear introns, when combined with cytochrome *b* in a supermatrix, were capable of resolving the evolutionary relationships among the rhinolophid species used in this study. The robust phylogeny (Fig 2.5), in which the deeper nodes are well supported and which differs from previous phylogenies in important ways, will be used in subsequent chapters to test various hypotheses about the evolution of echolocation call frequency in the Rhinolophidae.

CHAPTER 3

EVOLUTION OF ECHOLOCATION IN THE GENUS *RHINOLOPHUS*: A TEST OF THE ALLOTONIC FREQUENCY HYPOTHESIS

In days of old and insects bold
(Before bats were invented),
No sonar cries disturbed the skies –
Moths flew uninstrumented.

The Eocene brought mammals mean
And bats began to sing;
Their food they found by ultrasound
And chased it on the wing.

Now deafness was unsafe because
The loud high-pitched vibration
Came in advance and gave a chance
To beat echolocation.

Some found a place on wings of lace
To make an ear in haste;
Some thought it best upon the chest
And some below the waist.

Then Roeder's keys upon the breeze
Made Sphingids show their paces.
He found the ear by which they hear
In palps upon their faces.

Of all unlikely places!

(J. David Pye 1968 Nature 218, 797)

INTRODUCTION

Coevolution occurs when evolutionary change in one species influences, or drives, evolutionary change in another (Ridley 1996; Zimmer 2003). However, Jansen (1980) argues that coevolution, in the strictest sense of the word, only occurs when changes in the second species impose selective pressure on the first, resulting in a form of selection pressure reciprocity. Coevolutionary interactions may result in mutually beneficial interactions or can result in biological arms races. However, within the context of communities, the selection pressure may come from many species (Brewer 1998), resulting in diffuse coevolution with many species placing reciprocal selection pressures on many other species.

The interaction between bats and tympanate moths is often cited as an example of a coevolutionary arms race between predator and prey (Fenton & Fullard 1979; Fullard 1988; Rydell *et al.* 1995; Brewer 1998; Rydell *et al.* 2000; Fullard 2001; Hoagland *et al.* 2001; Miller & Surlykke 2001; Tallack 2003). To be more precise, as many tympanate moth species and many species of bat are involved, the interaction between bats and tympanate moths may be an example of diffuse coevolution (Rydell *et al.* 1995).

BATS AND MOTHS: PROTAGONISTS IN A COEVOLUTIONARY ARMS RACE

According to the fossil record, echolocating bats existed in the Eocene (Simmons & Geisler 1998; Teeling *et al.* 2002), by which time many moth families were well established (Gall & Tiffney 1983; Miller & Surlykke 2001). Fossil evidence shows that the main moth lineages which posses hearing species (the obtectomeran groups incorporating the Noctuoidea, Geometroidea and Pyraloidea) originated in the late Palaeocene (Kristensen & Skalski 1999). By this time echolocating bats had evolved. The evolution of echolocation represented an innovation that increased the effectiveness of bats in catching nocturnal insects, making them the principal predator of nocturnal insects. However, echolocation as a strategy has its limitations. It is only effective over short distances because of the physical laws that

govern sound transmission. Furthermore, to receive echoes from small targets (e.g. insect prey) bats must use high-frequency sounds (Pye 1993). However, high frequencies are subject to greater atmospheric attenuation than lower frequencies, and consequently generate weaker echoes (Lawrence & Simmons 1982). Thus, atmospheric attenuation places a limit on the maximum frequency a bat can use and still detect an echo. Frequencies of 20–60 kHz represent the optimum compromise between the range at which prey can be detected and the ability to locate small prey (Fenton *et al.* 1998).

Even in the 20–60 kHz frequency range, echolocation requires the broadcasting of intense pulses of ultrasound, potentially making bats conspicuous to any prey able to detect this sound. The predation pressure exerted by bats on nocturnal insects during the Eocene provided strong selection pressure on insects to evolve effective defences against bats (Miller & Surlykke 2001; Jones & Rydell 2003). This set the evolutionary stage for a potential predator-prey arms race between bat echolocation and insect hearing.

HAVE MOTH EARS EVOLVED IN RESPONSE TO BAT ECHOLOCATION?

Some nocturnal insects have evolved tympanate organs or 'ears' which are sensitive to the echolocation calls of foraging bats. These were first described in moths (Eggers 1919 in Jones & Rydell 2003) and many subsequent studies of insect hearing and bat echolocation have focused on moths, resulting in bat-moth interactions being the best researched examples of the interaction between bats and their tympanate insect prey. Nonetheless, tympanate organs have evolved independently in moths and nocturnal butterflies (Lepidoptera), crickets (Orthoptera), praying mantids (Dictyoptera), green lacewings (Neuroptera) and possibly within the flies (Diptera) and beetles (Coleoptera) (Spangler 1988a; Rydell *et al.* 1995; Forrest *et al.* 1997; Miller & Surlykke 2001). Furthermore, the fact that they can be located in at least ten different places on an insect's body suggests that tympanate ears have a polyphyletic origin (Fullard & Yack 1993).

The ability to detect an echolocating bat can provide tympanate moths with a 40% greater chance of evading bat predation than non-tympanate moths (Roeder 1967; Rydell 1992; Acharya & Fenton 1999). Similarly, moths which possess sensitive ears that are efficient at detecting bat echolocation calls (e.g. *Agrotis diplosticta*) are less likely to be preyed upon than moths with poorer hearing ability (e.g. *Haliophyle euclidias*) (Fullard 2001).

The similarities in the mechanisms underlying the audition and behaviour of tympanate insects (summarised by Miller & Surlykke 2001) suggests that the evolution of organs sensitive to ultrasound (although the ears are tone deaf and cannot discriminate frequencies - Fullard 1988) may be an example of convergent evolution in response to bat predation (e.g. Yack & Fullard 2000). There is much evidence in support of this hypothesis.

Firstly, tympanate organs have evolved in a diversity of physical locations in a diverse range of Lepidoptera and, more importantly, they have evolved in species that make no sounds of their own (i.e. they cannot fulfil a communication role - Fullard 1987; Waters 2003).

Secondly, upon hearing bat echolocation calls tympanate insects display negative phonotaxic responses, enabling them to take evasive action (Roeder 1967; Fullard 1982, 1987, Surlykke 1988; Fullard 1990; Miller & Surlykke 2001). This response involves the moth changing the direction of its flight path away from the sound source (the bat echolocation call), and can be so strong that it interrupts mating behaviour in pyralid moths (Acharya & McNeil 1998).

Thirdly, although some moths do use ultrasound and their tympanate organs for intraspecific communication (Spangler 1988b; Simmons & Conner 1996; Conner 1999; Greenfield & Weber 2000), the ears of moths are most sensitive to frequencies which coincide with the peak-frequency range of the most common echolocating bats, *viz* 20-60 kHz (Fullard 1982; 1987; 1988). Furthermore, at local

spatial scales, the sensitivity of moth ears reflects the echolocation frequencies of sympatric bats (Fenton & Fullard 1979; Fullard 1982; Fullard 1987; Göpfert & Wasserthal 1999). Some moth species that are sympatric with diverse bat communities in Africa can detect a greater range of frequencies than moths exposed to relatively species-poor bat communities. In addition to being able to detect frequencies in the 20-60 kHz range, moths in bat-rich areas are also capable of hearing frequencies of 5–25 kHz and 80-110 kHz, (frequencies used by some echolocating bats - Fullard 1982; Fullard 1988), although hearing sensitivity does decrease at frequencies above 65 kHz (Fenton & Fullard 1979).

Fourthly, moths not exposed to bat predation, but which, by virtue of being tympanate, presumably were exposed to such threats in their evolutionary history, appear to undergo auditory degeneration. Moths having tympanate organs and that occur in the absence of bats (e.g. on Tahiti - Fullard 1994) have reduced auditory sensitivity to high frequencies. Similarly, auditory degeneration is evident in diurnal moths which are not exposed to bat predation (Fullard *et al.* 1997), such as the diurnal geometrid *Archiearis parthenias* (Surlykke *et al.* 1998). Auditory degeneration is also evident among flightless moths which are less exposed than volant taxa to aerially foraging bats. For example, flightless female gypsy moths (*Lymantria dispar*) have reduced hearing capacity compared to their volant male counterparts (Cardone & Fullard 1988).

A tympanate moth which can detect an echolocating bat has an opportunity to avoid capture. Depending on the bat's proximity, the moth may fly in the opposite direction when the bat is far away (low intensity sound) or if the bat is closer (high intensity sound), the moth may employ evasive flight behaviours such as powered dives, erratic or looping flight, or dropping towards the ground (Roeder 1967; Jones & Rydell 2003). Even flightless moths respond to ultrasound by remaining motionless (Werner 1981). This would be beneficial in predator avoidance, because some bats, like the rhinolophids, rely on the acoustic signals given by the movement of insect wings to locate their prey (Neuweiler 2003).

In addition to being able to hear, some arctiid moths (Arctiidae) produce ultrasonic clicks in response to bat echolocation calls (Dunning & Roeder 1965). The functions of these clicks may be to startle the bat (Stoneman & Fenton 1988), to interfere with the bat's returning echoes by creating 'false echoes' (Fullard *et al.* 1979), or they may be aposematic in function (Surlykke & Miller 1985; Acharya & Fenton 1992).

HAVE BATS RESPONDED TO MOTH HEARING?

The evolution of tympanate organs in moths and their subsequent ability to detect echolocating bats, thereby reducing predation risk, may influence the foraging efficiency of bats (Fenton & Fullard 1979; Simmons 1995). It is likely, therefore, that the evolution of tympanate organs has exerted a reciprocal selection pressure on bats to evolve strategies to counter the hearing ability of moths.

To overcome moth defences, bats may alter their echolocation call characteristics or their foraging strategies. Bats which glean insects off a substratum possess echolocation calls characterized by short, broad-band calls of low intensity (Neuweiler 1989). The short duration and low intensity of these calls is necessary to reduce background echoes and overcome pulse-echo overlap when foraging in structurally complex ('cluttered') habitats (Schnitzler & Kalko 2001). However, these calls also provide a means of circumventing moth hearing, because a reduction in call intensity reduces the distance at which a moth can detect a bat (Fenton & Fullard 1979; Fullard 1992; Waters & Jones 1996). Examples of gleaning bats whose calls are relatively inaudible to moths include *Myotis evotis* (Faure *et al.* 1990) and *M. septentrionalis* (Faure *et al.* 1993). However, although low-intensity calls seemingly have a selective advantage in allowing bats to overcome moth defences, this advantage is probably secondary: it is unlikely that such calls evolved to reduce detection by moths. Low-intensity calls of short duration are suited to the foraging habitat and mode used by gleaning bats (Faure *et al.* 1990, 1993; Waters 2003) and probably evolved within this context to avoid pulse-echo overlap when foraging close to vegetation (Schnitzler & Kalko 2001).

Some gleaning bats use prey-generated sounds to locate their prey, and often do not use echolocation calls prior to attack (e.g. *Plecotus auritus* - Anderson & Racey 1991, 1993; Swift 1998). Because no echolocation calls are used, passive-listening bats are unlikely to be detected by tympanate moths. However the disadvantage of this foraging mode is that only moving prey are detectable (Anderson & Racey 1993). Other gleaners do use echolocation to locate their prey, but may stop echolocating before the attack and produce no terminal phase (Neuweiler 1989). Because no call is used during the final attack prior to capture, this may reduce the chance of escape behaviour by the prey.

Because moth ears are more sensitive to some frequencies than to others (Fenton & Fullard 1979) and their sensitivity decreases below 20 kHz and above 65 kHz (Fenton & Fullard 1979; Fullard 1987; Surlykke 1988; Fullard *et al.* 1997), a possible counter-adaptation by bats would be to exploit this limited hearing capacity of moths by using allotonic frequencies (frequencies above and below the greatest sensitivities of moth ears) (Novick 1977; Fullard 1987; Chapter 1). The Allotonic Frequency Hypothesis (AFH) proposes that bats have indeed responded evolutionarily to moth hearing, i.e. that the hearing ability of moths (which itself evolved in response to bat predation) has had a reciprocal selective effect in driving the evolution of allotonic frequencies in the calls of some bat species.

Most of the evidence in favour of the AFH comes from the support for the first prediction (*viz* the proportion of tympanate moths should be greater in diets of bats whose calls are dominated by allotonic frequencies; Chapter 1). Many studies have demonstrated a relationship between the proportion of moths in a bat's diet and its echolocation frequency (Jones 1992; Pavey & Burwell 1998; Bogdanowicz *et al.* 1999; Jacobs 2000; Schoeman & Jacobs 2003). Further evidence in support of this prediction comes from demonstrations that bats using low allotonic frequencies (below 20 kHz) are inaudible to tympanate moths (e.g. *Euderma maculatum* - Fullard & Dawson 1997).

In southern Africa, where 85% of moths that have been studied belong to families which possess tympanate organs (Fenton & Fullard 1979), there is a significant relationship between the proportion of moths in the diet and the peak frequencies of bat species in several communities, with the diets of species using allotonic frequencies comprising more moths (Schoeman 2006). In one community, which includes five rhinolophid species (*R. blasii*, *R. clivosus*, *R. darlingi*, *R. hildebrandti* and *R. simulator*) and two hipposiderid species (*Hipposideros caffer* and *Cloeotis percivali*), peak echolocation frequency is the best predictor of the proportion of moths in a bat's diet (i.e. bats echolocating at frequencies >60 kHz have a greater proportion of moths in the diet, Schoeman 2006). In addition to the nine *Rhinolophus* species studied by Jones (1992), the correlation between diet and peak frequency of the South African rhinolophids supports the first prediction of the AFH, namely that the proportion of moths incorporated into a bat's diet is greater in bats that use allotonic frequencies than in bats that do not (Schoeman 2006). However, the relationship between percentage moth in the diet and echolocation frequency does not necessarily support only the AFH because high frequencies may have evolved under other selective forces and then allowed an increase in moth consumption. If the hearing abilities of moths are driving the evolution of echolocation calls, evidence of this should be reflected in the bats' evolutionary history, i.e. allotonic frequencies should be a derived condition (the second prediction of the AFH, Chapter 1).

In this chapter I use a molecular phylogenetic approach to test the second prediction of the AFH, namely that high-frequency echolocation calls are more derived than low-frequency calls.

METHODS

EVOLUTION OF ECHOLOCATION

Echolocation data were recorded from South African rhinolophids captured at various sites and under conditions described in Chapter 2. For the South African rhinolophids, calls were recorded from hand-held bats because this eliminates any possible Doppler shift compensation (Heller & von Helversen 1989). A Pettersson D980 (Pettersson Electronik AB, Uppsala, Sweden) bat detector was used and the high-frequency output was recorded directly into a notebook computer via a DAQCard™ - 6062E high-speed soundcard (National Instruments, Austin Texas) with an anti-aliasing filter (F2000, Pettersson Electronik AB, Uppsala, Sweden). The recordings were analysed using BatSound Pro software (version 3.20; Pettersson Electronik AB, Uppsala, Sweden). In BatSound Pro, sampling was done at 500 kHz to avoid aliasing of calls, at a threshold of 16 from the FFT power spectrum, and at FFT sizes between 512 and 1024. A Hanning window was used because of the random occurrence of background noise. Sequences were selected in which the signal-to-noise ratio was high. I measured a minimum of ten calls per sequence. Peak frequency (the frequency of maximum intensity) was determined from the power spectrum.

Echolocation frequencies for rhinolophids from outside of South Africa were obtained from the literature. Bats echolocating at frequencies above 60 kHz and thus outside of the frequency range to which moth ears are most sensitive were considered to be using allotonic frequencies (after Fullard 1982). Although the sensitivity of most moth ears decreases sharply above 65 kHz (Fenton & Fullard 1979), some moths are still capable of detecting frequencies up to 110 kHz (Fullard 1982, 1988). The rhinolophids were therefore divided into the following three categories: 1) bats using frequencies ≥20 and ≤60 kHz; 2) bats using frequencies ≥61 and ≤110 kHz; 3) bats using frequencies ≥111 kHz. Although some species may echolocate at different frequencies in different geographical locations, I necessarily assume that there has been no change in echolocation between the

time of origin of a species and its present state. This assumption is necessary because the original state of echolocation within species is unknown.

As specimens from different localities within a species' distribution range reveal high sequence divergence values (Chapter 2), specimens of a given species that use different echolocation calls will be included. For example, *R. landeri* in South Africa is recorded as using frequencies of 107.3 kHz (this study), but specimens from Nigeria apparently use frequencies of 121 kHz (Roberts 1972). Where individuals from different geographic areas use different frequencies, the highest recorded frequency was used when mapping echolocation frequency onto the phylogeny.

MAPPING OF ECHOLOCATION CHARACTERS ONTO A PHYLOGENY

Taxon characteristics can be mapped onto trees to investigate patterns of evolution of these characters (Kirsch & Lapointe 1997; Eick *et al.* 2005). MacClade 4 version 4.07 (Maddison & Maddison 2005) was used to optimize the echolocation parameters onto the supermatrix Bayesian Inference topology recovered in Chapter 2 (Chapter 2, Fig. 2.5). Call parameters were mapped onto the molecular phylogeny using the 'trace character' option with both accelerated (ACCTRAN) and delayed (DELTRAN) transformations. The DELTRAN option delays changes away from the root and maximizes parallel changes. The ACCTRAN option maximizes early gains by accelerating changes towards the root, and can thereby force subsequent reversals (Maddison & Maddison 2005). ACCTRAN is the more conservative approach as it minimizes convergence and parallelisms in character states (Maddison & Maddison 2005). Parsimony was used to trace echolocation calls, coding echolocation call parameters as both ordered and unordered states. Only a few species in my phylogeny (Fig. 2.3, Chapter 2) use calls dominated by frequencies within the range where moth hearing is most sensitive. To obtain a broader view of the pattern of echolocation frequencies throughout the rhinolophids, I included all low-frequency (<60 kHz) and high-frequency species for which robust molecular data were available. This was done by constructing a rhinolophid topology based on a combination of my supermatrix tree with topologies where rhinolophid

relationships amongst terminal taxa were well supported (e.g. Cooper *et al.* 1998; Guillén *et al.* 2003; Li *et al.* 2006). These taxa were placed into the supermatrix topology based on their evolutionary relationships to those taxa included in the supermatrix topology. Thus taxa originally missing from the different subclades in the supermatrix topology were included in their prospective clades based on the evolutionary relationships recovered within these subclades (Cooper *et al.* 1998; Guillén *et al.* 2003; Li *et al.* 2006).

RESULTS

EVOLUTION OF ECHOLOCATION

The majority of rhinolophids use frequencies >60 kHz and <111 kHz (Table 3.1). The most parsimonious reconstruction of the evolution of call frequency on the supermatrix topology has a tree-length of 52 changes and a consistency index of 0.31 (Fig. 3.1). Echolocation data were not available for *R. maclaudi* and *R. monoceros*. Both the ACCTRAN and DELTRAN optimizations are shown. Ordered and unordered reconstruction did not differ substantially, and, as a result, ordered optimizations are shown. Although *R. maclaudi*'s frequency is not known, it is likely that only two changes to low frequency occurred among the African rhinolophids sampled (Fig. 3.1b). This is different to the ACCTRAN (Fig. 3.1a) optimization in which the ancestor of the *R. fumigatus-R. hildebrandti* clade evolved a low-frequency call, and *R. darlingi* subsequently evolved a high frequency.

Table 3.1 Peak echolocation frequencies (kHz) for *Rhinolophus* species, including location of where frequencies were recorded.

Species	Peak Frequency (kHz)	Location	Reference
R. acuminatus	91.6	Myanmar	Iain Mackie unpubl. data
R. affinis	73.8	Myanmar	Iain Mackie unpubl. data
R. alcyone	87	Uganda	Roberts 1972
R. arcuatus	66.5	Malaysia	Novick 1958
R. blasii	88.6	South Africa	This study
R.borneensis	92	Malaysia	Francis & Habersetzer 1998
R. capensis	83.9	South Africa	This study
R. clivosus	91.9	South Africa	This study
R. coelophyllus	77.5	Thailand	Robinson 1996
R. cornutus	108	China	Li *et al.* 2006
R. creaghi	68	Sabah	Francis & Habersetzer 1998
R. darlingi	87.9	South Africa	This study
R. denti	110.9	South Africa	This study
R. euryale	104	Europe	Heller & Von Helversen 1989
R. f. ferrumequinum	81	Europe	Heller & Von Helversen 1989
R. f. nippon	65.5	Japan	Taniguchi 1985
R. formosae	43.4	Taiwan	Chun-Chia Huang unpubl. data
R. fumigatus	53.8	South Africa	This study
R. hildebrandti	33.8	South Africa	This study
R. hipposideros	110	Europe	Heller & Von Helversen 1989
R. landeri	121	Nigeria	Roberts 1972
R. landeri	107.3	South Africa	This study
R. lepidus	100	Malaysia	Kingston *et al.* 2000
R. luctus	42	Malaysia	Roberts 1972
R. macrotis	57.5	Myanmar	Iain Mackie unpubl. data
R. malayanus	75.6	Myanmar	Iain Mackie unpubl. data
R. marshalli	39.3	Myanmar	Iain Mackie unpubl. data
R. megaphyllus	71	Australia	Fenton 1982
R. mehelyi	109	Europe	Heller & Von Helversen 1989
R. paradoxolophus	43.7	Guangxi, China	Zhao *et al.* 2003
R. pearsonii	57.5	Myanmar	Iain Mackie unpubl. data
R. philippinensis	36.6	Sabah	Francis & Habersetzer 1998
R. pusillus	116.4	Myanmar	Iain Mackie unpubl. data
R. rex	23.7	Guizhou, China	Zhao *et al*. 2003
R. rouxii	84	India	Schuller 1980
R. sedulus	67	Kuala Lompat	Kingston *et al*. 2000
R. shameli	75.1	Myanmar	Iain Mackie unpubl. data
R. simulator	80.6	South Africa	This study
R. sinicus	80-88.2	China	www.bio.bris.ac.uk/research/bats
R. stheno	93.2	Myanmar	Iain Mackie unpubl. data
R. subrufus	51	Philippines	Novick 1958
R. swinnyi	106.7	South Africa	This study
R. thomasi	86.9	Myanmar	Iain Mackie unpubl. data
R. trifoliatus	51.2	Sabah	Francis & Habersetzer 1998

The constructed rhinolophid topology (see methods) required 11 changes to optimize the echolocation calls and had a consistency index of 0.18 (Fig. 3.2). The frequencies of all bats included in this tree are known, except for *R. maclaudi* and *R. monoceros*. Once again the ACCTRAN (Fig. 3.2a) allows for reversal back to high frequencies among the African rhinolophids. Among the Eurasian-Oriental bats, the ancestor to the clade comprising *R. rex*, *R. paradoxolophus*, *R. philippinensis*, *R. marshalli* and *R. macrotis* was likely to have evolved a low frequency, with *R. malayanus* subsequently reverting back to a high frequency.

Few rhinolophids use frequencies above 110 kHz (e.g. *R. landeri* from Nigeria, 121 kHz) and these have evolved independently within both the Oriental and African rhinolophid clades. These very high frequencies may be derived as all species using these frequencies are close or on terminal branches (Fig. 3.1). Both topologies suggest that frequencies above 60 kHz are the basal condition, and that low frequencies (20-60 kHz) appear to be derived. Low frequencies have evolved independently in the two main rhinolophid lineages (African and Oriental clades) with one particular Oriental clade being characterized by low frequencies. Furthermore, sister taxa such as *R. philippinensis* (36.6 kHz; Francis & Habersetzer 1998) and *R. megaphyllus* (71 kHz; Fenton 1982), often have very different echolocation frequencies (Figs 3.1, 3.2).

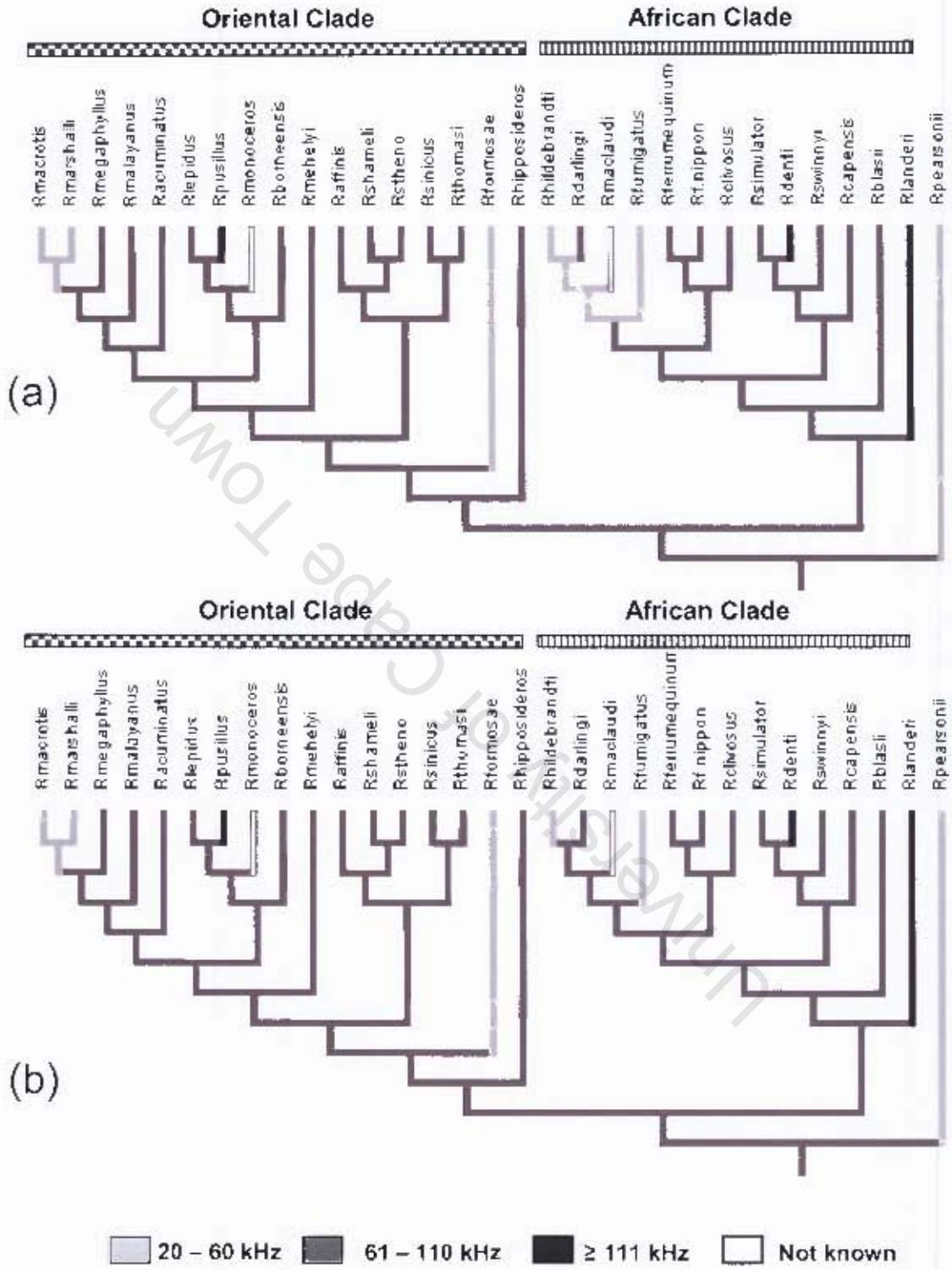


Fig. 3.1 Optimizations of echolocation call frequency onto the supermatrix topology. Three 'types' of call frequency were mapped based on moth hearing. Figure (a) represents characters mapped using ACCTRAN and Figure (b), DELTRAN.

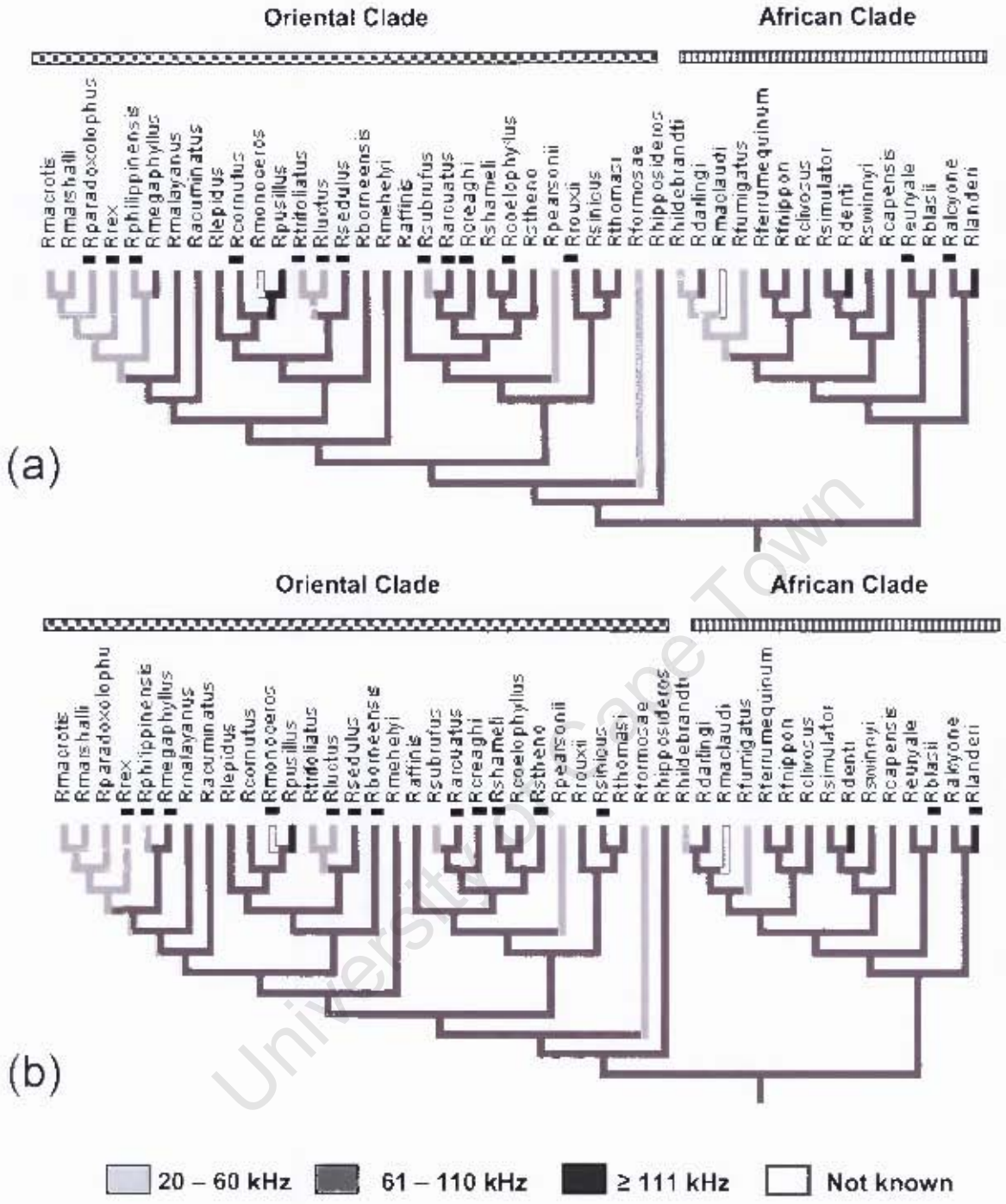


Fig. 3.2 Optimizations of echolocation call frequency onto a constructed topology, where ▬ indicates taxa 'placed' into the supermatrix topology. Three 'types' of call frequency were mapped based on moth hearing. Figure (a) represents characters mapped using ACCTRAN and Figure (b), DELTRAN

DISCUSSION

It is evident that the peak frequencies used by the majority of rhinolophid species fall outside the frequency range to which moth ears are most sensitive. Mapping of echolocation call frequency onto the phylogeny of the Rhinolophidae suggests that high frequencies are the ancestral condition. This is supported by the fact the sister family to the rhinolophids, the Hipposideridae, are also characterized by high frequencies (Heller & von Helversen 1989; Francis & Habersetzer 1998). It is therefore likely that the ancestor of these families was a bat using calls dominated by frequencies above 60 kHz. My results therefore do not support the second prediction of the Allotonic Frequency Hypothesis, namely that high-frequency echolocation should be derived, and low-frequency echolocation ancestral. Moth hearing cannot therefore explain the evolution of high frequencies in the Rhinolophidae. However, some moths can hear up to 110 kHz (Fullard 1982) which may suggest that the 'true' allotonic echolocators are those species using very high frequencies (>110 kHz). If this is the case, then species using frequencies above 110 kHz, and which are more derived, may provide support for the second prediction of the AFH.

If this is true, it suggests that the selection pressure that resulted in the high frequencies used by rhinolophids did not come from moth hearing. The correlation between bat peak frequencies and the proportion of moths in the diets of bats is therefore a secondary consequence of selection pressures from other sources. Although high frequencies evolved for reasons other than counteracting moth hearing, they have the added benefit of being allotonic, enabling high-frequency bats to exploit tympanate moths. Thus, high-frequency echolocation in the Rhinolophidae may be an exaptation, i.e. it evolved for reasons other than predation on moths (e.g. selective pressures imposed by living in cluttered habitats), but fulfils a secondary function in making these bats less audible to moths.

An increase in a bat's echolocation frequency has the potential cost of reduced detection distance for tympanate moths (Fullard 1987). For example, an increase in frequency from 30 kHz to 100 kHz reduces detection distance by 92% (Fullard 1987). Although some moths can hear up to 110 kHz (Fullard 1982), moth hearing sensitivity

nevertheless decreases with increasing frequency above 65 kHz, and as echolocation frequency increases to 110 kHz moths will have less time to react to an approaching bat. This seemingly explains the correlation between bat echolocation frequency and the proportion of moths in the diet, but does not require that high-frequency echolocation calls evolved to circumvent moth hearing. Instead, high frequencies selected in some other context functions secondarily in making bats less audible to moths. This could also explain deviations from the relationship between call frequency and the proportion of moths in a bat's diet. For example, *R. swinnyi* (106.9 kHz) does not incorporate more moths in its diet than *R. simulator* (80.6 kHz) or *R. blasii* (86.6 kHz) (Whitaker & Black 1976; Schoeman 2006). Similarly, some *Rhinolophus* species (e.g. *R. clivosus*, Schoeman 2006) using allotonic frequencies incorporate more beetles than moths in their diet, and others (e.g. *R. hildebrandti*, Schoeman 2006) which use syntonic calls consume large proportions of moths. The confounding factors here may be that the moths eaten by *R. hildebrandti*, for example, lack ears and the diets of bats are determined not only by the allotonic nature of their calls, but also by prey availability. However, among sympatric species that were sampled in the same place at the same time, and therefore had access to the same insect prey, some species (e.g. *R. hildebrandti* and *R. clivosus*, Schoeman 2006) deviate from the relationship whilst others do not.

In conclusion, phylogenetic analyses indicate that high-frequency echolocation in the Rhinolophidae is an ancestral, rather than derived condition. If this is the case, the current treatment of the divergence between Rhinolophid echolocation frequency and the auditory sensitivity spectrum of moths as reflecting reciprocal selection pressure (and thus being an ultimate evolutionary explanation) is wrong. Rather, this divergence appears to be a fortuitous, proximate exaptation driven extrinsically (i.e. by a different selection pressure) rather than intrinsically.

CHAPTER 4

ALTERNATIVE EXPLANATIONS FOR THE EVOLUTION OF HIGH FREQUENCIES IN THE RHINOLOPHIDAE: INFLUENCE OF BODY SIZE

"For there is a limit of size in all animals"

(Aristotle: De Generatione Animalium)

INTRODUCTION

Phylogenetic analyses suggest that moth hearing cannot explain the evolution of high-frequency echolocation calls in the Rhinolophidae (Chapter 3). Rather, the peak frequency of a bat's echolocation call may be the by-product of selective pressures acting on some other aspect of the bat's biology, for example, body size.

Body size, and in particular body mass, is integral in determining patterns associated with aspects of an organism's life history, ecology and evolution (Calder 1984). Many aspects of a species' life history can be scaled in the form of allometric relationships which describe life-history traits as power functions of body mass (Witting 1997). Furthermore, many physiological and biomechanical processes are dependent on an organism's body size (McNab 1983; Peters 1983; Schmidt-Nielsen 1983; Hayssen & Lacy 1985).

The body size of an animal is determined by the physical environment as well as by interspecific and intraspecific interactions (LaBarbera 1989). In bats, body size affects flight behaviour, roosting and reproductive behaviour, dietary selection, physiology (Swartz *et al.* 2004) and echolocation (Aldridge & Rautenbach 1987; Norberg & Rayner

1987; Barclay & Brigham 1991). Due to the physics of sound, the frequencies of certain objects (e.g. guitar strings or drum membranes) are inversely related to the linear dimensions of the object (Pye 1979). As linear size increases, lower frequencies are produced. Biological 'objects' are the same: higher frequencies are produced by smaller, narrower objects. Thus, small bats with a small vocal apparatus should be capable of producing higher frequency sounds than larger bats. Body size may therefore affect echolocation call frequency through the allometric scaling of organs involved in sound production.

BODY SIZE AND CALL FREQUENCY

The relationship between call frequency and body size has been demonstrated in vertebrates that utilize advertisement calls. Birds (Ryan & Brenowitz 1985) and frogs (Gerhardt 1994) show variation in call pitch associated with body size. Similarly, large toads, *Bufo* spp for example, have calls characterized by low frequencies relative to the calls of smaller species. Gerhardt (1994) suggests that because the dominant frequency in frog calls is correlated with body size in both intraspecific and interspecific studies, body size may constrain the evolution of call frequency.

Similarly, large bats (e.g. *Hipposideros commersoni,* Heller & von Helversen 1989) tend to emit longer calls with lower frequencies than smaller bats (Heller & von Helversen 1989; Barclay & Brigham 1991; Waters *et al.* 1995; Bogdanowicz *et al.* 1999; Jones 1999; Zhang *et al.* 2000). Echolocation call frequency scales negatively with body mass in at least five bat families - Rhinolophidae, Hipposideridae, Emballonuridae, Vespertilionidae, and Molossidae (Jones 1996).

The scaling of call frequency and body size is also evident in intraspecific studies. *Myotis adversus* individuals with long forearms produce lower frequency calls than do smaller conspecifics (Jones & Rayner 1991). Similarly, an inverse relationship between call frequency and size occurs in *H. larvatus* (Thabah *et al.* 2006). In *Rhinolophus philippinensis,* three distinct yet sympatric size morphs echolocate at different frequencies and call frequency scales negatively with body size. The largest morph uses the lowest harmonic of the fundamental frequency, and the smaller-sized morphs

use higher frequency harmonics of this fundamental frequency (Kingston & Rossiter 2004). In the hipposiderid *Asellia tridens*, females are smaller than males, and, as predicted by allometry, call at higher frequencies (Jones *et al.* 1993). By contrast, in *R. hipposideros*, where females are larger than males, females emit higher frequency calls than males (Jones *et al.* 1992). However, a recent study (Siemers *et al.* 2005) reported that call frequency was unrelated to either sex or forearm length in *R. hipposideros* (very small sample size), *R. euryale*, and *R. mehelyi* (also see Russo *et al.* 2007).

Given the apparent incidence of intraspecific exceptions to the allometric scaling of body size and call frequency, it is possible that the latter may not simply be the by-product of overall body size. If it was, all species should show the inverse relationship between sound frequency and size. More importantly, if the relationship between body size and peak frequency were to explain the high frequencies used by rhinolophid and hipposiderid bats, then these two families should on average, be smaller than other families of bats (see Jones 1996).

If echolocation frequency is influenced by the size of the sound-producing apparatus (rather than the size of the animal itself), then it could be predicted that morphological features directly involved in sound production and reception should scale more closely with frequency than does overall body size. The latter is a product of a variety of selection pressures, not solely those associated with sound production or audition, and may not therefore correlate strongly with peak frequency. Other morphological features (skulls, rostral chambers, noseleaves, ears etc.) associated directly with sound emission or reception may display stronger correlations with the frequencies of those sounds.

Horseshoe bats are nasal echolocators: their echolocation calls are emitted through their nostrils and not through their mouths. All nasal echolocators are characterized by having “noseleaves”, which are elaborate folds of skin, of varying shapes, situated in the nasal region. There is a significant negative correlation between call frequency and the breadth of the noseleaf (Bogdanowicz 1992).

Head size and the spacing of the ears are important for sound reception because they influence the temporal and frequency cues that are contained in the waveforms that reach the ears. Generally, larger animals, with larger heads, are more precise in the localization of sounds (Heffner & Heffner 1992). In bats, the shape and size of the pinnae and tragus play an important role in the directionality and sensitivity of detection of the returning echoes (Obrist *et al.* 1993). In two species of *Myotis* (Gannon *et al.* 2001), and in some Chinese rhinolophid and hipposiderid species (Zhao *et al.* 2003), larger pinnae are associated with lower frequencies and interspecific differences in call frequencies are correlated with pinnae shape and size (Zhao *et al.* 2003).

This relationship is not restricted to the outer ear. There is a strong negative relationship between call frequency and cochlea size in 15 rhinolophid species and 10 hipposiderid species, with wider cochleas being correlated with lower frequencies (Francis & Habersetzer 1998). In addition, echolocation frequency in two cryptic species of pipistrelle (formerly *Pipistrellus pipistrellus*) is correlated with skull morphology, whereby the larger skulled of the two cryptic taxa emitted the lower frequency calls and ate the larger prey (Barlow *et al.* 1997).

In this chapter I investigate the Allometry Hypothesis (Chapter 1), which proposes that high-frequency calls used by the rhinolophids are simply the result of allometric scaling. If so, high-frequency echolocation would simply be a by-product of other selective pressures on body size. I test this hypothesis by investigating the relationship between echolocation call frequency and morphology (specifically body size and morphological attributes that correlate with body size but are directly associated with sound production and audition). I also identify rhinolophid species that deviate from allometric predictions by having calls that are either higher or lower than predicted by body size. These species are used in subsequent chapters to investigate the reasons for their deviations from allometry.

In the past, morphological studies have made use of standard linear measurements, thereby obtaining a size variable or, as a residual, a shape variable. However, many of these studies investigate only size differences, often neglecting shape: this results in only a coarse resolution of morphological differences. For example, Gannon & Rácz

(2006) demonstrated that traditional linear morphometrics were less sensitive than geometric morphometrics in detecting character displacement between two *Myotis* species. Traditional morphometric analyses were unable to detect differences in jaw shape between males and females (*M. evotis* and *M. auriculus*), yet these differences were evident when using geometric morphometrics (Gannon & Rácz 2006). For these reasons, I adopt both traditional linear morphometrics and geometric morphometrics in the search for insight into interspecific morphological differences or similarities.

Studies investigating the inter- and intraspecific differences in call frequency are concerned with the changes in call frequency associated with the changes in size of various morphological features. The inverse relationship between call frequency and body size reflects a general trend, more than a hard-and-fast biological rule. Thus, the frequency of a bat's call may be influenced by shape rather than size. Because rhinolophids are nasal echolocators, the rostral area of the skull is directly related to the emission of the echolocation call. Furthermore, the cochlea, involved in sound reception, and the brain, involved in sound analysis, may influence the shape of a bat's skull. By analyzing the skull shape of the rhinolophid species occurring in South Africa, I will determine whether differences in skull shape are associated with echolocation frequency. If so, this would suggest that echolocation frequency might not simply be the result of body-size scaling but that selection has acted directly on echolocation frequency resulting in differences in the shapes of morphological features associated with sound production, reception and processing.

I focus on the South African rhinolophids in an attempt to understand better the relationship between body size, shape and echolocation frequency. The reasons for this choice are a) because skull data are available for South African rhinolophids, and b) I have comprehensive echolocation data for these species. I will address the following questions using both traditional and geometric morphometrics:

1) Is the relationship between echolocation frequency and body size present across families?
2) Is the echolocation frequency of the South African rhinolophids negatively correlated with body size, as observed in other bat species and families?
3) Which species deviate from the allometric relationship?

4) Is there an intraspecific relationship between echolocation frequency and body size in the South African rhinolophids?
5) Which linear skull parameters discriminate between South African rhinolophids, and are these correlated with echolocation frequency?
6) How does skull shape differ among South African rhinolophids, and are skull shapes associated with echolocation frequencies?
7) What is the best morphological predictor of echolocation frequency?

METHODS

BODY SIZE AND ECHOLOCATION CALL

Bat morphology and echolocation data

Capture methods and the study sites where bats were captured are detailed in Chapter 2. Forearm length (to the nearest 0.1 mm, measured using dial callipers) and body mass (measured, after ensuring that the gut was emptied, to the nearest 0.5 g, with a Pesola spring balance) of each individual bat was measured, and the sex of the bat recorded. Forearm length was used as the indicator of body size because mass can be highly variable depending on the time of day when the bats were caught, reproductive status (e.g. pregnancy) and whether or not the animal had been foraging. Where possible, ear length and noseleaf width (both to the nearest 0.1mm using dial callipers) were also measured. Ear length was measured in accordance with Skinner & Smithers (1990) and the width of the noseleaf was measured at the broadest part across the horseshoe. Only adult bats were used in analyses: sub-adults were identified by the presence of cartilaginous epiphyseal plates in their finger bones (Anthony 1988).

