Cospeciation of albatrosses and their chewing lice:

COI and 12S rRNA sequence data reveal different coevolutionary histories in four genera of albatross lice and their hosts

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Abstract

The study investigated the coevolutionary history of albatrosses (Procellariiforms:Diomedeidae) and their lice (Phthirpatera:Ischnocera). The louse phylogeny was produced using combined data from two mitochondrial genes; COI and 12S rRNA. Using this phylogeny, four genera of albatross lice were examined for evidence of phylogenetic congruence with host phylogenies. Using Jungle analysis, the costs of reconciling the louse and host trees were calculated for reconstructions that maximised cospeciation events and possible causes of incongruence between trees were examined. Cospeciation events appear to be more frequent in some genera of lice than in others. The genera *Docophoroides* and *Paraclisis* show quite clear patterns with the majority of species cospeciating with their hosts. The number of cospeciation events was significantly more than would be expected from chance alone ($p<0.000$). The patterns of association in the genera *Perineus* and *Harrisoniella* are less clear and may be confounded by incomplete sampling of host and parasite taxa. There are few cospeciation events in these genera and these fall within the number expected by chance agreement between tree topologies. There is an indication in these genera of a history containing the duplication of louse lineages, followed by sorting events and host switching. A discussion of the ecological basis of coevolutionary history in albatrosses and their lice suggests some of the factors that may have caused the differences in phylogenetic congruence. This work highlights the need for additional ecological data to complement the coevolutionary analysis.

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Introduction

A background to coevolutionary studies

Host-parasite systems have been the target for recent coevolutionary studies (e.g. Hafner & Nadler 1988, Paterson *et al.* 1993) after early work in parasitology took notice of the apparent correspondence between parasite and host phylogenies (Klassen 1992). Price (1980) describes parasites as 'adapted to exploit small, discontinuous environments' and sees colonisation of new hosts as a hazardous undertaking. The interaction of parasites with their environment, i.e. their host, can therefore be very applicable to studies of reciprocal adaptation and cospeciation. This is especially the case with those parasites that spend their entire life cycle on the body of the host.

The evolutionary consequences of interspecific interactions can be described as coevolution when there is reciprocity of selection pressure (Majerus *et al.* 1996). Coevolution between parasites and hosts has often been described as an evolutionary arms race where evolution is continually driven by repeated rounds of parasite attack followed by host defence (Majerus *et al.* 1996). The nature of the arms race may explain longevity in the relationship between parasites and hosts if host defences are costly. When these defences act, parasite numbers may decrease and as a result costly defences may also decline (Majerus *et al.* 1996). Alternatively, many parasites may permanently exist in small populations that have little effect on their host. The host may simply have nothing to gain by removing them (Clayton 1991). Coevolution may exist as parasites respond to changes in host morphology or behaviour that have nothing to do with the interaction between the two.

Ectoparasitic lice

Ectoparasitic lice have been used in many studies as the model system for parasite-host coevolution (e.g. Hafner & Nadler 1988, Paterson *et al.* 1993, Page *et al.* 1998). Lice (Phthiraptera) are wingless obligate ectoparasites of birds and mammals that lack free-living stages and intermediate hosts. They form a tightly linked system together with the body of their host, as they are incapable of independent mobility and quickly suffer high rates of mortality when removed from the host (Nelson & Murry 1971). Lice are in fact the insects that are most completely committed to parasitism (Askew 1971).

Striking adaptations for their lifestyle include the loss of wings, dorso-ventral flattening, and a tough integument. They are dependent on the warmth of the host's body for reproduction (Clayton 1991).

The taxonomy and systematics of the Phthiraptera has been under debate for some time (e.g. Lyal 1985, Johnson & Whiting 2002) and is still not fully understood. Traditionally, Lice have been split into four suborders: the Anoplura, the Ryncophthirina, the Amblycera and the Ischnocera (Cruickshank et al, 2001). Of the suborders, the Anoplura are ectoparasitic on mammals while the other groups are found on both mammals and birds, although avian lice make up the majority of the Ischnoceran suborder. In the past all suborders except the Anoplura ('sucking lice') have often been amalgamated into a group known as the Mallophaga ('chewing lice') a division based on feeding morphology. However (Lyal, 1985) regarded the Mallophaga as a paraphlyletic group. Cruickshank *et al*. (2001) suggests that the phylogenetic analysis of partial sequences of elongation factor 1α identifies the Ischnocera as paraphyletic. Subsequently, Johnson & Whiting (2002) have shown that 18S ribosomal RNA sequence data, as well as combined analysis of three gene regions provides strong support for Ischnoceran monophyly. Relationships of families within these suborders are also been examined and discussed using morphological (Smith 2001) and molecular data (Cruickshank *et al.* 2001, Johnson *et al.* 2001, Johnson & Whiting 2002).

Host-parasite coevolution: empirical studies using lice

Early work in parasitology took notice of the apparent correspondence between parasite and host phylogenies. Klassen (1992) provides a review of the work of the early parasitologists and researchers such as Kellogg, Metcalf and Eichler. A great deal of emphasis was placed at this time upon the few rather anecdotal examples of correspondence between parasite and host taxonomic relationships. It was the accepted view that host and parasite phylogenies are closely correlated, leading to the formation of several rules, including Farenholz's Rule (Eichler 1948 as cited by Askew 1971).This states that there should be cospeciation between parasites and their hosts.

In the late 1980s the development of molecular techniques made a large impact on phylogenetic work and it was at this point that empirical studies began to examine host-parasite coevolution in detail. There has been debate over whether it is better to use

morphological or molecular characters for constructing phylogenies. The decision may depend on the question being examined. When searching for coevolution between parasite and host trees, molecular data can provide useful homologous characters. This can allow the examination of rates of evolution and thus whether or not there is a 'molecular clock'. If there is a molecular clock, speciation events in parasite and host trees can be assessed for temporal congruence and what processes any incongruence may be due to (Page 1994).

Given the usefulness of molecular data, which sections of DNA have been the subjects of molecular analysis in the study of coevolution between lice and their hosts? Mitochondrial DNA (mtDNA) has been found to be a 'powerful subject for evolutionary studies' (Hoy 1994) and a majority of studies use mtDNA sequences (Majerus *et al.* 1996). A number of attributes make it useful. It has a relatively rapid rate of sequence divergence and is small in size compared to the nuclear genome (Hoy 1994). It also has the advantage of ease of purification and manipulation in the laboratory (Quinn 1997) and appears to be selectively neutral (Majerus *et al.* 1996). However, it is worthwhile noting that at least one study shows a pattern of non-neutral selection and departure from the 'molecular clock hypothesis' along *Drosophilia simulans* lineages (Ballard & Kreitman 1994). Which sections of mtDNA have been used to test lice-host cospeciation? Genes have been used where insect-specific primers exist and in addition louse-specific primers have been developed especially for these studies. Mitochondrial cytochrome *b* has been used for *Dennyus* lice (Page et al. 1998) and mt ribosomal RNA has been used for several studies (Paterson *et al.* 2000). Ribosomal RNA's contain regions that are more conserved than other genes (Hoy 1994) and have therefore been useful for resolving lice phylogenies.

The main empirical studies of host-parasite coevolution, described below, have found evidence for varying amounts of cospeciation in different associations. Hafner and Nadler's (1988, 1990) work on the cophylogeny between pocket gophers and their chewing lice has become the text book example of host-parasite cospeciation (e.g. Majerus *et al.* 1996). Phylogenetic trees, created using protein electrophoretic data, with a high degree of concordance in branching pattern and branch lengths were also very similar where the pattern was identical (Hafner & Nadler, 1998). In this case, the fossorial lifestyle of the hosts may promote specialisation and coevolution. Shortly after this, a study by Barker (1991) found little evidence of cospeciation between

Amblyceran lice and their rockwallaby hosts. The geographic ranges of parasites and hosts were found to be incongruent and there is evidence of widespread host switching and speciation of the lice independent of host speciation. Paterson *et al.* (1993) studied the lice of seabirds, finding evidence that there has been intrahost speciation and many sorting events but little host switching.

Theoretical methods in the study of host-parasite coevolution

In order to test cospeciation there must be confidence in the robustness of the host and parasite trees. Once this has been achieved, trees can then be compared for congruence. In the early stages of research this was only done subjectively. The next stage was to develop methods that assessed whether there was statistically significant congruence between host and parasite trees. Hafner & Nadler (1988) calculated the probability of the observed level of topological similarity occurring by chance alone using the component-replication method of Nelson & Platnick. However, as several researchers have pointed out (e.g. Paterson *et al.* 1993) the transmission of parasites from one host lineage to another (host switching) may obscure evidence of cospeciation. Other events that may occur in the historical association of hosts and parasites e.g. the extinction of parasite lineages, may also lead to incongruence without a lack of some amount of cospeciation occurring. Page (1990) describes the method of reconciliation of host and parasite trees. The number of evolutionary events required to reconcile the trees can be calculated and in this way any two trees can be reconciled. If the fit between the two trees is not significantly better than a tree drawn at random from the set of all possible host cladograms, the hypothesis of cospeciation between the two can be rejected. Reconciled trees can make inferences about ‘sorting events’ (Page & Charleston 1998) when there is an absence of a parasite on a host species where one was expected. For example, a sorting event could be an extinction of a parasite on a particular host or when due to chance, a parasite is missing from a host at a founding or speciation event (known as ‘missing the boat’). In reconciled trees, host switching by parasites is not permitted in the analysis. As this is not realistic, this limitation was taken into account with the development of Brooks parsimony analysis (BPA) (Brooks 1981). BPA finds the most parsimonous reconstruction of the parasite tree onto the host phylogeny, taking into account possible cospeciation, host switching and sorting events. However the BPA does not take into account possible duplication (intrahost speciation) events and Page (1998) states that BPA does not always produce biologically reasonable reconstructions.

Charleston (1998) developed the use of ‘Jungles’, mathematical structures which present all the possible ways in which a parasite tree can be mapped onto the host tree, incorporating the processes of cospeciation, duplication (within host speciation), sorting events and host switching. The jungle assigns costs to each of these processes, allowing comparison of jungle solutions to find the least costly reconstruction of the parasite and host association.

In consideration of the various different methods of examining coevolution, Paterson & Banks (2001) show that for an example of *Halipeurus* lice on their seabird hosts, all methods used provided the same conclusion of extensive cospeciation. This is not to say that all methods are equally good, the applicability of the different methods will differ with the occurrence of various phylogenetic events. Paterson & Banks (2001) state that the methods used to examine the robustness of data supporting the cophylogenetic events are effectively still in their infancy.

Rates of evolution and the timing of evolutionary events

A study on swiflet lice (Page *et al*. 1998) where the phylogenies for both hosts and parasites were based on mitochondrial cytochrome b DNA sequences, compared rates of sequence divergence and found cytochrome *b* to be evolving 2-3 times faster in the lice than in the birds. In fact, the louse mtDNA is evolving so rapidly that in one example, two species show 25-30% DNA sequence divergence. A recent study by Paterson et al. (2000) showed that lice have evolved at ~ 5.5 times the rate of seabirds. Why would lice be evolving more rapidly than their hosts? Possible explanations could be differences in generation times, different selective pressures and different population sizes (for instance, lice may be undergoing repeated founder events), (Page *et al.* 1998).

Page (1990) showed that information on the timing of cladogenetic events can increase the power of analysis of congruence. Given a molecular clock in both parasite and host lineages (which may tick at different rates) the timing of cospeciation events can be compared. Hafner & Nadler (1988) described ‘pseudo-cospeciation’, when an association is cladistically congruent but when speciation in the parasite post-dates that of the hosts. Temporal information can also help to explain cladistic incongruence (Page 1990).

The ecology of host-parasite coevolution

When examining the evolution of host-parasite relationships we find that close associations between insects and vertebrates have independently arisen in seven insect orders (Waage 1979). However the paucity of fossil records for insects results in a lack of evidence for specific pathways of evolution from free-living taxa to the current parasitic lifestyle (Waage 1979).

