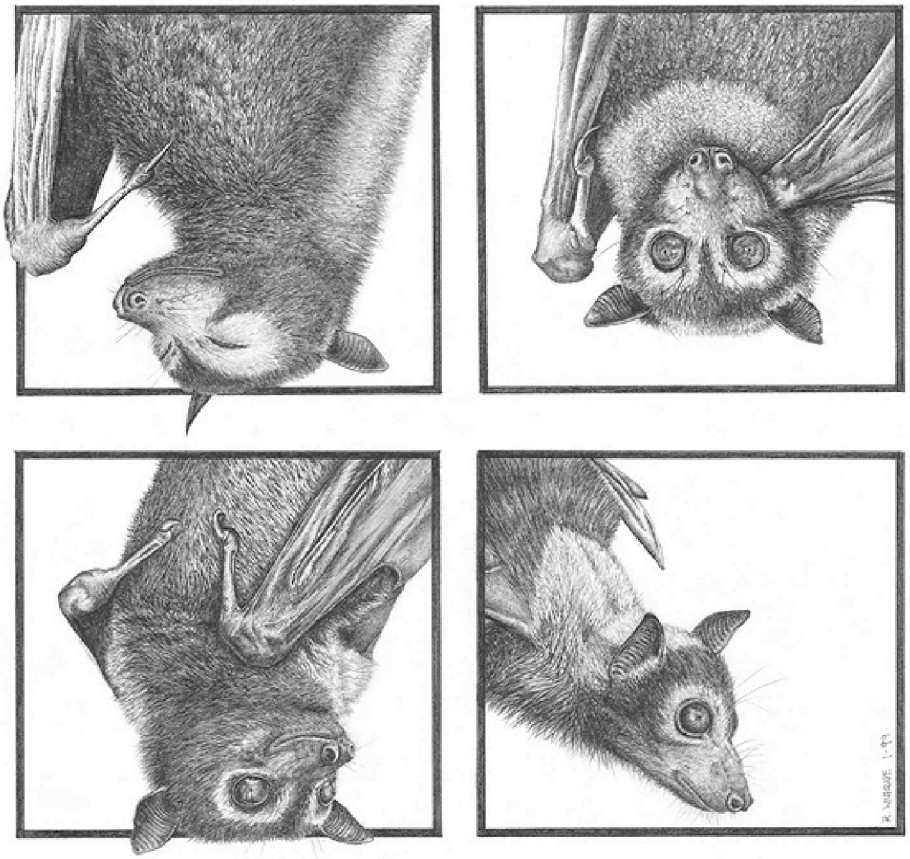
Population structure in the spectacled flying fox, *Pteropus conspicillatus*: A study of genetic and demographic factors



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Thesis submitted by

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For the degree of Doctor of Philosophy

In the Schools of Tropical Biology, and Tropical Environment Studies and Geography, James Cook University, Townsville

In nature's infinite book of secrecy

A little I am read

~ William Shakespeare, 'Anthony and Cleopatra'

Statement of sources

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any University or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references given.

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Abstract

The spectacled flying fox (*Pteropus conspicillatus*) is a difficult species to manage due to its dual status as an agricultural pest and a native species under threat. Like other flying fox species, the spectacled flying fox is very mobile, roosts in colonies during the day in largely inaccessible places, and is active at night. These factors make the spectacled flying fox a difficult species to study and are in part responsible for the lack of knowledge on the biology and ecology of this species. The population structure of the spectacled flying fox was examined using two genetic markers (highly polymorphic microsatellites, and a portion of the mtDNA D-loop) and cementum layers around the root of canine teeth to determine age structure. Incorporating both genetic and demographic factors, as well as examining population structure across several temporal and spatial scales, provided a more comprehensive understanding of this species.

A new hypothesis for the origin and evolution of flying foxes in Australia is presented: that flying foxes are an old lineage in Australia and that they colonised PNG from Australia rather than the other way around. The spectacled flying fox has experienced a tumultuous history, including population expansion and contraction as a result of climatic and geographic events. These events have aided in shaping the contemporary structure of highly connected colonies within a single panmictic population in the Wet Tropics region, along with an isolated population at Iron Range and populations of unknown status in Papua New Guinea. High allelic and haplotypic diversity suggest an old lineage within Australia, and the patterns of diversity suggest colonisation from Australia to PNG. Introgression between black flying foxes and spectacled flying foxes suggests a close association of these two species. The possibility of incomplete lineage sorting also suggests that *Pteropus alecto* and *Pteropus conspicillatus* might still be in the process of diverging. *Pteropus poliocephalus* might also belong to such a species complex, although no relevant data are yet available. Although high levels of gene flow occur among colonies within the Wet Tropics region, some sub-structuring in the form of kin groups within colonies is indicated, with several cohorts of

young remaining with their mothers before the young reach sexual maturity. High rates of mortality and low reproductive output may be putting this species at risk of decline, especially as their average longevity is considerably less than expected.

This study highlights the need for spectacled flying foxes to be managed on a regional scale. In addition, mortality rates need to be investigated throughout the Wet Tropics to determine the applicability of mortality rates estimated in this study, across the range of the species.

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Chapter One – studying population structure in the spectacled flying fox

The spectacled flying fox, *Pteropus conspicillatus* Gould 1850 (Andersen 1912), plays an important ecological role throughout its range as a seed and pollen disperser and is recognised as a significant component of the biodiversity of the Wet Tropics World Heritage area of north Queensland (Westcott *et al.* 2001). However, it is also considered an agricultural pest due to the loss of fruit from foraging flying foxes. In 2002 the spectacled flying fox was listed as a vulnerable species under the Australian Commonwealth Government's Environmental Protection and Biodiversity Conservation Act (EPBC Act). This was in part due to a lack of information on the population biology of this species, as well as the apprehension of conservationists and scientists that the species is in decline. The paucity of information on even the basics of population dynamics and ecology of this species has been recognised as a significant obstacle to developing appropriate management or recovery plans (Duncan *et al.* 1999; Westcott *et al.* 2001). An improved understanding of these aspects of species biology will inform environmental managers of patterns and processes affecting this species in order to balance the apparently contradictory requirements of pest mitigation and species conservation. Specifically, an understanding of connectivity between colonies will allow management agencies to refine the scale of management. A greater knowledge of the species will also answer many questions posed by fruit growers, which may then enable them to implement novel, or at least better, damage mitigation strategies. For farmers, understanding the connectivity of colonies will enable them to appreciate where foraging flying foxes are coming from. For conservationists, knowing the genetic health of the spectacled flying fox may allow them to build a more comprehensive picture of the status of the species.

Current knowledge of the spectacled flying fox is restricted to diet and feeding ecology (Richards 1990b; Eggert 1994; Parsons 2005), conservation status (Garnett *et al.* 1999; WPSQ 2000; ABS 2001; Tidemann and Vardon 2001;

Westcott *et al.* 2001), and roost sites and distribution (Ratcliffe 1932; Richards 1990a). Although research is being undertaken currently by the Commonwealth Science and Industrial Research Organisation (CSIRO) on movement patterns of spectacled flying foxes within the Wet Tropics World Heritage Area, nothing is known about population structure, broad-scale movement patterns throughout its distribution, or historical and contemporary processes that have affected the current geographical pattern of colony distribution. Current threats, including shooting by fruit farmers, tick paralysis, habitat loss, barbed wire fences and powerlines, are believed to be contributing to the general decline of this species. However, nothing is known of the effects of threats such as these on population demographics and therefore whether they are putting the species at risk.

Research to date has relied on observational techniques. However, many of the questions posed regarding population structure cannot be resolved with the use of observation alone. Hence, this project was instigated to examine questions of population structure across several spatial and temporal scales, using alternate approaches. The objective was to present an inclusive understanding of spectacled flying fox population dynamics. This chapter provides background on the Megachiroptera and the origins and relationships of Australian *Pteropus* species, to establish the historical setting within which to question the evolution and origin of Australian *Pteropus* species. This is followed by a description of the method used in this thesis to study population structure in a flying fox species.

Megachiroptera

The sub-order Megachiroptera comprises a single family, the Pteropodidae, which is distributed throughout tropical and subtropical Africa, Asia and Australia [Fig. 1.1 – reproduced from Hill and Smith (1984)]. Megachiropterans are especially diverse in Asia and the Australo-Papuan region where approximately 70% of the living species of Pteropodidae can be found. The family includes the largest of extant bats, with species of *Acerodon* and *Pteropus* achieving wing spans of 1.5 metres or more (Hill and Smith 1984).

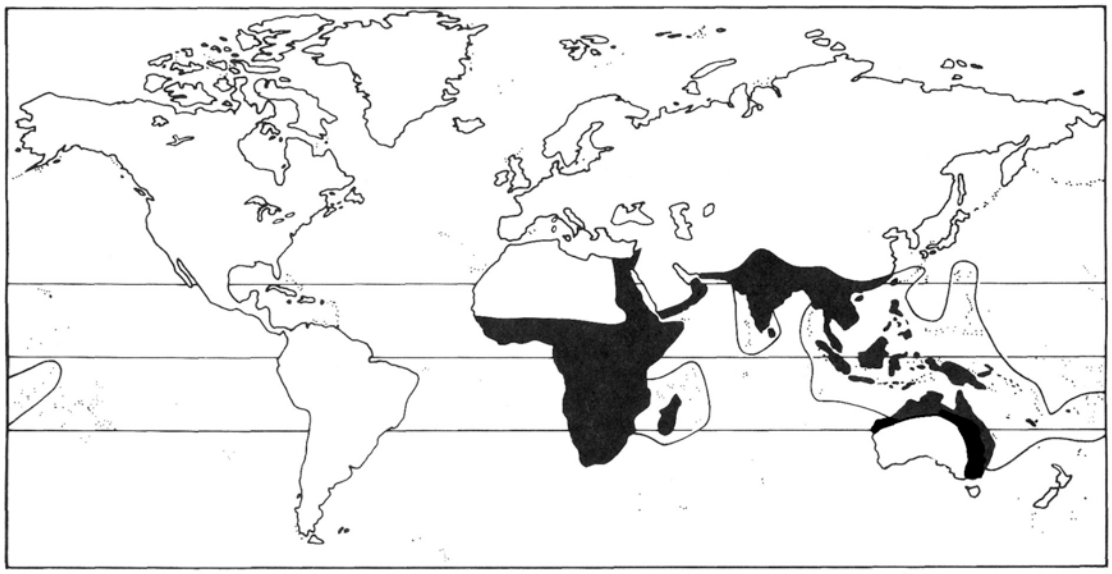

Figure 1.1 Distribution of the Pteropodidae (amended from Hill and Smith (1984)). Horizontal lines indicate position of the equator (middle), Tropic of Cancer (upper) and Tropic of Capricorn (lower).

Determining the evolutionary history of the Chiroptera has proved difficult due to the limited fossil record and incomplete or conflicting phylogenies (Teeling *et al.* 2005). Current phylogenetic analyses support the hypothesis that the order Chiroptera is monophyletic (Ammerman and Hillis 1992; Simmons 1994; Pettigrew and Kirsch 1995; Hutcheon *et al.* 1998; van den Bussch *et al.* 1998; Miyamoto *et al.* 2000; Nikaido *et al.* 2000; Jones *et al.* 2002; Teeling *et al.* 2005) and that the Microchiroptera and Megachiroptera diverged approximately 50 million years ago (mya) (Teeling *et al.* 2005). Previous alternative hypotheses held that the Chiroptera were diphyletic and that the Megachiroptera were more closely related to primates than to the Microchiroptera (Pettigrew 1987; Pettigrew *et al.* 1989; Pettigrew 1995). This view is now considered unlikely based on recent molecular evidence (Ammerman and Hillis 1992; Simmons 1994; Hutcheon *et al.* 1998). Within the Megachiroptera, taxonomic relationships are still disputed, with a lack of agreement on the splitting of the Pteropodidae into subfamilies (Alvarez *et al.* 1999; Colgan and da Costa 2002; Giannini and Simmons 2003) and poor resolution among the basal lineages (Colgan and da Costa 2002). A recent paper using morphological and molecular characters by Giannini and Simmons (2005) presents the most comprehensive analysis to date of associations within the Megachiroptera.

It has been proposed that the Megachiroptera originated in SE Asia – Melanesia (Colgan and da Costa 2002; Giannini and Simmons 2003; Teeling *et al.* 2005) or the Indo-Australo-Pacific region, where they are most diverse (Kirsch *et al.* 1995). Based on the absence of a fossil record, Australian megachiropterans have often been regarded as recent immigrants from Papua New Guinea (PNG) (Schodde and Calaby 1972). However, the fossil record of megachiropterans in general is particularly poor. Calculating the basal branch length for each chiropteran lineage that is unrepresented in the fossil record, Teeling *et al.* (2005) estimated that 98% of the megachiropteran fossil history is missing, probably because of their fragile bone structure and the fact that their roosting sites are generally not situated above substrata that favour the preservation of bones. The only megachiropteran fossil found in the Australo-Papuan region is that of a bat from the Pliocene (5–1.6 mya) found in PNG (Hall 1981).

McKean (1970) argues that the paucity of endemic Australo-Papuan Chiropteran genera, indicates that all bats, including the Megachiroptera, are 'recent' arrivals to the region. Unfortunately, McKean (1970) does not define what he means by 'recent'. The fact that, at a species level, the Australo-Papuan region has more megachiropteran species than any other region in the world (Hall 1984) is difficult to reconcile with such a view. The oldest chiropteran fossils in Australia are from an Eocene deposit in Murgon, southeast Queensland, and are believed to be 55 million years old (Hand *et al.* 1994). Although these are microchiropterans, the fossils show unambiguously that bats were resident in Australia during the Eocene epoch. With megachiropterans having a higher degree of endemicity in the Australo-Papuan region than microchiropterans, megachiropterans are proposed to have entered the region before microchiropterans (Hand 1984).

Australian flying foxes

There are five genera of pteropodids, containing 13 species, within Australia. These are commonly called flying foxes, fruit bats, blossom bats, and tube-nosed bats (Hall and Richards 2000). Although eight species of flying fox have been recorded historically in Australia, there are currently five species; four of the genus *Pteropus* and one of the genus *Dobsonia* (see Fig. 1.3 for the *Pteropus*

spp distribution). The other three species of *Pteropus* are now not considered to occur in Australia. The dusky flying fox (*Pteropus brunneus*) was only recorded once in 1874 on Percy Island, off the Central Queensland coast, and is now considered extinct (Hall and Richards 2000). *Pteropus banakrisi*, the Torresian flying fox, recorded as endemic to Moa Island, has recently been suggested to be erroneously based on subadult individuals of the black flying fox (*Pteropus alecto*) (Helgen 2004). Finally, Australian records of *Pteropus macrotis*, the large-eared flying fox, may be due to misidentifications of the little red flying fox (*Pteropus scapulatus*) (Helgen 2004).

The Australian Commonwealth Government's Environment Protection and Biodiversity Conservation Act (EPBC Act) lists two of the four Australian species of *Pteropus* as 'vulnerable to extinction' following IUCN guidelines. These are the spectacled flying fox (*Pteropus conspicillatus*) and the grey-headed flying fox (*Pteropus poliocephalus*). The EPBC Act aims to protect the environment, particularly in matters of national environmental significance. A listing as vulnerable is recognition that a species is under threat and likely to be declining in numbers. The act requires that a recovery plan be developed for listed species and that key threatening processes are recognised. In some instances, threat abatement plans are implemented.

The spectacled flying fox—*Pteropus conspicillatus* Gould, 1850

The spectacled flying fox is a large species so named for the straw coloured rings of fur around its eyes (Churchill 1998). Similar straw-coloured fur forms a mantle/ruff on the back of the neck and the shoulders. Elsewhere, the fur is black (Fig. 1.2). *Pteropus conspicillatus* has the smallest distribution of the four Australian *Pteropus* species (Fig. 1.3). Its range primarily covers the Wet Tropics World Heritage Area in North Queensland, between Cardwell in the south and Bloomfield in the north (Fig. 1.4). There is a small population in the Iron Range National Park on the Cape York Peninsula (Fig. 1.3), of which little is known. From limited observations it appears that the colony comprises a small number of individuals possibly numbering a couple of hundred (pers. obs.). No research has been undertaken on this population other than that during this study. Until August 2004 it was not known that the population as a whole leave

the park during the winter months. It is also not known where they go when they leave. They appear to return to the park during early summer (C. McMonagle, QPWS Ranger, Lockhart River, pers. obs.). It is assumed that the movement out of the park is food–driven. However, during August 2004 when the colony was absent, there was an abundant amount of *Grevillea* pollen available as well as *Eucalyptus tessellaris* in flower and *Ficus macrocarpa* in fruit. There are anecdotal reports of *P. conspicillatus* occurring in the McIlwraith Ranges, south of Iron Range, but there have not been any recent reported sightings, even though suitable habitat exists. From the Torres Strait Islands between PNG and Australia, there is only a single record of *P. conspicillatus* from Nepean Island (Andersen 1912). Although the distribution of *P. conspicillatus* on Cape York seems distinctly limited, there are large tracts of land inaccessible to researchers where *P. conspicillatus* camps may yet be discovered. No colonies occur west of the Great Dividing Range. *Pteropus conspicillatus* also occurs on the north and east coasts of PNG and on outlying islands. Although permanent colonies in towns, such as Madang or Lae, appear stable (Bonaccorso 1998), many of the colonies reputed to occur on outlying islands are poorly known. The current status of *P. conspicillatus* in PNG needs reassessment. Animals from PNG are regarded as conspecific with animals from Australia due to similarities in their pelage (Flannery 1995a; Churchill 1998). It is not known whether there is movement of animals between PNG and Australia, although with their known ability to fly 50 km in a single night when in search of food (Shilton & Westcott, unpublished data), movement between PNG and Australia is feasible.

The distribution of *P. conspicillatus* colonies in the Wet Tropics region is closely associated with that of rainforest vegetation having a high mean annual rainfall. Richards (1990a) reported that colonies were never more than 6.5 km away from rainforest and were located in areas with a mean annual rainfall of 1400 mm or more. Even though these two factors are highly correlated with the location of a *P. conspicillatus* camp, at present there is little understanding of other factors that may influence choice of campsite. Many campsites are found in locations that are unexpected. For example, some permanent *P. conspicillatus* campsites are found in city parks or small remnant blocks of forest (pers. obs.).

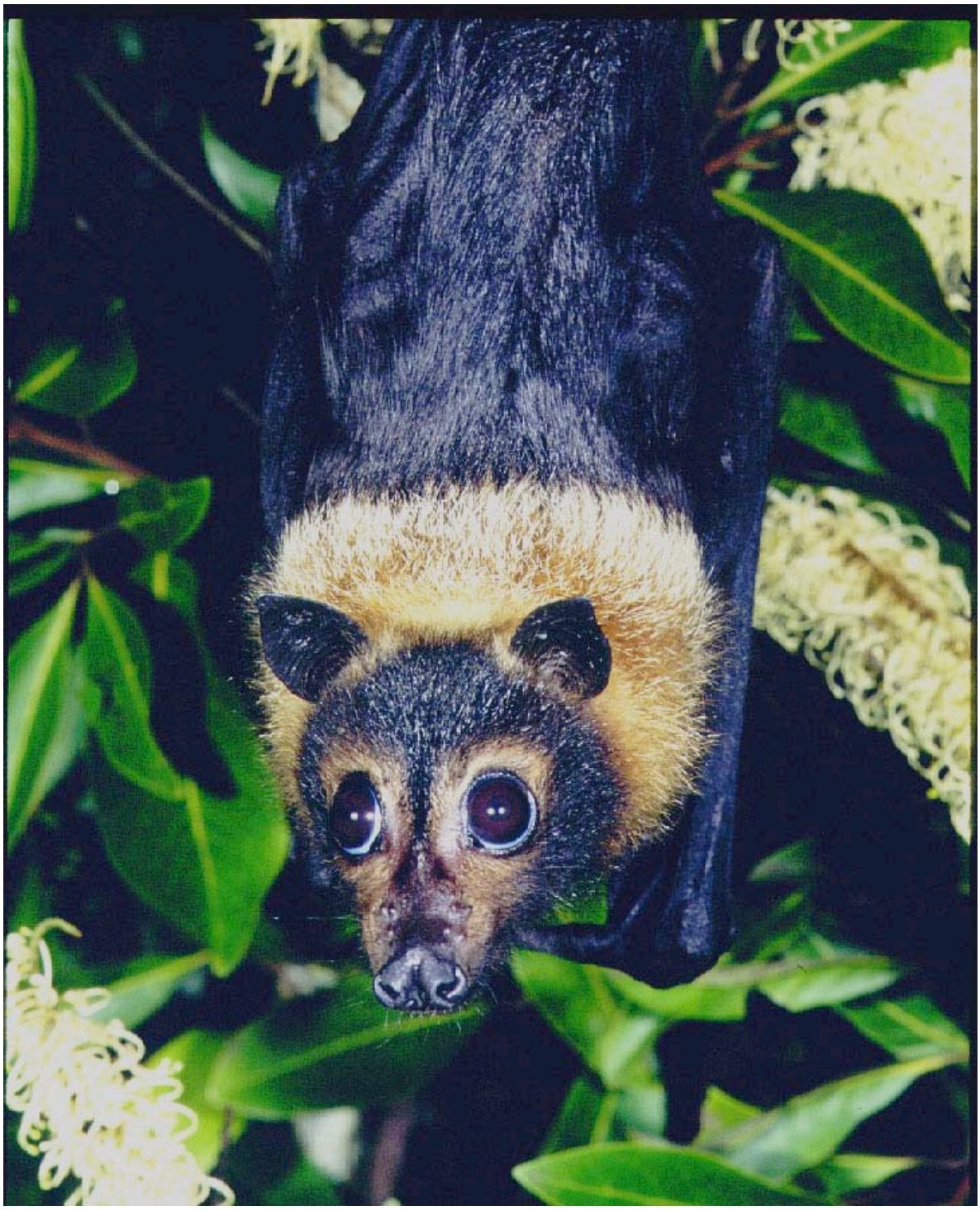

Figure 1.2 The spectacled flying fox, *Pteropus conspicillatus.* Photo: Bruce Thomson. Used with permission.

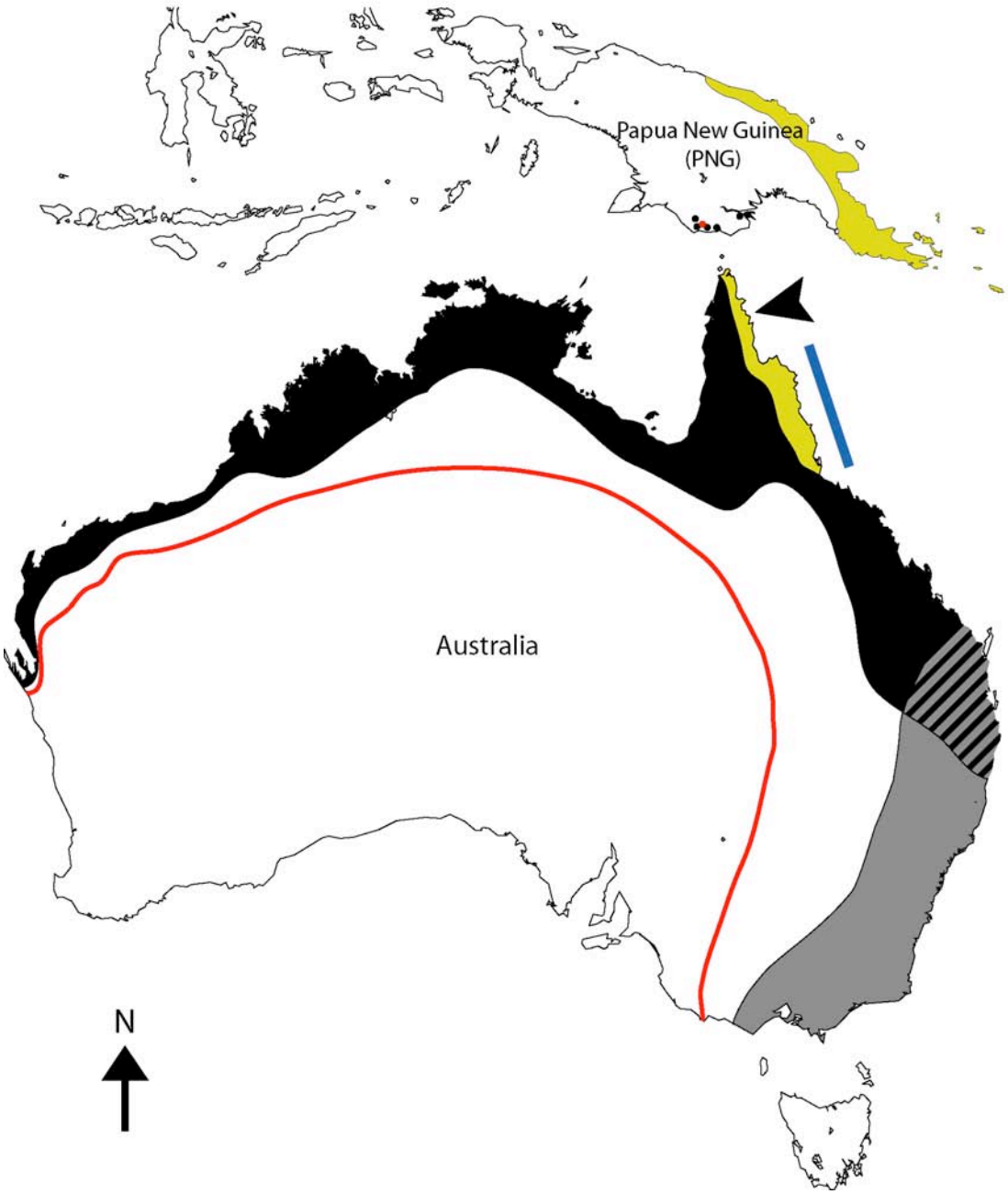

Figure 1.3 Distribution of Australia's flying foxes (after Hall and Richards 2000). *Pteropus conspicillatus* (spectacled flying fox), yellow area; *Pteropus alecto* (black flying fox), black area; *Pteropus scapulatus* (little red flying fox) inland limit, red line; *Pteropus poliocephalus* (grey-headed flying fox), grey area. Black and red dots on map of PNG = records of occurrence of black and little red flying foxes respectively in PNG. Arrow indicates approximate position (on land) of Iron Range. Blue line signifies the Wet Tropics World Heritage Area (WTWHA).

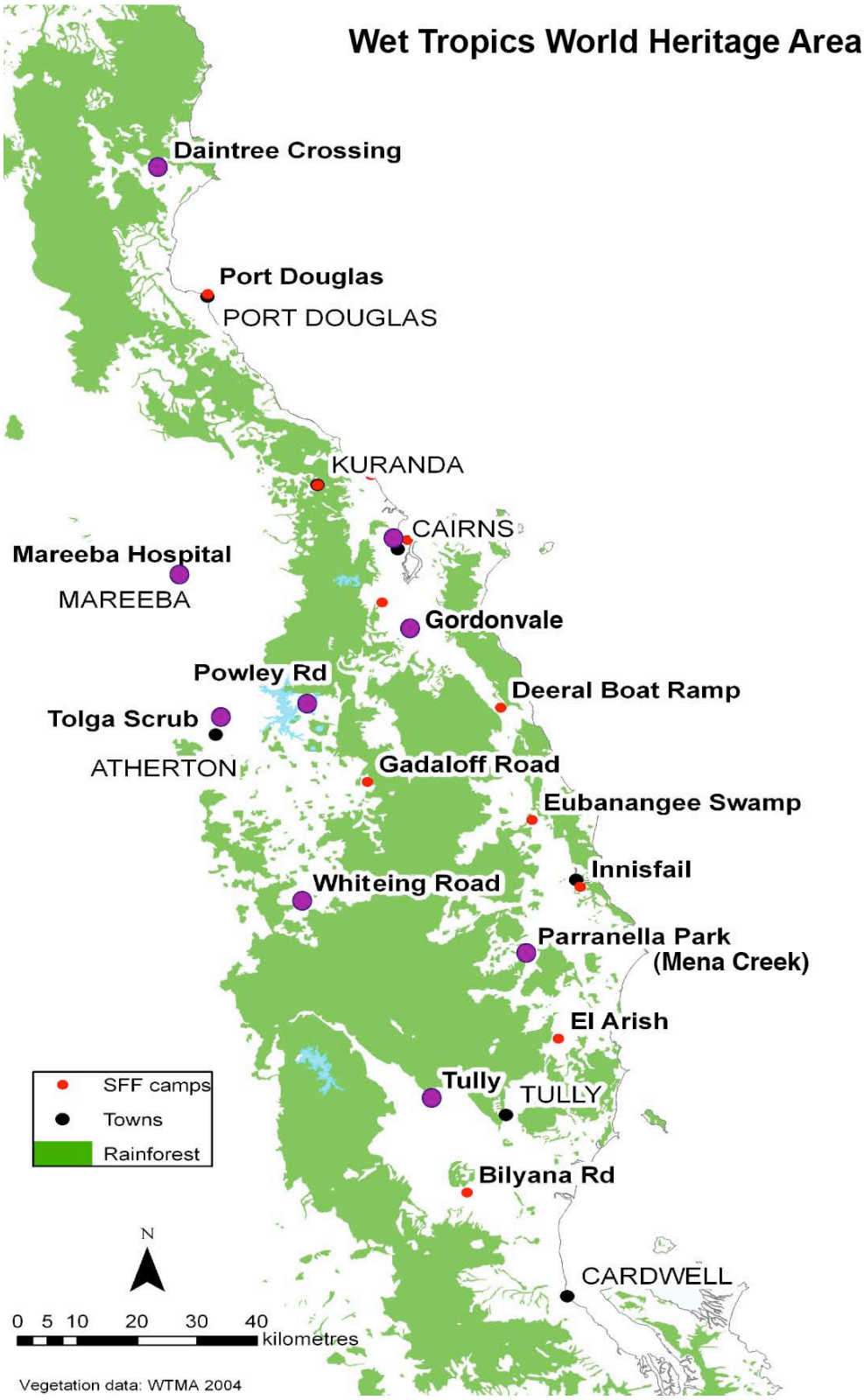

Figure 1.4 Known colony locations of *Pteropus conspicillatus* (spectacled flying fox) in the Wet Tropics region, principal area of its range in Australia. Red dots are camps not sampled in this study, purple dots are camps that were sampled. Atherton Tablelands colonies comprise Mareeba, Tolga Scrub, Powley Rd and Whiteing Rd.

Estimates of spectacled flying fox population size have been made every year since 1998 (Fig. 1.5) using a visual census conducted by volunteers in the Wet Tropics World Heritage Area. This initiative, started by a group of conservationists and scientists, aimed to establish if this species was in decline. Estimating the size of most mammal populations is difficult, but even more so for a nocturnal, volant mammal such as a flying fox, where many of the usual methods of estimation, such as mark–recapture, are ineffective (Nelson 1965a; Pierson and Rainey 1992). The only report to mention the number of spectacled flying foxes historically is that of Francis Ratcliffe (1932) who stated vaguely that in the early 1900s he only encountered *P. conspicillatus* in Cairns and on the Atherton Tablelands. The latter area appeared to have five colonies, three of which he visited, and two of which appeared to be 'sometimes large' (meaning >50,000 individuals).

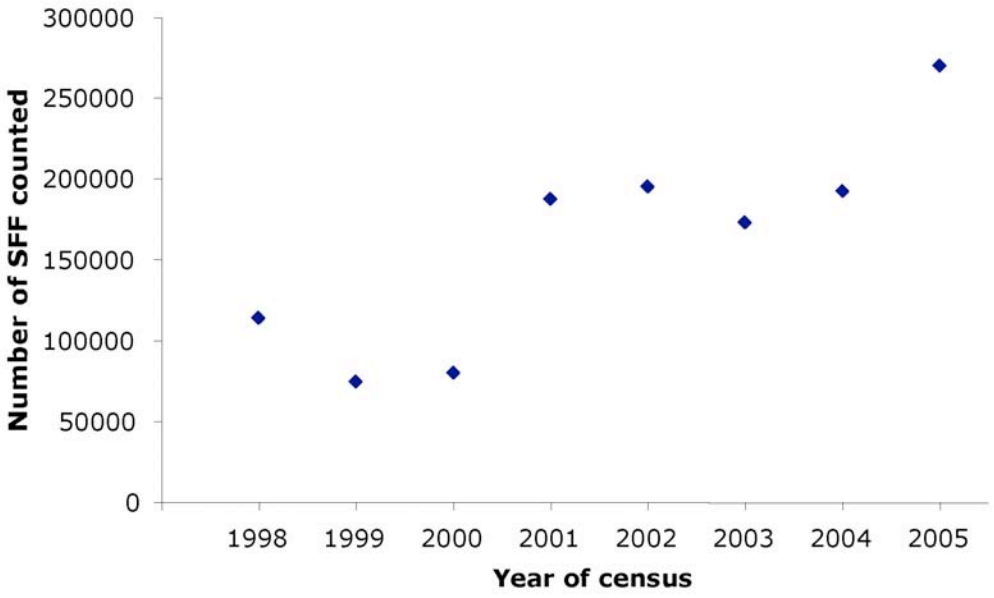

Figure 1.5 Census results for 1998–2005; counts of spectacled flying fox (SFF) colonies in the Wet Tropics region during December. Data from Threatened Species Network, Queensland Parks and Wildlife Service.

Because of the lack of dependable historical records of population size for *P. conspicillatus* prior to the start of these surveys, it is not possible to determine whether they are in decline. Periodic counts of some colonies were undertaken during the late 1980s to the early 1990s suggesting an estimated count of 784,000 (ABS 2001), however camp counts were not carried out simultaneously,

with no correction made for immigration between colonies affecting the final tally. It is from a comparison of this figure with that reported from the count in 1998 that the initial concern of decline and impetus to have the spectacled flying fox listed occurred. The census approach cannot give an accurate measure of how many spectacled flying foxes there actually are but can only act as a guide. Reservations concerning the census data result from the uncertainty of knowing whether all colonies have been located and therefore all animals in the region counted. To test the census methodology, researchers at the CSIRO on the Atherton Tablelands carried out a study to compare counts by volunteers, with that of video-based counts. The counters were always within ± 15% of the video-based count and more often than not underestimated the count rather than overestimated (Westcott and McKeown 2004). Because spectacled flying foxes in the Wet Tropics region have access to large tracts of rainforest that are inaccessible to people, the problem of "missing" colonies is always likely to be an issue. The large apparent increase in numbers from 2000 to 2001 and from 2004 to 2005 (Fig. 1.5) is almost certainly a result of immigration from unknown colonies. Such annual population increases cannot be due to successful recruitment alone (Chapter 6).

Although the annual census gives an indication of the number of individuals in a population, it is the effective population size (N_e) that is important in understanding the genetic diversity, and the loss of it, in a species. The effective population size is defined as the size of an idealised population that would lose genetic diversity at the same rate as the actual population (Frankham *et al.* 2002), and has become a central parameter in the field of conservation genetics.

Understanding population structure in *Pteropus conspicillatus*

Coloniality in flying foxes

All Australian flying foxes are colonial, roosting together during the day in groups of variable size from several hundred to tens of thousands (Ratcliffe 1932; Nelson 1965a; Eby *et al.* 1999; Freeman 2004). Colony size and structure is not static (Parry-Jones and Augee 1992; Garnett *et al.* 1999; Tidemann *et al.* 1999; Westcott *et al.* 2001), with tracking studies showing that animals often move from one roost site to another (Tidemann and Nelson 2004). Eby *et al.* (1999)

found that *Pteropus poliocephalus* moves depending on the extent of local food resources. This was confirmed by Parry-Jones & Augee (2001) who found a colony of *P. poliocephalus* in Sydney remained at the Gordon camp-site due to a permanent local supply of food. Size of colony has also been found to vary throughout the year depending on the local availability of foraging opportunities (Ratcliffe 1932; Nelson 1965a; Richards 1990a; Eby 1991; Parry-Jones and Augee 1992; Eby *et al.* 1999; Westcott *et al.* 2001).

Coloniality occurs in fish (de Fraipont *et al.* 1993), reptiles (Trillmich and Trillmich 1984), mammals (Campagna *et al.* 1992; Baldi *et al.* 1996) and most commonly in birds (Anderson and Hodum 1993; Rolland *et al.* 1998), where it is estimated one species in eight breeds in colonies (Lack 1968). However, the majority of these species only form colonies to breed, unlike flying foxes. Theory behind the function of colonial living in flying foxes includes the need to find a mate and raise young, predator protection and information exchange (Tidemann *et al.* 1999), all of which are also principal hypotheses given for coloniality in other groups. However, where members of other animal taxa will only come together for a short period of time to mate and/or give birth, flying foxes in Australia roost permanently in colonies.