The methods used to obtain echolocation data for South African rhinolophids are described in Chapter 3. Specimens from different localities within a species' distribution range can reveal high molecular sequence divergence values (Chapter 2), and populations of species in different geographical areas sometimes use different echolocation calls. Where information for individuals from different geographical locations was available it was included in regression analyses. For example, *R. landeri* in South Africa echolocates at 107 kHz, but individuals from west Africa echolocate at

121 kHz. There was one exception to this: at two localities in South Africa, different peak frequencies for *R. hildebrandti* have been recorded (Pafuri: 44 kHz, n=1; and Sudwala: 33 kHz, n=14). Because only one bat using the higher frequency call was recorded, this individual was excluded from subsequent analyses.

Data on peak echolocation frequency, skull morphology and body size of rhinolophids that occur outside of South Africa were obtained from the literature (Novick 1958; Roberts 1972; Kingdon 1974; Novick 1977; Schuller 1980; Fenton & Bell 1981; Fenton 1982; Medway 1983; Smithers 1983; Strahan 1983; Taniguchi 1985; Aldridge & Rautenbach 1987; Norberg & Rayner 1987; Lekagul & McNeely 1988; Heller & Volleth 1989; Heller & von Helversen 1989; Barclay & Brigham 1991; Robinson 1996; Francis & Habersetzer 1998; Kingston *et al.* 2000; Borissenko & Kruskop 2003; Csorba *et al.* 2003; Kingston *et al.* 2003; Yasuma *et al.* 2003; Zhao *et al.* 2003; Matveev 2005).

Body size and peak echolocation frequency

If the relationship between body size and peak frequency explains the high frequencies used by rhinolophid and hipposiderid bats then these two families should, on average, be smaller than other families of bats. To investigate the relative body size of rhinolophids with respect to other bat families I used the body mass data in Norberg & Rayner (1987) and Barclay & Brigham (1991). To provide an accurate reflection of the size range within each family, I increased the sample size of the Rhinolophidae and Hipposideridae using data that included the smallest and largest species in these families (Kingston *et al.* 2000; this study). I also included more families than were used by Jones (1996) in a similar study, but unlike Jones (*op. cit.*) I did not include the non-echolocating Pteropodidae. The following families were used in the analyses: Emballonuridae, Hipposideridae, Megadermatidae, Molossidae, Mormoopidae, Noctilionidae, Nycteridae, Phyllostomidae, Rhinolophidae, Rhinopomatidae and Vespertilionidae. Body mass was $\log_{10}(x + 1)$ transformed to ensure normality and an analysis of variance (ANOVA) was used to determine whether the families differed significantly in body mass.

Peak echolocation frequency was regressed against body mass, forearm length, noseleaf width and ear length for all *Rhinolophus* species for which data were

available, including my data on the South African rhinolophids. All morphological parameters and peak frequencies were $\log_{10}(\log x + 1)$ transformed such that the units were comparable. All statistical analyses were performed using STATISTICA 7 (StatSoft 2004) unless otherwise stated. Descriptive statistics (mean, variance, standard deviation etc.) were computed for the South African rhinolophids.

Controlling for phylogeny

Closely related species may share characters due to common descent rather than independent evolution, thus variables for each species may not be statistically independent (Flesenstein 1985; Purvis & Rambaut 1995). It is therefore important to take phylogeny into account when investigating whether two variables are correlated. I used the Comparative Analysis by Independent Contrasts (CAIC version 2.0.0; Purvis & Rambaut 1995) software to control for phylogeny when analyzing relationships between call frequency and morphology. The phylogeny I used for the global rhinolophids was obtained from my phylogeny in Chapter 2 or from Guillén *et al.* (2003), Cooper *et al.* (1998) and Li *et al.* (2006) for species absent from my phylogeny. I used the Crunch algorithm in CAIC, because the variables I was comparing were continuous (Purvis & Rambaut 1995). Garland *et al.* (1992) have demonstrated that the use of different branch-length definitions can produce the same results. Because I lacked branch-length information for the species I used from Guillén *et al.* (2003), branch lengths for the global phylogeny were set as equal. When branch lengths are equal, a branch length is defined by the number of steps along higher branches as indicated by the cladistic analysis (Garland *et al.* 1992).

The method of independent contrasts results in fewer independent points than the number of species used, because nodes (pairs of related species) are compared (Felsenstein 1985). Relationships between contrasts were tested using least-square regressions on log-transformed values, through the origin (Harvey & Pagel 1991). Where regressions for the data in which I did and did not control for phylogeny were both significant I used the data in which I did not control for phylogeny to generate the regression slopes, allowing me to include all species.

Skulls for the ten South African species were obtained from the Northern Flagship Institution (Loan number B1214: a list of museum codes is provided in Appendix 1). In an exploratory analysis, three-dimensional coordinates for landmarks were taken using a Polhemus 3Space Digitizer. However, because of the small size of the rhinolophid skulls (<20 mm in length) certain landmarks could not be accurately replicated in repeated measures, with the result that measurement errors were greater than interspecific differences. To obviate this problem, digital images of the skulls were used (digital images have been used successfully in other geometric morphometric studies where the specimens were very small, Querino *et al.* 2002; Baylac *et al.* 2003). Because the images can be enlarged, it was easier to obtain reliable repeated measurements from them, as indicated by a test for measurement repeatability.

Digital photographs were taken of individuals for each of the South African *Rhinolophus* species using a Fuji Finepix S1PRO.E camera (Fuji Photo Film Co. Ltd, Tokyo, Japan) with 105 mm macro lens (AF Micro Nikkor 1:2:8) set to manual setting; white balance = custom; ASA=320; resolution = fine; number of pixels = 3040; F-stop = F45 with lens fully extended; dark field compensation = -2.0; exposure = 0.25 seconds at F32; set to aperture priority. The subject-to-lens distance was 420 mm for *R. capensis* (n=10), *R. clivosus* (n=10), *R. darlingi* (n=11), R. *fumigatus* (n=8), *and R. hildebrandti* (n=12) and 380 mm for *R. blasii* (n=8), *R. denti* (n=9), *R. landeri* (n=10), *R. simulator* (n=10), and *R. swinnyi* (n=5). Dorsal, ventral, and lateral views of the cranium and dorsal and lateral views of the mandible were taken. To standardize measurement, each picture was calibrated with a 10 mm section of measuring tape.

Traditional morphometrics

I investigated skull shape in South African rhinolophids, following traditional morphometric techniques, by measuring the distance between characteristic points (Thomas 1997; Csorba *et al.* 2003) on the digitized skull images. All images were calibrated using the 10 mm scale before measurements were taken. Distances and areas were calculated using the SigmaScan Pro software (SPSS Inc. 1999).

Measurements were taken from both the dorsal, lateral, and ventral views of the skull images.

The following skull parameters were measured (Fig. 4.1):

Dorsal view: (DSL) dorsal skull length – the greatest length from the occipital to where the saggital crest meets the rostral depression; (MW) mastoid width – the greatest distance across the mastoid region; (ZW) zygomatic width – the greatest distance across the zygoma; (IOW) interorbital width - the minimum width of the interorbital constriction; (ALSW) the greatest width of the anterior lateral swellings; (AMSW) width of the anterior median swellings; (DRW) rostral width – the outer width across the maxillae taken at the posterior edges of the upper canines; (AR) area of the rostrum including the rostral depression, anterior lateral swellings and anterior median swellings

Lateral view: (GSL) maximum skull length - from the posterior-most part of the occipital to the anterior-most point of the maxilla; (RH) rostral height – the maximum height between M^1 and the highest point of the bulbous nasals; (SH) skull height - taken perpendicular to a line placed at base of skull; (TB) tympanic bulla length - taken perpendicular from a line at the base of the tympanic bulla

Ventral view: (ASL) anterior skull length - the maximum length from the posterior-most part of the occipital to where the palatal meets the premaxilla; (PL) palatal length; (BW) bullar width - greatest width from the external auditory meatus to the inner margin of bulla, perpendicular to the longitudinal axis of the skull; (BOW) basioccipital width – the least distance between the inner margins of the bulla; (VRW) rostral width - measured between the outer crowns of M^3; (UTRL) upper tooth row length – the length from the anterior edge of the upper canine to the posterior border of M^3; (AVENT) the area of the auditory bulla.

I tested for measurement repeatability by selecting 10 skulls at random from across all species and measuring the dorsal, lateral and ventral parameters for each skull three times. In order to obviate bias being introduced by skull familiarity, no repeat measurements were made on any species' skull without at least one other species' skull having been measured in the interim. Variables in which the inter-individual variation in measurement was greater than the variation among repeated

measurements of the same individual were considered to be repeatable (Barlow *et al.* 1997). Non-repeatable variables were excluded from analyses because there is a risk they may increase background variance and hence obscure the correct biological interpretation of morphological analyses (Bailey & Byrnes 1990). Furthermore, skull parameters in which intraspecific variation was greater than interspecific variation were also excluded from analyses.

Skull morphology and peak echolocation frequency

Descriptive statistics were computed for the skull parameters of ten South African rhinolophids. ANOVA and Tukey tests were used to identify differences in skull parameters between bats using high- and low-frequency echolocation calls. Peak echolocation frequency was regressed against skull size. Multivariate analyses of variance (MANOVA), followed by post-hoc Tukey tests, were used to evaluate interspecific and sex-linked differences in skull parameters: species and sex were used as categorical predictors. Data were $\log_{10}(x+1)$ transformed to enhance normality. Levene's test for homoscedacity and the Komolgorov-Smirnov test for normality were used to ensure that data met the assumptions required for the analysis of variance (Zar 1999).

Multivariate comparisons of species

To identify the degree of variation between the different species, Principal Components Analysis (PCA) was performed on log-transformed skull parameters that were found to be repeatable. PCA highlights the combinations of morphological features underlying interspecific variation, and provides an indication of their relative importance. It is therefore useful for identifying which skull parameters contribute to the three-dimensional spacing between the South African rhinolophids. To preserve the original relationships among species in multivariate space, the components were calculated from the covariance matrix (e.g. Kingston *et al.* 2000).

To identify which body size or skull parameter is the best predictor of peak frequency in South African rhinolophids I used Generalized Linear Mixed Models (GLMMs) in Genstat 8.1 (8th Edition, Lawes Agricultural Trust, Rothamsted, 2005). GLMMs were used because they allow the inclusion of repeated measures as random terms.

Furthermore, variables that are closely associated with one another can be incorporated individually into the model using a forward elimination. This illustrates the effect of adding a new variable on the other variables already included in the model. For example, variable x and y are associated: variable x influences y, but variable y has no effect on x.

I ran two GLMMs. The first determined the best predictor of peak frequency at the level of the individual, and the second the best predictor of peak frequency at the species level. At the individual level, forearm length, mass, noseleaf width and ear length were included. Skull data could not be used because they were from different individuals (i.e. call frequency data were not obtained from specimens where skull measurements were made). To determine the best predictor of peak frequency at the species level, the mean peak frequency for each species of South African rhinolophid and the mean value for noseleaf width, ear length and each of the following skull parameters were used: area of rostral chamber (AR), dorsal rostral width (DRW), area of auditory bulla (AVENT), skull height (SH), rostral height (RH), mastoid width (MW) and dorsal skull length (DSL). Both models used forward elimination and made use of an identity link function with a normal distribution, and with peak frequency as the response variable.

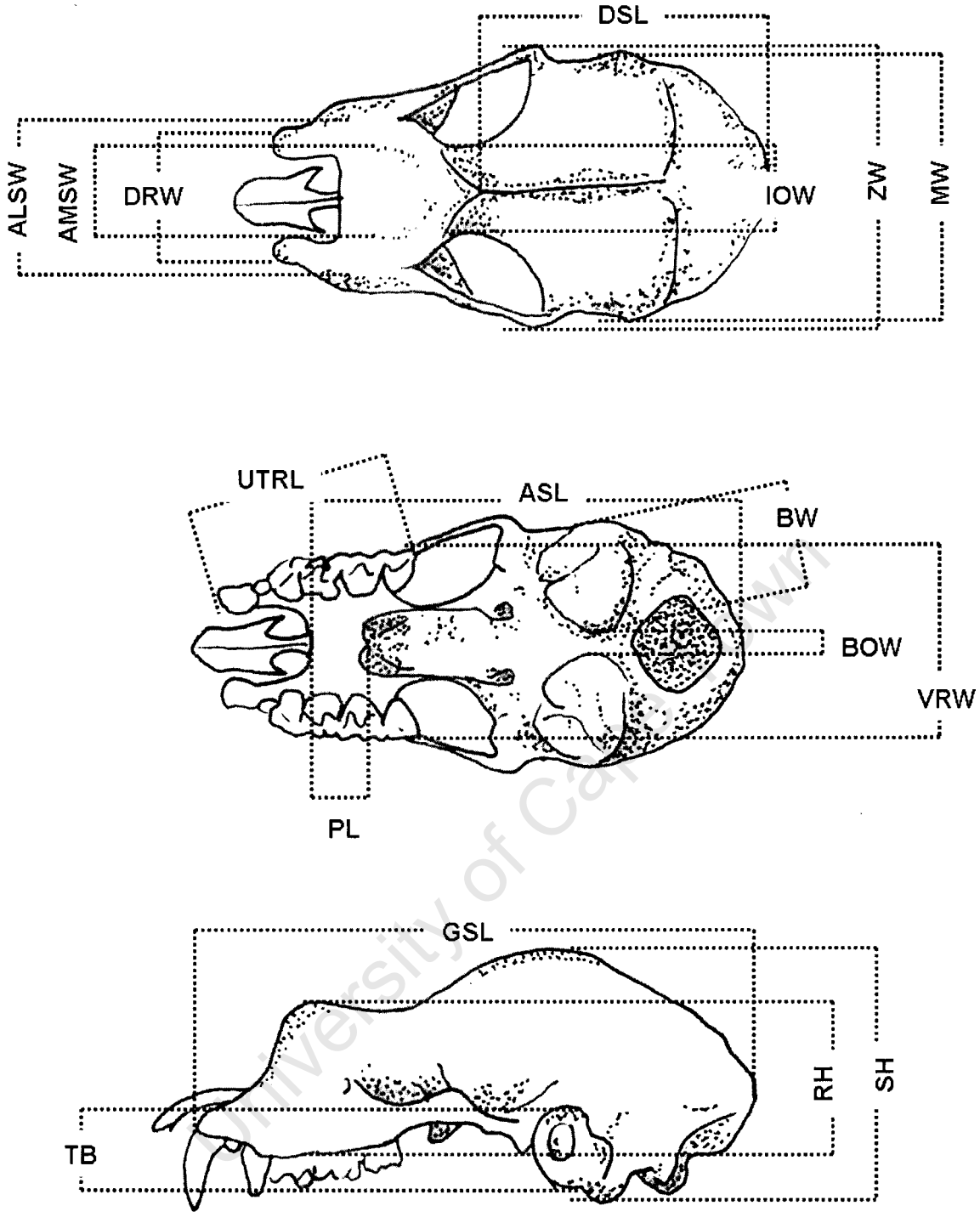


Fig. 4.1 A *Rhinolophus* skull showing the various linear measurements that were taken for traditional morphometric analyses. Variable legends together with a description of the variables measured are detailed above.

Geometric morphometrics

Geometric morphometrics were used to gain insight into skull shape differences between the 10 South African *Rhinolophus* species. The thin-plate spline digitizing analysis software (tpsDIG, Rohlf 2004) was used to set landmarks on digital images of the skulls. Landmarks were chosen according to the following criteria: marks/characters that can be clearly and reliably located on skulls for all individuals and species (such as suture marks), and positions that can be accurately obtained from repeated measures (Pavlinov 2001). Thus all landmarks on one skull corresponded to all landmarks on another skull.

I examined 95 specimens representing 10 species of South African rhinolophids - *R. blasii* (n=9), *R. capensis* (n=10), *R. clivosus* (n=10), *R. darlingi* (n=12), *R. denti* (n=9), R. *fumigatus* (n=8), *and R. hildebrandti* (n=12), *R. landeri* (n=10), *R. simulator* (n=10), and *R. swinnyi* (n=5). Fourteen landmarks (Fig. 4.2) were collected from the left half dorsal view of the skull of each specimen to avoid any effects of bilateral asymmetry (Bogdanowicz & Owen 1996).

The landmark configurations (the set of landmarks for each specimen) were aligned (i.e. rotated and scaled to match one another) using the generalized orthogonal least-squares (GLS) procedure in tpsSMALL (Rohlf 2003). In this analysis one configuration is fitted over another by minimizing the sum of squared distances between homologous landmarks (Rohlf & Slice 1990). The average configuration of landmarks resulting from this procedure is referred to as the reference configuration, and was used in subsequent analyses. The program tpsSMALL (Rohlf 2003) was also used to calculate the centroid size for each specimen. Centroid size is a measure of overall size and is calculated as the square root of the sum of squared distances between each landmark and the centre of the object (Slice *et al.* 1996). A multivariate analysis of variance (MANOVA), followed by post-hoc Tukey tests, was used to evaluate interspecific and sexual differences in centroid size.

tpsRELW (Rohlf 2005) was used to obtain partial warp scores. Partial warp scores were calculated using the reference configuration obtained in tpsSMALL. Partial warp scores are the displacement of each landmark from the reference configuration and

may be used as shape variables in multivariate analyses (Rohlf *et al.* 1996; Monteiro 1999).The scaling option used was α=0. This weights all the landmarks equally and is considered appropriate for exploratory studies and studies involving different species (Bookstein 1996). tpsRELW was also used to compute the weight matrix of the landmark data. The weight matrix is the matrix of partial warps scores together with the uniform shape component for a sample of shapes (Slice *et al.* 1996).

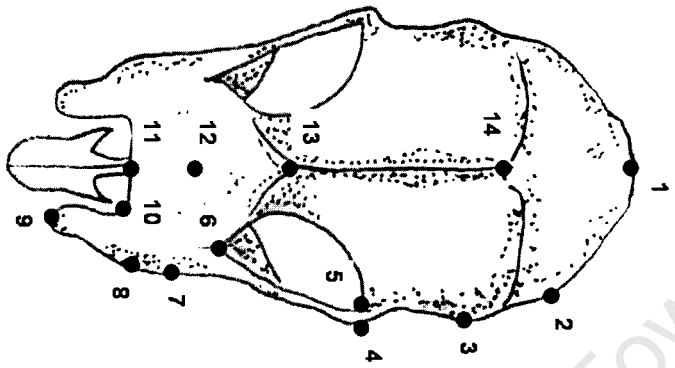


Fig. 4.2 The location of the fourteen landmarks used in the geometric morphometric analyses.

The weight matrix calculated using tpsRELW was saved as an .NTS file to be used in the Canonical Variates Analysis (CVA). CVA was used to investigate the level of variation between species, relative to the average variation found within species. These analyses made use of the aligned landmark configurations (from GLS analysis). NTSYSpc (version 2.1, Applied Biostatistics Inc.) was used to calculate the means and the average within-group covariance matrix. The weight matrix computed using tpsRELW was used as the input file to compute the group means, group size, and the pooled within-group covariance matrix. These files were then used as the input files in the CVA to compute canonical scores and vectors. The first and second canonical axes were used together with the original landmark configurations (skull shapes) as the input files in tpsREG (Rohlf *et al.* 1996; Cavalcanti *et al.* 1999). This program enables the shape changes associated with the canonical variate axes to be visualized as deformations from the mean shape by making use of thin-plate splines.

RESULTS

BODY SIZE AND ECHOLOCATION CALL

The 11 bat families analysed differed significantly in body mass (ANOVA, $F_{(10,213)}$=10.17, p<0.001). Tukey tests revealed that the Rhinolophidae had a significantly smaller body mass than the Molossidae (p<0.003), Phyllostomidae (p<0,001) and the Megadermatidae (p<0.005). The Megadermatidae, Molossidae, Noctilionidae, and Phyllostomidae had the largest mean body sizes (Fig. 4.3). Despite the Rhinolophidae having a much higher mean peak call frequency (77.1 kHz) than the Vespertilionidae (49.2 kHz) they are not, on average, smaller (Tukey HSD test, p=0.98; Fig. 4.3). Furthermore, the Rhinolophidae and Hipposideridae, which had the highest mean echolocation frequencies, did not have the smallest mean body mass (Fig. 4.3). Similarly, the Phyllostomidae (74.2 kHz), which echolocate at higher mean peak call frequencies than the Vespertilionidae (49.2 kHz), were significantly larger (Tukey HSD test, p<0.0001). Furthermore the mass ranges of the Rhinolophidae (3.5-29 g) and the Hipposideridae (3.8-89 g) overlap considerably with that of other families that use lower echolocation frequencies, e.g. Vespertilionidae (1.5-34.5 g) and Molossidae (5.8-89.2 g).

On a global basis, body mass of *Rhinolophus* spp was inversely related to echolocation frequency (r= -0.493, $F_{(1,37)}$=11.88 p<0.0014; Fig. 4.4), as was forearm length (r= -0.6493, $F_{(1,37)}$=26.965, p<0.0001; Fig. 4.5, Table 4.1). Both relationships remained significant after controlling for phylogeny (CAIC: forearm length: r=0.782, p<0.001; mass: r=0.656, p<0.002). Many species which deviated from the allometric relationship between frequency and body mass (e.g. *R. clivosus, R. fumigatus, R. ferrumequinum R. macrotis*, and *R. paradoxolophus*) also deviated from the allometric relationship between frequency and forearm length (Fig. 4.4 & 4.5). Because mass can vary greatly, e.g. with season, pregnancy and time since foraging, using mass data from the literature where collection descriptions are not provided can result in incomparable data. Consequently, forearm length may be more consistently comparable between species than is mass, and the former was the body-size measure used in subsequent analyses.

Table 4.1 Forearm length, mass, ear length, noseleaf width and peak echolocation frequency for rhinolophids from localities other than South Africa. For forearm length, mass, ear length and noseleaf width, the values used were calculated as the average between the minimum and maximum values provided in the literature. Data were obtained from: Novick 1958; Roberts 1972; Kingdon 1974; Novick 1977; Schuller 1980; Fenton & Bell 1981; Fenton 1982; Medway 1983; Smithers 1983; Strahan 1983; Taniguchi 1985; Aldridge & Rautenbach 1987; Norberg & Rayner 1987; Lekagul & McNeely 1988; Heller & Volleth 1989; Heller & von Helversen 1989; Barclay & Brigham 1991; Robinson 1996; Francis & Habersetzer 1998; Kingston *et al.* 2000; Borissenko & Kruskop 2003; Csorba *et al.* 2003; Kingston *et al.* 2003; Yasuma *et al.* 2003; Zhao *et al.* 2003; Matveev 2005; Li *et al.* 2006. ~ = no data.

SPECIES	Forearm length (mm)	Mass (g)	Ear length (mm)	Noseleaf width (mm)	Peak frequency (kHz)
R. acuminatus	48	9.9	19.1	9	91.6
R. affinis	50	13.8	19.5	9.9	73.8
R. alcyone	55	~	22.3	9.65	87
R. arcuatus	45	10.0	19.6	8.85	66.5
R. blasii	47	~	19.7	7.8	94
R. borneensis	46	9.2	17.75	8.7	92
R. coelophyllus	44.4	~	19.75	9.75	77.5
R. cornutus	36.9	~	17.5	6.45	108
R. creaghi	50	12	21.9	10.6	68
R. denti	42	~	20	~	110
R. euryale	47.4	10.9	21.4	6.05	104
R. ferrumequinum	56	22.6	23.25	8.2	81
R. f.nippon	61.1	~	25	~	65.5
R. fumigatus	55	12.9	24	9.6	47
R. hildebrandti	65	24.3	33	13.5	33.8
R. hipposideros	40	6.8	15.5	6.15	110
R. landeri	44	~	18	6.9	121
R. l. lobatus	45.3	9.0	17	~	80
R. lepidus	39.5	6.6	16.8	7	98
R. luctus	73.5	26.3	38.5	17.25	42
R. macrotis	46.5	7.5	22.25	8.4	48
R. malayanus	40.9	6.7	17.5	8.45	75
R. megaphyllus	47	9.8	21	8.75	71
R. mehelyi	50.3	16.5	20.5	5.8	109
R. paradoxolophus	52.9	12	32.9	12.5	43.7
R. parcus	37	~	16	~	107
R. pearsonii	50	15.3	26.2	11.9	56
R. philippinensis	51.5	10	30.4	11.8	36.6
R. pusillus	38.3	5.0	16.55	7	116
R. refulgens	39.8	6.3	15.5	7	98
R. rex	55.4	~	32.05	11.1	23.7
R. robinsoni	44	8.7	18	~	67
R. rouxii	48	~	18.25	8.1	84
R. sedulus	40.3	7.7	22.5	10.3	66.8
R. shameli	46.2	10	19	9	76
R. simulator	43	~	19.5	~	78
R. stheno	48.8	8.9	18.25	8.8	93.2
R. subrufus	55	~	24.2	12.55	51
R. swinnyi	40.9	7.0	16.8	~	107
R. thomasi	44.6	~	17.7	8.05	80
R. trifoliatus	53	14.7	24.5	11.45	51.2

Table 4.2 Mean ± SD of morphological and echolocation parameters for the South African rhinolophids. Ranges are given in parentheses.

		Body mass (g)	Forearm length (mm)	Noseleaf width (mm)		Ear length (mm)		Peak frequency (kHz)	
R. blasii	n=2	10.0 ± 1.0 (9.0 - 10.0)	46.7 ± 1.5 (45.6 - 47.8)	~	~	n=2	17.6 ± 0.8 (17.0 - 18.2)	n=2	86.6 ± 0.7 (86.1 - 87.1)
R. capensis	n=59	11.5 ± 1.2 (9.5 -16.0)	49.4 ± 1.02 (47.0 - 51.9)	n=37	8.4 ± 0.4 (7.8 - 9.4)	n=6	20.6 ± 1.2 (18.2 - 21.5)	n=40	83.9 ± 0.8 (81.9 - 85.5)
R. clivosus	n=58	18.4 ± 1.97 (15.0 - 23.0)	54.2 ± 2.1 (47.0 - 57.4)	n=44	7.7 ± 0.4 (7.0 - 8.5)	n=29	19.97 ± 1.4 (17.7 - 21.8)	n=55	91.9 ± 0.8 (89.6 - 93.2)
R. darlingi	n=18	9.2 ± 1.6 (7.5 - 13.0)	47.1 ± 1.3 (43.7 - 49.9)	n=10	7.96 ± 0.4 (7.4 - 8.5)	n=12	19.3 ± 1.2 (17.3 - 20.0)	n=18	87.9 ± 2.1 (83.7 - 91.0)
R. denti	n=14	5.9 ± 0.4 (5.0 - 6.5)	43.4 ± 0.8 (42.1 - 45.0)	n=14	6.8 ± 0.1 (6.5 - 7.0)	n=14	18.4 ± 0.4 (18.0 - 19.0)	n=14	110.9 ± 1.7 (108.2 - 115.4)
R. fumigatus	n=2	12.3 ± 0.4 (12.0 - 12.5)	52.3 ± 2.1 (50.8 - 53.7)	~	~	~	~	n=2	53.8 ± 0.2 (53.6 - 53.9)
R. hildebrandti	n=16	29.0 ± 2.3 (25.0 - 33.0)	66.8 ± 1.1 (65.4 - 68.9)	n=10	15.8 ± 0.3 (15.1 - 16.2)	n=14	34.9 ± 3.1 (30.0 - 43.8)	n=15	33.8 ± 3.2 (31.4 - 44.8)
R. landeri	n=2	7.5 ± 0.7 (7.0 - 8.0)	44.1 ± 0.6 (43.7 - 44.5)	~	~	~	~	n=2	107.3 ± 2.1 (105.8 - 108.7)
R. simulator	n=13	7.8 ± 1.5 (6.0 - 10.5)	45.0 ± 1.1 (42.8 - 46.5)	n=5	7.9 ± 0.5 (7.0 - 8.2)	n=6	19.9 ± 1.5 (17.9 - 22.0)	n=13	80.6 ± 0.96 (78.3 - 81.9)
R. swinnyi	n=24	7.8 ± 0.5 (7.0 - 9.0)	43.8 ± 0.9 (42.3 - 45.9)	n=24	6.7 ± 0.2 (6.4 - 7.5)	n=22	17.5 ± 0.7 (16.3 - 18.8)	n=24	106. 98 ± 0.8 (105.1 - 108.2)

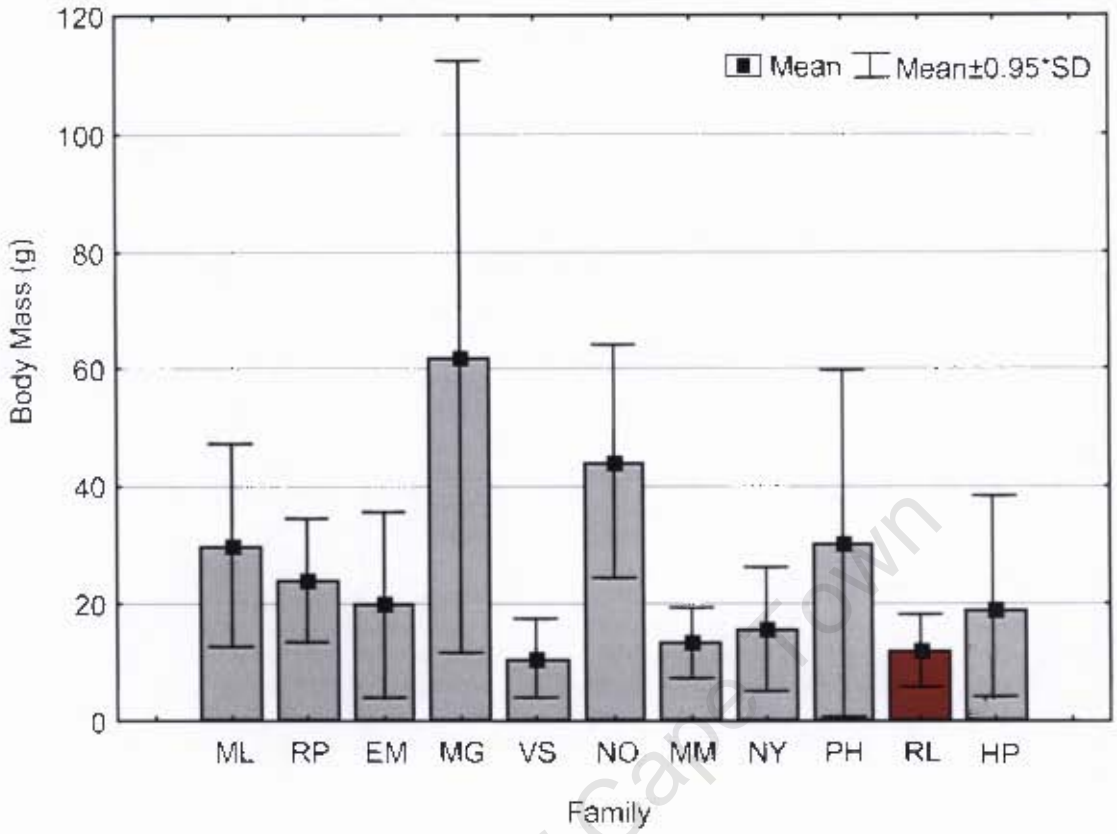


Fig. 4.3 A comparison of the mean body sizes for different families of echolocating bats. The Rhinolophidae are highlighted in red. The families are: Molossidae (ML, n=23); Emballonuridae (EM, n=12); Vespertilionidae (VS, n=91); Phyllostomidae; (PH, n=34); Rhinolophidae (RL, n=36); Hipposideridae (HP, n=12); Noctilionidae (NO, n=2); Megadermatidae (MG, n=3); Mormoopidae (MM, n=5); Nycteridae (NY, n=4); and Rhinomopatidae (RP, n=2). Bat families are arranged from left to right in order of increasing mean echolocation frequency. Echolocation data for species in each family were obtained from the literature. The mean peak echolocation frequencies for each family were: Molossidae 25 kHz (n=15); Rhinopomatidae 35 kHz (n=2); Emballonuridae 39.7 kHz (n=12); Megadermatidae 42.8 kHz (n=2); Vespertilionidae 49.2 kHz (n=47); Noctilionidae 65 kHz (n=2); Mormoopidae 71.4 kHz (n=7); Nycteridae 73.5k Hz (n=4); Phyllostomidae 74.2 kHz (n=26); Rhinolophidae 77.1 kHz (n=33); and Hipposideridae 121.2 kHz (n=20).

There was a negative relationship between forearm length and echolocation frequency of South African rhinolophids ($r= -0.8842$, $F_{(1,8)}=28.673$, $p<0.001$; Fig. 4.6, Table 4.2). This relationship remained significant after controlling for phylogeny (CAIC: $r=0.897$, $p<0.01$).

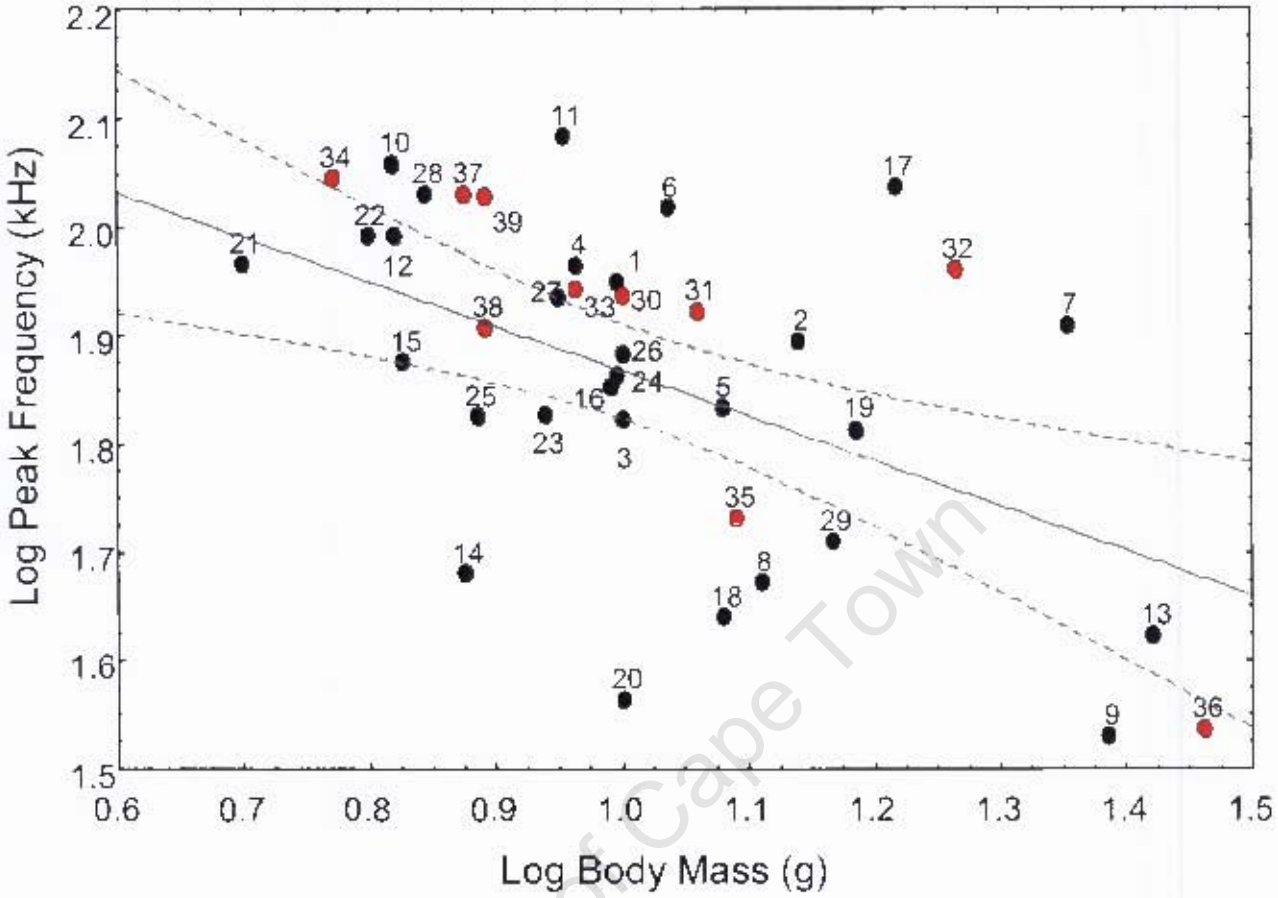


Fig. 4.4 Regression of the log of peak frequency against the log of body mass for *Rhinolophus* species around the world. Except for South African rhinolophids (in red), data were obtained from the literature. The solid line represents the line of best fit, where Log Peak Frequency = 2.2809 – 0.4138*Log Body Mass. Dashed lines represent the 95% confidence intervals. The species are: *R. acuminatus* (1); *R. affinis* (2); *R. arcuatus* (3); *R. borneensis* (4); *R. creaghi* (5); *R. euryale* (6); *R. ferrumequinum* (7); *R. fumigatus* (8); *R. hildebrandti* (9); *R. hipposideros* (10); *R. l. lobatus* (11); *R. lepidus* (12); *R. luctus* (13); *R. macrotis* (14); *R. malayanus* (15); *R. megaphyllus* (16); *R. mehelyi* (17); *R. paradoxolophus* (18); *R. pearsonii* (19); *R. philippinensis* (20); *R. pusillus* (21); *R. refulgens* (22); *R. robinsoni* (23); *R. rouxii* (24); *R. sedulus* (25); *R. shameli* (26); *R. stheno* (27); *R. swinnyi* (28); *R. trifoliatus* (29); *R. blasii* SA (30); *R. capensis* SA (31); *R. clivosus* SA (32); *R. darlingi* SA (33); *R. denti* SA (34); *R. fumigatus* SA (35); *R. hildebrandti* SA (36); *R. landeri* SA (37); *R. simulator* SA (38); and *R. swinnyi* SA (39).

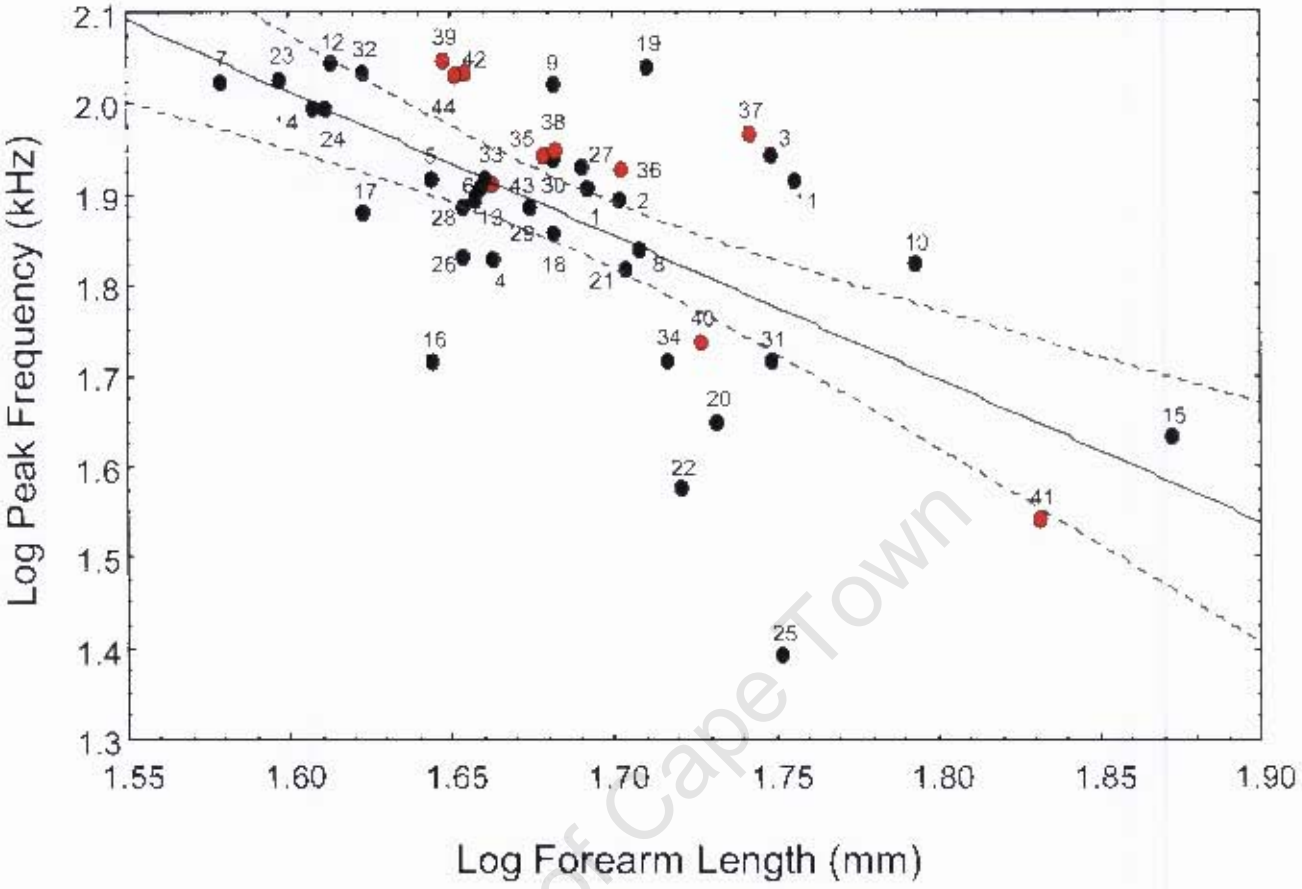


Fig. 4.5 Regression of the log of peak frequency against the log of forearm length for *Rhinolophus* species around the world. Except for South African rhinolophids (in red), data were obtained from the literature. The solid line represents the line of best fit, where Log Peak Frequency = 4.5441 – 1.5822*Log Forearm Length. Dashed lines represent the 95% confidence intervals. The species are: *R. acuminatus* (1); *R. affinis* (2); *R. alcyone* (3); *R. arcuatus* (4); *R. borneensis* (5); *R. coelophyllus* (6); *R. cornutus* (7); *R. creaghi* (8); *R. euryale* (9); *R. f. nippon* (10); *R. ferrumequinum* (11); *R. hipposideros* (12); *R. l. lobatus* (13); *R. lepidus* (14); *R. luctus* (15); *R. macrotis* (16); *R. malayanus* (17); *R. megaphyllus* (18); *R. mehelyi* (19); *R. paradoxolophus* (20); *R. pearsonii* (21); *R. philippinensis* (22); *R. pusillus* (23); *R. refulgens* (24); *R. rex* (25); *R. robinsoni* (26); *R. rouxii* (27); *R. sedulus* (28); *R. shameli* (29); *R. stheno* (30); *R. subrufus* (31); *R. swinnyi* (32); *R. thomasi* (33); *R. trifoliatus* (34); *R. blasii* SA (35); *R. capensis* SA (36); *R. clivosus* SA (37); *R. darlingi* SA (38); *R. denti* SA (39); *R. fumigatus* SA (40); *R. hildebrandti* SA (41); *R. landeri* SA (42); *R. simulator* SA (43); and *R. swinnyi* SA (44).

Intraspecific analyses of South African rhinolophids were performed on seven of the ten species: the sample sizes for *R. blasii, R. landeri,* and *R. fumigatus* were too small. In six out of seven species of South African rhinolophid, no relationship existed between individual forearm length and individual echolocation frequency (*R. capensis* r= -0.0702, $F_{(1,41)}$=0.203, p>0.5; *R. darlingi* r=0.2789, $F_{(1,12)}$=1.013,, p>0.1; *R. denti* r= -0.2851, $F_{(1,12)}$=1.062, p>0.1; *R. hildebrandti* r= -0.2449, $F_{(1,12)}$=0.7658, p>0.1; *R. simulator* r= -0.5254, $F_{(1,4)}$=1.526, p>0.1; *R. swinnyi* r= -0.0789, $F_{(1,22)}$=0.138, p>0.5). A significant relationship between echolocation and call frequency existed only for *R. clivosus* (r= -0.3043, $F_{(1,44)}$=4.489, p<0.05).