Waage (1979) considers the differences in feeding habits between different orders of lice to be the result of an evolutionary trend from feeding on host epidermis to facultative and finally obligate blood feeding (as seen in the Anoplura). Members of different suborders feed on either skin, feathers, sebaceous exudates or blood or a combination of these (Lyal 1985). One example of specialisation for a particular feeding habitat is seen in the louse *Columbicola columbae* found on rock doves. It rarely feeds on the host's skin preferring instead to consume the barbules of feathers, which it metabolises with the aid of symbiotic bacteria (Clayton 1991). The feeding habits of ectoparasites can be harmful to hosts and can reduce host fitness by causing injury, through feather damage, blood loss and also through infection and disease transmission (Waage 1979). The effect of parasites on host reproductive success has been demonstrated by a number of studies on birds, for example in mate choice (Møller 1991). Specifically looking at ectoparasites, Clayton & Tompkins (1995) show experimentally that mites (Acari) decrease host reproductive success. However in they also found lice had no effect on host reproductive success, even though feather damage is known to have detrimental energetic consequences (Booth *et al.*1993).

The morphology of lice reflects the adaptations to their particular parasitic lifestyle. In particular, the morphology of Ischnoceran lice appears to reflect adaptation to their use of microhabitats on the host. The Ischnocera of the head and neck region on birds are often slow moving, not greatly flattened in body shape, and have rounded bodies and large heads to allow for large mandibular muscles (Askew 1971). This is in contrast to the Ischnocera who inhabit the back and wings of birds. Living in these positions, they are vulnerable to preening and they are flattened, elongated and capable of rapid movement (Smith 2001). Lice in other Phthiriaptean suborders are also specialised to particular microhabitats on the host's body. For instance, certain species of Amblycera

are very highly adapted to live inside the quills of the feathers of the Curlew (Askew 1971).

Other types of ectoparasites have also been shown to be adapted to life on feathers. Jovani & Serrano (2001) show that ectoparasitic feather mites (Astigmata) avoid the moulting wing feathers of passerine birds. This adaptive behaviour allows them to be unharmed from the complete moult. It would be interesting to examine whether the avian Phthiraptera also show this behaviour.

Avian host defences have evolved in response to the negative effect of ectoparasites. Grooming is an important defence and is effective in reducing parasite loads (Clayton 1991). Selection has been shown to promote efficient preening and act against bill deformities in pigeons, while preening selects for small body size in lice (Clayton *et al* 1999). Many species of passerine birds carry out a behaviour known as 'anting'. Species of ants that produce formic acid are rubbed onto the plumage of the bird and this has been suggested to be an adaptation for louse eradication (Askew 1971). Richner & Heeb (1995) showed that clutch and brood size patterns in birds may have evolved as a response to the lifecycle length of their most common ectoparasite in order to reduce parasite load and thus increase the survival of their nestlings.

Host-specificity of lice

Host-specificity is considered to be a feature of the lice-host relationship. Most species of Ischnocera have been found to be very host-specific (Clayton 1991). For example, a study of Neotropical bird lice showed lice to be extremely host-specific, with nearly all species being restricted to a single species of host (Clayton, Gregory & Price 1992), another study found high host-specificity in *Strigiphulus* owl lice (Clayton 1990). Interestingly, a recent analysis of host specificity in Columbiform wing and body lice, two groups of Ischnoceran lice that are not closely related to one another (Cruickshank *et al.* 2001), found that body lice are considerably more host-specific than wing lice (Johnson *et al.* 2002).

However, host-specific parasites are not evidence of coevolution, they could be incapable of dispersing among host taxa (Clayton *et al.* in press). Tompkins & Clayton (1999) showed experimentally that host-specificity in Amblyceran lice on cave swiflets is due to adaptations to their particular host species, not simply because of their low

dispersal ability. They found that experimentally transferred lice had lower survival rates and concluded that this was a result of changes in feather barb size between hosts. Adaptation in the lice constrains them to a particular host or at least to a host with a particular feather barb size. This particular adaptation is likely to be linked to the ability to stay on the host, especially during flight and also the ability to move round the host environment (and thus avoid preening). This relationship has also been shown for chewing lice and their pocket gopher hosts (Morand *et al.* 2000). Using the method of independent contrasts, they found a relationship between host and parasite body size and linked this to the positive relationship between gopher hair-shaft diameter and louse head-groove width. As the chewing lice of mammals grasp the hair of the host in their head-groove, having the correct size of head-groove will allow a louse to clamp on tightly and prevent removal when the host is grooming. This may explain why certain species of lice appear unable to survive on certain species of host in transfer experiments (Morand *et al*. 2000).

Reed & Hafner (1997) carried out transfer experiments on the host specificity of pocket gopher lice. They showed that the colonisation of new hosts diminished with increasing phylogenetic distance from the natural host of each louse. They suggest that cospeciation patterns result mainly from the lack of colonisation opportunities, but even when these opportunities do occur, survival on new hosts may be impossible and this further reinforces cospeciation.

Parasite Transmission

The high levels of host specificity described above, mean that the successful transmission of lice is more likely within host species rather than between host species. The work of Morand *et al.* (2000) and Tompkins & Clayton (1999) could also suggest that lice can transfer between host species, but that successful transfer is limited to hosts with similar body sizes. Understanding the mechanisms of parasite transmission is necessary in coevolutionary studies of hosts and parasites. As lice are unable to survive for long periods way from the host environment due to their low vagility, transmission must mainly occur via host-host body contact. This is obvious more likely to occur between hosts of the same species, through parent-offspring contact (vertical transmission) or through host mating (Hillgarth 1996). Hafner & Nadler (1988) concluded that as closely related taxa of lice sometimes occur on hosts that are distantly

related, maternal transmission alone is an insufficient explanation of distribution. Demastes *et al.* (1998) tested the hypothesis that lice are transmitted maternally in pocket gophers by comparing the distribution of mitochondrial DNA haplotypes in the pocket gophers to the distribution of louse populations. They found no significant concordance between the two, evidence against a strict maternal transmission hypothesis.

Ròzsa et al (1996) found that colonial rooks harboured more species –rich loads and greater abundance of ectoparasites than territorial rooks. This may result from the increased frequency of horizontal transmissions through greater body-to-body contacts among colonial rooks. They also found that the frequency distributions of lice on rooks were less aggregated (overdispersed) than crows, this may have implications when considering factors preventing coevolution.

Clayton & Johnson (2001) discuss the feasibility of determining the identity of brood parasite foster parents from host-specific lice. However, previous studies (Hahn *et al.,* 2000) found only 4.5% of lice on the brood parasitic cowbirds could be assigned unambiguously to a single foster species. If lice found on adult cowbirds are in fact their own host-specific lice then this raises the question of how these lice are transmitted. The most likely possibility according to Clayton & Johnson (2001) is that the lice are transmitted from adult to juvenile birds after they have left the nest. Common cuckoos also have their own specific lice, a likely transmission route being from adult to juvenile birds during feeding aggregation on the wintering grounds (Brooke & Nakamura 1998).

Phoresis (transport of lice from one host to another) on hippoboscid flies has been noted for lice on several host species (Keirans 1975). This could be an important mode of transmission from dead hosts. Clayton *et al*. (in press) suggests that transmission via phoresis may have an important role in the coevolutionary history of host-parasite associations.

Procellariiform and louse coevolution

The procellariiforms are an avian taxonomic order containing albatrosses, petrels, storm petrels and diving petrels. Their evolutionary relationships have been discussed by several researchers (Sibley & Ahlquist 1990, Nunn & Stanley 1998) and the albatrosses are considered a monophyletic group. The albatrosses have traditionally been put into

four natural groups: (1) southern mollymawks, (2) North Pacific albatrosses, (3) great albatrosses, and (4) sooty albatrosses (Tickell 2000). Nunn *et al.* (1996) examined evolutionary relationships among the 14 extant albatrosses using complete cytochrome –*b* sequences. They concluded that early in the evolution of the Diomedeidae two lineages arose, each then diverged to produce the four groups. The phylogeny indicated for the first time that the traditional genus *Diomedea* (Great albatrosses, North Pacific albatrosses and Southern Mollymawks) is paraphyletic. They revise the taxonomy of the group according to the phylogenetic tree, eliminating the paraphyly of *Diomedea* by renaming the Southern Mollymawk genus *Thalassarche*.

Evolutionary-rate calibrations were also made on the basis of fossil evidence that indicated the presence of a member of the *Thalassarche* existed around 10 million years before present (MYBP), and an albatross that shared affinities with *Phoebastria* existed around 15 MYBP. Cytochrome -*b* third-position rate estimates were 1.58% and 2.86% per million years, for *Phoebastria* and *Thalassarche* respectively, comparing them with their sister groups in the phylogeny. These rates are considerably slower than those previously found in mammals (Nunn *et al.* 1996). Further work by Nunn & Stanley (1998) indicated the lack of clock-like evolution in the procellariiforms as a whole, with rates of mitochondrial DNA evolution being slower for larger taxa.

Procellariiforms are parasitised by several genera of Ischnoceran lice. Given the high host-specificity of Ischnoceran lice and the small amount of physical contact between seabird hosts making host switching less likely, a strong pattern of cospeciation may be expected. Pthirapteran lice are highly host-specific on seabird hosts (Furness & Palma 1991, Smith 2001) and in general, louse presence reflects procellariiform phylogeny (Paterson *et al.* 1993, Paterson *et al.* 2000).

Paterson *et al.* (1993) discussed whether louse presence reflects the phylogeny of seabirds (petrels and penguins). Component analysis, presence/absence data and reconciliation analysis were used to compare host and parasite trees. They found evidence for several intrahost speciation events and many sorting events, but as expected, little evidence for host-switching. A recent paper by Paterson *et al*. (2000) compared louse trees derived from 12S rRNA to seabird (Procellariiformes and Spenisciformes) phylogenetic trees using Brooks Parsimony Analysis (BPA) and reconciliation analysis. They found that the number of cospeciation events was

significantly more than would be expected by chance. In addition they found no evidence for rate heterogeneity for seabirds and lice. The cospeciation events appear to have occurred synchronously.

This study: aims and methodology

In this study the host-parasite relationships of albatrosses and their chewing lice are examined and discussed. Albatrosses host 6 genera of chewing lice. *Perineus, Docophoroides* and *Paraclisis* are found on albatrosses and other procellariiforms while *Harrisoniella* and *Episbates* are unique to albatrosses. *Saemundssonia*, which are mostly found on charadriiform seabirds, are also found on some albatrosses. Within each genus, species found on albatrosses are not found on any other procellariiform taxa.

This study examined four of these groups (*Docophoroides, Harrisonella, Paraclisis & Perineus*) for which we have a large enough data set. The phylogenetic relationships in these louse groups and genera that are found on other procellariiform hosts are discussed. For each of the four groups parasite trees are compared to trees for their hosts and assessed for statistically significant cospeciation. Where they are incongruent, possible events causing this are assessed in terms of their evolutionary cost and biological likelihood. The results are discussed in the context of the ecology of hosts and parasites. An assessment of the validity of the results is made and discussed in the context of the present state of studies in this field.

Methods

Specimens

Lice were collected from live birds in the field. Collector's names and exact localities for each sample are listed on LOUSE BASE (http://r6-page.zoology.gla.ac.uk/lousebase/2/). Collection technique is generally by manual examination or through the use of a 'delousing chamber' (for description of methods see Ramili *et al.* 2000). Once collected, lice are sent to the University of Glasgow where they are stored in tubes in 95% alcohol.

Laboratory work was carried out to obtain DNA sequences for two genes, cytochrome oxidase subunit I (COI) and 12S ribosomal RNA, from samples of lice taken from procellariiform hosts. For a complete list of lice species used, as well as the host they were taken from, see Appendix 2. For a detailed report on laboratory procedures carried out (DNA extraction, PCR, gel electrophoresis and gel extraction), including laboratory protocols, see Appendix 1.

Phylogenetic Analysis

Alignment of sequences

The sequences were aligned using the program Clustal X (version 1.81). This involves establishing which regions of the sequences are homologous and can be compared. Insertions and deletions in DNA complicate the procedure by creating gaps. The task is to find the alignment with the lowest cost, effectively the smallest number of changes, with gaps carrying a higher cost than subsitutions (Page & Holmes 1998). Aligned sequences were then added to LOUSEBASE.

Taxa sampled

Sequences for the two genes for lice with Procellariiform hosts were taken from LOUSEBASE for phylogenetic analysis. In addition, for 10 species, sequences were obtained from lice sampled from more than one host species. Sequences from one individual were taken for 24 species, 2 individuals for 7 species, 3 individuals for 2

species and 5 individuals for 1 species. The data set also included sequences from 5 individuals that were only identified to the level of genus. In total, the data set therefore contained 54 individuals; 34 species and 5 unknowns. Sections of poor alignment in the sequences were discarded. An outgroup was used consisting of the following genera; *Anatoecus, Anaticola, Ardeicola, Docophoroides, Trabeculus*, & *Pectinopygus*.