Colonial living in flying foxes may be correlated with the annual reproductive cycle: finding a mate and conceiving young; gestation and the need to learn to locate good food resources; birth of young and the protection of the colony for new mothers; and the protection of the colony for newly weaned and independent young, learning to fly and finding where to go for food. Almost as soon as the young are weaned and independent, the females once again become receptive to mating and the cycle repeats itself (Hall and Richards 2000). The predator avoidance/protection theory alone is unlikely to be applicable to flying foxes, as although a single roosting flying fox is difficult to detect in the canopy, a colony of several thousand individuals is very obvious. In a review paper on group living, Danchin & Wagner (1997) demonstrated that predation protection models are not universally applicable and that it appears to hold for some prey species and not others. Wilkinson & South (2002) found no reduction of mortality risk as a result of predator dilution in bat species that roosted in large colonies. However, Nelson (1965b) believed the advantage of living in a

large colony was one of protection, with the presence of disturbance of any kind quickly conveyed from one individual to the rest of the camp. The information transfer theory is likely to apply to flying foxes as the passing on of information about blossom and fruit location in a patchy landscape is believed to occur (Ratcliffe 1932; Eby *et al.* 1999). However, none of these theories have been investigated directly in the spectacled flying fox.

Colony structure in Australian flying foxes, with regard to sex ratio and age structure, appears to change throughout the year. Colonies can be divided into permanent or maternity colonies, and temporary or satellite colonies (Richards 1990a; Eby 1999; Tidemann *et al.* 1999). *Pteropus poliocephalus* was found to peak in numbers in a Sydney colony each year during the period when adults were mating (Eby 1991; Parry-Jones and Augee 1992). Nelson (1965a) found that from September to December, when females were giving birth, the ratio of the sexes of adult *P. poliocephalus* approached equality, but that after conception had occurred, during winter months, colonies were often sexually segregated. It was also common at this time to find smaller camps containing mainly immature animals. Small satellite colonies of black flying foxes (*P. alecto*) in the Northern Territory were found to have a significant bias towards males (Tidemann *et al.* 1999). The very mixed results of reported sex ratios in Australian flying fox colonies (Hayden 1992; Bull 1993; Eby *et al.* 1999; Vardon and Tidemann 1999; Tidemann and Vardon 2001; Westcott *et al.* 2001) may indicate that there is a constant change in sex ratios depending on the type of colony and time of year.

The age structure of individuals in a colony might suggest a number of processes that are occurring con-currently. A colony of relatively young individuals may indicate a growing population and/or a species that has a short lifespan. A colony with a greater proportion of old individuals may indicate little recruitment to the colony and/or a species that has an extended lifespan. A population with a bimodal age structure may indicate years of decline in population size and the following recovery. This knowledge is important in assessing a populations'/species' capacity for growth in the form of reproductive output. However, with a highly mobile animal that appears to move regularly between colonies, assessment of a single colony at a single point in time may not necessarily be applicable to the species as a whole, and this

should be considered in interpretation of results. No studies have been undertaken on the age structure of flying fox colonies.

Definition of a 'Population'

Research on movement and gene flow in other species of flying fox in Australia (Sinclair *et al.* 1996; Webb and Tidemann 1996) show panmixia across large spatial scales in *P. poliocephalus*, *P. alecto* and *P. scapulatus*. There is thus an *a priori* expectation that colonies of spectacled flying foxes within the Wet Tropics region form a single panmictic 'population' Throughout this thesis 'colony' is used to refer to a group of flying foxes roosting together, 'population' is used in more general discussion, while 'camp-site' refers more specifically to the location of where the animals roost during the day.

Studying colony/population structure

There are two main components determining population structure: demographic factors such as sex ratios, age structure, mating strategy, birth and death rates, longevity and reproductive output; and genetic factors including gene flow, genetic diversity, relatedness, evolution and historical processes, and effective population size (Slatkin 1994). Census and observation may provide information on some demographic factors, although factors such as age estimation require long-term monitoring of animals or destructive sampling. Other factors may be inferred from an understanding of population genetic structure.

The overall contribution of both demographic and genetic factors that influence population structure may be broken down into five main components (Fig. 1.6):

- the evolutionary history of the population and species under study, including biogeographic history, species boundaries and introgression, and historical structuring due to past events such as bottlenecks.

- the size and distinct nature of current populations.

- the scale and nature of gene flow, including dispersal and migration patterns and frequency.

- the relatedness of individuals within the population as a result of mating behaviour, the resulting level of inbreeding and fitness this confers.

- the demographic factors as have been outlined previously.

By studying all of these factors in concert, an inclusive and comprehensive understanding of population structure and dynamics may be obtained.

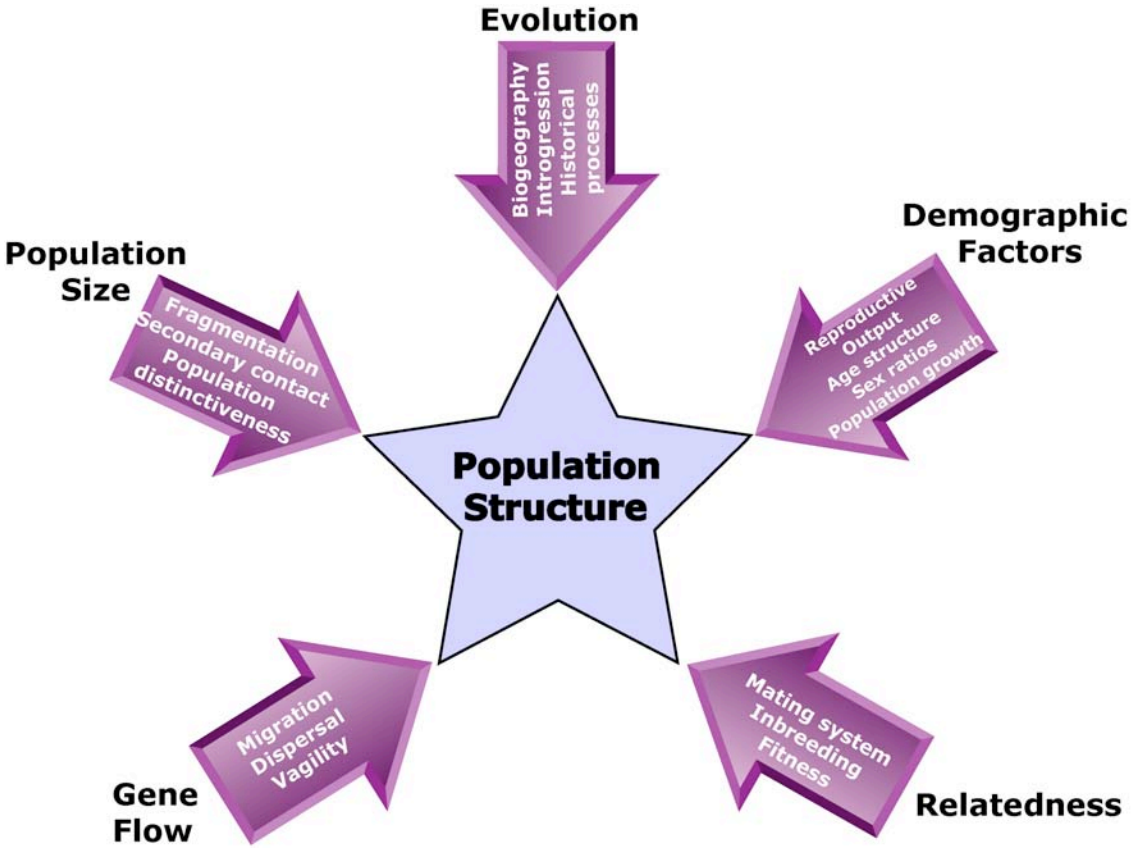

Figure 1.6 Contribution of demographic and genetic factors affecting population structure.

Demographic and genetic components of assessing population structure combine to explain patterns more fully. By considering either factor alone, or a subset of the factors that contribute to determining population structure, the results and the interpretations possible, are limited. For example, male reproductive success in birds was historically studied by observing males in their territories, and the number of females that they mated with and guarded

within that territory (Birkhead *et al.* 1987). This often lead to the assumption that only males within a territory were successful and that males without a territory did not breed. However, with the use of genetic analysis of parentage, it has been found that extra-pair copulations result in resident males having a high variability in reproductive success due to satellite males sneaking in to mate with a female while the resident male is distracted (Kupper *et al.* 2004; Blomqvist *et al.* 2005). In this case, the observed aspect of the mating system was only giving half of the story. Genetic information on parentage provided otherwise missing information, providing a fuller picture. Therefore, as many factors as are logistically possible should be considered, and a combination of demographic and genetic data should be obtained if an accurate picture of population structure is sought.

In addition to revealing cryptic factors that influence a population's structure, combined datasets provide information that may be used to understand current and historic population processes simultaneously. Although the demographic aspects of population structure are reflected in the current time frame (such as sex ratios seen in a colony during the breeding season), some genetic data will reveal a signature of historical events and processes that may have contributed to the contemporary structure (such as an historical bottleneck of a species with low vagility due to habitat fragmentation, which has resulted in reduced genetic diversity). Genetic analysis across differing spatial scales provides insight into the difference of impacts on a broad spatial scale compared to a local spatial scale. The following two sections examine the use of these two components, demographic and genetic, in studying population structure.

Demographic factors

Although the mating system of a species is considered to be a demographic factor, it has important consequences for gene flow, relatedness of individuals and ultimately, genetic diversity in a population. Because of this, knowledge of a species' mating system can be invaluable when determining the effective population size (Nunny and Elan 1994). A population of randomly mating individuals (panmixia) exhibits a greater degree of heterozygosity and genetic diversity than would otherwise be the case. Alternately, species which exhibit

extreme polygynous behaviour, where a single resident male mates with a large harem of females, may have reduced genetic diversity due to a smaller proportion of the adult male population reproducing e.g. southern elephant seals, *Mirounga leonina* (Fabiani *et al.* 2004), common quail, *Coturnix coturnix* (Rodriguez-Teijeiro *et al.* 2003) or some seahorse species (Wilson *et al.* 2003). Polygyny can also affect the population in that the sex ratio of adults found in a single population may be skewed. In such a case, a population of reproducing individuals may have a large number of females and a much smaller number of males, while non-breeding populations may solely consist of bachelor males and possibly non-breeding females, creating a local skew in sex ratios. Some mating systems may lead to an increased risk of inbreeding depression and reduced heterozygosity and genetic diversity, while other mating systems include strategies to avoid inbreeding depression such as dispersal of male or female offspring (Stow and Sunnucks 2004). The above suggest ways that a single demographic parameter, the mating system, can influence many aspects of population structure such as gene flow, relatedness of individuals, genetic diversity, effective population size, sex ratio, population growth rate, inbreeding and dispersal in a species.

Apart from aspects of colony structure such as total population size and sex ratios (Richards 1990a; Hayden 1992; Bull 1993), no data on demographic factors in the spectacled flying fox exist. Many of the demographic parameters for spectacled flying foxes have been inferred from those calculated for other Australian flying fox species. In many instances this is reasonable as traits such as number of reproductive events per year (one), gestation time (6 months) and the number of young born (one) are uniform across flying fox species (Hall and Richards 2000). Other traits, such as age structure, sex ratios, reproductive output and population growth, that are more fluid within populations and are affected by intrinsic as well as extrinsic factors, may vary from population to population, as well as from species to species. As with other Chiropteran species, flying foxes are believed to be long lived based on the observation that they live long in captivity (Hall and Richards 2000), and that they have other life history traits (such as slow growth; late sexual maturation; only produce a single, large young each year; high maternal investment in young (Tuttle and Stevenson 1982; Kurta and Kunz 1987; Read and Harvey 1989; Austad and

Fischer 1991; Barclay *et al.* 2004) that are associated with extended longevity. However, average longevity has never been assessed empirically in wild flying foxes, and as this trait is linked to other important factors such as reproductive output and population growth, it needs to be understood in light of general life history traits.

Effective techniques for determining age in flying foxes have been demonstrated using cementum layers around the root of teeth (Cool *et al.* 1994) and present an opportunity to develop an understanding of population age structure if teeth from a sufficient number of animals are available.

Genetic factors

To determine the genetic structure of a population, it is important to understand the extent of genetic variation in the species by evaluating the genotypes of individuals (Slatkin 1994). By comparing these genotypes across different spatial scales, genetic diversity, gene flow and degree of inbreeding can be determined, as well as relatedness patterns, dispersal ability and the mating system. Understanding the extent of gene flow between populations is important in determining the independence of each individual population. If there is extensive gene flow between populations they may function collectively as a single unit and the genetic structure within each population will be similar. With reduced rates of gene flow, each population acts more independently and the genetic structure may be markedly different between populations (Slatkin 1994). If different temporal scales are also investigated, historical events that shaped the contemporary structure can be established. For example, mtDNA datasets can be analysed to determine whether the population has been through an historical bottleneck or population expansion.

Two classes of genetic markers that are extensively used by population geneticists to cover multiple spatial and temporal scales, are various portions of the mitochondrial genome, and multilocus genotypes based on repetitive DNA known as microsatellites, in the nuclear genome. Mitochondrial DNA gives a broad-scale picture, as well as permitting historical population structure to be determined. Microsatellites allow fine-scale variability to be detected and measured. By using these two classes of markers together, population structure,

and many of the processes that are currently affecting it, and have affected it historically, can be assessed.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) is a circular genome located within mitochondria in the cytoplasm of cells (Avise 2000). It is transmitted maternally without recombination and therefore has a clonal mode of inheritance. As a result, mtDNA typically reflects the maternal evolutionary lineage, and together with nuclear markers, can be used to infer the extent of gender-biased dispersal. Mitochondrial DNA has a rate of mutation approximately 5–10 times greater than nuclear DNA (Brown *et al.* 1979) and is highly variable (Frankham *et al.* 2002). The characteristics of mtDNA, especially its rapid rate of sequence evolution, make it a valuable tool for studying both inter- and intra-specific relationships and it is commonly used in studies of phylogeny, phylogeography and population genetics in animals. Molecular surveys of mtDNA frequently detect phylogeographic patterns that record the effects of long-standing population structure (Avise 2000).

Many species-level genetic studies incorporate a phylogeny into their analysis to determine intraspecific variation (Avise 2000) and to test for monophyly of the study species. A usual assumption is that the study species is monophyletic with respect to the alleles at the locus studied (Funk and Omland 2003). That is, that DNA sequence alleles analysed are more closely related to others from the study species than to any alleles that exist in any other species (Funk and Omland 2003). It is becoming apparent that this assumption, in many instances, may not be met. Gene trees (association of alleles) may not be the same as species trees (the association of individuals). Species-level polyphyly or paraphyly is detected when gene trees reveal an allele from one species to be more closely related to alleles in a different species than any conspecific alleles. This anomaly, however, is not detectable unless closely related species are analysed alongside the study species, which in many cases is not done. Besides misidentified specimens or poor phylogenetic resolution, there are two processes that can confer species-level paraphyly or polyphyly; introgression and incomplete lineage sorting. Introgression occurs when alleles from one

species penetrate the gene pool of another through interspecific mating, followed by backcrossing of hybrids into the parental populations (Harrison 1993). Incomplete lineage sorting results when two diverging species retain copies of the same ancestral alleles that have not been completely sorted, or lost, through genetic drift. Once sorting of the lineages is finished, speciation is often regarded as complete. The effects of introgression and incomplete lineage sorting on evolutionary inferences about species can be great and involve inflation or deflation of inferred times and rates of evolutionary divergence, as well as compromising population genetic and phylogeographic studies by leading to mistaken conclusions about demography and evolutionary processes that are based on allelic frequencies and relationships (Funk and Omland 2003). Conversely, if detected, poly- or paraphyly can give great insight into historical processes and may put other results into correct context. Intensive sampling of individuals and close congeneric species should ensure that detection of a deviation from species-level monophyly can be interpreted accurately.

Microsatellites

Microsatellites have become the genetic marker of choice for many population geneticists investigating contemporary gene flow within and between populations (Beaumont and Bruford 1999; Balloux and Lugon-Moulin 2002). Microsatellites are short tandemly repeated sequences (e.g. AT_{11}) (Queller *et al.* 1993) that are relatively abundant and uniformly distributed throughout the non-coding regions of the genome (Hancock 1999). Microsatellites are particularly useful because repeats are often lost or gained via slippage during DNA replication or recombination (Hancock 1999), creating a great deal of variation at the population level in the number of repeats at each locus. Microsatellites are generally considered to have a rate of mutation several orders of magnitude higher than other nuclear DNA markers (Nikitina and Nazarenko 2004), making them useful in both inter- and intrapopulation studies (Goldstein and Schlotterer 1999). The mutation process is highly heterogeneous, however, with distinct differences between species. Within species mutation rates differ among loci with different repeat numbers, among alleles at a single locus and also among individuals of different sex and age (Nikitina and Nazarenko 2004). Microsatellites are biparentally inherited

codominant markers and therefore individual genotypes reflect both male and female parents.

Initial isolation of microsatellites is expensive, and in some groups difficult. However, cross-species amplification of microsatellite loci may be possible (Lowe *et al.* 2005). This increases the cost effectiveness of the use of microsatellites. For example, seven out of eight polymorphic microsatellite markers isolated for the brown treecreeper (*Climacteris picumnus*) also amplified successfully in the white-throated tree creeper (*Cormobates leucophaeus*) (Doerr 2005). As these were the first genetic markers developed for this passerine family, they are expected to be useful for genetic studies in any Australian treecreeper species (Doerr 2005).

Microsatellites are used to investigate many aspects of population structure, including; gene flow (Girman *et al.* 2001), genetic diversity (Gautschi *et al.* 2003; Baucom *et al.* 2005; Jedrzejewksi *et al.* 2005), inbreeding (Eldridge *et al.* 1999; Bjorklund 2003; Andersen *et al.* 2004), bottlenecks (Leijs *et al.* 1999; Miller and Hedrick 2001; Al-Rabah'ah and Williams 2004), relatedness and kinship (Bentzen *et al.* 2001; Burland *et al.* 2001; Gerlach *et al.* 2001; Kerth *et al.* 2002b; Garner and Schmidt 2003), as well as mating systems (Heckel and Von Helversen 2003), reproductive success (Ortega *et al.* 2003), social structure (Storz *et al.* 2000), and divergence between populations and species (Petren *et al.* 2005). Estimates of rates of gene flow help in answering broad-scale, between-population questions relating to migration, dispersal and vagility. Estimates of genetic diversity are useful in determining factors such as population resilience, fitness and inbreeding. Relatedness can be considered at different spatial scales depending on whether the mating system of species is being considered (broad-scale) or whether single groups of individuals are being assessed for kinship (small scale).

Whereas mtDNA is used principally to infer historical events at broad spatial scales, microsatellites are used to examine more contemporary events over smaller spatial scales. Use of these two markers together allows for robust genetic analysis of processes that affect population structure. For example, use of both mtDNA and microsatellite data allowed the global genetic structure of

sperm whales to be determined (Lyrholm *et al.* 1999). If only mtDNA had been used, the results would have suggested that there was significant genetic differentiation between oceans. However, data from microsatellites made it clear that there was little differentiation between oceans and the pattern seen in the mtDNA was the result of female philopatry (Lyrholm *et al.* 1999).

Population structure, conservation and management

Genetic analysis of populations has become an important component of strategies used for the conservation and management of threatened species. The realisation that genetics can inform management on important aspects of a species' ecology and biology, such as its probable capacity to cope with stochastic environmental events (genetic diversity) (Bowyer *et al.* 2002; Ruedi and Castella 2003; Andersen *et al*. 2004), its distinctiveness from other closely related species (phylogeny) (Jones *et al*. 2002; Carstens *et al*. 2004; Morando *et al*. 2004), reasons for reduced reproductive output and less fit offspring (inbreeding depression) (Eldridge *et al.* 1999; Slate *et al*. 2000; Miller and Hedrick 2001), evidence of past reduction in population size (historical bottleneck) (Al-Rabah'ah and Williams 2004; Lucchini *et al*. 2004), and the likely effects of translocating populations (gene flow, introgression, genetic diversity) (Goossens *et al.* 2002; Nguyen *et al*. 2002; Mock *et al.* 2004), has meant that genetic techniques are now important tools used in conservation biology. Many aspects of a species biology that are critical to its effective conservation are often unknown, or difficult and time consuming to determine directly (Frankham *et al*. 2002). Hence, the inclusion of genetic analysis in conservation and management of threatened species, although relatively recent, is now standard.

Impetus for this study and aims of the thesis

Following debate regarding the status and apparent decline of the spectacled flying fox in the Wet Tropics of north Queensland, this project was devised to establish baseline knowledge for this species and through the use of genetic and demographic techniques, infer population processes. It was believed that by using a different approach to census and observation techniques, this project could offer an alternative and independent assessment of the status of the spectacled flying fox.

By incorporating demographic and genetic data, this study investigated population structure in the spectacled flying fox across varying geographic and temporal scales throughout its distribution. The specific aims of this thesis are to:

1. Investigate the historical population structure of the spectacled flying fox and determine whether it reflects the contemporary geographical structure. Interpret the historical structure of the spectacled flying fox in context of its evolution in the Australo-Papuan region and its colonisation of Australia (Chapter 3).

2. Examine the genetic relatedness of spectacled flying foxes across their range to determine movement patterns, gene flow and genetic diversity across the three core areas of their distribution (Chapter 4).

3. Investigate the level of relatedness of individuals within a colony during the pupping season to determine whether there is any social structure within colonies occurring (Chapter 5).

4. Investigate aspects of the life history of a wild population of spectacled flying foxes and, through the use of life tables, investigate rates of population growth, mortality and reproductive output (Chapter 6).

Chapter Two – Isolation and characterisation of polymorphic microsatellite markers in the spectacled flying fox

Population genetic markers of high sensitivity allow genetic tracking of individuals and investigation of population–scale gene flow. For this reason, microsatellites are commonly used. To date, there has been a single published report of microsatellite markers isolated in the family Pteropodidae, for species within the genus *Cynopterus* (Storz 2000). Species–specific microsatellite markers were isolated from the spectacled flying fox (*Pteropus conspicillatus*) for the purpose of determining levels of gene flow, scales of movement and relatedness of individuals within and between colonies. In addition, microsatellites isolated, but not published, for other species of Pteropodidae developed by G. McCracken (pers. comm.), were tested to investigate across-group amplification of microsatellite loci.

A microsatellite repeat enriched DNA library was constructed using the Hamilton linker system to prevent and identify concatenated genomic fragments in the final products (Hamilton *et al.* 1999). DNA was extracted from the wing tissue of one individual spectacled flying fox using a DNeasy Tissue kit (Qiagen™) according to manufacturers instructions. Multiple extractions were performed on the same individual until 3–5 µg of high molecular weight DNA was gained. DNA extracts were combined, ethanol precipitated and eluted in a suitable volume for a subsequent, combined *Nhe*I and *Xmn*I restriction digestion following manufacturer's instructions. Digested DNA was sonicated until most DNA fragments fell within the 300–1200 bp size range, then size selected by gel purification using a Qiagen™ Minelute Gel Extraction Kit. Following the methods and PCR conditions of Hamilton *et al.* (1999), SNX linkers were ligated to these fragments and the ligated products PCR amplified. This amplified product was enriched for $(AC)_{14}$ repeats using streptavidin-

magnetic capture following the methods of Glen *et al.* (2000). The enriched product PCR was amplified, producing a broad smear between 300–1200 bp on a 1.5% agarose gel. Enrichment amplicons were cloned using a pMOS Blue blunt ended cloning kit (Amersham Biosciences) according to manufacturer's instructions and white colonies were picked using pipette tips into 96 well plates containing 50 µL molecular grade water per well. Plasmid DNA was extracted by boil-lysis (Sambrook and Russell 2001). Plasmid inserts were PCR amplified using T7 and U19 vector primers in a 15 µL reaction containing 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100 (1X Thermopol buffer), 0.13 µM each primer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.5 units *Taq* DNA polymerase with 1 µL of boil-lysed plasmid template. Thermocycler conditions were 94°C for two minutes followed by 35 cycles of 94°C for one minute, 55°C for one minute, 72°C for two minutes and a final extension of 72°C for five minutes. All subsequent sequencing was carried out using 1 µL of this (uncleaned) PCR product as template.

Detection of plasmids containing microsatellite motifs was performed by hybridisation of [γ-^{32}P] dATP labelled common di, tri and tetra-mer oligonucleotide repeat motifs (as listed in Glen *et al.* 2000) to dot blots (Sambrook and Russell 2001) of PCR amplified plasmid inserts. Dot blot images were developed using a phosphor-imaging system (Molecular Dynamics). Hybrid positives were sequenced using Amersham ET DYnamic© sequencing chemistry according to manufacturer's instructions and analysed on a MegaBACE 1000 (Amersham Biosciences).

Of the 1312 clones screened, 62 hybrid positive clones were sequenced, 25 of which contained microsatellite repeats. Twelve of these did not have sufficient flanking region for primer design. Primers were initially designed using Primer 3 (Rozen and Skaletsky 2000). The best five primers for each putative locus were then analysed for potential hairpin and primer dimer amplification using OLIGO v 4.0 (National Biosciences Inc.). Primers were tested across a bank of four individual spectacled flying foxes known to readily PCR amplify. Of the 12 primers, eight amplified readily across all four individuals. Optimisation of PCR conditions for each primer pair was conducted following a Taguchi approach (Cobb and Clarkson 1994). A group of 96 individuals was screened

from populations across the range of the species to determine if the primers detected polymorphisms. Four primers did not detect polymorphisms (Table 2.1) and were not screened further. The remaining four primers were screened across more than 400 individuals to determine average levels of polymorphism (Table 2.1). All four loci were highly polymorphic with 15-21 alleles per locus. Three loci conformed to Hardy-Weinberg expectations for average levels of heterozygosity. One locus, PC36c2, did not conform to expectations at the 5% significance level but did at the 10% level. This locus contains a complex repeat $(GTCTCTCT)_4$. Among the samples analysed were a bank of mother-baby pairs with these individuals verifying the co-dominant inheritance of all four primer pairs, and negating the presence of null alleles.

In addition to the novel microsatellite markers generated by this study, markers designed for *Pteropus rodricensis, P. hypomelanus, P. samoensis* and *P. tonganus* by G. McCracken (pers. comm.) were tested against individuals of the spectacled flying fox from two colonies. Six loci were tested and all amplified across the 210 individuals analysed (Table 2.2). Two loci (c6, ph9) have been incorporated into the main genetic study of *P. conspicillatus*. Of the four loci not used in the study, one was a complex repeat that was difficult to score (D1), and the remaining three (A1, A2, A3) showed indications of null alleles being present. This highlights the success of across-group amplification of microsatellite loci.

Microsatellites are becoming the genetic marker of choice for many population genetic studies as they can answer a multitude of questions: extent of gene flow and inbreeding (Andersen *et al*. 2004; Garoia *et al*. 2004; Durand *et al*. 2005), relatedness of individuals (Bentzen *et al.* 2001; Kerth *et al*. 2002b), genetic diversity and detection of bottlenecks (Leijs *et al.* 1999; Bowyer *et al.* 2002), to name a few. In a species that is listed as vulnerable, and is difficult to study due to its mobility and inaccessibility, the use of this indirect method to answer ecological, demographic and social questions, reduces the impact on the species as well as allowing greater depth of knowledge. The principal drawback with microsatellites has been the difficulty and cost of isolating them (Lowe *et al.* 2005) and the fact that they are often species-specific. Four microsatellite loci are reported here that have been isolated from the spectacled flying fox for use in this study. I also report successful cross-species amplification of microsatellite

loci isolated for other *Pteropus* species. These six loci will be used to investigate gene flow, colony differentiation, genetic diversity and relatedness in the spectacled flying fox (*Pteropus conspicillatus*).

Table 2.1 Attributes of eight microsatellites developed for *Pteropus conspicillatus.* **Ta** refers to final annealing temperature. **N** is the number of samples scored for each locus. **Ho** & **He** are the observed and expected heterozygosity calculated by GenAlEx v6.0 (Peakall and Smouse 2005).

Locus Accession No	Repeat	Primers: 5' to 3'	Ta (°C)	No. of alleles	Size range (bp)	N	Ho	He
PC25b6	TG(11)	F: GCTCAAACACCTACAGGAGCA R: GCTCGGGGCTTGTTTCTATT	50	21	234-278	711	0.762	0.857
PC26a7	TG(17)	F: TTCAGAGTTAGTAGGGGCAAAG R: TTTCCTTGAAAAGCAGCAAA	55	17	136-166	702	0.806	0.904
PC36c2	GTCTCTCT(4)	F: AGTTGAAGGGCACTCATGCT R: CAGAGACAGAGACAGAGACAGAGA	50	15	134-222	453	0.689	0.702*
PC31h4	TG(9)	F: CATTCCCCATTTGAGTGTTT R: ACAGCACCTGAAACCAAACC	55	21	111-149	408	0.745	0.818
PC25c3	AC(8)	F: GCCTGGTGATTACAGTTAAT R: ATTCCCACAATCAAGTTCAT		1	120	96	-	-
PC35e11	AC(13)	F: TCTTTCAAAGCACCCTTCAGA R: TGGGCGAGTCAAATAACAGC		1		96	-	-
PC33g9	TA(5)	F: TTTCTCGGAAGTCTGCGATT R: CATGCTGAATTGTGGCTCTG		1	240	96	-	-
PC40b4	AC(8)	F: ATCCACACCAAACAATTTTC R: AGTGGCAGCAAGTCATCCAA		1	156	96	-	-

* significant departure from overall HWE at 5% confidence interval but not at 10%.

Table 2.2 Attributes of six polymorphic microsatellite loci designed for *Pteropus* species by G. McCracken but tested against individuals of *P. conspicillatus*

Locus	N	Na	Size range	Ho	He
D1	208	31	104-348	0.70	0.65*
C6	206	14	126-300	0.70	0.77
A1	210	20	114-354	0.85	0.88
Ph9	209	22	170-254	0.89	0.90
A2	198	11	128-218	0.28	0.79*
A3	200	20	120-208	0.58	0.86*

*significant departure from overall HWE at 5% confidence interval.

Table 2.3 Volumes of reagents used for each primer in the PCR reaction

Locus	10x buffer	$MgCl_2$ 25mM	dNTP's 10mM	Primer 10mM	Taq	DNA 3ng/ul	H_2O	Thermocycler program
PC25b6	1.5ul	0.9ul	0.15ul	0.45ul	0.08ul	2.5ul	8.97ul	58TD50
PC26a7	1.5ul	0.9ul	0.15ul	0.9ul	0.08ul	2.5ul	8.07ul	STD55
PC36c2	1.5ul	-	0.3ul	0.6ul	0.08ul	2.5ul	9.42ul	58TD50
PC31h4	1.5ul	0.9ul	0.3ul	0.3ul	0.08ul	1.5ul	10.12ul	STD55
c6	1.5ul	0.9ul	0.3ul	0.3ul	0.08ul	2.5ul	9.12ul	50TD44
ph9	1.5ul	-	0.15ul	0.3ul	0.08ul	1.0ul	11.67ul	STD55

*Details of amplification in each thermocycle program are listed in Appendix 3

Chapter Three – Phylogeography, historical population expansion and introgression inferred from mtDNA

Introduction

The four species of flying fox found on mainland Australia are sympatric except for the spectacled flying fox (*Pteropus conspicillatus*) and the grey-headed flying fox (*Pteropus poliocephalus*), which are allopatric (Fig. 1.3). The phylogenetic relationship of these four species, and their evolutionary history within Australia, is unknown; in fact the relationships among species within the genus *Pteropus* remains unresolved. In one of the earliest phylogenetic interpretations of relationships among the species within *Pteropus* (Andersen 1912), the four Australian species were placed in different groups, each aligned more closely with other species than with each other. One study found *P. conspicillatus* most closely related to *Pteropus neohibernicus*, the greater flying fox that occurs in PNG (Colgan and Flannery 1995), while in another it was thought to be most closely related to *Pteropus ocularis*, the Ceram flying fox that occurs in the central Moluccas, Indonesia (Jones *et al.* 2002). Flannery (1995b) stated that *P. conspicillatus* was most closely related to *Pteropus tonganus*, the Pacific flying fox, to which it bears a resemblance. Electrophoretic studies on these two species apparently support this assertion (Flannery 1995b).

Determining the phylogenetic relationships among Australia's flying foxes may help understand the evolution and colonisation of Australia by this group. A comparison of the vertebrate fauna of the major tracts of rainforest in Australia shows the fauna in the Wet Tropics region (Fig. 1.2) to be more closely associated with fauna from the tract of rainforest from south eastern Queensland – northern New South Wales, while the fauna of Cape York Peninsula lies closely with the fauna of the Fly River area of New Guinea

(Schodde and Calaby 1972). Accordingly, the appearance of *P. conspicillatus* in the Wet Tropics region is apparently anomalous. However, its colonisation of this area from the north is commonly stated to be a recent occurrence (Schodde and Calaby 1972) (Fig. 3.1). Although colonisation of Australia by *P. conspicillatus* is proposed to be a result of historical migration from PNG (Schodde and Calaby 1972), it is unknown whether there is any current and/or ongoing movement between these countries.

With previous phylogenetic analyses of *Pteropus* species indicating a close, but unresolved, relationship within the genus, it is also appropriate to include geographically local congeners in any evolutionary study of Australian *Pteropus* species. Three of the four species of flying fox found in Australia have also been recorded in Papua New Guinea (PNG): *P. conspicillatus, Pteropus alecto* and *Pteropus scapulatus*. While *P. conspicillatus* is believed to be widespread and common in PNG, *P. alecto* has a limited distribution and is uncommon, and *P. scapulatus* is only known from a single collection from 1973 (Fig. 1.3) (Bonaccorso 1998). There are also three other large *Pteropus* species found in PNG that do not occur in Australia: *Pteropus hypomelanus* (the variable flying fox), *Pteropus macrotis* (the big-eared flying fox) and *P. neohibernicus* (the greater flying fox) (Flannery 1995a). Moreover, there are another six *Pteropus* species that are not found on the PNG mainland but are commonly found on offshore islands (Bonaccorso 1998), and another 29 species found in the South-west Pacific and Moluccan islands (Flannery 1995b). Although Australian flying fox species are believed to have colonised Australia from PNG, there are no records of the other three large flying fox species in PNG attempting to migrate to Australia. Understanding historical processes may clarify past movement patterns that have influenced the contemporary distributions of Australasian *Pteropus* species.

The earliest study of flying foxes in Australia noted that the distribution of the three large species may be indicative of the sequence of their colonisation into Australia, with *P. poliocephalus* arriving first (based on its most southerly distribution from the point of entry, and its endemic status), followed by *P. alecto*, and finally, more recently, by *P. conspicillatus* (Ratcliffe 1932). *Pteropus poliocephalus* is Australia's only endemic flying fox, so it seems counter-intuitive

to suggest that it has arrived from elsewhere, especially as it is also proposed that flying foxes are recent immigrants in Australia. Despite the propensity for these three species to be separated in previous phylogenies (Andersen 1912; Colgan and Flannery 1995; Jones *et al.* 2002), their possible syntopic relationship led Webb & Tidemann (1995) to investigate their genetic relatedness. Allozyme electrophoresis found very little differentiation between *P. alecto* and *P. poliocephalus*, or between *P. alecto* and *P. conspicillatus* (Webb and Tidemann 1995). However, with the large degree of morphological variability between individuals in *P. alecto* and *P. conspicillatus*, the authors commented that inferred hybrids could have been misidentified. People working closely with *P. alecto* and *P. conspicillatus*, however, believe that these two species hybridise successfully (H. Spencer; J. Maclean, pers. comm.).

Phylogeography is the study of the geographic distribution of genealogical lineages, especially those within and among closely related species (Avise 2000). With the advent of appropriate molecular methods, it has become possible to trace evolutionary relationships among species, as well as within and among populations of a single species (Lowe *et al.* 2005). Phylogeography permits interpretation of historical processes, such as vicariance and dispersal, which have left their signature in contemporary geographic distributions of species. The growing interest in the field of phylogeography has seen the literature base grow from 300 published articles on phylogeography or phylogeny in 1999 (Avise 2000), to more than 26,000 (Web of Science search) in 2005.