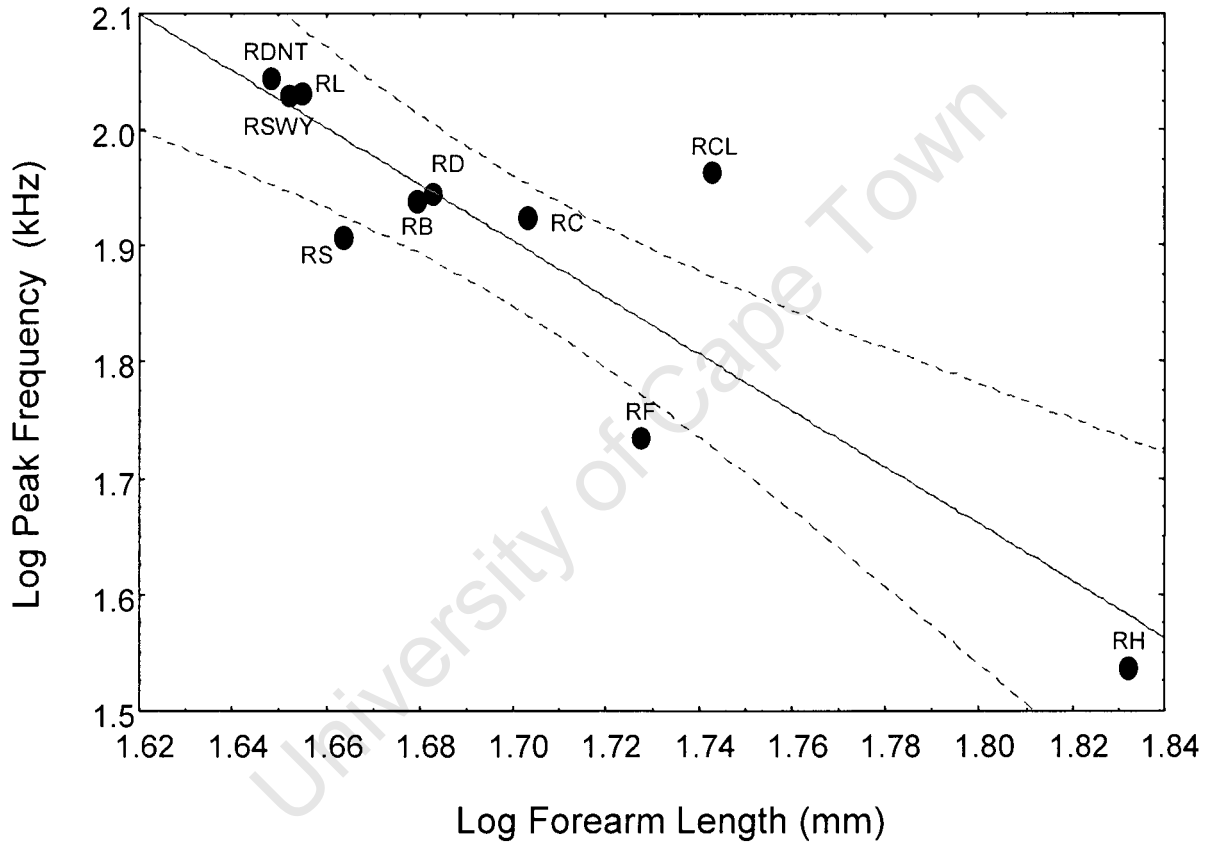


Fig. 4.6 Regression of the log of forearm length and the log of peak echolocation frequency for the South African *Rhinolophus* species: *R. blasii* (RB), *R. capensis* (RC), *R. clivosus* (RCL), *R. darlingi* (RD), *R. denti* (RDNT), *R. fumigatus* (RF), *R. hildebrandti* (RH), *R. landeri* (RL), *R. simulator* (RS), and *R. swinnyi* (RSWY). The solid line represents the best fit, where Log Peak Frequency = 6.0537 – 2.4407*Log Forearm Length. Dashed lines represent the 95% confidence intervals.

Noseleaf width was inversely related to peak frequency (r=-0.8276, $F_{(1,39)}$=84.774, p<0.001; Fig. 4.7) and positively related with echolocation wavelength (r=0.8166, $F_{(1,39)}$=78.0485, p<0.001. Similarly, a negative relationship existed between ear length and peak echolocation frequency (r= -0.8417, $F_{(1,49)}$=119.08, p<0.001; Fig. 4.8). These relationships remained significant after controlling for phylogeny (CAIC, r=0.81, 0.78, 0.81 respectively, all p<0.01).

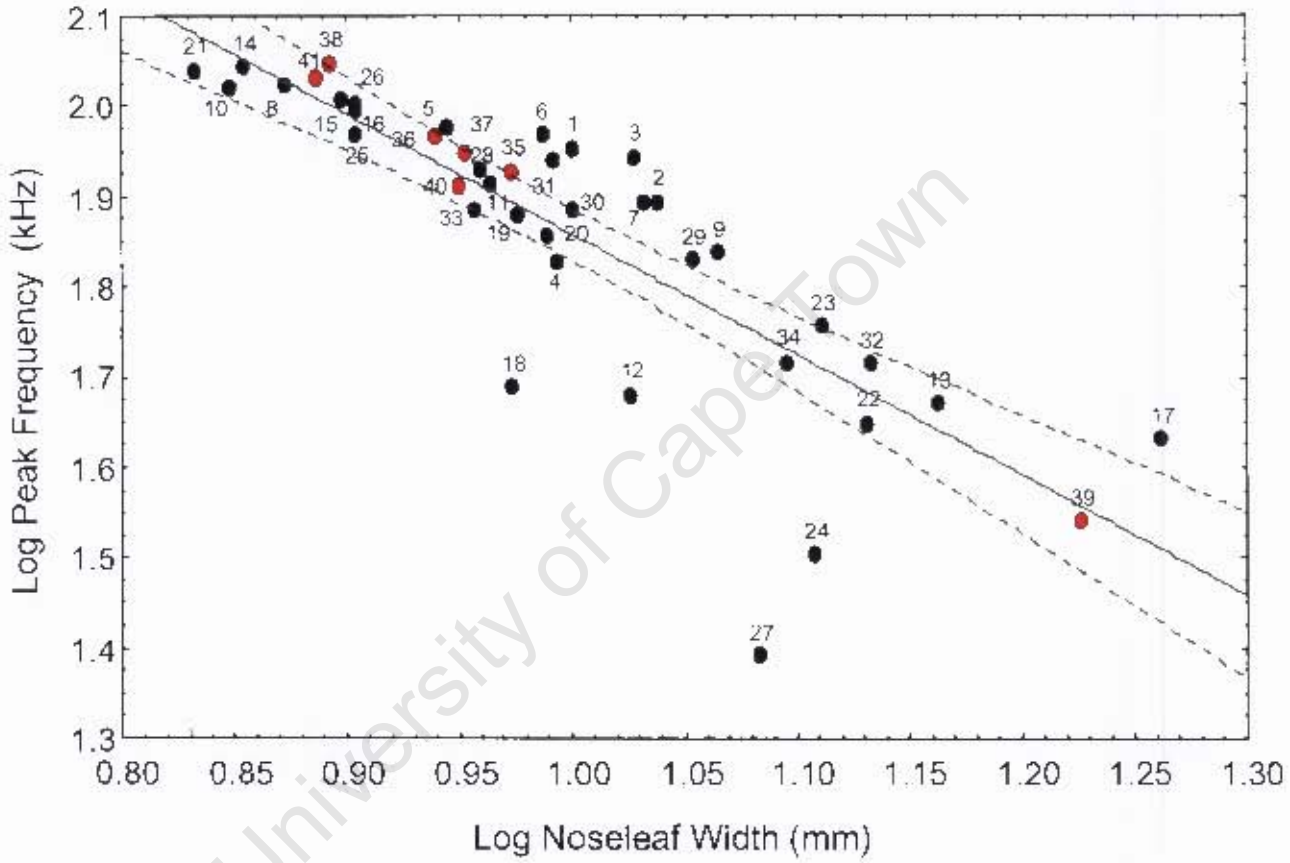


Fig. 4.7 The regression of log noseleaf width and log peak frequency for rhinolophids from around the world. Except for the South African rhinolophids (in red), data were obtained from the literature. The solid line represents the line of best fit, where Log Peak Frequency = 3.1905 – 1.3323*Log Noseleaf Width. Dashed lines represent the 95% confidence intervals. The species are: *R. acuminatus* (1); *R. affinis* (2); *R. alcyone* (3); *R. arcuatus* (4); *R. blasii* (5); *R. borneensis* (6); *R. coelophyllus* (7); *R. cornutus* (8); *R. creaghi* (9); *R. euryale* (10); *R. ferrumequinum* (11); *R. fumigatus* (12); *R. hildebrandti* (13); *R. hipposideros* (14); *R. landeri* (15); *R. lepidus* (16); *R. luctus* (17); *R. macrotis* (18); *R. malayanus* (19); *R. megaphyllus* (20); *R. mehelyi* (21); *R. paradoxolophus* (22); *R. pearsonii* (23); *R. philippinensis* (24); *R. pusillus* (25); *R. refulgens* (26); *R. rex* (27); *R. rouxii* (28); *R. sedulus* (29); *R. shameli* (30); *R. stheno* (31); *R. subrufus* (32); *R. thomasi* (33); *R. trifoliatus* (34); *R. capensis* SA (35); *R. clivosus* SA (36); *R. darlingi* SA (37); *R. denti* SA (38); *R. hildebrandti* SA (39); *R. simulator* SA (40) and *R. swinnyi* SA (41).

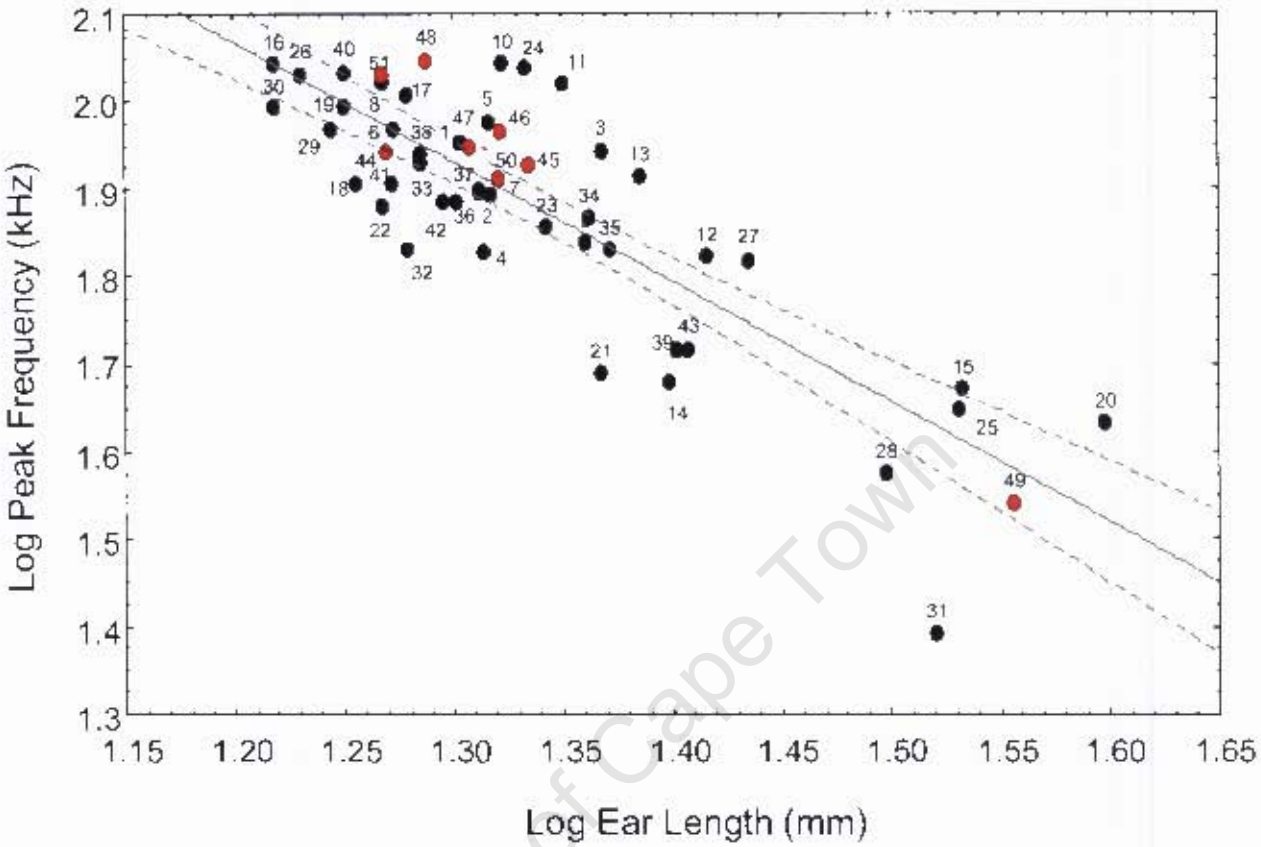


Fig. 4.8 The regression of log ear length against log peak frequency for rhinolophids from around the world. Except for the South African rhinolophids (in red), data were obtained from the literature. The solid line represents the line of best fit, where Log Peak Frequency = 3.7166 – 1.3733*Log Ear Length. Dashed lines represent the 95% confidence intervals. The species are: *R. acuminatus* (1); *R. affinis* (2); *R. alcyone* (3); *R. arcuatus* (4); *R. blasii* (5);*R. borneensis* (6); *R. coelophyllus* (7); *R. cornutus* (8); *R. creaghi* (9); *R. denti* (10); *R. euryale* (11); *R. nippon* (12); *R. ferrumequinum* (13); *R. fumigatus* (14); *R. hildebrandti* (15); *R. hipposideros* (16); *R. landeri* (17); *R. l. lobatus* (18); *R. lepidus* (19); *R. luctus* (20); *R. macrotis* (21); *R. malayanus* (22); *R. megaphyllus* (23); *R. mehelyi* (24); *R. paradoxolophus* (25); *R. parcus* (26); *R. pearsonii* (27); *R. philippinensis* (28); *R. pusillus* (29); *R. refulgens* (30); *R. rex* (31); *R. robinsoni* (32); *R. rouxii* (33); *R. rouxii* (34); *R. sedulus* (35); *R. shameli* (36); *R. simulator* (37); *R. stheno* (38); *R. subrufus* (39); *R. swinnyi* (40); *R. thomasi* (41); *R. thomasi* (42); *R. trifoliatus* (43); *R. blasii* SA (44); *R. capensis* SA (45); *R. clivosus* SA (46); *R. darlingi* SA (47); *R. denti* SA (48); *R. hildebrandti* SA (49); *R. simulator* (50); and *R. swinnyi* SA (51).

Skull morphology and echolocation

Skull length was negatively correlated with echolocation frequency (r= -0.6051, $F_{(1,37)}$=21.374, p<0.0001; Fig. 4.9). The relationship remained significant after controlling for phylogeny (CAIC: r=0.965, p<0.05).

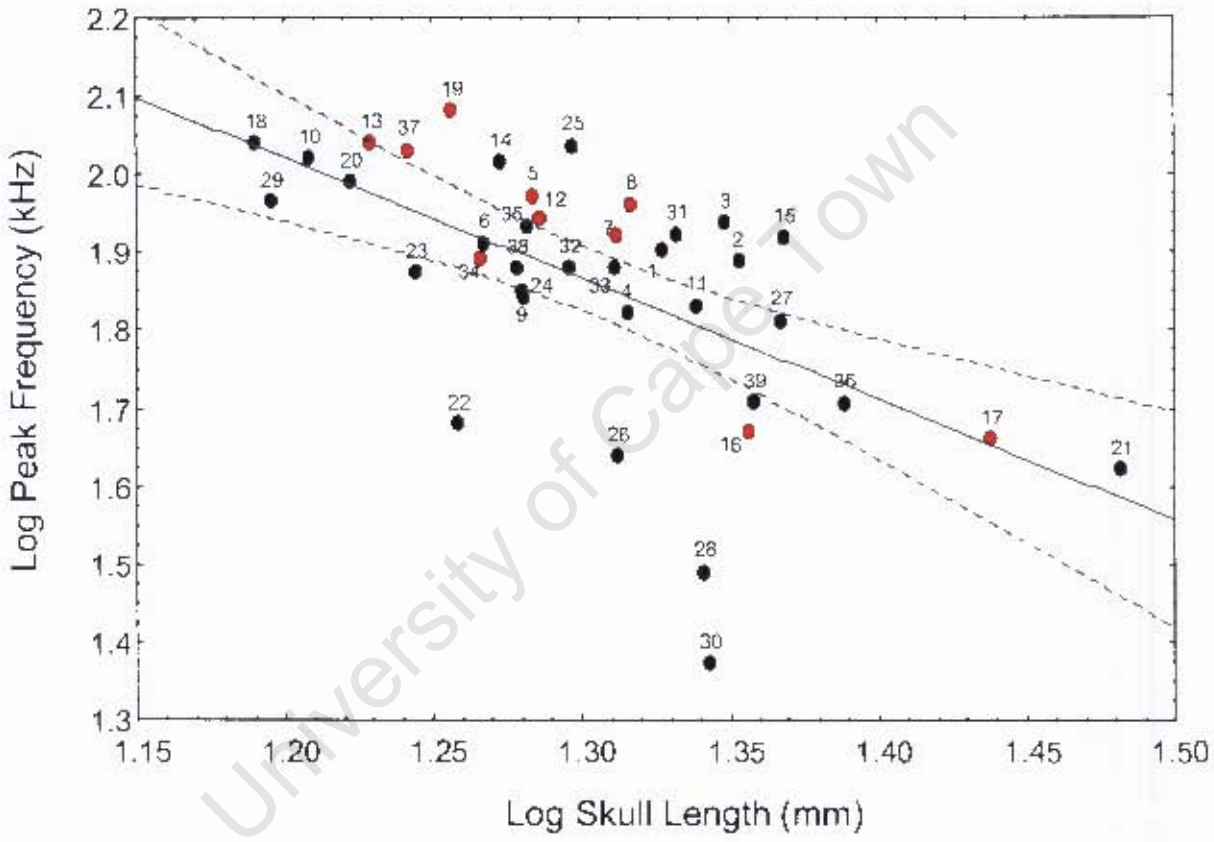


Fig. 4.9 Regression of the log of skull length against the log of peak frequency for *Rhinolophus* species. Except for South African rhinolophids (in red), data were obtained from the literature. The solid line represents the line of best fit, where Log Peak Frequency = 3.8762 – 1.5461*Log Skull Length. Dashed lines represent the 95% confidence intervals. The species are: *R. acuminatus* (1); *R. affinis* (2); *R. alcyone* (3); *R. arcuatus* (4); *R. blasii* (5); *R. borneensis* (6); *R. capensis* (7); *R. clivosus* (8); *R. coelophyllus* (9); *R. cornutus* (10); *R. creaghi* (11); *R. darlingi* (12); *R. denti* (13); *R. euryale* (14); *R. ferrumequinum* (15); *R. fumigatus* (16); *R. hildebrandti* (17); *R. hipposideros* (18); *R. landeri* (19); *R. lepidus* (20); *R. luctus* (21); *R. macrotis* (22); *R. malayanus* (23); *R. megaphyllus* (24); *R. mehelyi* (25); *R. paradoxolophus* (26); *R. pearsonii* (27); *R. philippinensis* (28); *R. pusillus* (29); *R. rex* (30); *R. rouxii* (31); *R. sedulus* (32); *R. shameli* (33); *R. simulator* (34); *R. stheno* (35); *R. subrufus* (36); *R. swinnyi* (37); *R. thomasi* (38); and *R. trifoliatus* (39).

Species differed significantly in skull parameters (Wilk's λ=0.000001, $F_{(198,395)}$=9.01, $p<0.001$) but no differences occurred between males and females (Wilk's λ=0.634, $F_{(22,45)}$=1.177, $p>0.3$; Table 4.3). The interactive term species*sex was not significant (Wilk's λ=0.020701, $F_{(198,395)}$=1.178, $p>0.08$). Because there was no sexual dimorphism in any of the species (Wilk's λ=0.021, $F_{(198,395)}$=1.18, $p>0.08$) males and females were analysed together.

Traditional morphometrics

Skull parameters excluded from analyses due to measurement error among species included BOW (basioccipital width - least distance between the inner margins of the bulla) and AMSW (width of anterior median swellings in dorsal view). These were identified as having a greater variance for repeat measures of a single skull compared with variances between skulls of different species.

The three principal components extracted from the log-transformed linear skull measurements for the 10 South African rhinolophids accounted for 96.845 % of the variation among species (Table 4.4). PC4 only explained an additional 0.68% of the variation.

Table 4.3 Mean ± SD (mm) of skull parameters for *R. blasii* (RB), *R. capensis* (RC), *R. clivosus* (RCL), *R. darlingi* (RD), *R. denti* (RDNT), *R. fumigatus* (RF), *R. hildebrandti* (RH), *R. landeri* (RL), *R. simulator* (RS), and *R. swinnyi* (RSWY). Ranges are given in parentheses.

SKULL PARAMETER	RB	RC	RCL	RD	RDNT
	n = 8	n = 10	n = 10	n = 12	n = 9
Dorsal skull length (DSL)	11.6 ± 0.20 (11.3 – 11.9)	12.3 ± 0.27 (11.9 – 12.7)	13.1 ± 0.34 (12.2 – 13.4)	11.7 ± 0.45 (10.5 – 12.4)	11.0 ± 0.21 (10.5 – 11.2)
Mastoid width (MW)	9.1 ± 0.10 (9.0 – 9.3)	9.9 ± 0.10 (9.7 – 10.0)	10.1 ± 0.16 (9.8 – 10.3)	9.1 ± 0.24 (8.6 – 9.4)	8.4 ± 0.13 (8.2 – 8.6)
Zygomatic width (ZW)	8.9 ± 0.19 (8.6 – 9.2)	10.4 ± 0.22 (10.1 – 10.8)	11.6 ± 0.23 (11.1 – 12.0)	9.9 ± 0.47 (8.8 – 10.4)	8.6 ± 0.18 (8.3 – 8.9)
Interorbital width (IOW)	2.5 ± 0.08 (2.3 – 2.6)	2.4 ± 0.18 (2.1 – 2.7)	2.8 ± 0.18 (2.6 – 3.2)	2.6 ± 0.12 (2.4 – 2.8)	2.1 ± 0.25 (1.4 – 2.2)
Anterior lateral swellings (ALSW)	5.1 ± 0.06 (5.0 – 5.2)	5.1 ± 0.17 (5.0 – 5.5)	5.6 ± 0.17 (5.4 – 5.9)	5.0 ± 0.14 (4.7 – 5.2)	4.0 ± 0.13 (3.9 – 4.3)
Anterior median swellings (AMSW)	2.9 ± 0.14 (2.8 – 3.2)	3.0 ± 0.18 (2.7 – 3.2)	3.2 ± 0.19 (2.8 – 3.5)	3.0 ± 0.31 (2.5 – 3.5)	2.5 ± 0.14 (2.3 – 2.8)
Dorsal rostral width (DRW)	4.3 ± 0.16 (4.0 – 4.5)	4.1 ± 0.09 (4.0 – 4.2)	4.7 ± 0.18 (4.5 – 5.0)	4.0 ± 0.26 (3.4 – 4.3)	3.0 ± 0.08 (3.0 – 3.2)
Rostrum area (AR)	16.3 ± 0.57 (15.7 – 17.4)	16.8 ± 0.37 (16.3 – 17.4)	19.8 ± 0.64 (18.9 – 20.8)	16.1 ± 0.42 (15.2 – 16.8)	10.2 ± 0.17 (10.0 – 10.5)
Greatest skull length (GSL)	19.1 ± 0.26 (18.8 – 19.5)	20.6 ± 0.34 (20.0 – 21.0)	22.0 ± 0.43 (20.9 – 22.4)	19.2 ± 0.55 (18.1 – 20.0)	17.2 ± 0.28 (16.9 – 17.5)
Rostral height (RH)	5.3 ± 0.17 (5.0 – 5.4)	6.0 ± 0.21 (5.7 – 6.3)	6.6 ± 0.37 (6.0 – 7.2)	5.9 ± 0.22 (5.5 – 6.3)	4.5 ± 0.17 (4.3 – 4.8)
Skull height (SH)	8.5 ± 0.18 (8.2 – 8.7)	8.9 ± 0.32 (8.5 – 9.5)	9.6 ± 0.31 (9.1 – 10.2)	8.4 ± 0.26 (8.0 – 8.9)	7.5 ± 0.23 (7.3 – 8.0)
Tympanic bulla length (TB)	2.8 ± 0.23 (2.5 – 3.2)	3.0 ± 0.23 (2.6 – 3.3)	2.8 ± 0.13 (2.6 – 3.0)	2.7 ± 0.15 (2.4 – 3.0)	2.5 ± 0.07 (2.4 – 2.6)
Anterior skull length (ASL)	16.0 ± 0.22 (15.8 – 16.4)	16.8 ± 0.18 (16.6 – 17.1)	17.8 ± 0.28 (17.5 – 18.4)	15.9 ± 0.45 (15.1 – 16.7)	14.2 ± 0.22 (13.9 – 14.7)
Palatal length (PL)	2.5 ± 0.23 (2.3 – 3.0)	2.5 ± 0.21 (2.3 – 2.9)	2.3 ± 0.25 (2.0 – 2.9)	2.3 ± 0.17 (2.0 – 2.6)	1.8 ± 0.14 (1.5 – 2.0)
Left bullar width (BW L)	4.3 ± 0.11 (4.2 – 4.6)	4.7 ± 0.10 (4.6 – 4.8)	4.6 ± 0.15 (4.4 – 4.9)	4.2 ± 0.13 (4.1 – 4.5)	3.9 ± 0.08 (3.8 – 4.0)
Basioccipital width (BOW)	0.7 ± 0.11 (0.5 – 0.8)	0.7 ± 0.04 (0.6 – 0.7)	0.9 ± 0.12 (0.7 – 1.1)	0.6 ± 0.10 (0.5 – 0.8)	0.6 ± 0.06 (0.6 – 0.8)
Right bullar width (BW R)	4.3 ± 0.13 (4.1 – 4.5)	4.6 ± 0.12 (4.4 – 4.8)	4.6 ± 0.16 (4.4 – 4.9)	4.2 ± 0.13 (4.0 – 4.4)	3.9 ± 0.09 (3.7 – 4.0)
Ventral rostral width (VRW)	6.6 ± 0.07 (6.4 – 6.6)	7.7 ± 0.14 (7.5 – 7.9)	8.7 ± 0.27 (8.3 – 9.2)	7.5 ± 0.37 (6.5 – 7.9)	6.3 ± 0.18 (5.9 – 6.5)
Left upper tooth row length (UTRL L)	6.8 ± 0.17 (6.4 – 7.0)	7.5 ± 0.30 (6.8 – 7.9)	8.3 ± 0.12 (8.0 – 8.4)	7.1 ± 0.25 (6.6 – 7.4)	5.9 ± 0.13 (5.8 – 6.1)
Right upper tooth row length (UTRL R)	6.8 ± 0.17 (6.6 – 7.1)	7.6 ± 0.18 (7.3 – 7.8)	8.3 ± 0.13 (8.0 – 8.4)	7.1 ± 0.32 (6.4 – 7.6)	5.9 ± 0.09 (5.8 – 6.1)
Left auditory bullar area (AVENT L)	11.0 ± 0.74 (10.4 – 12.7)	12.6 ± 0.46 (12.0 – 13.4)	11.2 ± 0.87 (9.1 – 12.3)	10.3 ± 0.75 (9.1 – 11.2)	9.0 ± 0.35 (8.3 – 9.4)
Right auditory bullar area (AVENT R)	10.9 ± 0.69 (10.3 – 12.4)	12.2 ± 0.59 (11.1 – 13.1)	11.0 ± 1.06 (8.1 – 11.9)	10.2 ± 0.69 (9.3 – 11.2)	8.8 ± 0.43 (8.2 – 9.5)

Table 4.3 Continued.

SKULL PARAMETER	RF	RH	RL	RS	RSWY
	n = 8	n = 12	n = 10	n = 10	n = 5
Dorsal skull length (DSL)	12.8 ± 0.64 (12.0 – 13.7)	15.3 ± 0.65 (14.1 – 16.1)	10.5 ± 0.23 (10.3 – 10.9)	11.1 ± 0.56 (10.2 – 11.9)	11.1 ± 0.11 (11.0 – 11.3)
Mastoid width (MW)	10.4 ± 0.25 (10.1 – 10.9)	12.2 ± 0.67 (10.7 – 13.4)	8.2 ± 0.25 (7.9 – 8.6)	8.9 ± 0.31 (8.2 – 9.3)	8.9 ± 0.07 (8.8 – 9.0)
Zygomatic width (ZW)	11.5 ± 0.20 (11.3 – 11.9)	13.7 ± 0.73 (11.6 – 14.3)	8.5 ± 0.16 (8.3 – 8.9)	8.9 ± 0.27 (8.4 – 9.2)	9.0 ± 0.11 (8.8 – 9.1)
Interorbital width (IOW)	2.8 ± 0.24 (2.5 – 3.1)	3.1 ± 0.22 (2.7 – 3.4)	2.2 ± 0.20 (1.8 – 2.5)	2.4 ± 0.18 (2.1 – 2.6)	2.3 ± 0.07 (2.2 – 2.4)
Anterior lateral swellings (ALSW)	6.1 ± 0.26 (5.8 – 6.6)	7.3 ± 0.43 (6.5 – 8.0)	4.5 ± 0.18 (4.2 – 4.8)	4.5 ± 0.12 (4.4 – 4.8)	4.4 ± 0.28 (3.9 – 4.6)
Anterior median swellings (AMSW)	3.7 ± 0.36 (3.0 – 4.3)	4.3 ± 0.29 (3.8 – 4.7)	2.5 ± 0.36 (2.2 – 3.2)	2.6 ± 0.27 (2.3 – 3.2)	2.3 ± 0.20 (2.0 – 2.5)
Dorsal rostral width (DRW)	4.9 ± 0.16 (4.7 – 5.1)	5.8 ± 0.37 (4.8 – 6.2)	3.3 ± 0.10 (3.1 – 3.5)	3.5 ± 0.17 (3.2 – 3.7)	3.5 ± 0.09 (3.4 – 3.7)
Rostrum area (AR)	27.9 ± 0.98 (26.6 – 29.6)	37.3 ± 3.80 (31.6 – 45.2)	13.6 ± 0.33 (12.9 – 13.9)	15.4 ± 0.31 (14.7 – 15.8)	12.9 ± 0.31 (12.3 – 13.2)
Greatest skull length (GSL)	22.6 ± 0.46 (22.1 – 23.2)	27.0 ± 1.68 (23.2 – 29.8)	17.2 ± 0.19 (16.9 – 17.5)	18.6 ± 0.67 (17.4 – 19.7)	17.9 ± 0.09 (17.8 – 18.1)
Rostral height (RH)	7.3 ± 0.25 (6.9 – 7.7)	8.6 ± 0.70 (7.4 – 9.9)	4.7 ± 0.14 (4.6 – 5.0)	5.2 ± 0.27 (4.7 – 5.5)	4.8 ± 0.16 (4.6 – 5.0)
Skull height (SH)	10.1 ± 0.35 (9.7 – 10.7)	11.6 ± 0.55 (10.6 – 12.4)	7.3 ± 0.27 (6.9 – 7.8)	8.0 ± 0.37 (7.4 – 8.5)	7.7 ± 0.13 (7.4 – 7.8)
Tympanic bulla length (TB)	3.3 ± 0.14 (3.2 – 3.6)	3.6 ± 0.24 (3.3 – 4.1)	2.4 ± 0.09 (2.3 – 2.6)	2.8 ± 0.15 (2.5 – 3.0)	2.4 ± 0.16 (2.3 – 2.6)
Anterior skull length (ASL)	19.2 ± 0.42 (18.4 – 19.7)	22.8 ± 1.42 (19.6 – 25.3)	14.3 ± 0.23 (13.9 – 14.7)	15.4 ± 0.45 (14.3 – 15.9)	14.6 ± 0.19 (14.5 – 15.0)
Palatal length (PL)	3.1 ± 0.51 (2.8 – 4.3)	3.6 ± 0.40 (3.0 – 4.3)	2.1 ± 0.21 (1.9 – 2.6)	2.3 ± 0.19 (2.0 – 2.5)	1.9 ± 0.11 (1.7 – 2.0)
Left bullar width (BW L)	4.9 ± 0.19 (4.7 – 5.3)	5.7 ± 0.33 (5.1 – 6.3)	3.8 ± 0.22 (3.3 – 4.1)	4.2 ± 0.16 (3.9 – 4.4)	4.0 ± 0.16 (3.8 – 4.2)
Basioccipital width (BOW)	0.6 ± 0.10 (0.5 – 0.7)	0.8 ± 0.15 (0.6 – 1.1)	0.6 ± 0.11 (0.4 – 0.8)	0.5 ± 0.10 (0.4 – 0.7)	0.6 ± 0.05 (0.6 – 0.7)
Right bullar width (BW R)	4.9 ± 0.14 (4.7 – 5.1)	5.7 ± 0.30 (5.3 – 6.2)	3.8 ± 0.20 (3.5 – 4.0)	4.2 ± 0.21 (3.7 – 4.5)	4.0 ± 0.10 (3.8 – 4.1)
Ventral rostral width (VRW)	8.8 ± 0.23 (8.4 – 9.2)	10.4 ± 0.49 (9.1 – 10.9)	6.4 ± 0.17 (6.3 – 6.7)	6.6 ± 0.21 (6.3 – 7.0)	6.7 ± 0.10 (6.6 – 6.8)
Left upper tooth row length (UTRL L)	8.4 ± 0.11 (8.3 – 8.6)	10.0 ± 0.53 (8.5 – 10.6)	6.3 ± 0.14 (6.1 – 6.5)	6.8 ± 0.24 (6.4 – 7.1)	6.3 ± 0.11 (6.2 – 6.5)
Right upper tooth row length (UTRL R)	8.4 ± 0.11 (8.3 – 8.5)	10.0 ± 0.56 (8.4 – 10.6)	6.2 ± 0.18 (5.8 – 6.5)	6.7 ± 0.23 (6.3 – 7.1)	6.3 ± 0.13 (6.1 – 6.5)
Left auditory bullar area (AVENT L)	14.9 ± 0.66 (13.8 – 15.7)	18.7 ± 1.43 (17.1 – 21.9)	8.5 ± 0.48 (7.7 – 9.2)	10.5 ± 0.88 (8.6 – 11.6)	9.8 ± 0.47 (9.3 – 10.4)
Right auditory bullar area (AVENT R)	14.7 ± 0.74 (13.7 – 15.8)	18.1 ± 1.59 (16.3 – 21.0)	8.4 ± 0.47 (7.8 – 9.2)	10.7 ± 1.06 (8.6 – 12.1)	9.7 ± 0.67 (9.2 – 10.8)

Table 4.4 Factor loadings, eigenvalues, and the percent variation of the first three principal components (PC) obtained from the principal components analysis of log-transformed skull parameters.

	PC 1	PC 2	PC 3
Skull parameters:			
Greatest skull length (GSL)	-0.058	-0.004	0.005
Dorsal skull length (DSL)	-0.043	-0.007	0.009
Mastoid width (MW)	-0.046	-0.001	0.007
Zygomatic width (ZW)	-0.060	-0.015	0.008
Interorbital width (IOW)	-0.037	-0.010	-0.007
Greatest width anterior lateral swellings (ALSW)	-0.063	-0.006	-0.004
Dorsal rostral width (DRW)	-0.066	-0.010	-0.004
Rostrum area (AR)	-0.151	0.001	-0.023
Rostral height (RH)	-0.075	-0.009	0.000
Skull height (SH)	-0.057	-0.005	0.004
Tympanic bulla length (TB)	-0.040	0.012	0.006
Anterior skull length (ASL)	-0.060	-0.001	0.001
Palatal length (PL)	-0.060	0.022	-0.018
Left bullar width (BW L)	-0.043	0.003	0.007
Right bullar width (BW R)	-0.042	0.004	0.006
Rostral width (VRW)	-0.062	-0.016	0.006
Left upper tooth row length (UTRL L)	-0.060	-0.009	0.002
Right upper tooth row length (UTRL R)	-0.061	-0.008	0.001
Left auditory bullar area (AVENT L)	-0.093	0.020	0.013
Right auditory bullar area (AVENT R)	-0.090	0.020	0.013
Eigenvalue	0.093	0.002	0.002
Total variance explained (%)	92.717	2.429	1.700
Cumulative variance (%)	92.717	95.146	96.845

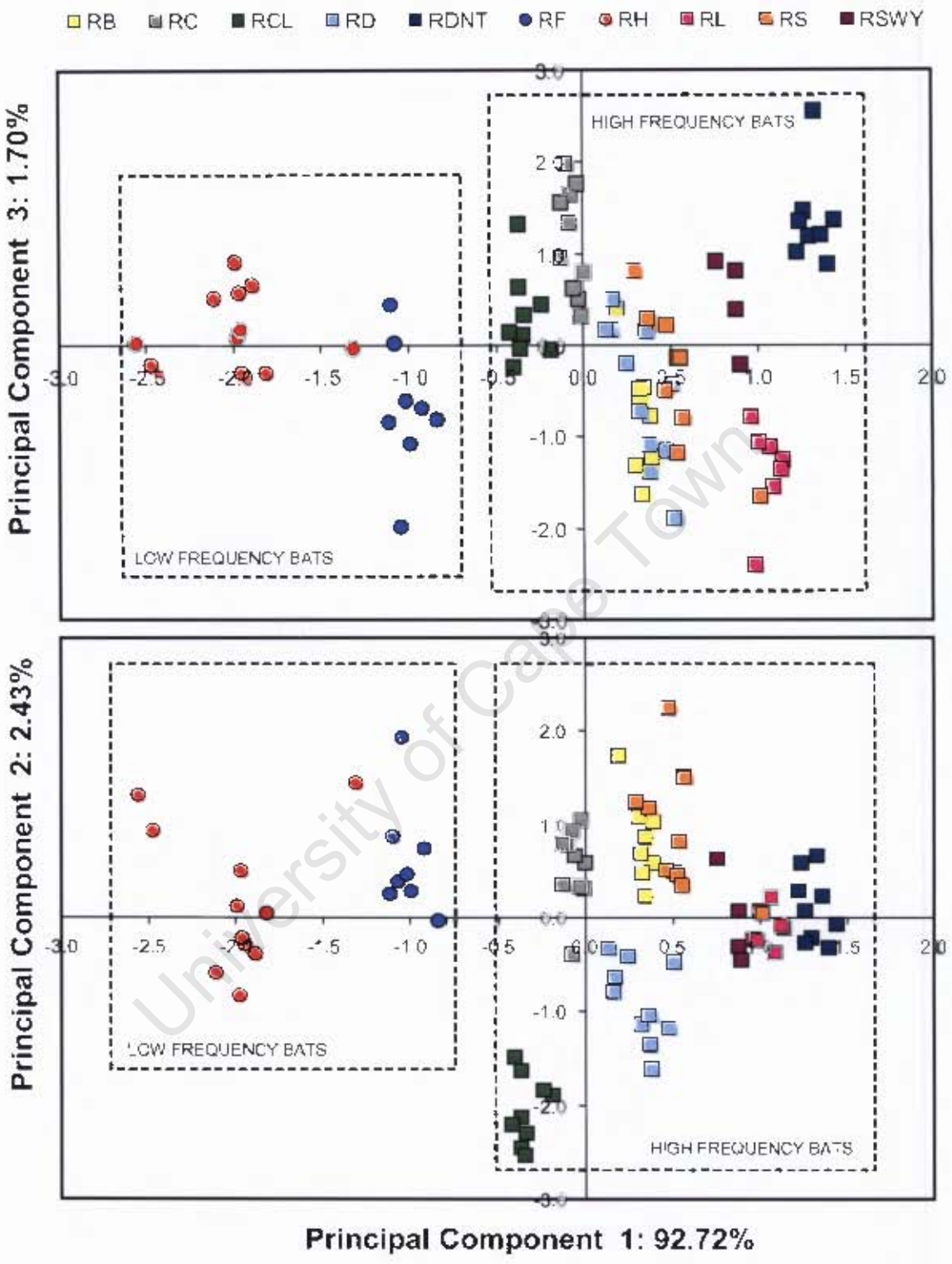


Fig. 4.10 Plot of the component scores for *R. blasii* (■), *R. capensis* (■), *R. clivosus* (■), *R. darlingi* (■), *R. denti* (■), *R. fumigatus* (●), *R. hildebrandti* (●), *R. landeri* (■), *R. simulator* (■), and *R. swinnyi* (■). Circles indicate bats with frequencies < 60 kHz, and squares indicate bats using frequencies > 60 kHz.

The first principal component accounts for 92.72% of the interspecific variation and is associated with the area of the rostrum (AR; Fig. 4.10). Species loading low on PC1 generally have large rostra, whereas species loading high on PC1 are characterized by smaller rostra (Fig. 4.10, Table 4.3). PC2 accounts for 2.43% of the variation among species (Fig. 4.10), and no clear separation among the rhinolophids is apparent. However, *R. clivosus* and *R. darlingi* load low on PC2 and are separated from *R. blasii*, *R. simulator,* and *R. capensis*. PC3 only accounts for a small amount of variation (1.7%) but does separate the highest frequency echolocating bats (*R. denti*, *R. swinnyi,* and *R. landeri*). *Rhinolophus denti* and *R. swinnyi* load positively on PC3, whereas *R. landeri* loads negatively. Palatal length appeared to make the largest contribution to the distribution of species along PC3 (Table 4.3). *Rhinolophus landeri* has a much longer palate than *R. denti* and *R. swinnyi* (Table 4.3). Furthermore, bats appear to be separated on the basis of size along PC1 where the largest bat, *R. hildebrandti* loads lowest, and the smallest bat *R. denti* loads highest (Table 4.2).

When the distribution of the rhinolophids in multivariate space was considered in relation to the peak frequency used by each species, low-frequency (< 60 kHz) and high-frequency (> 60 kHz) rhinolophids showed a distinct separation along PC1 (Fig. 4.10). Low-frequency echolocators (*R. fumigatus* and *R. hildebrandti*) tended to have lower PC1 scores. The highest echolocating bat (*R. denti*, Table 4.2) loaded highest on PC1, and the bat with the lowest frequency (*R. hildebrandti*, Table 4.2) loaded lowest. Bats using high (>60 kHz) or low (<60 kHz) frequency echolocation calls had significantly different sized skulls (Wilk's λ=0.082, $F_{(22,63)}$=32.1, $p<0.01$). All skull parameters differed significantly between high- and low-frequency bats (Tukey HSD test, all p's<0.05), except basioccipital width (BOW, Tukey HSD test, p>0.05). Low-frequency bats tended to have larger values than high-frequency bats for each skull parameter (Table 4.3).

Morphology and echolocation call

Noseleaf width, followed by ear length, was identified as the best predictor of peak frequency at the level of the individual (Table 4.5). Noseleaf width (Wald statistic = 327.57, df=1, p<0.001) was also the best predictor of peak frequency at the level of

species. Of the skull parameters included in the second GLMM, rostral area was identified as the skull parameter that best predicts peak frequency (Table 4.6).

Table 4.5 GLMM analysis of morphological parameters associated with peak frequency at the level of the individual. Data were from all South African rhinolophid individuals for which measurements were available.

MODEL TERM			
Full Model	**Wald Statistic**	**df**	**p**
Log Noseleaf Width (mm)	175.82	1	< 0.001
Log Ear Length (mm)	10.23	1	0.001
Minimal Model	**Average Effect**	**s.e**	
Constant	1.942	0.0028	
Log Noseleaf Width (mm)	-0.3277	0.1025	
Log Ear Length (mm)	-1.139	0.0859	

Table 4.6 GLMM analysis of morphological parameters associated with peak frequency at the species level. Data used were mean values calculated for each of the ten South African *Rhinolophus* species.

MODEL TERM			
Full Model	**Wald Statistic**	**df**	**p**
Noseleaf Width (mm)	327.57	1	< 0.001
AR (rostral area, mm^2)	14.61	1	<0.001
MW (mastoid width, mm)	1.16	1	0.281
DSL (dorsal skull length, mm)	0.07	1	0.794
Minimal Model	**Average Effect**	**s.e**	
Constant	1.904	0.016	
Noseleaf Width (mm)	-1.224	0.6915	
AR (rostral area, mm^2)	-0.8321	0.2177	
MW (mastoid width, mm)	-2.727	2.5307	
DSL (dorsal skull length, mm)	-0.5268	2.0199	

Geometric morphometrics

The skulls of South African rhinolophids differed significantly in centroid size (MANOVA, $F_{(19,73)}=92.95$, $p<0.01$) but the interactive term species*sex was not significant (MANOVA, $F_{(9,73)}=0.74$, $p>0.67$). The low-frequency echolocating bats, *R. hildebrandti* (RH) and *R. fumigatus* (RF) had the largest overall skulls (Fig. 4.11). *Rhinolophus hildebrandti* had a significantly larger centroid size compared to the other South African rhinolophids (Tukey HSD test, p's<0.001). *Rhinolophus fumigatus* and *R. clivosus* had similar centroid sizes (Tukey HSD test, p=0.17), as did *R. darlingi*, *R. simulator* and *R. blasii* (Tukey HSD tests p's>0.5). Similarly the bats echolocating at the highest frequencies, *R. denti, R. swinnyi,* and *R. landeri*, did not differ significantly in centroid size (Tukey HSD test, p's>0.8).