Phylogenetic reconstruction

Sequences were analysed in PAUP* (version 4.0, Swofford 2001), using Neighbour-joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) methods.

NJ is a distance method that works by starting with a completely unstructured tree and then adds internal branches to improve the fit to the data (Saitou & Nei 1987). Distance methods convert aligned sequences into a pairwise distance matrix before inputting this into a tree building program (Page & Holmes 1998). NJ is a widely used method for tree building. As well as producing a single tree, it is also very quick to generate results (Page & Holmes

The 'whole-tree' (or discrete) methods of ML and MP work by comparing whole trees to the fit of the data. ML calculates the probability of observing alternative trees based on the composite probability of observing all events in the tree (Felsenstein 1981). ML is a useful method as it can incorporate explicit models of sequence evolution. The program MODELTEST (version 3.06, Posada 1998) was used to estimate parameters for ML branch swapping. MODELTEST tests the null hypotheses of equal base frequencies, transition rate equals transversion rate, equal transition rates and equal tranversion rates, rates equal among sites and no invariable sites. The program chooses the model that best fits the data according to the resulting p-values of the likelihood ratio tests performed (Posada & Crandell 1998). The model selected by MODELTEST was TVM +I+Γ, which has unequal base frequencies (T>A>G>C), 5 subsitution rates, invariable sites and gamma distributed rates at variable sites.

MP is less computationally intensive than ML. Using individual nucloetide sites as data points, it finds the tree that requires the minimum number of independent mutation events at each site to explain it (Majerus *et al* 1996). MP can produce more than one solution with an equal number of steps. A strict consensus tree was created for MP trees. Consensus trees summarise information common to two or more trees. The strict

consensus method includes only those groups that occur in all trees being considered (Page & Holmes 1998).

NJ and MP trees were given bootstrapping values to test the robustness of the trees obtained. Bootstrapping resamples trees a set number of times and displays the number of times each branch on the original tree is found in these trials (Felsenstein 1985). These values are not absolute confidence intervals but can give a general idea of the robustness of the tree created. MP and NJ trees were bootstrapped with 1000 replicates.

Reconciliation analysis

Reconciliation analysis of host and parasite trees was performed using TreeMap (version 2.0.1, Charleston & Page 2001). Reconciliation analysis embeds the parasite tree within the tree host tree, displays congruence and attempts to explain incongruence between the two by postulating evolutionary events such as duplications of parasite lineages, sorting events and host-switching. The total number of events the reconciled tree postulates is the ‘cost’ of the tree (Page & Holmes 1998). TreeMap was used to compare the phylogenetic trees of 4 Ischnoceran genera (*Docophoroides*, *Paraclisis*, *Perineus* and *Harrisoniella*) with their host (Procellariiform; Diomedaea) phylogenies. The albatross phylogeny was taken from Nunn & Stanley. (1998). TreeMap was used to compare each parasite group in turn to the host phylogeny using Jungle analysis (Charleston 1998). Jungle Analysis examines all possible scenarios and selects the outcome(s) that maximise the number of cospeciation events between the host and parasite trees. The maximum number of cospeciation events was then tested for statistical significance by comparison with a distribution of cospeciation events derived from generating 1000 random parasite trees and comparing them with the host tree. Statistical testing was carried out on TreeMap (version 1.0b, Page 1995).

Results

DNA sequencing

Appendix 2 shows the results of the laboratory work. Of the 21 samples, 5 were sequenced successfully for both 12S rRNA and COI. The success rate for producing PCR product was 51% (14 successes, 27 failures). For 4 of these successes, the Sequencing Unit were unable to complete the sequencing procedure. When carrying out PCR, on failure to obtain PCR product from the reactions, the amount of DNA in the PCR mix was increased. Appendix 2 shows that increasing the amount of DNA in the PCR mix only once lead to producing PCR product where before there had been none. A second strategy – to extract DNA from another louse in the sample, was never successful in eliciting PCR product. This may have been to do with problems in collection or storage of the samples. However, when a different sample for the same species was tried (n=2) these also did not lead to successful PCR.

Phylogenetic Relationships of louse taxa

Figures 1 & 2 show Neighbour-joining (NJ) trees for sampled louse taxa, with figure 1 presented as a phylogram and figure 2 showing bootstrap values for the branches. Figure 1 shows a fairly well resolved tree at the level of genera. It shows that procellariiform lice as a group are not monophyletic, in agreement with detailed studies of louse phylogeny (e.g. Smith 2001). The genera *Naubates* is polyphyletic, with two species (*Naubates harrisoni* and *Naubates fuliginosus*) differing significantly from the others and shown as a sister group to *Haliperus*. *Paraclisis* and *Docophoroides* are monophyletic groups. *Perineus* is a paraphyletic group, containing *Episbates pederiforms* and is a sister group to *Haffernia* and *Harrisoniella. Perineus circumfasciatus* sampled from different hosts is shown to be genetically undifferentiated, whereas all other species sampled from different hosts show very small amounts of genetic differentiation (much smaller than for between different species on the tree). The bootstrap values shown on Figure 2 support the divisions of the different genera of lice as described above, but are <50% for several of the divisions of species within these groups. For instance within *Docophoroides*, the relationships of the three species are unresolved. Within the *Haffneria/Harrisonella* group relationships are

well resolved, with bootstrap values of 70% on every branch. The split of *Naubates* has high bootstrap support.

Figure 3 shows the phylogram for the louse taxa created using the Maximum Parsimony (MP) method. There is resolution at the base of the tree, but figure 4 showing MP bootstrap values shows there is no support for relationships at this level as in the NJ tree. In contrast to the NJ tree, the genus *Docophoroides* is now within the monophyletic group of procellariiform lice, and is a sister group to the clade containing *Perineus* and the *Haffneria/Harrisonella* group, although bootstrapping does not support this relationship. *Naubates* is polyphyletic as in the NJ tree. In agreement with the NJ tree, *Perineus* is paraphyletic and a sister group to the *Haffneria* and *Harrisoniella*, with 73% bootstraping support for this relationship although the position of *Haffneria* in this clade is unresolved. Once again, the phylogram shows that differentiation between the same species on different host is much less than any differentiation between species of louse. Relationships within genera of lice are generally well supported by bootstrap values. However, there is a polytomy again in *Docophoroides* and relationships in the *Haliperus* clade are unresolved at the deeper level although there are high bootstrap values for resolving relationships between individual pairs or trios of the species in several cases.

The Maximum Likelihood (ML) analysis produced 2 trees the only difference between them was in the *Docophoroides* genus. The phylogram of these trees is identical and shown in Figure 5. The tree shows good resolution at all levels without any other polytomies. *Docophoroides* is a sister group to the clade containing all other procellariiform lice in agreement with the NJ tree. *Naubates* is again polyphyletic. The genus *Harrisonella* is monophyletic, in agreement with the MP tree. All other relationships between genera broadly agree with the NJ and MP analyses.

Relationships within the genera *Docophoroides, Paraclisis, Harrisonella* and *Perineus*

As the aim of the study is to compare relationships within these four genera to the relationships of their albatross hosts, the differences in relationships shown by different methods of phylogenetic analysis (NJ, MP and ML) merit detailed description. The relationship between species of *Docophoroides* differs between tree building methods. However, both the NJ and ML trees agree on the relationship between *D. brevis* and the

other two species of *Docophoroides* although the bootstrap support for the branch is only 52%. The MP tree differs at this branch but the bootstrap is >50%. We can conclude that the true relationship is more likely to be expressed in the ML and NJ trees. All trees suggest that *D. brevis* obtained from different host species are very similar, and that *D. brevis* extracted from *Diomedea epomophora* is a sister to *D. brevis* from the other two species of host. Within the genus *Paraclisis*, *P.confidens* and *P. miriceps* form a terminal group in all trees, with *P. hyalina*, as a sister group to these two. In all three trees *P.diomedaea* is shown as a sister to the clade containing all other species of *Paraclisis*, with high bootstrap support for this relationship in both the NJ and MP trees. The five samples of *P. diomedaea* taken from different hosts consistently show, with high bootstrap support, *P.diomedaea* extracted from *Phoebetria palpebrata* (Light-mantled sooty albatross) branching off from those extracted from *Diomedea* hosts which are grouped closely together.

In the genus *Harrisonella* the NJ and MP trees agree on the relationships between the three species, with *H. densa* as a sister group to the other two species and bootstrap values of >70% support this. The ML tree shows *H. ferox* as the sister group and *H. densa* is placed within the two species of *H. hopkinsi* extracted from different hosts. The ML phylogram (Figure 5) shows that the samples of *H. hopkinsi* from different hosts are very similar whereas *H. densa* is highly genetically differentiated from these two.

The genus *Perineus* is not monophyletic in any of the trees, as *Episbates pederiformes* is placed with this group in all trees and *Haffernia grandis* is also placed here in all trees apart from the NJ tree. In all trees *P. circumfasciatus*, *E. pederiformes*, and *P. nigrolimbatus* form a clade. In the NJ and ML trees *E. pederiformes* is a sister group to the other two species, I n the MP tree the relationship is unresolved due to low bootstrapping values (Figure 4). This group is then placed next to *P. concinnoides* in the NJ and MP trees but in the ML tree is placed next to *Haffernia grandis*. This relationship has >50% support in the NJ tree and only weak support in the MP tree (65%).

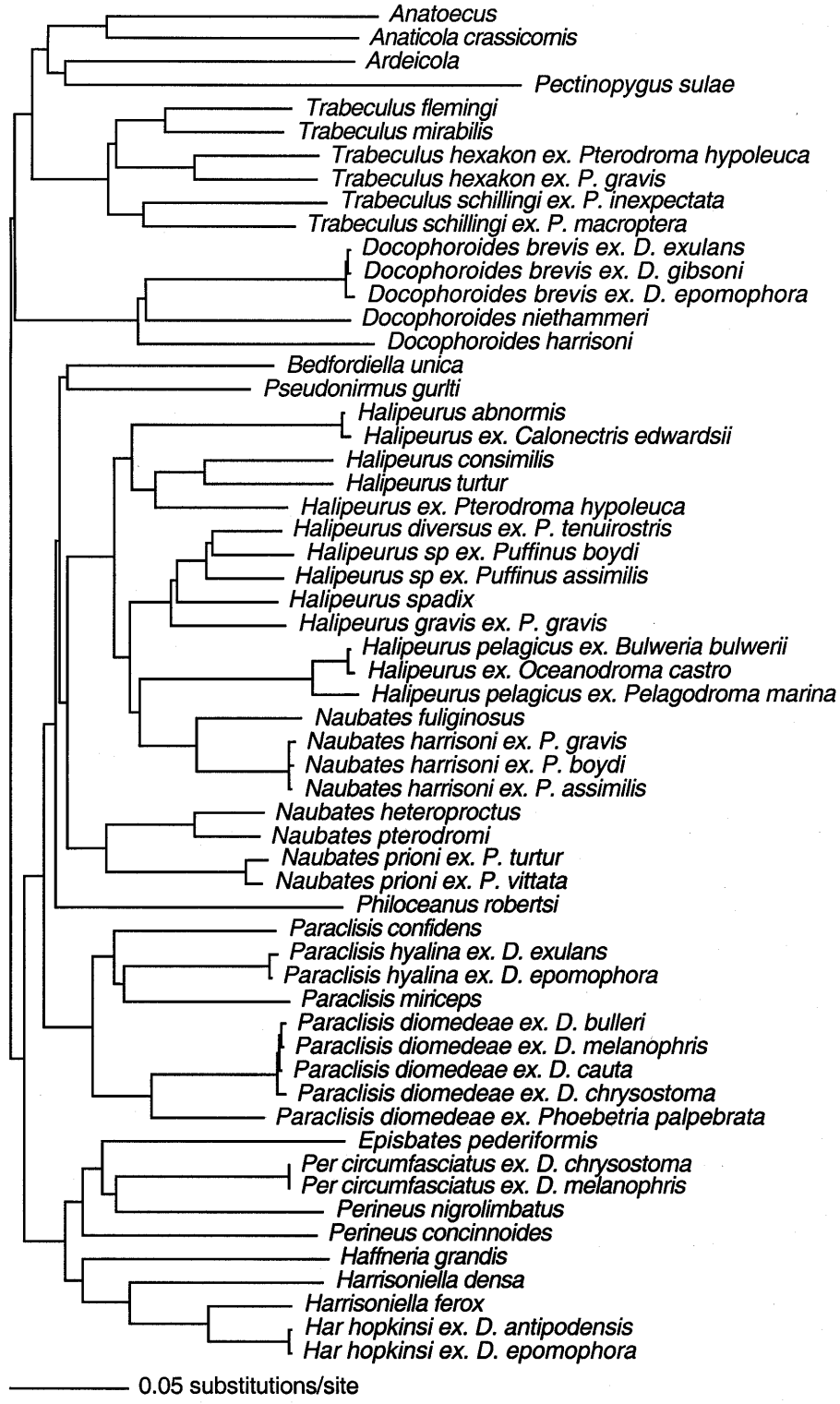


Figure 1. Neighbour-joining phylogram for procellariiform lice using COI and 12S rRNA sequence data. Host species are shown where a louse species has been sampled from more than one species of host.