Animal mitochondrial DNA (mtDNA) has proven to be an ideal genetic marker for phylogenetic analysis due to its high rate of mutation, uniparental inheritance and lack of recombination (Lowe *et al.* 2005). Clonal inheritance means that interpretation of evolutionary processes is more straightforward than would be the case with the nuclear genome. It also means that mtDNA is suitable for determining historical tokogenetic associations (genetic relationships among individuals), something that is not possible with nuclear DNA in sexually reproducing species because of recombination (Avise 2000). Mitochondrial DNA and phylogeography have been widely used in trying to resolve the relationships of bat groups (Colgan and Flannery 1995; Kirsch *et al.*

1995; Springer *et al.* 1995; Alvarez *et al.* 1999; Nikaido *et al.* 2000; Castella *et al.* 2001; Colgan and da Costa 2002; Jones *et al.* 2002; Giannini and Simmons 2003; Campbell *et al.* 2004; Teeling *et al.* 2005).

Mitochondrial DNA is also useful for detecting introgression, with its uniparental mode of inheritance allowing identification of introgression events through the maternal lineage. Introgression is the incorporation of genetic material from one distinct species into the genome of another, and results from the formation of fertile hybrids (Barton and Gale 1993) that subsequently backcross with one or both of the parent species (Harrison 1993). Introgression appears to be fairly common in some animal lineages (Haig *et al.* 2004; Morando *et al.* 2004; Shapiro *et al.* 2004; Barrowclough *et al.* 2005; Helbig *et al.* 2005; Uthicke *et al.* 2005), especially in birds. Introgression can be inferred if individuals of one species group with individuals of another species in a phylogeny constructed using appropriate sequence data. Mitochondrial DNA sequences are particularly useful for this as their maternal mode of inheritance allows tracking of the genealogical history back to the most recent common ancestor.

A signature in the phylogeny that suggests introgression, however, may also be due to the retention of ancestral polymorphism (incomplete lineage sorting). Incomplete lineage sorting occurs when two recently separated polyphyletic or paraphyletic sister taxa are still in the process of diverging from a common ancestral species, and sequences present in the ancestral species are still present in both diverging species (Frankham *et al.* 2002). It can be difficult to determine which of these two processes, introgression or incomplete lineage sorting, is operating if no prior knowledge of historical events or the general ecology of the two species is available. In some cases, both processes may be occurring. For example, in the *Liolaemus darwinii* complex of lizards in Argentina, isolation of populations during the Pleistocene resulted in the divergence and speciation of several new species. Subsequent range expansion and secondary contact of these species has resulted in introgression of one species into two others, and incomplete lineage sorting in two species (Morando *et al.* 2004).

Mitochondrial DNA can also detect population expansion events by analysing the distribution of mutation frequencies using a coalescent-based approach focussed primarily on the neutral evolution of genes (Ramos-Onsins and Rozas 2002), or the distribution of the number of pairwise differences in nucleotides between DNA sequences (Harpending 1994). Detection of an historical expansion event may clarify historical distribution patterns of *P. conspicillatus*, as well as indicate when *P. conspicillatus* colonised Australia.

In this chapter, partial mtDNA D-loop sequences are used to investigate historical population structure in the spectacled flying fox and compare it with contemporary colony geographical location. The phylogenetic relationship of *P. conspicillatus* and a sympatric congener, *P. alecto*, is also examined.

Methods

Laboratory protocols

Tissue samples from a total of 50 individual spectacled flying foxes from five different areas across the entire range of *P. conspicillatus* were used in the phylogenetic analysis (see Fig. 3.1). In order to balance suitable sample sizes with the cost of sequencing, it was decided to use 10 samples from 5 areas across the species range. These 50 samples were taken from the larger pool of samples used in the microsatellite analysis. Details regarding obtaining these samples can be found in Chapter Four. Samples were also collected from seven *P. alecto* (black flying fox) individuals to use as an outgroup. Five of the seven samples came from a permanent colony of black flying foxes on Thursday Island in the Torres Strait, and the remaining two samples came from individuals in Townsville, north Queensland.

Wing membrane tissue for DNA extraction was collected from the plagiopatagium using 6 mm biopsy punches from Steifel Laboratories, and stored in 5 M NaCl-saturated 20% DMSO and refrigerated. DNA was extracted using a QIAGEN DNeasy tissue kit and gel-quantified against Lambda standards of known concentration.

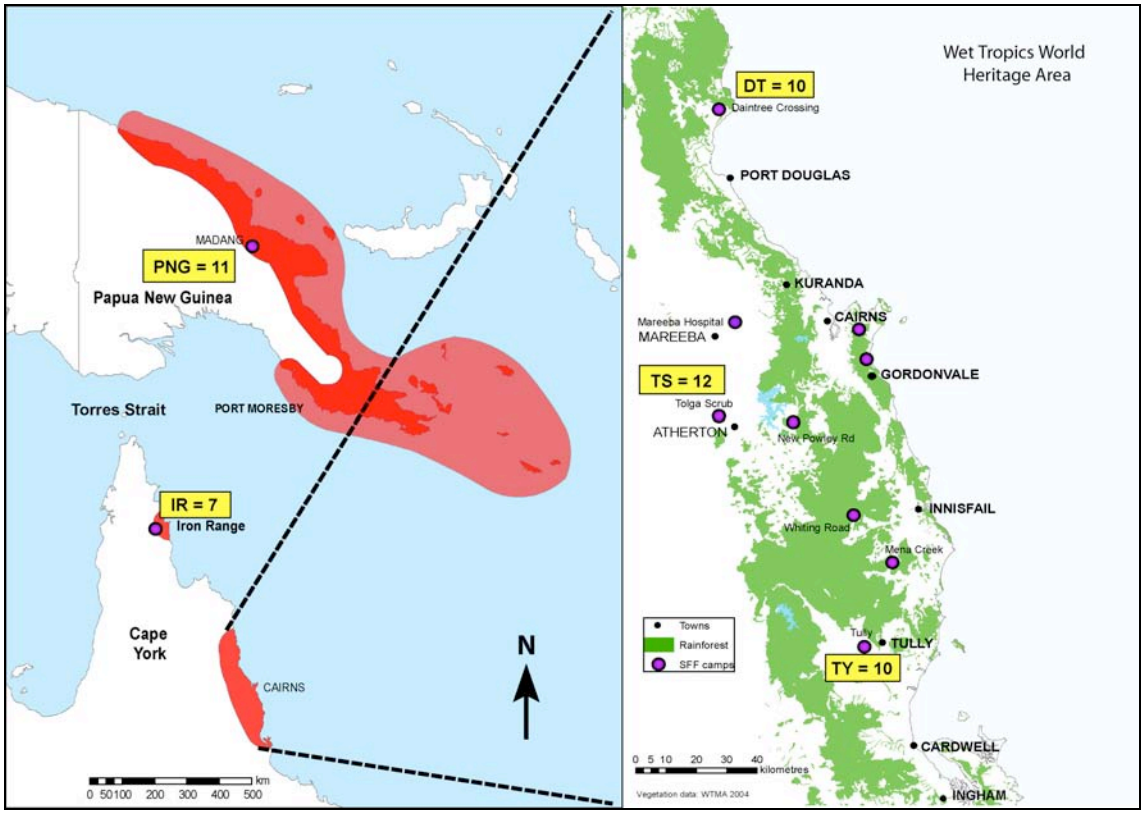

Figure 3.1 Distribution and sampling strategy map for mtDNA phylogeographic analysis. Purple circles = spectacled flying fox colonies. Yellow boxes contain codes for each colony sampled and sample size. Population/colony codes: PNG, Papua New Guinea; IR, Iron Range; DT, Daintree; TS, Tolga Scrub; TY, Tully.

A portion of the mitochondrial D-loop was amplified using primers PVDLPL (5′-AACACCCAAAGCTGATATTCTACT-3′) and PVDLPR (5′-CGTATGCGTATGCGTATGTC-3′), sequenced in both directions. These primers were designed for *Pteropus vampyrus* and provided by Kevin Olival (Columbia University, New York). The 25 µL PCR reaction mix contained 15 ng DNA, 1 x PCR buffer (containing Tris-HCl and KCl), 1 mM $MgCl_2$, 0.10 mM of dNTPs in equal concentrations, 0.2 µM of each primer and 0.5 units of Platinum Taq DNA polymerase (Invitrogen). The PCR included an initial denaturation step of 94°C for 3 minutes, 32 cycles of 94°C for 60 s, 57°C for 60 s and 74°C for 60 s, with a final 7 minute extension at 74°C. PCR products were gel-purified using a MinElute™ Gel Extraction kit (QIAGEN) according to manufacturer's instructions. Sequencing reactions were performed using DYEnamic ET dye terminator for MegaBACE (Amersham BioSciences) and unincorporated nucleotides, salts and other carryover PCR amplification components were removed by spinning through Sephadex–G50. DNA sequencing products were analysed on a MegaBACE 1000 (Amersham Biosciences) at the Advanced

Analytical Centre, James Cook University. Sequences were edited in SEQUENCHER™ v 4.5 (Gene Codes Corporation), and manually aligned using SEAL v 2.0a11 (Rambaut 2002) (Appendix 1).

Data analysis

The most appropriate model of nucleotide substitution was determined using MODELTEST 3.5 (Posada and Crandall 1998) and PAUP* 4.0b10 (Swofford 1998) across 100 bootstrap replicates. Base frequencies and the transition/transversion ratio were calculated using MODELTEST. Maximum likelihood analysis was implemented in PAUP* 4.0b10. The tree produced was rooted by outgroup (*Pteropus alecto*). Intraspecific haplotype diversity (*h*) and nucleotide diversity (π) were calculated for each of the five spectacled flying fox colonies included in this study (Fig. 3.1). The nucleotide sequence divergence (*d*) was calculated between clades with the Kimura 2-parameter algorithm using MEGA version 3.1 (Kumar *et al.* 2004). An analysis of molecular variance (AMOVA) was calculated across three regions (Wet Tropics, Iron Range and PNG) in ARLEQUIN 3.0 (Excoffier *et al.* 2005). An additional AMOVA was carried out on the dataset once the three main clades were identified. As clade 3 only contained three *P. conspicillatus* individuals, it was not included in any further analyses. The lack of structure in the phylogeny according to current geographical locations of colonies, indicated that additional analysis such as an isolation-by-distance analysis and nested-clade-analysis would not be informative.

Historical demography was investigated using mismatch analysis and neutrality tests. Mismatch analysis was performed using ARLEQUIN 3.0 with 1000 bootstrap replicates to assess the statistical significance of Harpending's raggedness (*rg*) statistic (Harpending 1994). Mismatch distribution has been shown to have low power in detecting signals of population growth (Ramos-Onsins and Rozas 2002), while neutrality tests, especially Fu's F_S, are more robust. Fu's F_S was calculated in ARLEQUIN 3.0 using 1000 coalescent simulations to assess statistical significance. Whereas mismatch distributions analyse pairwise nucleotide differences, Fu's F_S uses information from the distribution of mutations (Fu and Li 1993; Fu 1997) to test whether the

population is evolving in a neutral manner. Fu and Li (1993) also showed that while Fu's F_S is most powerful for detecting population growth, other neutrality tests, specifically Fu & Li's F* and D*, are more powerful for detecting genetic hitchhiking (where neutral mutations 'hitchhike' to high frequency when situated close to sites under positive selection), a process that leaves a similar signature in the data. If Fu & Li's F* and D* are significant and Fu's F_S is not, then the signature seen is due to genetic hitchhiking. If Fu's F_S is significant but Fu & Li's F* and D* are not, then the signature is due to a population expansion (Fu and Li 1993). Fu & Li's F* and D* were calculated using DNASP 4.1 (Rozas *et al.* 2003). Mismatch distribution and neutrality tests were carried out on the dataset split into the two major clades found in the phylogeny. Where a population expansion was suggested, the mismatch distribution was used to estimate the time since expansion (τ) scaled in mutational units. The relationship $\tau = 2ut$, where t is the time in generations and $u = \mu k$, where μ is the mutation rate per million years and k is the sequence length, was used to rescale to absolute time (Rogers 1995). As a comparison, a very simple method of working out time to most recent common ancestor was used. The total number of substitutions in clade 1 was calculated as a proportion of change in the number of base pairs in the sequence length analysed. The rate of mutation in the D-loop is highly variable, so this was compared to a conservative rate of D-loop mutation published for mammals (2% per million years) (Brown *et al.* 1979), as well as a rate of D-loop mutation published for bats (20% per million years) (Petit *et al.* 1999). The effective population size of females was calculated using the relationship $\theta = 2Ne_{(f)}\mu$. Theta (θ) was obtained from MIGRATE (Beerli and Felsenstein 1999; Beerli and Felsenstein 2001), which uses a coalescence maximum likelihood approach to calculate migration rate and population size.

Results

Sequence analysis

In total, 732 base pairs (bp), containing 165 polymorphic sites, of the D-loop in the control region in *Pteropus conspicillatus* were sequenced. Sequences were all of exceptional quality making editing simple and unambiguous. Sequence variation included 6 insertions and/or deletions (indels), each 1 bp in length,

making alignment unequivocal. The ratio of transitions to transversions (ts/tv) was 9.6:1 for samples overall. Average base composition (C=0.26, T=0.26, A=0.31, G=0.17) was similar to that of other mammals (Conroy and Cook 2000). The hierarchical likelihood tests and Akaike information criterion from MODELTEST both selected the TrN+I+G model as the best fit for the data (-LogLikelihood=3577.57; AIC 7137.95; Nst=6; Rmat=(1.0000 77.6637 1.0000 1.0000 48.2607)) with variable substitution along the sequence (Rates=gamma), a moderate alpha value (Shape=0.7175), and the proportion of invariable sites = 0.5715.

Phylogenetic analysis

The Maximum Likelihood (ML) analysis revealed 136 equally likely trees, with the variability in tree topology predominantly occurring at the tip nodes. The ML tree most similar to the majority rule tree was chosen. The chosen ML tree divided the dataset in to one well-resolved clade (clade 1) and two smaller groupings (clade 2 and 3), as well as the outgroup (Fig. 3.2). Some individuals also fell out separately away from the main clades (Fig. 3.2 a+b). Several *P. alecto* individuals were placed as an outgroup to all *P. conspicillatus* individuals as would be expected of a different species. However, four of the seven black flying fox individuals fell within the spectacled flying fox clades. The phylogeographic structure depicted by the mtDNA data does not reflect the current geographic relationships of the colonies, as is evident by the lack of structure observed across the colonies throughout the phylogeny (Fig. 3.2).

Molecular diversity

The D-loop in *P. conspicillatus* is characterised by very high levels of haplotypic diversity (Table 3.1). Among a total of 50 individuals, 46 different haplotypes were identified.

Table 3.1 Molecular diversity indices for all colonies. n = number of individuals sequenced, n_h = number of haplotypes, *h* = haplotype diversity, π = nucleotide diversity.

Colony	n	n_h	*h*	π (± SD)
PNG	11	8	0.935	0.054 ± 0.029
Iron Range	7	7	1.000	0.048 ± 0.027
Daintree	10	10	1.000	0.045 ± 0.025
Tolga Scrub	12	12	1.000	0.072 ± 0.038
Tully	10	10	1.000	0.045 ± 0.024
Overall	50	46*	0.996	0.050 ± 0.025

* Note: Total number of haplotypes is less than the sum of n_h due to one haplotype being shared between Iron Range and Daintree.

Although overall haplotype diversity was high (*h*=0.996), overall sequence diversity was relatively low (π=0.050). Forty-three of the 46 different haplotypes occurred as singletons, with two of the remaining three haplotypes each being found in only two individuals. The remaining haplotype was found in three individuals from PNG. One of the two haplotypes found in two individuals was shared between the Iron Range colony and the Daintree colony. Haplotype diversity in PNG appeared to be lower than all the Australian colonies (Table 3.1). Mean sequence divergence between clade one and two was 0.055 (S.E.=0.008).

Population genetic structure

The AMOVA generated on the three regions (Wet Tropics, Iron Range and PNG) showed little differentiation between the regions (5.3%), with most variability occurring within regions (94.7%). An overall fixation index of 0.053 supports the lack of distinction among populations found with the AMOVA. Pairwise F_{ST} values also showed no distinctive genetic difference between the five colonies (Table 3.2).

Table 3.2 Pairwise F_{ST} values between colonies (lower diagonal) and their correspondent significance values (upper diagonal).

	PNG	Iron Range	Daintree	Tolga Scrub	Tully
PNG	-	0.153	0.297	0.234	0.054
Iron Range	0.072	-	0.153	0.117	0.054
Daintree	0.023	0.039	-	0.973	0.486
Tolga Scrub	0.032	0.046	-0.058	-	0.631
Tully	0.076	0.114	-0.018	-0.026	-

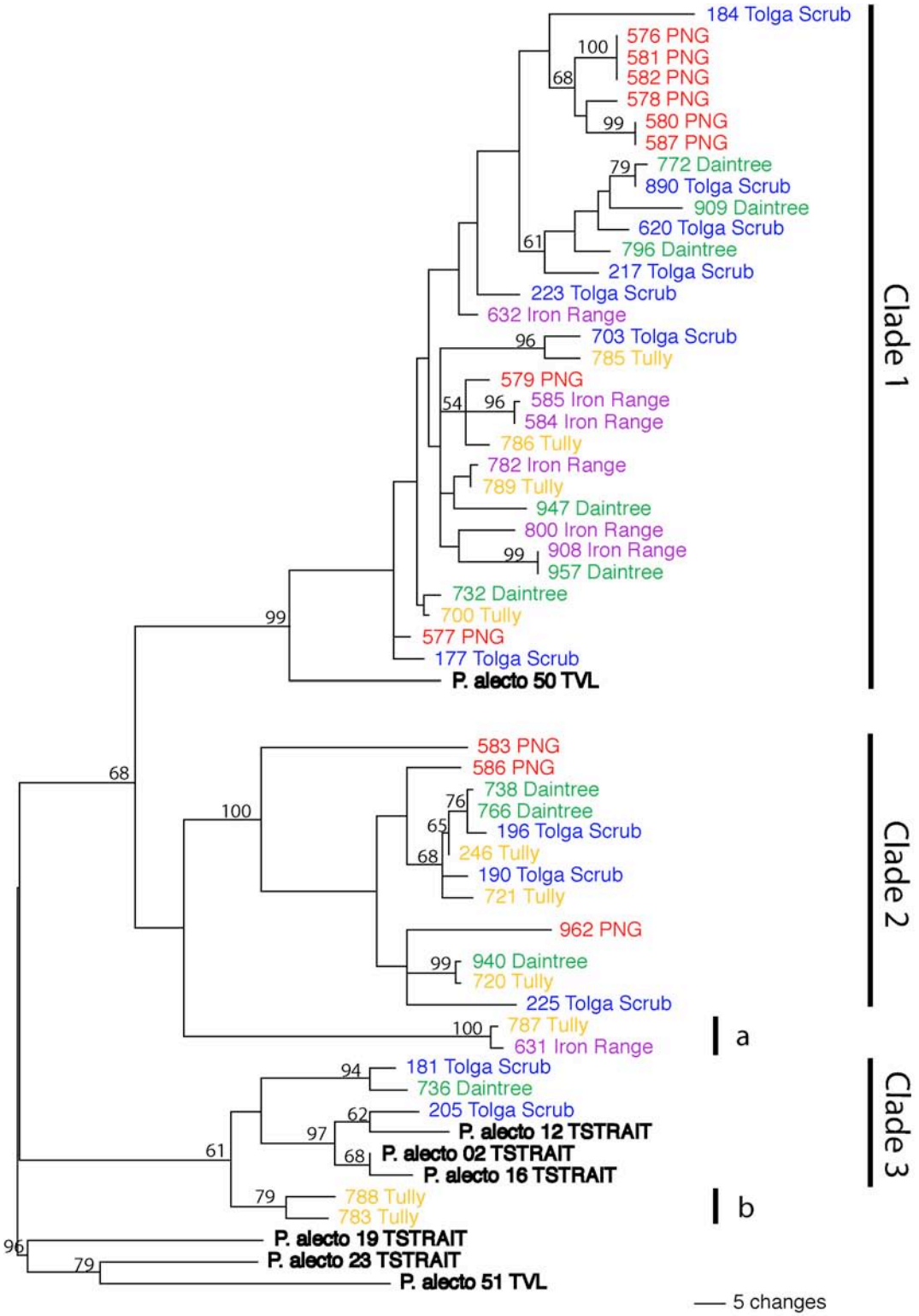

Figure 3.2 Maximum likelihood tree of D-loop mtDNA sequences from *Pteropus conspicillatus* individuals throughout their range, with a closely related congeneric species (*Pteropus alecto*) as the outgroup. Sample names comprise the unique individual identifier followed by the colony from which the sample was collected. Numbers on branches represent percentage bootstrap replicate support. Locality codes: PNG, Papua New Guinea; TSTRAIT, Torres Strait; TVL, Townsville.

An AMOVA, carried out on data separated into the two main clades, found that nearly two-thirds of the variability in the data was between clades (64.48%) while the remaining 35.52% variability was within clades. A fixation index of 0.64 supports the distinctiveness of these two clades.

Demographic analyses

With the dataset separated into two clades, the mismatch distribution analysis for clade 1 was unimodal and fit the expected distribution under a model of population expansion (Fig. 3.3). The raggedness statistic (*rg*) was low but not significant (*rg* = 0.0078, P = 0.52). Clade 2 demonstrated a ragged distribution that fit the expected distribution under a model of stationarity (*rg* = 0.066, P = 0.13) (Fig. 3.3). Fu's F_S strongly supported a population expansion in clade 1 (Fu's F_S = -18.27, P = 0.0000). Fu and Li's F* and D* were however, not significant for clade 1 (F* = -1.577 p > 0.10, D* = -1.265 p > 0.10). Fu's F_S was not signficant for clade 2 (F_S = -2.482, P = 0.073), while Fu & Li's F* and D* were also not significant (F* = -0.7645 p > 0.10, D* = -0.06667 p > 0.10).

Estimates of time since expansion were initially taken from the mismatch distribution with Tau (τ) = 15.713 for clade 1. Assuming a mutation rate of 2% divergence in sequences per million years (0.02 per base pair per million years), and a generation time of five years (see Chapter 6) suggests a time since expansion of 1.43 mya. When this same equation is completed with a mutation rate of 20% per million years as published by Petit *et al*. (1999) for bats, the result is an order of magnitude different (143,367 years). Using a simple equation of time to last common ancestor using number of substitutions and mutation rate, the complete spectacled flying fox dataset suggests a time of 12.5 mya while clade 1 data suggest a time since divergence of 8.5 mya. Again, when a less conservative mutation rate is used in these equations, the results are an order of magnitude different, with time to the last common ancestor becoming 1.25 mya and the time since divergence for clade 1 becoming 850,000 years.

Using the relationship $\theta = 2Ne_{(f)}\mu$, with $\theta = 0.426$ and the mutation rate reported for D-loop in mammals (2% per million years (Brown *et al.* 1979)), and the comparative mutation rate reported for the hyper-variable portion of the

control region in bats (20% per million years (Petit *et al.* 1999)), the effective female population size was calculated to be 38 868 and 3887 respectively.

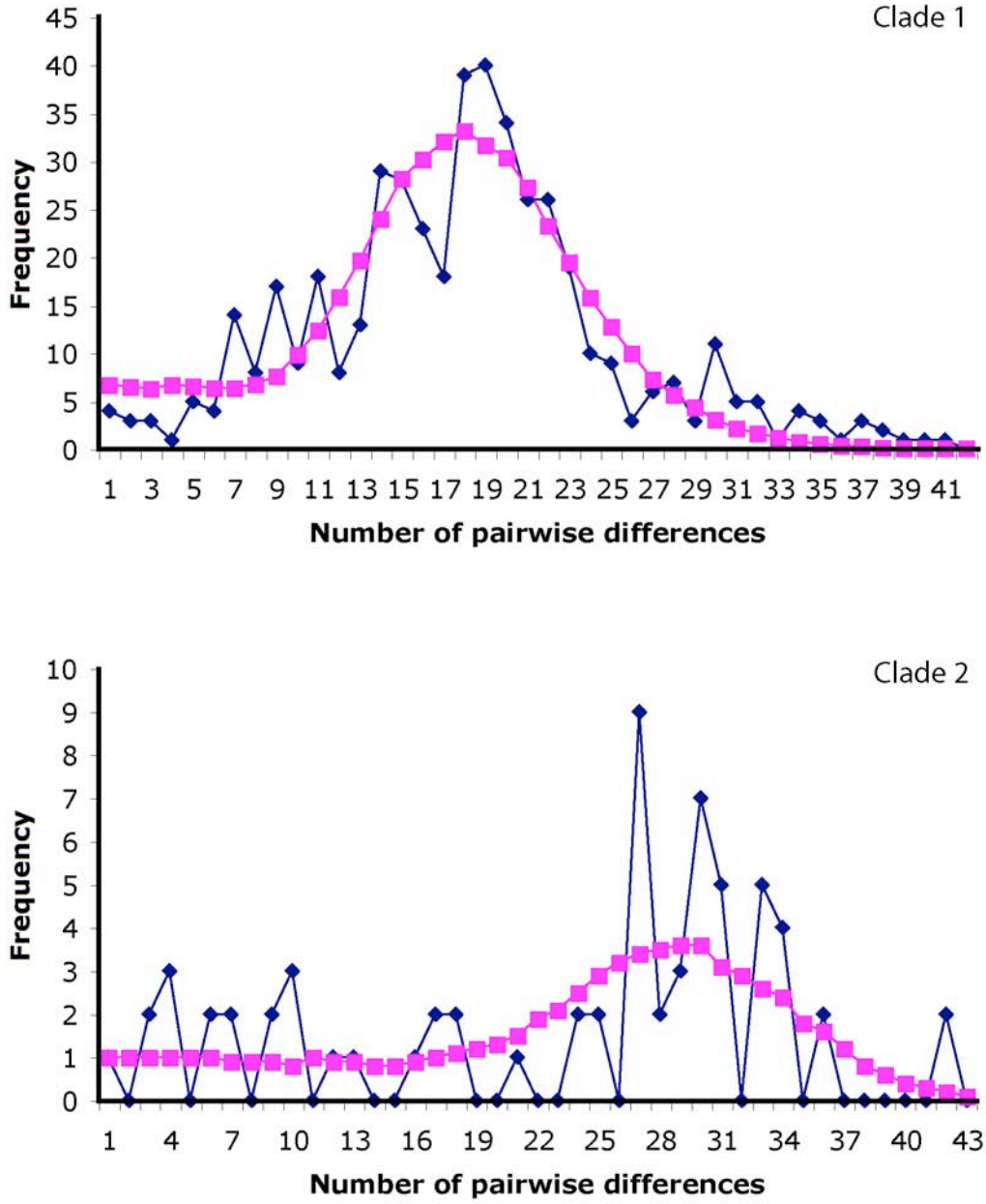

Figure 3.3 Mismatch distribution analysis for clade 1 and 2. Clade 1 fits the expected model of population expansion while clade 2 follows a model of stationarity. Blue points are the distribution of the data for Clade 1 and 2, while the pink points are the expected distribution under that particular model (expansion model for Clade 1 and stationarity model for Clade 2)

Discussion

The phylogeographic structure of spectacled flying foxes

The phylogeographic analysis of *Pteropus conspicillatus* revealed a complex structure with a single large, well-supported clade and two smaller clades with varying degrees of support. Unfortunately the small number of individuals that were in clade 3 meant additional analysis of this clade was not possible

The current geographical location of spectacled flying fox colonies bears no direct relation to the historical (mtDNA) genetic structure, suggesting that the current geographical locations of spectacled flying fox colonies, especially in the Wet Tropics region, may be a relatively recent phenomenon. The lack of structure also corresponds with the general pattern of panmixia found in other Australian flying fox species (Sinclair *et al.* 1996; Webb and Tidemann 1996). Moreover, there is also no obvious structure in the phylogeny of colonies outside the core distribution of the Wet Tropics region. Iron Range individuals are scattered throughout the phylogenetic tree with the rest of the Australian colonies. This may indicate that spectacled flying fox colonies were historically contiguous throughout north Queensland and Cape York Peninsula. If this were the case, and there was once suitable habitat for colonies throughout this area, movement throughout north Queensland, and between Australia and PNG would have been more likely. This may have occurred during one of the warm, moist inter-glacial periods when more suitable habitat would have been present to support the movement of flying foxes. While it could be proposed, then, that movement between PNG and Australia should also be occurring now as it is currently an inter-glacial period, human impact has also had an effect on available habitat, and therefore, current vegetation distribution might not be directly comparable with other inter-glacial periods.

Introgression between *P. alecto* and *P. conspicillatus*

The placement of several individuals of *P. alecto* within the *P. conspicillatus* clades suggests historical introgression or retention of ancestral polymorphism. That several *P. alecto* individuals were placed well outside the main

P. conspicillatus clades also implies that *P. alecto* is genetically differentiated as a separate species. The occurrence of introgression or incomplete lineage sorting, are discussed below.

Theoretically, introgression results in haplotypes of one species being shared with a sympatric but distinct species. Although the two species would have had to be sympatric in the past for the introgression to occur, they will not necessarily still be sympatric. Thus, it is possible that the similarity seen between *P. conspicillatus* and *P. alecto* represents shared haplotypes from old introgression events (Morando *et al.* 2004). The extent of introgression is not known. Whether the introgression is reciprocal is also unknown. That more than half of the limited number of *P. alecto* individuals sequenced fell within *P. conspicillatus* clades, indicates that the extent of introgression between these two species may be high. It is possible that the main clade (clade 1) is a central spectacled flying fox clade, and one of the smaller clades (clade 3) could contain original black flying fox sequences introgressed into spectacled flying fox individuals.

The pattern seen may not be a case of two different species coming together but more a case of a single polymorphic species in the process of divergence. The placing of black flying foxes within spectacled flying fox clades could represent retention of ancestral polymorphisms (or incomplete lineage sorting) between black flying foxes and spectacled flying foxes. Lineage sorting eliminates ancestral polymorphisms over time such that sister species become reciprocally monophyletic (Tajima 1983). However, if these two species are still in the process of diverging from a common ancestor, there will still be ancestral polymorphisms present in the sequences of both species. Phenotypically, some black flying foxes and spectacled flying foxes share similar markings and colouring in the extent of the spectacles occurring and the colouring of the ruff around the neck.

There is no single method currently able to distinguish between introgression and incomplete lineage sorting; two processes that produce similar patterns (Masta *et al.* 2003). Incomplete lineage sorting and introgression due to cycles of isolation and subsequent contact has been suggested in South American lizards

(Morando *et al.* 2004), while incomplete lineage sorting in Caribbean phyllostomid bat species, due to island founder events, produced lower genetic divergence among islands than expected (Carstens *et al.* 2004).

It is difficult to determine which of the above explanations for the pattern seen in the phylogeny is most appropriate. However, sharing of tip haplotypes, or similarity at gene tree tips, between two syntopic species is more indicative of introgression than of shared ancestral polymorphisms, where older, more interior haplotypes (basal) are expected to be shared (Maddison 1997). Although most of the black flying foxes share tip haplotypes, *P. alecto* 50 TVL is more basal and may indicate shared ancestral polymorphisms. So there is evidence to suggest that both processes may be occurring. Many more sequences of both species need to be analysed to determine which explanation can be confidently assigned. As there is also evidence that black and grey-headed flying foxes hybridise (Webb and Tidemann 1995) it would be very informative to include grey-headed flying foxes in future work to determine if these three species form a species complex. This information may also help determine whether introgression or the retention of ancestral polymorphism is occurring.

Pre- or post zygotic mechanisms usually prevent introgression between syntopic species. A common post-zygotic mechanism is production of sterile F1 offspring. However, the discovery that black flying fox individuals appear to be carrying historical spectacled flying fox mtDNA, implies that these two species produce fertile hybrid offspring. There is also anecdotal evidence to this effect (H. Spencer, pers. comm.). A number of possible pre-zygotic mating avoidance mechanisms exist in flying foxes, including a species-specific secretion produced by the scapular gland of males (Martin *et al.* 1995); vocalisations; and grooming. In addition, most flying fox species do not regularly roost together in the same site, and within a camp of conspecifics during the mating season, males will drive off any interloping males, and females will also fend off unwelcome males (Hall and Richards 2000). Ratcliffe (1932) noted that 'not once' did he encounter *P. conspicillatus* roosting with another flying fox species. During 18 monthly camp visits to spectacled flying fox camps in the Wet Tropics, no co-occupation by black flying foxes and spectacled flying foxes was

witnessed (Shilton, pers. comm.). Conversely, Welbergen (2004) undertook behavioural observations in a colony containing both black and grey-headed flying foxes. All of the above factors make it reasonable to suggest that there are at least some barriers in place to prevent introgression occurring between these species.

It is possible that introgression between black flying foxes and spectacled flying foxes occurs through males forcing females of the other species to mate. As these two species appear rarely to roost together, forced copulation may occur during overlapping feeding ranges of the two species. Most flying fox species are believed to have a polygynous mating system where a male defends a territory and mates with the females within that territory (McCracken and Wilkinson 2000; Markus 2002). Males without a territory and, therefore, females to mate with, may take whatever opportunity is open to them. As black flying foxes and spectacled flying foxes also share the same mating and parturition time in the Wet Tropics (Hall and Richards 2000), males in search of a female to mate with will encounter females of both species in oestrus. Forced copulation has mainly been recorded in bird species (Dunn *et al.* 1999; Low 2005), but also in frogs (Byrne and Roberts 2004**)** and snakes (Shine *et al.* 2003) although this is usually intraspecific. Behavioural observations suggest that female flying foxes often resist the copulatory attentions of even a conspecific male to the point of screaming and struggling. Although this may appear forced from an anthropogenic viewpoint, the fact that the female remains in the vicinity of the male following the act prevents behavioural ecologists from calling it 'forced' (Markus, pers. comm.). During interspecific interactions, females in captive colonies appear to be indifferent to the specific identity of a potential mate (H. Spencer, pers. comm.).

Support for an historical population expansion

The pattern of high haplotype diversity and low nucleotide diversity is indicative of an historical population expansion. This same pattern of molecular diversity was observed in the Mexican free-tailed bat (Russell *et al.* 2005), which was found to have undergone a population expansion. The unimodal distribution of pair-wise nucleotide differences in the mismatch analysis, and

neutrality tests strongly support this assertion for clade 1. That Fu's F_S was significant, while Fu and Li's F* and D* were not, indicates that the signature in the data is likely to be due to a population expansion rather than the influence of background selection. Clade 2 was not significant for any of the population expansion analyses, suggesting that it has not undergone a significant expansion event.

The movement of bats into Australia is believed to have followed two routes: from Papua New Guinea down through Cape York Peninsula, and from the islands of Indonesia into the Kimberley/Arnhem land region (Hall 1984). The diversity and geographic distribution of Australian bat species as we see them today is believed to have been the result of many migration events (Hall 1984; Hand 1984). Although the general agreement in the literature is that flying foxes are recent immigrants to Australia, the high haplotypic diversity reported in this chapter suggests an old lineage, rather than one that arrived relatively recently. The higher haplotypic diversity in Australia compared with PNG also suggests that spectacled flying foxes have colonised PNG from Australia rather than the other way around. However, the influence of the introgression between black flying foxes and spectacled flying foxes on the haplotype diversity is unknown and should be kept in mind when comparing diversity between PNG (which has a limited occurrence of black flying foxes) and Australia.

Sequence divergence between clades is high in comparison with other mammal species (Conroy and Cook 2000; Demboski and Cook 2001; Miller *et al.* 2006). The mean D–loop divergence value of 0.055 (5.5%) is greater than divergence values reported between different shrew species (0.031 – (Demboski and Cook 2001). This degree of sequence divergence between clades is indicative of an extensive period of isolation before secondary contact occurred, supporting the assertion of an old lineage in Australia rather than a recent one. Past glacial maxima or geological events may have resulted in a contraction of flying fox habitat, preventing migration of individuals in to, or out of, the Wet Tropics.