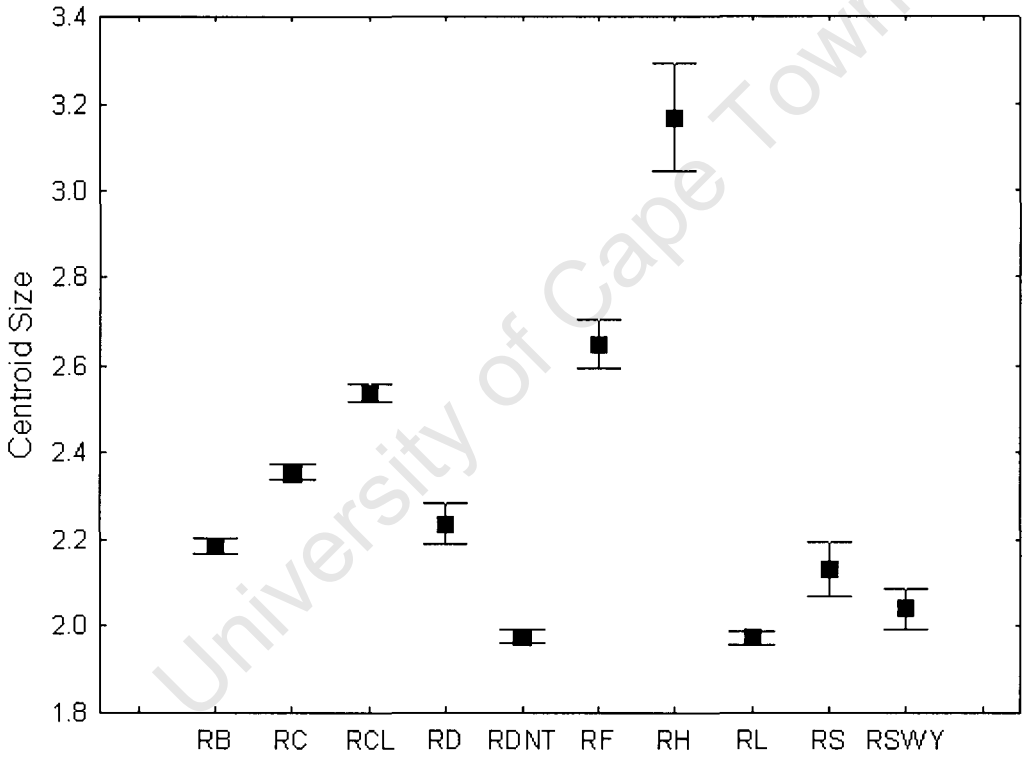


Fig. 4.11 Centroid size for each of the South African rhinolophids. Species are: *R. blasii* (RB), *R. capensis* (RC), *R. clivosus* (RCL), *R. darlingi* (RD), *R. denti* (RDNT), *R. fumigatus* (RF), *R. hildebrandti* (RH), *R. landeri* (RL), *R. simulator* (RS), and *R. swinnyi* (RSWY). Filled squares represent the mean centroid size for each species, and bars represent 95% confidence intervals.

Centroid sizes were negatively correlated with echolocation frequency (r= -0.9148, $F_{(1,8)}$=41.06, p<0.005; Fig. 4.12). This relationship remained significant after controlling for phylogeny (CAIC: r=0.94, p<0.005). *Rhinolophus fumigatus* fell within the 95% confidence intervals for its frequency when centroid size as opposed to forearm length, was used (cf Figs 4.6, 4.12). Relative to their overall skull sizes, *R. clivosus* had a higher-than-expected call frequency and *R. simulator* had a slightly lower-than-expected call frequency (Fig. 4.12). *Rhinolophus clivosus* differed greatly from the allometric prediction of peak call frequency for South African rhinolophids when both skull centroid size (Fig. 4.12) and forearm length (Fig. 4.6) were used.

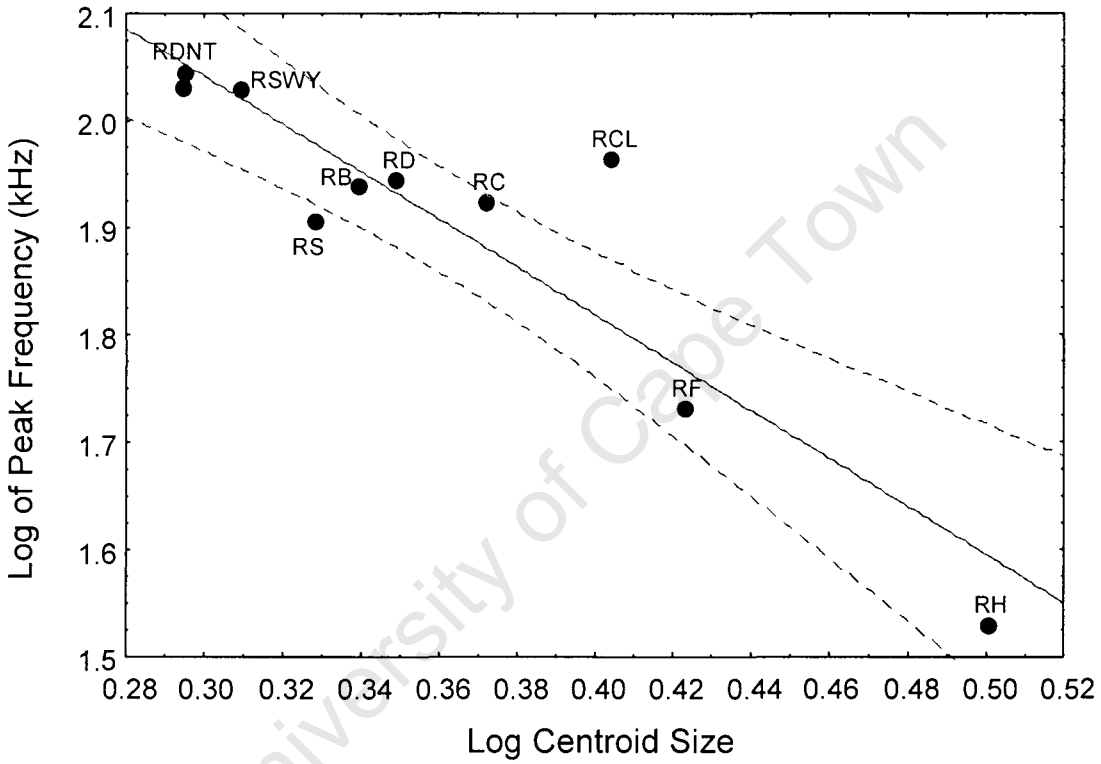


Fig. 4.12 Regression of the log of centroid size and the log of peak frequency for *Rhinolophus* species. Species are: *R. blasii* (RB), *R. capensis* (RC), *R. clivosus* (RCL), *R. darlingi* (RD), *R. denti* (RDNT), *R. fumigatus* (RF), *R. hildebrandti* (RH), *R. landeri* (RL), *R. simulator* (RS), and *R. swinnyi* (RSWY). Solid line represents the line of best fit, where Log Peak Frequency = 2.712 – 2.2342*Log Centroid Size. Dashed lines represent the 95% confidence intervals.

The weight matrix was used in a Canonical Variates Analysis to investigate the level of variation in skull shape among species. *Rhinolophus landeri* loaded high on canonical variate (CV) 1 and low on CV 2, whereas *R. fumigatus* and *R. darlingi* loaded high on both CV 1 and CV 2 (Fig. 4.13). *Rhinolophus blasii* fell in the centre of the plot, suggesting that it has the mean shape for both axes. That the low-frequency bats (*R. fumigatus* and *R. hildebrandti*) did not group with one another in this plot of shape suggests that there is no overall skull shape associated with emitting a low-frequency echolocation call. To interpret the orientation of species in relation to one another, it is necessary to examine the skull shapes associated with the negative and positive loadings on the axes.

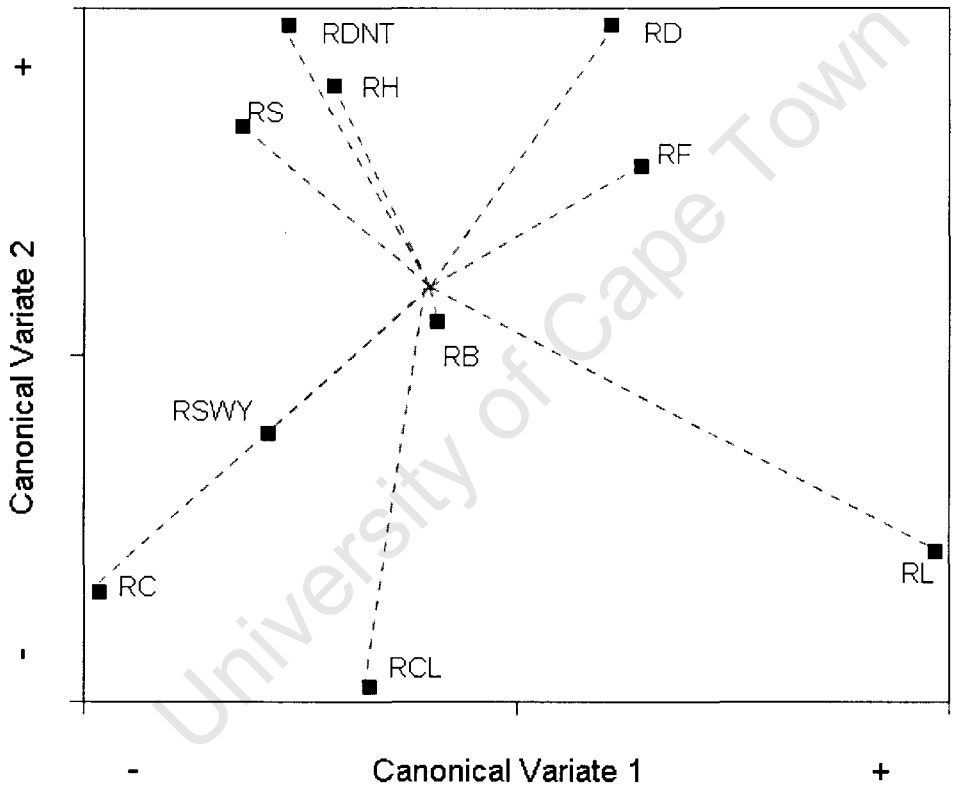


Fig. 4.13 The orientation of the ten South African *Rhinolophus* species along the first and second canonical axes resulting from the Canonical Variates Analysis on skull shape. Species are: *R. blasii* (RB), *R. capensis* (RC), *R. clivosus* (RCL), *R. darlingi* (RD), *R. denti* (RDNT), *R. fumigatus* (RF), *R. hildebrandti* (RH), *R. landeri* (RL), *R. simulator* (RS), and *R. swinnyi* (RSWY).

Bats which scored positively on CV 1 (*R. darlingi*, *R. fumigatus*, and *R. landeri*; Fig. 4.13) were characterized by having wide rostral areas and short braincases (Fig. 4.14). Bats scoring negatively on CV 1 (*R. blasii, R. capensis, R. clivosus, R. denti, R. hildebrandti, R. simulator,* and *R. swinnyi*) had narrower rostral regions and longer braincases. On CV 2, positive loadings were associated with skulls in which the mastoid and zygoma are of similar width. Negative loadings were associated with a narrower mastoid relative to zygomatic width (Fig. 4.15). Thus, bats loading positively on CV 2 (*R. blasii, R. darlingi, R. denti, R. fumigatus, R. hildebrandti,* and *R. simulator*) had much wider braincases than those species with negative loadings (*R. capensis*, *R. clivosus*, *R. landeri,* and *R. swinnyi*).

Rhinolophus simulator and *R. denti* (which are sister taxa) had similar-shaped skulls, although *R. denti* had a broader braincase and slightly shorter skull. *Rhinolophus capensis* and *R. swinnyi* (also sister taxa) both had narrow rostral regions, but *R. capensis* had much the narrower and longer braincase (Fig. 4.13 in conjunction with Figs 4.14, 4.15) causing it to be displaced further from the mean configuration. *Rhinolophus darlingi*, *R. hildebrandti* and *R. fumigatus* are closely related species (Chapter 2) yet did not group together based on skull shape, indicating that sister taxa can be highly divergent in terms of skull shape, body size, and echolocation frequency. *Rhinolophus blasii* appeared to have the 'average' skull shape for the South African rhinolophids included in this study, and *R. landeri* appeared to be quite different from the other South African rhinolophids. Both these species are basal to the clades containing the rest of the South African rhinolophids (Chapter 2). Skull shape thus supported the ancestral position of *R. blasii* to all of the African rhinolophids, except *R. landeri*, and *R. blasii* probably diverged from *R. landeri* before giving rise to the other African rhinolophids (Chapter 2).

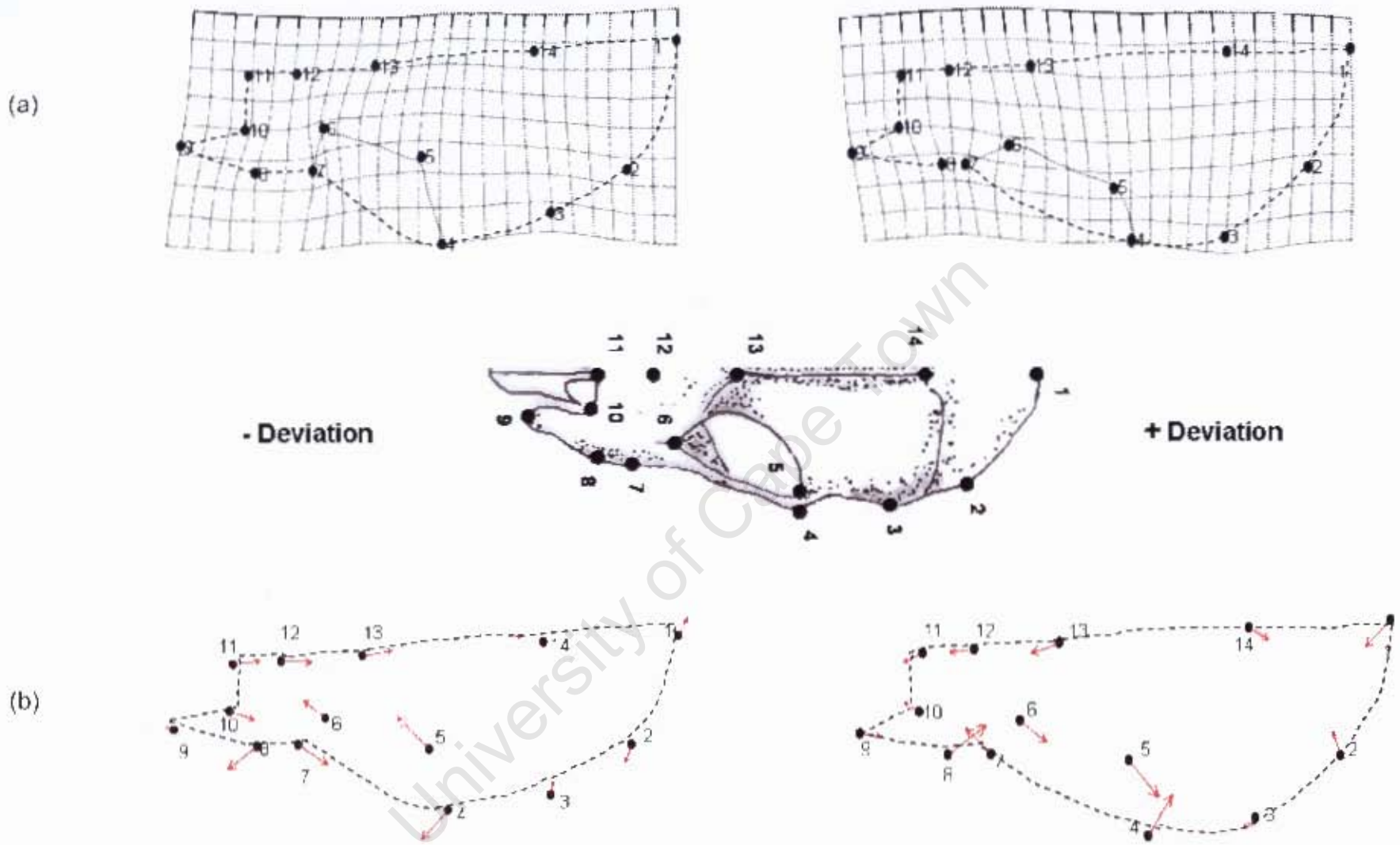


Fig. 4.14 Results of the regression of the partial-warp scores onto the first canonical variate axis. The centre figure shows the mean landmark positions, and numbers in (a) and (b) correspond to these landmarks. (a) Shape changes are shown as deformations using thin-plate spline diagrams. Negative deformations are shown in the diagram on the left, and positive deviations are shown on the right. The figures in (b) use vectors to show the direction of landmark displacements. To make the changes more visible, the magnitude of the change in shape was exaggerated. Figures to the left demonstrate shape changes for negative deviations from the mean, and figures to the right, show positive deviations from the mean.

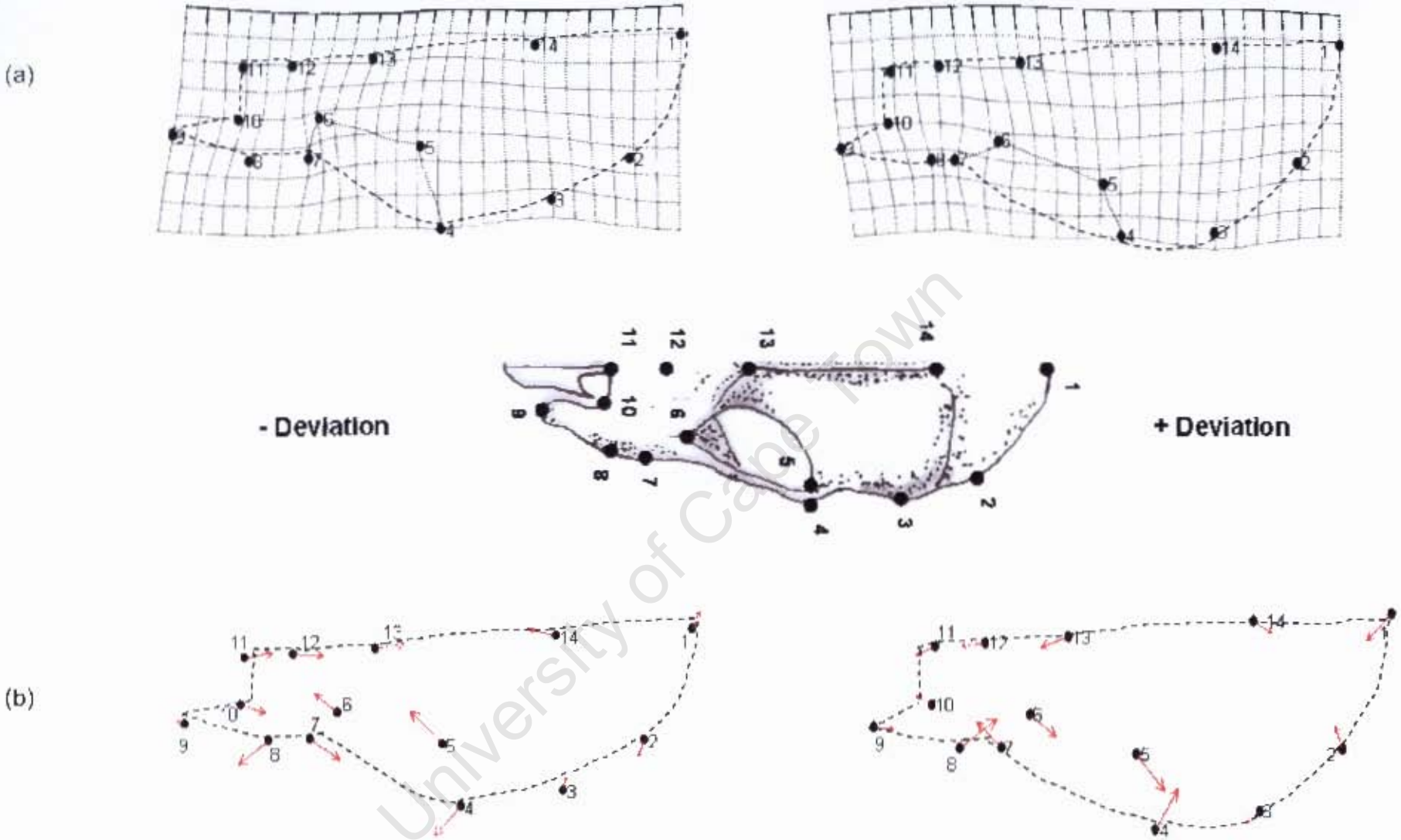


Fig. 4.15 Results of the regression of the partial-warp scores onto the second canonical variate axis. The centre figure shows the mean landmark positions, and numbers in (a) and (b), correspond to these landmarks. (a) Shape changes are shown as deformations using thin plate spline diagrams. Negative deviations are shown in the left diagram and positive deviations in the right. The figures in (b) use vectors to show the direction of landmark displacements. To make the changes more visible, the magnitude of the change in shape was exaggerated. Figures to the left demonstrate shape changes for a negative deviations from the mean, and figures to the right, show positive deviations from the mean.

DISCUSSION

My results confirm findings of earlier studies (e.g. Heller & von Helversen 1989; Jones 1996, 1999; Zhang *et al.* 2000), that within the rhinolophids, and in particular within the South African rhinolophids, echolocation frequency is negatively correlated with body size. Both forearm length and skull length correlate with call frequencies for species of *Rhinolophus* from throughout the Old World (Figs 4.5, 4.9) for which data were available. Forearm length and call frequency are also correlated for the 10 *Rhinolophus* species sampled in South Africa.

Despite this, body size alone does not fully explain the evolution of high-frequency echolocation in the Rhinolophidae. Although significant allometric relationships between body size and echolocation frequency exist within families (Jones 1996; this study), this significance does not persist when body sizes and echolocation frequencies are compared across families. My analyses show that bat families such as the Rhinolophidae and Hipposideridae, which have on average higher frequency echolocation calls than other families, have similar or larger average body sizes than families of bats that use much lower echolocation frequencies (e.g. Vespertilionidae). Although the body masses of the different families overlap (see also Jones 1996), for a given body mass the rhinolophids and hipposiderids echolocate at higher frequencies than other bats (Jones 1996). One reason why the rhinolophids and hipposiderids emit frequencies much higher than that of similar sized bats in other families is that they place most of the energy into the second harmonic of the call. However, the hipposiderids are on average slightly larger than the rhinolophids, yet they use higher echolocation calls (Jones 1996). This suggests that despite the relationship between body size and call frequency within families, body size cannot predict and ergo cannot explain the generally higher frequencies used by the rhinolophids relative to other families of similar body size.

Intraspecific analyses recovered no relationship between body size (as measured by forearm length) and echolocation frequency. The body size – echolocation frequency relationship may be absent at this level because, within the species of South African Rhinolophidae for which I had comprehensive data, echolocation frequency varies much less than does body size. This suggests that echolocation call frequency is under stronger selection pressure than body size.

Within the Rhinolophidae, noseleaf width predicted echolocation frequency better than did forearm length, mass or ear length (Table 4.5). Wider noseleaves correlate with lower frequencies. Noseleaf width is also highly correlated with the wavelength of the peak frequency. A significant negative relationship also existed between the length of the bat's ear and its peak frequency (Table 4.5) as reported by Obrist *et al.* (1993), Gannon *et al.* (2001) and Zhao *et al.* (2003). These results support the prediction that there should be a closer relationship between echolocation frequency and sound-generating/sound-processing apparatus than between frequency and body size *per se* (Francis & Habersetzer 1998). This is demonstrated by *R. clivosus*, the South African rhinolophid whose peak echolocation frequency is considerably higher than predicted by body-size allometry, lying well outside the 95% confidence limits (e.g. Fig. 4.12). Because noseleaf width is the best predictor of echolocation frequency, *R. clivosus* should have a narrower noseleaf width relative to smaller bats with high echolocation frequencies. *Rhinolophus capensis* and *R. simulator* are both smaller than *R. clivosus*, yet echolocate at frequencies lower than *R. clivosus*. Although larger than both these bats, *R. clivosus* has a narrower noseleaf, which corresponds with its higher frequency echolocation (Table 4.2).

Significant differences occur in the cranial morphology between nasal and oral emitting bats (Pedersen 1993) suggesting that skull morphology may be linked to echolocation call frequency. This study identified significant correlations between call frequency and skull morphology (*contra* Goudy-Trainor & Freeman 2002). Multivariate analyses highlighted certain aspects of skull morphology which distinguish the 10 species of South African rhinolophid, separating high- from low-frequency echolocating species on the first principal component (Fig. 4.10).

This separation was primarily a function of size, with bats echolocating at low frequencies tending to have larger skulls than high-frequency echolocators.

Geometric morphometric analyses show that overall skull size, as expressed by centroid size, is negatively correlated with frequency. Although no general skull shape is associated with separating low-frequency bats from those using high frequencies, the use of geometric morphometrics did highlight various skull structural features that appear to be associated with one another. Two aspects of skull shape, relating to landmarks plotted along the first two canonical variate axes (Figs 4.14, 4.15) indicate that placement of species in shape-space is influenced by linked variation in shape of the rostral region and the braincase. On CV 1, bats with wide nasal regions tend to have short braincases (*R. darlingi*, *R. landeri* and *R. fumigatus*). On CV 2, positive scores correspond with a mastoid and zygoma of similar width resulting in wider braincases. Bats with narrow nasal regions (e.g. *R. clivosus, R denti* and *R. swinnyi*) are more likely to use high-frequency calls and, together with wide braincases (linked presumably to large brains and hence enhanced information processing capacity, Reader & Laland 2002), this may allow them to exploit complex (cluttered) habitats. Bats with wide braincases tend to be among the behaviourally more flexible species, capable of exploiting new and complex environments (Ratcliffe *et al.* 2006).

Traditional morphometric analyses show that the area of the rostrum (nasal chamber), area of the auditory bulla, width of anterior lateral swellings, dorsal rostral width, and rostral height are largest in bats emitting the lowest frequency calls (Fig. 4.10 & Table 4.3). Bats echolocating at higher frequencies (e.g. *R. clivosus*, and *R. denti*) are characterized by smaller rostral areas, longer braincases and wider mastoids (Tables 4.2, 4.3). The GLMMs identified rostral area as being the skull parameter most closely associated with echolocation frequency. This corresponds with the finding that noseleaf width is the best external morphological predictor of echolocation call frequency. The rhinarial cartilaginous skeleton of the noseleaf is associated with muscles attached to the rostrum that allow a bat to change the size of the nostril, thereby controlling the passage of air during call emission, as well as to move the position of the nostril

in relation to the orientation of the noseleaf (Göbbel 2000, 2002). This is important because the position of the nostrils can modulate the frequency and intensity of the echolocation call (Hartley & Suthers 1987).

Although the majority of *Rhinolophus* species conform to the relationship between body size and echolocation frequency (Fig 4.5), there are notable exceptions, both within and between species. For example, female *R. rouxii* produce higher frequency calls than males, yet there is no sexual size dimorphism (Neuweiler *et al.* 1987). Even less predictably, female *R. hipposideros* produce higher frequency calls than the smaller males (Jones *et al.* 1992, but see Siemers *et al.* 2005).

Among the South African rhinolophids, *R. fumigatus* and *R. clivosus* deviate from the predictions of allometry. *Rhinolophus clivosus* echolocates at a higher than predicted frequency. On average, *R. clivosus* is slightly larger than *R. fumigatus* (which calls at 53 kHz, a frequency much lower than the *ca* 65 kHz predicted from body-size allometry - Figs 4.5, 4.6, Table 4.2). Echolocation frequency/body size allometry predicts *R. clivosus* should echolocate around 50 kHz - as opposed to its 91 kHz. However, *R. clivosus* has the narrow noseleaf typical of high-frequency echolocators. Similarly, the call frequency of *R. fumigatus* could have been fairly accurately predicted from skull size (Fig. 4.12), although not from body size (Fig 4.6). Numerous other rhinolophids are outliers from the echolocation frequency/body size relationship. For example, *R. stheno*, *R. euryale, R. mehelyi*, *R. swinnyi* all have higher than predicted peak echolocation frequencies (Fig. 4.5). By contrast, *R. macrotis, R. rex*, and *R. trifoliatus* have much lower frequency calls than predicted. It is species such as these, that deviate from the predictions of allometry, which may provide the all-important insights into why many *Rhinolophus* species use seemingly 'aberrant' echolocation call frequencies.

In conclusion, the allometric relationship between body size and echolocation frequency within the Rhinolophidae cannot explain the evolution of high frequencies in this family. There is a complete overlap in body sizes between the

Rhinolophidae (and Hipposideridae) and families such as the Vespertilionidae which use lower echolocation frequencies. Furthermore, among the rhinolophids echolocation frequency has stronger allometric relationships with morphological characters that are directly associated with sound production, emission, and reception. This contrast strongly suggests that selection has acted directly on echolocation rather than on body size in the Rhinolophidae. This is supported by the basal phylogenetic position of the high-frequency *R. blasii* (Chapter 2), the proximity of *R. blasii* to the centroid skull shape for the ten South African species analysed (Fig. 4.14) and the similar sizes of the species within the same clade, despite their divergent call frequencies (Tables 4.2, 4.3). Both of these results suggest that selection on echolocation played a major role in the divergence of the species comprising this clade. This supports proposals that echolocation constrains body size rather than *vice versa* (Barclay & Brigham 1991; Jones 1996). Thus, selection pressure acting directly on echolocation, with complimentary changes in morphology (but not necessarily size) may explain why morphological allometry predicts echolocation call frequency better than does body-size allometry. A necessary corollary of this is that alternatives to the Allometry Hypothesis need to be found before the evolution of high-frequency echolocation in the Rhinolophidae can be explained. These possibilities are explored in the following chapters.

APPENDIX 1

Northern Flagship Institution codes for the bat skulls photographed:

Rhinolophus blasii – TM2888, TM7076, TM7076, TM7080, TM7083, TM13960, TM13962, TM13996, TM13997, TM13998

Rhinolophus capensis – TM29063, TM29064, TM29067, TM29077, TM29080, TM40566, TM40568, TM40570, TM40573, TM40576

Rhinolophus clivosus – TM25613, TM41266, TM41267, TM41268, TM41270, TM41272, TM41264, TM41275, TM41276, TM41278

Rhinolophus darlingi – TM30037, TM30046, TM39708, TM39709, TM39710, TM39714, TM39716, TM39717, TM39719, TM39733, TM46648*, TM46648*

Rhinolophus denti – TM36032, TM36076, TM36077, TM40649, TM40652, TM40657, TM40659, TM40661, TM40663

Rhinolophus fumigatus – TM30472, TM36608, TM36789, TM40827, TM40833, TM40835, TM41726, TM41730

Rhinolophus hildebrandti – TM36612, TM36788, TM37073, TM37084, TM37897, TM37939, TM40826, TM40831, TM40832, TM40836, TM46641*, TM46642*

Rhinolophus landeri – TM34909, TM34910, TM34911, TM34913, TM34915, TM34917, TM34919, TM34921, TM34922, TM34924

Rhinolophus simulator – TM39775, TM39777, TM39779, TM40837, TM41322, TM41323, TM41325, TM41327, TM46639*, TM46640*

Rhinolophus swinnyi – TM36580, TM36582, TM36584, TM40504, TM40505

* Skulls from voucher specimens collected on our field trips.

CHAPTER 5

THE EVOLUTION OF HIGH FREQUENCIES: HISTORICAL BIOGEOGRAPHY OF THE GENUS *RHINOLOPHUS* AND THE ACOUSTIC ADAPTATION HYPOTHESIS

INTRODUCTION

Body size (as measured by forearm length) appears to be a good predictor of peak echolocation call frequency in rhinolophid bats (Chapter 4) although many species do deviate from the allometric relationship between peak frequency and body size (Chapter 4). External morphological (e.g. noseleaf and ears) and cranial (e.g. rostral chamber) features are, in fact, more closely associated with peak frequency than is forearm length (Chapter 4). Thus, body size cannot explain why most rhinolophids have evolved echolocation frequencies higher than those of other families of bats (e.g. Vespertilionidae) with similar ranges of body sizes. Alternative explanations are therefore needed to explain high frequencies in the Rhinolophidae. Such explanations may be found through an understanding of rhinolophid biogeography.

Species' distributions are not random and range limits are usually governed by the species' ecologies (Ridley 1996). Mammal species richness in general is related to vegetation diversity (Avery 1993) and, for small mammals in particular, species richness and distributions are strongly correlated with vegetation and climatic parameters (Andrews & O'Brien 2000). Small mammal distributions and diversity, for example, are strongly correlated with woody plant species and therefore seasonal rainfall (O'Brien 1993), whereas temperature is a more important range determinant for larger mammals (Andrews & O'Brien 2000).

Habitat structure (typically vegetation cover) has been considered a main factor in shaping the evolution of bird song (Boncoraglio & Saino 2007), and the link between the two is the focus of the Acoustic Adaptation Hypothesis (Morton 1975; Hansen 1979). This hypothesis is based on the assumption that bird songs are shaped by habitat-driven selection to enhance both sound propagation and sound constancy, which in turn vary depending on the physical structure of the habitat (Boncoraglio & Saino 2007). Bats use their echolocation calls primarily for orientation and prey capture, rather than for e.g. territorial/mate advertising as in the case of birds, but echolocation calls are nonetheless subject to the same physical constraints due simply to the physics of sound and sound transmission. Habitat structure may play an important role in determining the range of echolocation frequencies that can function effectively in any particular environment. For example, low-frequency calls, which have long wavelengths, do not provide sufficient echo resolution to enable a bat to forage successfully within a cluttered habitat (Neuweiler 1989). Similarly, high-frequency, shorter wavelength calls do not provide the long detection ranges necessary for efficiently locating insect prey in open habitats. If the Acoustic Adaptation Hypothesis is correct, there should be a relationship between vegetation structure and echolocation frequencies, i.e. bats that occur in cluttered habitats such as forests should emit higher frequency calls than bats hunting in more open habitats. This assumes that a gradient of selection pressure operating over a gradient of increasing habitat clutter results in an increase in echolocation frequency.

Environmental and physical factors other than vegetation cover may also influence the call frequency best suited to a given environment. Physical factors (such as humidity) may set limits on the range of frequencies that function effectively under different environmental conditions (Griffin 1971). As sound travels through air, sound waves spread as spherical wave fronts, and the sound energy dissipates with increasing distance from the source (spreading loss - Griffin 1971). Atmospheric attenuation (the additional decrease in sound intensity not attributable to spreading loss – Griffin 1971) also reduces sound energy, but

whereas spreading loss is independent of frequency, atmospheric attenuation increases exponentially with increasing frequency (Lawrence & Simmons 1982) and may be an important constraint on echolocation frequencies. Atmospheric attenuation has played a role in the evolution of bird songs (Boncoraglio & Saino 2007) and may have influenced the evolution of echolocation frequencies within bats (Griffin 1971; Guillén et *al.* 2000). High-frequency sounds experience the greatest atmospheric attenuation, with a rapid increase in attenuation for frequencies above 90 kHz (Lawrence & Simmons 1982).

Increasing humidity causes increased atmospheric attenuation (Griffin 1971; Lawrence & Simmons 1982). By contrast, warm, dry air enhances sound transmission (Harris 1966). Echolocation is at best a short-range detection system and the frequency of echolocation calls in a given environment may have been selected for by a trade-off between low frequencies (which reduce atmospheric attenuation) and high frequencies (which increase resolution). Unlike humidity, changes in ambient temperature between 15° to 30°C have a negligible effect on the atmospheric attenuation of sound (Lawrence & Simmons 1982).

Although high-frequency calls are best suited to cluttered habitats due to their resolving power, in humid areas, high frequencies will be subject to strong atmospheric attenuation, and lower frequency calls may be favoured under such conditions. Indeed, an inverse relationship exists between call frequency and environmental humidity (measured as local mean annual rainfall) (Guillén *et al.* 2000). Similarly, bats living in dry areas tend to have higher call frequencies than expected on the basis of body size (Heller & von Helversen 1989), and rain forest species have lower frequency calls than similarly sized species living in drier habitats (Guillén *et al.* 2000).

The distribution of *Rhinolophus* species using high- or low-frequency echolocation calls may provide some insight into the influence that environmental factors have on call frequency. For example, humidity may favour individuals using certain frequencies such that bats using low frequencies should occur in

regions where humidity is high, and bats of equivalent size using high frequencies should occupy less-humid areas.

Although the distributional limits of a species are set by a species' ecological attributes (Ridley 1996), ecological and environmental factors alone cannot fully explain the current distribution of species. Historical factors need to be considered because distributions may have been influenced through past climatic events (e.g. glaciations), and can be altered through dispersal (Ridley 1996).

Rhinolophids occur throughout the Old World in the Afrotropical, Eurasian, Oriental, and Australasian biogeographic regions (Csorba *et al.* 2003). A few species have very large-scale distributions (e.g. *R. ferrumequinum* and *R. clivosus)* and a few occur in more than one biogeographic zone (e.g. *R. blasii* and *R. megaphyllus*), but most are confined to a single biogeographic region (Csorba *et al.* 2003). Ten species of *Rhinolophus* are distributed throughout the major biomes in South Africa (Skinner & Smithers 1990; Taylor 2000; Csorba *et al.* 2003). Of the ten species, *R. clivosus* has the widest distribution, ranging from the south-western Cape through to Egypt and into the Arabian Peninsula (Skinner & Smithers 1990; Taylor 2000; Csorba *et al.* 2003). *Rhinolophus capensis* probably has the most restricted distribution, being confined to the Cape Floristic Kingdom (Fynbos biome) at the south-western tip of South Africa (Skinner and Smithers 1990; Taylor 2000; Csorba *et al.* 2003).

Three hypotheses have been proposed for the centre of origin of the horseshoe bats. One hypothesis proposes that the rhinolophids originated in the humid tropical rainforests of south-east Asia (Bogdanowicz 1992), with subsequent dispersal into Europe and Africa. A second hypothesis proposes that they originated in Europe, with subsequent immigration into Africa and Asia (Guillén *et al.* 2003). The third proposes that the Rhinolophidae originated in Africa and subsequently colonised the remainder of the Old World (Eick *et al.* 2005).

In this chapter I use the phylogeny recovered in Chapter 2 to identify the centre of origin for the Rhinolophidae and, by superimposing call frequencies and

habitat on this phylogeny, attempt to determine whether the Acoustic Adaptation Hypothesis is an adequate explanation for the subsequent evolution of call frequencies. On a more local scale I investigate whether the distributions of the low-frequency South African rhinolophids (*R. hildebrandti* and *R. fumigatus*) correspond with habitats characterized by higher humidity (and thus experiencing greater sound attenuation) than those occupied by other South African rhinolophids.

In an attempt to better understand the relationship between echolocation frequency and environmental factors linked to species' distributions, I will address the following questions:

1) Where did the Rhinolophidae originate?

2) Does the distribution of low-frequency rhinolophids correspond with humid regions?

3) Do South African rhinolophids using high-frequency echolocation calls show patterns in their distribution ranges that correspond with habitat structure?

METHODS

BIOGEOGRAPHICAL ANALYSES

The phylogenetic tree based on the supermatrix data set (Chapter 2, Fig. 2.5), together with the dates of species' divergence estimated using the relaxed Bayesian clock method (Chapter 2), was used to reconstruct ancestral distributions of extant rhinolophids. For the dispersal-vicariance (DIVA) analysis I used DIVA version 1.1 (Ronquist 1996). Four geographic areas were defined based on the main zoogeographic regions (Cox 2001), namely Afrotropical (A), Eurasian (B), Oriental (C), and Australasian (D). The current distribution of each species was coded as present or absent in each of the geographic areas. Where species occur in more than one zoogeographic region both regions were included in the analyses. Where species occur on or close to the boundary of each region, analyses were done in which both regions were included unless the species distribution did not extend into the region further than the boundary. Current

species distributions were obtained using the distribution data and maps in Csorba *et al.* (2003). The analysis was run without constraining the number of "maxareas" (the maximum number of unit areas allowed in ancestral distributions) following Eick *et al.* (2005). To represent distributions of species among the four zoogeographic reqions, current species distributions were mapped onto the supermatrix tree using MacClade 4 version 4.07 (Maddison & Maddison 2005). Both accelerated (ACCTRAN) and delayed (DELTRAN) transformations were used and species' distributions were unordered.

South African rhinolophid distributions

To investigate the distribution of South African rhinolophids in relation to vegetation biome and rainfall seasonality, distribution maps were created using ArcView GIS (version 3.3). Data on species' distributions were obtained from Keith (2004) and from field trips undertaken during my studies. Bat distributions were mapped onto vegetation biome and rainfall seasonality overlays.

Vegetation biomes were chosen as a surrogate for various climatic conditions. Climate plays an important role in shaping vegetation patterns (Akin 1991) on both a local and sub-continental scale. Differences in climate are reflected in vegetation, with biomes being characterized by variations in rainfall, temperature, and humidity, and the interactions between these variables (Schulze 1997). Temperature and water appear to be the most important climatic factors that affect plant form and thus vegetation structure (Box 1981; Rutherford & Westfall 1986). A correspondence between vegetation and animals should exist because vegetation determines the structural nature of a habitat (Odum 1971). Furthermore, biomes have evolved over long periods of time and may better reflect the environmental conditions bats were exposed to in evolutionary history. Present-day vegetation distributions within southern Africa reflect climatic changes that have occurred since the Cretaceous (Partridge 1997). I have chosen not to use rainfall and temperature records because these records (which are, in any case, spatially patchy) usually do not go back for more than a few decades (Rutherford & Westfall 1986) and are not good representations of

conditions (including humidity) experienced by the bats over their evolutionary history.

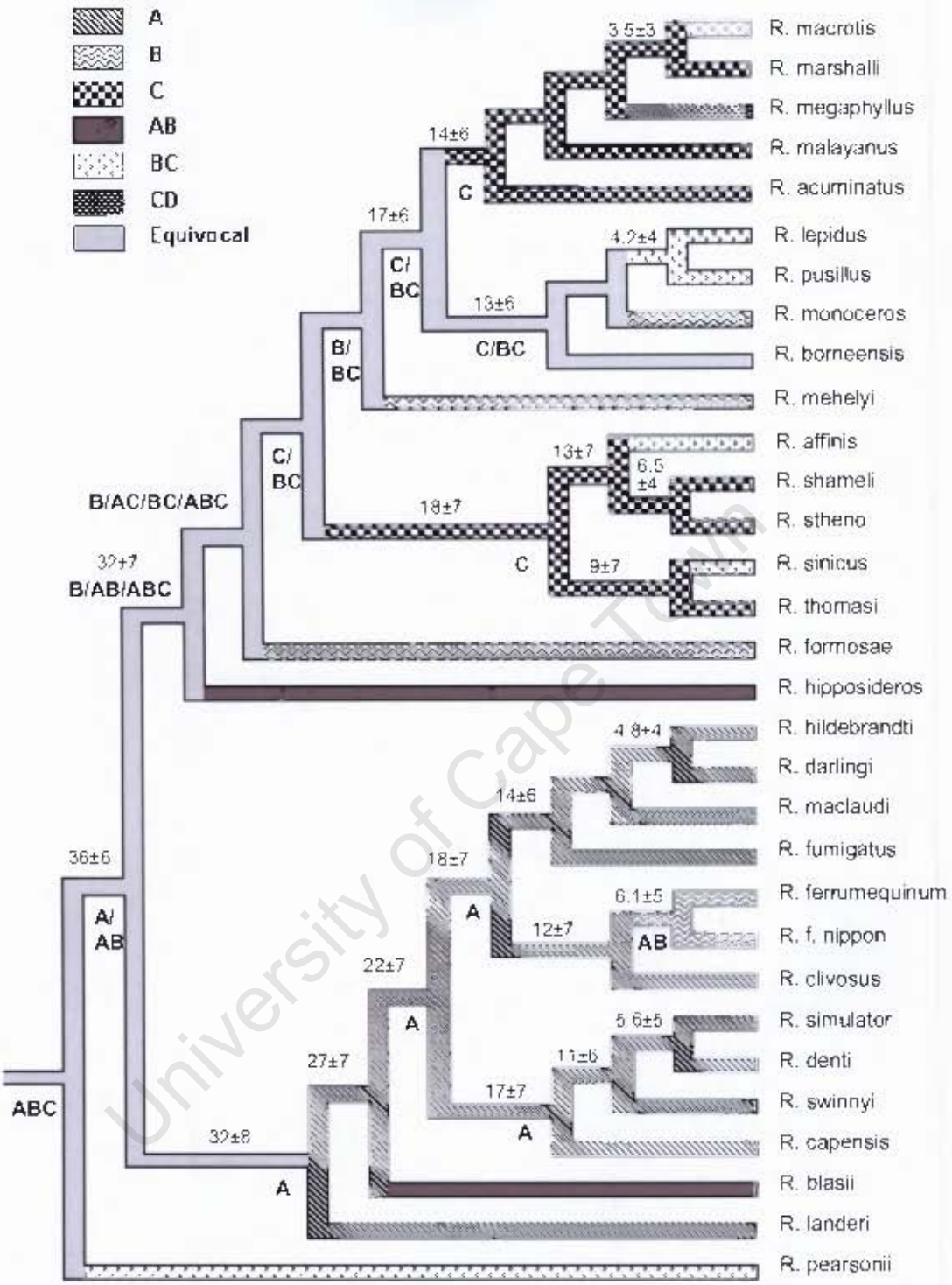
RESULTS

HISTORICAL BIOGEOGRAPHY OF THE RHINOLOPHIDAE

Results from the DIVA analysis cannot place the centre of origin for the rhinolophids. Based on this analysis, the Rhinolophidae are equally likely to have originated in the Afrotropical, Eurasian or Oriental biogeographic regions. The optimal reconstruction required 13 dispersal events (Fig. 5.1). The rhinolophids split from the hipposiderids during the Eocene (Fig. 5.1; Chapter 2), with subsequent radiation during the Miocene into the six subgenera defined by Guillén *et al.* (2003). Using insertion and/or deletion (indel) events in the nuclear introns sequenced in Chapter 2, it is likely that the Rhinolophidae originated in Asia. Within the African clade, *R. landeri* occupies the most basal position, followed by *R. blasii* (Fig. 5.1; Fig. 2.6, Chapter 2). Indels which are shared by bats in the Oriental clade are also shared with *R. landeri* and *R. blasii*, but are not present in the rest of the African taxa (Table 2.5, Chapter 2). For the THY nuclear intron, two independent, three-base-pair (bp) insertions are shared by the Oriental taxa and *R. landeri* and *R. blasii*. A 6 bp insertion and an 8 bp insertion are shared by the Oriental taxa and *R. landeri*. Furthermore in the PRKC1 nuclear intron a 4 bp insertion characterizes the Oriental taxa and is also present in *R. landeri* and *R. blasii*. These indel events are additionally shared with taxa in the genera *Hipposideros* (Hipposideridae) and *Triaenops* (Hipposideridae), as well as with *Rhinopoma hardwickei* (Rhinopomatidae) and *Rousettus aegyptiacus* (Pteropodidae) – taxa in two families within the Pteropodiformes to which the Rhinolophidae belong. Among the African rhinolophids these shared indels are found only in *R. landeri* and *R. blasii*, suggesting that the African rhinolophids are more derived than their Oriental counterparts.