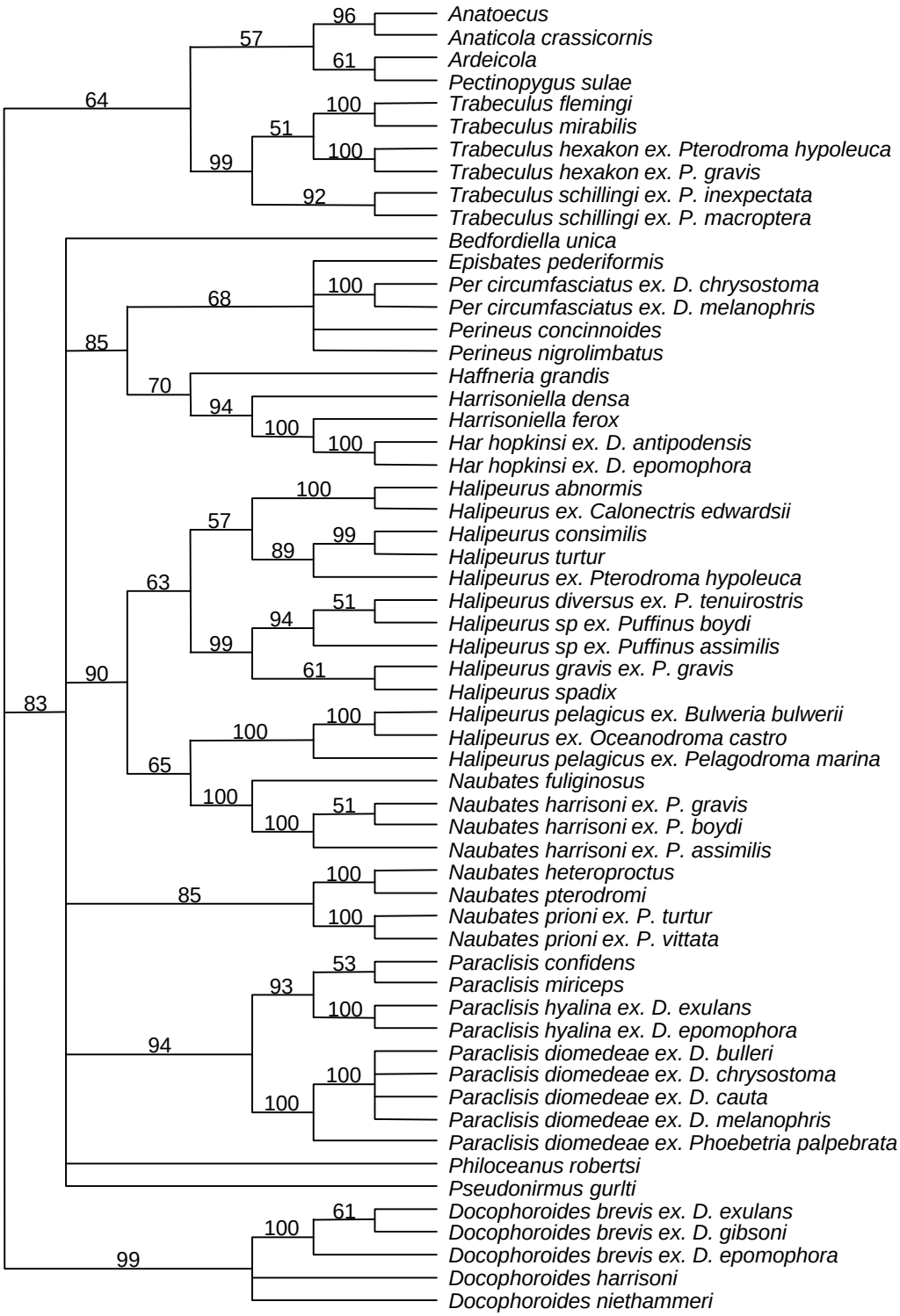


Figure 2. Neighbour-joining cladogram for procellariiform lice showing bootstrap values > 50% for branches, based on 1000 replicates. Host species are shown where a louse species has been sampled from more than one species of host.

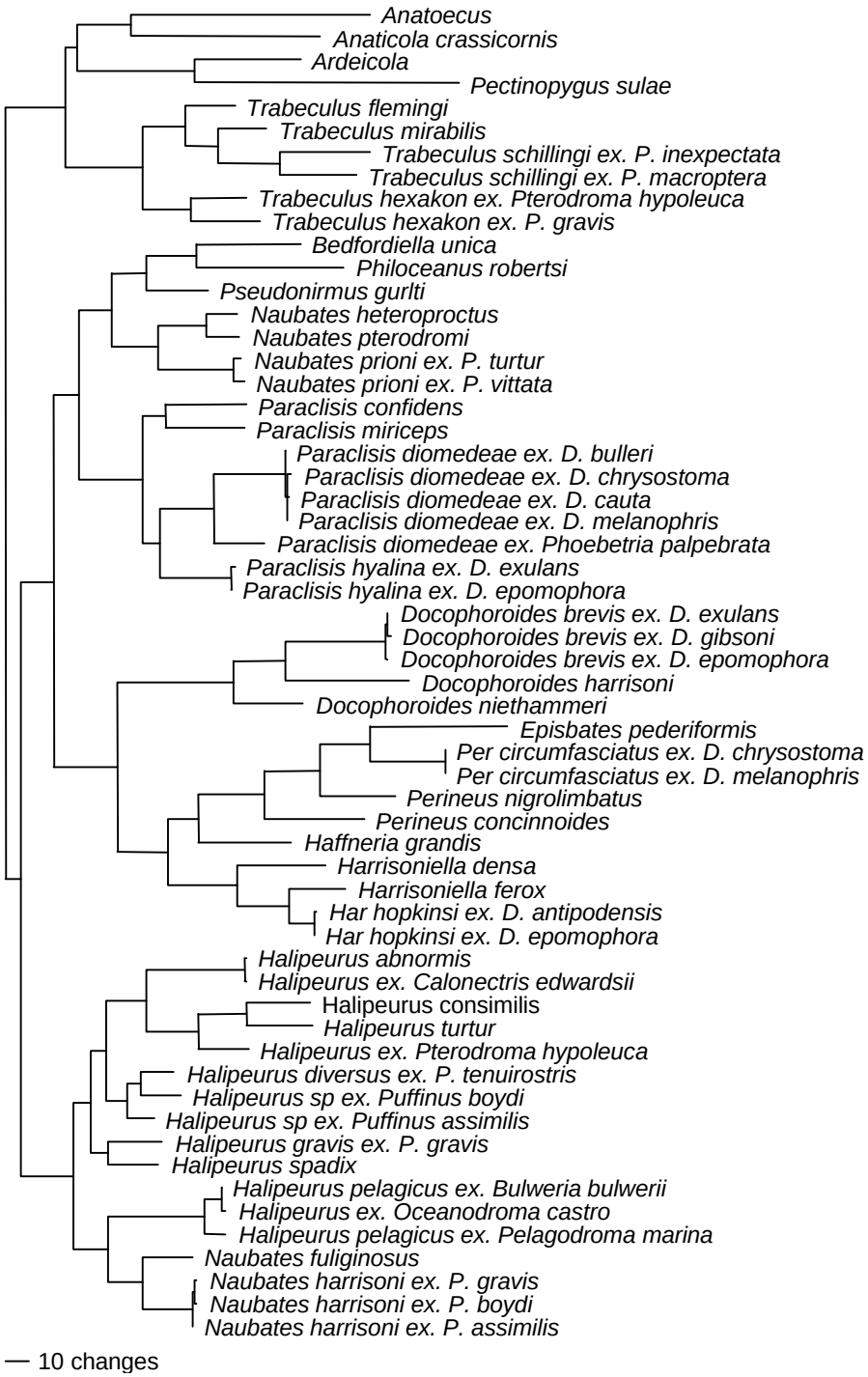


Figure 3. Maximum Parsimony phylogram for procellariiform lice based on COI and 12S RNA sequence data. The phylogram is a strict consensus of 25 Maximum Parsimony trees. Host species are shown where a louse species has been sampled from more than one species of host.

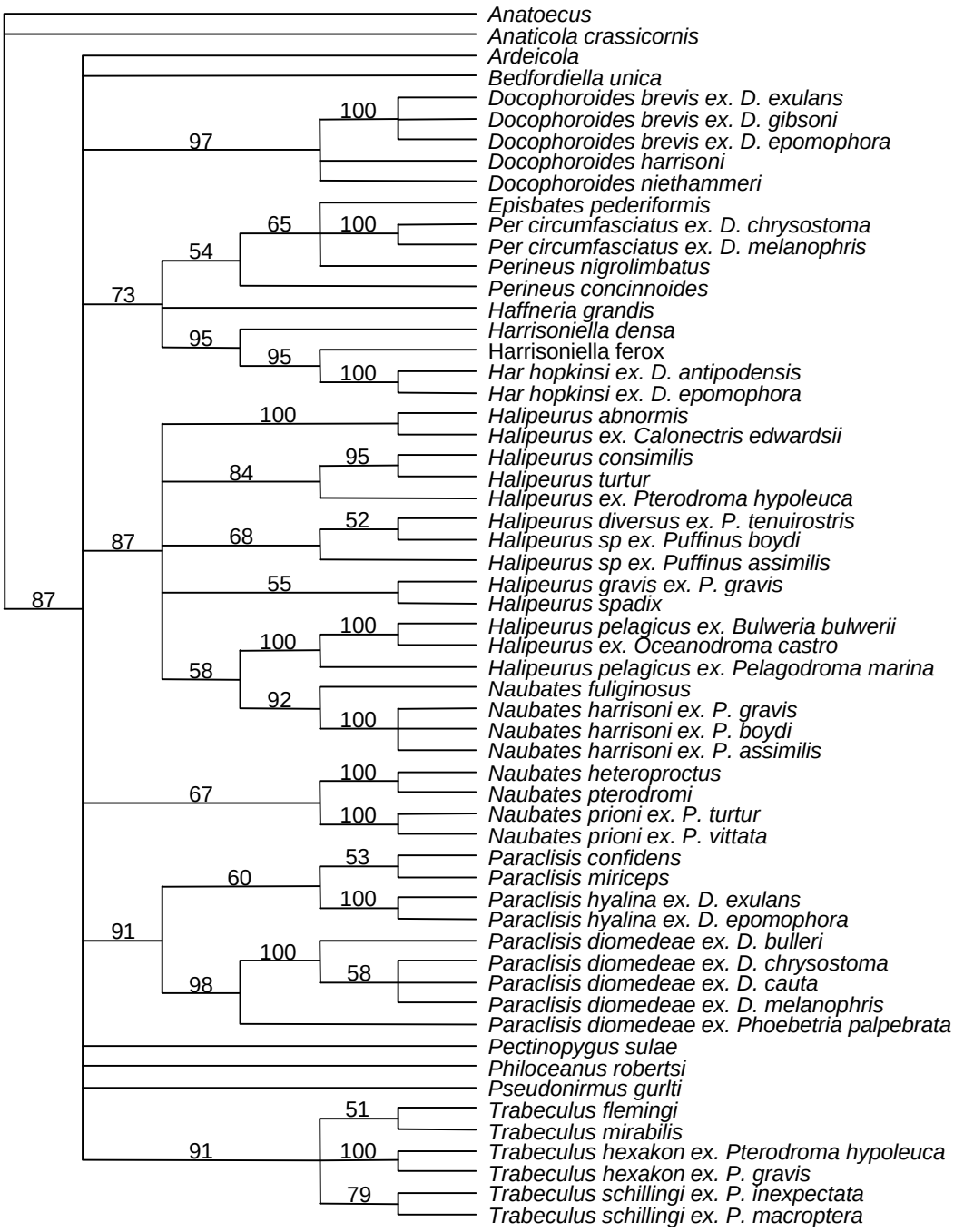


Figure 4. Maximum Parsimony cladogram for procellariiform lice, showing bootstrap values > 50% for branches, based on 1000 replicates. Host species are shown where a louse species has been sampled from more than one species of host.