The current limited distribution of the spectacled flying fox in the Wet Tropics region has also been suggested to be a result of its recent colonisation (Ratcliffe

1932; Schodde and Calaby 1972). The current distribution pattern in spectacled flying foxes may be due to some aspects of flying fox ecology. Richards (1990b) suggested that their current distribution was the result of a reliance on rainforest. The most southerly geographical boundary of the spectacled flying fox distribution coincides with the southern-most boundary of Wet Tropics rainforest, perhaps explaining the range of this species. Conversely, recent work on movements of *P. conspicillatus* within the Wet Tropics region (Shilton, pers. comm.) and its feeding ecology (Parsons 2005), show that they appear to be more habitat generalists than previously supposed and move continuously between, and feed in, many vegetation types other than rainforest.

A further explanation for the current limited distribution of *P. conspicillatus* is competition. It is rare that more than three species of flying fox are sympatric, and in such cases they usually differ in size (McKean 1970). *P. alecto* and *P. conspicillatus* are of a similar size and overlap in distribution and dietary requirements. *P. scapulatus*, which is smaller than *P. alecto* and *P. conspicillatus*, but nevertheless feeds on the same nectar and blossom-producing species, also overlaps with these two species. It is possible that outside of the current distribution of *P. conspicillatus*, there are insufficient food-resources to sustain three flying fox species, forcing it to retain its northerly distribution. Competition may also be preventing other PNG species of *Pteropus* colonizing Australia (*P. neohibernicus* and *P. hypomelanus* are both large, widespread flying foxes in PNG that have not flown the short distance to Australia). If competition is the factor preventing *P. conspicillatus* from extending its distribution south from the Wet Tropics region, then its present limited distribution is no indication of recent arrival. There may also be other behavioural and/or physiological adaptations that restrict *P. conspicillatus* to its present distribution.

Time since expansion

The data suggest a population expansion of spectacled flying foxes occurred in Australia somewhere in the early to middle Pleistocene. The Pleistocene epoch was characterised by cycles of glacial maxima and interglacial periods, which resulted in broad scale habitat changes (Schneider *et al.* 1998). The warmer, wet interglacials were characterised by expansion of rainforest, while the cooler

drier glacial periods saw rainforests contract. During the last glacial period the rainforest disappeared completely, and the habitat became dominated by sclerophyllous vegetation (Truswell 1990; Kershaw 1994). However, the estimated time to expansion can only be used as a guide because of a number of simplifying assumptions in the model that are easily violated (Rogers 1995). The error rates around this estimate are therefore likely to be large. Although it can be inferred that the expansion occurred during one of the Pleistocene glacial cycles, it is not possible to determine which one, or whether it occurred during a glacial or interglacial period.

The estimates of time since expansion using the two different rates of mutation were greatly different, highlighting one factor that may introduce variability into the final estimate. As the conservative mutation rate reported by Brown *et al*. (1979) covers the entire mitochondrial genome and was estimated for primates, it is likely that the mutation rate reported by Petit *et al*. (1999) for the hyper-variable section of the control region in bats, is a more accurate estimate to use in this case. That said, the estimate of 143,367 years since the expansion of clade 1 took place, can only provide an approximation of when the expansion took place. The error in these estimations was recently highlighted in a study on *Rhinolophus monoceros* (Chen *et al*. 2006), which, after using a mutation rate of 20% per million years, found a population expansion occurred in this bat species 30,000 years ago, with 95% CI ranging from 16,000 – 72,000 years.

Teeling *et al*. (2005) estimated that *Pteropus* and *Rousettus* diverged from a common ancestor 18-28 mya. The estimated time to the most recent common ancestor for *P. conspicillatus* is 1.25 mya (using a mutation rate of 20%/Myr). As the data presented here suggests that spectacled flying foxes are an old lineage in Australia, this divergence could have occurred within Australia. The obviously close association between black flying foxes and spectacled flying foxes, and the possible incomplete lineage sorting, also support this assertion. The suggestion of clade 1 diverging 0.85 mya may indicate an historical event that caused extensive isolation of spectacled flying fox populations. The simplicity of the method used should be considered as a caveat to interpreting these results, with an accurate estimate unlikely. However, it does give an

indication of the time scales to be considered in the timing of major divergence events in this species. A more accurate estimate of time since expansion could be gained with a more exact measure of mutation rate of the D-loop in flying foxes.

Mitochondrial DNA is haplotypic and passed clonally from mother to offspring, so any effective population size estimate will only reflect that of the female population. Only Australian samples were considered in the estimate of theta (θ) so it is likely that this estimate reflects an historical average effective population size of females within Australia through time. The ratio of effective population size (Ne) to population census size (N) has been calculated to be about 1/10 (Frankham *et al.* 2002), therefore the ratio of the female effective population size (Ne_f) is going to be 1/20 of the census size. The effective population size of females (Ne_f) of 3887 would therefore give a population census size of 77,740, indicating that the historical census size was smaller than it is currently. As a comparison, using the 2005 census results for spectacled flying foxes in the Wet Tropics region, the current effective population size for females can be calculated by dividing this census figure by 20. This results in a Ne_f of 13,486: three and a half times that of the historical female population size. As anecdotal information on population sizes of spectacled flying foxes in the early 1900's suggested that there were many more bats than currently (Ratcliffe 1932), it is possible that spectacled flying fox numbers increased from this historical level to one much higher than seen presently. Much like the estimate of time since expansion, the estimate of Ne_f is only ever as accurate as the variables used in the calculation, therefore it is likely that the error rates around these figures are large.

Conclusion

Analysis of mtDNA in the spectacled flying fox indicated a close historical relationship with the black flying fox (*P. alecto*) through hybridisation or incomplete lineage sorting, or possibly both. The age of the species may indicate whether incomplete lineage sorting was occurring—species of recent origin would exhibit incomplete lineage sorting. Hybridisation could be ruled out if there was reproductive isolation, but this does not appear to be the case

with these two species. The mating period, distribution and feeding areas overlap. Hybrid offspring of the two species are also believed to be fertile (H. Spencer, pers. comm.).

The recent colonisation of flying foxes in Australia from PNG is questioned. The endemic status of the grey-headed flying fox suggests that flying foxes have been in Australia for an extensive period. The high haplotypic diversity in the spectacled flying fox also suggests an old lineage in Australia. The greater haplotypic diversity in Australian individuals in comparison to PNG individuals does not support colonisation of spectacled flying foxes from PNG but rather the reverse. The rare occurrences of black and little red flying foxes in southern PNG also does not support the theory of Australia's flying foxes originating in PNG. That *P. neohibernicus*, *P. macrotis*, and *P. hypomelanus* have not been recorded in Australia further opposes the current theory of Australian flying fox origins in PNG.

This study has highlighted the fact that the relationship of spectacled flying foxes to their syntopic and sympatric species is unknown and needs further exploration. Further analyses with larger sample sizes of both *P. conspicillatus* and *P. alecto* are required to discern whether the pattern seen in the mtDNA is due to hybridisation or retention of ancestral polymorphisms. But even more interesting would be to extend the study to include all possible closely related congeners in the Australo-Papuan region.

Chapter Four – Genetic diversity across the range of the spectacled flying fox

Introduction

Analyses undertaken using mtDNA reported in Chapter 3 provided insight into historical population patterns and processes of the spectacled flying fox. However, knowledge of contemporary movements at different spatial scales, and the effect of this movement on population structure in the spectacled flying fox is limited. Historically, the only information available on long distance movement was an untested belief that there is seasonal migration from the coastal lowlands to the uplands of the Atherton Tablelands in the Wet Tropics region of north Queensland in the summer wet season, and *vice versa* in the dry season (Ratcliffe 1932; Richards 1990a). This was assumed following the discovery of several known Atherton Tablelands colony sites being unoccupied during winter (Ratcliffe 1932). The recent use of radio and satellite telemetry to track the movements of spectacled flying foxes within the Wet Tropics region refutes the idea of this seasonal migration. Instead there appears to be regular and recurring aseasonal movement between upland and lowland areas (Shilton, pers. comm.), and some animals seem to travel throughout the whole of the Wet Tropics region.

The extent of movement of the spectacled flying fox between its three principal areas of distribution (Wet Tropics region, Iron Range area and PNG) is unknown. Other than the small colony of spectacled flying foxes that is usually found within the Iron Range National Park, there is no known camp on the Australian mainland north of the Wet Tropics region and no known camps of spectacled flying foxes on any of the Torres Strait islands. As a comparison, the closely related black flying fox (*Pteropus alecto*) has a permanent camp on Thursday Island, in the Torres Strait, as well as seasonal camps at Coen and Weipa on the Cape York Peninsula. Travel has been documented in *P. alecto* between Thursday Island (located in the Torres Strait between the Australian

mainland and the PNG mainland, see Fig. 4.1) and PNG (H. Field, pers. comm.). East–west movement between the Weipa and Coen camps has also been documented in this species (H. Field, pers. comm.).

Although direct observation of the movements of highly vagile species such as flying foxes is difficult, movement patterns can be assessed indirectly by investigating the pattern of genetic variation across the species' range. Allozyme electrophoresis has been used to study genetic subdivision between colonies in the other three *Pteropus* species on mainland Australia (*P. alecto, P. poliocephalus* and *P. scapulatus*). Sinclair *et al.* (1996) examined genetic variation between six colonies of the little red flying fox (*P. scapulatus*) from central NSW to north Queensland and the Northern Territory. They found only 5% of the total genetic variation occurred among populations and an overall F_{ST} value of 0.028 indicated little genetic differentiation. Webb & Tidemann (1996) investigated genetic variation in the black flying fox (*P. alecto*) and the grey-headed flying fox (*P. poliocephalus*) from colonies across the entire range of both species. Like Sinclair *et al*. (1996), Webb & Tidemann (1996) found low levels of differentiation among colonies (F_{ST}: *P. alecto* – 0.023; *P. poliocephalus* – 0.014). Both studies concluded that large-scale movement within the ranges of all three species occurs, suggesting panmixia. Apart from inclusion in two phylogenetic studies (Colgan and Flannery 1995; Jones *et al.* 2002), no genetic research on *P. conspicillatus* has been reported.

Studying species movement with the aid of genetic tools allows a concomitant analysis of genetic diversity and gene flow (Feldheim *et al.* 2001; Girman *et al.* 2001; Garoia *et al.* 2004). By analysing the amount of genetic differentiation among geographically defined populations, population structure can be revealed at varying spatial scales (Ridgway *et al.* 2001; Perez-Losada *et al.* 2002; Taylor *et al.* 2003; Anderson *et al.* 2004; Manel *et al.* 2004; Dannewitz *et al.* 2005). The IUCN recognises genetic diversity as one of three levels of biodiversity that need to be conserved (McNeely *et al.* 1990). The principal reasons for this are two–fold: firstly, genetic diversity is considered to confer adaptive potential, which allows a species to better respond to environmental change, although the speed of the response depends on generation time (for example, the spread of industrial melanism in moths (Majerus 1998) happened relatively quickly due

to their short generation time). Species with a long generation time, which typically also have a low reproductive output, will take much longer to adapt. Secondly, low genetic diversity has been linked to reduced reproduction and survival (i.e. reproductive fitness) (Reed and Frankham 2001; Frankham *et al.* 2002; Hansson and Westerberg 2002; Reed and Frankham 2003). For example, in a wild population of red deer, heterozygosity was shown to be positively correlated with adult lifetime breeding success (Slate *et al.* 2000). Determining the level of genetic diversity in a species gives insight into that species' resilience to stochastic environmental change as well as its probable reproductive fitness.

Typically, levels of gene flow and genetic diversity within and between populations are analysed through multi-locus genotypes with the use of microsatellite markers. As microsatellites also allow examination of recent descent through kinship and can detect the genetic effect of a population bottleneck, they have become popular in population genetic and conservation genetic studies. With their great variability, ease of use and consistent, unambiguous score ability, microsatellites are an ideal genetic marker to examine population genetic structure at a contemporary time scale, while allowing the investigation of several spatial scales.

The signature of an historical bottleneck can be detected through low levels of genetic diversity or an excess of heterozygosity in the microsatellite dataset (Amos and Balmford 2001; England *et al.* 2003). A bottleneck occurs when there is a sudden decrease in population size, resulting in a reduction of total genetic variability (Lincoln *et al.* 1998). Populations that have been through a bottleneck are at increased risk of extinction (Diamond 1989; Groombridge 1992; Caughley and Gunn 1996; Purvis *et al.* 2000a; Purvis *et al.* 2000b; Regan *et al.* 2001; Frankham *et al.* 2002), and identifying these populations is particularly important for threatened species. The 'signature' of a bottleneck only remains in the genetic makeup of a population for between 0.2–4 effective population size (N_e) generations (Piry *et al.* 1999). The severity and duration of the bottleneck, as well as the mutation rate and mutation model of the loci being studied, can reduce or increase this time frame (Cornuet and Luikart 1996), but eventually

mutation and drift remove the signature of the bottleneck from the population (Luikart and Cornuet 1998).

Microsatellites have been widely employed in the genetic analysis of the Microchiroptera (Rossiter *et al.* 1999; Worthington-Wilmer *et al.* 1999; Rossiter *et al.* 2000b; Rossiter *et al.* 2000a; Vege and McCracken 2001; Vonhof *et al.* 2001; Kerth *et al.* 2002a; Kerth *et al.* 2002b; Vonhof *et al.* 2002; Heckel and Von Helversen 2003), but published work on megachiropterans is limited to *Cynopterus sphinx* (Storz *et al.* 2000; Storz *et al.* 2001a; Storz *et al.* 2001b; Storz 2002; Storz and Beaumont 2002). Research on *C. sphinx* has concentrated on the genetic consequences of social structure and mating systems. Microsatellite loci that were isolated for *P. rodricensis*, *P. samoensis*, *P. tonganus* and *P. hypomelanus* (G. McCracken, unpublished data) have been lodged with GenBank, but no study using these primers has been published. A single study employing McCracken's primers and those used by Storz has considered the genetic consequences of captive breeding in the highly endangered *P. rodricensis* (O'Brien *et al*, in press). Considering the extensive use of microsatellites in conservation genetic studies (Beaumont and Bruford 1999), and the fact that 41% of megachiropteran bats are considered at risk of extinction (Mace and Balmford 2000), it is surprising that there have not been more studies involving the use of microsatellites on this group. Genetic studies on megachiropteran bats have used either allozymes (Schmitt *et al.* 1995; Sinclair *et al.* 1996; Webb and Tidemann 1996; Juste *et al.* 2000) or mtDNA gene sequences (Alvarez *et al.* 1999; Jones *et al.* 2002; Giannini and Simmons 2003). While mtDNA analysis is a powerful tool for providing insights into phylogeography and historical patterns and processes (Moritz *et al.* 1987), it does not allow the fine spatial scale analysis sometimes required in population studies. Allozymes remain one of the most popular classes of markers used in ecological genetic studies (Butlin and Tregenza 1998), but due to a low number of alleles detected per locus, their use is declining (Lowe *et al.* 2005). Compared to allozymes, species-specific microsatellites are the more powerful and precise tool for investigating contemporary population structure and genetic differentiation.

In this chapter microsatellite markers are used to investigate contemporary gene flow within and among populations of the spectacled flying fox

(*P. conspicillatus*). These data are used to assess population genetic structure and gene flow across the geographic range of the species.

Methods

Sample collection

Samples were collected from 718 individual bats from 11 spectacled flying fox colonies distributed across the entire range of the species (Fig. 4.1). Samples comprised a 6 mm diameter portion of wing membrane taken from the plagiopatagium with a Stiefel Laboratories biopsy punch (Worthington-Wilmer and Barratt 1996). Samples from the Tolga Scrub, Powley Rd and Whiteing Rd colonies on the Atherton Tablelands came from dead or moribund animals affected by tick paralysis between the months of September and January, in years between 2001 and 2004. Most samples obtained from other colonies in the Wet Tropics region were collected in the same months from orphaned young in care, although some samples came from animals brought into vets (Cairns, Daintree). Samples from Tully and Iron Range were obtained from bats captured for this study in mist nets. PNG samples were obtained from specimens kept at the Australian Museum, SA Museum and Australian National Wildlife Collection (n=10, see Appendix 4 for Museum registration numbers), with the remainder of the PNG specimens being collected by Dr. A. Mack from Wildlife Conservation Society (n=5). Tissue samples were stored in 5 M NaCl-saturated 20% DMSO (Dimethyl Sulphoxide) and refrigerated at 4°C. Samples were collected under two QPWS permits (WISP00556402 and WITK00554802) and cleared under James Cook University animal ethics clearance # A723_02.

DNA extraction and amplification

DNA extraction was carried out using a QIAGEN DNeasy tissue extraction kit according to manufacturer's instructions. The DNA extract was quantified by gel electrophoresis by comparison with DNA lambda standards of known-concentration using the software ImageJ vers 1.33s (Rasband 2004). DNA extracted from punch samples often required concentrating and this was done

by precipitation with 2.2 volumes of 100% ethanol and 0.1 volume of 2.5 M sodium acetate. DNA was diluted to a concentration of 3 ng/μL prior to microsatellite amplification using Polymerase Chain Reaction (PCR) with species specific primers (see Chapter 2).

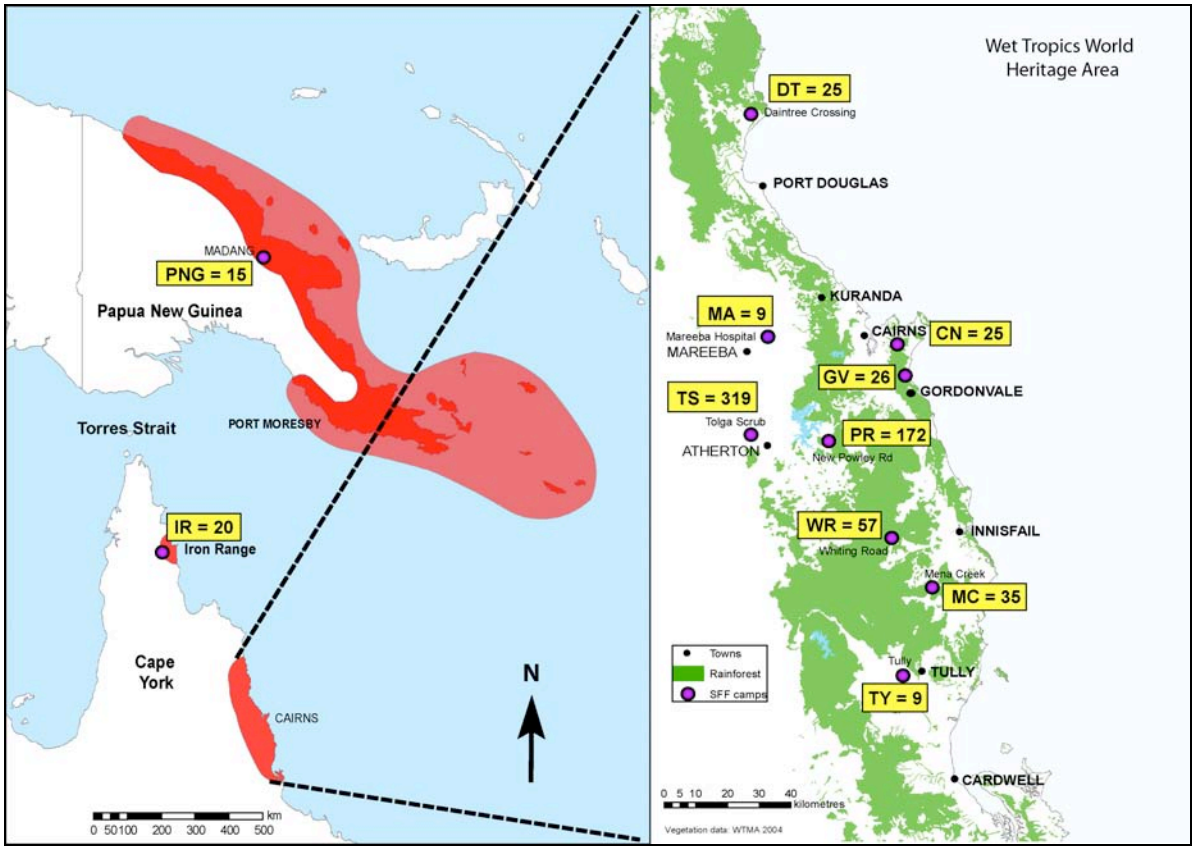

Figure 4.1 Map of the distribution of the spectacled flying fox (*Pteropus conspicillatus*) including detail of colonies within the Wet Tropics region (sample sizes from each colony in yellow boxes). Population/colony code: PNG, Papua New Guinea; IR, Iron Range; DT, Daintree; CN, Cairns; GV, Gordonvale; MA, Mareeba; TS, Tolga Scrub; PR, Powley Rd; WR, Whiteing Rd; MC , Mena Creek; TY, Tully.

Of 10 microsatellite loci tested, four were monomorphic in *P. conspicillatus* and were not used further (see Table 2.1). Samples were PCR amplified and scored at six microsatellite loci: one dinucleotide (Ph9) and one trinucleotide (C6) locus isolated for other *Pteropus* species (McCracken, unpublished data); and three dinucleotide (PC25b6, PC26a7, PC31h4) and one compound repeat (PC36c2) loci isolated from *P. conspicillatus* (see Chapter 2). The 15 μL PCR reaction mix contained varying amounts of reagents depending on the loci and underwent different thermal cycling conditions (see Table 2.3 and Appendix 4). Primers

were labelled with fluorescent tags of HEX, FAM or TET. PCR products were purified by centrifuging through 300 μL of Sephadex, before being analysed on a MegaBace 1000 Genetic Analyser (Amersham BioSciences) at the Advanced Analytical Centre, James Cook University, using the ET 400-Rox (Amersham BioSciences ™) internal size standard. Allele sizes were verified using the program FRAGMENT PROFILER v 1.2 (Amersham BioSciences ™).

Data analysis

Allele frequencies and the mean number of alleles were calculated using GENALEX v 6.0 (Peakall and Smouse 2005). As both statistics are biased by sample size (Hedrick 2000), and there is great variability in sample sizes in this study, allelic richness was also calculated in FSTAT v 2.9.3.2 (Goudet 1995). This measures the number of alleles for each locus and population and corrects for different sample sizes using rarefaction. Allelic richness was averaged across populations (to give mean allelic richness by locus) and across loci (to give mean allelic richness by population). Expected and observed heterozygosity, effective number of alleles and frequency of less common alleles (<25% occurrence) were calculated in GENALEX. The list of frequency of less common alleles was tabulated for each population to determine the extent of occurrence. To determine whether the unequal sample sizes among colonies had an influential effect on allele frequency statistics, the two colonies with the largest sample sizes (Tolga Scrub, n=319 and Powley Rd, n=172) had a random sample of 50 individuals taken from the total sample, and the effective number of alleles were compared between the reduced sample and the total sample. The expected genotype frequencies according to Hardy Weinberg equilibrium (HWE) predictions were tested for each population by locus, and locus pairs using GENALEX (Peakall and Smouse 2005). A sequential Bonferroni correction was used to correct the significance level ($\alpha = 0.05$) for multiple comparisons (Dunn-Sidak method (Sokal and Rohlf 1995)). As the inclusion of close relatives can bias HWE results, all known offspring of females were removed and the HWE analysis was run again. As this made no difference to the outcome, results reported are for the entire sample pool. Linkage disequilibrium between pairs of loci, private alleles and number of migrants (Nm) per generation using the private alleles method (Slatkin 1985) were calculated using GENEPOP V3.4

(Raymond and Rousset 1995). The number of migrants between populations was also calculated using Wright's island model (Wright 1951) according to the equation

$$\mathbf{Nm = (1-F_{ST})/4F_{ST}}$$

There is continuing debate as to the most appropriate statistical analyses to determine genetic relationships among populations using microsatellite markers (Weir and Cockerham 1984; Cockerham and Weir 1993; Slatkin 1994; Slatkin 1995; Michalakis and Excoffier 1996; Rousset 1996; Neigel 2002; Lowe *et al.* 2005). Standard *F* statistics (Wright 1951) are calculated from measures of heterozygosity that assume conformation to Hardy Weinberg equilibrium, which in turn assumes that rates of mutation are negligible. This assumption may be inappropriate when employing markers such as microsatellites that are prone to high rates of mutation (Lowe *et al.* 2005). However, F_{ST} is considered to be more conservative and robust than other measures of genetic differentiation (Lowe *et al.* 2005). Two principal mutation models have been proposed for microsatellites. The step-wise mutation model (SMM) assumes mutation results in the addition or loss of a single repeat unit, whilst the infinite alleles model (IAM) allows for conversion of an allele into any other unique allele by the loss or gain of any number of repeats (Goldstein and Schlotterer 1999). Calculation of an analog of F_{ST}, that is R_{ST} (Slatkin 1995), assumes the SMM model but also assumes equal sample sizes (Lowe *et al.* 2005). Likewise, an analog of R_{ST}, that is Rho_{ST} (ρ_{ST}) (Michalakis and Excoffier 1996), is calculated as the ratio of the estimated variance due to differences among populations over the estimated total variance, based on an analysis of molecular variance (AMOVA) and requires no assumptions on linkage or Hardy Weinberg equilibrium. ρ_{ST} is calculated on microsatellite repeat number rather than allele size or identity, with the added advantage of allowing for uneven sample sizes in populations. The drawbacks to using R_{ST} and ρ_{ST} are that range constraints of allele size lead to overestimations of differentiation when sample sizes are large, variances often tend to be large, and these statistics ignore the influence of migration (Lowe *et al.* 2005). As a consequence these statistics are not necessarily a more reliable estimate of differentiation than are traditional measures (Pearse and Crandall 2004). To accommodate the differences in opinion, authors are

beginning to present more than one of these options as a comparison (Donnelly and Townson 2000; Garoia *et al.* 2004). Both F_{ST} and R_{ST} were calculated in GENALEX and ρ_{ST} calculated in GENEPOP, for intra-population differentiation to ascertain whether there are significant differences between these statistics in my data.

An analysis of molecular variance (AMOVA), which accounts for gene frequencies and number of mutations, was performed in GENALEX (Peakall and Smouse 2005). F_{ST} and R_{ST} were used to compare populations separated into three geographically discrete regions–PNG, Iron Range and the Wet Tropics region. An analysis of isolation-by-distance was performed using a Mantel test in GENALEX (Peakall and Smouse 2005). The genetic distance matrix was produced using Nei's distances (Nei 1972) and the geographic matrix was calculated as the shortest distance between any two colonies. A neighbour-joining tree was constructed in PAUP v4 (Swofford 1998) using the Nei's genetic distance matrix used in the isolation-by-distance test. A Bayesian population assignment protocol implemented in STRUCTURE v2.1 (Pritchard *et al.* 2000) was used to infer the number of populations in the data and to assign individuals to those populations. Models were run for 1–15 populations. Conditions for running STRUCTURE included a burn in of 10 000 replicates with 10 000 Markov Chain Monte Carlo (MCMC) simulations, using the admixture model with allele frequencies correlated between populations. A total of 10 iterations for each putative number of populations (K) were performed. Evidence of population expansion/contraction was tested using the program BOTTLENECK v 1.2.02 (Piry *et al.* 1999). This program evaluates deviations from an theoretical mutation-drift equilibrium and is expressed as the difference between the observed heterozygosity (H_e), and the heterozygosity expected at mutation-drift equilibrium (H_{eq}). H_{eq} is calculated from the allele number (which is reduced faster than H_e in bottlenecked populations). BOTTLENECK also evaluates whether data adhere more closely to the IAM or SMM. The one-tailed Wilcoxon's test for heterozygosity excess and the sign test were used for both mutation models. Estimations were made over 1000 replications. To reduce the bias in allele frequencies from parent-offspring groups, any offspring with a known parent in the dataset was removed. Each population was tested individually and then all Wet Tropics populations were combined and treated as a single population.

Results

Genetic diversity, Hardy-Weinberg and linkage disequilibrium

The number of alleles recorded ranged from 14 in locus C6 to 22 in locus Ph9. Twenty-three private or rare alleles were detected across all populations and loci (Table 4.1). Locus PC31h4 had more private alleles than any of the other loci (n=7).

Table 4.1 Private or rare alleles found in each population by locus. Calculated in GENEPOP v3.4 (Raymond and Rousset 1995). n = sample size for each population.

	n	PC25b6	PC26a7	PC36c2	C6	Ph9	PC31h4
PNG	15			1			1
Iron Range	20				1		1
Daintree	25		2				1
Cairns	25						
Gordonvale	26	1				1	
Mareeba	9						
Mena Creek	35						
Powley Rd	172		1				1
Tolga Scrub	319	1		2	3	2	3
Tully	9						
Whiteing Rd	57				1		

Significant departures from Hardy-Weinberg expectations following sequential Bonferroni correction were recorded in the Powley RD (PR), Tolga Scrub (TS) and Whiteing Rd (WR) populations for one, four and two loci respectively. There was no pattern to the loci that were out of Hardy Weinberg equilibrium. Of 15 locus pairs, two (PC36c2 and PC31h4, Ph9 and PC31h4) showed significant linkage after correction for multiple tests (p=0.002). As PC31h4 was the common locus in both inferred linkages, it was removed from the data and pair-wise F_{ST} figures were re-calculated to determine whether linkage affected the results. No difference was observed between the two sets of results, and therefore PC31h4 was retained for further analyses.

Table 4.2 Summary table of average standard allele frequency statistics by population. Na = number of alleles; Ne = effective number of alleles; Ho = observed heterozygosity; He = expected heterozygosity; A. Rich = allelic richness.

	Na	Ne	Ho	He	A. Rich.
PNG	7.5	4.6	0.74	0.75	5.74
Iron Range	10.0	6.3	0.79	0.79	6.91
Cairns	10.3	6.0	0.81	0.81	6.31
Daintree	11.5	7.4	0.78	0.83	7.21
Gordonvale	11.0	6.6	0.78	0.82	6.81
Mareeba	6.7	4.5	0.80	0.75	6.02
Mena Creek	10.8	6.7	0.78	0.82	6.65
Powley Rd	14.0	6.6	0.78	0.82	6.59
Tolga Scrub	16.2	6.8	0.78	0.82	6.61
Tully	7.5	5.1	0.82	0.77	6.68
Whiteing Rd	12.3	6.2	0.76	0.81	6.48

The average number of alleles per population (across all loci) ranged from 10.0 to 16.2 except for the three populations that had small sample sizes (PNG, Mareeba and Tully) where they ranged from 6.7 to 7.5 (Table 4.2). Less common alleles (<25%) ranged in presence from 99% in the Tolga Scrub population to 37% in the Mareeba population. Understandably, the two populations with the smallest sample size (Mareeba and Tully) had lower proportions of the less common alleles. Notably, PNG had a similar proportion of the less common alleles (40%) to populations with much smaller sample size. This pattern was repeated with the effective number of alleles, where PNG and Mareeba both had the least effective number of alleles. When the effective number of alleles from the reduced sample of Tolga Scrub (Ne=6.5) and Powley Rd (Ne=6.1) were compared with that of the total population (TS Ne=6.8, PR Ne=6.6), little difference was observed. Mean allelic richness by population over all loci ranged from 5.7 to 7.2. There was no significant difference in mean allelic richness across populations (Fig. 4.2a), but mean allelic richness was significantly lower in locus PC36c2, C6 and PC31h4 (Fig. 4.2b), according to non-overlapping error bars.

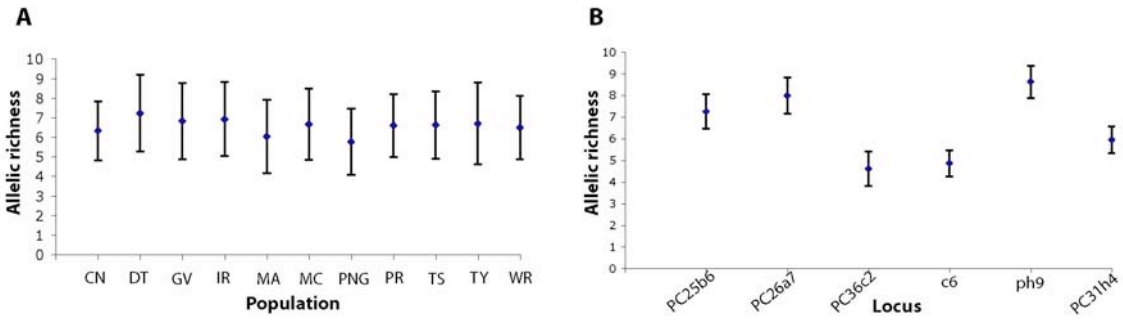

Figure 4.2 a. Mean allelic richness over all six loci by population (+/- SD). b. Mean allelic richness across all 11 populations by locus (+/- SD) Population codes: CN, Cairns; DT, Daintree; GV, Gordonvale; IR, Iron Range; MA, Mareeba; MC, Mena Creek; PNG, Papua New Guinea; PR, Powley Rd; TS, Tolga Scrub; TY, Tully; WR, Whiteing Rd.

Overall mean expected heterozygosity (H_e) across populations was 0.79 and ranged from 0.83 (SE 0.043) in the Daintree (DT) population to 0.75 (SE 0.045) in PNG (PG) (Table 4.2). Overall mean observed heterozygosity (H_o) across populations was 0.78 and ranged from 0.82 in the Tully (TY) population, to 0.74 in PNG (PG) (Table 4.2). F_{IS} figures by population and locus demonstrate that some populations either harbour a heterozygosity excess or deficit at some loci (Table. 4.3). No single population or locus consistently deviated from zero and of the three populations that showed significant deviations, two were those with the smallest sample sizes. It is likely that the F_{IS} figures indicate stochastic effects brought about by small sample sizes and highly polymorphic microsatellite loci. F_{IS} averaged over all populations and loci (0.051) was low and not significant, indicating little likelihood of any inbreeding occurring.

Table 4.3 F_{IS} by population (rows) and locus (columns) following Weir and Cockerham (1984). * denotes significant F_{IS} values according to bootstrapping over loci (95% CI)

	PC25b6	PC26a7	PC36c2	C6	Ph9	PC31h4	Pop mean
PNG	-0.0030	0.3708	0.1867	-0.1857	0.0446	-0.1879	0.0376
Iron Range	0.1618	0.2177	-0.3333	0.0720	0.0312	-0.1153	0.0290
Cairns	0.1661	0.0931	-0.0596	-0.1655	0.0414	-0.0519	0.0120*
Daintree	0.1273	0.1080	-0.0360	0.0075	0.1217	0.0716	0.0730
Gordonvale	0.2419	-0.0144	-0.0435	0.0798	0.0170	0.0865	0.0615
Mareeba	-0.0090	-0.1163	-0.2500	0.1864	-0.0079	0.0427	-0.0150*
Mena Creek	0.0779	0.1057	-0.0173	0.0880	0.0786	0.0149	0.0610
Powley Rd	0.1241	0.0924	-0.0203	0.0453	0.0287	0.0419	0.0540
Tolga Scrub	0.0797	0.0946	-0.0400	0.0049	0.0520	0.0749	0.0480
Tully	0.1667	-0.1250	0.1579	-0.3196	-0.0746	0.1040	-0.0110*
Whiteing Rd	0.1323	0.1250	0.0814	0.1148	-0.0026	0.0263	0.0790

Genetic differentiation

In the 3-region comparison (PNG, IR, WT), AMOVA generated an F_{ST} value of 0.043, with most variability found among individuals within populations (96%). 4% of the total variation was attributed to that occurring among regions. However, when AMOVA was calculated using R_{ST}, a larger proportion of the variability was found among-regions (34%). As R_{ST} is calculated incorporating the mutation model (and therefore the number of alleles and allele distribution), allele distribution for each locus was graphed to determine why R_{ST} would infer regional differentiation when the more conservative F_{ST} did not. The allele size distribution expanded from PNG to Iron Range to the Wet Tropics in 4 of the 6 loci (Fig. 4.3). This may reflect the different sample sizes between the regions (PNG, 15; Iron Range, 20; Wet Tropics, 683), although if this were the case, the majority of allele distributions for PNG and Iron Range (with the smaller sample sizes) would be more widely spaced, reflecting the presence of the common alleles but the absence of rarer alleles. This occurs at some loci (e.g. PC25b6 for PNG) although there are also a number of loci where the alleles are bunched tightly together, indicating that all alleles are likely to have been sampled, but that there is a gradient in allelic diversity from PNG through to the Wet Tropics region. The 'spreading tails' of Wet Tropics samples for loci PC36c2 and C6 represent rare alleles that are not picked up in the two regions with small sample sizes.

F_{ST}, R_{ST} and ρ_{ST} for pair-wise analyses of populations were not significant after correction for multiple comparisons. As the three methods give the same result, only the more robust and conservative *F*-statistics will be employed from here on (Table 4.4).