Horseshoe bats are clutter-foragers and their preferred habitats are dominated by either forest or woodland (Csorba *et al.* 2003). The majority of species within the Oriental clade occur in forest habitat, whereas the majority of species within the (more derived) African clade occur in woodland or savanna-woodland habitats (Fig. 5.2). However, despite the fact that the majority of species within the Oriental clade occupy forested habitats, some of these species echolocate at low frequencies, i.e. below 60 kHz (Fig. 5.2; Fig. 3.2, Chapter 3).

There appeared to be no relationship between call frequency and biome. Most Asian and African rhinolophids echolocate between 61 and 110 kHz, even though the former are found in forests and the latter in savanna woodland. Similarly, the Asian (*R. macrotis* and *R. marshalli*) and African (*R. hildebrandti* and *R. fumigatus*) species which echolocate at lower frequencies (20-60 kHz) also occur in forest and savanna woodland, respectively. Finally, African *R. landeri* and *R. denti* both use frequencies > 110 kHz but the former occurs in tropical forests and the latter in arid habitat.



latter in arid habitat.

Fig. 5.1 Summary of the optimal reconstruction of ancestral distributions of extant *Rhinolophus* species. At each node the optimal distribution is given with alternative equally optimal distributions separated by a forward slash. Biogeographical regions correspond to the Afrotropics (A), Eurasia (B), the Orient (C), and Australasia (D). Combined letters indicate species whose distributions span more than one biogeographic region. Estimates of divergence times (MYA) from a relaxed Bayesian clock (Chapter 2) are indicated above nodes.

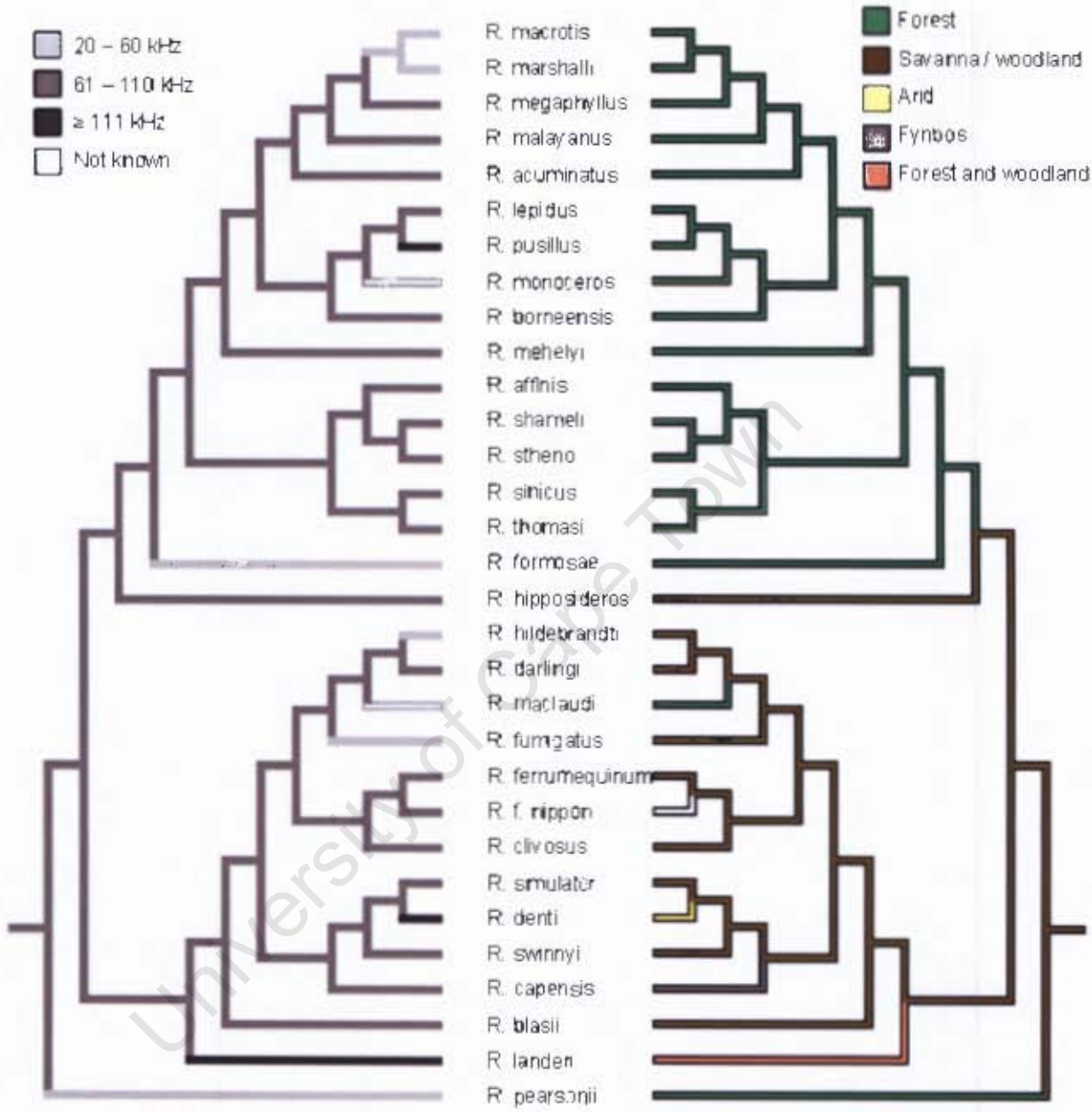


Fig. 5.2 Summary of the optimal reconstruction of habitat preference of *Rhinolophus* species in relation to echolocation frequency. Echolocation data are from Chapter 3, data on the habitat of *Rhinolophus* species were obtained from the literature (Bontadina *et al.* 2002; Csorba *et al.* 2003; Russo *et al.* 2005; Kingston *et al.* 2006).

Echolocation and rhinolophid distributions in South Africa: support for the Acoustic Adaptation Hypothesis?

Maps relating species' distributions to vegetation biome and rainfall seasonality are provided in Fig. 5.3 and Fig. 5.4 respectively. The most speciose biome is the Savanna where all nine *Rhinolophus* species (except the Fynbos-endemic *R. capensis* - Walker 2006; Jacobs *et al.* 2007) occur. *Rhinolophus clivosus* has the broadest distribution and occurs in all biomes, and *R. darlingi* has a broad distribution, but is absent from the Fynbos biome. Although some species' distributions do extend into the grasslands and/or the Nama-Karoo, no species have their distributions restricted to these biomes.

Closely related species appear to have allopatric or parapatric ranges within South Africa. For example, the sister taxa *R. denti* and *R. simulator* do not overlap in their ranges, and there is only a small overlap between *R. capensis* and *R. swinnyi* where the Fynbos, Thicket and Nama-Karoo biomes meet. The distribution of *R. swinnyi* does not, however, extend into the region characterized by year-round rainfall (Fig 5.4b), in which *R. capensis* does occur.

Within South Africa, bats using high frequencies and low frequencies co-occur in the same biome. Both *R. fumigatus* (53.8 kHz, this study) and *R. hildebrandti* (34.4 kHz, this study) occur in the savanna biome, along with the high-frequency *R. landeri* (107.3 kHz, this study). Both *R. fumigatus* and *R. hildebrandti* were caught at the same locality in Pafuri, suggesting they forage in similar habitats, whereas *R. landeri* was caught at a different location within the Pafuri area. Throughout its African distribution, *R. fumigatus* is restricted to open woodland habitats (Csorba *et al.* 2003) in which its low-frequency calls provide enhanced detection distance.

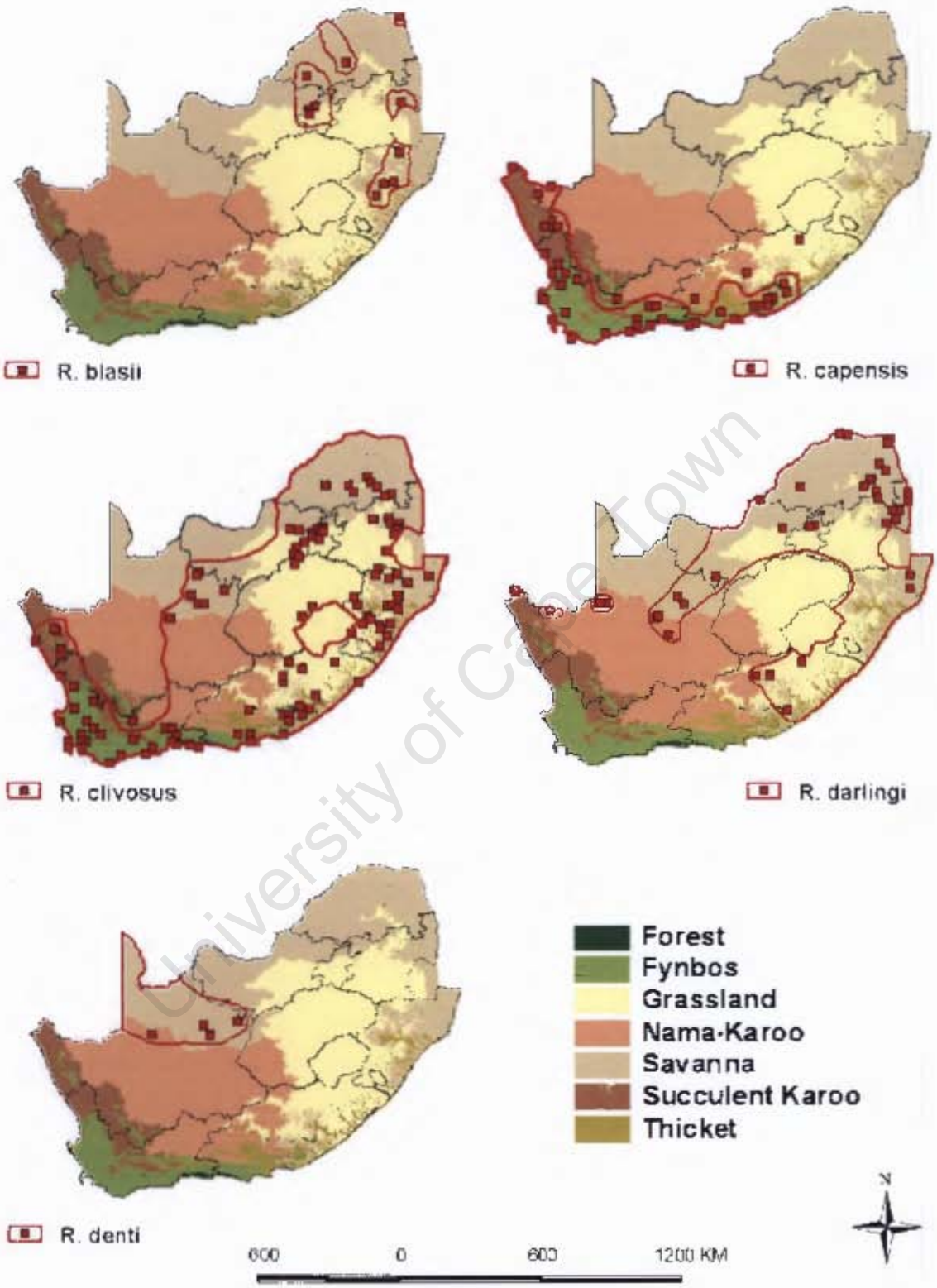


Fig. 5.3a Distribution of *Rhinolophus* species in South Africa in relation to vegetation biome.

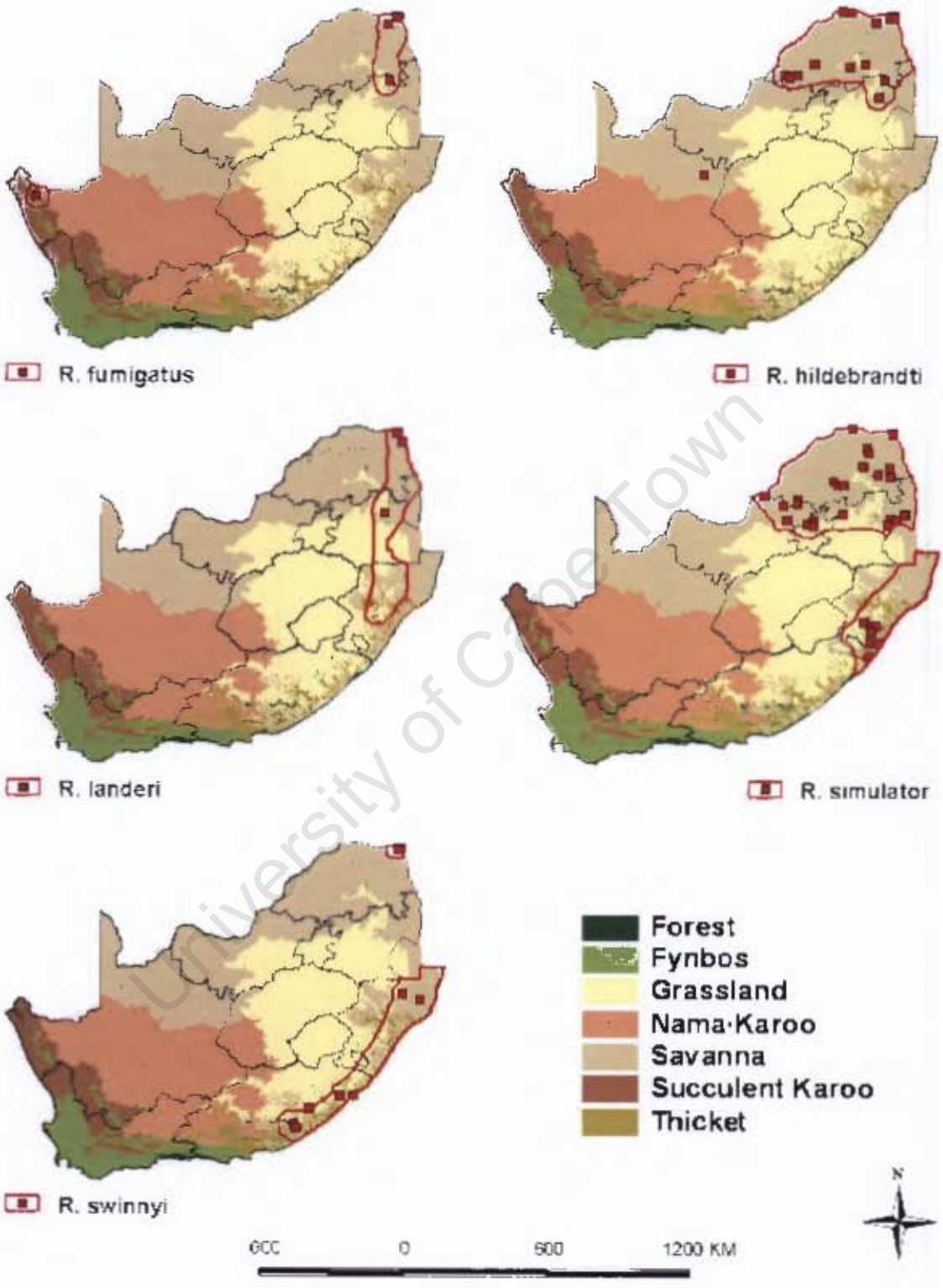


Fig. 5.3b continued.

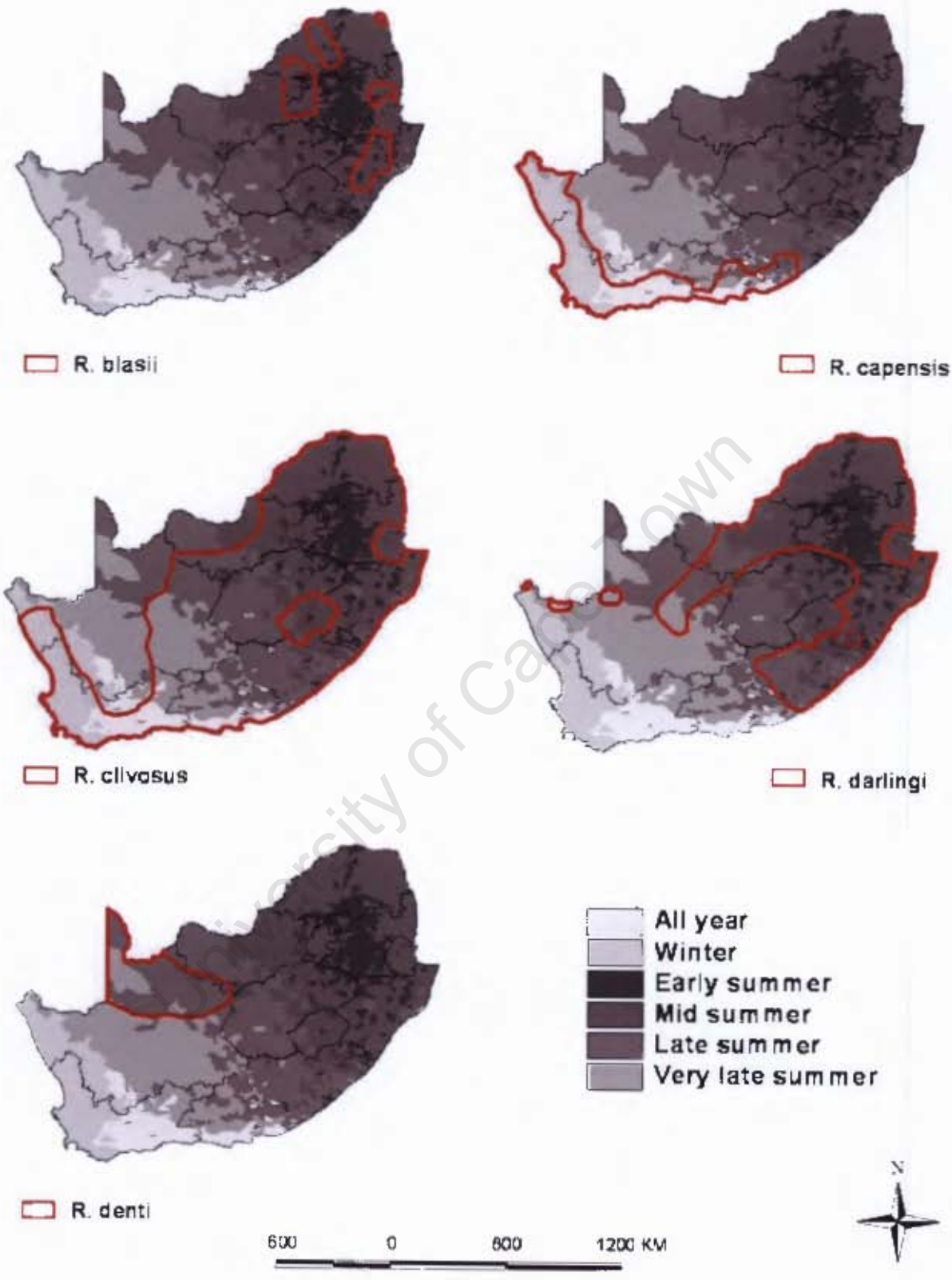


Fig. 5.4a Distribution of *Rhinolophus* species in South Africa relative to rainfall seasonality. Rainfall seasonality from Schulze *et al.* (2006).

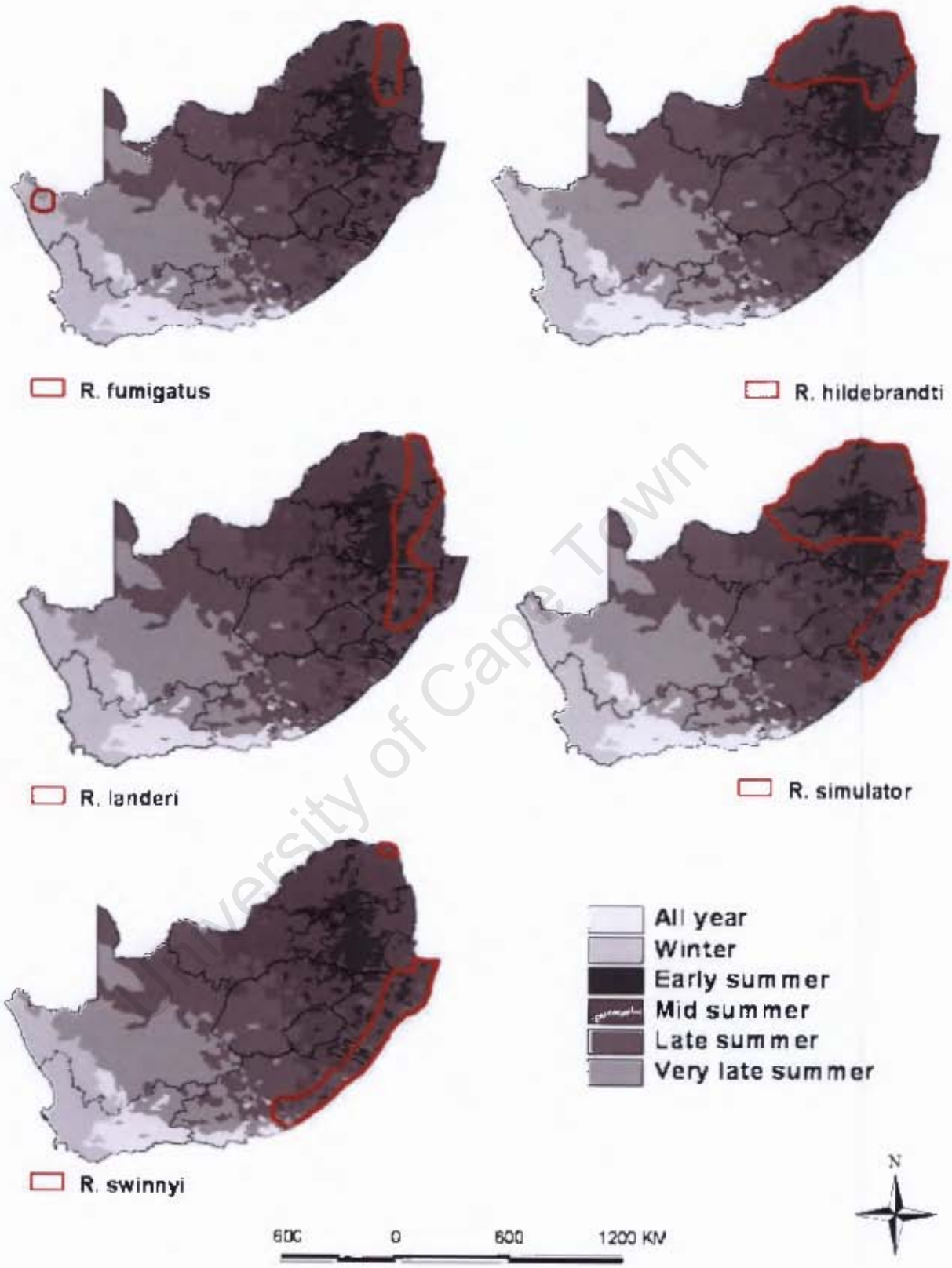


Fig. 5.4b continued.

Two of the South Africa species which echolocate at the highest frequencies, *R. denti* (110.9 kHz, this study) and *R. swinnyi* (106.9 kHz, this study), have disjunct distributions. *Rhinolophus denti* occurs in the arid Nama-Karoo and Savanna region of South Africa, characterized by late summer rainfall. Although occupying an arid habitat, *R. denti* was observed foraging close to vegetation or just above the ground. On the other hand, *R. swinnyi* occurs along the eastern side of South Africa in the more mesic Thicket biome, where rainfall peaks in early to mid summer (Figs 5.3b, 5.4b), yet also forages close to the vegetation or just above the ground (E. Kelly, personal communication).

Within southern Africa, another high-frequency echolocator, *R. landeri* (107.3 kHz, this study), occurs in savanna woodland habitats, but in western Africa it is a forest-dwelling species (Csorba *et al.* 2003). The difference in echolocation frequency between these two regions could be interpreted as meaning that the higher frequencies used by *R. landeri* in West Africa (121 kHz, Roberts 1972), serves to provide greater resolution in the more cluttered forest (vs. savanna) habitats. However, at such high frequencies, the 13 kHz difference equates to a very small difference in wave-length (*ca* 0.37 mm) and is unlikely to result in marked differences in the discrimination of two objects

Although some co-occurring rhinolophid species do use similar echolocation frequencies, others do not. *Rhinolophus blasii*, *R. darlingi* and *R. simulator* use similar echolocation frequencies of *ca* 80-88 kHz (Table 3.1, Chapter 3) and all occur within the savanna biome. *Rhinolophus darlingi* does however, extend into more arid regions (Fig 5.3) with populations occurring within Nama-Karoo, and Namibia. With the exception of *R. capensis*, all *Rhinolophus* species within South Africa occur in regions characterized by mid-to-late summer rainfall (Fig. 5.4). Only *R. clivosus* occurs in regions with rainfall seasonality ranging from summer, through all-year, to winter. *Rhinolophus capensis* is the only rhinolophid in South Africa absent from summer rainfall areas, and apart from *R. clivosus* is the only species that occurs in winter and year-round rainfall regimes.

As was the case with rhinolophids in general, for southern African rhinolophids no pattern is evident between echolocation frequency (high or low) and the vegetation biome in which a species occurs. Similarly, there is no clear link between echolocation frequency and the rainfall seasonality that defines a given region.

DISCUSSION

HISTORICAL BIOGEOGRAPHY OF THE RHINOLOPHIDAE

The sparse fossil record for bats (Hand 1984) has made it difficult to reconstruct the area of origin for the Chiroptera, and as a result many, often-opposing hypotheses exist. Paleontological evidence proposes that the Chiroptera originated in Laurasia (Cracraft 1973), with the fossil record biased towards Europe (Savage & Russel 1983). Recent studies propose that bats originated in Laurasia (Teeling *et al.* 2005) or in Africa (Eick *et al.* 2005).

In searching for the geographical origins of the Rhinolophidae, morphological analyses have been inconclusive, suggesting either an African or Asian origin for the rhinolophids (Bogdanowicz 1992; Bogdanowicz & Owen 1992). By contrast, molecular analysis of cytochrome *b* suggests a European origin for the family Rhinolophidae with subsequent expansion into Asia and Africa (Guillén *et al.* 2003). More recently, Eick *et al.* (2005) also made use of molecular characters (necessarily confining analyses to extant bat families), whereas Teeling *et al.* (2005) made use of both molecular and morphological characters, thus permitting the inclusion of extinct, fossil taxa. These studies also made opposing hypotheses about the origins of the Rhinolophidae. Eick *et al.* (2005) propose an African origin for the Rhinolophidae, whereas ancestral reconstructions by Teeling *et al.* (2005) support an Asian origin for the suborder of bats (Pteropodiformes or Yinpterochiroptera) that includes the Rhinolophidae.

My results date the divergence between the Rhinolophidae and Hipposideridae at *ca* 40.5 MYA (Chapter 2): this is in agreement with previous studies placing the divergence during the middle Eocene, between 55 and 37 MYA (Maree & Grant 1997; Teeling *et al.* 2003; Eick *et al.* 2005; Teeling *et al.* 2005). Results from the DIVA analysis are equivocal, suggesting an equally likely African, Eurasian, or Oriental origin for extant rhinolophid taxa. However, evidence from indels in two nuclear introns (Chapter 2) suggests that the African taxa are more derived and that the Rhinolophidae are likely to have originated in the tropical rainforests of Asia during the Eocene.

This finding is in keeping with other evidence that suggests Asia as the centre of origin for early mammals (Bowen *et al.* 2002). Furthermore, eastern Asia, and in particular south-eastern Asia experienced a phase of increased faunal endemism during the Late Eocene *ca* 37-33 MYA (Tsubamoto *et al.* 2004). Both the Rhinolophidae and Hipposideridae are characterized by high-frequency echolocation calls which are well adapted for cluttered habitats such as the tropical forests which characterized both Africa and Asia during the Early to Middle Eocene, *ca* 50 MYA (Janis 1993), when rhinolophids evolved. The warm climate during this period facilitated a continuous flora comprising both tropical and sub-tropical forests extending throughout Eurasia (Wolf 1975). This forest corridor would have allowed ancient rhinolophid species to disperse throughout the southern Palaearctic towards Europe and into Africa, where the basal lineages within the African clade (e.g. *R. landeri*; Chapter 2) are thought to have been linked to tropical forest habitats (Guillén *et al.* 2003).

Rhinolophids are incapable of crossing large bodies of water (Koopman 1970) and would therefore, along with other, larger land mammals, have required land-bridges between continents to facilitate faunal dispersal and colonization. During the Late Eocene to Early Oligocene (*ca* 34 MYA), the disappearance of the Obik Sea, which separated Asia and Europe (Cox 2000), would have provided a land bridge by which many mammals, including bats, could have moved from Asia into Europe. The likely immigration into Europe by rhinolophids during this period is supported by fossil data. Hand & Kirsh (1998) propose that *Rhinolophus* may be

the sister group to *Vaylatsia*, an extinct genus with fossils from the Late Eocene to Early Oligocene (*ca* 34 MYA) in Europe. Another fossil genus, *Palaeonycteris,* assumed to be closely affiliated with the modern-day *Rhinolophus* genus, dates back to the Middle Miocene (*ca* 20 MYA) of Europe (Hand 1984). Rhinolophids were well established in Europe by the Miocene, as supported by numerous *Rhinolophus* fossils from Middle Miocene deposits, particularly in Bavaria (Ziegler 2003).

The uplift of the Higher Himalaya during the Miocene (*ca* 17 MYA - Amano & Taira 1992), and the uplift of the Himalaya-Tibetan plateau during the Late Miocene (*ca* 10 MYA - An *et al.* 2001) would have acted as a large ecological barrier for dispersing species, as it does today for migrating birds (Irwin & Irwin 2005). By the Late Miocene, the tropical forests of Africa had receded to the equatorial regions and were replaced in the north by Mediterranean-type woodland, and in the south by paratropical forests (forests with a dry season) and woodland savanna (Janis 1993). At the same time the oriental regions of Asia were still characterized by tropical forest in the south, and paratropical forests to the north (Janis *op. cit.*). Furthermore, the cooling which occurred during the Late Tertiary and Early Pleistocene would have resulted in the fragmentation of forest habitat throughout Eurasia, with many forest patches being forced southward (Wolfe 1975; Tiffney 1985). This suggests that any further dispersal of rhinolophid species would have occurred in the southern Palaearctic and Oriental regions where suitable habitat was available, such as the forests which remained in Central China, Japan, southern India, and south-east Asia (Janis 1993). Extant species diversity in the Oriental region is high, with many rhinolophid species in India, mainland and insular south-east Asia, including the island archipelagos of Indonesia (Csorba *et al.* 2003). The most plausible explanation for the distribution of the rhinolophids throughout these latter islands would be that they colonized islands on the south-east Asian continental shelf during periods of low sea levels when the islands were connected to the mainland by land bridges (Cox 2000), and when Indonesian islands were repeatedly linked with Asia (Voris 2000; Inger & Voris 2001).

Only two rhinolophid species occur in Australia, both of which have distributions extending into the Oriental region and surrounding islands (Csorba *et al.* 2003). As is assumed of other bat species (Hamilton-Smith 1974; Flannery 1989), *R. megaphyllus* and *R. philippinensis* probably entered Australia from Asia using the islands as 'stepping stones'.

Dispersal into and out of Africa

The disappearance of the Obik Sea would have allowed immigration into Africa from Asia during the Early Oligocene (*ca* 34 MYA), a time during which the ancestor to the African rhinolophid clade existed (Approximately 32 MYA; Fig. 5.1). It is likely that the rhinolophids entered via the forest corridor linking Africa with Asia (but see Butler 1978). Many groups of mammals (e.g. Juste *et al.* 1999) and some birds (e.g. Bowie 2003) also used this forest corridor to enter Africa. It is unlikely that rhinolophids entered Africa via Gibraltar because this land-bridge was not available until the Late Miocene (*ca* 10.MYA) (Steiniger *et al.* 1985), by which time many sub-Saharan *Rhinolophus* species had already evolved (Fig. 5.1).

The most basal lineages within the African clade comprise species linked to tropical forest habitats, such as *R. landeri* (Guillén *et al.* 2003; Chapter 2), which possibly entered Africa via the forest corridor. Global cooling began in middle Miocene (Zachos *et al.* 2001), leading to increased seasonality. This in turn resulted in the contraction of forests and the expansion of savanna woodlands and grasslands (Janis 1993; Lindsay 1998). It is plausible that the radiation of African taxa from forest ancestors occurred in the more arid areas in the eastern and southern parts of Africa, where they are presently distributed.

Multiple dispersals into and out of Africa have been recorded for fruit bats (Juste *et al.* 1999), the African tree frog, *Chiromantis* (Wilkinson *et al.* 2002), and many species of bird (Voelker 1999, 2002; Bowie 2003). The same may be true of some African rhinolophids.

Rhinolophus blasii is also basal within the African clade and has a distribution which extends throughout eastern and south-eastern Africa, the Mediterranean, Middle East and Morocco (Csorba *et al.* 2003). It is likely that *R. blasii* evolved within the more open habitats of Africa and dispersed into Europe. Its disjunct distribution in North Africa (Csorba *et al.* 2003) may be due to a second dispersal event into Africa across the Gibraltar land-bridge, *ca* 10 MYA (Steiniger *et al.* 1985).

Rhinolophus clivosus, which is closely related to other African rhinolophids, is distributed throughout eastern and southern Africa, Israel, Jordan and Arabia (Csorba *et al.* 2003). This species would have dispersed from Africa to Arabia, possibly via the Red Sea land bridges, during the middle to late Miocene (Orszag-Sperber *et al.* 2001), or more recently via the Sinai Peninsula or across the Bab-el-Mandeb Strait in the southern Red Sea (Fernandes *et al.* 2006). *Rhinolophus ferrumequinum*, the sister taxon to *R. clivosus*, has one of the largest rhinolophid distributions, extending throughout the southern Palaearctic and northern Africa. It is plausible that the occurrence of *R. ferrumequinum* in Africa is due to dispersal via the Gibralter land-bridge during the Late Miocene (Steiniger *et al.*1985). This is in concordance with a fossil Morrocan rhinolophid from the Mio-Pliocene *ca* 1.8 MYA that is assumed to be closely related to *R. ferrumequinum* (Lavocat 1961).

South African rhinolophids

The onset of global cooling in the middle Miocene, and the sharp increase in seasonality during the Miocene/Pliocene boundary resulted in many seasonal woodland/ forest habitats being replaced by more open woodland (Cerling *et al.* 1997). The changes in vegetation and the increased habitat diversity within the Miocene may have facilitated the radiation of the African rhinolophids, particularly south of the Sahara. *Rhinolophus capensis*, *R. darlingi*, *R. denti*, *R. fumigatus*, *R. hildebrandti*, *R. simulator* and *R. swinnyi* are probably endemic to sub-Saharan Africa, and a result of two separate radiations (Fig 2.6, Chapter 2).

Two sibling species pairs (Fig 2.4, Chapter 2) occur within South Africa: *R. capensis* and *R. swinnyi*, and *R. simulator* and *R. denti*. Their present distributions are mostly allopatric, with an area of contact between three species occurring in Northern Zimbabwe (Csorba *et al.* 2003).

The distribution of *R. swinnyi* extends from Tanzania south to South Africa. This is a much more extensive range than that of its sister taxon *R. capensis*, which is endemic to the Fynbos biome. The disjunct ranges of these taxa make it likely that changes in rainfall seasonality and vegetation led to their speciation. Although now confined to the Cape Floristic Region (Fynbos biome; Skinner & Smithers 1990; Taylor 2000), the historical range of *R. capensis* appears to have been much more extensive within South Africa, with a fossil from Makapansgat, in north-eastern South Africa (and far from the Fynbos) dated to the Late Pliocene (De Graaf 1960). Rainfall seasonality appears to be an important factor separating the distributions of these species today (Fig. 5.4).

Rhinolophus simulator occurs from the Sudan south towards the savanna biome of southern Africa. Its sibling taxon *R. denti* is more restricted to arid regions of the western part of southern Africa, but their ranges overlap in northern Zimbabwe (Csorba *et al.* 2003). It is likely that these species diverged from an ancestor in southern Africa, with *R. denti* moving into more arid habitats, and *R. simulator* remaining in savanna woodland areas. The arid Karoo shrublands may have acted as a barrier preventing these species from meeting within South Africa.

Within South Africa, *R. darlingi* has a widespread distribution. This species may be separated from *R. capensis* by the plateau. It is likely that *R. darlingi* spread to the arid western regions of southern Africa via river systems in Botswana and South Africa. The Karoo acts as a present day barrier preventing movement between populations from this arid region and the eastern side of South Africa, which may account for the large sequence divergence values between these populations (Table 2.9, Chapter 2).

ECHOLOCATION FREQUENCY AND DISTRIBUTION: THE ACOUSTIC ADAPTATION HYPOTHESIS

Throughout the Old World, the majority of *Rhinolophus* species occur in either forested or woodland areas. The high call frequencies used by these bats are well suited to foraging in these cluttered habitats. Bats in the Oriental clade use both high and low frequencies, and many of them occur in the tropical rainforests of south-east Asia. It is likely that the ancestral condition of high frequencies (Chapter 3) arose in the ancestors of Rhinolophidae to facilitate foraging in cluttered habitats. Many species living in the tropical forests of the Oriental region use frequencies between 60 kHz and 90 kHz and as such, humidity may not exert huge selection pressure on these bats through increased atmospheric attenuation. However, high-frequency sounds experience the greatest atmospheric attenuation, with a rapid increase in attenuation for frequencies above 90 kHz (Lawrence & Simmons 1982). The fact that the upper frequency used by the Oriental bats appears to be capped at 90 kHz suggests that humidity may be imposing one selective pressure – *viz* the upper limit to echolocation call frequency. On a global, present-day scale, neither habitat nor vegetation biome explains the variation in high frequencies seen among the rhinolophids, nor do they explain why some species have calls higher or lower than would be predicted based on body size.

On the basis of rainfall seasonality and vegetation biomes, no clear pattern exists to explain the distribution of high- and low-frequency calls among South African rhinolophids. This suggests that the environmental variables which define vegetation biomes on a regional scale (rainfall quantity and seasonality) are not driving the evolution of high or low frequencies within the rhinolophids. The question thus remains as to why species occupying similar habitats are using very different frequencies. An explanation may lie in the way in which different species use their foraging habitats. Within a particular biome, many different types of habitat exist on meso- and micro-scales. Even in very open areas, bats which forage close to the available vegetation or the ground are 'experiencing' a

cluttered environment. Within England, cryptic species of pipistrelle (*Pipistrellus pipistrellus* and *P. pygmaeus*) are characterized by different echolocation frequencies, and select different foraging micro-habitats (Davidson-Watts *et al.* 2006) within the same geographical area. Thus, within a particular region, the selection of different foraging habitats may be driving the evolution of divergent high frequencies, particularly if habitat is placing a selective pressure on wing design (because wing design and echolocation call have been presumed, in the past, to form an adaptive complex). Small differences in wing design can influence flight performance (Bogdanowicz 1992) and may therefore define optimum foraging habitat.

However, rhinolophids are clutter-specialists (Findley *et al.* 1972; Norberg & Rayner 1987; Jones & Rayner 1989) and their wing design and high-frequency, high-duty cycle echolocation calls are adapted for foraging close to or within dense vegetation. Even species that occur in more arid habitats (e.g. *R. denti*), in which vegetation is sparser, use their habitat in ways which lead them to experience the habitat as being cluttered.

In conclusion, rhinolophids arose in tropical forests to which their wing design and high-frequency echolocation calls are well adapted. However, differences in habitat structure and climatic variables cannot explain differences in echolocation call frequency and therefore the Acoustic Adaptation Hypothesis cannot explain the higher-than-expected call frequencies used by this family of bats. In the next chapter I explore alternative explanations for the high frequencies used by rhinolophids.

CHAPTER 6

THE EVOLUTION OF HIGH FREQUENCIES: THE FORAGING HABITAT AND ACOUSTIC COMMUNICATION HYPOTHESES

INTRODUCTION

Aerial insectivorous bats forage in habitats ranging from the open (i.e. uninterrupted airspace) to the cluttered (i.e. within dense vegetation). In analyses of habitat use, three general habitats have been recognized - open, clutter/edge and clutter (summarised by Schnitzler & Kalko 1998).

It has been suggested that wing morphology may be the primary factor structuring bat communities (Aldridge & Rautenbach 1987). However, wings and echolocation calls have previously been assumed to form part of the same adaptive complex (Aldridge & Rautenbach 1987), and morphology, in conjunction with echolocation call parameters, can be used to predict various aspects of habitat selection and utilization by bats (Aldridge & Rautenbach 1987; Saunders & Barclay 1992; Bowie *et al.* 1999). Bats with low aspect ratios and wing loadings typically use high-frequency echolocation calls because these provide maximum resolution and directionality in cluttered habitats (Neuweiler 1989; Rydell *et al.* 1995). Behavioural flexibility may be constrained by morphology (Fenton 1995), but echolocation frequency is nonetheless predicted to vary to suit particular foraging modes (Aldridge & Rautenbach 1987).

Horseshoe bats (Rhinolophidae) forage in cluttered habitats and have a uniform foraging mode (Fenton 1972) and an echolocation system that is uniquely adapted to overcoming the perceptual problems associated with cluttered

habitats. The structure of rhinolophid echolocation calls (a long, constant-frequency component with a short frequency-modulated tail) is common to all rhinolophid species and allows for Doppler shift compensation. The use of Doppler shift echolocation enables these bats to filter the echoes of background clutter and distinguish prey echoes from background echoes by detecting the fluttering of insect wings (Schuller 1984; Neuweiler 1989; Fenton 1990; Chapter 1). Furthermore, rhinolophid calls are dominated by high frequencies which are more directional and provide greater resolution than low frequencies in cluttered habitats (Neuweiler 1989; Rydell *et al.* 1995). Although call structure is very similar across the rhinolophids, the peak frequencies of species' calls vary greatly, despite the fact that they all occupy habitats with similar physical properties (Chapter 5).

Rhinolophids arose in tropical forests to which their high-frequency echolocation calls are well adapted (Chapter 5). However some species use calls that are higher-than-predicted from allometry, and may differ according to geographic location (e.g. *R. landeri* Chapter 5). These seemingly 'aberrant' echolocation call frequencies may be due to habitat-induced selective pressures on wing morphology if wing design and echolocation call are under linked selection (i.e. form an adaptive complex). Small changes in wing morphology that allow a bat to forage more manœuvrably within a particular foraging habitat (e.g. forest vs. savanna) may be linked with small changes in echolocation frequency.