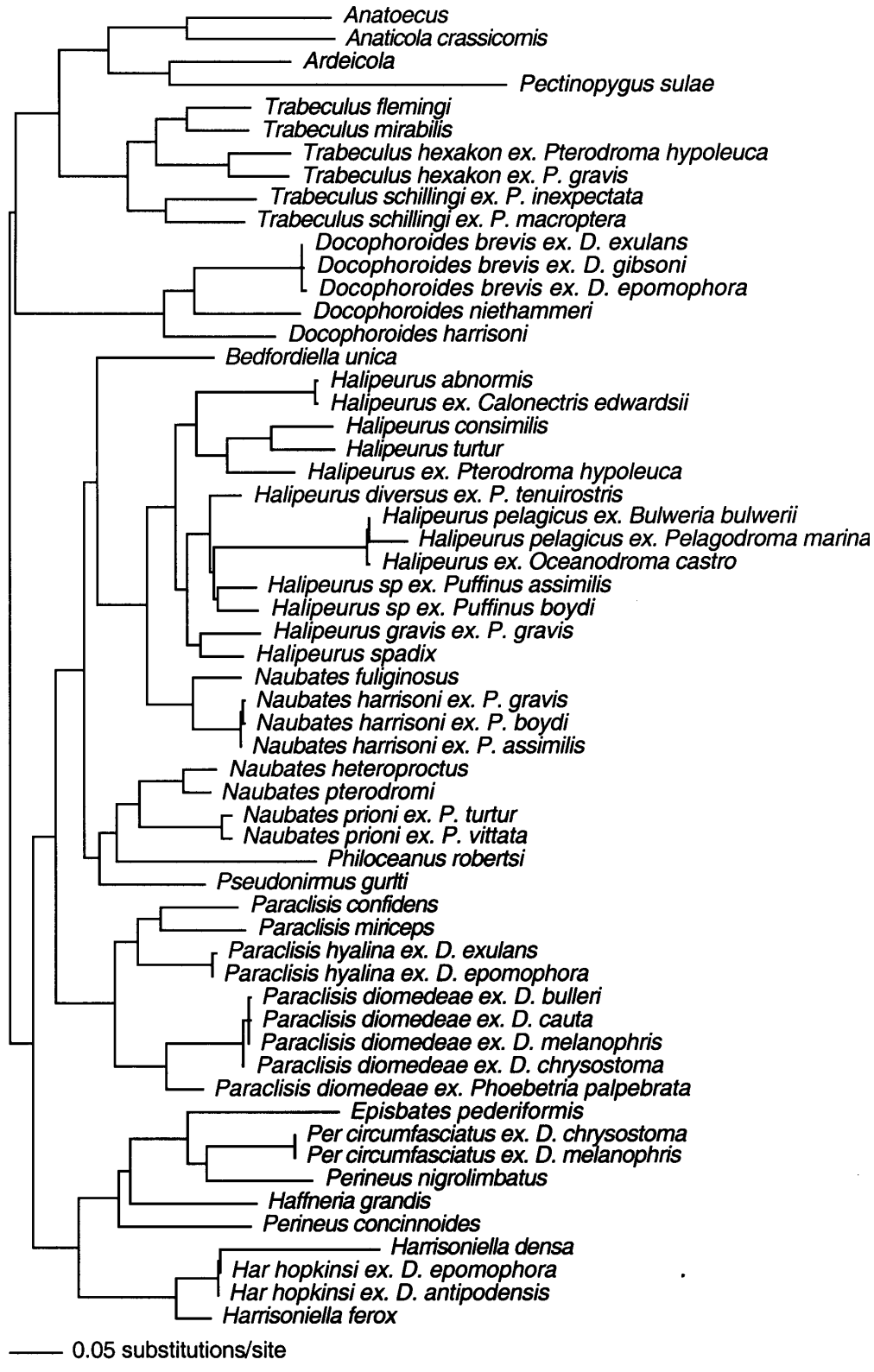


Figure 5. Maximum Likelihood phylogram for procellariiform lice based on COI and 12S rRNA sequence data. Host species are shown where a louse species has been sampled from more than one species of host.

Reconciliation Analysis

Docophoroides

Figure 6 shows host and parasite associations for the genus *Docophoroides* and their albatross hosts. As the host and parasite trees mirror each other, the Jungle Analysis produced one solution (Figure 7) which shows 4 cospeciations, no duplications, no sorting events and no switches. The reconstruction has a cost of zero. Using TreeMap the 4 cospeciations were tested for statistical significance. The histogram produced (Figure 8) shows that obtaining the 4 observed cospeciation events is unlikely to be due to chance alone (p = 0.000).

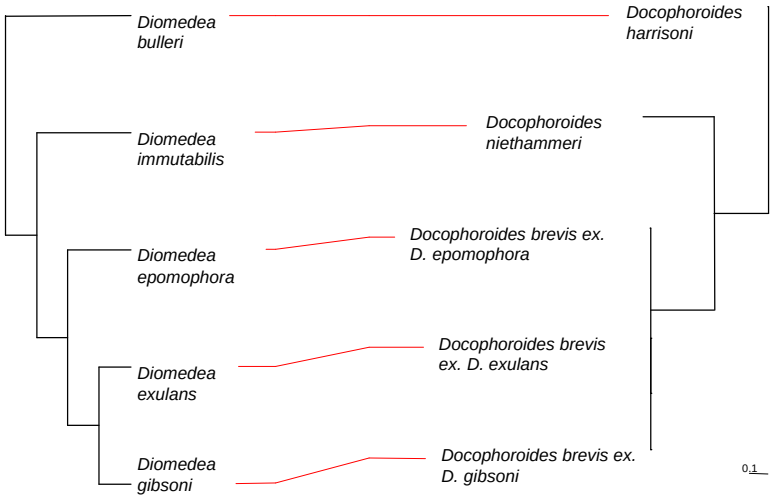


Figure 6. Phylogram for the louse genus *Docophoroides*, shown with host phylogram (Nunn & Stanley 1998). Connecting red lines show host-parasite associations.

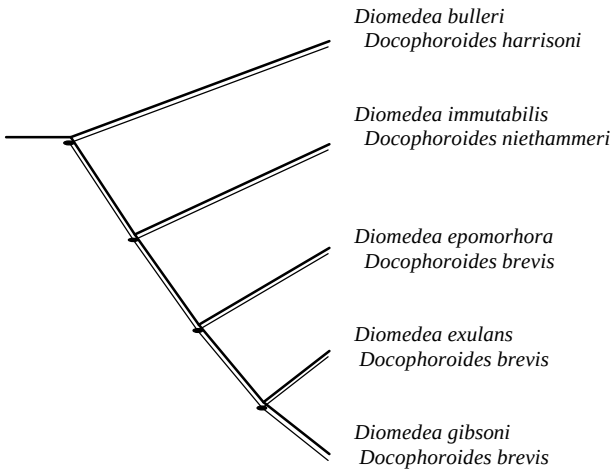


Figure 7. Host and Parasite associations for the genus *Docophoroides* and *Diomedeae* hosts. Black circles indicate cospeciation events. The lower thin lines indicate the parasite phylogeny.

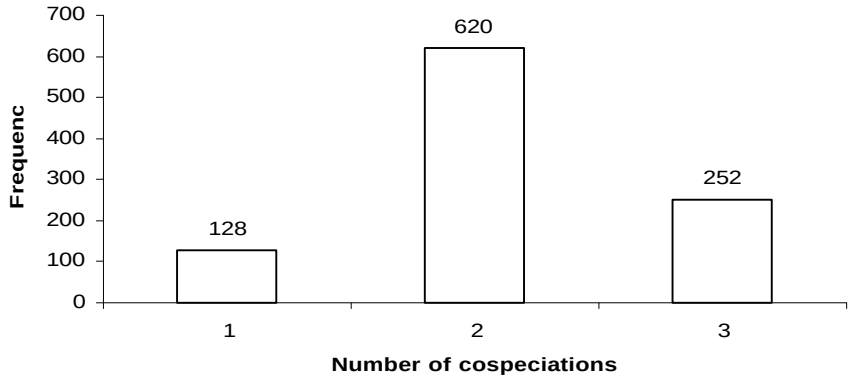


Figure 8. Histogram of the number of cospeciation events between *Docophoroides* lice and albatross hosts generated from 1000 random parasite trees using the Yule (Markovian) model.

Harrisonella

Figure 9 shows phylograms for the genus *Harrisonella* and their albatross hosts. Jungle analysis produced 5 optimal solutions from 37 possible solutions. These are shown in Figures 10(a)-(e). Which solution (or possible solutions) that we decide is most likely, may depend on both the cost of that evolutionary scenario (as given in the Jungle analysis) and on the biology of the host-parasite system. Some solutions may seem more likely than others given our knowledge of ecology. As discussed above, in procellariiforms host-switching could be considered to be unlikely to occur in comparison to sorting events. All but one (Figure 10(b)) of the 5 optimal solutions involves a host-switching event. The maximum number of cospeciation events between albatrosses and *Harrisonella* chewing lice, shown by the reconstructions is 2, supported by Figures 10(b), (c) & (d). These are also the reconstructions with the least number of host-switches and the lowest cost. Figure 10(b) postulates 3 sorting events and one duplication, whereas (c) & (d) contain only 1 sorting event each. The differences between these two reconstructions simply lies in the placement of the sorting event and the direction of the host-switch. In Figure 10(c) *H. hopkinsi* colonises *D. epomorpha*, whereas in 10(d) *H. hopkinsi* is associated with *D. epomorpha* by descent and colonises *D. immutabilis*.

To test whether 2 cospeciations could be obtained by chance alone, 1000 random parasite trees were generated using the Yule (Markovian) model. The resulting distribution shows that 2 cospeciation events were generated 484 times in 1000 pairs of random host and parasite trees. This suggests that the 2 observed cospeciations in Figures 10(b)-(d) may be due to chance alone ($p>0.05$).

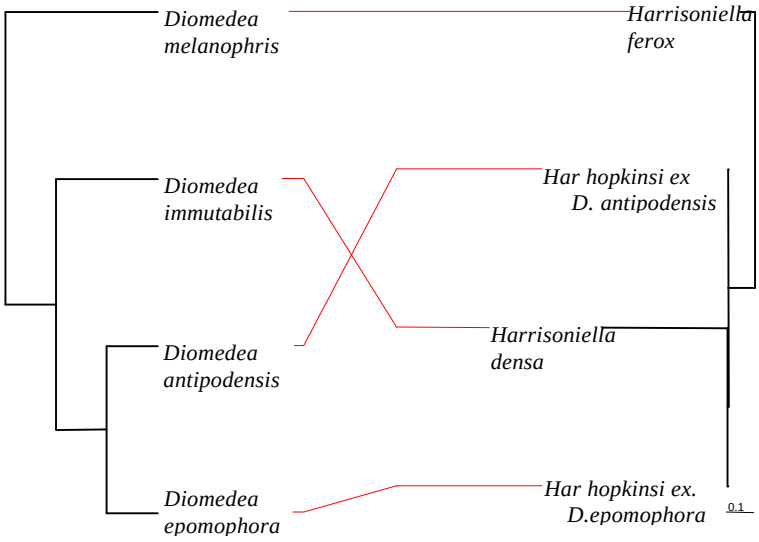
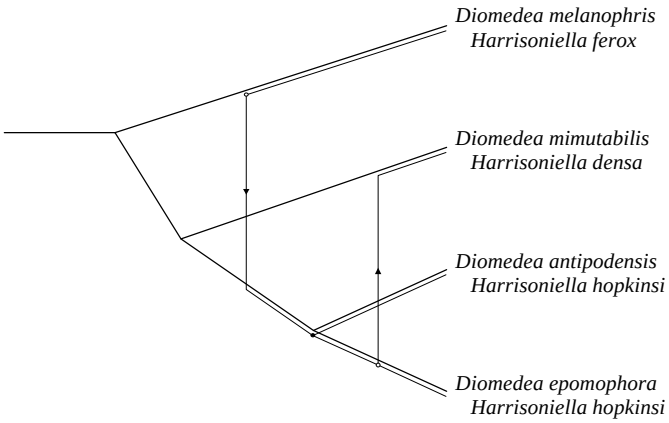
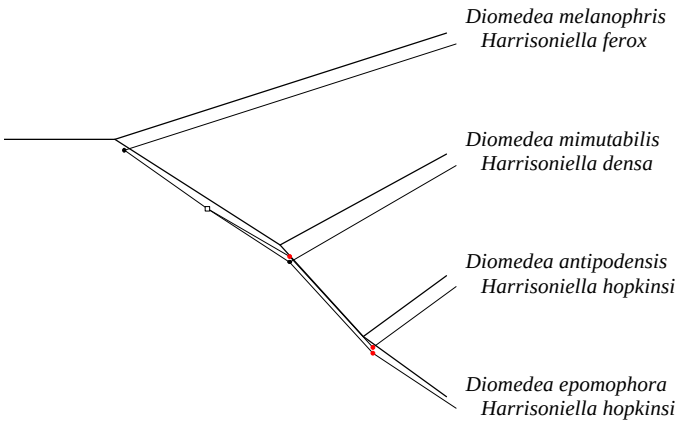


Figure 9. Host-parasite phylograms for *Harrisoniella* lice. Host-parasite associations are indicated by connecting red lines. Host phylogram from Nunn & Stanley (1998).