No isolation-by-distance was found within the range of *P. conspicillatus* (r^2=0.16, p=0.11). The neighbour joining tree indicated little genetic distance between the Wet Tropics populations, but those from PNG and Iron Range stand slightly apart (Fig. 4.4). STRUCTURE results did not support the occurrence of multiple populations as all individuals share characteristics of multiple populations when K>1. The number of migrants per generation was calculated to be 8.09 based on private alleles and after correction for sample size. This suggests high

gene flow occurs between all populations. The same statistic calculated using F_{ST} put the number of migrants at 65. As the private alleles method is considered to be conservative and the F_{ST} method is less robust, it is likely that the most accurate value falls somewhere between these two figures.

Statistical analysis of allele frequencies using the program BOTTLENECK indicated that the majority of populations adhered to the IAM, with four or five of the six loci being retained by both models in four populations. No significant excess of heterozygosity was found in any populations other than Tolga Scrub, Powley Rd and Whiteing Rd. Powley Rd and Whiteing Rd populations both produced a significant result under the IAM for the sign test and the one-tailed Wilcoxon test (Table 4.5). The Tolga Scrub population was only significant for the one-tailed Wilcoxon test under the IAM model. When combined, all Wet Tropics populations showed a significant result for a bottleneck under the Wilcoxon test.

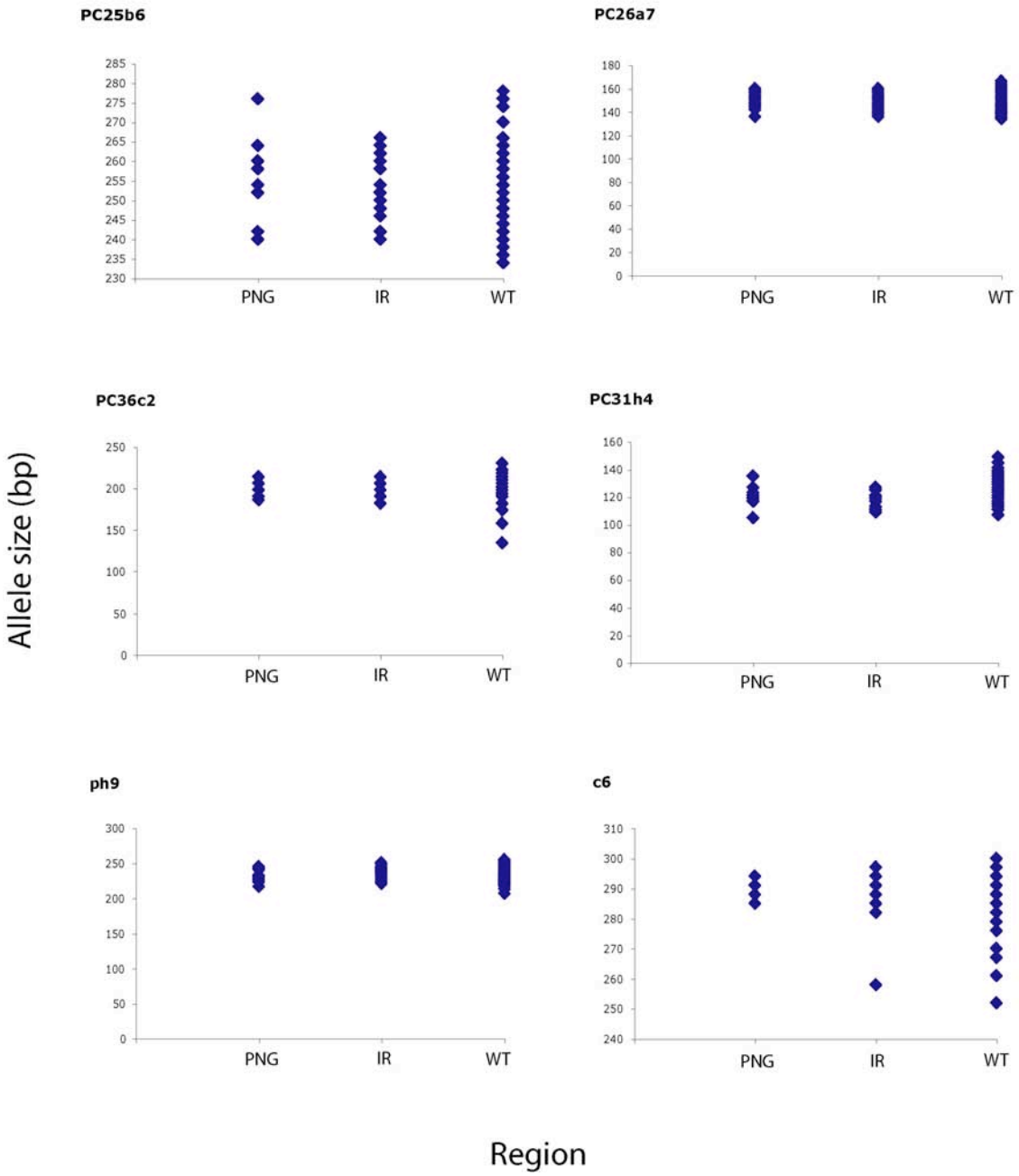

Figure 4.3 Microsatellite allele size distribution by locus for each of three regions of *Pteropus conspicillatus*. Region codes: PNG, Papua New Guinea; IR, Iron Range; WT, Wet Tropics

Table 4.4 Pairwise population F_{ST} values (below diagonal) and pairwise population R_{ST} (above diagonal). None were significant after Bonferroni correction for multiple comparisons (Dunn Sidak method - (Sokal and Rohlf 1995)). Population codes: PNG, Papua New Guinea; IR, Iron Range; CN, Cairns; DT, Daintree; GV, Gordonvale; MA, Mareeba; MC, Mena Creek; PR, Powley Rd; TS, Tolga Scrub; TY, Tully; WR, Whiteing Rd.

Pop	PNG	IR	CN	DT	GV	MA	MC	PR	TS	TY	WR
PNG		0.391	0.159	0.039	0.072	0.280	0.018	0.029	0.023	0.143	0.046
IR	0.094		0.221	0.359	0.281	0.172	0.406	0.409	0.457	0.316	0.333
CN	0.060	0.042		0.146	0.035	0.000	0.101	0.036	0.051	0.139	0.029
DT	0.038	0.062	0.012		0.085	0.138	0.048	0.043	0.037	0.079	0.052
GV	0.040	0.041	0.005	0.009		0.036	0.043	0.021	0.033	0.045	0.001
MA	0.063	0.043	0.021	0.015	0.011		0.099	0.013	0.023	0.139	0.021
MC	0.030	0.053	0.012	0.007	0.000	0.011		0.009	0.010	0.024	0.027
PR	0.035	0.047	0.011	0.008	0.002	0.005	0.001		0.000	0.051	0.001
TS	0.034	0.048	0.010	0.005	0.003	0.005	0.002	0.000		0.059	0.005
TY	0.035	0.071	0.018	0.012	0.000	0.013	0.000	0.000	0.003		0.071
WR	0.035	0.047	0.009	0.005	0.001	0.006	0.003	0.000	0.001	0.004	

Table 4.5 Sign and Wilcoxon results from BOTTLENECK. [a] populations conform to the SMM. [b] populations conform to the IAM. [a+b] BOTTLENECK retained both models for the population. * Marginally significant (p=0.05). **Significant (p<0.05) *** Highly significant (p<0.01).

Population	Sign Test	Wilcoxon Test (One-tailed for H excess)
Cairns [a+b]	0.2029	0.9218
Daintree [a+b]	0.1961	0.9218
Gordonvale [a+b]	0.2088	0.9218
Iron Range [b]	0.5023	0.9218
Mareeba [b]	0.5136	0.5781
Mena Creek [a]	0.4809	0.6562
PNG [a+b]	0.2043	0.9453
Tolga Scrub [b]	0.2494	0.0234**
Powley Rd [b]	0.0473*	0.0078***
Tully [b]	0.4117	0.9453
Whiteing Rd [b]	0.2437	0.0390**
All Wet Tropics [b]	0.2291	0.0156**

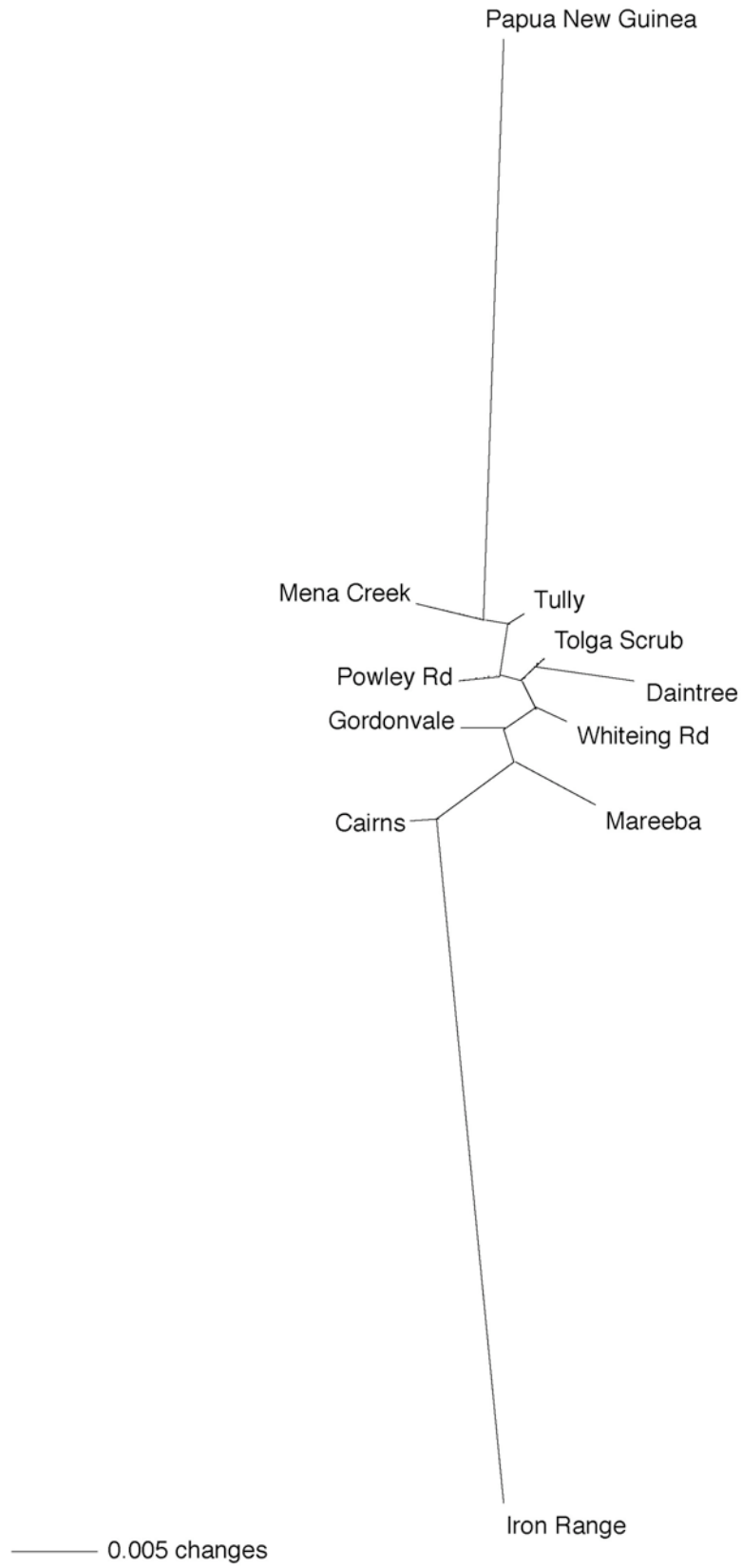

Figure 4.4 Unrooted neighbour-joining tree of pairwise F_{ST} values among populations for multilocus microsatellite genotypes of the spectacled flying fox

Discussion

High levels of genetic diversity

All six microsatellite loci, including those originally isolated from other *Pteropus* species, were highly polymorphic. Cross-species amplification of microsatellite loci is usually associated with reduced microsatellite polymorphism (White and Powell 1997; Lowe *et al.* 2005). However, the allelic diversity of loci isolated from *Pteropus samoensis* and *Pteropus tonganus*, but amplified in *Pteropus conspicillatus*, were comparable with that of loci isolated from *P. conspicillatus* itself. As ease of isolating microsatellite loci between groups differs (Goldstein and Schlotterer 1999), this suggests that there may also be significant group variability in the effectiveness of cross-species amplification.

The overall mean expected heterozygosity value (0.79) and overall mean observed heterozygosity (0.78) indicate high levels of genetic diversity, especially when compared with levels reported in other species of Megachiroptera. *Cynopterus sphinx* and *Cynopterus brachyotis* in India exhibit expected heterozygosity values of 0.68 and 0.65 respectively (Storz and Beaumont 2002) while *Pteropus rodricensis* was reported to have a mean expected heterozygosity of 0.63 (O'Brien *et al*. in press). Reduced expected heterozygosity reported in these species may be due to the mating strategy of the *Cynopterus* species (both are highly polygynous, harem-forming species (Storz *et al.* 2001a; Storz *et al.* 2001b; Storz and Beaumont 2002)) and the critically endangered status of *P. rodricensis* (O'Brien *et al*. in press). A mating strategy, such as polygyny, that biases alleles passed on to the next generation, and reduced population size as a result of a bottleneck, can reduce genetic variability in a population. The mean expected heterozygosity of spectacled flying foxes was similar to that of other highly mobile animals such as fin whales (0.81 - (Berube *et al.* 1998), bigeye tuna (0.78 - (Durand *et al.* 2005), lemon sharks (0.78 - (Feldheim *et al.* 2001), wolves (0.73 - (Jedrzejewksi *et al.* 2005), Horsfield's Bronze-cuckoo (0.71 - (Adcock *et al.* 2005), and Northern Goshawks (0.81 - (de Volo *et al.* 2005). Greater genetic diversity can also be a function of large population size. A greater number of individuals means a larger number of mutations occurring in the population, with a reduced effect of genetic drift

in removing new mutations but an increased effect of selection in removing deleterious mutations (Frankham *et al.* 2002). With the Wet Tropics region supporting approximately 270 000 spectacled flying foxes in 2005 (Fig. 1.4), the high genetic diversity may be a result of large numbers, and high gene flow between colonies due to considerable mobility. The possible introgression between black flying foxes and spectacled flying foxes (Chapter 3) may also have an effect on the nuclear genetic diversity.

No population differentiation due to high gene flow

Broad-scale analysis revealed a high rate of gene flow throughout the entire distribution of the spectacled flying fox, suggesting a single panmictic population. This was supported by the AMOVA, F_{ST}, isolation–by–distance and number of migrants per generation (Nm) results. This result was also supported by Bayesian simulation methods (STRUCTURE), which assigned all individuals to a single population. No genetic differentiation was found across populations when broken down by locus and there was little differentiation according to the pair-wise population comparisons of F_{ST}. This is consistent with results reported from other flying fox species of mainland Australia (Sinclair *et al.* 1996; Webb and Tidemann 1996), although both of these studies used allozymes, a less powerful molecular marker. The high rate of gene flow found in spectacled flying foxes is supported by findings from the CSIRO. In a study of spectacled flying foxes using radio and satellite tracking researchers found large-scale movement throughout the Wet Tropics region (Shilton, pers. comm.).

Panmixia in animal populations is commonly observed. Animals with high mobility are more likely to be panmictic than those with a lesser capacity to disperse. Volant animals such as birds (Chan and Arcese 2003; Rocha *et al.* 2004) as well as many marine species such as fish (Gerlach *et al.* 2001; Garoia *et al.* 2004), turtles (Lopez-Castro and Rocha-Olivares 2005; Mockford *et al.* 2005), sharks (Schrey and Heist 2003; Keeney *et al.* 2005), dolphins (Harlin *et al.* 2003) and whales (Lyrholm *et al.* 1999) are found to be panmictic. Animals with a highly dispersive larval phase such as corals (Ridgway *et al.* 2001) and eels (Dannewitz *et al.* 2005) or wind-assisted dispersal (Schuster and Mitton 2000) are also often found to be panmictic. Although species within the Chiroptera

are highly volant, most appear to have some degree of genetic structuring, often due to female philopatry (Worthington-Wilmer *et al.* 1994; Castella *et al.* 2001; Kerth *et al*. 2002; Miller-Butterworth *et al*. 2003; Rivers *et al*. 2005). Exceptions to this pattern seem to be the large flying foxes (Sinclair *et al*. 1996; Webb and Tidemann 1996) and migratory species such as Tadarida brasiliensis (Russell *et al.* 2005). The high gene flow and low among-population differentiation found in this study on spectacled flying foxes suggest this species is capable of maintaining genetic contact throughout a highly fragmented habitat beyond the rainforests previously associated with habitat preferences of this species.

Although there appears to be large-scale movement of individuals within the Wet Tropics region (Shilton, pers. comm.), the lack of clear genetic differentiation between Australian populations and those of PNG is more surprising. It is possible that as yet unidentified colonies of spectacled flying foxes occur on Cape York Peninsula, providing a link across the Torres Strait. In the absence of such colonies, contact would only be made through exceptionally long flights between Australia and PNG. It is unlikely that intentional flights of this kind would occur by individuals, as the social nature of flying foxes means they would likely avoid solitary life or travel. The alternative is that they travel in groups. There is some anecdotal evidence of large group movement when colonies of spectacled flying foxes have vacated a roost together on a single night but then returned as a group a week later (C. Sabag, pers. comm., Tully colony April 2004; C. Kroger, pers. comm., Mena Creek colony April 2004). Large-scale movements of grey-headed flying foxes in NSW were correlated with the availability of food resources (Eby 1991; Eby *et al.* 1999). It is more likely that groups of spectacled flying foxes would make long distance travel in response to fruit, nectar or pollen pulses, especially if local resources were scarce.

Flying foxes may be aided in their long distance dispersal by trade winds. The seasonal winds on Cape York Peninsula are dominated by south-easterly trades during the dry season and episodically active north-westerlies during the summer monsoon (Colls and Whitaker 2001). If flying foxes were travelling in the same direction as the winds were blowing, either wind system could aid cross–strait commuting. Cyclonic winds can contribute to extralimital

occurrences of volant animals (Daniel 1975) and could cause an individual to be blown off course. The main formation areas of tropical cyclones and the courses they track (Colls and Whitaker 2001) dictate that bats would only be blown from PNG to Australia and not vice versa. That said, the usual behaviour of a flying fox in cyclonic conditions is to hang lower in the tree and to not fly out to feed (Luly, pers. comm.). As there has been no direct study on large-scale movement throughout the range of the spectacled flying fox, these suggestions are speculative.

A small number of migrants between populations each generation is required to create sufficient gene flow to erase the differentiating effects of isolation. In the case of the spectacled flying fox, this means a few immigrants every five years (see Chapter 6 for generation-time estimate) would homogenise emerging differences between colonies. At a regional scale, considering the mobility of these animals, this could simply be the result of a group of individuals following a food resource pulse outside the normal boundaries of neighbourhood movement, or strong winds blowing a number of animals off course. The neighbour-joining tree suggests that some systematic difference can be discerned between Wet Tropics populations, and those of Iron Range or PNG. Although the number of changes (length of the branches) indicates that the difference is small, that there is a difference at all may indicate a reduced amount of inter-regional gene flow or movement, compared to intra-regional movement within the Wet Tropics. However, no isolation-by-distance was detected indicating that there is no difference between the most southerly population (Tully) and the most northerly (PNG).

PNG exhibits low allele richness, low effective number of alleles, and a low proportion of the less common alleles and a smaller distribution of allele sizes indicating an overall reduction in genetic diversity. This pattern was also reported in Chapter 3, where PNG had lower haplotypic diversity in the mtDNA compared to Iron Range or the Wet Tropics. The high gene flow between PNG and Australia suggested by analyses may be a function of a high proportion of common alleles in the PNG dataset resulting in a close association with Australia. Fragmentation of ideal habitat in Australia, which may be preventing migration between the two countries, is geologically recent, so that

while migration between PNG and Australia may no longer occur, the genetic markers used may not have had time to become sorted through genetic drift. If this is the case the actual rate of migration between the two countries will be much lower than estimated by the statistics.

A factor that should be considered with microsatellite data when population differentiation is less pronounced than predicted, is the possibility of microsatellite allele size homoplasy (Estoup *et al.* 2002). Homoplasy occurs when two alleles that are identical in state are not identical by descent. Homoplasy is generated through mutation events (Estoup and Cornuet 1999), and is therefore related to the way mutation produces new alleles and also to the mutation rate. Rapidly mutating markers have higher levels of allele size homoplasy than more slowly changing ones (Estoup *et al.* 2002). Homoplasy is thought to occur most often at loci that conform to the Stepwise Mutation Model (SMM), and other analogous models but not with the Infinite Alleles Mutation model (IAM) (Estoup *et al.* 2002). The effect of microsatellite size homoplasy is a reduction in the observed number of alleles per population, the proportion of heterozygous individuals and in gene diversity (Estoup *et al.* 2002). However, size homoplasy is not believed to affect values of F_{IS} and has a weak effect on values of F_{ST} (Estoup *et al.* 2002), two of the most commonly used genetic differentiation statistics. It is also believed that the effect of mutational processes decreases with increased rates of migration between populations (Estoup *et al.* 2002) due to the ratios of coalescent times of genes within and between populations being different for shorter periods of time. None of the effects of microsatellite allele size homoplasy are evident in data from this study, with high allele number, heterozygosity and gene diversity documented. Along with the amount of migration that is known to occur within the Wet Tropics region, it is unlikely that allele size homoplasy has had a significant effect on the results reported here.

Population bottleneck in the Wet Tropics

That the three Atherton Tablelands populations did not conform to Hardy Weinberg Equilibrium (HWE) was initially surprising. These three colonies provided the largest number of samples. It is normally supposed that small

sample size and highly polymorphic microsatellite loci can bias population allele frequencies. The significant departures from HWE may be the result of a number of factors:

- Departures could result from the presence of null alleles. Comparing mother and offspring genotypes is a robust method of checking for the occurrence of null alleles. This was tested across 76 mother-offspring pairs with no evidence of null alleles occurring in any of the six loci used in this study.

- Mating strategy could bias the frequency of alleles found in the populations. This commonly occurs in species that do not follow a random mating strategy. Species that follow extreme polygyny where males mate with a harem of females, such as elephant seals (Hoelzel *et al.* 1993), could show biased frequency of alleles and therefore departure from HWE. The mating system of flying foxes in general seems to favour some form of polygyny, with high variability in the type of polygyny adopted, while a few species remain monogamous (McCracken and Wilkinson 2000). Some appear to adopt both strategies, as seems to be the case with *P. poliocephalus* (Nelson 1965b; Martin *et al.* 1995). To date, flying fox species have not been found to be extreme polygynists. Although there is no evidence to confirm or refute the extent of polygyny in spectacled flying foxes, as the departure from HWE is not observed in all populations it is unlikely that the mating strategy is the cause of the this departure.

- If colonies contained strong sub-structuring in the form of kin-groups that were essentially populations in their own right, and there was little gene flow between them, sampling from several of these groups can create a Wahlund effect, resulting in a heterozygosity deficit relative to HWE. With the amount of gene flow recorded between spectacled flying fox colonies, this is extremely unlikely.

Results from both Hardy Weinberg equilibrium analysis and tests for heterozygosity excess using the program BOTTLENECK, suggested that only the three populations on the Atherton Tablelands have suffered from an historical

population bottleneck. Limitations of the method pointed out by Luikart and Cornuet (1998) include reduced power to detect a bottleneck when fewer than 10 loci are used or sample sizes are small, and a greater tendency for the IAM model to detect a bottleneck than the SMM. The authors suggest that the Wilcoxon's test is more appropriate and powerful for datasets with fewer than 20 loci. Although only six loci were used in this analysis, the highly polymorphic nature of all loci increased the power of the test. Luikart and Cornuet (1998) also recommend adopting both mutation models to be statistically conservative, since it is believed that the true microsatellite mutation model lies somewhere between the extremes of the SMM and the IAM. However, one of the assumptions of the models is that all loci conform to a particular mutation model, and for all three populations showing a significant bottleneck result, three or four of the six loci did not conform to the SMM model. Small sample size is not a problem with the inferred bottlenecked populations but it may reduce power to detect a bottleneck in the other eight populations analysed. With colonies in the Wet Tropics exhibiting a panmictic structure, it appears unlikely that only a few colonies would have experienced a bottleneck. With this in mind, as well as the lack of power to detect a bottleneck in populations with a small sample size, all the colonies in the Wet Tropics region were analysed as a single population using the program BOTTLENECK. The highly significant Wilcoxon's test suggests that spectacled flying foxes within the Wet Tropics region as a whole have been through a bottleneck. Luikart and Cornuet (1998) suggest caution when interpreting significant results with loci that do not conform to HWE, due to mechanisms such as over-dominance. The use of selectively neutral markers in this study should remove any risk of biasing results through over-dominance. Finally, it is possible that the heterozygosity excess could result from sampling of kin-groups, creating sub-structuring within populations. As a significant result was still obtained when all juveniles and one year olds (non-sexually mature individuals—data from Chapter 6) were removed from the dataset this is unlikely to be the cause.

Attempting to determine when a population contraction may have occurred, and resulted in a genetic bottleneck, is difficult. Typically, the signature of a bottleneck remains in genetic data for a relatively short period of time; from $0.2N_e$–$4N_e$ generations (Cornuet and Luikart 1996). The effective population size

(N_e) is that of the population before the contraction occurred and generation time in spectacled flying foxes is estimated to be approximately five years (see Chapter 6). In species where a population bottleneck has been recorded, such as in the northern elephant seal (Hoelzel *et al.* 1993), or there is general knowledge of an event that created a decline (Andersen *et al.* 2004), it is easier to calculate an effective population size at the time of the bottleneck event. In the case of the spectacled flying fox this could have been thousands of years ago, based on the high genetic diversity currently observed. The habitat of the spectacled flying fox has undergone radical transformations in the past, with the most dramatic accompanying climatic change through the last glacial-interglacial cycle. Pollen (Kershaw 1994) and macroscopic charcoal analyses (Hopkins *et al.* 1993) indicate that at the peak of the last glacial maximum (approx. 18 000 BP) rainforest had virtually disappeared from the Atherton Tablelands. The extent of the reduction was such that no rainforest refugia have yet been identified (Hopkins *et al.* 1993), and it is likely that rainforest was confined to creek lines and valleys. Although climatic recovery was rapid, rainforest expansion was significantly delayed (Kershaw 1994). It is likely that a climatically driven bottleneck would be diffuse rather than intense. England *et al*. (2003) compared the effects of an intense bottleneck (small population sizes for brief periods) with those of a diffuse one (larger population sizes but experiencing population stress for prolonged periods) on genetic diversity and short-term evolutionary potential. England *et al*. (2003) found that intense bottlenecks had a lower probability of retaining alleles compared with diffuse bottlenecks but that short-term evolutionary potential was similar. With the high genetic diversity found in spectacled flying foxes, it is possible that the species went through a diffuse bottleneck as habitat contracted over thousands of years. The slow regeneration of forested habitat could have extended the bottleneck further. Population bottlenecks in response to habitat contraction resulting from the last glacial maximum are common, with bottlenecks found in taxa with lower dispersal abilities, such as the Lost Pines ((Al-Rabah'ah and Williams 2004), Lumholtz tree kangaroo (Bowyer *et al.* 2002), and wolves (Lucchini *et al.* 2004) as well as those with significant dispersal abilities like the North American songbird (Mila *et al.* 2000) and the larger mouse-eared bat (Ruedi and Castella 2003). If the bottleneck in spectacled flying foxes was a result of past climate change, then it is likely that other species of flying fox would also have suffered

the same fate. The reliance of flying foxes on a certain type of habitat for protection from the elements, as well as for food, would have meant that all species of flying foxes would have been under threat from habitat contraction and, therefore, a population bottleneck. This could be a subject of further research, and would strengthen conclusions reached in this study.

More than one measure of genetic differentiation was adopted in this study to determine whether there was a difference in the results they gave and whether one was more appropriate for use in microsatellite studies. Results from this study suggest that the use of more than one statistical method may have merit with some data. Several analyses in this study suggested little differentiation between spectacled flying fox colonies, however the R_{ST} result suggested apparent structure. Once the allele distribution was established, it was evident that a combination of different sample sizes between the regions and a slight gradient of allelic diversity from PNG to Iron Range to the Wet Tropics, resulted in the structure suggested by the R_{ST} result. This suggests not only that further investigation of PNG and Iron Range populations may be warranted to determine whether there is reduced allelic diversity in these areas compared to the Wet Tropics region, but also that the use of more than one genetic differentiation statistic is of value in understanding trends in the data.

Conclusion

Microsatellite analyses suggest that high rates of gene flow between colonies of the spectacled flying fox have resulted in little population differentiation. This species is therefore best described as a single panmictic population within the Wet Tropics World Heritage Area. While analyses suggest that movement of individuals occurs between Australian and PNG populations, this is likely to happen infrequently. Although genetic diversity is high, there is evidence to suggest that spectacled flying foxes throughout the Wet Tropics region have been through a diffuse bottleneck that may have permitted the retention of many alleles likely to be lost under an intense bottleneck model. The slow contraction and expansion of rainforest during the last glacial maximum is a likely candidate for the cause of the bottleneck in Wet Tropics spectacled flying foxes.

Chapter Five – kin group sub-structuring found within spectacled flying fox colonies

Introduction

Population ecologists have long known that many populations consist of sub-groups or demes exhibiting varying levels of relatedness (Sugg *et al.* 1996). Social organisation in some species may confer mating advantages to particular individuals (Emlen and Oring 1977). Alternatively, social structure may result from, or lead to, incomplete or sex-biased dispersal (Coltman *et al.* 2003). Living in family groups can also bestow advantages in the form of behaviours such as co-operative breeding (Gilchrist *et al.* 2004; Morton *et al.* 2004; Radford 2004), co-operative feeding (Wilkinson 1984), and social grooming (Wilkinson 1986). Understanding the social organisation of a species requires knowledge of the mating system, parentage, and relatedness within and between social groups. These factors can be particularly difficult to study using behavioural methods in wild populations but even more so in such mobile and inaccessible organisms as bats. Mating or parental behaviour may be difficult to observe, while multiple copulations or shared parental care may make observations unreliable (Burland *et al.* 2001). There are also many questions that cannot be answered by observation alone. This has been highlighted in many recent studies of paternity, especially in birds, which have shown a high prevalence of extra-pair copulations, resulting in paternity of young by non-resident males (Kupper *et al.* 2004; Schamel *et al.* 2004; Blomqvist *et al.* 2005; Perez-Staples and Drummond 2005). To understand the actual relationships of interacting individuals, such as true mating success, it is necessary to analyse the genetic relationships of individuals in the population.

There have been numerous genetic studies on mating systems and relatedness in bat species (McCracken and Wilkinson 1988; Petri *et al.* 1997; Kerth *et al.* 2000; McCracken and Wilkinson 2000; Rossiter *et al.* 2000b; Storz *et al.* 2000; Burland

et al. 2001; Burland and Worthington-Wilmer 2001; Storz *et al.* 2001a; Kerth *et al.* 2002b; Rossiter *et al.* 2002; Ortega *et al.* 2003; Willis and Brigham 2004), although most have been conducted on microchiropteran species. These studies showed varying levels of polygyny within the Chiroptera, with females in many microchiropteran species being philopatric. Research on megachiropteran species has largely been confined to observation and behavioural studies (Ratcliffe 1932; Nelson 1965a; Nelson 1965b; Wiles 1987; Pierson and Rainey 1992; Markus 2002). An exception is Storz (Storz *et al.* 2001a; Storz *et al.* 2001b) who reports detailed studies on polygyny and its genetic consequences in the Indian fruit bat, *Cynopterus sphinx*. With the use of microsatellite loci, this harem-forming, highly polygynous species was shown to exhibit variable rates of mating success by resident males, as well as complete natal dispersal by both sexes.

Members of the order Chiroptera are generally gregarious with most species forming colonies at some stage during their life cycle (Burland and Worthington-Wilmer 2001). In tropical species enormous variation in the pattern of colony formation occurs. Colonies are typically formed at least during the mating period, and/or the parturition and lactation period in most species (McCracken and Wilkinson 2000). Australian flying fox species, however, are year-round colony occupiers, although there appears to be extensive movement of individuals between colonies (Chapter 4 of this study; Shilton, pers. comm.; Tidemann and Nelson 2004).

The majority of bat species are polygynous but a few appear to be monogamous (McCracken and Wilkinson 2000). Polygyny in bats is highly variable, with some species employing year-round harem systems (Wiles 1987), while others create short-term random groups of males and females (Nelson 1965a). There also appear to be many intermediate forms, creating a 'polygyny continuum' in this group (McCracken and Wilkinson 2000).

Research on Australian flying fox mating systems and social interactions has considered three of the four species: the grey-headed flying fox, *P. poliocephalus* (Ratcliffe 1932; Nelson 1965a; Nelson 1965b); the black flying fox, *P. alecto* (Ratcliffe 1932; Nelson 1965a; Nelson 1965b; Vardon and Tidemann 1999;

Markus 2002); and the little red flying fox, *P. scapulatus* (Ratcliffe 1932; Nelson 1965a; Nelson 1965b). Research by Ratcliff and by Nelson provides much of our preliminary knowledge. Colony size and sex ratios varied at different times of the year (Ratcliffe 1932; Nelson 1965a; Vardon and Tidemann 1999). Basic reproductive biology of each species (male & female reproductive cycle, time of year for mating & parturition, number of young, time spent nursing young, etc) has been recorded (Ratcliffe 1932; Nelson 1965a; Nelson 1965b). Territoriality and courtship have been discussed, noting that both *P. alecto* and *P. poliocephalus* appear to follow a polygynous mating system (Nelson 1965b; Markus 2002). In all accounts there appears to be a similarity in behaviour of these two species, and in many instances these are in agreement with anecdotal information on *Pteropus conspicillatus* (Spencer, pers. comm., Maclean, pers. comm.). With *P. alecto* and *P. poliocephalus* having similar morphological and ecological characteristics to *P. conspicillatus*, and with preliminary evidence of introgression between *P. alecto* and *P. conspicillatus* (Chapter 3), it is likely that *P. conspicillatus* will also exhibit a polygynous mating system.

Aside from sexual interactions, and colony size and structure, there is little published information on social interactions within colonies of Australian flying foxes. Colony structure outside the mating and parturition periods has been briefly studied but only to the extent of sex ratios and numbers present being recorded (Ratcliffe 1932; Nelson 1965a; Nelson 1965b; Richards 1990a; Eby 1991; Hayden 1992; Parry-Jones and Augee 1992; Eby *et al.* 1999; Tidemann *et al.* 1999; Vardon and Tidemann 1999; Westcott *et al.* 2001). No genetic studies have investigated possible sub-structuring or the relatedness of individuals to each other within a colony.

In this chapter microsatellite markers are employed to determine whether spectacled flying fox colonies show evidence of genetic sub-structuring within or between colonies. Any sub-structuring will then be investigated to determine the degree of relatedness within and between colonies as well as the composition of individuals within a sub-group.

Methods

Data for microsatellite analysis of relatedness were the same as that reported in Chapter 4 (i.e. genotypes of individuals consisting of six polymorphic microsatellite loci). Initial analyses were conducted on the entire microsatellite dataset consisting of 11 populations with 718 individuals (Fig. 5.1). More detailed analyses were conducted on the largest population samples from three maternity colonies on the Atherton Tablelands: Tolga Scrub (n=319), Powley Rd (n=172) and Whiteing Rd (n=57). These samples were mostly collected during the summer months from October–January in 2002, when females were giving birth and caring for young. Great care was taken to collect, and keep track of, samples from mother/baby pairs (n=78; TS=53, PR=20, CN=2, WR=2) from the Tolga Scrub colony and the Powley Rd colony, that came into care at the Tolga Bat Hospital. These two colonies are approximately 7 km apart in a direct line and analyses reported in Chapter 4 showed them not to be genetically differentiated.