THE FORAGING HABITAT HYPOTHESIS

The foraging habitat hypothesis (FHH; Jones & Barlow 2004) proposes that echolocation and wing design have evolved to allow bats to forage successfully in their particular habitat. As a result a negative relationship should exist between echolocation frequency and wing loading (Jacobs *et al.* 2007) because high frequencies and low wing loading are suited to cluttered habitats and high wing loadings and low frequencies are better adapted to open habitats. I will test this prediction using rhinolophids from South Africa as well as species from Europe and Asia. Furthermore, if echolocation frequency is linked to selection acting on

wing morphology, then species which deviate from the allometric relationship between body size and peak frequency (e.g. *R. clivosus, R. fumigatus*; Chapter 4), should not deviate from the allometric relationship between peak frequency and wing loading (Jacobs *et al.* 2007). However, if echolocation frequency is being selected for independently of wing morphology, then bats deviating from the allometric relationship between peak call frequency and body size should not deviate from the relationship between body size and wing design (wing area and wingspan).

An example of a species deviating from echolocation frequency/body size allometry predictions is *R. clivosus*, which has a higher-than-expected echolocation frequency for its body size (Jacobs *et al.* 2007, Chapter 4). However, *Rhinolophus clivosus* does not deviate from the allometric relationship between body mass and either wingspan or wing area. This suggests that echolocation call is being selected for independently of wing design (Jacobs *et al.* 2007). Provided *R. clivosus* is not just an exception to the rule, other species which deviate from the relationship between body size and peak frequency should also not deviate from the allometric predictions of the relationships between body size and wing parameters. This would suggest that foraging habitat does not explain deviations in peak frequency. Furthermore, these patterns should occur for species with calls of both higher and lower frequencies than predicted from body size.

For bats that do not deviate from allometry in wing parameters but do in peak frequency, as in the case of *R. clivosus* (Jacobs *et al.* 2007), it is possible that the evolution of echolocation frequency has been influenced by sympatric bat species. Horseshoe bats are capable of identifying conspecifics by their echolocation calls (Möhres 1967) and the latter may also serve a social communication function (Barclay 1982, Leonard & Fenton 1984; Fenton 1986; Neuweiler *et al.* 1987). Thus, communities comprising more than one rhinolophid species may be partitioning their sonar frequency bands (Heller & von Helversen 1989; Guillén *et al.* 2000) to ensure effective conspecific communication, the so-called Acoustic Communication Hypothesis (ACH, Jacobs *et al.* 2007).

THE ACOUSTIC COMMUNICATION HYPOTHESIS

The Acoustic Communication Hypothesis (Jacobs *et al.* 2007) proposes that deviations in echolocation call frequency from allometric predictions based on body size which do not show corresponding deviations between body size and wing morphology, may have evolved to ensure effective communication among conspecifics. Thus, if echolocation calls evolved due to selection pressure stemming from social interactions, this should result in frequency partitioning among co-occurring species (Heller & von Helversen 1989). Social information would have to be encoded in relatively small changes in frequency for effective communication (Jacobs *et al.* 2007) because the auditory fovea of rhinolophids is closely tuned to their peak echolocation frequencies (Schuller & Pollak 1979). This is supported by the relatively small amount of variation in peak frequency between individuals of the same species for some Asian and European rhinolophids (Heller & von Helversen 1989). If echolocation frequency has evolved to ensure effective communication among conspecifics through the partitioning of sonar bands, I predict that the amount of variation in peak frequency between individuals of the same species should be small and there should be no overlap in peak echolocation frequency between South African rhinolophid species that co-occur in the same habitat. By the same token, closely related species (e.g. sister taxa) that occur in sympatry should use frequencies that do not overlap.

Kingston & Rossiter (2004) proposed that the different frequencies used by three morphs of *R. philippinensis* were due to 'harmonic-hopping'. Within *R. philippinensis* calls are arranged along a harmonic series with the largest morph using the lowest harmonic of the fundamental frequency and the smaller morphs using higher harmonics of the same fundamental frequency (Kingston & Rossiter 2004). Species within the same clade as *R. philippinensis* (e.g. *R. celebensis*, *R. virgo*, *R. megaphyllus* and *R. borneensis*) also call at differing harmonics of the large morph's fundamental frequency (Kingston & Rossiter *op. cit.*). If closely related South African species are sympatric, the evolution of frequencies which

occupy different sonar bands and are thus 'partitioned', may also have occurred through the utilization of different harmonics, i.e. via the same route of 'harmonic hopping'.

The aims of this chapter are to test the predictions made by the Foraging Habitat Hypothesis and the Acoustic Communication Hypothesis. If foraging habitat is influencing the evolution of echolocation calls through selection on wing morphology, a relationship should exist between peak echolocation frequency and wing loading. However, if no relationship exists, the evolution of call frequency may have evolved to partition frequencies to allow for efficient communication between conspecifics.

I will make use of data on the South African rhinolophids, as well as data collected from the literature for European and Asian rhinolophids to answer the following questions:

1) Does a relationship exist between wing loading and peak frequencies for the South African rhinolophids and for a global set of rhinolophids?
2) Do bats that deviate from the allometric relationship between peak frequency and body size (e.g. *R. clivosus*, *R. euryale*, *R. ferrumequinum*, *R .fumigatus*, *R. macrotis*, *R. mehelyi*, *R. stheno*, and *R. trifoliatus*; Chapter 4) show similar deviations between wing design and body size; i.e. are wing morphology and echolocation frequency under linked selection?
3) Among South African rhinolophids, is variation in call frequency within species less than variation in call frequencies between species?
4) Do sympatric South African rhinolophid species overlap in their peak echolocation frequencies?
5) Can 'harmonic-hopping' explain the different frequencies used by closely related taxa?

METHODS

THE FORAGING HABITAT HYPOTHESIS

Wing morphology

Forearm length (to nearest 0.1 mm) and body mass (to nearest 0.5 g) of each individual bat was measured, and the sex and age of the bat recorded. Photographs were taken of the extended right wing of each bat (Saunders & Barclay 1992) using a digital camera (Fuji Finepix S1PRO.E, Fuji Photo Film Co. Ltd, Tokyo, Japan or Olympus C730 digital). The camera was positioned at 90° above the wing to prevent angular distortion, so that length measurements and wing area could be calculated using the software programme, SigmaScan Pro 5 (version 3.20). The wing was extended on graph paper to provide a reference for the calibration of the software programme. I evaluated wing design using wingspan (B), wing area (S), aspect ratio ($AR=B^2/S$), and wing loading ($WL=Mg/S$ where M is body mass and g is gravitational acceleration; Norberg & Rayner 1987). Wingtip shape index ($I=Ts/TL-Ts$) was used to evaluate flight manœuvrability. A higher index indicates a more rounded wing and thus, enhanced ability to hover and to fly slowly in clutter (Norberg & Rayner 1987). Analysis of wing morphology data was done on ten randomly selected adults (five females and five males) per species. If the sample size for any species was less than ten, all individuals were included. Wing morphology data for rhinolophids which do not occur in South Africa were obtained from the literature

Statistical analyses

I used a multivariate analysis of variance (MANOVA) and post-hoc Tukey tests on wingspan, wing area, aspect ratio, wing loading and wingtip shape to evaluate interspecific differences in wing design, as well as inter-sexual differences, using species and sex as categorical predictors. Levene's test for homoscedacity and the Kolmogorov-Smirnov test for normality were used to ensure that the data met the assumptions required for the analysis of variance. I regressed wing

parameters against body mass and peak frequency for all *Rhinolophus* species for which data were available. Although forearm length was used as an indicator of body size in other chapters (e.g. Chapter 4), in this chapter body mass was used instead of forearm length because forearm length is automatically incorporated into wingspan and wing area measurements. All statistical analyses were performed using STATISTICA 7 (StatSoft 2004) unless otherwise stated. Descriptive statistics (mean, variance, standard deviation, and standard error etc.) were computed for the South African rhinolophids.

Controlling for phylogeny

I used the Comparative Analysis by Independent Contrasts (CAIC version 2.0.0; Purvis & Rambaut 1995) software to control for phylogeny when analyzing the relationships between wing morphology, body size and peak frequency (details in Chapter 4).

THE ACOUSTIC COMMUNICATION HYPOTHESIS

Echolocation data

Echolocation data were recorded from South African rhinolophids captured at various sites and under conditions described in Chapter 2. Calls were recorded according to the specifications described in Chapter 3. Echolocation sequences (a minimum of ten calls per sequence) were selected in which the signal-to-noise ratio was high.

The following parameters were measured for each call in a sequence:

1) Peak frequency (PF; kHz) - the frequency of maximum intensity determined from the power spectrum;
2) Inter-pulse interval (IPI; ms) - measured from the end of one call to the beginning of the next call in the sequence;
3) Call duration (DUR; ms) – the time from beginning to end of the call (determined from the oscillogram);

4) Bandwidth (BW; kHz) – the difference between peak frequency and the minimum frequency of the frequency-modulated tail (measured from the spectrogram); and

5) The number and peak frequency of harmonics present for each call.

Finally, frequency wavelength (FWL) was calculated as the speed of sound in air (347.65 $m.s^{-1}$) divided by the call frequency (Kingston *et al.* 2004).

To avoid pseudo-replication when comparing calls between individuals, a single call from the ten calls measured for each bat was used. The call with parameters closest to the mean of the ten calls for each bat was chosen. This ensured that actual echolocation call parameters rather than statistical ones were used in comparison between individuals. Analysis of echolocation data was done on ten randomly selected individuals (five females and five males) per species. If the sample size for any species was less than ten, all individuals were included.

Statistical analyses

Multivariate analysis of variance (MANOVA) and post-hoc Tukey tests were used on peak frequency, wavelength, bandwidth, interpulse interval and duration to evaluate interspecific differences in echolocation calls, as well as inter-sexual differences in South African rhinolophids using species and sex as categorical predictors. Levene's test for homoscedacity and the Kolmogorov-Smirnov test for normality were used to ensure that the data met the assumptions required for the analysis of variance. Standard deviations for individuals within each species were calculated using the ten calls per individual bat.

RESULTS

THE FORAGING HABITAT HYPOTHESIS

All wing parameters were normally distributed (K-S, p all >0.1) except for wing area (K-S $d=0.25$, $p<0.05$), which was $\log_{10}(x+1)$ transformed to ensure normality. Wings were significantly different among South African *Rhinolophus*

species (Wilk's λ=0.000965, $F_{(49,258)}$=15.42, p<0.001; Table 6.1), but the interactive term species*sex did not explain a significant proportion of the variation in wings (Wilk's λ=0.3014, $F_{(49,258)}$=1.40, p=0.051). Wing-tip shape was also not significantly different among species (p all >0.2), but wingspan (MANOVA, $F_{(17,56)}$=46.35, p<0.001), wing area (MANOVA, $F_{(17,56)}$=82.11, p<0.001), aspect ratio (MANOVA, $F_{(17,56)}$=2.83, p<0.01) and wing loading (MANOVA, $F_{(17, 56)}$=14.78, p<0.001) differed significantly.

Rhinolophus hildebrandti had a significantly larger wingspan (Tukey HSD test, p all <0.001) and wing area (Tukey HSD test, p all <0.001) than all other South African rhinolophids (Table 6.1). Similarly, *R. clivosus* differed significantly from all other rhinolophids in wingspan (Tukey HSD test, p all <0.03) and wing area (Tukey HSD test, p all <0.02). *Rhinolophus denti*, *R. blasii*, *R. simulator*, *R. landeri* and *R. swinnyi* all had similar wingspans (Tukey HSD test, p all >0.08) and wing areas (Tukey HSD test, p all >0.6). Similarly *R. capensis*, *R. darlingi,* and *R. fumigatus* all had similar wingspans (Tukey HSD test, p all >0.8). *Rhinolophus capensis* differed significantly from *R. hildebrandti, R. landeri,* and *R. simulator* in aspect ratio (Tukey HSD test, p all <0.02). Aspect ratios were also significantly different between *R. denti, R. landeri*, and *R. simulator* (Tukey HSD test, p all <0.04). Wing loading was significantly different among some rhinolophids. *Rhinolophus blasii* differed significantly only from *R. denti* (Tukey HSD test, p<0.005), whereas similar wing loadings were shared by *R. hildebrandti*, *R. blasii*, and *R. clivosus* (Tukey HSD test, p all>0.1), and by *R. denti, R. landeri* and *R. swinnyi* (Tukey HSD test, all p>0.2).

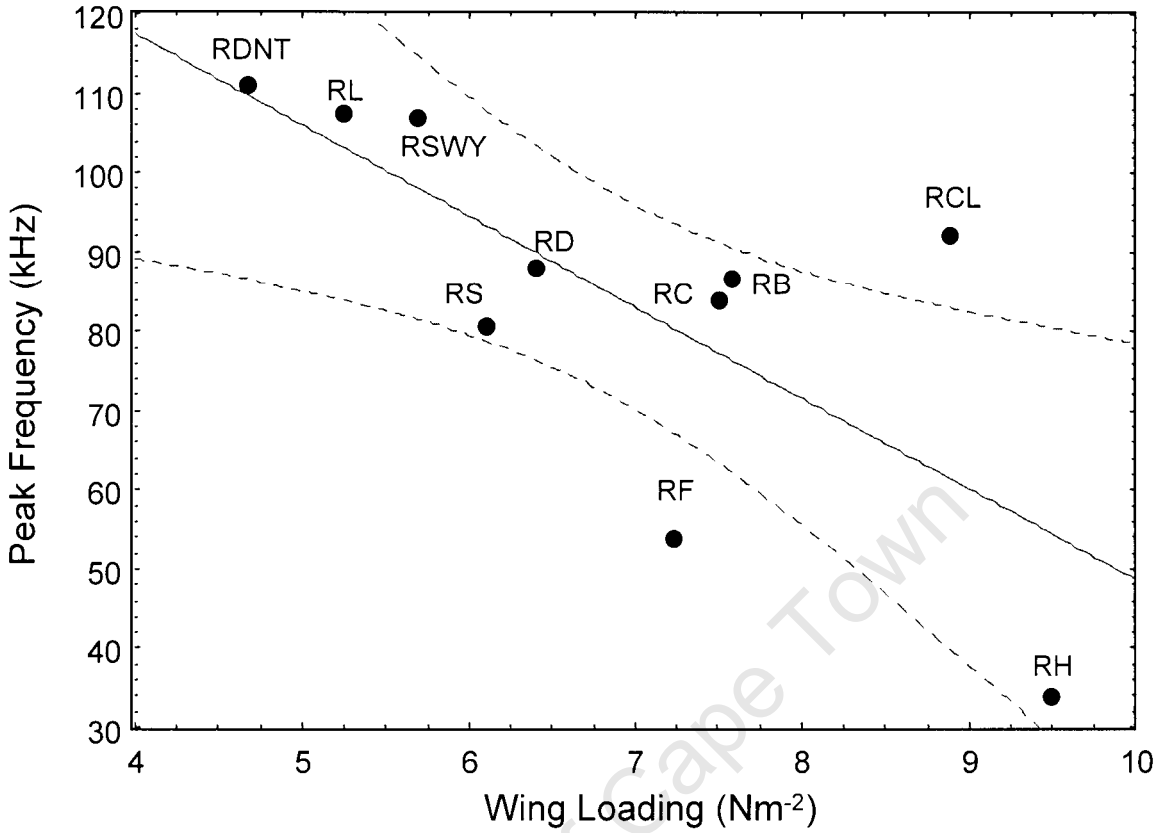


Fig. 6.1 The relationship between echolocation frequency and wing loading for South African rhinolophids. The solid line represents the line of best fit, where Peak Frequency = 163.28 – 11.46*Wing loading. Dashed lines represent the 95% confidence intervals. The species are: *R. blasii* (RB), *R. capensis* (RC), *R. clivosus* (RCL), *R. darlingi* (RD), *R. denti* (RDNT), *R. fumigatus* (RF), *R. hildebrandti* (RH), *R. landeri* (RL), *R. simulator* (RS) and *R. swinnyi* (RSWY).

Peak call frequency was inversely correlated with wing loading for South African rhinolophids (r=-0.731; $F_{(1,8)}$=9.17; p=0.016; Fig. 6.1). This relationship remained significant after controlling for phylogeny (CAIC: r=0.92, p<0.004). However if *R. hildebrandti*, which has the highest wing loading and lowest peak echolocation frequency, is removed from the analysis, peak frequency is no longer correlated with wing loading for the South African rhinolophids (r= -0.521, $F_{(1,7)}$=2.61, p=0.15).

Table 6.1 Mean ± SD of wing parameters for *R. blasii* (RB), *R. capensis* (RC), *R. clivosus* (RCL), *R. darlingi* (RD), *R. denti* (RDNT), *R. fumigatus* (RF), *R. hildebrandti* (RH), *R. landeri* (RL), *R. simulator* (RS) and *R. swinnyi* (RSWY). Ranges are given in parentheses.

WING PARAMETER	SPECIES									
	RB	RC	RCL	RD	RDNT	RF	RH	RL	RS	RSWY
	n=2	n=59	n=58	n=18	n=14	n=2	n=16	n=2	n=13	n=24
Body mass (g)	10.0 ± 1.0 (9.0 - 10.0)	11.5 ± 1.2 (9.5 -16.0)	18.4 ± 1.97 (15.0 - 23.0)	9.2 ± 1.6 (7.5 - 13.0)	5.9 ± 0.4 (5.0 - 6.5)	12.3 ± 0.4 (12.0 - 12.5)	29.0 ± 2.3 (25.0 - 33.0)	7.5 ± 0.7 (7.0 - 8.0)	7.8 ± 1.5 (6.0 - 10.5)	7.8 ± 0.5 (7.0 - 9.0)
Forearm length (mm)	46.7 ± 1.5 (45.6 - 47.8)	49.4 ± 1.02 (47.0 - 51.9)	54.2 ± 2.1 (47.0 - 57.4)	47.1 ± 1.3 (43.7 - 49.9)	43.4 ± 0.8 (42.1 - 45.0)	52.3 ± 2.1 (50.8 - 53.7)	66.8 ± 1.1 (65.4 - 68.9)	44.1 ± 0.6 (43.7 - 44.5)	45.0 ± 1.1 (42.8 - 46.5)	43.8 ± 0.9 (42.3 - 45.9)
Wing span (cm)	27.0 ± 1.0 (26.3 - 27.7)	30.7 ± 1.5 (27.6 - 34.1)	33.98 ± 1.4 (31.8 - 37.9)	28.7 ± 1.3 (26.9 - 31.9)	26.98 ± 0.8 (25.9 - 28.3)	30.8 ± 2.1 (29.3 - 32.3)	40.3 ± 1.4 (37.6 - 42.1)	26.96 ± 0.08 (26.9 - 27.02)	26.9 ± 1.08 (25.4 - 28.9)	27.7 ± 0.6 (26.5 - 28.7)
Wing area (cm^2)	130.3 ± 8.4 (124.4 - 136.3)	156.3 ± 11.8 (131.8 - 177.8)	200.3 ± 15.8 (167.9 - 230.9)	147.9 ± 10.9 (133.1 - 167.5)	121.7 ± 7.4 (106.3 - 136.4)	169.6 ± 37.5 (143.1 - 196.1)	291. 0 ± 18.2 (263.5 - 316.5)	140.06 ± 0.08 (140.0 - 140.1)	132.1 ± 8.4 (121.9 - 145.8)	132.8 ± 5.3 (123.2 - 143.9)
Aspect ratio	5.6 ± 0.1 (5.5 - 5.7)	6.05 ± 0.4 (5.08 - 6.87)	5.8 ± 0.5 (5.02 - 7.1)	5.6 ± 0.3 (5.2 - 6.3)	5.98 ± 0.2 (5.7 - 6.2)	5.7 ± 0.5 (5.3 - 6.0)	5.6 ± 0.2 (5.2 - 5.9)	5.2 ± 0.04 (5.16 - 5.2)	5.5 ± 0.3 (5.1 - 6.08)	5.8 ± 0.2 (5.5 - 6.3)
Wing loading (Nm^{-2})	7.6 ± 1.6 (6.5 - 8.7)	7.3 ± 0.9 (6.13 - 10.2)	9.04 ± 1.02 (7.3 - 10.98)	6.1 ± 0.8 (4.9 - 7.9)	4.7 ± 0.4 (3.9 - 5.5)	7.2 ± 1.4 (6.3 - 8.2)	9.8 ± 0.8 (8.05 - 10.8)	5.3 ± 0.5 (4.9 - 5.6)	6.1 ± 1.1 (4.8 - 8.3)	5.8 ± 0.4 (5.08 - 6.7)
Tip length ratio	1.2 ± 0.1 (1.1 - 1.3)	1.2 ± 0.08 (0.97 - 1.4)	1.1 ± 0.07 (1.02 - 1.3)	1.1 ± 0.08 (0.9 - 1.2)	1.2 ± 0.02 (1.1 - 1.2)	1.2 ± 0.01 (1.14 - 1.15)	1.2 ± 0.1 (1.07 - 1.4)	1.2 ± 0.08 (1.1 - 1.2)	1.1 ± 0.09 (0.96 - 1.2)	1.2 ± 0.04 (1.08 - 1.3)
Tip area ratio	0.8 ± 0.0 (0.77 - 0.82)	0.7 ± 0.06 (0.6 - 0.9)	0.7 ± 0.06 (0.6 - 0.8)	0.7 ± 0.06 (0.6 - 0.8)	0.7 ± 0.03 (0.65 - 0.79)	0.7 ± 0.09 (0.6 - 0.8)	0.7 ± 0.08 (0.6 - 0.8)	0.7 ± 0.05 (0.68 - 0.75)	0.7 ± 0.1 (0.6 - 0.9)	0.7 ± 0.05 (0.6 - 0.8)
Tip shape index	1.8 ± 0.1 (1.7 - 1.9)	1.8 ± 0.7 (1.1 - 4.4)	1.6 ± 0.3 (1.16 - 2.3)	1.8 ± 0.5 (1.3 - 2.9)	1.5 ± 0.2 (1.3 - 2.08)	1.6 ± 0.5 (1.2 - 1.95)	1.6 ± 0.3 (1.3 - 2.2)	1.7 ± 0.6 (1.3 - 1.08)	1.9 ± 0.5 (1.3 - 3.08)	1.5 ± 0.2 (1.1 - 1.9)

No significant relationship existed between frequency and wing loading for the global set of rhinolophids for which wing loading and frequency data were available (r=0.155, $F_{(1,20)}$=0.493, p=0.49; Fig. 6.2 & Table 6.2). However, this may be due to the fact that the two major clades comprising the Rhinolophidae (Chapter 2) may have experienced different selection pressures. However, when the two main clades were analysed separately, there was still no significant relationship between frequency and wing loading for either the African (r=0.481, $F_{(1,10)}$=3.01, p=0.11) or the Oriental clade (r=0.2216, $F_{(1,9)}$=0.4646, p=0.51).

Table 6.2 Wing parameters and body mass for rhinolophids occurring outside of South Africa. Data were obtained from Norberg & Rayner (1987) and Kingston *et al.* (2000). ~ = missing data.

Species	Wingspan (cm)	Wing Area (cm^2)	Wing Loading (Nm^{-2})	Mass (g)
R. affinis	31.2	120	11.3	13.8
R. euryale	28.5	132	8.1	10.9
R. ferrumequinum	33.2	182	12.2	22.6
R. hipposideros	23.1	94	7.1	6.8
R. luctus	39.6	283	9.1	26.3
R. macrotis	28.4	139	5.3	7.5
R. megaphyllus	28.1	130	7.4	9.8
R. mehelyi	~	139	11.6	16.5
R. refulgens	24.5	103	6	6.3
R. sedulus	26	125	6	7.7
R. stheno	28.6	137	6.4	8.9
R. trifoliatus	32.4	188	7.3	13.9

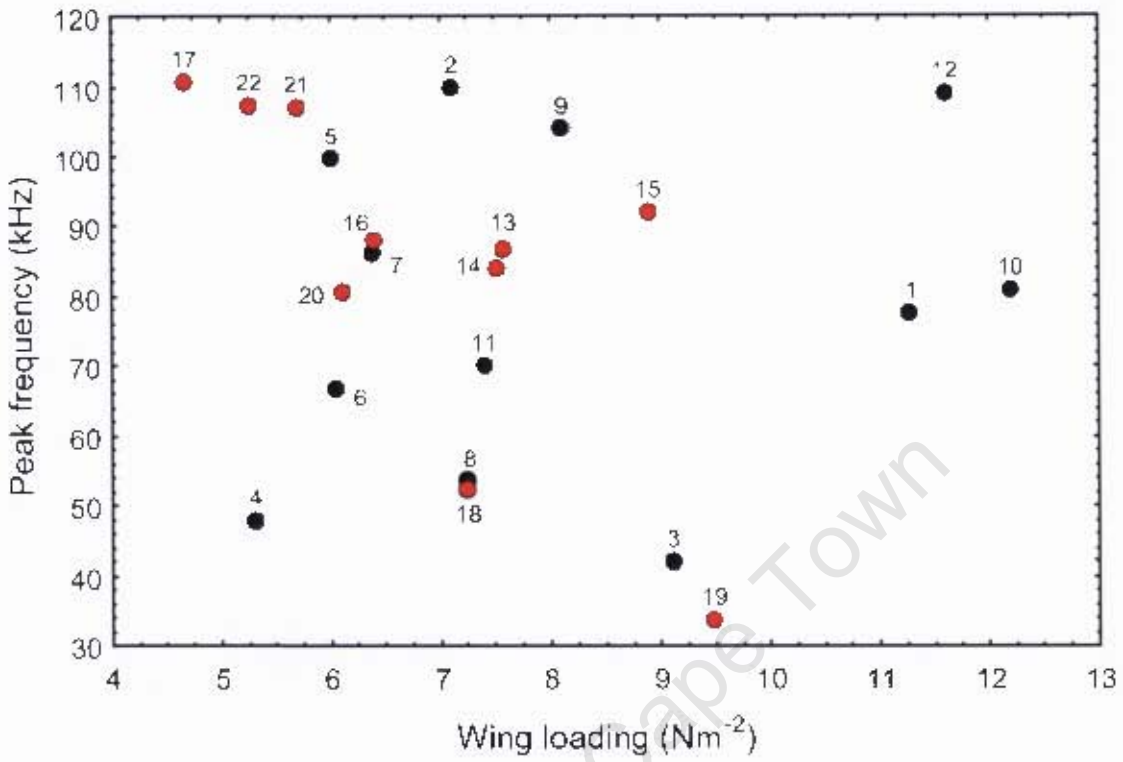


Fig. 6.2 Relationship between echolocation frequency and wing loading for rhinolophids from around the world. Except for South African rhinolophids (in red), data were obtained from the literature, where Peak Frequency = 94.65 – 1.75*Wing loading. The species are: *R. affinis* (1), *R. hipposideros* (2), *R. luctus* (3), *R. macrotis* (4), *R. refulgens* (5), *R. sedulus* (6), *R. stheno* (7), *R. trifoliatus* (8), *R. euryale* (9), *R. ferrumequinum* (10), *R. megaphyllus* (11), *R. mehelyi* (12), *R. blasii* (13), *R. capensis* (14), *R. clivosus* (15), *R. darlingi* (16), *R. denti* (17), *R. fumigatus* (18), *R. hildebrandti* (19), *R. simulator* (20), *R. swinnyi* (21) and *R. landeri* (22).

Regression of wing design against body size reveals a significant relationship between body mass and wingspan ($r=0.939$, $F_{(1,19)}=140.25$, $p<0.0001$; Fig. 6.3) and wing area ($r=0.852$, $F_{(1,20)}=53.19$, $p<0.0001$; Fig. 6.4) for the global set of rhinolophids. After controlling for phylogeny both relationships remained significant (CIAC: wingspan – $r=0.94$, $p<0.0001$; wing area – $r=0.91$, $p<0.0001$).

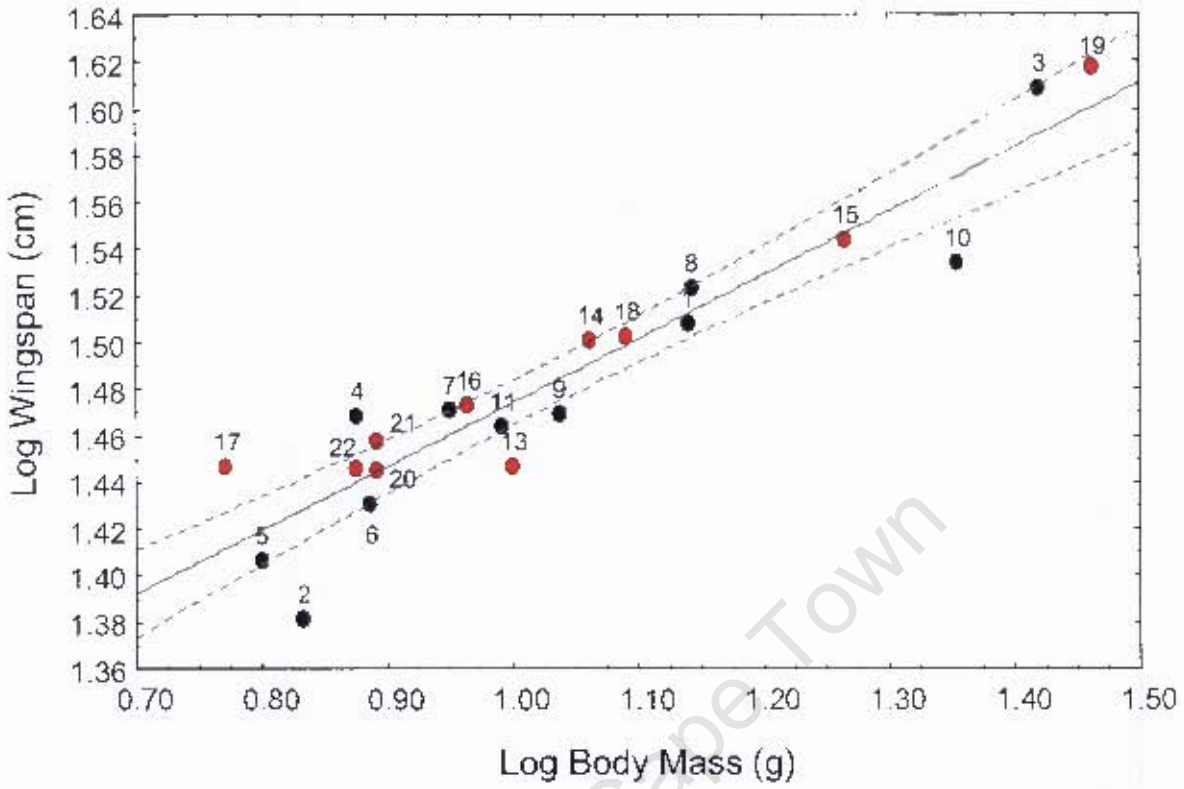


Fig. 6.3 Relationship between body mass and wingspan for rhinolophids from around the world. Except for South African rhinolophids (in red), data were obtained from the literature. The solid line represents the line of best fit, where Log Wingspan = 1.2011 + 0.273*Log Body Mass. Dashed lines represent the 95% confidence intervals. The species are: *R. affinis* (1), *R. hipposideros* (2), *R. luctus* (3), *R. macrotis* (4), *R. refulgens* (5), *R. sedulus* (6), *R. stheno* (7), *R. trifoliatus* (8), *R. euryale* (9), *R. ferrumequinum* (10), *R. megaphyllus* (11), *R. mehelyi* (12, not included), *R. blasii* (13), *R. capensis* (14), *R. clivosus* (15), *R. darlingi* (16), *R. denti* (17), *R. fumigatus* (18), *R. hildebrandti* (19), *R. simulator* (20), *R. swinnyi* (21) and *R. landeri* (22).

Although deviations from the allometric relationship between body size and wing design did exist, it is interesting that in many these were not the species which deviate from the allometric relationship between peak frequency and body size (Chapter 4). Of the South African rhinolophids, *R. clivosus* and *R. fumigatus* showed great deviations from the call frequency-body size relationship in having calls that were respectively higher and lower than predicted from body size

(Chapter 4, Fig. 4.6). However neither species deviated from the wing design-body size relationship for the Rhinolophidae (Figs 6.3, 6.4).

Similarly, *R. capensis*, *R. darlingi*, and *R. swinnyi* did not deviate from the relationships between body size and either wingspan or wing area for the rhinolophids (Figs 6.3, 6.4) although they did deviate from the allometric relationship between peak call frequency and body size. For *R. stheno*, call frequency was higher than predicted by body size (Fig 4.5, Chapter 4), but wingspan and wing area (relative to body size) were within the 95% confidence intervals for the Rhinolophidae.

Species that did deviate in call frequency from predictions of both body size and wing morphology allometry included *R. mehelyi*: this species echolocated at a higher-than-expected frequency for its body size, but had a smaller wing area (Fig. 6.4). *Rhinolophus trifoliatus* echolocated at a low frequency for its body size, but had a larger-than-expected wing area (Figs 6.3, 6.4). However, only *R. ferrumequinum* and *R. euryale* showed deviations from the allometric relationship for both wingspan and wing area (Figs 6.3, 6.4; Fig. 4.5, Chapter 4). These deviations were not in the directions predicted by the FHH. Frequencies higher than predicted by body size did not corresponded with smaller wingspans and larger wing areas.

Among the other *Rhinolophus* species, those showing deviations from the relationship between wing design and body size (e.g. *R. affinis, R. hipposideros*, *R. hildebrandti*, and *R. luctus*), did not show corresponding deviations from the allometric relationship between peak frequency and body size (Figs 6.3, 6.4; Fig. 4.5, Chapter 4).

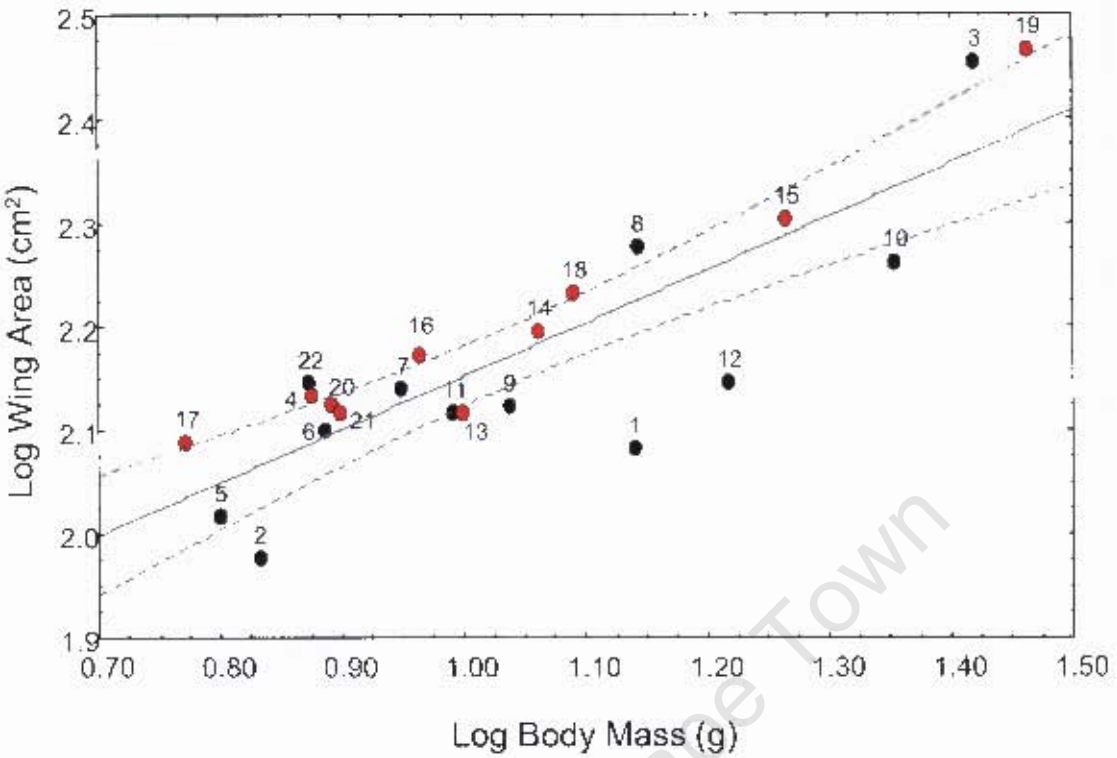


Fig. 6.4 Relationship between wing area and body mass for rhinolophids from around the world. Except for South African rhinolophids (in red), data were obtained from the literature. The solid line represents the line of best fit, where Log Wing area = 1.6403 + 0.512*Log Body Mass. Dashed lines represent the 95% confidence intervals. The species are *R. affinis* (1), *R. hipposideros* (2), *R. luctus* (3), *R. macrotis* (4), *R. refulgens* (5), *R. sedulus* (6), *R. stheno* (7), *R. trifoliatus* (8), *R. euryale* (9), *R. ferrumequinum* (10), *R. megaphyllus* (11), *R. mehelyi* (12), *R. blasii* (13), *R. capensis* (14), *R. clivosus* (15), *R. darlingi* (16), *R. denti* (17), *R. fumigatus* (18), *R. hildebrandti* (19), *R. simulator* (20), *R. swinnyi* (21) and *R. landeri* (22).

THE ACOUSTIC COMMUNICATION HYPOTHESIS

Echolocation parameters in the South African rhinolophids were normally distributed (Kolmogorov-Smirnov Test, p all >0.1; Table 6.3). Echolocation calls were significantly different between *Rhinolophus* species (Wilk's λ=0.00014, $F_{(42,172)}$=23.15, p<0.001), but the interaction term species*sex was not significant (Wilk's λ=0.421, $F_{(42,172)}$=0.832, p>0.75). Inter-pulse interval did not differ significantly between species (p all >0.2), but peak frequency (MANOVA, $F_{(17,41)}$=417.62, p<0.001), bandwidth (MANOVA, $F_{(17,41)}$=4.23, p<0.001), wavelength (MANOVA, $F_{(17,41)}$=96.32, p<0.001) and duration (MANOVA, $F_{(17,41)}$=4.34, p<0.001) all differed significantly. *Rhinolophus denti* and *R. capensis* differed significantly in bandwidth (Tukey HSD test, p<0.005), with *R. denti* having a longer bandwidth and thus longer frequency-modulated tail at the end of the call. The calls of *R. hildebrandti* were characterized by significantly shorter bandwidths than *R. clivosus, R. denti, R. landeri, R. simulator* and *R. swinnyi* (Tukey HSD tests, p all >0.02).

Both *R. denti* and *R. swinnyi* used shorter calls than the other rhinolophids. Call duration of *R. denti* differed significantly from those of *R. capensis*, *R. clivosus*, *R. darlingi*, *R. fumigatus* and *R. hildebrandti* (Tukey HSD tests, p<0.05). Similarly, the call duration of *R. swinnyi* differed significantly from those of *R. capensis*, *R. clivosus* and *R. hildebrandti* (Tukey HSD tests, p<0.02). *Rhinolophus fumigatus* and *R. hildebrandti* differed significantly in frequency wavelength (Tukey HSD test, p<0.001) and both had significantly longer frequency wavelengths than all other South African rhinolophids (Tukey HSD tests, p all <0.001). Although *R. denti* and *R. swinnyi* had wavelengths of similar magnitude (Tukey HSD test, p>0.99) both species had shorter wavelength calls than *R. capensis*, *R. clivosus* and *R. simulator* (Tukey HSD tests, p all <0.05).

Table 6.3 Mean ± SD of echolocation parameters for *R. blasii* (RB), *R. capensis* (RC), *R. clivosus* (RCL), *R. darlingi* (RD), *R. denti* (RDNT), *R. fumigatus* (RF), *R. hildebrandti* (RH), *R. landeri* (RL), *R. simulator* (RS) and *R. swinnyi* (RSWY). Ranges are given in parentheses. Echolocation parameters: peak frequency (PF; kHz), bandwidth (BW; kHz), inter-pulse interval (IPI; ms), duration (DUR; ms) and wavelength of frequency (FWL).

	ECHOLOCATION PARAMETERS				
SPECIES	PF	BW	IPI	DUR	FWL
RB (n=2)	86.6 ± 0.7 (86.1 — 87.1)	17.1 ± 7.1 (12.1 — 22.1)	506.5 ± 559.3 (111.0 — 902.0)	26.9 ± 11.5 (18.7 — 35.0)	4.0 ± 0.0 (4.0 — 4.1)
RC (n=10)	83.7 ± 1.0 (82.1 — 84.7)	12.9 ± 1.2 (11.0 — 14.5)	103.5 ± 42.7 (15.0 — 150.5)	41.2 ± 3.8 (35.6 — 45.9)	4.2 ± 0.0 (4.1 — 4.2)
RCL (n=10)	91.3 ± 1.1 (89.6 — 92.5)	19.0 ± 4.9 (10.2 — 25.5)	225.4 ± 337.3 (6.7 — 942.0)	37.6 ± 6.3 (27.5 — 45.5)	3.8 ± 0.0 (3.8 — 3.9)
RD (n=7)	87.6 ± 2.9 (83.7 — 91.0)	16.0 ± 4.3 (10.7 — 22.3)	43.5 ± 25.7 (4.5 — 92.1)	33.1 ± 7.2 (23.0 — 40.8)	4.0 ± 0.1 (3.8 — 4.2)
RDNT (n=10)	110.9 ± 1.9 (108.2 — 115.4)	22.1 ± 5.1 (12.0 — 29.0)	39.6 ± 43.8 (3.7 — 107.7)	22.0 ± 4.5 (17.8 — 30.0)	3.1 ± 0.1 (3.0 — 3.2)
RF (n=2)	53.8 ± 0.2 (53.6 — 53.9)	10.3 ± 0.9 (9.6 — 10.9)	91.2 ± 47.0 (57.9 — 124.4)	42.3 ± 2.8 (40.3 — 44.3)	6.5 ± 0.0 (6.5 — 6.5)
RH (n=10)	34.4 ± 3.7 (31.9 — 44.8)	6.2 ± 3.9 (1.5 — 12.8)	63.2 ± 30.4 (9.3 — 109.9)	40.8 ± 9.8 (27.7 — 61.6)	10.2 ± 0.9 (7.8 — 10.9)
RL (n=2)	107.3 ± 2.1 (105.8 — 108.7)	24.8 ± 0.1 (24.7 — 24.8)	24.3 ± 26.1 (5.8 — 42.7)	39.9 ± 5.3 (36.1 — 43.6)	3.2 ± 0.1 (3.2 — 3.3)
RS (n=6)	80.6 ± 1.3 (78.3 — 81.9)	19.9 ± 3.5 (17.4 — 22.4)	429.2 ± 401.5 (76.4 — 1101.0)	29.1 ± 5.4 (21.2 — 38.1)	4.3 ± 0.1 (4.3 — 4.4)
RSWY (n=10)	106.9 ± 0.7 (105.6 — 107.7)	18.9 ± 5.7 (4.2 — 22.7)	44.8 ± 35.8 (3.9 — 108.8)	25.2 ± 9.6 (17.2 — 47.2)	3.3 ± 0.0 (3.2 — 3.3)

Variation in peak frequency within species was low with coefficients of variation of the former ranging from 0.007 (*R. swinnyi*) to 0.024 (*R. darlingi*). Within calls from a single individual, the coefficients of variation for peak frequency were low, ranging from 0 to 0.008. The lowest standard deviations were found in *R. hildebrandti* (0–0.21 kHz; coefficient of variation = 0.09) and *R. fumigatus* (0–0.2 kHz; coefficient of variation = 0.003).

Rhinolophus blasii did not differ significantly in peak call frequency from *R. capensis*, *R. clivosus* and *R. darlingi* (Tukey HSD tests, p all >0.1). *Rhinolophus capensis and R. simulator* had similar peak frequencies (Tukey HSD tests, p all >0.1; Table 6.3). Furthermore, *R. landeri* did not differ significantly in peak frequency from *R. denti* or from *R. swinnyi* (Tukey HSD tests, p all >0.5).

The range of peak frequencies within species for the South African rhinolophids (Table 6.3) spans less than 5 kHz, corresponding to a deviation of less than 5% from the mean for each species (as found by Heller & von Helversen 1989 for European and Asian bats), with the exceptions of *R. denti, R. hildebrandti* and *R. darlingi*. *Rhinolophus denti* uses the highest frequency and overlaps at the lower end of its range only slightly with the upper end of the range of *R. landeri*. *Rhinolophus hildebrandti* has a large range due to the inclusion of an individual from Pafuri which echolocated at 44 kHz. If this individual is removed, *R. hildebrandti*'s frequency range is reduced to less than 5 kHz. Thus, only one South African rhinolophid really causes interspecific overlap in peak call frequency – *R. darlingi*. The overlap of peak frequencies between *R. darlingi* and *R. blasii, R. capensis*, and *R. clivosus* is due to *R. darlingi*'s large frequency range rather than large frequency ranges of any of the other three species (Table 6.3, Fig. 6.5).