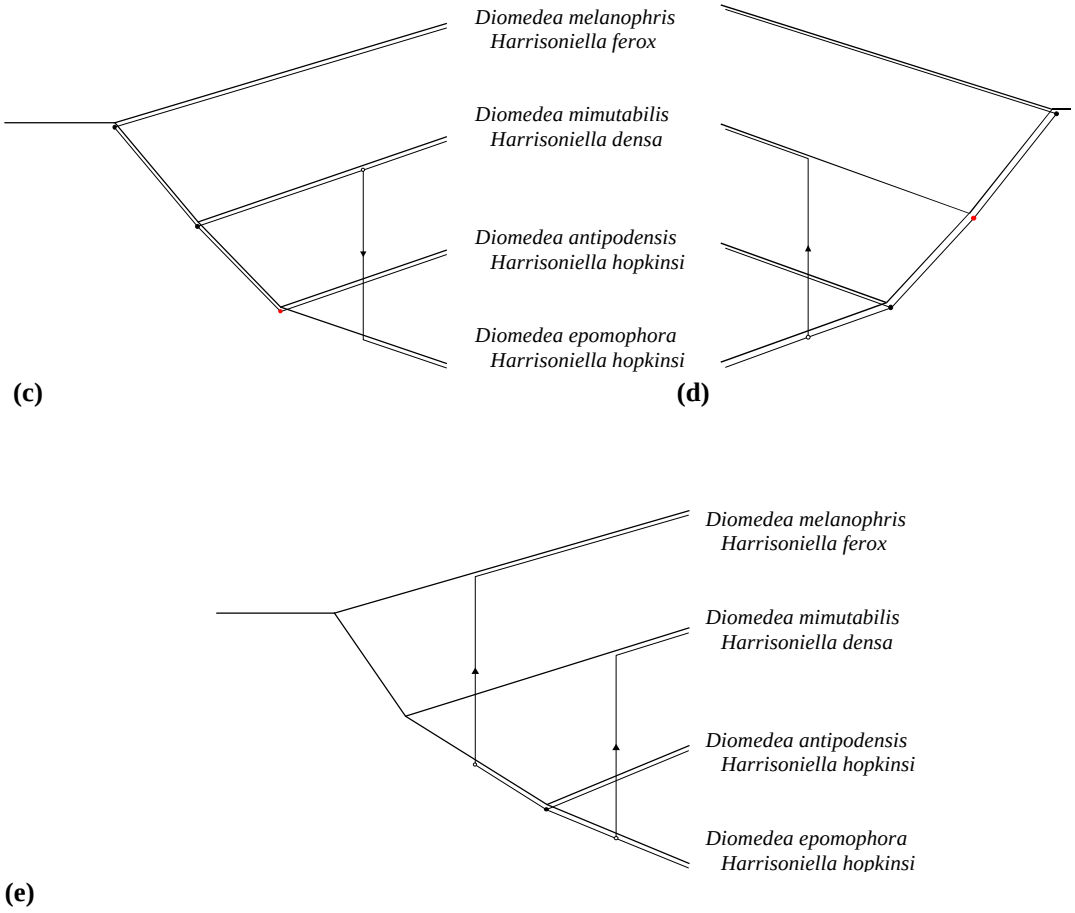


(a)



(b)

Figures 10 (a)-(e). The five optimal reconstructions of the albatross-*Harrisonella* associations. Thin lower lines indicate the parasite phylogeny. Filled circles indicate cospeciations, open circles show host switches, red circles show sorting events and open squares indicate duplication events.



Figures 10 (a)-(e). The five optimal reconstructions of the albatross-*Harrisonella* associations. Filled circles indicate cospeciations, open circles show host switches, red circles show sorting events and open squares indicate duplication events.

Perineus

Figure 11 shows associations between the *Perineus* clade of lice and their hosts. Jungle analysis produced 5 optimal solutions from 84 possible solutions, shown in Figures 12(a)-(e). The number of cospeciations postulated by these solutions ranges from 2-3, with the cost of solutions given as 18,23,20,15,15 (12(a)-(e) respectively). The taxonomic relationships of the hosts immediately suggests host switching of lice between the *Diomedea* hosts and *Catharacta skua* and *Fulmarus glacialis*. This is supported by Figures 12(a), (d) & (e) which are the lowest in cost. These 3 solutions all postulate 2 cospeciations, Figures 12(d) & (e) minimise sorting events, but both have 3 host switches, while (a) has 2 host switches, 3 sorting events and one duplication.

To test whether 2 cospeciations could be obtained by chance alone, 1000 random parasite trees were generated. The resulting distribution found 584 pairs of host and

parasite trees where there were 2 cospeciation events. This suggests that the postulated cospeciation events may be due to chance alone (p>0.05).

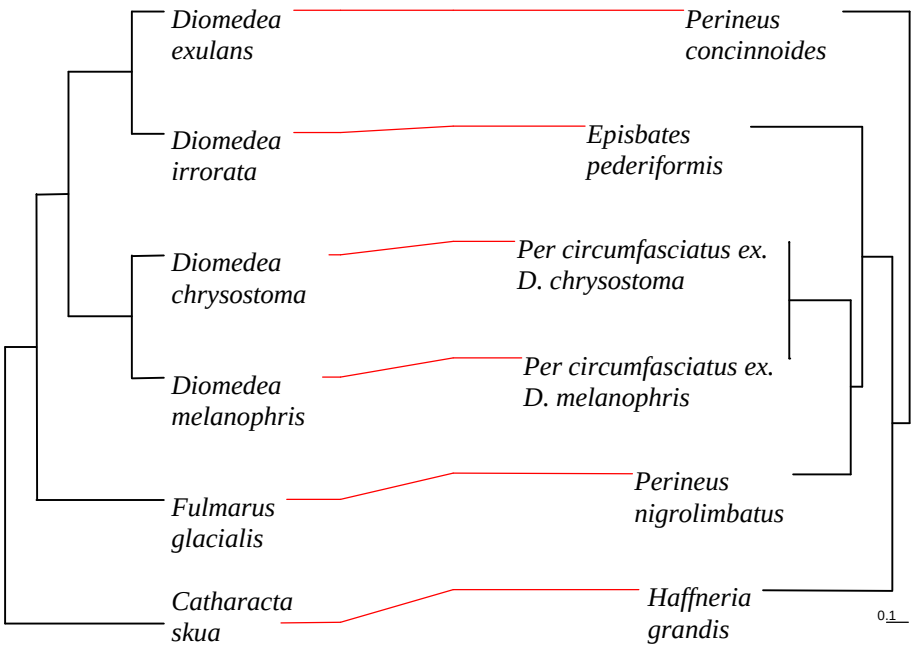
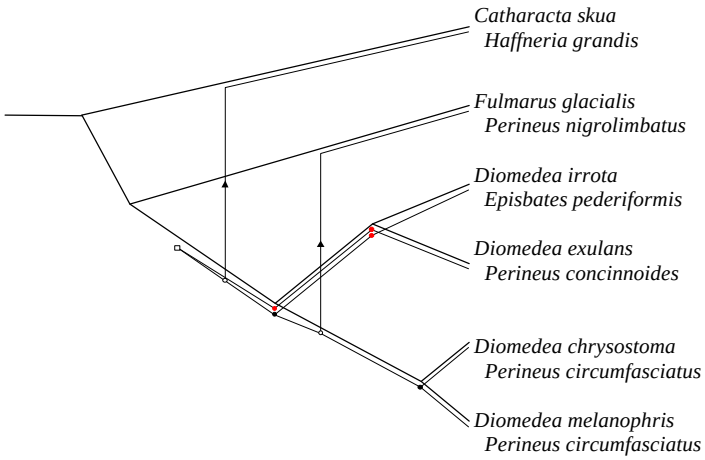
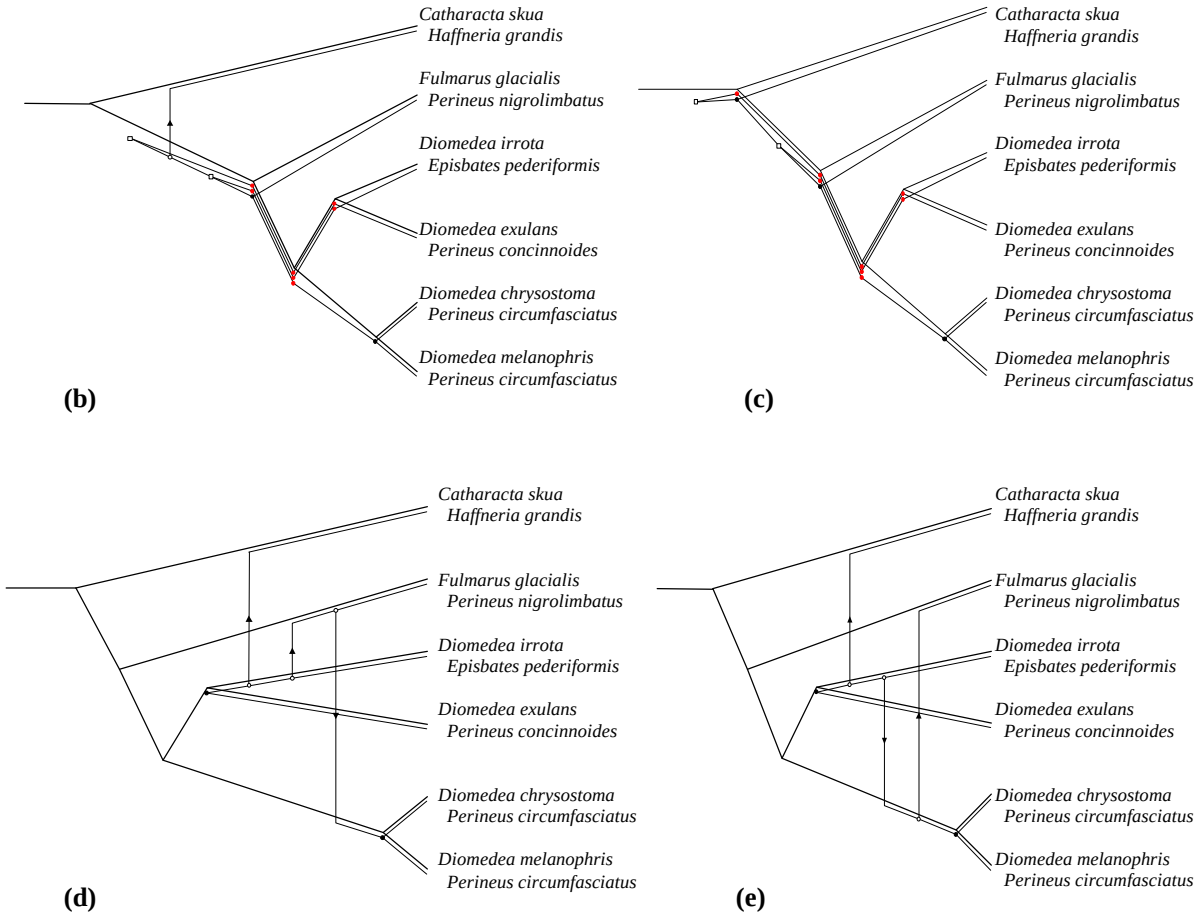


Figure 11. Phylograms for *Perineus* lice and hosts. Host-parasite associations are indicated by connecting red lines. Host phylogram from Nunn & Stanley (1998).