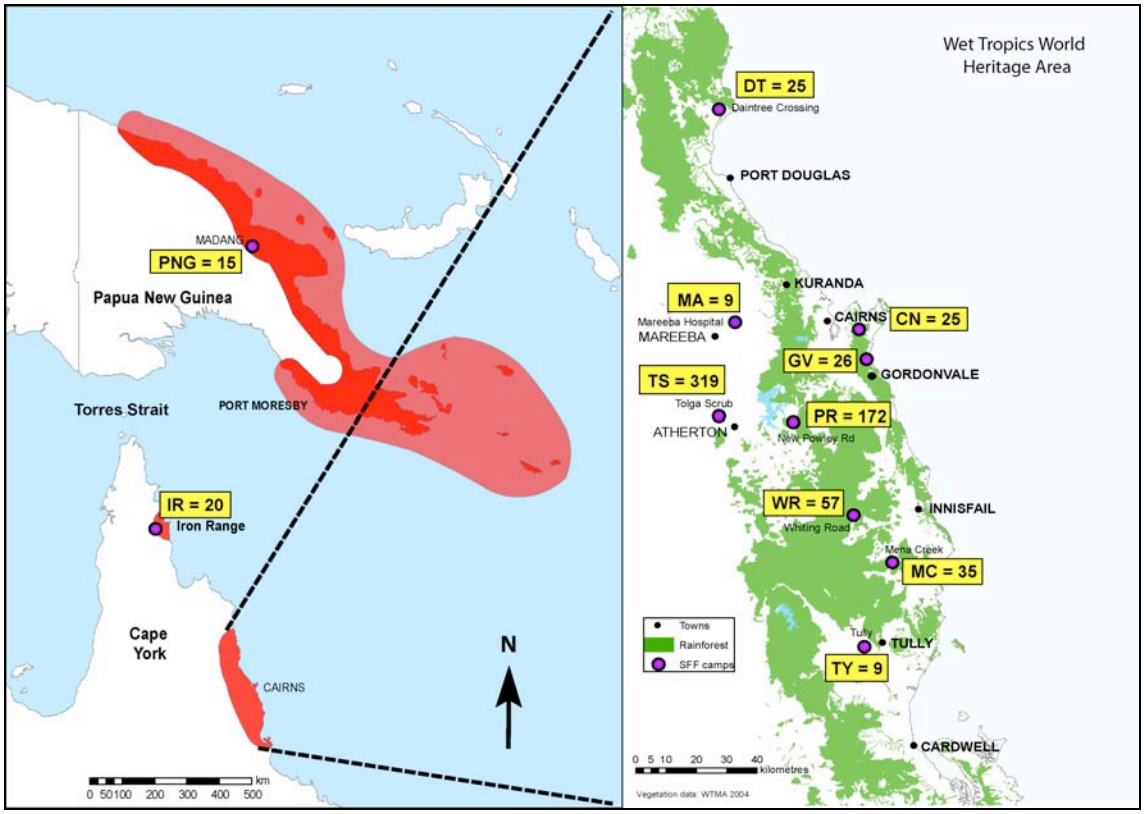

Figure 5.1 Distribution map of the spectacled flying fox showing sampling regime for this study. Broad scale comparisons use all colonies. Colony Codes: PNG, Papua New Guinea; IR, Iron Range; DT, Daintree; CN, Cairns; GV, Gordonvale; MA, Mareeba; TS, Tolga Scrub; PR, Powley Rd; WR, Whiteing Rd; MC, Mena creek; TY, Tully. Detailed analyses only use Tablelands colonies TS, PR and WR.

Data analysis

Broad-scale, among-population level analysis of relatedness was conducted using the program MARK v2.0 (Ritland and Travis 2004) because of its ability to incorporate within-population pairwise relatedness into a larger scale, among-population pairwise relatedness measure. Using a method-of-moment estimator, relatedness coefficients were calculated on the entire microsatellite dataset (11 populations) using MARK v2.0 (Ritland and Travis 2004). Individual pairwise relatedness was calculated in MARK using the D2 estimator that is most appropriate for Simple Sequence Repeats (microsatellites). Average relatedness across all pairwise comparisons within and between each population pair was calculated, and then used in a population pairwise comparison. All unrelated pairwise comparisons were scored as zero. Pairwise relatedness values and

individual population comparisons were sorted into within- and between-population comparisons for the total output and for each population, using FILEMAKER PRO V7.0 (© 1984-2004 Filemaker, Inc.). Graphs comparing within- and between-comparisons were drawn using SIGMAPLOT v9.01 (Systat Software Inc. 2004).

Average relatedness [R] across all individuals within each of the three Atherton Tableland maternity colonies (TS, PR and WR), and between colonies, was calculated using the program RELATEDNESS v5.0.8 (Queller and Goodnight 1989), a commonly used estimator of relatedness between individuals that uses an identity-by-descent approach. Pairwise relatedness was calculated separately for all possible pairwise combinations within each colony using RELATEDNESS. Pairwise relatedness is a relative measure that is calibrated for the allele frequency composition and the number of individuals that are present in the population analysed. The basic form of the calculation follows

$$r = (P_y - P^*) / (P_x - P^*)$$

where P* is the frequency of allele A in the population as a whole, P_x is the frequency of allele A in the reference individual, and P_y is the frequency of allele A in the group/individuals to be compared to P_x. For diploid individuals the allele frequency in the reference individuals, P_x, must be 0.5 or 1.0. Unless the comparative group/individuals are closely related and have similar allele frequencies, the value for P_y is likely to be much smaller. Hence, with unrelated comparisons the *r* value will be negative. Positive values signify a degree of relatedness. An unweighted pair group method using arithmetic averages (UPGMA) tree was constructed based on the transformed (-ln) pairwise relatedness matrix using PAUP* v4.0 (Swofford 1998). A UPGMA tree clusters all the most closely related individuals in accordance with the relatedness values from the matrix. The overall topology of the resulting UPGMA tree for each colony was similar, and therefore, a single colony was used as an example typical of the others in further analysis. For all further analysis the 172 samples from the Powley Rd (PR) colony collected in 2002 were used. A sub-group of samples that clustered closely together on the tree was analysed separately to determine how closely related to each other individuals within the cluster were.

The sub-group was also used to test hypotheses about particular pedigree relationships using the program KINSHIP v1.3.1 (Goodnight and Queller 1999). Hypotheses tested were that alleles were identical by descent as a result of direct maternal descent (R_M=1.0, R_P=0.0) or direct paternal descent (R_M=0.0, R_P=1.0), with the null hypothesis being that alleles were not identical by descent because pups were not related to mothers/fathers within the group (R_M=0, R_P=0). Full sibling (R_M=0.5, R_P=0.5) and half sibling (maternal: R_M=0.5, R_P=0.0/paternal: R_M=0.0, R_P=0.5) relationships were also tested. Significance values were obtained using 10 000 simulated pairs.

A smaller identified kin group of individuals was then analysed to determine the sex and age composition of the group. Data on age and sex of individuals was obtained from Chapter 6, although ages could only be obtained for individuals that had died as a consequence of tick envenomation. To determine whether there was a degree of inbreeding occurring within the colony, F_{IS} was calculated using the program GENEPOP (Raymond and Rousset 1995).

Results

Histograms of pairwise relatedness comparisons within- and between-colonies (Fig. 5.2) calculated using MARK, show the same pattern of distribution of relatedness values for within-colony comparisons as for between-colony comparisons.

Individual plots of the frequency distribution of pairwise relatedness values within each colony (Fig. 5.3) show that most colonies conform to a Poisson-type distribution, although small sample sizes in some colonies produced skewed or erratic relatedness distributions. Whereas most colonies have a mode around the 0.2–0.3 point, PNG and WR show positive kurtosis with modes around 0.5. The three Tablelands colonies (TS, PR, WR), which have the largest sample sizes, show smooth and even distributions similar to that for the aggregated dataset (Fig. 5.2).

The population-by-population average pairwise relatedness comparison (Fig. 5.4) shows PNG having the highest average relatedness in comparisons with

other colonies (7 of 11 comparisons) and IR had the lowest average pairwise relatedness in comparisons with the other colonies (10 of 11 comparisons).

Average relatedness within a colony across all individuals calculated with RELATEDNESS was very low (PR: R=-0.006, SE=0.0001; TS: R=-0.0032, SE=0.0001; WR: R=-0.0184, SE=0.0004). Likewise, average relatedness between colonies was also low (TS vs PR: R=-0.0021, SE=0.0000; TS vs WR: R=-0.00028, SE=0.0000; PR vs WR: R=-0.0046, SE=0.0001).

UPGMA trees inferred from the pairwise related matrix (calculated in RELATEDNESS) had the same composition for all three colonies, with widely spaced individuals at the top and bottom of the tree being those not closely related to others within that colony, and tight clusters of individuals throughout the tree suggesting possible kin groups (Fig. 5.5). Smaller scale analysis of a sub-group from the PR UPGMA tree shows high levels of relatedness within one of the tight clusters of individuals (Fig. 5.6a). When this cluster is analysed for age and sex of individuals, it appears the group is comprised of a single older male, two older females, several offspring from that year (2002) and a number of sub-adults from the previous two years (Fig. 5.6b).

The F_{IS} result for the within-colony inbreeding coefficient was low (0.038) suggesting no occurrence of inbreeding in the PR colony.

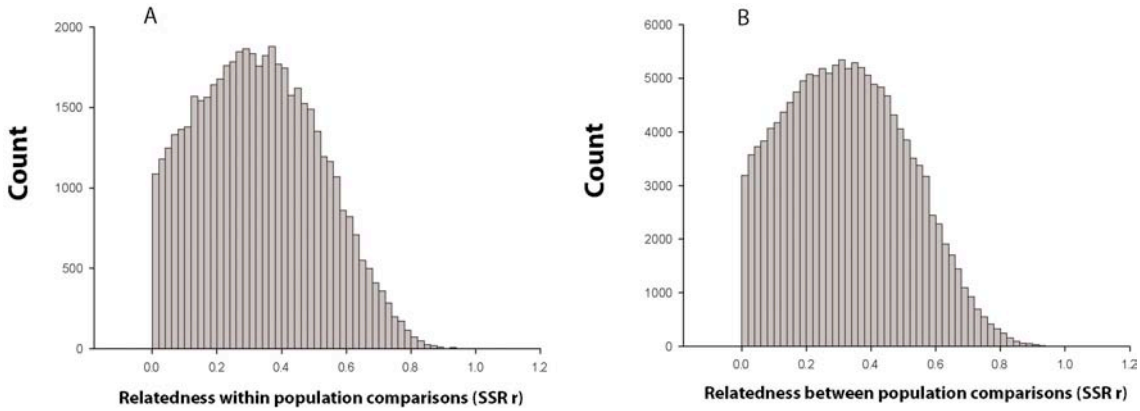

Figure 5.2 Frequency distribution of all pairwise relatedness values across 11 colonies of the *Pteropus conspicillatus* (A) within and (B) between colony comparisons, calculated by MARK. All non-related values (zero or negative) are excluded.

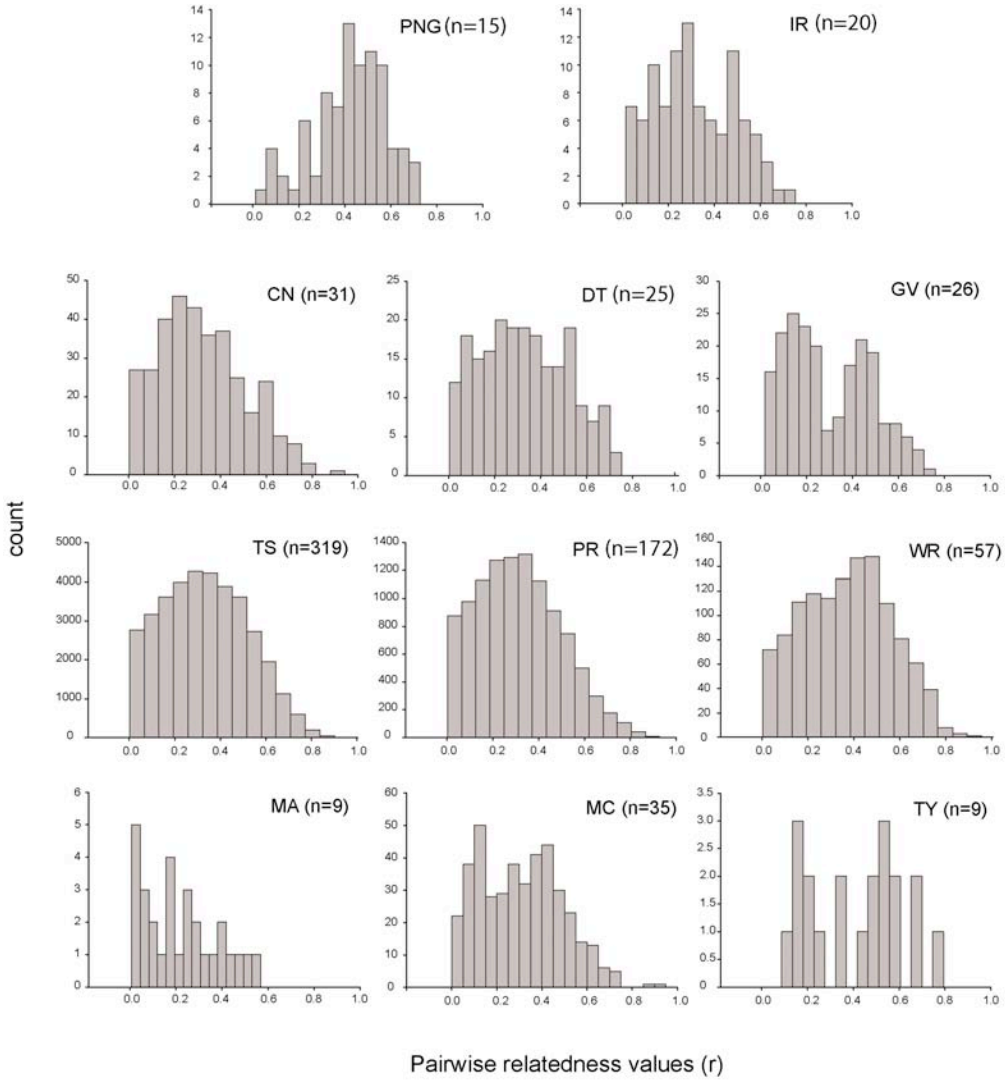

Figure 5.3 Frequency distribution of all pairwise relatedness values between individuals within colonies, for all colonies across the range of *Pteropus conspicillatus* (unrelated values removed). Calculated using the SSR relatedness calculated using MARK (Ritland 2004).

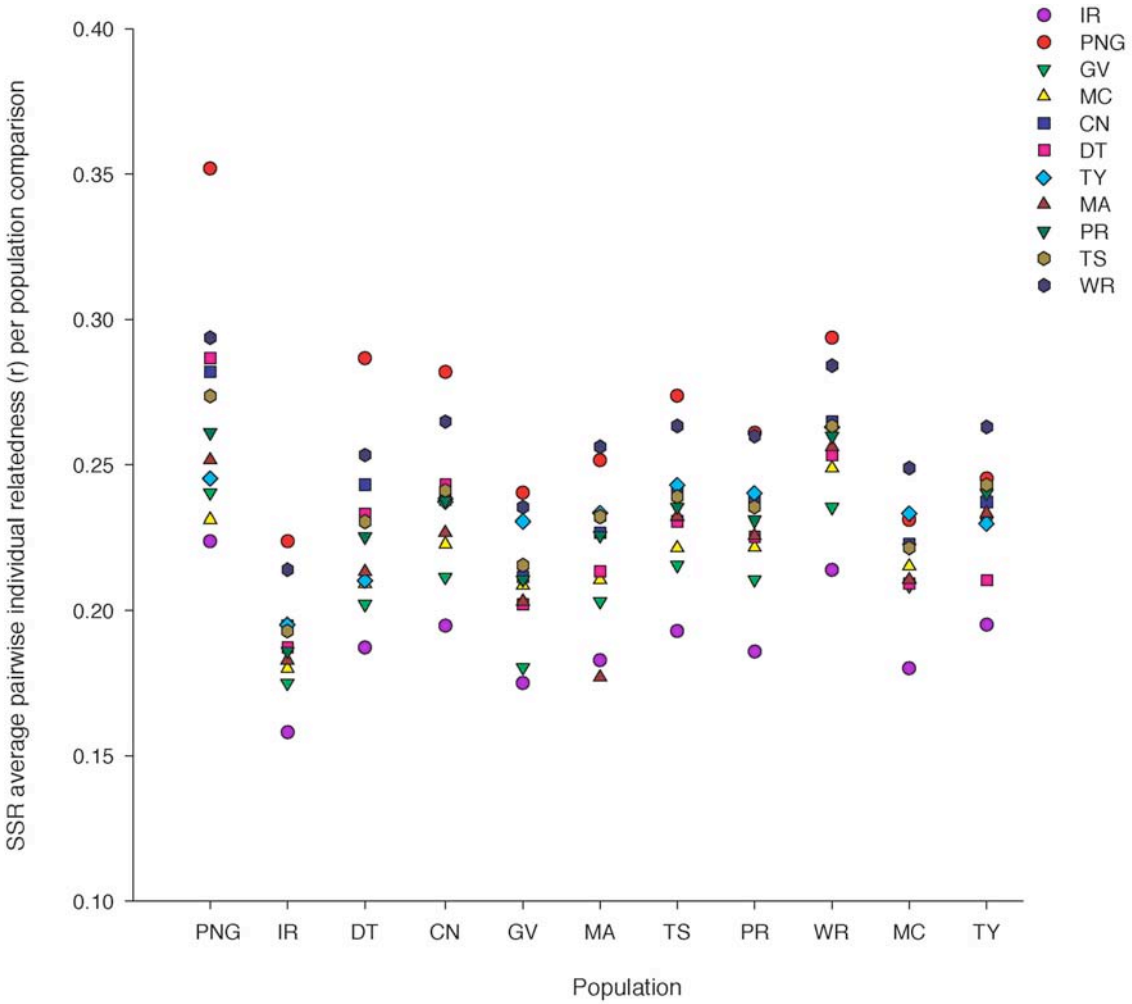

Figure 5.4 Population-by-population average pairwise relatedness for all within- and between-population pairwise individual relatedness comparisons (D2 estimates from MARK – (Ritland and Travis 2004)).

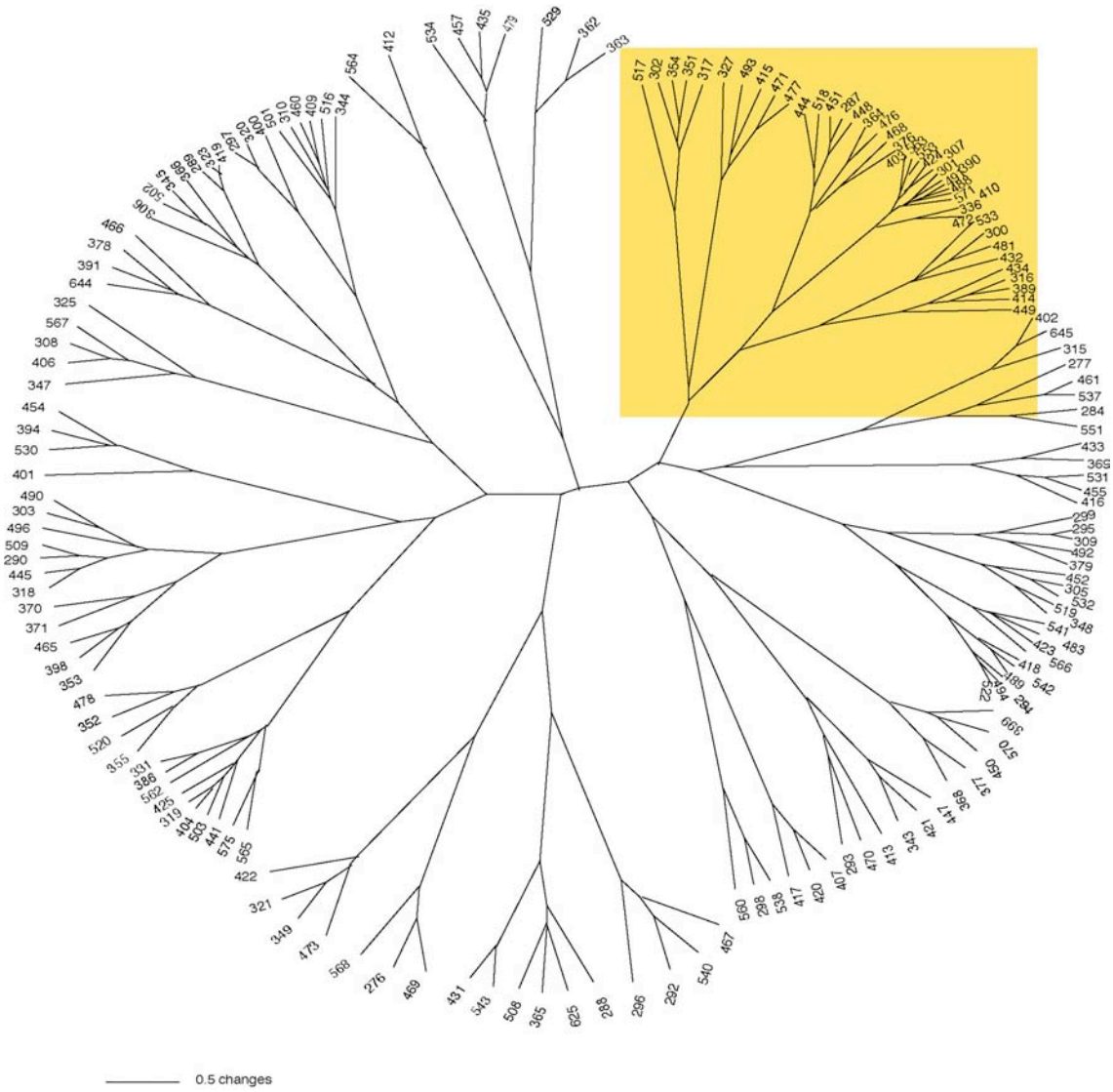

Figure 5.5 UPGMA tree of all individuals (n=172) sampled from PR colony, clustered relative to their pairwise relatedness values. Highlighted box indicates the sub-group investigated in more detail (see Fig. 5.6). Numbers at the end of each branch indicate the unique identifier for each individual.

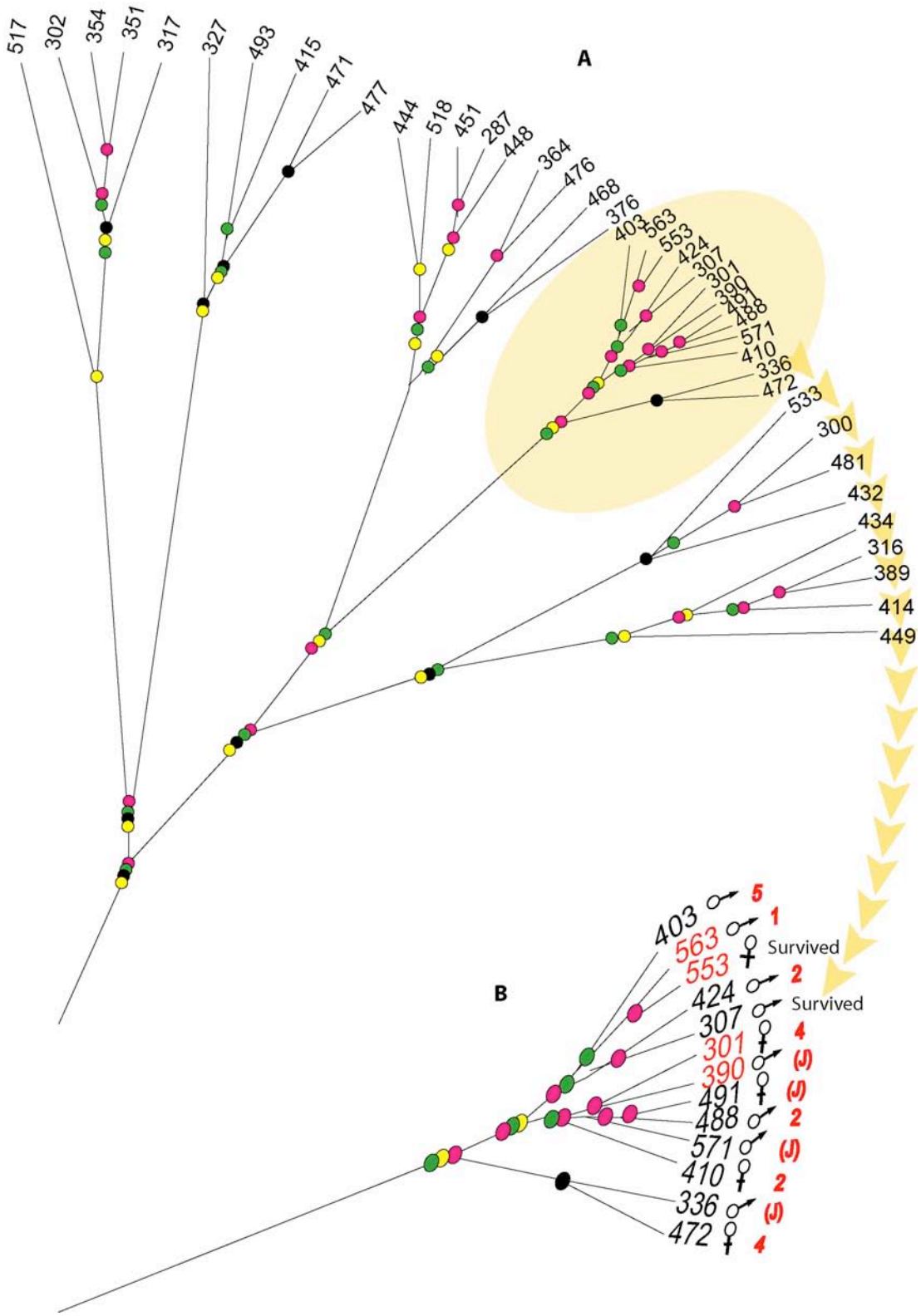

Figure 5.6 Sample sub-group from PR colony showing scale of relatedness between individuals (A), with family group highlighted showing age and sex of family members (B). Scale of pairwise relatedness values ● >= 0.50; ● 0.25-0.5; ● 0-0.25; ● Not related. Red individuals in (B) are mother/offspring pairs determined both by relatedness values and at the time of collection of samples. (J) = juvenile

Kinship results on the PR sub-group (Fig. 5.6) found eight pairs of individuals related by direct maternal or paternal descent at the level of p=0.01 and four pairs of individuals at the p=0.001 level, which equated to four of the five known mother-offspring pairs. Seven pairs of individuals were found to have significant identity by descent for half siblings at the p=0.01 level and 17 pairs of individuals had significant identity by descent for half siblings at the p=0.001 level. Eleven pairs of individuals were related at the full sibling level (p=0.01) while 14 pairs of individuals were related as full siblings at the p=0.001 level. Several pairs of individuals appeared in more than one group.

Discussion

Broad-scale view of relatedness across populations of the spectacled flying fox

Analysis of pairwise relatedness is commonly conducted on groups of individuals (Rossiter *et al.* 2000b; Bentzen *et al.* 2001; Burland *et al.* 2001; Kerth *et al.* 2002b), but it is limited to the extent of comparing relationships between pairs of individuals. The use of frequency plots based on the pairwise relatedness matrices allows additional insight into the results of these individual genotype comparisons. The plot of the frequency distributions of pairwise relatedness values for between-colonies and within-colonies indicated equivalent distribution of relatedness within colonies compared to between colonies. If there were strong patterns of relatedness within colonies, different distributions of pairwise relatedness between these two comparisons would be expected. That there is no relatedness structuring within colonies also suggests that there is random mating between colonies. This is supported by results in previous chapters showing high gene flow among colonies and little genetic differentiation throughout the range of the spectacled flying fox. Plots for within- and between-colony comparisons also showed that the modal value of relatedness (excluding zero and negative values) was similar at approximately 0.3.

The frequency distribution of individual pairwise relatedness values within colonies indicated that the majority of colonies appear to have a comparable

pattern of low within-colony relatedness, although in colonies with small sample size, and therefore erratic relatedness distributions, this can be more difficult to discern. The positive skew in the relatedness distribution in PNG is likely to reflect a greater abundance of common alleles and few rare or less common alleles, creating the illusion of high average relatedness within the PNG dataset. This is reinforced by the population-by-population average pairwise relatedness results, where PNG seems to have the greatest average pairwise relatedness compared to almost every other colony. This is also supported by data from Chapter 3 and 4 that show PNG as having low haplotypic and allelic diversity compared to Australia.

That IR falls at the bottom of the population-by-population average pairwise relatedness graph, signifying the smallest degree of relatedness to other colonies, may be a result of the opposing allele pattern to PNG, in that it has more rare and uncommon alleles, with individuals therefore standing apart from each other as well as from individuals from other colonies. This is suggested in the result of the compilation of less common alleles (<25%) found in the Iron Range population (Chapter 4), where 60% of the alleles found in the Iron Range colony fell into the less common category. Small sample size will also compound this effect by reducing the chance of sampling related individuals.

Low average colony relatedness not indicative of colony social structure

Overall average relatedness values within and between colonies calculated with RELATEDNESS were low. However, the UPGMA tree based on pairwise relatedness values between the 172 individuals sampled from the PR colony, suggested that spectacled flying fox colonies may contain kin groups. This may indicate that overall average relatedness values are not indicative of the actual social structure of a colony. Low average relatedness in colonies of closely related individuals has been found previously in other bat species (Wilkinson 1985; Burland *et al.* 2001; Storz *et al.* 2001a; Kerth *et al.* 2002b; Rossiter *et al.* 2002). Wilkinson (1985) attributed low average relatedness values in *Desmodus rotundus* to immigration into the colony 'diluting' overall relatedness. However, in colonies of *Myotis bechsteinii*, where colonies of females were considered

closed units with a high degree of relatedness between individuals, a low average overall colony relatedness was still found (Kerth *et al.* 2002b). Kerth *et al.* (2002b) suggested that this could be due to the majority of colony members not being paternally related. It is possible that both of these factors influence the overall average colony relatedness values in spectacled flying foxes. Movement into and out of colonies is likely to be continual. This would be consistent with the finding that a number of individuals within the PR colony are not related to the main kin clusters. Likewise, with some form of polygyny likely to be the dominant mating system, it is unlikely that females would mate with the same male each year and, therefore, the likelihood of paternal relatedness between individuals within each kin group would be low. Lukas *et al*. (2005) in their study of kin groups in chimpanzees, found that high average relatedness was only ever found in very small groups. A polygynous mating system with low paternal relatedness between individuals in kin groups, along with large group size is probably the reason for the low overall average relatedness found in this study. These results highlight the need for fine scale analysis of relatedness to understand the true level of kinship within a colony.

Pairwise relatedness indicates possible kin structuring in the Powley Rd colony

Results from both RELATEDNESS and KINSHIP show clusters of kin-level relatedness among individuals within the PR colony. These clusters of closely related individuals that appear together on the UPGMA tree, suggest that this colony includes at least several kin groups with varying degrees of relatedness and different numbers of individuals. The fact that the other two Atherton Tablelands colonies showed the same overall topology of the UPGMA tree suggests that this pattern may not be unique to the PR colony. The high levels of relatedness found within the sub-group using KINSHIP indicate the presence of large numbers of half and full siblings. KINSHIP also identified four of five known mother-offspring relationships. The fifth mother-offspring pair was identified as a half-sibling relationship. Goodnight and Queller (1999) note that KINSHIP cannot distinguish between types of relationships that have the same *R*-values, such as parent-offspring and half siblings. This would explain why many pairs of individuals appeared in more than one relationship category (i.e.

some pairs of individuals were found to be full siblings as well as half siblings) and that the results were the same for mother-offspring tests as for father-offspring tests. In fact, one father-offspring relationship was discovered. For the purposes of this study, it is only important that a pair be shown to be closely related. As five mother-offspring pairs were already identified at the time of sample collection, this was a useful test to show that KINSHIP can identify this level of relatedness over others. However, it was noted that several apparently close relationships identified with RELATEDNESS were not found with KINSHIP. As the pairwise relatedness values are relative and comprise a scale of values, it is often difficult to discern exactly where the boundaries between 'close relative' and 'more distant relative' lie. This suggests that pairwise relatedness values calculated with RELATEDNESS should be interpreted with caution. These results are useful for determining whether there are closely related individuals within a group, but the relationship of individuals cannot be accurately assigned using this method. KINSHIP was designed for this purpose.

Given the sampling regime for the collection of this data (mainly from animals that had fallen with tick paralysis), the fact that kin groups have been uncovered by the data may suggest that kin groups travel and forage together, and are, as a result, exposed to paralysis ticks together as well. It should be noted that the introduced *Solanum mauritianum* (wild tobacco), from which it is believed that the flying foxes pick up the majority of the paralysis ticks (Eggert 1994), is a widespread food resource to flying foxes on the Atherton Tablelands and appears to be a major component in spectacled flying fox diets (Parsons 2005). Thus, it is also possible that kin may be foraging apart while still feeding on wild tobacco and, therefore, pick up the paralysis tick in different places, while not necessarily travelling together . There is also the likelihood that not all members of the kin groups were sampled, and therefore that some had not picked up ticks, suggesting that the kin group may not forage together; or that even when foraging together, not every bat picks up a tick in the same area. Despite these caveats, co-operative foraging remains a possibility.

Co-operative foraging has been reported in many animal groups (Heinsohn and Packer 1995; Courchamp *et al.* 2002; Blundell *et al.* 2004) although the individuals are not always related. While co-operative foraging as exhibited by

other animal groups may not occur in flying foxes, overlapping foraging grounds may exist where young learn the feeding range of their mother and incorporate this feeding range into their own range as independent adults. This information transfer, where individuals share knowledge about location and quality of food patches, has been shown in other bat species (Wilkinson 1992b; Brooke 1997) and is exhibited by one individual following the activities of another individual (Wilkinson 1995). Although this has only so far been shown to occur in microchiropteran species, there is anecdotal evidence that flying foxes also 'share' foraging information (Ratcliffe 1932; Eby *et al.* 1999).

Detailed description of the smaller family group showing sex and age of kin group members, suggests that offspring from several cohorts may live with their mother until the offspring achieve sexual maturity. In the kin cluster analysed, there were two sexually mature females with offspring from that year and also a sexually mature female who was lactating but had lost her baby. The majority of remaining members of the kin group were sub-adult (sexually immature) individuals who could be offspring from previous years' cohorts. Unfortunately not all the members of the family appear to be present in the sample and so it is difficult to work backwards through the genealogy to determine which individual is related to which to obtain a complete picture. An example of how this could be done is shown with female #553 who survived tick paralysis (and hence there are no age data for her) and was shown to have a mother-offspring relationship with male #563 who was one year old. This was shown both in the pairwise relatedness value clustering on the UPGMA tree and in the results from KINSHIP. Female #553 was not lactating in 2002 when her sample was collected, hence there is no offspring from the current cohort. KINSHIP also showed that female #553 had a mother/offspring relationship with female #468, a six year old female located on the neighbouring cluster of the same extended family. Female #468 was also not lactating that year and therefore did not have young in the 2002 cohort. If female #468 was mother of female #553, this would indicate that three generations of the same family were sampled.

No evidence of inbreeding in the spectacled flying fox

The low F_{IS} value implies that inbreeding does not occur in the PR colony, suggesting that inter-kin group mating occurs, whether within a colony or between-colonies. This was supported not only by the broad-scale analyses already discussed but also by the observation that individuals from one kin cluster can have a high pairwise relatedness value with individuals from other kin clusters. Along with the high degree of movement and gene flow between colonies and the likelihood of a polygynous mating system, random mating between kin groups would create an effective inbreeding avoidance strategy. Inbreeding is often prevented by one sex dispersing upon reaching sexual maturity (Pusey 1987; Lyrholm *et al.* 1999; Petit and Mayer 1999; Kerth *et al.* 2002a; Radespiel *et al.* 2003; Stow and Sunnucks 2004) while the other remains philopatric. Most mammals exhibit male-biased dispersal, especially those with a polygynous mating system (Prugnolle and de Meeus 2002). There is no evidence to suggest philopatry in female spectacled flying foxes and the combined results outlined in chapters 3 and 4 show this not to be the case. Genetic differentiation between colonies measured using AMOVA with both microsatellite data (Chapter 4) and mtDNA (Chapter 3) showed there to be no colony differentiation with either molecular marker. It is common to compare the genetic differentiation between populations using a bi-parentally inherited marker (microsatellites) with results from a uni-parentally inherited marker (mtDNA), to determine whether females are philopatric and males disperse (Prugnolle and de Meeus 2002). If this were the case, there would be little differentiation between colonies according to the microsatellite data but strong differentiation indicated by the mtDNA data, since mtDNA is maternally inherited. This was not found in the spectacled flying fox.