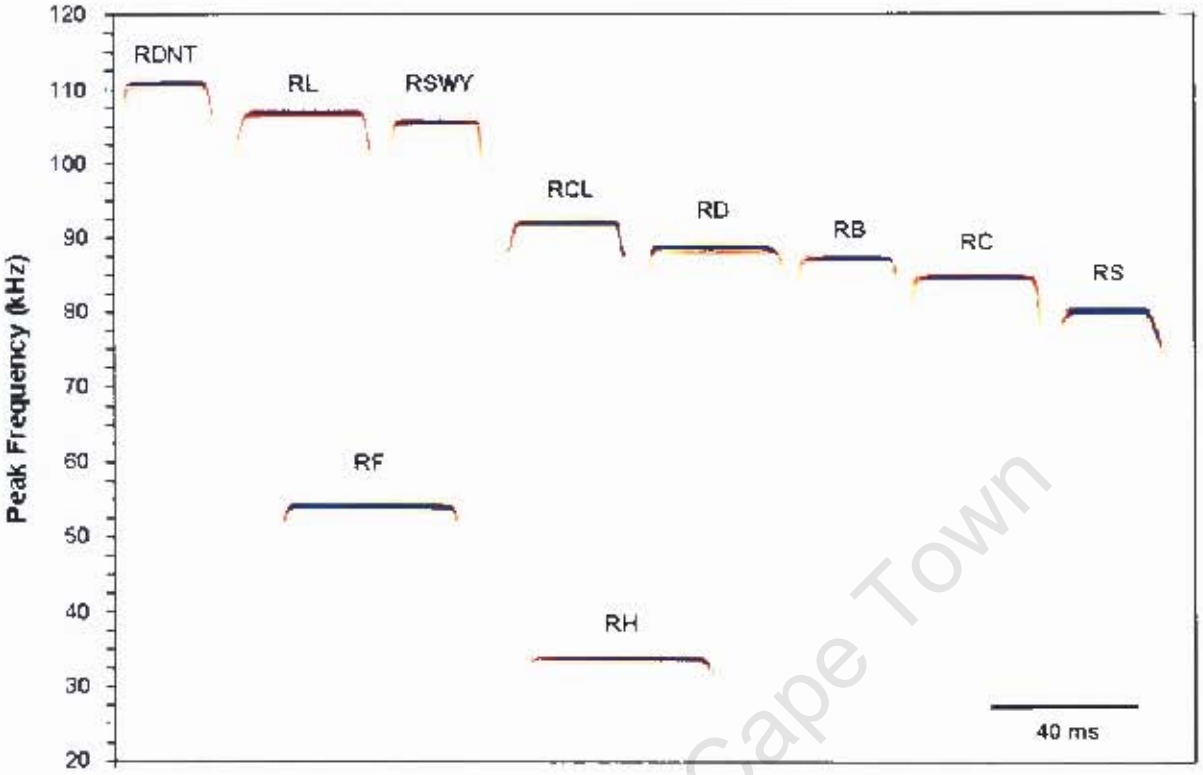


Fig. 6.5 Mean peak echolocation frequency for the ten South African rhinolophids taken from the spectrogram in BatSound Pro software with a FFT of 256. The species are: *R. blasii* (RB), *R. capensis* (RC), *R. clivosus* (RCL), *R. darlingi* (RD), *R. denti* (RDNT), *R. fumigatus* (RF), *R. hildebrandti* (RH), *R. landeri* (RL), *R. simulator* (RS) and *R. swinnyi* (RSWY).

For species where only the dominant harmonic was present in the echolocation call, higher order harmonics were calculated. Only one *R. clivosus* individual had 'good' calls containing the fifth harmonic (all others were calculated). Many species had very similar fundamental frequencies e.g. *R. blasii*, *R. capensis* and *R. darlingi* (all about 42-43 kHz) and *R. swinnyi* and *R. landeri* (54 kHz; Table 6.4).

Table 6.4 The peak frequency for each of the harmonics that were measured in South African rhinolophids. Harmonics are multiples of the fundamental frequency. Values in red were measured from call sequences. Values in black were calculated.

SPECIES	1st Harmonic (Fundamental)	2nd Harmonic (Peak Freq.)	3rd Harmonic	4th Harmonic	5th Harmonic
R. blasii	43.2	86.6	129.2	172.4	216
R. capensis	42.2	83.7	126.5	168	211
R. clivosus	45.6	91.3	136.4	182.2	227.5
R. darlingi	43.3	87.6	129.3	171.8	216.5
R. denti	55.4	110.9	165.8	221	277
R. fumigatus	26.9	53.8	80.7	107.6	134.5
R. hildebrandti	17.0	34.4	50.1	68	85
R. landeri	53.7	107.3	161.1	214.8	268.5
R. simulator	40.3	80.6	120.9	161.2	201.5
R. swinnyi	53.6	106.9	159.8	221	268

On the other hand, closely related South African rhinolophid species make use of very different peak frequencies (Table 6.3; Fig 2.3, Chapter 2), that are not harmonics of the same fundamental frequency. Based on cytochrome *b* sequences (Fig 2.3, Chapter 2), *R. capensis* (83.7 kHz) and *R. swinnyi* (106.9 kHz) are well supported sister-taxa, as are *R. denti* (110.9 kHz) and *R. simulator* (80.6 kHz). Both these species-pairs have very divergent peak call frequencies. Similarly, *R. hildebrandti* (33.8 kHz), *R. fumigatus* (53.8 kHz) and *R. darlingi* (87.6 kHz) are closely related but are characterized by very different peak frequencies. The same is true of *R. clivosus* (91.3 kHz), *R. f. ferrumequinum* (81 kHz) and *R. f. nippon* (65.5 kHz).

DISCUSSION

THE FORAGING HABITAT HYPOTHESIS

I found no support for the FHH in the form of a relationship between wing loading and peak frequency for a global set of rhinolophids. Although a significant relationship exists for the South African rhinolophids, removal of one of the ten species (*R. hildebrandti*) results in a non-significant relationship. The absence of a significant relationship between peak frequency and wing loading, coupled with some examples of species which do not show support for the FHH (e.g. *R. ferrumequinum*) suggest that causes and processes that lead to divergence of echolocation frequencies and wing morphology within the Rhinolophidae may be different for each species.

Similarly, the processes causing the divergence of echolocation call frequencies may also be different for each subclade. Although Kingston and Rossiter (2004) found evidence of harmonic hopping in the *R. philippinensis* clade in Asia, I found no evidence of this mechanism for speciation in the African clade. Sibling species (e.g. *R. capensis* and *R. swinnyi,* and *R. denti* and *R. simulator*) had very divergent echolocation frequencies that were not harmonics of the same fundamental frequency.

The absence of a relationship between wing loading and echolocation frequency and deviations from the allometric relationship between body size and wing design (wingspan and/or wing area) in species such as *R. capensis*, *R. clivosus, R. darlingi, R. fumigatus, R. swinnyi* and *R. stheno* (which have frequencies either higher or lower than predicted from body size; Fig. 4.5, Chapter 4), suggests that selection acts on echolocation frequency independently of wing morphology. Furthermore, *R. affinis, R. hipposideros* and *R. luctus* echolocate at frequencies that could be predicted from their body size (Fig. 4.5, Chapter 4), yet they deviate from the body size-wing design relationship (Figs 6.3, 6.4).

Deviations in wing design without corresponding deviations in echolocation call (or *vice versa*) in the majority of the rhinolophid species used in these analyses, together with the absence of a relationship between wing loading and peak echolocation frequency within the Rhinolophidae, suggest that wing morphology and echolocation frequency may not be under linked selection. Thus the putative adaptive complex that ostensibly links wing morphology and echolocation call frequency may not be that important within a particular foraging habitat and a certain level of flexibility in either wing design or call frequency may not have a huge impact on how a bat forages in a cluttered habitat

Similarly, a change in frequency at the high end of the frequency spectrum, results in a negligible change in wavelength. These changes are so small as to be biologically unimportant for discriminating between prey items (Chapter 1): as such, they should not have a negative effect on a bat's perception of its habitat. Given this flexibility in their apparent ability to evolve higher frequencies, bats which deviate from the allometric relationship between body size and peak frequency, may be evolving higher frequencies to ensure efficient communication amongst conspecifics via the partitioning of communication channels (i.e. frequency bands).

ACOUSTIC COMMUNICATION HYPOTHESIS

The small variation in echolocation frequency within South African rhinolophids, coupled with the limited overlap of peak frequencies between sympatric species, supports the predictions of the ACH. Only two species (*R. landeri* and *R. darlingi*) appear to cause some overlap in peak frequencies among the ten rhinolophids within South Africa. However, neither of these two species is syntopic with either of the species with which their call frequencies overlap, and genetic analyses suggest that *R. darlingi* may contain cryptic species (Chapter 2).

The echolocation frequency of *Rhinolophus landeri* overlaps with those of both *R. denti* and *R. swinnyi* (Table 6.3). However, within South Africa, *R. denti* and *R. landeri* are allopatric (Csorba *et al.* 2003; Chapter 5). The distributions of *R. landeri* and *R. swinnyi* overlap at the northern border of South Africa with Mozambique and Zimbabwe. However, the *R. swinnyi* individuals used in these analyses are not from populations that are sympatric with *R. landeri* (Chapter 5). Echolocation data for both species in the zone of apparent overlap on the northern border of South Africa is needed before final conclusions can be drawn.

The echolocation frequency of *R. darlingi* overlaps with *R. blasii, R. capensis* and *R. clivosus* (Table 6.3). It is not sympatric with *R. capensis*. It may be sympatric with *R. clivosus* and *R. blasii*, but the high intraspecific variability in echolocation calls of *R. darlingi* (a deviation of >5% from the mean, Table 6.3) and the marked genetic differences between *R. darlingi* from different parts of South Africa (Chapter 2, Fig 2.3) strongly suggest the existence of cryptic species. Resolution of the systematics of the *R. darlingi* species complex may remove the apparent overlap in the echolocation frequency of populations of *R. darlingi* with that of sympatric populations of *R. clivosus* and *R. blasii*.

The well-documented bat community at Sudwala (Mpumalanga, South Africa) comprises five of the ten South African species of rhinolophid (*R. blasii*, *R. clivosus*, *R. darlingi*, *R. hildebrandti* and *R. simulator*; Schoeman 2006). The peak frequencies used by these five species offer some support for the ACH.

The frequencies of *R. hildebrandti* and *R. simulator* do not overlap with the other three species. The frequencies used by the Sudwala population of *R. darlingi* ranges from 83.7 – 89.2 kHz (n=11) and those of *R. clivosus* range from 90.2 – 93.1 kHz (n=19). Thus, within this community, these two species do not overlap in peak frequency. Although *R. darlingi* still overlaps with *R. blasii* (86.1 – 87.1 kHz), *R. blasii* was trapped in a cave in which it co-occurs with *R. clivosus*, but we did not collect or record *R. darlingi* from the same cave. Although the

frequencies of *R. blasii* and *R. darlingi* overlap, it appears that they do not roost in the same caves and may not therefore be syntopic. Similar local-scale parapatry has been reported in Europe between two species echolocating at similar frequencies (*R. hipposideros* and *R. mehelyi*; Heller & von Helversen 1989).

There is some evidence that harmonic hopping may explain the divergence in echolocation frequency within at least one clade. Within the *hildebrandti-fumigatus-darlingi* clade the fundamental of *R. hildebrandti* is 17 kHz. This means that if harmonic hopping has occurred there should be a harmonic series of 17, 34, 51, 68, and 85 kHz. Within this clade, *R. hildebrandti* echolocates at 34 kHz, *R. fumigatus* at 54 kHz, and *R. darlingi* at 88 kHz (Table 6.4). This is very close to the expected series with small deviations (<5 kHz) from the predicted harmonic series possibly being the result of drift (Gareth Jones, personal communication).

At high frequencies, small changes in peak frequency translate into negligible differences in the sizes of prey detectable to a bat (Chapter 1); ergo resource partitioning cannot explain small differences in peak frequency. The same may be argued for habitat use. Small differences in frequency (even 10 kHz among frequencies above 60 kHz) may not translate into biologically meaningful differences in the way a bat uses its particular habitat. However, these differences in peak frequency are sufficient to enable sonar band partitioning, thus enabling conspecifics to use echolocation calls in a social context, as well as to avoid 'eavesdropping' by non-conspecifics.

In conclusion, the echolocation data from South African rhinolophids support predictions of the Acoustic Communication Hypothesis and also show support for the hypothesis that the high frequencies used by many rhinolophid species have evolved to enable efficient conspecific communication. Furthermore, Jacobs *et al.* (2007) have shown that the variation around the relationship between call

frequency and body size within the rhinolophids is smaller than among *Myotis* species. This would be expected if communication between conspecifics was driving the evolution of frequencies, rather than ecology, which is the case within *Myotis* (Fenton & Bogdanowicz 2002). Data on European and Asian rhinolophid communities also support the notion that frequency differences have evolved to partition communication channels (Heller & von Helversen 1989; Russo *et al.* 2007).

Finally, if echolocation call frequency has evolved to enable efficient communication between, and recognition of conspecifics, acoustic character displacement should occur among sympatric species using similar echolocation frequencies (Russo *et al.* 2007). A future test of the ACH using South African rhinolophids should address whether acoustic character displacement (without corresponding displacement in body size, i.e. forearm length) is present in rhinolophid species that occur in allopatric populations and in sympatric populations with other species using calls of similar echolocation frequency. If communication is driving the evolution and divergence of call frequencies, then species using similar frequencies should demonstrate greater divergence in frequency in sympatry than in allopatry.

CHAPTER 7

CONCLUSIONS

When compared to other bat families of similar body size, the Rhinolophidae are characterized by higher frequency echolocation calls than would be predicted by allometry. The primary aims of this study were to use a molecular phylogenetic approach to:

1. Construct a robust molecular phylogeny for the genus *Rhinolophus*;
2. Using the above, test the predictions of the Allotonic Frequency Hypothesis; and
3. Investigate the influence of body size and habitat on the evolution of rhinolophid echolocation call frequency, testing the Allometry, Acoustic Adaptation, Foraging Habitat and Acoustic Communication hypotheses.

The supermatrix data set consisting of one mtDNA gene (cytochrome *b*) and three nuclear introns (TG, THY and PRKC1) produced a well-resolved phylogeny for the rhinolophids, with good bootstrap and posterior probability support between deeper nodes in the topology (Chapter 2). Mapping of echolocation call frequencies onto this phylogeny indicates that high-frequency echolocation calls are the ancestral condition in the Rhinolophidae, and that the subsequent evolution of low frequencies in some species is therefore a derived character (Chapter 3).

THE ALLOTONIC FREQUENCY HYPOTHESIS

If moth hearing was driving the evolution of high frequencies as part of a co-evolutionary arm's race, then low-frequency echolocation would be the ancestral condition and high frequencies would characterise more recent taxa. I found no evidence to support the second prediction of the Allotonic Frequency Hypothesis, *viz* that species using allotonic frequencies are more derived (Chapter 3).

Furthermore, results from this study question the validity of the view that there is a coevolutionary arms race between bats and moths (Janzen 1980); at least as far as high frequencies in the Rhinolophidae are concerned. Although moths have evolved tympanate organs in response to bat echolocation calls so as to avoid predation, the evolution of tympanate organs in moths does not appear to have driven the secondary evolution of the high/allotonic frequencies used by the Rhinolophidae. Thus, in this specific case, there is no evidence of selection pressure reciprocity, but rather of prey (moth hearing) evolving under strong, ecological selection pressure imposed by predatory bats. Moth hearing is thus not the ultimate factor driving the evolution of high frequencies in the horseshoe bats.

THE ALLOMETRY HYPOTHESIS

Although an allometric relationship exists between body size and echolocation frequency within the Rhinolophidae, body size alone cannot explain the evolution of high frequencies in this family. In a comparison of five bat families (Jones 1996), rhinolophids echolocate using the second highest frequencies for their size, and as such should on average be characterised by the second smallest body size. This was not the case (Chapter 4). There is complete overlap in body size between the Rhinolophidae (and equally high-frequency Hipposideridae) and families such as the Vespertilionidae which use lower echolocation frequencies. The slopes of the relationships between frequency and body mass for five bat families are different (Jones 1996) which suggests that within each family, selection acts on echolocation call frequency independently of body size. Furthermore, among the rhinolophids, echolocation frequency has stronger allometric relationships with morphological characters directly associated with sound production, emission, and reception. This contrast strongly suggests that selection has acted directly on echolocation rather than on body size in the Rhinolophidae and supports proposals that echolocation constrains body size rather than *vice versa* (Barclay & Brigham 1991; Jones 1996). Thus, selection pressure acting directly on echolocation frequency, with complimentary changes

in morphology (but not necessarily size), may explain why morphological allometry predicts call frequency better than does body-size allometry (Chapter 4).

THE ACOUSTIC ADAPTATION HYPOTHESIS

The Rhinolophidae split from their sister family the Hipposideridae, at a time when both Africa and Asia where dominated by tropical forests (Janis 1993). Results of a DIVA analysis are equivocal, suggesting that the rhinolophids could have originated in Africa, Asia or Europe. However, indels in nuclear introns suggest that the African clade may be more derived (Chapter 2) and that the Rhinolophidae originated in the tropical forests of Asia as proposed by earlier authors (Koopman 1970; Bogdanowicz 1992). Rhinolophids are clutter-specialists (Findley *et al.* 1972; Norberg & Rayner 1987; Jones & Rayner 1989) and their wing design and high-frequency, high-duty cycle echolocation calls are adapted for foraging close to or within dense vegetation. The ancestral condition of high-frequency, high-duty cycle echolocation may have been an adaptation to the tropical forests in which the horseshoe bats arose (Chapter 5). For present-day taxa differences in habitat structure and climatic variables cannot explain differences in echolocation call frequency, nor why some species are using echolocation calls of much higher frequency than would be predicted by the allometric relationship between call frequency and body size. Both low-frequency and high-frequency species occupy syntopic habitats and because of this, habitat alone cannot explain the high frequencies used by this family of bats.

THE FORAGING HABITAT HYPOTHESIS

Limited support was found for the FHH, with only a few species showing corresponding deviations between wing design and body size and echolocation frequency and body size. No relationship exists between peak echolocation frequency and wing loading (Chapter 6). Deviations in wing design without

corresponding deviations in echolocation call (or *vice versa*) in the majority of the rhinolophid species used in these analyses suggests that selection may be acting on echolocation and wing design independently, and thus selection for different wing design properties which can affect flight performance are not linked with corresponding changes in echolocation frequency (Chapter 6). Furthermore the absence of a relationship between wing loading and peak echolocation frequency in the Rhinolophidae, and therefore the implication that selection operates independently on these two variables, questions the validity of an 'adaptive complex', at least within a particular habitat (e.g. clutter). Changes in frequency do not necessarily have to be accompanied by corresponding changes in wing design for a bat to use its foraging habitat effectively. This suggests that the evolution of frequencies higher than those predicted from body size may facilitate effective communication amongst conspecifics, without linked changes occurring in wing morphology.

THE ACOUSTIC COMMUNICATION HYPOTHESIS

Data on European and Asian rhinolophid communities support the notion that frequency differences have evolved to partition communication channels between sympatric rhinolophid species (Heller & von Helversen 1989; Russo *et al.* 2007). Echolocation data from South African rhinolophids also support predictions of the Acoustic Communication Hypothesis and additionally show support for the hypothesis that the high frequencies used by many rhinolophid species have evolved to enable efficient conspecific communication.

Rhinolophid echolocation calls are characterized by Doppler shift compensation (DSC), and high frequencies appear to be best adapted for Doppler shift compensation because they require less precise shifts in call frequency (Waters 2003). This may explain why the variation in an individual bat's peak frequency is smaller in species using low-frequency calls that in species making use of higher frequencies.

Higher frequencies demonstrate greater Doppler shifts and also require a wider acoustic fovea (Waters 2003), which may drive the evolution of calls in bats using DSC towards higher frequencies. This in turn suggests that bats which deviate from the allometric relationship between call frequency and body size (by evolving higher frequencies) have done so to enable partitioning of communication channels amongst sympatric taxa.

CONCLUSIONS

In summary, high-frequency echolocation calls in the Rhinolophidae appear to have evolved as an adaptation to allow efficient foraging in the cluttered tropical forests in which they first arose, rather than as an adaptation to circumvent moth hearing (Chapter 3). Subsequent divergence in echolocation frequency within the Rhinolophidae was driven by the partitioning of sonar frequency bands for effective intraspecific communication (the Acoustic Communication Hypothesis, Chapter 6) and to a lesser extent by selection on body size (Chapter 4). The greater correlation between echolocation frequency and features directly related to the production and reception of echolocation signals than that between echolocation frequency and body size (Chapter 4) suggests an evolutionary decoupling of echolocation call characteristics and body size. Such decoupling may have been caused by selection pressure for non-overlapping echolocation frequencies within bat assemblages to allow effective intraspecific communication. Surprisingly, differences in habitat structure and climate (the Acoustic Adaptation Hypothesis, Chapter 5) appear to have exerted little influence on the evolution of echolocation in the Rhinolophidae.

CHAPTER 8

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Nuclear Introns – Appendix 2

Alignment of the nuclear intron sequence data used in conjunction with cytochrome *b* to construct the supermatrix. Character partitions are as follows: TG (Thyroglobulin), THY (Thyrotropin beta chain precursor), and PRKC1 (Protein kinase C, iota 1). 'N' indicates missing data, and '-' indicates a gap.

TG


```
[                              10        20        30        40        50        60        70        80]
[                              .         .         .         .         .         .         .         .]

C_percivali__cmrc2        cgggtgg-cttgctggaa-ttag-agaccagggaccgtactgccccgaggaaggcttgctcggcctgtaactggcaggg-   [76]
H_caffer_cmrH2            tgggcag-ctcactggta-ttag-acaccagggaccgtactgccccaaggaacgcttgctcagcctgtaactgacaggg-   [76]
R_acuminatus_cmr93        NNNNcgg-catgctggaa-ttgg-agaccagggaccgtgctgtcctgaggaaggcttgctcagcctgtaactgacaggg-   [76]
R_affinis_cmr31           tgggcgg-cgtgctggaa-ttgg-agaccagggaccgtactgccctgaggaaggcttgctccgcctgtaactgacaggg-   [76]
R_affinis_cmr87           tgggcgg-cgtgctggaa-ttgg-agaccagggaccgtactgccctgaggaaggcttgctccgcctgtaactgacaggg-   [76]
R_blasii_cmr30            tgggcgg-catgctggaa-ttgg-agaccagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_blasii_cmr49            tgggccg-catgctggaa-ttgg-agaccagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_borneensis_cmr129       tgggtgg-catgctggaa-ttgg-agaccagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_capensis_cmr10          tgggcgg-catgctggaa-ttgg-aaacgagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_clivosus_cmr1           tgggcgggcatgctggaa-ttgg-aggccagggaccgtactgccccgaggaaggcttgctcggcctgtaactgacaggg-   [77]
R_darlingi_cmr76          tgggctg-catgctggaa-ttgg-aggccagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacagggg   [77]
R_denti_cmr56             tgggcgg-catgctggaa-ttgg-agacgagggaccgtacggccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_ferrumequinum_cmr51     tgggcgg-catgctggaaattgg-aggccagggaccgtactgccccgaggaaggcttgctcagcctgtaactgacaggg-   [77]
R_formosae_cmr119         tgggcag-cacgctggaa-ttgg-agaccagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_fumigatus_cmr12         tgggcgg-catgctggaa-ttgg-aggccagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_fumigatus_cmr80         NNNNNNNNcatgctggaa-ttgg-aggccagggaccgtactgccctgaggaaggcttgcgcggcctgtaactgacaggg-   [77]
R_hildebrandti_cmr4       tgggcgg-catgctggaa-ttgg-aggccagggaccgtactgccctgaggaaagct-gctcggcctgtaactgacaggg-   [75]
R_hildebrandti_cmr83      tgggcgg-catgctggaa-ttgg-aggccagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_landeri_cmr13           tgggcgg-catgctggaa-ttgg-agaccagggaccatactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_lepidus_cmr91           Ngggcgg-catgctggaa-ttgg-agaccagggaccgtactgccctgaggaaaNatggctcggcctgtaactgacaggg-   [76]
R_maclaudi_cmr78          tgggccg-catgctggaa-ttgg-aggccagggactggactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
```

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R_megaphyllus_cmr36     tgggcgg-catgctggaa-ttgg-agaccagggaccgcactgccctgaggaaggcttgctcggcctgtaactgacagtg-   [76]
R_mehelyi_cmr50         tgggcgg-catgctggaa-ttgg-agaccagggactgtactgccctgaggaaggcttgctcggcctgtagctgacaggg-   [76]
R_monoceros_cmr121      tgggcgg-catgctggaa-ttgg-agaccagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_f_nippon_cmr118       tgggcgg-catgctgNaa-tNNa-gggccagggaccgtactgccccgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_pussilus_cmr96        Ngggcgg-cttgctggaa-ttgg-agaccagggaccgtactgccctgaggattaatggctctccctgta-ctgacaggg-   [75]
R_shameli_cmr92         tgggcag-catgctggaa-ttgg-agaccagggaccgtactgccctgaggaaggcttgctccgcctgtaactgacaggg-   [76]
R_simulator_cmr27       tgggcgg-catgctggaa-ttgg-agacgagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_simulator_cmr82       tgggcgg-cttgctggaa-ttgg-agacaagggaccgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_swinnyi_cmr60         tgggcgg-catgctggaa-ttgg-aaacgcgggactgtactgccctgaggaaggcttgctcggcctgtaactgacaggg-   [76]
R_thomasi_cmr94         NNNNNNNNNatgctggaa-ttgg-agaccagggaccgtactgtcctgaggaaggcttgctcggcctgtaactgacaggg-   [77]
T_persicus_cmr153       tcggcga-cttgctggaa-ttag-agaccagggaccgtactgccctgaggaagg-aagctcggcctgtaactgacaggg-   [75]

[                                90        100       110       120       130       140       150       160]
[                                .         .         .         .         .         .         .         .]

C_percivali__cmrc2      tgtggggcctgtggagcaacaatgagcaagtatgtcaggttaaggagtttgatgagcacagaagaccaaggtgga-----   [151]
H_caffer_cmrH2          tgtggggcctggagagaaacaataagcaagcgtgt-----------------------------------------   [111]
R_acuminatus_cmr93      cgtggggcctgcggagaaacaataagaaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_affinis_cmr31         cgtggggcctgtggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_affinis_cmr87         cgtggggcctgtggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_blasii_cmr30          cgtggggcctgcggagaaacaataagcaagtctgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_blasii_cmr49          cgtggggcctgcggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_borneensis_cmr129     cgtggggcctgtggagaaacaataagcaagtgtgtcagcttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_capensis_cmr10        catggggcctgcggagaaacaatgagcaagtgtgtcaggttcaggagtttaatgagcacagaagcccaagatggaagctc   [156]
R_clivosus_cmr1         cgtggggcctgcggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [157]
R_darlingi_cmr76        cgtggggcctgcggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [157]
R_denti_cmr56           catggggcctgtggagaaacaatgagcaagtgtgtcaggttcaggagttaaatgagcacagaagcccaagatggaagcgc   [156]
R_ferrumequinum_cmr51   cgtggggcctgcggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagagggaagctc   [157]
R_formosae_cmr119       tgtggggcctgcggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_fumigatus_cmr12       cgtggggcctgcggagaaacaatcagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_fumigatus_cmr80       cgtggggcctgcggagaaacaataagcaagtgtggcaggttcaggagtttaatgagcacagaagaccaagacggaagctc   [157]
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R_hildebrandti_cmr83    cgtggggcctgcggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_landeri_cmr13         cgtggggcctgcggagaaacaattagcaagtgtgtcaggttcaggagtttaat-agcacagaagaccaagatggaagctc   [155]
R_lepidus_cmr91         cgtggggcctgcagataaacaataagcaggtgtgtcagcttcaggagtttaatgggcacagaagaccaagatggaagctc   [156]
R_maclaudi_cmr78        cgtggggcctgcggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_megaphyllus_cmr36     cgtggggcctgtggagaaacaatacgcaggtgtgtcaggttcaggagtttaatgagcatagaagaccaagatggaagttt   [156]
R_mehelyi_cmr50         cgtggggcctgcggagaaacaatcagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
```

```
R_monoceros_cmr121      cgtggggcctgcagagaaacaataagcaggtgtgtcagcttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_f_nippon_cmr118       cgtggggcctgcggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagctc   [156]
R_pussilus_cmr96        cgtggggcctgcgtagaaaaaataagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaacatggaagctc   [155]
R_shameli_cmr92         cgtggggcctgtggagaaacaataagcaagtgtgtcaggctcagaagtttaatgagcacagaagaccaagatggaagctc   [156]
R_simulator_cmr27       catggggcctgcggagaaacaatgagcaagtgtgtcaggttcaggagttaaatgagcacagaagcccaagatggaagcgc   [156]
R_simulator_cmr82       catggggcctgcggagaaacaatgagcaagtgtgtcaggttcaggagttaaatgagcacagaagcccaagatggaagcgc   [156]
R_swinnyi_cmr60         catggggcctgcggagaaacaatgagcaagtgtgtcaggttcaggagtttaatgagcacagaagaccaagatggaagcgc   [156]
R_thomasi_cmr94         cgaggggcctgcggagaaacaataagcaagtgtgtcaggttcaggagtttaatgagcacagaaaaccaagatggaagctc   [157]
T_persicus_cmr153       cgtggggcctgaggagacacaataagcaagtatgtcaggttaaggagtttgatgagcacagaagaccaaggtggacactc   [155]

[                                170       180       190       200       210       220       230       240]
[                                .         .         .         .         .         .         .         .]

C_percivali__cmrc2      ------------------------------------------------------------------------------   [151]
H_caffer_cmrH2          --------------------------------tacagccacagatcctatcacgtctaaacagagggcatccccatcact   [159]
R_acuminatus_cmr93      -gtgagcatccaagctcta-tataaaaccttctacagccagagatccgatcacgtctacacagagagaaaccccatcac-   [233]
R_affinis_cmr31         -gtgagcatccaagctcta-tataaaaccttctacagccagagatcccatcacgtctacacagagagaaaccccatcact   [234]
R_affinis_cmr87         -gtgagcatccaagctcta-tataaaaccttctacagccagagatcccatcacgtctacacagagagaaaccccatcact   [234]
R_blasii_cmr30          -gtgagcgtccaagctcca-tat-aaa---tctacagccagagatcccatcacgtctacacagagagaaacccc------   [224]
R_blasii_cmr49          -gtgagcgtccaagctcca-tataaaacct---acagccagagatcccatcacgtctacacagagagaaacccc------   [225]
R_borneensis_cmr129     -gtgagcgtccaagctcta-tataaaaccttctacagccagagatcccatcacgtctacacagagagaaaccccatcact   [234]
R_capensis_cmr10        aatgagcatccaagctcca-tataaaacct---acagccagagatcccatcacgtctacacagagagaaacccc------   [226]
R_clivosus_cmr1         aatgagcatccaagctcca-tataaaacct---acagccagagatcccatcacgtctacacagagagaaaccccat----   [229]
R_darlingi_cmr76        aatgagcatccaagctcca-tataaaacct---acagccagagatcccatcacgtctacacagagagaaaccccat----   [229]
R_denti_cmr56           aatgagcatccaagctcca-tataaaaccc---acagccagagatcccatcacgtctacacatagagaaaccccat----   [228]
R_ferrumequinum_cmr51   aatgagcatccaagctcca-tattaaacct---acagccagagatcccatcacgtctacacagagagaaacccc------   [227]
R_formosae_cmr119       -gtgagtgtccaagctcta-cataaaaccttctacagccagagatcccatcacatctacacagagagaaaccccatcact   [234]
R_fumigatus_cmr12       aatgagcatccaagctcca-tataaaacct---acagccagagatcccatcacgtctacacagagagaaaccccac----   [228]
R_fumigatus_cmr80       actgagcatccaagctcca-tgtaaaacct---acagccagagatcccatcacgtctacacagagagaaaccccat----   [229]
R_hildebrandti_cmr4     aatgagcatccaagctcca-tataaaacct---acagccagagatcccatcacgtctacacagagagaaaccccat----   [227]
R_hildebrandti_cmr83    aatgagcatccaagctcca-tataaaacct---acagccagagatcccatcacgtctacacagagagaaaccccat----   [228]
R_landeri_cmr13         -gtgagcgtccaagctcca-tataaaa---tctatagccagagatcccactatgtctacacagagagaaaccccatcgcg   [230]
R_lepidus_cmr91         -gggagcgtccaagctcta-tacaaaaccttctacagccagagatcccatcacgtctacacagagagaaaccccgtcact   [234]
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R_megaphyllus_cmr36     -gtgagcatccaagctcta-tataaaaccttctacagccagagagcccatcacgtctacacagagagaaaccccatcact   [234]
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R_blasii_cmr49          ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggccccac-gccctgtcagt   [601]
R_borneensis_cmr129     ttggtcttctgtgctcattaaactcctgaagctgacacacttgcttattgtttctccacaggccccac-gccctgtcagt   [605]
R_capensis_cmr10        ttgggcttctgtgctcattaaactcctgaacctgacacacttgctcattgtttctccgcaggccccat-gccctgtcagt   [580]
R_clivosus_cmr1         ttggtcttctgtgctcattaaactcctgaatctgacacacttgcttattgtttctccgcaggccccac-gccctgtcagt   [606]
R_darlingi_cmr76        ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggccccacgcccctgtcagt   [607]
R_denti_cmr56           ttgggcttctgtgctcatttaactcctgaacctgacacacttgctcattgtttctccgcaggccccat-gccctgtcagt   [605]
R_ferrumequinum_cmr51   ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggccccac-gccctgtcagt   [582]
R_formosae_cmr119       ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggccccac-accctgtcagt   [605]
R_fumigatus_cmr12       ttggtcttctgtgctcattaaactcctgaacctgccacacttgctgattgtttctccgcaggccccac-gccctgtcagt   [605]
R_fumigatus_cmr80       ttggtcttctgtgctcattaaactcctgaacctgccacacttgcttattgtttctccgcaggccccac-gccctgtcagt   [606]
R_hildebrandti_cmr4     ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggccccac-gccctgtcagt   [604]
R_hildebrandti_cmr83    ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggccccac-gccctgtcagt   [605]
R_landeri_cmr13         ttggtcttctgtgct-attaaactcctgaacctgacacacttgctaattgtttctccgcaggccccac-gccctgtcagt   [615]
R_lepidus_cmr91         ttggtcttctgtgcccattaaactcctgaagctgacacacctgcttattgtttatctgcaggccccac-gccctgtcagt   [624]
R_maclaudi_cmr78        ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggccccac-gccctgtcagt   [605]
R_megaphyllus_cmr36     ttggtcttctgtgctcattaaactcctgaacctgacacacctgcttattgtttctccacaggccccac-gccctgtcagt   [623]
R_mehelyi_cmr50         ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggccccac-gccctgtcagc   [617]
R_monoceros_cmr121      ttggtcttctgtgctcattaaactcctgaagctgacacacctgcttattgtttctctgcaggccccac-gccctgtcagt   [605]
R_f_nippon_cmr118       ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggccccac-gccctgtcagt   [603]
R_pussilus_cmr96        ttggtcttctgtgctcattaaactcctgaacctgacacacttgcttatttttttctacgcaggccccac-gccctgtcag-   [621]
R_shameli_cmr92         ttggtcttctgtgctcattaaacttctgagcctgacacacttgcttattgtttctccacaggccccac-gccctgtcagt   [623]
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R_simulator_cmr27       ttgggcttctgtgctcatttaactcctgaacctgacacacttgctcattgtttctccgcaggccccat-gccctgtcagt   [604]
R_simulator_cmr82       ttgggcttctgtgctcatttaactcctgaacctgacacacttgctcattgtttctccgcaggccccat-gccctgtcagt   [605]
R_swinnyi_cmr60         ttggtcttctgtgctcattaaactcctgaacctgacacacttgctcattgtttctccgcaggccccat-gccctgtcagt   [605]
R_thomasi_cmr94         ttggttttctgtgctcattaaactcctgaacctgacacacttgcttattgtttctccgcaggcccctc-gccctgtcagt   [624]
T_persicus_cmr153       ttggtcttctgtgctcatcaaactccttaacctgacatacttgcttattgtgtctcctcaggccccac-gccctgtcagt   [551]


[                               650       660       670       680       690       700       710       720]
[                               .         .         .         .         .         .         .         .]

C_percivali__cmrc2      tacaggccgagcaagccttcctcggggcagtacggtccctggtct-ctaa-ttccagcaagc-cacccgctctctccagt   [398]
H_caffer_cmrH2          tacaggctgagcaagcgttccttggggcagtacggtccctggtgt-ctaa-taccagtgagc-tgcccactctctccagt   [477]
R_acuminatus_cmr93      tacaggctgagcaagccttcctcaggacagcacggtccctggtct-ccaa-ttccagcatgc-cgNNNNNNNNNNNNNN   [692]
R_affinis_cmr31         tacaggcggagcaagccttcctcagggcagtacggtccctggtct-ccaa-ttccagcacgc-cgcccactctctccaNN   [700]
R_affinis_cmr87         tacaggcggagcaagccttcctcagggcagtacggtccctggtct-ccaa-ttccagcacgc-cgcccactNNNNNNNNN   [700]
R_blasii_cmr30          tacaggccgagcaagccttcctcagggcagtacggtccctggtct-ccaa-ttccagcatgc-cgcccactctctccagt   [651]
R_blasii_cmr49          tacaggccgagcaagccttcctcagggcagtacggtccctggtct-ccaa-ttccagcatgc-ggcccactNNNNNNNNN   [678]
R_borneensis_cmr129     tacaggccgagcaagccttcctcagggcagtacggtccctggtct-ccaa-ttccagcatgc-cacccactctgtccaNN   [682]
R_capensis_cmr10        tacaggccgagcaagccttcctcagggcagtacggtccctcgttt-ccaa-ttccagcatgc-cgcccactctctccagt   [657]
R_clivosus_cmr1         tacaggccgagcaagccttcctcggggcagtacggtccctggcct-ccaa-ttccagcatgcccgcccactctctccagt   [684]
R_darlingi_cmr76        tacaggccgagcaagccttcctcagggcagtacggtccctggcct-ccaa-ttccagcatgc-agcccactctNNNNNNN   [684]
R_denti_cmr56           tacaggccgagcaagccttcctcagggccgtacggtccctcgtct-ccaa-ttccagcatgc-cgcccactctctccagt   [682]
R_ferrumequinum_cmr51   tacaggctgagcaagccttcctcggggcagtacggtccctggcct-ccaatttccagcatgc-cgcccaNNNNNNNNNN   [660]
R_formosae_cmr119       tacaggccgagcaagccttcctcagggcagtacggtccctggtct-ccaa-ttccagcgtgc-tgcccactctctccagt   [682]
R_fumigatus_cmr12       tacaggccgagcaagccttcctcagggcagtacggtccctggcct-ccaa-ttccagcatgc-cgcccactctctccagt   [682]
R_fumigatus_cmr80       tacaggccgcgcaagccttcctcagggcagtacggtccctggcct-ccaa-ttccagcatgNNNNNNNNNNNNNNNNN   [684]
R_hildebrandti_cmr4     tacaggccgagc-agctttcctcagggcagtacggtccctggcct-ccaa-ttccagcatgc-cgcccactctctccaNN   [680]
R_hildebrandti_cmr83    tacaggccgagcaagccttcctcagggcagtacggtccctggcct-ccaa-ttccagcatgc-cgcccactctNNNNNNN   [682]
R_landeri_cmr13         tacaggccgagcaagccttcctcagggcagtatggtccctggtct-ccaa-ttccagcatgc-cgcccactctctccaNN   [692]
R_lepidus_cmr91         tacaggccgagccatNtttcctcagggcagtacggtccctggtct-ccaa-ttccagcatgc-cgcccNNNNNNNNNNN   [701]
R_maclaudi_cmr78        tacaggccgagcaagccttcctcagggcagtccagtccctggcct-ccaa-ttccagcatgc-ggcccactctttccagt   [682]
R_megaphyllus_cmr36     tacaggccgagcaagccttcctcagggcagtgcggtccctggtct-ccaa-ttccagcatgc-cgcccactct-tccagt   [699]
R_mehelyi_cmr50         tacaggccgagcaagccttcctcagggcagtacggtccctggtct-ccaa-ttccagcatgc-cgcccactctctccagt   [694]
R_monoceros_cmr121      tacaggccgagcaagccttcctcagggcagtacggtccctggtct-ccaa-ttccagcatgc-cgcccactctctccagN   [682]
R_f_nippon_cmr118       tacaggccgagcaagccttcctcggggcagtagggtccctggcct-ccaa-ttccagcatgc-cgcccactctctNcagN   [680]
R_pussilus_cmr96        tacagggagagccattaatcctcagggcagtacggtccctggtct-ccaa-ttccagcaagc-cgcccNNNNNNNNNNN   [698]
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R_shameli_cmr92          tacaggcggagcaagccttcctcagggcagtacggtccctggtct-ccaa-ttccagcatgc-tgcccaNNNNNNNNNNN   [700]
R_simulator_cmr27        tacaggccgagcaagccttcctcagggcagtacggtccctcgtct-ccaa-ttccagcatgc-cgcccactctctccagt   [681]
R_simulator_cmr82        tacaggccgagcaagccttcctcagggcagtacggtcccttgtct-ccaa-ttccagcaagc-cgcccaNNNNNNNNNNN   [682]
R_swinnyi_cmr60          tacaggccgagcaagccttcctcagggcagtacggtccctcgttt-ccaa-ttccagcatgc-cgcccactctctccagN   [682]
R_thomasi_cmr94          tacaggccgagcaagccttcctcaggacagtacggtccctggtct-ccaa-ttccagcatNNNNNNNNNNNNNNNNNNN   [702]
T_persicus_cmr153        tacaggccgagctt-ccttcctcagggcagtacggtccctggtct-ctaa-ttccagcaagt-cgccgactctcNNNNNN   [627]


[                         ]
[                         ]

C_percivali__cmrc2       N   [399]
H_caffer_cmrH2           N   [478]
R_acuminatus_cmr93       N   [693]
R_affinis_cmr31          N   [701]
R_affinis_cmr87          N   [701]
R_blasii_cmr30           N   [652]
R_blasii_cmr49           N   [679]
R_borneensis_cmr129      N   [683]
R_capensis_cmr10         N   [658]
R_clivosus_cmr1          N   [685]
R_darlingi_cmr76         N   [685]
R_denti_cmr56            c   [683]
R_ferrumequinum_cmr51    N   [661]
R_formosae_cmr119        c   [683]
R_fumigatus_cmr12        N   [683]
R_fumigatus_cmr80        N   [685]
R_hildebrandti_cmr4      N   [681]
R_hildebrandti_cmr83     N   [683]
R_landeri_cmr13          N   [693]
R_lepidus_cmr91          N   [702]
R_maclaudi_cmr78         N   [683]
R_megaphyllus_cmr36      N   [700]
R_mehelyi_cmr50          c   [695]
R_monoceros_cmr121       N   [683]
R_f_nippon_cmr118        N   [681]
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R_pussilus_cmr96          N    [699]
R_shameli_cmr92           N    [701]
R_simulator_cmr27         N    [682]
R_simulator_cmr82         N    [683]
R_swinnyi_cmr60           N    [683]
R_thomasi_cmr94           N    [703]
T_persicus_cmr153         N    [628]
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THY

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[                                10        20        30        40        50        60        70        80]
[                                .         .         .         .         .         .         .         .]