(a)

Figures 12 (a)-(e). The five optimal reconstructions of host-*Perineus* associations. Filled circles indicate cospeciations, open circles show host switches, red circles show sorting events and open squares indicate duplication events.



Figures 12 (a)-(e). The five optimal reconstructions of host-*Perineus* associations. Filled circles indicate cospeciations, open circles show host switches, red circles show sorting events and open squares indicate duplication events.

Paraclisis

The host-parasite associations between *Paraclisis* and their albatross hosts are shown in Figure 13. Jungle analysis found 2025 solutions, 23 of which were optimal. The 23 solutions ranged in cost from 9-25 and contained between 3-7 cospeciation events. All solutions bar one involved at least one instance of host-switching and many involved between 3-5 host-switches. The solutions optimising cospeciation events are shown in Figures 14(a) – (c). All of these postulate a sorting event to explain why *D. immutablis* does not host any *Paraclisis* lice. Figures 14(a) & (b) postulate host-switching in *P.diomedeae* whereas (c) explains the distribution of *P.diomedeae* on different host species through a duplication event and 3 subsequent sorting events.

To test whether 7 cospeciation events could be generated by chance alone, 1000 random parasite trees were generated. The resulting distribution showed that obtaining the 7 observed cospeciation events is unlikely to be due to chance alone ($p = 0.000$).

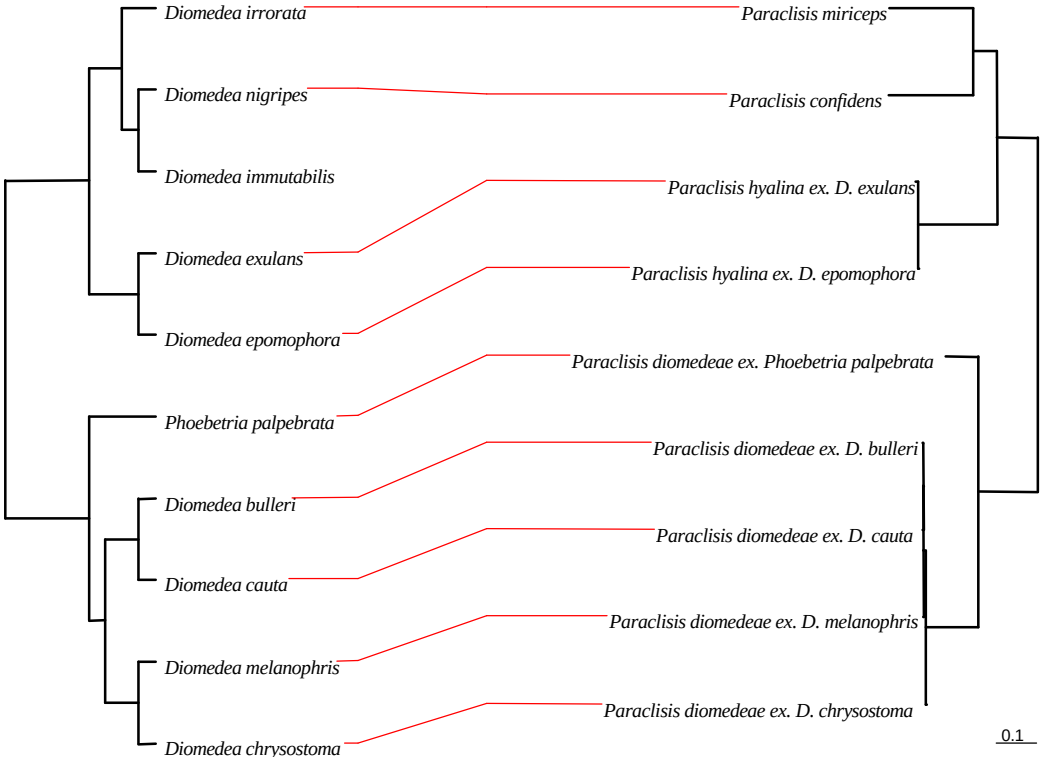
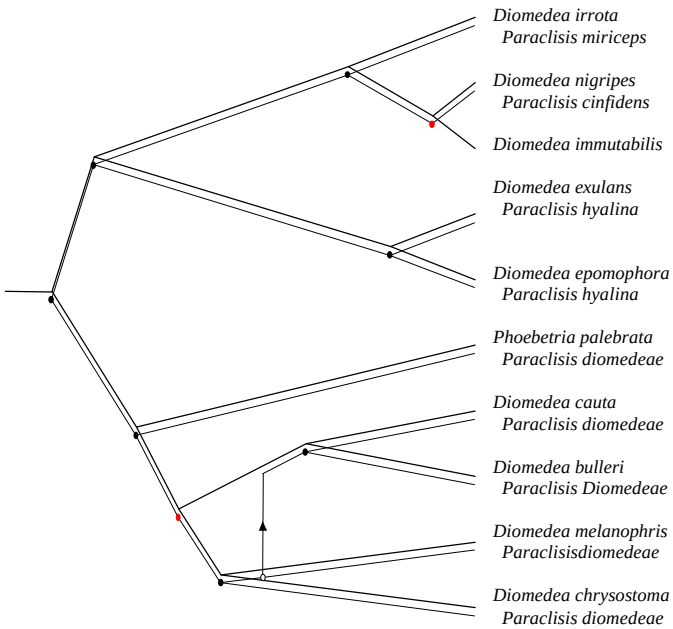
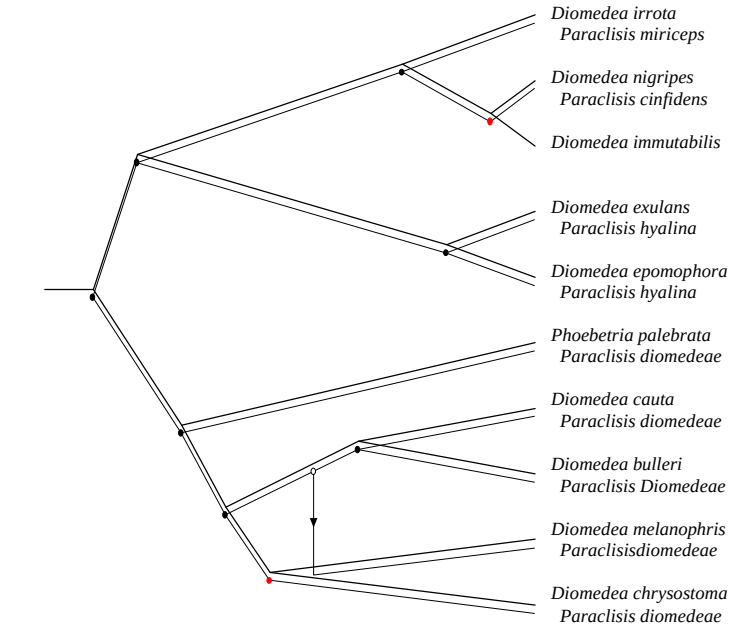


Figure 13. Phylograms of albatross hosts and *Paraclisis* lice. Host-parasite associations are indicated by connecting red lines.

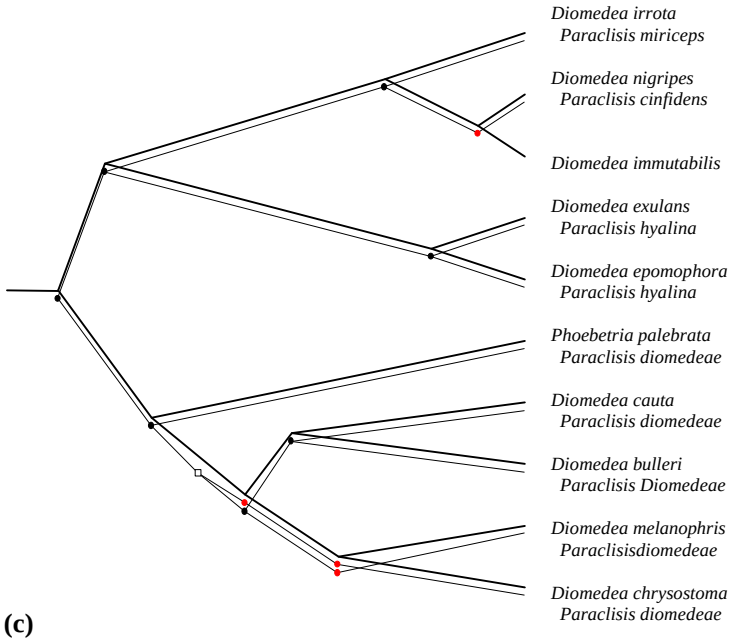


(a)

Figures 14 (a)-(c). The optimal reconstructions of host-*Paraclisis* associations that maximise cospeciation events. Filled circles indicate cospeciations, open circles show host switches, red circles show sorting events and open squares indicate duplication events.



(b)



(c)

Figures 14 (a)-(c). The optimal reconstructions of host-*Paraclisis* associations that maximise cospeciation events. Filled circles indicate cospeciations, open circles show host switches, red circles show sorting events and open squares indicate duplication events.

Discussion

Cospeciation in four genera of Ischnoceran Lice and their albatross hosts

Analysis of the host and parasite trees of the four genera of Ischnoceran lice on albatrosses show that the degree of cospeciation differs between groups. In the genus *Docophoroides* there is a high level of congruence between host and parasite trees with a statistically significant number of cospeciation events. *D.brevis* from three different host species, which are all Great Albatrosses (a monophyletic group, Nunn *et al*. 1996), show some genetic differentiation. Previous to Nunn *et al.* (1996) *D.gibsoni*, was seen as a subspecies of *D.exulans*. The louse phylogeny reflects the closer relationship between *D.gibsoni* and *D.exulans* relative to the third host species *D.epomophora*. Although the Great Albatross species breed at the same sites, the nests are very scattered (Tickell 2000) and this may faciliate cospeciation between *Docophoroides* and hosts. Transmission of lice between species of Great Albatross may be rare, thus decreasing the chances of host-switches occurring.

The genus *Harrisoniella* shows less phylogenetic congruence with the host phylogeny, with a maximum of two cospeciation events and these may be due to chance alone. Many of the reconstructions show host-switching, especially between lice on *D.epomorpha* and *D.immutablis* and between the louse *H. ferox* on *D.melanophris* and the common ancestor of *H. hopkinsi* on *D.antipodensis* and *D.epomorpha*. The pattern of associations is hard to explain. A switch of hosts between *D.epomophora* and *D.immutabilis* seems unlikely, as their distributions do not overlap. *D.immutabilis* (the laysan albatross) is in the North Pacific Ocean, very rarely crossing the tropics and *D.epomophora* in the Southern Ocean. The only reconstruction that does not show this switch (Figure 10(b)) shows a duplication in the louse phylogeny, indicating that the procellariiform ancestor hosted two lineages of lice, one that lead to *D.immutabilis* and *H.hopkinsi* on *D.epomophora* and one that lead to *H.hopkinsi* on *D.antipodensis*. Three sorting events explain the loss of descendants of each of the louse lineages on several of the host species. Sorting events could be considered to be fairly likely, given seabird ecology. However, some evidence suggests there is little confidence in the topology of the parasite tree for the *Harrisoniella* genus. It seems unlikely that *H.densa* and *H.hopkinsi* from one species of host are more closely related than *H.hopkinsi* extracted

from each host are to each other. The NJ and MP trees indicate the opposite conclusion in this case and with high bootstrap values in support.