Conclusion

This study is the first to examine genetic relatedness of individuals within and between colonies in a *Pteropus* species. Results have highlighted the value of incorporating several types of analysis at several spatial scales to accurately understand relatedness patterns and processes within a species. Broad-scale analysis has reinforced results from previous chapters regarding colony

structure, gene flow, movement and mating structure, while fine-scale genetic analysis gave the most comprehensive understanding of relationships within a single flying fox colony. Spectacled flying fox colonies appear to contain sub-structuring in the form of kin-groups that comprise females and several cohorts of immature sub-adult offspring. As there is no evidence of female philopatry, it seems likely that close kin groups may travel together between colonies. This period of time where sub-adults remain with their mother may constitute a period of information transfer and learning in offspring, to find food resources at different times of the year by following their mother. Results reported in this chapter give greater insight into the dynamics of a colony and also help managers to understand why mass movements of colonies occur.

Chapter Six – Life history data reveal negative growth and short life-span in the spectacled flying fox

Introduction

The life history of a species includes parameters such as fecundity, mortality rate and the age structure of a population. A species' life history can have an enormous influence on population resilience. Some species are able to quickly return to previous numbers after suffering a decline, but often the species that are most affected and take longer to recover, are those that have a delayed sexual maturity, are large bodied, long lived and have a reduced capacity for increase due to a low reproductive potential (Purvis *et al.* 2000a; Jones *et al.* 2003). The potential reproductive capacity of individuals is intrinsically linked to the potential rate of increase for that population (Krebs 2001), and is therefore critically important in determining recovery time. For example, comparing the life history traits of an elephant (mean age at birth of first offspring – 14 yrs; mean birth interval - 4.5 yrs; 1 young at a time) (Moss 2001), with that of a rabbit (mean age at birth of first offspring - 16 weeks; mean birth interval - 30 days; average litter size - 5) (von Holst *et al.* 2002) makes it clear that rabbits would recover from a decline quickly, whereas elephants would take decades.

Despite their small size, species of the order Chiroptera exhibit characteristics that place them at the 'slow' end of the life history continuum. They are believed to be long-lived, living up to three times longer than non-flying eutherian mammals of comparable size (Austad and Fischer 1991; Wilkinson and South 2002; Brunet-Rossinni and Austad 2004), with late sexual maturation (Pierson and Rainey 1992), low reproductive output (Barclay and Harder 2003; Barclay *et al.* 2004) and long periods of maternal care (Kurta and Kunz 1987).

Oli (2004) found that the projected population growth in 'slow' species, such as bats, was most sensitive to changes in juvenile and adult survival rates, while Jones *et al*. (2003) found that the low reproductive rate in flying foxes was one of two main predictors of extinction risk in megachiropterans.

Although bats are the second most speciose order of mammals (Koopman 1993), over 25% of modern species are classified as extinct or threatened (Hilton-Taylor 2000). Demographic data are important for understanding life-history processes, are often the basis of management and conservation planning for a species, and can be used to explore wider patterns of population structure and intraspecific relationships. With their high vagility, their nocturnal habits and their tendency to roost in inaccessible places, bats remain a difficult group for demographic study.

Demographic data, in the form of age structure, are often analysed using life tables (Cailliet 1992; Schwartz *et al.* 1998; Moss 2001; Richard *et al.* 2002; Belcher 2003; Banks *et al.* 2005). Obtaining the initial dataset of aged individuals to construct a life table requires either many years of observation (Schwartz *et al.* 1998; Moss 2001; Richard *et al.* 2002), mark-recapture data (Belcher 2003; Banks *et al.* 2005) or the ability to remove a sample of a population randomly (Cailliet 1992). Because of the logistical difficulties in obtaining demographic data for flying foxes, extensive age-structure data do not exist and therefore life tables have not been constructed for this group. In one recent attempt to circumvent this lack of field data, McIlwee & Martin (2002) estimated the intrinsic capacity of increase and modelled population growth of Australia's flying foxes using estimated demographic parameters. They suggested that mortality rates of flying foxes being culled in fruit orchards were unsustainable for a species with such a low reproductive rate. Given the import of this conclusion and the necessary reliance on estimated demographic parameters, there is an obvious need to produce data from wild populations to calculate basic demographic parameters.

Although reproductive output in flying foxes is believed to be low, maternal investment in each young produced is high. Like other species of *Pteropus*, spectacled flying foxes give birth to a single pup once a year (Hall and Richards

2000; Barclay and Harder 2003). Spectacled flying foxes are physiologically capable of giving birth at two years of age, although it is believed that they generally do not reproduce successfully until three years of age (Maclean, pers. com.). The extent of reproduction occurring in two-year-olds of this species has not been studied. Maternal investment in young is extensive, with six months gestation followed by three months lactation (Hall and Richards 2000). Young are still dependent on their mothers for some months post-parturition, so most of the year is taken up in some stage of reproduction for females.

Determining a species' longevity is important to understanding their life-time reproductive potential. Flying foxes have been known to live as long as 30 years in captivity but there is no accurate record of how long they live in the wild (Hall and Richards 2000).

In this study, estimates of basic demographic parameters gained from field-based data are provided, and the age structure of a wild flying fox population is described. Annual increments in cementum layers in the teeth of flying foxes collected dead from a colony as a result of tick paralysis, were used to construct an age distribution of the sample population. Dental and cementum annuli around the root of a tooth give a reliable indicator of age provided there are known-aged individuals to validate the periodic deposition of layers of cementum or dentine (Cool *et al.* 1994). The formation of appositional growth layers in hard tissues such as teeth has been attributed to periodic or seasonal changes in nutrition, hormonal activity, growth rates and mechanical forces (Klevezal and Kleinenberg 1969; Lieberman 1993). Because accurate age estimation is based on the relationship between chronological and physiological age, the regularity of the cementum increments will potentially differ from one species to the next. Cool *et al*. (1994) were the first to investigate the use of cementum layers to age captive-bred Australian flying foxes, while also looking at the possible use of dentine and bones for reliably aging individuals. Cementum gave the most consistently reliable results. This study on spectacled flying foxes and Divlijan *et al.* (In press) are the first to apply this technique to aging wild bats, although the focus in Divlijan *et al.* (In press) is investigating the technique itself rather than its application. The study that this chapter

describes is the first to develop life tables from field-based data for a flying fox species and to estimate the intrinsic capacity for increase for that species.

Methods

Data collection

Animals were aged using cementum layers around the root of the canine tooth, based on the methods of Cool *et al.* (1994). Some modifications were implemented to accommodate the available equipment and to improve the technique as processing of teeth progressed.

Teeth were taken from 361 spectacled flying foxes (Female=196, Male=165) that had died from tick paralysis at the Tolga scrub colony on the Atherton Tablelands in north Queensland in 2001 and 2002. Tick paralysis season corresponds with the time of year when females give birth, which is October-December. Spectacled flying foxes are seasonal breeders, therefore, as teeth were collected at the same time as parturition, all animals that were used in this study had just had another birthday—an important consideration when aging an animal using an annually incremental feature. Each individual flying fox was given a numerical identifier, and gender, reproductive status and morphological measurements were recorded. Female reproductive status was recorded in three categories: i) lactating at the time, meaning she had given birth that season, ii) had given birth in previous years but was not lactating at time of death, and, iii) had never suckled young. The difference between a female that had lactated previously and one that had never lactated was obvious by the elongation of the nipples caused by offspring remaining attached to them for several months.

Teeth

To make tooth removal easier, carcasses were frozen for 24 hours. The two upper canine teeth, and their roots, were then removed intact from the jaw with a sharp knife and a pair of side cutters. Teeth were stored in Dimethylsulphoxide (DMSO) but were washed and placed in 10% formaldehyde before processing began.

Tooth preparation and sectioning

Before processing, the entire exposed tooth and lower apical section of the root were cut off, leaving only the upper portion of the root (Fig. 6.1). This served a number of purposes: it made decalcification quicker, it made embedding of the tooth in wax easier and it ensured that all cross-sections were taken from the same point of the root—the midpoint. The midpoint of the root was targeted, as it is this area that is thought to provide the best approximation of known age (Cool *et al.* 1994).

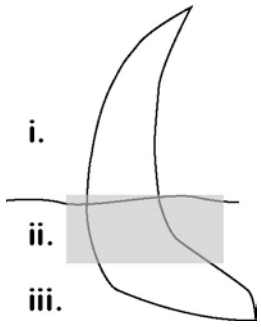

Figure 6.1 Diagram of tooth sectioning. i. Above gum, exposed tooth, removed before processing. ii. Mid-section of root below gum-line, removed to be embedded in wax. iii. Lower apical section of root removed before processing.

The remaining part of the tooth root was decalcified in 10% formic acid for 48 hours, after which the acid was changed and the tooth left in the new acid for another hour. An Arnim's test (Arnim 1935) then determined whether the end-point of decalcification had been reached; if not, the tooth was returned to the acid bath. Once this point was reached, teeth were washed in water for three hours before being placed in a Shandon Hypercentre overnight to be dehydrated through a series of alcohols and xylene washes before finally being coated in wax. Teeth were then manually embedded in a wax block, for sectioning.

Sectioning was carried out using a Leitz manual rotary microtome. Initially, sections were cut at room temperature but quality sections were difficult to obtain. Thereafter, the exposed face of the tooth root was placed on tissue paper soaked in 10% ammonium hydroxide (on ice), for softening prior to sectioning. Sections were cut 8-10µm thick and placed on microscope slides. Slides were then placed in an oven at 37°C for approximately 72 hours to allow sections to

adhere to the slides and the wax to melt away. Tooth sections were then stained following the Mayer's Haematoxylin and Young's Eosin–Erythrosin staining procedures (Woods 1994). Samples were again oven-dried before being fixed onto the slide using a cover slip and DPX (dibutyl phthalate with xylene) and placed in a 37°C oven for approximately 72 hours. Many sections lifted from slides during the staining process. Using poly-1-lysine slides with an adhesive, to maintain permanent contact between the section and the slide, rectified this problem. This markedly improved the number of sections retained on slides during staining. This was improved further by putting the slides in a 37°C oven for three days rather than following the standard 60°C for 24 hours. The slower but extended drying time allowed the sections to adhere to the slides properly before going through the staining process.

Counting cementum layers

Cementum layers were viewed and counted on a double-headed Olympus light microscope (Fig. 6.2). Several sections of each tooth were taken and each section was viewed to check for consistency in the number of cementum layers exhibited. Counts were made in areas that exhibited clear layering throughout the entire width of the section. Any sample for which it was difficult to assign a number of layers, was not used in the analysis. Sections were viewed and layers counted, at 40x magnification. In some species, such as humans, the permanent dentition does not erupt until several years have passed, requiring cementum layer counts (years) to be adjusted for the number of years prior to eruption. This is unnecessary in flying foxes as the permanent dentition of flying foxes erupts soon after birth (Hall and Richards 2000).

Analysis

The frequency of individuals in each age class was plotted to determine the shape of the age distribution for the overall population, the shape for males and females separately, and a comparison between years. Lactation data in females were graphed against age data to determine at what age females first produced young, and the proportion of females breeding each year.

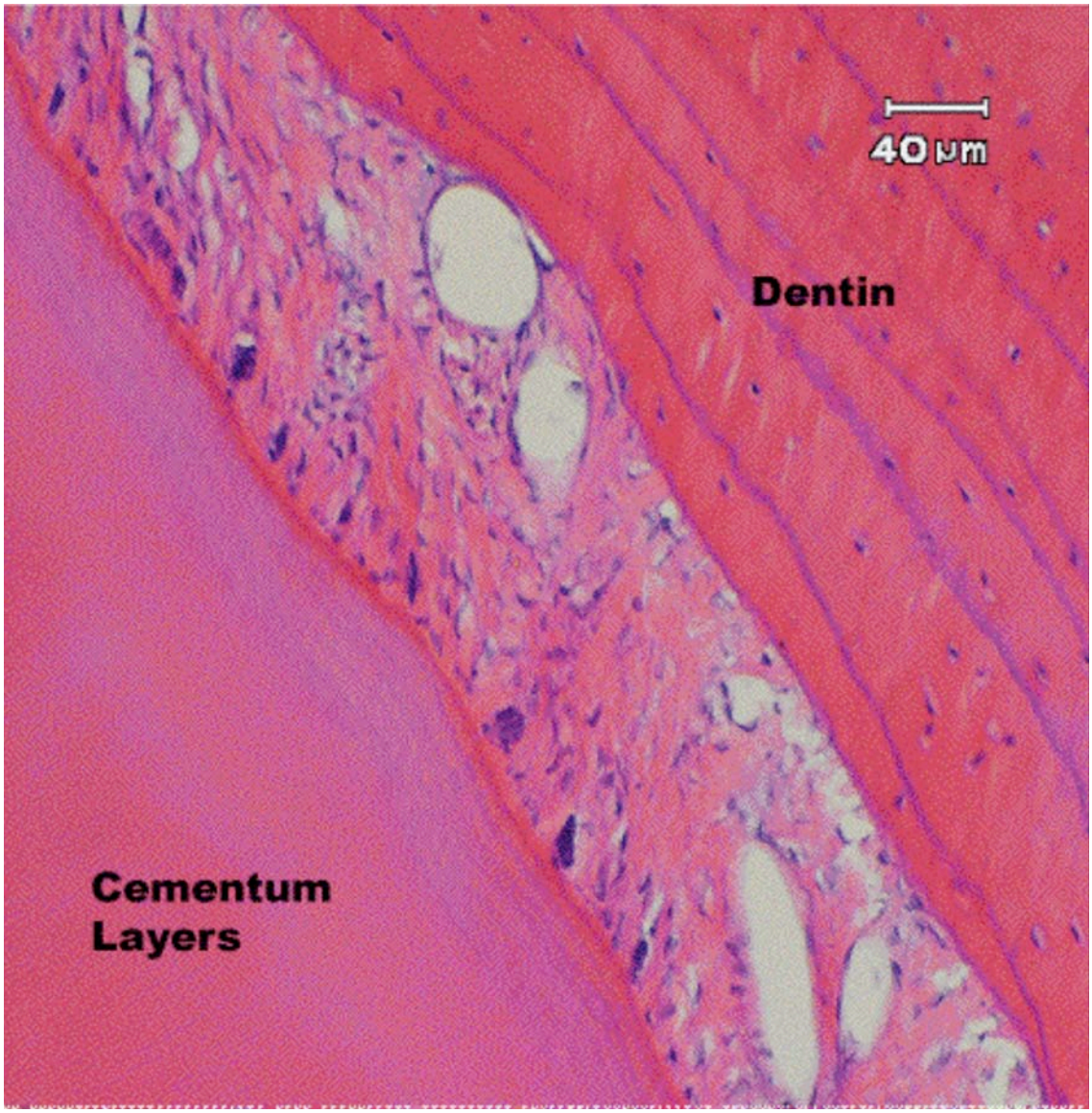

Figure 6.2 Photo of cementum layers surrounding root of tooth taken from a spectacled flying fox. There are 6 rings in this photo indicating an individual of 6 years.

Life tables

A life table was constructed for the Tolga Scrub colony of spectacled flying foxes on the Atherton Tablelands following Krebs (2001) and Caughley (1977). As the fecundity of females limits population growth, life tables calculating an intrinsic capacity for increase are completed using data on females only (Caughley 1977; Stearns 1992; Caughley and Gunn 1996; Krebs 2001). The maximum age (longevity) used was that of the oldest individual aged in this study (13 years). Instantaneous mortality estimates were gained by plotting age in years against the natural log of the frequency of individuals in each age class (see Ricker 1975). This method was originally used to calculate mortality rates

of fish populations caused by fishing, and is called a 'catch curve'. The slope of the regression was taken as the instantaneous mortality rate for all animals aged two years and above (40%). Juveniles in the first year age category were assigned a mortality rate of 50% due to the inability to estimate a mortality rate for this age class from the data. Typically, juvenile estimates of mortality are taken as double the adult rate (Cailliet 1992), which in this case would be 80%. Although possibly realistic, a conservative estimate of 50% was chosen instead. In comparison, Vardon & Tidemann (2000) estimated that juvenile black flying foxes (*P. alecto*) had a mean mortality rate of 68%, and Sibley *et al*. (1997) found juvenile mammals had a mean mortality rate of 56%. Tuttle & Stevenson (1982) also found that mortality rates for bats were highest in juveniles. These rates were used to estimate the natural survivorship function ($\mathbf{l_x}$)

$$\mathbf{l_x = N_0(e^{-Mx})}$$

where $\mathbf{M}x$ is the instantaneous mortality rate at time x and $\mathbf{N_0}$ is the number of animals at time 0. Annual rate of mortality ($\mathbf{d_x}$) was estimated as

$$\mathbf{d_x = 1\text{-}e^{-Mx}}$$

Age at maturity, and fecundity were calculated from the dataset in this study. Comparison of reproductive status data with age gave the proportion of females reproducing each year in this sample (fecundity), and the age that females commenced producing young. Fecundity $\mathbf{(b_x)}$ is the number of female offspring born per female per year and is calculated as

b_x = # of young born per season x age-specific natality x ratio of female to male offspring

The product of the survivorship function $\mathbf{(}l_x\mathbf{)}$ and fecundity $\mathbf{(}b_x\mathbf{)}$ gives average lifetime reproductive success $\mathbf{(}l_x b_x\mathbf{)}$. The nett reproductive rate $\mathbf{R_0}$ is calculated as

$$\mathbf{R_0 = (\Sigma}\ l_x b_x\mathbf{)}$$

and is the number of viable female offspring produced by a single female in a lifetime. Mean generation time (G) is the average age of mothers of new-born offspring and is calculated as

$$\mathbf{G = (\Sigma\, } l_x b_x \mathbf{)}\, x \,/\, \mathbf{R_0}$$

where x = the age of individuals. The intrinsic capacity for increase (r_m) is an instantaneous rate and is derived from the generation time (G), which is only an estimate (Krebs 2001), making r_m also only an approximation when generations overlap. A more accurate result is given with the finite rate of increase (λ), following an iterative calculation using the Euler-Lotka equation. The finite rate of increase (λ) was calculated as

$$\lambda = e^r$$

which is the exponential of r (or r_m) (Krebs 2001). The age distribution for 2001 was compared with that of 2002 using a Kolmagorov-Smirnov two-sample test (Zar 1998) to determine whether there was a significant difference between the years. Life tables were completed for all females pooled across both years. Data for 2001 and 2002 were also separated and calculated independently. Additional life tables were completed with amended mortality and natality rates to estimate levels needed to obtain a stable population.

Results

Validation of aging technique

Three hundred and sixty one (361) animals were aged using cementum layers, with several factors allowing a 1:1 ratio of cementum layers to age in years to be attributed. Three known-aged animals demonstrated an exact match between age and the number of cementum layers. The known-aged animals were two, five and fifteen years old, the last indicating that even older animals, which are more difficult to age due to the cementum layers being closer together, could be aged accurately using this method. One-year old animals could be identified by their smaller forearm measurements [*Pteropus conspicillatus* does not attain full adult size until approximately 18 months of age (Hall and Richards 2000)], and

their first-year status was also corroborated by aging using cementum layers. Female lactation data were also consistent with the aging method being accurate, with a low proportion of two year olds, based on cementum layers, found to be reproductively active. Divljan *et al.* (In press) also found a 1:1 relationship between cementum layers and age in years for grey-headed flying foxes (*Pteropus poliocephalus*).

Age structure and demographic parameters

The age distribution of this sample of *P. conspicillatus* showed that 93% of the population was six years old and under (Fig. 6.3). The maximum age of an individual from this sample was 13 years. The data for 2001 and 2002 were pooled once it was determined that the age distributions for females for these two years were not significantly different (Kolmagorov-Smirnov two-sample test, D=0.2308, p=0.8793).

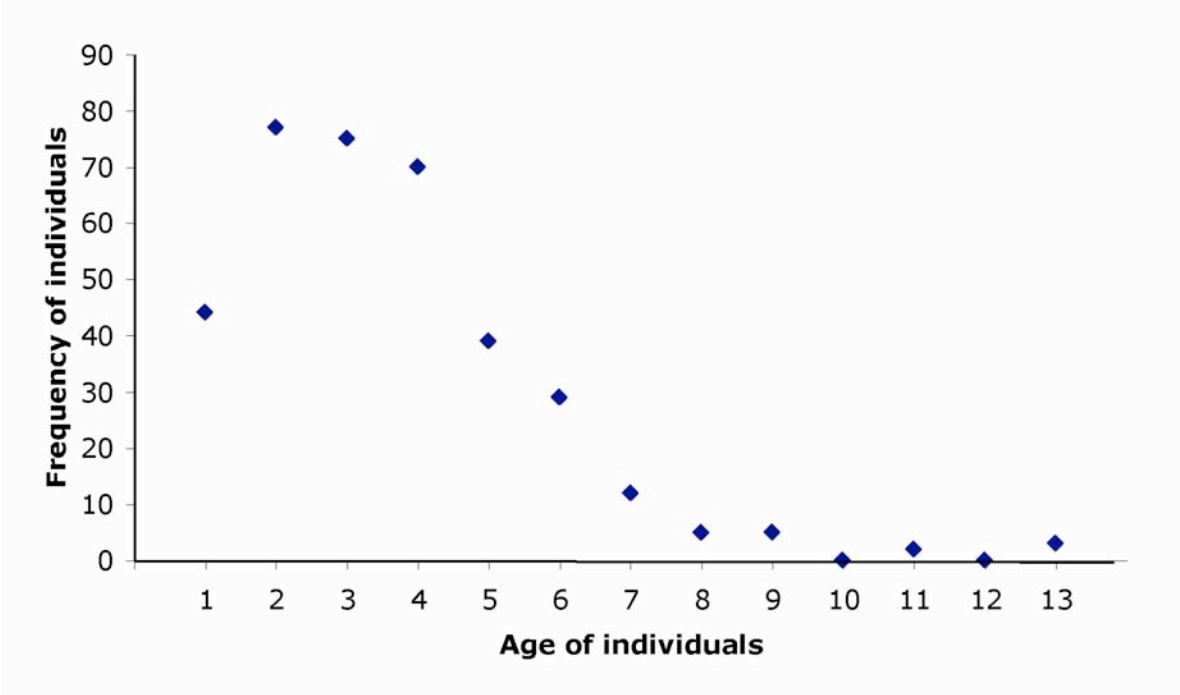

Figure 6.3 Age distribution for sample population from Tolga Scrub colony on the Atherton Tablelands for 2001-2002 (males and females combined). Young-of-year of collection not included.

Thirty nine percent (39%) of two year old females had given birth but most females did not reproduce for the first time until three or four years (Fig. 6.4). Not every female reproduced each year, with an average reproductive rate of

0.89 (89% of females producing a pup each year) for females up to seven years. Reproductive rates for females above seven years were not calculated as sample sizes were low.

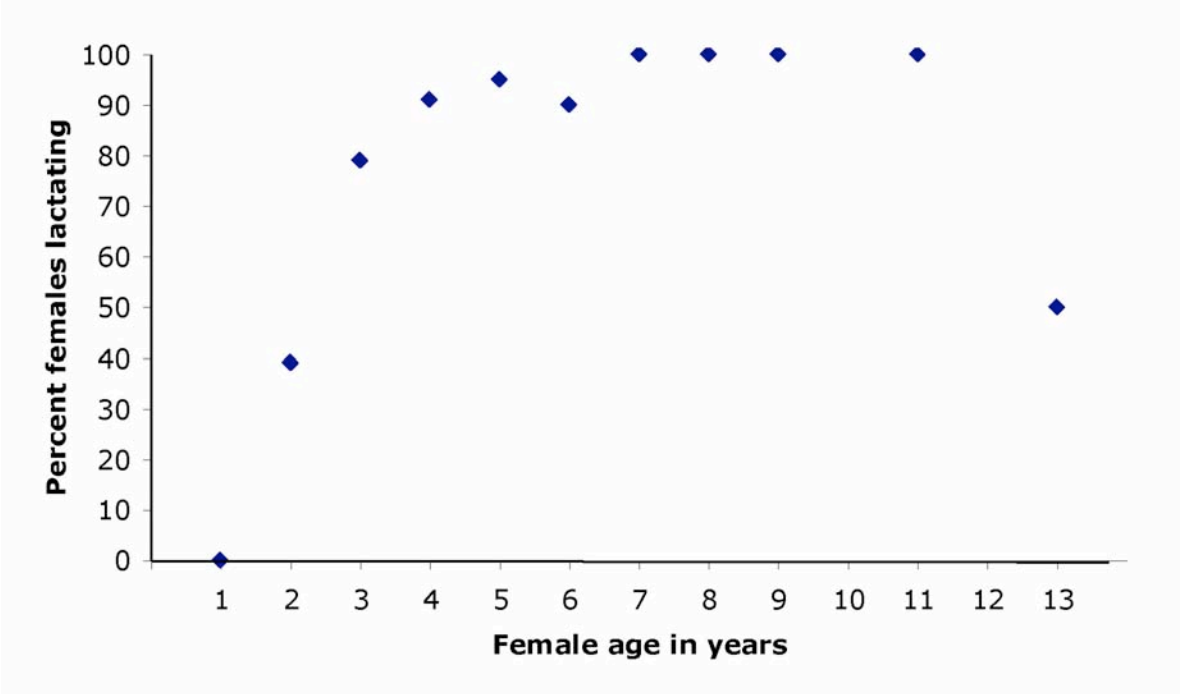

Figure 6.4 Proportion of females lactating in each age class. Sample sizes for age classes 8 years onward are low (8, n=2; 9, n=4; 11, n=2; 13, n=2).

The instantaneous mortality rate was 43% for the population overall and 40% for females alone. When this was converted to an annual mortality rate, the overall rate was 35% and 33% for females only. When survival was compared between males and females with a log rank test, females had a significantly greater survival rate than males ($\chi^2_{358,\,1}$=5.55, p<0.05). The overall sex ratio did not differ from 1:1 ($\chi^2_{358,\,1}$=2.52, p<0.05, Yate's correction applied).

Life history tables

The life table using pooled age data from 2001 and 2002 (Table 6.1) indicates a population growth rate of –16.23% for that period. The net reproductive rate ($\mathbf{R_o}$) was estimated to be 0.364 per generation. The generation time (G) was approximately 5.0 years. The intrinsic (or instantaneous) rate of population increase (r_m) was –18%. For this sample of the population during 2001-2002, the more precise finite rate of increase (λ) was –16.23%. When the two years of data were treated independently, the finite rate of increase (λ) was –15.1% for 2001,

and –12.01% for 2002. Manipulation of the mortality rate and fecundity rate parameters suggested that this sample population would have been stable if the mortality rate was halved to 20%. If the fecundity rate was increased to 100% for all females from the age of three years, in addition to the reduced mortality rate, the sample population would have increased by 1.6% per year.

Table 6.1 Life history table for female *Pteropus conspicillatus.* Symbols for columns: *x*, Age in years; l_x, survivorship (mortality was set at 50% for the first year of life and 40% for the remainder; b_x, natality; $l_x b_x$, age-specific reproductive rate; R_o, net reproductive rate per generation; *G*, mean generation time; *r*, intrinsic rate of increase of population.

x	l_x	b_x	$l_x b_x$	$l_x b_x x$	R_o	G	r
0	1.000	0.000	0.000	0.000	0.364	5.049	-0.1814
1	0.607	0.000	0.000	0.000			
2	0.407	0.000	0.000	0.000			
3	0.273	0.395	0.108	0.323			
4	0.183	0.455	0.083	0.332			
5	0.122	0.475	0.058	0.291			
6	0.082	0.445	0.037	0.219			
7	0.055	0.500	0.028	0.193			
8	0.037	0.500	0.018	0.148			
9	0.025	0.500	0.012	0.111			
10	0.017	0.500	0.008	0.083			
11	0.011	0.500	0.006	0.061			
12	0.007	0.500	0.004	0.045			
13	0.005	0.500	0.002	0.032			

Discussion

The analysis of population dynamics through the use of life history traits and life tables is widely used in planning for the conservation and management of wildlife. For some animal groups such as highly mobile, inaccessible and nocturnal species like flying foxes, acquiring the data needed for these analyses is extremely difficult (Pierson and Rainey 1992; Hall and Richards 2000; Vardon and Tidemann 2000; McIllwee and Martin 2002; Barclay and Harder 2003). One of the principal reasons for this is that standardised, random sampling methods such as mark-recapture are difficult to implement with these animals. Thus, this is the first life table reported for a flying fox species based on actual field data. Although these data are not without their own biases, which are explained below, some important messages can still be drawn from them.

Before considering the conclusions that can be drawn from the data, the following assumptions should be noted. Most importantly, the analysis

assumes that the data collection is unbiased i.e. that individual animals have been sampled randomly. In this case, this assumes that the effect of the paralysis tick on the target species was completely random, with no sex or age bias. Though there was little direct data with which to assess this assumption, examination of the arguments suggest that the assumption holds. Firstly, some believe that young, inexperienced, and older, or sick animals are more likely to feed on *Solanum mauritianum* berries, where they are exposed to ticks, because it is widely available although possibly a poor 'second best' choice to native fruits. If this was the case, the age distribution graph should show a larger number of young and older animals, and a deficit of individuals in the mid-range age groups. This is not what is seen, either in the overall sample distribution graph or the separate male and female age distribution data. Secondly, independent and random exposure to ticks also assumes independent foraging. During fly–outs from flying fox camps, individuals tend to 'peel off' from the main stream, making their way to a foraging destination. Flying foxes generally do not like to share their foraging locations with others. There is also no evidence to suggest that *Solanum mauritianum* attracts more animals than any other food source. A recent study of the use of food resources in spectacled flying foxes found individuals from the Tolga Scrub colony had a higher proportion of *Solanum mauritianum* in their diet compared with other colonies on the Atherton Tablelands and on the lowlands (Parsons 2005). This was suggested to be a direct result of the amount of cleared land and access to wild tobacco at the Tolga Scrub colony, whereas other colonies have greater access to rainforest and sclerophyllous foraging opportunities. However, in Chapter 5 it was suggested that kingroups may be foraging together. Therefore, if there is a bias, it is that family groups have been sampled that have been foraging together in a patch of *Solanum mauritianum*. Thirdly, the sampled sex ratio of males to females was 1:1. The understanding of sex ratios within flying fox camps at different times of the year is limited generally, and is not known for the Tolga Scrub colony at this time of the year during the two years studied. However, Nelson (1965a) found sex ratios in *P. poliocephalus* nearing equality at this time of the year. It is also believed that 100% mortality occurs in untreated animals that are envenomated by a paralysis tick (Maclean, pers. comm.). In the two years that these data were collected, all animals that fell within the colony were collected, so size or age bias in collection is unlikely. It is also noted that

the shape of the age distribution curve is that expected of a mammal species with low reproductive output, high juvenile mortality and constant mortality throughout the age classes. The lower than expected (Promislow and Harvey 1990) number of one year olds is likely to result from the sample being taken from a maternity colony during the pupping season where it is unlikely that most one-year olds would be found. Ratcliffe (1932), Nelson (1965a) and Vardon & Tidemann (1999) all reported colonies of Australian flying foxes containing sub-adult animals only, suggesting that at certain times of the year sub-adults form temporary camps. The decreased number of animals in this age class means a bias away from this group in the age distribution graph. However, as this group is not usually considered in calculations of overall population mortality (because their losses exceed all other groups) and they are not reproductive, the absence of a realistic estimate in this class does not affect the final life table calculations. All of the above arguments make a strong case for the assumption of unbiased data collection being met.

Other assumptions made for this analysis are that there is constant mortality within each age group, that recruitment is constant for all age groups and that there is a stable age distribution. These are standard assumptions made in the use of life tables even though they are believed to be relatively unrealistic in nature (Caughley 1977; Krebs 2001). However, although Stearns (1992) stated that natural populations are rarely in a stable age distribution, he believed that when they are not, they are usually returning to one. Therefore, "as a reference point representing the average situation, the stable age distribution is a good choice" (Stearns 1992).

Age at first reproduction

In the study sample, the age at first reproduction was two years but only a minority of females gave birth when they initially became physically capable of it. In fact, it was not until females reached four years of age that they started to reproduce at a rate approximating the mean reproductive rate (89%) of females for the sample population. Additionally, the generation figure calculated from the life tables indicated that the average age of mothers with new-born offspring was five years, suggesting that this is the peak age of reproductive

output in the colony. To have the same age group as the beginning of the major reproductive phase and also the peak of the reproductive phase does not bode well for overall reproductive output and population increase.

Delaying first breeding may incur an immediate cost but provide benefits in terms of lifetime reproductive output or quality of offspring. For example, most female sifaka (*Propithecus verreauxi verreauxi*), a Madagascan primate, delay reproduction for several years after reaching sexual maturity (Richard *et al.* 2002). Although physically capable of giving birth at three years only a small number did so, with most females delaying reproduction until seven years of age because of a highly variable environment and female 'bet-hedging' by investing in personal growth and maintenance in resource-deficient years. Delayed sexual maturity is usually offset by greater adult survival and the chance to reproduce many times in years to come. Stearns (1992) found this pattern of delayed sexual maturity and variable reproductive rate from year to year, and increased longevity, to be true of 'bet-hedging' species that experienced climatic unpredictability and therefore fluctuating abundance in resources. This strategy results in more offspring being born to bigger, healthier mothers with a greater probability of survival. With bats exhibiting 'slow' end life history traits, it is likely that following this strategy could prove advantageous (Harvey and Zammuto 1985; Harvey *et al.* 1989; Read and Harvey 1989; Promislow and Harvey 1990; Austad and Fischer 1991; Jones and MacLarnon 2001; Barclay and Harder 2003; Barclay *et al.* 2004; Brunet-Rossinni and Austad 2004; Oli 2004). It has also been argued that delayed age at first reproduction in bats may be linked to flight constraints and that time required for adult skeletal development may postpone the age of sexual development (Jones and MacLarnon 2001; Barclay and Harder 2003; Brunet-Rossinni and Austad 2004). This is consistent with the notion that females may postpone their first reproductive event in favour of increased physical development and maintenance. However, the resulting reduction in overall reproductive output from delayed age at first reproduction, and the slow reproductive rate is believed to be a significant predictor of extinction in the Megachiroptera (Jones *et al.* 2003).

Longevity shorter than previously thought

Although there is extensive literature on the long life spans of bat species (Read and Harvey 1989; Austad and Fischer 1991; Pierson and Rainey 1992; Ransome 1995; Hall and Richards 2000; Racey and Entwhistle 2000; Jonsson and Ebenman 2001; McIllwee and Martin 2002; Barclay and Harder 2003; Jones *et al.* 2003; Barclay *et al.* 2004; Brunet-Rossinni and Austad 2004) it may be that few individuals will achieve these ages. Although the oldest spectacled flying foxes sampled were 13 years of age, 93% of animals were six years of age or less. This appears to be a consistent pattern across Pteropids. Divljan *et al*. (In press) aged their oldest grey-headed flying fox at 23 years but found that 85% of their sample of wild grey-headed flying foxes (*P. poliocephalus*) were six years of age or less. Vardon & Tidemann (2000) in their study of black flying foxes (*P. alecto*) in the Northern Territory recorded their oldest known-aged bat at 4.5 years of age but were unable to give average age at death. In a comparative study of early and late breeding greater horseshoe bats (*Rhinolophus ferrumequinum*), Ransome (1995) found that although his late–breeding females could still reproduce at the age of 29 years, their average age at death was 8.1 years. While some animals reach the older age groups most die well before they reach double figures. This will have obvious consequences for demographic models in that actual and expected reproductive output will be quite different. This pattern will be different for animals in captivity, highlighting the fact that wild animals should be used for demographic analysis.

Reproductive rate

The average annual reproductive rate of 89% for female spectacled flying foxes up to seven years of age in this study, indicates most females produce young each year. This figure is consistent with studies on other species of *Pteropus* (Vardon and Tidemann 1998; Barclay *et al.* 2004). *Pteropus tonganus*, a species believed to be most closely related to *P. conspicillatus* (Flannery 1995a), was shown to have a reproductive rate of 0.87 (Barclay *et al.* 2004) and *Pteropus rodricensis* was found to breed every alternate birth cycle (West 1986). Vardon and Tidemann (1998) found that only 70% of black flying foxes (*Pteropus alecto*) in the Northern Territory were breeding, although one of the years studied was

drought-affected and a lack of resources may have prevented many females from breeding that year.