H_caffer                  TTCATCTTACTTCTTTCAGTTGTAAATTAGGTAAACCTGGACACTGTCCATTTCTATCCAGCAA--------TGAGATAA   [72]
H_ruber                   NTCATCTTACTTCTTTCAGCTGTAAATCAGGTAAACCTGGACACTGTCCATTTCTATCCAGCAA--------TGAGATAA   [72]
R_acuminatus_cmr93        TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
Rous_aegyptiacus          NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCTAGACTCAATCC-TTTCTATCCAGAAAGGAGGAATTGAGATAA   [79]
R_affinis_cmr31           TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATGA   [77]
R_affinis_cmr87           TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATGA   [77]
R_blasii_cmr30            TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]
R_blasii__cmr49           TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]
R_borneensis_cmr129       TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
R_capensis_cmr10          NNNNNNNNNNNNNNNNNNNTGTTTAT-AGAC-AACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [75]
R_clivosus_cmr1           NNNNNNNNNNNNNNNNNNNNNNNNNNNAGA----CCTGNACACAATCNATTTNTNNNCAGAAAAG---NAANGAGATNA   [73]
R_darlingi_cmr76          NNNNNNNNNNNNCTTTCGGCNGTAAATTANACAAACCTGGANACANTCCATTTNTGTNCAAAAAAG---AARTGAGATNA   [77]
R_denti_cmr56             TTNATCTTANTNNTTNNGNNNGTAAATATGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]
R_formosae_cmr119         TTTATNTTACTTTTTTCGGGTGTAAATTAGACAAACCTGGACACAGTTCATTTCTGTCCCGAAAAG---AAATGAGATAA   [77]
R_fumigatus_cmr12         TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGT-CaTTT-TgTaCAtgaGAG---AAAtGAGA-AA   [74]
R_fumigatus_cmr80         TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]
R_hildebrandti_cmr4       TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]
R_hildebrandti_cmr83      TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]
R_hipposideros_cmr142     TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
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R_landeri_cmr13          TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAGCCTGGACACAGTCCATTTCTGTCTGGAAAA----AAATGAGATAA   [76]
R_lepidus_cmr91          TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
R_maclaudi_cmr78         NTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACATGAGAT---AAATGAGATAA   [77]
R_macrotis_cmr89         TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
R_malayanus_cmr86        TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
R_marshalli_cmr90        TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
R_megaphyllus_cmr36      TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
R_monoceros_cmr121       TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
R_pearsoni_cmr95         TTCATCTTACTTCTNTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---AAATGAGATAA   [77]
Rp_hardwickei            NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCTAGACACAGTCCATTTCTATCCAGAAAAG---AAATGAGATAA   [77]
R_pussilus_cmr96         TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTATCCAGAAAAG---AAATGAGATAA   [77]
R_simulator_cmr27        TTCATCTTACTTCTTTCAGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]
R_simulator_cmr82        TTCATCTTACTTCTTTCAGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]
R_sinicus_cmr126         TTCATCTTACTTCTTTCGGCTGTAACTTAGACAAACCTGGACACAGTCCATTTCTGTCCAGAAAAG---ACATGAGATAA   [77]
R_swinnyi_cmr60          TTCATCTTACTTCTTTCGGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]
R_thomasi_cmr94          TTCATCTTACTTCTTTCAGCTGTAAATTAGACAAACCTGGACACAGTCCATTTCTGTACAGAAAAG---AAATGAGATAA   [77]

[                                  90        100       110       120       130       140       150       160]
[                                  .         .         .         .         .         .         .         .]

H_caffer                 ATCACA---ACCTAATTCCCCAAATATAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [149]
H_ruber                  ATCACA---ACCTAATTCCCCAAATATAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [149]
R_acuminatus_cmr93       ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
Rous_aegyptiacus         ACCACA----CCTCATTTCCCAAATCTAATGGTTATTGGCTTCTTAGAGGTCGAGTGCGCAGGTTACAATATTATGTGGA   [155]
R_affinis_cmr31          ATCACA---ACCTCATTTCCCAAATGTAATGGTTAGTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATCATACAGA   [154]
R_affinis_cmr87          ATCACA---ACCTCATTTCCCAAATGTAACGGTTAGTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATCACACAGA   [154]
R_blasii_cmr30           ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_blasii__cmr49          ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_borneensis_cmr129      ATCACA---ACCTCATTTCCCAAATATAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_capensis_cmr10         ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [152]
R_clivosus_cmr1          ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [150]
R_darlingi_cmr76         ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTCAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_denti_cmr56            ATCACA---ACCTCATTTCCCAAATGAAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATGTTATATGGA   [154]
R_formosae_cmr119        ATCACA---ACCTCATATCC-AAATGTAATGGTTATTGGTTCCGTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [153]
R_fumigatus_cmr12        AtCACA---aCCTCaTTTCcAAAATgAAAtGGTTatTGGCTCcTtAGaGgtAGaGtgCactGGTtACAATAtTATAtGGA   [151]
R_fumigatus_cmr80        ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_hildebrandti_cmr4      ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_hildebrandti_cmr83     ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
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R_hipposideros_cmr142    ATCACA---ATCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_landeri_cmr13          ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [153]
R_lepidus_cmr91          ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_maclaudi_cmr78         ATCACA---GCCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_macrotis_cmr89         ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_malayanus_cmr86        ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTGGAGTGCACTGGTTACAATATTATATGGA   [154]
R_marshalli_cmr90        ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_megaphyllus_cmr36      ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTACACTGGTTACAACATTATATGGA   [154]
R_monoceros_cmr121       ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_pearsoni_cmr95         ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTGAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
Rp_hardwickei            ATCATT---TCCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAG--TGCACAGGTTACA-TATTATATGGA   [151]
R_pussilus_cmr96         ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_simulator_cmr27        ATCGCA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATGTTATATGGA   [154]
R_simulator_cmr82        ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_sinicus_cmr126         ACCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_swinnyi_cmr60          ATCACA---ACCTCaTTtcCcAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]
R_thomasi_cmr94          ATCACA---ACCTCATTTCCCAAATGTAATGGTTATTGGCTCCTTAGAGGTAGAGTGCACTGGTTACAATATTATATGGA   [154]

[                                 170       180       190       200       210       220       230       240]
[                                 .         .         .         .         .         .         .         .]

H_caffer                 TCTACTCAGCACAACGAGTAAAGATAATTTTC--------TAACAGTTTACTTCCAAACTTTATTTAAACCTTATCTTGT   [221]
H_ruber                  TCTACTCAGCACAACGAGTAAAGATAATTTTC--------TAACAGTTTACTTCCAAACTTTATTTAAACCTTATCTTGT   [221]
R_acuminatus_cmr93       TCTGCTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTCCAACCTTATCTTGT   [226]
Rous_aegyptiacus         TCTACTTGGCATAATGGGTACAGATAATTTTA--------TAGCAGTTTACTTCCAAACTTTATTTAAACCTTATATTAT   [227]
R_affinis_cmr31          TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTACTTCAACCTTATCTTGT   [226]
R_affinis_cmr87          TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]
R_blasii_cmr30           TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [226]
R_blasii__cmr49          TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [226]
R_borneensis_cmr129      TCTACTCAGCACAATGGGTACAGATAATTTTG--------TAACAGTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]
R_capensis_cmr10         TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [224]
R_clivosus_cmr1          TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [222]
R_darlingi_cmr76         TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [226]
R_denti_cmr56            TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [226]
R_formosae_cmr119        TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [225]
R_fumigatus_cmr12        TCtaCTCAGCACAAGGGGtACAGATAATTTTT--------taACAGTTGATTTCCAAATTTTATTTCAACATTATCTTGT   [223]
R_fumigatus_cmr80        TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [226]
R_hildebrandti_cmr4      TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [226]
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R_hildebrandti_cmr83     TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [226]
R_hipposideros_cmr142    TCTACTCAGCACAGTGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACCTGATCTTAT   [226]
R_landeri_cmr13          TTTACTCAGCACAATGGGTACAGATAATTTTC--------TGACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [225]
R_lepidus_cmr91          TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]
R_maclaudi_cmr78         TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTAT   [226]
R_macrotis_cmr89         TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]
R_malayanus_cmr86        TCTACTCAGTACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAANCTTATCTTGT   [226]
R_marshalli_cmr90        TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]
R_megaphyllus_cmr36      TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]
R_monoceros_cmr121       TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]
R_pearsoni_cmr95         TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAATCTTATCTTGT   [226]
Rp_hardwickei            TCTACTCAACACGATGGGTACAGATAATT---ATATTATATAACAGTTCTCTTCCAAACTTTATTTAAACCTTATCTTGT   [228]
R_pussilus_cmr96         TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]
R_simulator_cmr27        TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCTAAACTTTATTTCAACATTATCTTGT   [226]
R_simulator_cmr82        TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTTTCTTGT   [226]
R_sinicus_cmr126         TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACACTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]
R_swinnyi_cmr60          TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACAGTTGACTTCCAAACTTTATTTCAACATTATCTTGT   [226]
R_thomasi_cmr94          TCTACTCAGCACAATGGGTACAGATAATTTTC--------TAACACTTGACTTCCAAACTTTATTTCAACCTTATCTTGT   [226]

[                                 250       260       270       280       290       300       310       320]
[                                 .         .         .         .         .         .         .         .]

H_caffer                 TCCTATAATCAAGGATAAAAGGCCACTTTTGTCTCCATGGGACTCAGTGAGGATGTGTTGAGTTGGTGTTGGGAAATGGG   [301]
H_ruber                  TCCTATAATCAAGGATAAAAGGCCACTTTTGTCTCCATGGGACTCAGTGAGGATGTGTTGAGTTGGTGTTGGGAAGTGGG   [301]
R_acuminatus_cmr93       TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAATGAGGATGTGTTGAGTTGGTATTCGGGAATGGG   [306]
Rous_aegyptiacus         TCCCATGATCAGGGATAAAAGGCCACTTTTGTCTCTCTTGGGTACAATGAAGTTGTG-TAAGTTGACATTGGGGAATGGG   [306]
R_affinis_cmr31          TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGATGTGTTGAGTTGGTATTCGGGAATGGG   [306]
R_affinis_cmr87          TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGATGTGTTGAGTTGGTATTCGGGAATGGG   [306]
R_blasii_cmr30           TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [306]
R_blasii__cmr49          TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [306]
R_borneensis_cmr129      TCCTATGATCAAGGATAAAAGGCCGCTTTTGTCTCCCTGGGATTCAATGAGGATGTGTTGAGTTGGTATTTGGGAACGGG   [306]
R_capensis_cmr10         TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [304]
R_clivosus_cmr1          TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [302]
R_darlingi_cmr76         TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [306]
R_denti_cmr56            TCCTATGATCAAGGATAAA-GGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTTGGAAATGGG   [305]
R_formosae_cmr119        TCCCATGATCAAGGATAAA-GGCCACTTTTGTCTCTCTGGGATTCAGTGAGGATGTGTCGAGTTGGTATTCGGGAATGGG   [304]
R_fumigatus_cmr12        TCCTATGATCAAGGA-AAA-GGCCACTTTTGTC-CCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTTTT-GGAAATGGG   [299]
R_fumigatus_cmr80        TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [306]
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R_hildebrandti_cmr4      TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [306]
R_hildebrandti_cmr83     TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [306]
R_hipposideros_cmr142    TCCCGTGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGATGTGCTGAGTTGGTATTCGGGAATGGG   [306]
R_landeri_cmr13          TCCCATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [305]
R_lepidus_cmr91          TCCTATGATCAAGGATAAGAGGCCACTTTTGTCTCCCTGGGATTCAATGAGGATGTGTTGAGTTGGTATTCGGGAATGGG   [306]
R_maclaudi_cmr78         TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [306]
R_macrotis_cmr89         TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAATGAGGATGTGTTGAGTTGGTATTCGGGAATGGG   [306]
R_malayanus_cmr86        TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAATGAGGATGTCTTGAGTTGGTATTCGGGAATGGG   [306]
R_marshalli_cmr90        TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAATGAGGATGTGTTGAGTTGGTATTCGGGAATGGG   [306]
R_megaphyllus_cmr36      TCCCATGATCAAGGATAAAAGGCCACTTTTGTCTCCTTGGGATTCAATGAGGATGTGTTGAGTTGGTATTCGGGAATGGG   [306]
R_monoceros_cmr121       TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAATGAGGATGTGTTGAGTTGGTATTCGGGAATGGG   [306]
R_pearsoni_cmr95         TCCCATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGATGTGTTGAGTTGGTATTCGGGAGTGGA   [306]
Rp_hardwickei            TCCTGTGATCAAAGATAAAAGGGCACTTTTGTCTCCATGGGATTCACTGAGGATGTGTCGAGTTGGGATTGGGGAATGGG   [308]
R_pussilus_cmr96         TCCTATGATCAAGGATAAGAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGATGTGTTGAGTTGGTATTCGGGAATGGG   [306]
R_simulator_cmr27        TCCTATGATCAAGGATAAA-GGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTTGGAAATGGG   [305]
R_simulator_cmr82        TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCNGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [306]
R_sinicus_cmr126         TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAATGAGGGTGTGTTGAGTTGGTATTCGGGAATGGG   [306]
R_swinnyi_cmr60          TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAGTGAGGACGTGTTGAGTTGGTATTCGGAAATGGG   [306]
R_thomasi_cmr94          TCCTATGATCAAGGATAAAAGGCCACTTTTGTCTCCCTGGGATTCAATGAGGGTGTGTTGAGTTGGTATTCGGGAATGGG   [306]

[                                 330       340       350       360       370       380       390       400]
[                                 .         .         .         .         .         .         .         .]

H_caffer                 GCTAAGGGATC-TTCTCCC----------------AGTCCTATTTGTGACAAAGGAATATAACCAAATTAAGTTTT---   [360]
H_ruber                  GCTAAGGGATC-TTCTCCC----------------AGTCCTATTTGTGACAAAGGAATATAACCAAATTAAGTTTT---   [360]
R_acuminatus_cmr93       ACTAAGGCATC-TTCCCCA----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
Rous_aegyptiacus         ACTA---------CTCCCCAATGGGAATCTTCCTCCATTCCTATTTGTGATAAAGGAATGTAAGTGAATTAATTTGTATT   [377]
R_affinis_cmr31          ACTAAGGCATC-TTCCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
R_affinis_cmr87          ACTAAGGCATC-TTCCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
R_blasii_cmr30           ACTAAGGCATCGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [369]
R_blasii__cmr49          ACTAAGGCATTGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [369]
R_borneensis_cmr129      ACTAAGGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
R_capensis_cmr10         ACTAAGGCATCGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGGGAATTAAGTTTTATT   [367]
R_clivosus_cmr1          ACTAAGGCATTGTCTCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [365]
R_darlingi_cmr76         ACTAAGGCATCGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGGATATAAGTGAATTAAGTTTTATT   [369]
R_denti_cmr56            ACTAAGGCATCGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGGGAATTAAGTTTT---   [365]
R_formosae_cmr119        ACTAAGACATC-TTCCCC----------------------TATTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [360]
R_fumigatus_cmr12        ACTAAGGCATTCCCCCCCC----------------AGTCCTGTTTGTGCAAAAGGGATAAAAGT-AAATAAGTTTTATT   [361]
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R_fumigatus_cmr80       ACTAAGGCATCATCCCCCC----------------AGTCCTGTTTGTGACAAAGGGATATAAGTGAATTAAGTTTTATT   [369]
R_hildebrandti_cmr4     ACTAAGGCATTGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGGATATAAGTGAATTAAGTTTTATT   [369]
R_hildebrandti_cmr83    ACTAAGGCATTGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGGATATAAGTGAATTAAGTTTTATT   [369]
R_hipposideros_cmr142   ACTAAGGCATCTTCCCCCC----------------AGTCCTATTTGTTACAAAGGAATATAAGTGAATTAAGTTTTATT   [369]
R_landeri_cmr13         ACTAAGGCATCGTCCCCCC----------------AGTCCTATTTGTGACAAAGGAATACAAGTGAATTAAGTTTTATT   [368]
R_lepidus_cmr91         ACTAAGGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
R_maclaudi_cmr78        ACTAAGGCATCGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGGATATAAGTGAATTAAGTTTTATT   [369]
R_macrotis_cmr89        ACTAAGGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
R_malayanus_cmr86       ACTAAGGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATTAGTGAATTAAGTTTTATT   [368]
R_marshalli_cmr90       ACTAAGGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
R_megaphyllus_cmr36     ACTAAGGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
R_monoceros_cmr121      ACTAAGGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
R_pearsoni_cmr95        ACTAAGGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAACTTTT---   [365]
Rp_hardwickei           ACTAAGGAATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATAGAAGTGAAGTAATTTTTATT   [370]
R_pussilus_cmr96        ACTAAGGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAAGTTTTATT   [368]
R_simulator_cmr27       ACTAAGGCATCGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGGGAATTAAGTTTT---   [365]
R_simulator_cmr82       ACTAAGGCATCGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGGGAATTAAGTTTT---   [366]
R_sinicus_cmr126        ACTA-GGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAACTTTTATT   [367]
R_swinnyi_cmr60         ACTAAGGCATCGTCCCCCC----------------AGTCCTGTTTGTGACAAAGGAATATAAGGGAATTAAGTTTTATT   [369]
R_thomasi_cmr94         ACTA-GGCATC-TTCCCCC----------------AGTCCTATTTGTGACAAAGGAATATAAGTGAATTAACTTTTATT   [367]

[                              410       420       430       440       450       460       470       480]
[                              .         .         .         .         .         .         .         .]

H_caffer                -GTTTCCATTATCTATATGTGTCCTAAAGTCCTATCACACTGTGCTCCTTTTCCTGTTCTTCCTC-AGGATATCAATGGC   [438]
H_ruber                 -GTTTCCATTATCTATATGTGTCCTAAAGTCCTATCACACTGTGCTCCTTTTCCTGTTCTTCCTC-AGGATATCAATGGC   [438]
R_acuminatus_cmr93      TGTTTCCATTATCTAGATGTGTCCTGAAGTCCTATCACACTACGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
Rous_aegyptiacus        CGTTTCCATTATCCATATGTATTTTAAAATCCTATCACATT-----CCTTTTTCTGCTCTTCCTT-AGGATATCAACGGC   [451]
R_affinis_cmr31         TGTTTCTATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
R_affinis_cmr87         TGTTTCTATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
R_blasii_cmr30          TGTTTCCATTATCTAGATGTGTCCTCAAGCCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [448]
R_blasii__cmr49         TGTTTCCATTATCTAGATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [448]
R_borneensis_cmr129     TGTTTCCATAATCTAGATGTGTCCTAAAGTCCTATCACGCTATGTTCCTTTTTCTGTTTTTCCTCTAGGAGATCAATGGC   [448]
R_capensis_cmr10        TGTTTCCAATATCTAGATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [446]
R_clivosus_cmr1         TGTTTCCATTATCTAGATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [444]
R_darlingi_cmr76        TGTTTCCATTATCTAGAGGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTGTTTCCTC-AGGATATCAATGGC   [448]
R_denti_cmr56           -GTTTCCAATATCTAGATGTGTCCTCAATTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [443]
R_formosae_cmr119       TGTTTCCATTATCTGGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTATTTTTCCTC-AGGATATCAATGGC   [439]
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R_fumigatus_cmr12        TGTTTCCATTATCTAGATGGTTCCTCAAGTCCTATCACACTAGGTTCCTTTTTCGGCTTTTCCTC-AGGATATCAATGGC   [440]
R_fumigatus_cmr80        TGTTTCCATTATCTAGATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [448]
R_hildebrandti_cmr4      TGTTTCCATTATCTAGATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [448]
R_hildebrandti_cmr83     TGTTTCCATTATCTAGATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [448]
R_hipposideros_cmr142    TGTTTCCATTATCTAAATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [448]
R_landeri_cmr13          TGTTTCCATTATCTATATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
R_lepidus_cmr91          TGTTTCCATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
R_maclaudi_cmr78         TGTTTCCATTATCTAGATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [448]
R_macrotis_cmr89         TGTTTCCATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
R_malayanus_cmr86        TGTTTCCATTATCTAGATGTAGCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
R_marshalli_cmr90        TGTTTCCATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
R_megaphyllus_cmr36      TGTTTCCATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
R_monoceros_cmr121       TGTTTCCATTATCTAGATGTGTCCTAAAGTCCTATCACACTATATTCCTTTTTCTGTTTTTCCTCTAGGAGATCAATGGC   [448]
R_pearsoni_cmr95         -GTTTCCATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATACCAATGGG   [443]
Rp_hardwickei            TGTTTCCATTATCTATATGTGTCCTAAAGTCCTATCTCACGATGCTCCTTTTTCTGTTCTTCCTC-AGGATATCAACGGC   [449]
R_pussilus_cmr96         TGTTTCCATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [447]
R_simulator_cmr27        -GTTTCCAATATCTAGATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [443]
R_simulator_cmr82        -GTTTCCAATATCTAGATGTGTCCTCAATTCCTATCACACTATGTACCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [444]
R_sinicus_cmr126         TGTTTCCATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTTTTCTATTTTTCCTC-AGGATATCAATGGC   [446]
R_swinnyi_cmr60          TGTTTCCAATATCTAGATGTGTCCTCAAGTCCTATCACACTATGTTCCTTTTTCTGTTTTTCCTC-AGGATATCAATGGC   [448]
R_thomasi_cmr94          TGTTTCCATTATCTAGATGTGTCCTAAAGTCCTATCACACTATGTTCCTTCTTCTATTTTTCCTC-AGGATATCAATGGC   [446]

[                                490       500       510       520]
[                                .         .         .         . ]

H_caffer                 AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [479]
H_ruber                  AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTT-G   [478]
R_acuminatus_cmr93       AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [488]
Rous_aegyptiacus         AAGCTGTTTCTCCCCAAATATGCTCTGTCTCAGGATGTTTG   [492]
R_affinis_cmr31          AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [488]
R_affinis_cmr87          AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [488]
R_blasii_cmr30           AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [489]
R_blasii__cmr49          AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [489]
R_borneensis_cmr129      AAGCT-TTTCTCCCCAAATATACTCT-TCCCAGGANGTAGG   [487]
R_capensis_cmr10         AAGCTGTTTCTCCCCAAATATGCCCTGTCCCAGGATGTTTG   [487]
R_clivosus_cmr1          AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [485]
R_darlingi_cmr76         AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTNG   [489]
R_denti_cmr56            AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [484]
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R_formosae_cmr119        AAGCTGTTTCTCCCCAAATATGCTCT-TCCCAGGATGTTTG   [479]
R_fumigatus_cmr12        AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [481]
R_fumigatus_cmr80        AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [489]
R_hildebrandti_cmr4      AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [489]
R_hildebrandti_cmr83     AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTT-G   [488]
R_hipposideros_cmr142    AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTT-G   [488]
R_landeri_cmr13          AAGCTTTTGCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [488]
R_lepidus_cmr91          AAGCTGTTTCTCCCCAAATATGCTCTGTCTCAGGATGTTTG   [488]
R_maclaudi_cmr78         AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTT-G   [488]
R_macrotis_cmr89         AAGCTGTTTCTCCCCAAATATGCTCTGTCTCAGGATGTTTG   [488]
R_malayanus_cmr86        AAGCTGTTTCTCCCCAAATACGCTCTGTCCCAGGATGTTTG   [488]
R_marshalli_cmr90        AAGCTGTTTCTCCCCAAATATGCTCTGTCTCAGGATGTTTG   [488]
R_megaphyllus_cmr36      AAGCTGTTTCTTCCCAAATATGCTCTGTCCCAGGATGTTTG   [488]
R_monoceros_cmr121       AAGCTGTTTCTCCCCAAATATGCTCTGTCTCAGGATGTTTG   [489]
R_pearsoni_cmr95         AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [484]
Rp_hardwickei            AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [490]
R_pussilus_cmr96         AAGCTGTTTCTCCCCAAATATGCTCTGTCTCAGGATGTTNG   [488]
R_simulator_cmr27        AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [484]
R_simulator_cmr82        AAGGAGTTTCTCCCCAAATATTNTCTGTCCCCNNNNGTTTG   [485]
R_sinicus_cmr126         AAGCTGTTTCTCCCCAAATATGCTCT-TCCCAGGATGTTTG   [486]
R_swinnyi_cmr60          AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [489]
R_thomasi_cmr94          AAGCTGTTTCTCCCCAAATATGCTCTGTCCCAGGATGTTTG   [487]
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PRKC1

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[                          10        20        30        40        50        60        70        80]
[                          .         .         .         .         .         .         .         .]

H_caffer                 TTGGCTT-AGTATACCAT-----TTCAGAGAAAACCACAATGTGTGTGCCTTATTATATCAGTCTACTTTTCTAAATACT   [74]
H_ruber                  NNNNNTTANGTATACCAT-----TTCAGAGAAAACCACAATGTGTGTGCCTTATTGTATCAGTCTACTTTTCTAAATACT   [75]
R_acuminatus_cmr93       TTGGCTT-AGTGTACCAG-----TTCAGATAAAACTGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [72]
Rous_aegyptiacus         ttg-cataagtgtaccat-----ttcagagaaaacctcaatgtctgtgccttattatttcagcctgcttttctaaata--   [72]
R_affinis_cmr31          TTGGCTT-AGTGT-CCAG-----TTCAGATAAAACTGCAATATTTGTACCTTATTACTTCAGTCTA--TATCTAAATA--   [69]
R_affinis_cmr87          TTGGCTT-AGTGTACCAG-----TTCAGATAAAACTGCAATATTTGTACcTTATTACTTCAGTCtACTTTTCTAAATA--   [72]
R_blasii_cmr30           TTGGCTT-AGTGC-CCAGTTCAGTTCAGATAAAACCTCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [76]
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R_blasii_cmr49          TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCTCAATATTTGTACCTTATTACTTTAGTCTACTTTTCTAAATA--   [77]
R_borneensis_cmr129     TTGGCTT-AGTGTACCAG-----TTCAGATAAAACTGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [72]
R_capensis_cmr10        TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [77]
R_clivosus_cmr1         TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [77]
R_darlingi_cmr76        TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [77]
R_denti_cmr56           TTGGCT-AAGTGCACCAGTTC-GTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCNGTCTACTTTTCTAAATA--   [76]
R_formosae_cmr119       TTGGCTT-AGTGTACCAG-----TTCAGATAAAACCGCAATATTTGTACCTGATTACTTCAATCTaCTTTTCTAAATA--   [72]
R_fumigatus_cmr12       TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAgTcTACTTTTCTAAATA--   [77]
R_fumigatus_cmr80       TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [77]
R_hildebrandti_cmr4     TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [77]
R_hildebrandti_cmr83    TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [77]
R_hipposideros_cmr52    TTGGCTT-AGTGCACCAG-----TTCCGGTAAAACCGCAATATTTGTACCTTATTACTTCCGTCTACTTTTCTAAATG--   [72]
R_landeri_cmr13         TTGGCTT-AGtGCACCAGTTCAGTTCAGATAAAACCTCAATATTTGTACCTTATcACTTCAATCTACTTTTCTAAATA--   [77]
R_maclaudi_cmr78        TTGGCTT-AGTGTACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [77]
R_macrotis_cmr89        TTGGCTT-AGTGTACCAG-----TTCAGATAAAACTGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [72]
R_malayanus_cmr86       NTGGCTt-AgTGTACCAG-----TTc-GATAAAACTGCAATATTTGTACCTTATTACTTCAGTCtaCTTTTCTAAATA--   [71]
R_marshalli_cmr90       TTGGCTT-AGTGTACCAG-----TTCAGATAAAACTGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [72]
R_megaphyllus_cmr36     TTGGCT--AGTGT-CCAG-----TTC-GATAAAACTGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [69]
R_mehelyi_cmr50         TTGGCTTAAGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTATACCTTATTATTTCAGTCTACTTTTCTAAATA--   [78]
R_monoceros_cmr121      NNGGCTTGAGTGT-CCAG-----TTCCGATAGAACTGCGATATTTGTACCTTATTACTTCAGTCTATNAGTCTAAATA--   [72]
R_f_nippon_cmr118       NNNNCTTAAGTGC-CCAGTTCAGTTGAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [77]
Rhp_hardwickei          ttggcttaagtataccat-----ttcagagcaaccctcagtgtttgtgccttattatttcaatctactttttctaaatac-   [74]
R_pussilus_cmr96        TTGGCTT-AGTGTACCAG-----TTCAGATAAAACTGCAATATTTGTACCTTATTACTTCAGTCtaCTTTTCTAAATA--   [72]
R_shameli_cmr92         TTGGCTT-AGTGTACCAG-----TTCAGATAAAACTGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [72]
R_simulator_cmr27       TTGACTTAAGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [78]
R_simulator_cmr82       TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTA----TTCAGTCTACTTTTCTAAATA--   [73]
R_sinicus_cmr126        TTGGCTT-AGTGTACCAG-----GTCAGATAAAACTGCAATATTTGTACCTTTTTACTTCAGTCTACTTTTCTAAATA--   [72]
R_swinnyi_cmr60         TTGGCTT-AGTGCACCAGTTCAGTTCAGATAAAACCGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [77]
R_thomasi_cmr94         NGGGCTT-AGTGTATCAG-----TTCAGATAAAACTGCAATATTTGTACCTTATTACTTCAGTCTACTTTTCTAAATA--   [72]

[                              90        100       110       120       130       140       150       160]
[                              .         .         .         .         .         .         .         .]

H_caffer                GTATATGCTTCAAACAG--TTATATAGGATGTATTTAAAGGAATGTGATGATTATTAGATTTTTAAAAAA-TAAAAATCA   [151]
H_ruber                 GTATATGCTTCAAACAG--TTATATAGGATGTATTTAAAGGAATGTGATGATTATTAGATTTTTAAAAAA-TAAAAATCA   [152]
R_acuminatus_cmr93      ---TATGCCTCAAACAGATTTATATGGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTTAAAAATAAAAATCA   [146]
Rous_aegyptiacus        ---tatgcttcaatcaga-ttatataggatatatctaaagtaatctaatgattattaaatt----aaaa---aaaaatca   [141]
R_affinis_cmr31         ---TATGCCTCAAGCAGATTTATATAGGATATCTTTAAAGGAAGCTGGTGG---TTAGATTTTTTAAAAA-TAAAAATCA   [142]
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R_affinis_cmr87          ---TATGCCTCAAGCAGATTTATATAGGATATATTTAAAGGAAGCTGGTGG---TTAGATTTTTTAAAAA-TAAAAATCA   [145]
R_blasii_cmr30           ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAATCA   [149]
R_blasii_cmr49           ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAATCA   [150]
R_borneensis_cmr129      ---TATGCCTCAAACAGATTTATATAGGaTGTATTTAAAGGAAGCTGATGG---TTAGATTTtTaaAAAA-TAAAAATCA   [145]
R_capensis_cmr10         ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTT-AAAAAGTAAAAAA--   [148]
R_clivosus_cmr1          ---TATGCCTCGAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAAA--   [148]
R_darlingi_cmr76         ---TATGCCTTGAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAAA--   [148]
R_denti_cmr56            ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTAAAAAA-TAAAAAA--   [147]
R_formosae_cmr119        ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAATCA   [145]
R_fumigatus_cmr12        ---TATGCCTCGAACAGATTTATATagGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAAA--   [148]
R_fumigatus_cmr80        ---TATGCCTCGAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAAA--   [148]
R_hildebrandti_cmr4      ---TATGCCTCGAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAAA--   [148]
R_hildebrandti_cmr83     ---TATGCCTCGAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAAA--   [148]
R_hipposideros_cmr52     ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGA---GTAGATTTTTTAAAAA-TAAAAATCA   [145]
R_landeri_cmr13          ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAATcA   [150]
R_maclaudi_cmr78         ---TATGCCTCGAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAAA--   [148]
R_macrotis_cmr89         ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-T------CA   [139]
R_malayanus_cmr86        ---TATGccTCAAACAGATTTATATAGGATataTTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAATCA   [144]
R_marshalli_cmr90        ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAATCA   [145]
R_megaphyllus_cmr36      ---TATGCCTCAAACAGATTTATATAGGATATATTTA--GC-AGCTGATGG---TTAGATTTTTTAAAAA-TAAAAATCA   [139]
R_mehelyi_cmr50          ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAAATGATGG---CTAGATTTTTTAAAAA-TAAAAATCA   [151]
R_monoceros_cmr121       ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTAAAAAAATAAAAATCA   [146]
R_f_nippon_cmr118        ---TATGCCTCGAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAAT--   [148]
Rhp_hardwickei           -tatatgcttcaagcagattt-tataggatatt--------aatatgg---ttattagatttttttataaataaaaatca   [141]
R_pussilus_cmr96         ---TATGCCTCAAACAGATTTATATAGGaTATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAAATAAAAATCA   [146]
R_shameli_cmr92          ---TATGCCTCAAGCAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAATCA   [145]
R_simulator_cmr27        ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTAAAAAA-TAAAAAA--   [149]
R_simulator_cmr82        ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTAAAAAA-TAAAAAA--   [144]
R_sinicus_cmr126         ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TAAAAATCA   [145]
R_swinnyi_cmr60          ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTT-AAAAAGTAAAAAA--   [148]
R_thomasi_cmr94          ---TATGCCTCAAACAGATTTATATAGGATATATTTAAAGGAAGCTGATGG---TTAGATTTTTTAAAAA-TCAAAATCA   [145]

[                                170       180       190       200       210       220       230       240]
[                                .         .         .         .         .         .         .         .]

H_caffer                 AACCAT---ATTATCAAGGGAAAGTGCTTGGTTGAGCTTTGTAAGAGTTTTCTAGATTGTTTAATAATATTGAAAGATTC   [228]
H_ruber                  AACCAT---ATTATCAAGGGAAAGTGCTTGGTTGAGCTTTGTAAGAGTTTTCTAGATTGTTTAATAATATTGAAAGATTC   [229]
R_acuminatus_cmr93       AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTGAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
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Rous_aegyptiacus        agccat---attattaagggaaag---ttgattgagctttgtaaaagttttctagactgttttgtaatattgaaagattt   [215]
R_affinis_cmr31         AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAG-TTTCTAGATTATTTAATAACATTGAAAGATTC   [218]
R_affinis_cmr87         AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [222]
R_blasii_cmr30          AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [226]
R_blasii_cmr49          AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTT-GTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [226]
R_borneensis_cmr129     AACTAT---ATTATCAAGGGAaAATACTTGGTTAaGCtTTGgAAAAGTTTTcTAGATTATTTAATAACATTGAAAGATTC   [222]
R_capensis_cmr10        --CTAT---ATTATCAAGGGAAAGTACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
R_clivosus_cmr1         --CTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
R_darlingi_cmr76        --CTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTaTTTAATAACATTGAAAGATTC   [223]
R_denti_cmr56           --CTAT---ATTATCAAGGGAAAGTACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [222]
R_formosae_cmr119       AACT-----ATTATCAAGGGGAAATACTTGGTTAAGCTTTGTAAAAGTTTTTTAGATTATCTAATAACATTGAAAGATTC   [220]
R_fumigatus_cmr12       -ACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [224]
R_fumigatus_cmr80       --CTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGACAGATTC   [223]
R_hildebrandti_cmr4     --CTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
R_hildebrandti_cmr83    --CTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
R_hipposideros_cmr52    AACTAT---ATTATCAAGGGAAAATACTTGCTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [222]
R_landeri_cmr13         AaTTAT---ATTATCAAGGGAAAACaCTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAGGATTC   [227]
R_maclaudi_cmr78        --CTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
R_macrotis_cmr89        AACTAT---ATTATCAAGGAAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [216]
R_malayanus_cmr86       AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTATTCTAAATTATTTAATAACATTGAAAGATTC   [221]
R_marshalli_cmr90       AACTGT---ATTATCAAGGGAAAATACTTGGATAAGCTT-GTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [221]
R_megaphyllus_cmr36     AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAACATTC   [216]
R_mehelyi_cmr50         AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [228]
R_monoceros_cmr121      AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
R_f_nippon_cmr118       --CTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTGAAAGTCTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
Rhp_hardwickei          aaccatattattatcaagggaaagtacttgattgagctttgtaaaagttttctagaccatttaataatattgaaagattt   [221]
R_pussilus_cmr96        AACTAT---ATAATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
R_shameli_cmr92         AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [222]
R_simulator_cmr27       --CTAT---ATTATCAAGGGAAAGTACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [224]
R_simulator_cmr82       --CTAT---ATTATCAAGGGAAAGTACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [219]
R_sinicus_cmr126        AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTGCTAGATTATTTAATAACATTGAAAGATTC   [222]
R_swinnyi_cmr60         --CTAT---ATTATCAAGGGAAAGTACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [223]
R_thomasi_cmr94         AACTAT---ATTATCAAGGGAAAATACTTGGTTAAGCTTTGTAAAAGTTTTCTAGATTATTTAATAACATTGAAAGATTC   [222]

[                               250       260       270       280       290       300       310       320]
[                               .         .         .         .         .         .         .         .]

H_caffer                ACTGGTGATGTTTTCCCCATTAGTATGTGTAAATTGTTGTTTCAT-ATTATCAATGCTTTTGATATTTAGCCTAGTTCAT   [307]
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H_ruber                 ACTGGTGATGTTTTCCTCTTTAGTATGTGTAAATTGTTGTTTTAT-ATTATCAATGCTTTTGATATTTAGCCTAGTTCAT   [308]
R_acuminatus_cmr93      ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGTTTTTGATATTTCGCCTAGTTCAT   [297]
Rous_aegyptiacus        actggtgRtatttttcttttt-gtata--taaattgtt---tcatgattatcattgcttttgatattcagcctagttaat   [289]
R_affinis_cmr31         ACTGGTGATGATTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [292]
R_affinis_cmr87         ACTGGTGATGATTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [296]
R_blasii_cmr30          ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [300]
R_blasii_cmr49          ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [300]
R_borneensis_cmr129     AcTGGtGATGTtTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAAtGTTTgtGGTATTTCGCCTAGTTCAT   [296]
R_capensis_cmr10        ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [297]
R_clivosus_cmr1         ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [297]
R_darlingi_cmr76        ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTTGCCTAGTTCAT   [297]
R_denti_cmr56           ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [296]
R_formosae_cmr119       ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [294]
R_fumigatus_cmr12       ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [298]
R_fumigatus_cmr80       ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [297]
R_hildebrandti_cmr4     ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [297]
R_hildebrandti_cmr83    ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [297]
R_hipposideros_cmr52    ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [296]
R_landeri_cmr13         ACTGGTGAtGTTTTtCCCTTT-GtAtA--tAAATTgTT---TCaTGATTATCAgTGCTTTTgATaTTtCgCCtAGTTCAT   [301]
R_maclaudi_cmr78        ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [297]
R_macrotis_cmr89        ACTGGTGATGTTTTTCCCTTT-GTA----TAAATTGTT---TCATGATTATCAATGTTTTTGATATTTCGCCTAGTTCAT   [288]
R_malayanus_cmr86       ACTGGTGATGTTTTTCCCTTT-GTGTA--TGAATTGTt---TCATGATTATCAATGTTTTTGATATTTCACCTAGTTCAT   [295]
R_marshalli_cmr90       ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGTTTTTGATATTTCGCCTAGTTCAT   [295]
R_megaphyllus_cmr36     ACTGGTGATGTTTTTCCCTTT-GTATA--TAAACTGTT---TCATGATTATCAATGTTTTTGGTATTTCGCCTAGTTCAT   [290]
R_mehelyi_cmr50         ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [302]
R_monoceros_cmr121      ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGTTTTTGATATTTCGCCTAGCTCAT   [297]
R_f_nippon_cmr118       ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAGTGCTTTTGATATTTCGCCTAGTTCAT   [297]
Rhp_hardwickei          agtagtggtgtttttcccttt-gtatg--taaattgttatttcttgattatcaatgcttttaatgtttagcctagttcat   [298]
R_pussilus_cmr96        ACTGGTGATGTTTTTCcCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGTTTTTGATATTTCGCCTAGTTCAT   [297]
R_shameli_cmr92         ACTGGTGATGATTTTCCCTTT-GTATA--TAAATTGTT---TCACGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [296]
R_simulator_cmr27       ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [298]
R_simulator_cmr82       ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [293]
R_sinicus_cmr126        ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAGTGCTTTTGATATTTCGCCTAGTTCAT   [296]
R_swinnyi_cmr60         ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [297]
R_thomasi_cmr94         ACTGGTGATGTTTTTCCCTTT-GTATA--TAAATTGTT---TCATGATTATCAATGCTTTTGATATTTCGCCTAGTTCAT   [296]

[                              330        340        350        360        370        380        390        400]
[                              .          .          .          .          .          .          .          .]
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H_caffer                AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATCATTTTAGTTTGACATAGATTTCTT   [387]
H_ruber                 AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGACTTATCATAGTTTTGATATCATTTTAGTTTGACATAGATTTCTT   [388]
R_acuminatus_cmr93      AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATTATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [377]
Rous_aegyptiacus        aaagacatttgtgataattgaattgctaagaacaaatttat-ataatttcgatg-------------NNNNNNNNNNNN   [355]
R_affinis_cmr31         AGTTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCACAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [372]
R_affinis_cmr87         AGTTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [376]
R_blasii_cmr30          AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [380]
R_blasii_cmr49          AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [380]
R_borneensis_cmr129     AATTGCATTTGTGATCTTTGAATTACTAAGAACAGATTTATTATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [376]
R_capensis_cmr10        AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [377]
R_clivosus_cmr1         AGTTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [377]
R_darlingi_cmr76        AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [377]
R_denti_cmr56           AATTGCATTTGTGATCTTTGAATTGCTAAAAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [376]
R_formosae_cmr119       AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [374]
R_fumigatus_cmr12       AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [378]
R_fumigatus_cmr80       AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [377]
R_hildebrandti_cmr4     AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTGTTGATATAATTTTAGTGTGACATAGATTTCTT   [377]
R_hildebrandti_cmr83    AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTGTTGATATAATTTTAGTGTGACATAGATTTCTT   [377]
R_hipposideros_cmr52    AATTGCATTTGTGATTTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [376]
R_landeri_cmr13         AATtGCaTTTGtGAtcTTtGAATTGCtAAAAAcAgATTtATCATaGTTTTGAtATAATTTtaGGGtGACATAgaTTTcTT   [381]
R_maclaudi_cmr78        AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [377]
R_macrotis_cmr89        AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATTATAGTTTTGATATCATTATAGTGTGACATAGATTTCTT   [368]
R_malayanus_cmr86       AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATTATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [375]
R_marshalli_cmr90       AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATTATAGTTTTGATATCATTATAGTGTGACATAGATTTCTT   [375]
R_megaphyllus_cmr36     AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATTATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [370]
R_mehelyi_cmr50         AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACACAGATTTCTT   [382]
R_monoceros_cmr121      AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATTATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [377]
R_f_nippon_cmr118       AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGAGTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [377]
Rhp_hardwickei          aatagcatttgtgatctttgaattgctaagaacaaatttatcatagttttgctatcattttagtttgacatagatttctt   [378]
R_pussilus_cmr96        AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN   [377]
R_shameli_cmr92         AGTTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [376]
R_simulator_cmr27       AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [378]
R_simulator_cmr82       AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [373]
R_sinicus_cmr126        AATTGCATTTGTGATCTTTGAATTTCTAAGAACAGATTTATTATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [376]
R_swinnyi_cmr60         AATTGCATTTGTGATCTTTGAATTGCTAAGAACAGATTTATCATAGTTTTGATATAATTTTAGTGTGACATAGATTTCTT   [377]
R_thomasi_cmr94         AATTGCATTTGTGATCTTTGAATTTCTAAGAACAGATTTATTATAGTTTTGATATCATTTTAGTGTGACATAGATTTCTT   [376]
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[                              410       ]
[                              .         ]
H_caffer                  TCATATTTT-AAAATAGGA   [405]
H_ruber                   TCATATTTT-AAAATAGGA   [406]
R_acuminatus_cmr93        TCATATTTT-AAAATAGGA   [395]
Rous_aegyptiacus          NNNNNNNNNNNNNNNNNN    [374]
R_affinis_cmr31           TCATATTTT-AAAATAGGA   [390]
R_affinis_cmr87           TCATATTTT-AAAATAGGA   [394]
R_blasii_cmr30            TCATATTTT-AAAATAGGA   [398]
R_blasii_cmr49            TCATATTTT-AAAATAGGC   [398]
R_borneensis_cmr129       TCATATTTT-AAAATAGGa   [394]
R_capensis_cmr10          TCATATTTT-AAAATAGGA   [395]
R_clivosus_cmr1           TCATATTTT-AAAATAGGA   [395]
R_darlingi_cmr76          TCATATTTT-AAAATAGGA   [395]
R_denti_cmr56             TCATATTTN-AAAATAGAA   [394]
R_formosae_cmr119         TCATATTTT-AAAATAGGA   [392]
R_fumigatus_cmr12         TCATATTTT-AAAATAGGa   [396]
R_fumigatus_cmr80         TCATATTTT-AAAATAGGC   [395]
R_hildebrandti_cmr4       TCATATTTT-AAAATAGGA   [395]
R_hildebrandti_cmr83      TCATATTTT-AAAATAGGA   [395]
R_hipposideros_cmr52      TCATATTTT-AAAATAGGA   [394]
R_landeri_cmr13           TCaTaTTTT-AAAAtAGGA   [399]
R_maclaudi_cmr78          TCATATTTT-AAAATAGGA   [395]
R_macrotis_cmr89          TCATATTTT-AAAATAGGA   [386]
R_malayanus_cmr86         tcATATTTT-AAAATAGGN   [393]
R_marshalli_cmr90         TCATATTTT-AAAATAGGA   [393]
R_megaphyllus_cmr36       TCATATTTT-AAAATAGGA   [388]
R_mehelyi_cmr50           TCATATTTT-AAAATAGGA   [400]
R_monoceros_cmr121        TCATATTTT-AAAATAGGA   [395]
R_f_nippon_cmr118         TCATATTTT-AAAATAGGA   [395]
Rhp_hardwickei            tcatatttttaaaatagNN   [397]
R_pussilus_cmr96          NNNNNNNNNNNNNNNNNN    [396]
R_shameli_cmr92           TCATATTTT-AAAATAGGA   [394]
R_simulator_cmr27         TCATATTTN-AAAATAGGA   [396]
R_simulator_cmr82         TCATATTTA-AAAATAGGA   [391]
R_sinicus_cmr126          -CATATTTT-AAAATAGGA   [393]
R_swinnyi_cmr60           TCATATTTT-AAAATAGGA   [395]
R_thomasi_cmr94           TCATATTTT-AAAATAGGA   [394]
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