While a reproductive rate of 89% may be a normal annual average due to miscarriages, unsuccessful matings or females not mating that year, resource limitation may also reduce reproductive output. This has been shown in grey-headed flying foxes (Eby 1999), and other mammal species (Schwartz *et al.* 1998; Richard *et al.* 2002; Bowen *et al.* 2003), although in some cases it is difficult to distinguish between females not breeding that year and females that have lost young as a result of inadequate nutrition or maternal inexperience. Although the reproductive rate for the period of this study was 89% it is likely that the reproductive output varies widely from year to year depending on several intrinsic and extrinsic factors.

High mortality rate in the year studied

The annual mortality rate of 35% for the sample population is consistent with mortality data for mammals in general (Sibly *et al.* 1997). However, the combination of low fecundity and late age at first reproduction means that a stable population can only be maintained if the majority of animals (especially females) have considerable longevity and low year-to-year mortality.

Historically, when the life history traits of this species were evolving, flying foxes, with the advantage of flight, would be expected to have had a low natural rate of extrinsic adult mortality, principally suffering from factors such as illness, disease, predation and severe weather anomalies. Modern flying foxes contend with a whole new suite of threats. There is no knowledge of threats faced by flying foxes in Australia before Europeans arrived. In the 1900s, when unprotected, spectacled flying foxes were shot for entertainment (Garnett *et al.* 1999). Although illegal shooting probably still occurs, it is likely to be of a lesser extent since this species has been listed as vulnerable. Habitat loss throughout the 1900s has reduced the carrying capacity in the Wet Tropics region for this species (Garnett *et al.* 1999) although the rate of recent habitat loss has eased. However, electric powerlines, barbed-wire fences, paralysis ticks and culling in orchards all increase their mortality rate. Contemporary extrinsic factors such as climate change have an unknown effect on flying foxes and their

food sources, although heat stress events cause mass mortality in flying fox colonies (Welbergen 2004). However, the difference between the annual mortality rate and the instantaneous mortality rate is likely to reflect the effect of tick paralysis on the colony at the time. Both 2001 and 2002 were considered 'bad' tick paralysis seasons (Maclean, pers. comm.). Considering the number of different threats that this species faces, and the fact that there is no knowledge of mortality rates as a result of each factor, it is impossible to say which aspect has the most effect on spectacled flying fox numbers. In the two years that the Tolga Scrub colony was studied however, it appears that the late age of first reproduction, low reproductive output, and high maternal investment in young, are no longer being compensated for by long life spans and low adult mortality.

Iteroparous organisms (those that reproduce several times during a lifetime) have been found to be more prone to extinction when adult survival rate varies (Jonsson and Ebenman 2001), and population growth rate in species at the 'slow' end of the continuum has been shown to be sensitive to changes in juvenile and adult survival rates (Oli 2004). McIlwee & Martin (2002) suggested that this species could not sustain a mortality rate any higher than 20%, irrespective of the fecundity rate. The mortality rate for the two years studied exceeded this figure. It may be hypothesised that these two years were anomalous and that the year-to-year mortality rate is usually more sustainable. To understand the extent of variability in the year-to-year mortality rate and how it affects the species as a whole, mortality rates require further comparative investigation between colonies and between years.

Negative population growth rate

The life history table analysis presented here suggests that, in the absence of immigration, the spectacled flying fox colony at Tolga Scrub suffered a negative growth rate as a result of high mortality rates during 2001 and 2002. Because sampling was restricted to a single colony and the analysis was conducted in the absence of any broader population data, it is impossible to tell whether this equates to a decline in general across the species. Similarly, without data over a longer time frame or on immigration to the colony, it is difficult to generalise

the consequences for the colony itself. This analysis gives us a 'snapshot' view of what happened at this colony during the breeding seasons of 2001 and 2002. What is observed in nature is an actual rate of population change, which is constantly varying between positive and negative in response to changes in population age and social structure, as well as genetic composition and changes in environmental factors (Krebs 2001). In a 32-year study of yellow-bellied marmots (*Marmota flaviventris*), Schwartz *et al*. (1998) found that although the life-table statistics returned a negative growth value, the population fluctuated around an average but remained relatively stable. What this result highlights is the fact that a negative growth value does not automatically mean a declining species, and that it is important to incorporate different methods into a demographic study to obtain an accurate and comprehensive understanding of the species.

If the annual census figures (Fig. 1.4) are an accurate approximation of population size throughout the Wet Tropics, the negative growth rate of the Tolga Scrub colony is not reflected by the rest of the colonies. The census undertaken in 2001 recorded the largest number of spectacled flying foxes in the Wet Tropics since the census began. A more than doubling in population size in a year is not possible in this species through recruitment alone, so this result will be due to flying foxes moving from unrecorded colonies to known colonies. This highlights the principal disadvantage of the census, in that it can only estimate numbers of flying foxes based on previously known colonies. There are abundant prospects for unrecorded colony sites in the Wet Tropics region, especially in the forested region that runs along the Great Dividing range from Cardwell to Cooktown. This influx of animals from unknown colonies has concealed any indication of a population reduction through high mortality.

Conclusion

Spectacled flying foxes exhibit life history traits expected of a species positioned at the 'slow' end of the life history traits continuum. However, the life table constructed from field data in this study suggests that longevity and life-time reproductive success may not have offset the effects of other traits such as late

sexual maturation and low reproductive output. Taken together these results indicate that this colony was not at equilibrium during the period of the study. The persistence of reproduction to an age of 13 years indicates that reproductive senescence has not been reached by this time, and that spectacled flying foxes are physically capable of reaching these older ages. Modern life spans, on the other hand, may be significantly shorter than expected from their life history traits. The age distribution shows a lower number of one year olds than would be expected, but as flying fox colonies are not static in structure and these samples were taken from a maternity colony, it may be that at that time of the year there were fewer sub-adults in the colony.

Australian spectacled flying foxes suffer from many threats (Richards and Hall 1998) but these results suggest that the mortality rate they experienced in the years 2001/2002 in the Tolga scrub colony was twice as high as they could realistically sustain. Elasticity analysis has shown that mammals at the 'slow' end of the continuum are more sensitive to changes in adult and juvenile survival rather than changes in reproductive parameters (Heppell *et al.* 2000; Jonsson and Ebenman 2001; Oli 2004). This means conservation efforts in trying to halt the decline of a mammal species with a 'slow' life history should focus on trying to reduce mortality rates.

Results from this research highlight an area that requires further study to ascertain whether the mortality rates witnessed in 2001 and 2002 are general throughout spectacled flying fox colonies and from year to year. If this proves to be the case, the reduction in mortality in this species will need to be urgently addressed by management agencies.

Chapter Seven – genetic and demographic insights into the past, present and future of the spectacled flying fox

This thesis presents results of a study that investigated both demographic and genetic influences on population structure in a single species, using the same individuals. The use of both genetic and demographic methods allowed population structure in the spectacled flying fox (*Pteropus conspicillatus*) to be investigated across a range of geographic and temporal scales. Mitochondrial DNA provided insight into historical and broad-scale factors that have shaped current population structure. Highly polymorphic microsatellite markers revealed current population genetic structure at local and regional scales, as well as exploring patterns of relatedness within flying fox colonies. Demography of colonies was investigated by aging individual animals through annually incremented cementum layers in the root of teeth, establishing the age profile of a single colony and a life table of current mortality rates and population growth. In combination, these data provide baseline information to infer processes that have affected populations historically, as well as processes currently in effect.

The Past

The origin of flying foxes in Australia

The biogeographic origin of flying foxes in Australia remains hypothetical although they are thought to have dispersed from a neighbouring centre of higher diversity and colonised Australia through intermittent waves of invasion. Published literature claims that flying foxes in Australia migrated from Papua New Guinea (PNG) (McKean 1970; Schodde and Calaby 1972; Hall 1984) and are relatively recent colonisers (McKean 1970; Schodde and Calaby 1972), especially in the Wet Tropics region. Hand (1984) states that bats

continuously filtered into Australia throughout the Cainozoic (65 mya to present).

This study provides empirical evidence of a long occupation of Australia by flying foxes. High mitochondrial nucleotide diversity (Chapter 3) and nuclear allelic diversity (Chapter 4) among Australian individuals of spectacled flying fox, suggest an old lineage rather than one recently arrived in Australia. The greater haplotypic (mtDNA) and allelic diversity (nDNA) in Australia compared to PNG also suggests movement from Australia to PNG, not in the other direction as sometimes supposed. This is corroborated by the relatively high proportion of common alleles in PNG individuals and the greater number of rare alleles in Australia. PNG also has the lowest allelic richness, observed and expected heterozygosity, and effective number of alleles (Chapter 4). Coupled with the endemic status of the grey-headed flying fox in Australia and the limited occurrence of black flying foxes and little red flying foxes in PNG, these results support a hypothesis of an old lineage of flying foxes in Australia colonising Australia well before migration to PNG occurred.

The case for recent colonisation of Australia by flying foxes, especially the spectacled flying fox, rests on three propositions; the lack of any fossil record of the Megachiroptera in Australia, the restricted northerly distribution of the spectacled flying fox, and the low species diversity of Megachiroptera in Australia. Each of the three arguments will be discussed, and alternative propositions presented.

The fossil record

The lack of any fossil record of Megachiroptera in Australia makes inference about the history of this group in Australia difficult. The fossil record of the Megachiroptera in general is impoverished; the fossil history of 98% of megachiropteran–lineages is estimated to be missing (Teeling *et al.* 2005). Despite the lack of detection of fossils in Australia, this does not necessarily equate to a complete absence, just that they have not been uncovered as yet. For example, much of the Riversleigh deposits, which have yielded over one thousand microchiropteran fossils (Hand 1984), remain to be searched and uncovered (Archer *et al.* 1991).

Although there are no megachiropteran fossils in Australia, the oldest microchiropteran fossil in Australia was found in 55 my old sediments near Murgon, southeast Queensland (Hand 1994). The majority of, and best preserved, bat fossils come from deposits on the Riversleigh station in northwest Queensland dating from 20 mya (Hand 1999). Due to the higher degree of endemicity of the Megachiroptera compared to the Microchiroptera in the Australo/Papuan region, it has been suggested that the Megachiroptera entered the region first (Hand 1984). If so, and the oldest microchiropteran fossil is 55 my old, then the arrival of megachiropterans in Australia should pre-date this time. The only megachiropteran fossil found in the Australo/Papuan region is that of a bat from the Pliocene (5–1.6 mya) found in PNG (Hall 1981). Thorough investigation of deposits with large numbers of fossils of small to medium–sized arboreal animals may yet turn up flying foxes.

A number of authors have queried the reliance on, and reliability of, the fossil record for inference of the biogeography and diversification of species (Hedges *et al.* 1996; Heads 2001). Molecular estimates of mammal and bird diversification dates have been found to be 50-90% earlier using molecular methods than estimates based on the fossil record (Hedges *et al.* 1996).

Restricted distribution of the spectacled flying fox

Previous research has shown that spectacled flying fox colonies occur in close proximity to rainforest (Richards 1990a). Australia had extensive tracts of rainforest up to 5 mya (Truswell 1990). The distribution of spectacled flying foxes may be due to an ecological requirement to be close to warm, wet forests, and has followed the contraction of Australia's tropical rainforests to current relictual areas. The distribution of *P. conspicillatus* is likely to have been more extensive historically. Although spectacled flying foxes are known to forage in many habitat types throughout the Wet Tropics region (Parsons 2005), and do not appear to be limited in that respect, there does appear to be some aspect of their association with the rainforests of northern Queensland that limit their distribution to this area. What these aspects might be are still poorly understood.

Low species diversity of Megachiroptera in Australia

Diversity of flying fox species is low in Australia despite high genetic diversity suggesting a long presence here. A similar example exists in the birds of paradise, which are most diverse in PNG with 38 species, with only three species found in Australia (Heads 2001). The lack of a fossil record suggested that they should be considered a young group (Heads 2001). However, Heads (2001) proposed that they are an old group that have originated from an ancestral bird group in Australia that inhabited mangroves, beach forests and coastal hinterlands. The extensive radiation of the birds of paradise in PNG is proposed to have resulted from vicariant events resulting from the tumultuous geological history of PNG, rather than dispersal (Heads 2001). This highlights the point that a centre of diversity is not necessarily the area of origin, and therefore, an area of low diversity does not always equate with an area of recent colonisation. The higher species diversity of the Megachiroptera in PNG is likely to have resulted from interchange with both Indonesia and Australia and subsequent vicariance.

Proposed origin of spectacled flying foxes in Australia

The consequences of the reinterpretation of spectacled flying fox history in Australia are manifold. Their entry into Australia is most likely to have been via an ancient connection with Indonesia as suggested for many plants that exploited stepping stones provided by continental fragments or platelets that moved ahead of the Australian plate (Audley-Charles 1987). Prior to ~10 mya, PNG was a low and undistinguished group of islands that were episodically uplifted or inundated by global tectonic activity (Pigram and Davies 1987). This early 'Papua New Guinea' would be poorly suited to development of the diversity exhibited by volant organisms such as bats. Microchiropteran fossils place microbats in Australia approximately 55 mya, and megachiropterans are suggested to have arrived prior to this time (Hand 1984), when PNG was no more than a single terrane (regions of rocks with a similar geological history and rock type) (Pigram and Davies 1987). The proposal that flying foxes colonised Australia via an ancient connection with Indonesia is supported by the greater haplotypic and allelic diversity (Chapters 3 and 4) of the spectacled

flying fox in Australia compared to PNG, suggesting movement of flying foxes from Australia to PNG. This finding may have implications to other species of megachiroptera in the Australo-Papuan region.

The combined evidence suggests that at some point during the Eocene epoch flying foxes arrived in Australia. The fossil record suggests that the vegetation along the south east coast of Australia comprised a mixture of complex rainforest and forest comprising *Nothofagus* species (Truswell 1990), providing suitable habitat for colonisation and diversification to have occurred. The north east coast has tentatively been suggested to comprise similar complex rainforest elements to those currently found north of the Daintree river. There is very little information about the north coast (Truswell 1990). Changing climate and the resultant, sometimes dramatic, shifts in vegetation structure would have provided opportunities for speciation to occur from ancestral stocks resulting in the extant diversity observed in flying foxes.

Black flying foxes and spectacled flying foxes are known to interbreed, with introgression and possible incomplete lineage sorting being shown in this study (Chapter 3). It is likely, therefore, that these two species and possibly the grey-headed flying fox have diverged from a common ancestor. There is nothing to indicate what sequence of events might have caused the ancestral species to diverge into the three extant species. However, the current distributions of the grey-headed and spectacled flying foxes reflect different ecological requirements or tolerances. Increasing niche specialisation of these two species would likely have caused their allopatric distributions and divergence from each other. *Pteropus conspicillatus*, at some time in the past 10 million years, has colonised PNG and dispersed westwards to West Papua where it has diverged into the subspecies *Pteropus conspicillatus chrysauchen*, which was not included in this study due to the difficulty in obtaining specimens.

Although Schodde and Calaby (1972) were the proponents of the recent arrival of flying foxes from PNG, they noted that the rainforest fauna and flora in eastern Australia south of the Cape York Peninsula are old and autochthonous (evolved *in situ*). They also stated that the effect of the Torres Strait as a barrier or land bridge in shaping the Australian and PNG faunal distributions, has

been minor and intermittent (Schodde and Calaby 1972). The nuclear and mitochondrial DNA diversity results are consistent with a view of flying foxes being long term members of the Australian biota that colonised the continent long before undergoing secondary dispersal to PNG and the Pacific.

Introgression/incomplete lineage sorting

The observation of introgression, or of incomplete lineage sorting, between *P. conspicillatus* and *P. alecto* (Chapter 3) may require the addition of data from *P. poliocephalus*, the third large *Pteropus* species in Australia, to clarify the relationship among these three species. Additional data from *P. alecto* across a broader geographic range, as well as from *P. poliocephalus,* may resolve the issue of whether the signature in the data is due to occasional introgression or incomplete lineage sorting.

Typically, in species where hybridisation has been documented, introgression occurs at the point of contact of the two taxa involved, forming a hybrid zone (Harrison 1993). In the spectacled flying fox, the observed haplotype relationships suggest hybrids occur but do not form a 'hybrid zone' as the extensive movements that this species undertake make this implausible. Evidence of this may be seen in that the two geographical locations from which the black flying fox samples came (Townsville, and Thursday Island from the Torres Strait) are outside the current recognised distribution of the spectacled flying fox in Australia.

The evidence of hybridisation observed in the mtDNA data could be a result of either current and ongoing introgression events, or ancient introgression events, or a combination of both. Introgression may be a widespread and continuing phenomenon between flying fox species in Australia, or the haplotypic relationships reported in Chapter 3 may have resulted from a few interspecific matings long ago. Branch lengths between some of the black flying fox and spectacled flying fox individuals on the phylogeny are quite short, indicating a fairly recent introgression event. However, the branch leading to *P. alecto* 50 TVL emerges from deep within the tree and comprises 25 bp changes, suggesting an older, historical relationship. These two examples demonstrate that introgression may have occurred continually throughout

time, and still be ongoing today. Without further sampling and analyses of both species, the timing of any introgression events remains unknown.

In the example of *P. alecto* 50 TVL, the long branch length may be a signal of incomplete lineage sorting rather than introgression. The evolutionary history of flying foxes in Australia remains unclear. In addition, the timing of the divergence of the three large flying foxes from a common ancestor is unknown. It is possible that the bat lineages in Australia are still in the process of sorting, and thus have not yet completely diverged. It appears likely that both introgression and incomplete lineage sorting may contribute to the mixing of haplotypes between *P. alecto* and *P. conspicillatus*.

Vicariance and dispersal

This study revealed a genetic signature of expansion in the mtDNA data (Chapter 3) and also the genetic signature of a bottleneck in the microsatellite data (Chapter 4). These conflicting results highlight the turbulent history of the spectacled flying fox in Australia. This species has likely undergone population expansions and contractions as a result of climatic and geological events. The east coast of Australia, although currently lacking active volcanoes, was an area of regular volcanism throughout Australia's geological history (Johnson 2004). Basalt eruptions began approximately 50 mya in northern Queensland, although most of the outcrops seen today resulted from activity between 8 mya and 13 000 years ago. In the Atherton basalt province, eight major volcanoes created the Atherton Tablelands approximately 3.9–1.6 mya (Johnson 2004). The eruption of the Undara volcano occurred 190 000 years ago and resulted in large areas of lava flow regionally (Johnson 2004). The volcanic activity in north Queensland would have removed large areas of forest, affecting the distribution of the spectacled flying fox and leading to population fragmentation. Such events can provide the mechanisms for generating the distinct clades seen in the phylogenetic analyses. Along with changes in habitat availability owing to climatic fluctuations, the likelihood of population expansions and bottlenecks throughout time is strong. This pattern is also likely to be seen in other members of the old Wet Tropics fauna. For example, two species of ringtail possum, *Pseudochirulus cinereus* and *P. herbertensis*, are

believed to be have diverged as a result of isolation in rainforest refugia during the last glacial maximum (Moritz *et al.* 1997).

The Present

Broad scale

Both microsatellite and mitochondrial analysis of spectacled flying fox colonies throughout their range suggests high gene flow and connectivity between colonies resulting in a single panmictic population and no genetic distinctiveness among colonies (Chapter 4). Although analysis suggests that there is currently high gene flow between Australia and PNG, there are elements of the data implying the opposite. Mitochondrial DNA haplotypic diversity (Chapter 3) in PNG is lower than in Australia. There is reduced allelic richness and genetic diversity at microsatellite loci in PNG (Chapter 4). The impression of an excess of common alleles (Chapter 5) was backed up by detailed description of the alleles occurring at less than 25% frequency in all colonies (Chapter 4), showing PNG to only have 40% of the less common alleles present. The PNG data demonstrated similar diversity to Wet Tropics colonies with almost half the number of samples. If occasional, but consistent, movement and gene flow were occurring between PNG and Australia, the occurrence of rare alleles in the PNG data should be greater than demonstrated. The combination of these more detailed analyses suggest that contemporary movement between PNG and Australia is unlikely or rare.

Iron Range appears distinct from the other Australian colonies. It has the lowest population-by-population pairwise relatedness value compared to almost every other colony (Chapter 5). This may be due to small sample size and a greater proportion of rare or uncommon alleles. The measure of allelic richness, which removes the bias as a result of sample size, revealed Iron Range had the second highest allelic richness of all colonies. From limited field observations of this colony, it appears to be very small in size, at most comprising several hundred individuals. It is difficult to determine if this is an unusual colony, or whether small sample size and a random sampling of alleles is biasing these diversity measures. However, when a comparison of the effective number of alleles was made by randomly subsampling the two Atherton Tableland populations with

large sample sizes (Chapter 4), with the total dataset, there was little difference. This may suggest that increasing the sample size in the Iron Range population would not change the results or the allelic diversity outcome. Episodic migration of a few spectacled flying foxes from PNG and/or the Wet Tropics region, followed by long periods of isolation, and increased genetic drift due to small population size, is likely responsible for the allelic pattern observed in the Iron Range sample.

Pteropus conspicillatus is principally found in the Wet Tropics region of north Queensland. The highly gregarious nature of this species, along with a patchy landscape of food resources, means there is considerable movement of individuals between colonies. A high degree of movement does not automatically confer abundant gene flow unless matings, and therefore offspring, result from these journeys. However, the lack of structure in the mtDNA (Chapter 3) and microsatellite data (Chapter 4), suggest that high gene flow does occur among colonies throughout the region. This implies that mating is random with regard to geographic location or colony, and that there is no bias in the dispersal of offspring from a colony (i.e. no evidence for female philopatry). This is in agreement with the two previous studies on gene flow using allozyme markers in the other three *Pteropus* species found on mainland Australia (Sinclair *et al.* 1996; Webb and Tidemann 1996).

Despite the genetic signature of a bottleneck in the Wet Tropics colonies (Chapter 4), there remains abundant genetic diversity in this region. Two processes are likely to have contributed to this pattern. Firstly, the panmictic structure of the Wet Tropics colonies means large-scale gene flow between colonies will ameliorate the effect of any bottleneck by reducing the effect of genetic drift. The loss of individuals or components of habitat are important, but the influence at the genetic level would be diluted across the landscape. The likely diffuse nature of the bottleneck will have resulted in a slower rate of loss of genetic variability. Secondly, the introgression of *P. alecto* into *P. conspicillatus* has likely increased the diversity of mtDNA and nDNA in *P. conspicillatus* in Australia. With the extent of the introgression being unknown, the effect of it on analyses involving allele frequencies or time since divergence is also unknown. As the analysis used to detect a bottleneck works on allele number

and heterozygosity, it is possible that the bottleneck suggested in the Wet Tropics colonies could actually be a result of the increased allelic diversity as a result of novel alleles from the black flying fox. Additional sampling of *P. alecto* across its range should assist in understanding its contribution to this pattern.

Local scale

Although many bat species exhibit panmixia, there is often some degree of inter-colony structure due to features of their mating system or dispersal pattern (Petri *et al.* 1997; Petit and Mayer 1999; Rossiter *et al.* 2000b; Castella *et al.* 2001; Ortega *et al.* 2003; Ruedi and Castella 2003). The mating strategy of *P. conspicillatus* was not studied directly in this investigation, although the lack of genetic structure among colonies (Chapter 3), as well as the indication of cross kin-group matings (Chapter 5), suggest random mating throughout the Wet Tropics region.

Female philopatry is common in mammals (Prugnolle and de Meeus 2002), and especially in female Microchiroptera (Wilkinson 1992a; Worthington-Wilmer *et al.* 1994; Petri *et al.* 1997; Entwhistle *et al.* 2000; Burland *et al.* 2001; Castella *et al.* 2001; Kerth *et al.* 2002b; Miller-Butterworth *et al.* 2003). No female philopatry has been reported in the Megachiroptera due to a lack of genetic studies on colony inter-relatedness. This study found no evidence of female philopatry in spectacled flying foxes (Chapters 3 and 4). However, preliminary evidence that sub-adults travel with their mothers to forage was observed (Chapter 5). The need to travel long distances to forage for patchy food resources provides impetus for this behaviour. In doing this, sub-adults would learn the location of valuable food resources at different times. The requirement for constant movement between colonies depending on the location of current food sources provides many opportunities for inter-colony matings. This study suggests such matings are common. In addition, despite the observation of family groups containing closely related sub-adults and mature females, there is no evidence of inbreeding or obvious within kin-group matings.

As the age structure of the Tolga Scrub colony indicates a population of mainly young animals, and the reproductive output of this colony is low, the ability of this population to achieve positive growth is low unless mortality rates are

reduced (Chapter 6). Although the year analysed (2002) was a relatively bad tick paralysis season (equating to approximately 5% loss of individuals from Atherton Tablelands colonies–J. Maclean, pers. comm.), the difference between the instantaneous mortality rate and annual mortality rate gives an indication of the effect of tick paralysis. The annual mortality rate (without the effect of tick paralysis), although in keeping with mortality rates for mammals in general, is higher than this species can realistically sustain, considering their short life span and low reproductive output.

Considering the short period of time over which this study was undertaken, it is difficult to make any statement about year-to-year variation in recruitment and mortality. Both reproductive output (birth) and mortality rate (death) are likely to vary from year to year, and these two processes are critical influences on current population size trends. Thus, it is important to have a detailed and comprehensive understanding of these demographic components. However, mortality rate is a difficult parameter to ascertain directly. Determining the ages of individuals through the use of cementum layers in teeth, although time consuming, may be the only realistic method of determining age structure for a flying fox colony, and therefore, mortality rate and population growth. However, new methods for aging animals using non-destructive techniques, such as the rate of telomere erosion throughout an organisms life, are now becoming available (Gan *et al.* 2001; Haussmann and Vleck 2002).

As flying fox colonies have such a fluid nature, the use of life tables to estimate demographic processes may be inappropriate, as the assumptions of these tables may not be met. Although it can be argued that the life table results from this study cannot be extrapolated to other colonies due to their fluid nature, other factors suggest that sampling flying foxes at this time of the year may be as close to random as can be hoped. That the sex ratio of samples taken was 1:1, and with the large-scale and apparent random nature of the movement between colonies, it could be argued that the sample taken would likely be an average across the Wet Tropics. This would need to be proven one way or the other by sampling across several colonies throughout the range of the spectacled flying fox at the same time.

Future

Conservation and management of the spectacled flying fox

The initial impetus for this study came from a need to provide basic information on the general biology and ecology of the spectacled flying fox to facilitate informed decisions regarding this species. For the management agencies these decisions involved creating management plans, for the fruit farmers it was how best to protect their crops without having to cull animals on a nightly basis. To conservationists, it was how best to help the species stabilise in light of their alleged decline.

To management agencies, the level of gene flow and connectivity between colonies observed in this study emphasises the need to manage the spectacled flying fox as a single management unit throughout the Wet Tropics region. It also highlights the fact that there appear to be very few barriers to movement in this region, and that their current distribution is not a reflection of their incapacity to move further afield, but likely due to other limiting ecological aspects, such as food resources.

The loss of individuals to tick paralysis each year is known to management agencies. However, these results reveal the effect of this loss on the population growth rate. There is a critical need to reduce the mortality rate, especially that due to tick paralysis, to improve population growth rates to a more sustainable level. The clarification of the age at first reproduction in females, and determining the reproductive output on an annual basis, will advise management of the capacity of this species to recover from a population decline.

The high connectivity of colonies found in the Wet Tropics region will answer many questions posed by fruit farmers. These results show that the removal of a single colony neighbouring a fruit farm would have no effect on mitigating the impact of foraging flying foxes, as individuals foraging on fruit crops would have come from colonies throughout the Wet Tropics region. The removal of a colony from the panmictic Wet Tropics population would have no immediate effect on genetic diversity in the species, although it would have an effect on

demographic factors such as overall reproductive output, which in the long term would likely affect genetic diversity.

This study also highlights that spectacled flying foxes are incapable of a boom and bust reproductive system, dismissing the 'flying rodents' claim made by many fruit farmers. As the majority of females give birth for the first time between the ages of three and four, produce a single young per year, and most likely only live to six or seven years of age, this species follows a typical 'slow' life history trait scenario, without the long life span to compensate for the low reproductive output (Chapter 6). It appears possible that in many years their mortality rate overshadows their reproductive output, resulting in either zero or even a negative population growth rate.

The high genetic diversity (Chapters 3 and 4) will confirm for conservationists that the spectacled flying fox has the genetic capability to cope with environmental change. High gene flow and connectivity of colonies ensure that loss of individuals through a localised mortality event will not have an immediate effect on genetic diversity within the population, although it will affect reproductive capacity and total population size.

Future research

This study has answered a number of questions but at the same time highlighted many others. The phylogeny of the spectacled flying fox needs to be extended to include greater representation of close congeners to elucidate an accurate history of *Pteropus* species that occur on mainland Australia. Increasing the sampling of *P. alecto* and including samples from *P. poliocephalus*, will extend the phylogeny of large Australian pteropodids and allow their relationship to each other to be determined. This will also provide information on the extent of introgression, whether it is reciprocal, and whether both introgression and incomplete lineage sorting are responsible for the mitochondrial poly- or paraphyly observed. By also including other close congeners from the Australo-Pacific region, the evolution of *Pteropus* species in this region may be clarified.

A social study on within-colony behaviour and relationships through observation may validate the hypothesis emerging from this study of kin-groups occurring within colonies. This type of study will also confirm whether the spectacled flying fox follows a polygynous mating strategy and the type of polygyny involved. A combination of observation of behaviour and genetic analysis to determine successful matings should clarify colony behavioural dynamics in this species.

It would be informative to compare nuclear genetic diversity among the black flying foxes, grey-headed flying foxes and spectacled flying foxes. Applying the microsatellite loci isolated and optimised for the spectacled flying fox would be an appropriate tool to examine the allelic range and distribution among these species. This may clarify the sequence of divergence of *Pteropus* in Australia as well as reveal whether the difference in genetic diversity between Australia and PNG is a result of introgression from black flying foxes or an indication of an older, ancestral lineage in Australia. The data obtained from such a study would also provide insight into the diversification of these apparently closely related species, as well as determine whether other flying fox species suffered from a bottleneck during the last glacial maximum.

Considering the aspects of population structure that appear to be most important to the conservation of this species, I believe the most important future research that could be completed on this species is to determine whether the mortality rates and population growth rates estimated in this study are applicable to the rest of the Wet Tropics population. Once this has been resolved, the degree of urgency for remedial action can be established.

The population of spectacled flying foxes occurring in the Iron Range National Park area appears to be an outlier within Australia and the data is equivocal as to whether there is current movement into or out of this colony. Little is known about this population. The movement of the entire colony out of the park during the winter months and the fact that this was unknown before August 2004, highlight the need for intensive field-based research, as well as more genetic samples, to accurately assess the status of this population.

While this study has concentrated on Australian populations of spectacled flying fox, it has highlighted the lack of knowledge on the populations in PNG. Little is known of the colonies of spectacled flying fox that occur in PNG, where only a couple of larger colonies close to towns can be verified as still in existence. As little is known about flying foxes in PNG in general, this would be a worthwhile, if ambitious, project in adding knowledge on the evolution of flying foxes in the Australo-Pacific region, a so-called hotspot of diversity for the Megachiroptera.

Conclusion

A new hypothesis for the origin and evolution of flying foxes in Australia is presented. The spectacled flying fox has experienced a tumultuous history, including population expansion and contraction as a result of climatic and geographic events. These events have aided in shaping the contemporary structure of highly connected colonies within a single panmictic population in the Wet Tropics region, along with an isolated population at Iron Range and populations of unknown status in PNG. High allelic and haplotypic diversity suggest an old lineage within Australia and colonisation from Australia to PNG. Introgression between black flying foxes and spectacled flying foxes suggests a close association between these two species. The possibility of incomplete lineage sorting also intimates that the three large species of *Pteropus* in Australia might still be in the process of diverging. Although high levels of gene flow among colonies within the Wet Tropics region suggest fluidity within and among colonies, some sub-structuring in the form of kin groups is indicated, with several cohorts of young remaining with mothers before the young reach sexual maturity. High rates of mortality and low reproductive output may be putting this species at risk of decline, especially as their average longevity is considerably less than expected.

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Appendix 1—mtDNA sequence alignment

Appendix 2—microsatellite genotypes

A CD is enclosed containing both appendices in two formats, first as either a text file or excel spreadsheet and second as a pdf.

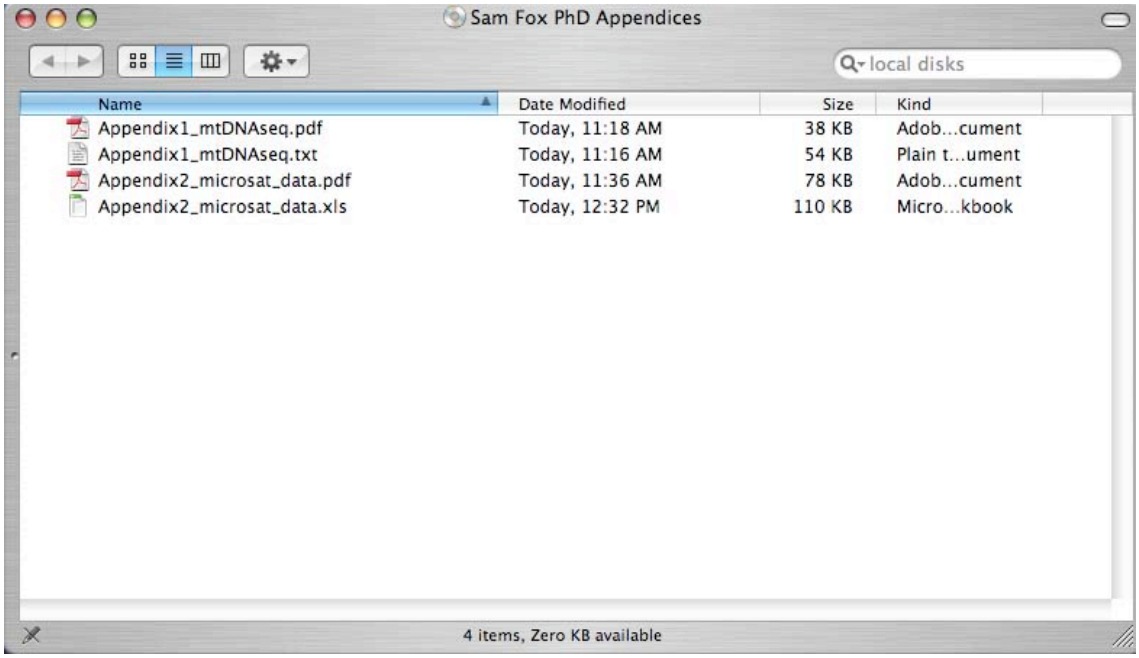

Figure A1. Contents of CD containing appendices

DATA APPENDICES ARE NOT AVAILABLE THROUGH THIS REPOSITORY

Appendix 3-amplification details for each thermocycle program

Program STD55

1. 95°C for 5 mins
2. 95°C for 45 secs
3. 55°C for 30 secs
4. 72°C for 45 secs
5. Goto step 2 and repeat 35 times
6. 72°C for 10 mins
7. 12°C HOLD

Program 58TD50

1. 94° for 3 mins
2. 94° for 30 secs
3. 58° for 40 secs
4. 72° for 1 min
5. Go to step 2 and repeat 4 times – then decrease temp by 2° and repeat 4x
6. 94° for 30 secs
7. 50° for 40 secs
8. 72° for 1 min
9. Go to step 6 and repeat 9 times
10. 72°C for 10 mins
11. 12°C HOLD

Program 50TD44

1. 94°C for 1 min
2. 94°C for 30 secs
3. 50°C for 40 secs
4. 72°C for 1min 30secs
5. Go to step 2 and repeat 4 times – then decrease by 2° and repeat 3x
6. 94°C for 30 secs
7. 44°C for 40 secs
8. 72°C for 1min 30secs
9. Go to step 6 and repeat 19 times
10. 72°C for 10 mins
11. 12°C HOLD

Appendix 4-registration numbers for *P. conspicillatus* samples obtained from Museums

Australian Museum:

M21243, M21582, M21583, M22538, M22539, M22540, M22564, M21669, M25292, M25291, M19446, M21584

South Australian Museum:

196056-1, 196056-2, 196056-3, 196056-4, 198805, 198821, 200211, 200226, 205734, B105, NQ79