Reconstructions for the association between the *Perineus* genus and their hosts showed very few cospeciation events and no evidence that these are not due to chance agreement in the topology of the host and parasite trees. Relationships in the genus are unclear, with two species outside the genus included in the group. There is also the presence of two non-albatross hosts, suggesting duplication in the ancestor of all species of *Perineus*. In one of the these lineages there is a host switch to the great skua (*Catharacta skua*) and either the reconstruction is incorrect or there is a taxonomic misalliance of the louse *Haffneria grandis*. The second host switch comes from the ancestor of *P. circumfasciatus* from a *diomedea* host to the northern fulmar (*Perineus nigrolimbatus*). Several sorting events are also suggested, two of them being in the split between the hosts *D. exulans* and *D. irrorata*. The distribution of these two host species provides evidence for the likelihood of these sorting events. *D. irrorata* (the gálapagos albatross) is the only tropical albatross, with a very restricted breeding range, whereas *D. exulans* (the wandering albatross) is a Great albatross of the Southern Oceans. The split between these two hosts would be very likely to cause a particular species of louse to 'miss the boat', effectively caused by a Founder effect in the host population. *P. circumfasciatus* is taken from two albatross hosts and Figures 5, 3 & 1 show that there is no genetic differentiation between lice taken from each host. As these two species of host, both in the monophyletic group of Southern Mollymawks (Nunn *et al.* 1996) are sympatric and can nest in overlapping colonies nesting close together (Tickell 2000), it is not surprising that they have not diverged with their hosts. As a whole the group shows little evidence of cospeciation.

Associations between albatrosses and lice in the genus *Paraclisis* show a large number of cospeciation events that are unlikely to be due to chance congruence between host and parasite tree topologies. *P.diomedeae* from different hosts shows a clear spilt, in all reconstructions, between cospeciation with *Phoebetria palpebrata*, the light-mantled sooty and a mixture of cospeciation, sorting events and host-switching within all other hosts of *P. diomedeae* (all Southern Mollymawks). The distributions and breeding islands of the Southern Mollymawks and the light-mantled sooty overlap, but the lack of host-switching and the greater genetic differentiation of *P. diomedeae* on the sooty could be explained by the separate breeding habitat of the sooties. They breed on much

steeper ground than other albatrosses (Tickell 2000) and this might decrease the chances of host switching onto non-sooties. The second clade of *Paraclisis* lice, comprising of *P. miriceps*, *P. confidens* and *P. hyalina*, shows a pattern of cospeciation with one sorting event, where the ancestor of *P. confidens* speciates with *D. nigripes* but is lost from the host *D. immutabilis*. However, it is unclear whether this sorting event is not just an artifact of incomplete data in this study. The laysan albatross, *D. immutabilis*, does host a louse from this genus; *P. giganticola*, which is missing from our database and should it be added, might be placed over this sorting event, cospeciating with *D. immutabilis*.

In summary, cospeciation appears to be greater in some genera of lice than others. The genera *Docophoroides* and *Paraclisis* show quite clear patterns, with a majority of species cospeciating with hosts. The patterns in the genera *Perineus* and *Harrisoniella* are less clear and may be confounded by incomplete sampling of host taxa and possible errors in the topology of the louse phylogeny. However there is an indication of a history containing duplication of louse lineages followed by sorting events and host-switches to unrelated seabirds.

Rates of evolution and the timing of evolutionary events

The comparison of host and parasite phylogenies can be greatly enhanced if the relative timing of evolutionary events is known. Even if parasite and host trees share the same topology, there may have been temporal variation in speciation events. For the genera of albatross lice showing cospeciation, it would be necessary to test the null hypothesis of identical speciation times

Knowledge of the relative time of divergence between host and parasite species can help to distinguish between the various explanations of incongruence presented by the Jungle analysis. For instance, it may be possible to distinguish between explanations based on host switching and extinction of parasite lineages.

The ecology of host-parasite coevolution in albatrosses and their lice

Why should louse genera differ in the amount of phylogenetic congruence they show with their hosts? Clayton *et al.* (in press) discuss using the ecology of extant host and parasite species and the associations between them to explain differences in the degree of congruence among related host-parasite systems. Albatrosses vary widely in the degree of sympatry and syntopy between species. Most species are colonial, breeding on small islands, but nest densities are variable with species and location (Tickell 2000). Colonies may be interspecific, or adjacent intraspecific colonies may overlap at boundaries (Tickell 2000). There are also temporal differences between some sympatric species in breeding season, this will influence the potential for contact between them. Where hosts are sympatric and syntopic, these distributional factors will promote phylogenetic congruence between lice and their hosts, both through failure to speciate and through host switching.

Previous studies (e.g. Paterson *et al.* 1993) have considered host switching unlikely in procellariiform lice due to a lack of physical contact between species. In the scenario of closely nesting colonial species however, it seems reasonable to suggest that host switching may occur. Although being able to move onto a foreign host does not necessarily mean being able to survive and reproduce on that host, as discussed below. Outside of breeding colonies, there are interspecific interactions of seabirds at pelagic food sources, whether these could facilitate host switching is not clear, but it seems unlikely that they play a major role.

Several studies have examined the distribution of procellariiform lice on hosts and found them to be patchy (Rékási *et al.* 1997, Paterson *et al.* 1999). Rékási *et al.* (1997) also showed that lice of colonial birds tend to form less variable subpopulations in comparison to territorial host species. Patchy distributions of lice will increase the likelihood of intrahost speciations and also increase the chances of lice ‘missing the boat’ (MTB) when hosts speciate (Clayton *et al.* in press). Paterson et al (1999) have suggested that the explanation for the reduced numbers of species of lice on New Zealand birds was that of many species going through MTB events when their hosts went through Founder events.

The abundance of lice on their hosts will also influence congruence between lice and their hosts through its effect on the likelihood of MTB and extinction events. If the prevalence and the mean intensity of lice are low then stochastic events will be more likely to occur. There is some evidence that this is the case in procellariiform lice (Paterson *et al.* 1999, Furness & Palma 1991). Whether abundances differ between genera of lice and between different species of host is unclear but there is a suggestion that host body mass may influence mean intensity of ectoparasite (Ròsza 1997). Albatrosses carry out both allo- and auto- preening and these may act together to decrease parasite loads from all parts of the body (and thus both wing and body lice) although there is no direct evidence for this (Tickell 2000).

Although the factors described above may influence the likelihood of a host switching event occurring, a switch to a foreign host also requires establishment on that host (Clayton *et al*. in press). Adaptations to a particular host may prevent survival on a foreign host, host-specific parasites may therefore find it harder to successfully establish on foreign hosts. Asymmetric competitive effects may explain why some lice are able to switch hosts, while others so not. Clayton *et al* (in press) show in an earlier experiment that wing lice compete badly in the presence of body lice, while body lice are unaffected by wing lice. Cruickshank *et al.* (2001) suggest that the host switch of procellariiform lice onto charadriiform hosts may have been facilitated by the differential adaptation to microhabitats on the host of the procellariiform and charadrriiform lice.

Examining the above factors in the genera of chewing lice on albatrosses may help to understand the different patterns of phylogenetic congruence that this study has found. In summary, there are a number of points in general that can be made about the ecology of the association between albatrosses and their lice. Where hosts are sympatric and syntopic, opportunities may exist for host switching. However these opportunities are likely to be infrequent and establishment on foreign hosts may be limited by competitive exclusion and maladaptive traits in the parasites. These factors will increase the amount of phylogenetic congruence between hosts and parasites. In contrast, other ecological aspects may favour events that reduce congruence between host and parasite phylogenies. The distribution of parasites is likely to be patchy which will increase the probability of duplication and MTB events occurring. Further work with this group could examine each louse genus to discuss how these ecological factors are affecting

their coevolutionary history and present associations. A large amount of ecological data on the above factors would be invaluable and would complement the coevolutionary analysis.

Previous work (Tompkins & Clayton 1999) has used experimental techniques to examine the questions of adaptations to specific hosts, asymmetric competitive effects and host switching. Although these transfer experiments may not be particularly practicable in the case of albatross hosts, in other host groups they have been the best way to provide answers to these questions.

Criticisms of the present study: Sampling and the validity of the phylogenetic analysis

As Page et al. (1996) state, one of the requirements in testing for cospeciation is exhaustive sampling of host and parasite clades. As discussed above, in this study there are several instances of missing louse taxa possibly leading to misanalysis of evolutionary events in the association between parasites and hosts. Filling in these gaps would give greater confidence in the observed patterns of association.

Confidence in the robustness of the parasite phylogeny is obviously important when testing for cospeciation. There is some evidence (Johnson *et al*. 2001) that the COI gene has a high amount of sequence divergence compared to other commonly used genes. As COI might be subject to more multiple substitutions at these divergences, methods that take into account rate differences, such as Maximum Likelihood which was used in this study, should provide a good estimate of phylogenetic relationships (Johnson *et al.* 2001).

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Appendix 1.

Laboratory Methods

Laboratory Protocols

All laboratory work was carried out under standard laboratory safety and experimental protocols and polymerase chain reaction (PCR) hygiene recommendations (Kwok & Higuchi 1989). This included the use of latex gloves (with frequent changes), sterilising all surfaces and tools with alcohol before use, using sterilised water, handling toxic substances in the fume cupboard and disposal of waste material in the appropriate bins. Equipment such as pipette tips and Eppendorf tubes were autoclaved before use. Gilson pipettes were kept separate and labelled as such for the pre-and-post PCR stages. The use of positive displacement pipettes with disposable tips decreased the chances of contamination by aerosols. When working in the UV room, protective facemasks were worn. Negative controls were carried out at all stages to check for possible contamination.

DNA Extraction

A single individual specimen was selected from each sample container for DNA extraction with the criteria of choosing a large, dark adult louse. This was to maximise DNA yield and to avoid misidentification of juvenile instars. Total genomic DNA was extracted using the DNAeasy Tissue Kit (Qiagen 2001). The extraction was carried out following the Qiagen DNAeasy Tissue Kit protocol, with modifications adapted for extracting louse DNA established by Cruickshank *et al.* (2001). The basis of this procedure is that of a buffer system that allows direct cell lysis, using Proteinase K, followed by selective binding of the DNA to the DNeasy membrane. After lysis, the centrifuging process removes all contaminants and enzyme inhibitors while binding the DNA to the column membrane. DNA is then eluted in water and is ready for use.

At the start of the procedure the specimen was placed on a glass slide and a scalpel used to separate the head from the body. A small paintbrush was used to carefully manipulate the specimens. The parts of the specimen were then both placed into a 1.5ml Eppendorf tube with 180µl of buffer ATL and 20µl of Proteinase K. This was mixed by vortexing

and incubated at 55°C in a water bath for two nights. After two nights, the liquid was removed and placed in a new tube. 50µl of water was added to the tube containing the specimens. This was then kept for future slide mounting as a morphological voucher specimen. 200µl of buffer AL was added to the extraction liquid, this was mixed by vortexing and was incubated at 70°C for 10 minutes. 200µl of ethanol was added and this was mixed by vortexing. The mixture was then pipetted into a DNeasy mini column sitting in a 2ml collection tube. This was centrifuged at 8000rpm for 1 minute. The flow-through and collection tube were discarded. The mini-column was placed in a new collection tube and 500µl of buffer AW1 was added. This was then centrifuged at 8000rpm for 1 minute. The collection tube was discarded and the column placed in a new tube. 500µl of AW2 buffer was added and the mixture was then centrifuged for 3 minutes at 13000rpm to dry the DNeasy membrane. The centrifuging steps ensure that any residual ethanol is removed from the column. The flow-through and collection tube were again discarded carefully, to ensure that the column did not contact the flow-through, as this would result in carryover of ethanol. The mini-column was then placed in a new 1.5ml microcentrifuge tube and 50µl of water was pipetted directly onto the membrane of the column. This was then incubated at room temperature for between 10 and 30 minutes. After this the column was centrifuged again, at 8000rpm for 1 minute, causing elution of the DNA that was bound to the filter column. The column was then thrown away and the tube kept containing the extracted DNA.

Throughout all of the above procedures an extraction negative was included that contained only the added reagents without a specimen. This was to provide a check for contamination and was also taken through the PCR and Gel Electrophoresis stages below.

Polymerase chain reaction (PCR) amplification of DNA

The PCR was carried out in order to produce large quantities of specific DNA for sequencing. The PCR first separates DNA into single strands (high temperatures split the hydrogen bonds). Specific primers then anneal to the DNA at the target sequence. DNA polymerase is then used to synthesise DNA, in the presence of dNTPs, from the primers onwards, in the correct direction for each strand so that the target sequence is generated. In this extension phase, the temperature is increased slowly to prevent sequences from denaturing before they have been fully synthesised. The cycle can be

repeated many times, in this way producing many short target sequences and also small amounts of long products. PCR now uses a DNA polymerase (known as Taq) derived from the hyperthermophilic bacterium *Thermus aquaticus*. As this can withstand the high temperatures required to separate DNA strands, it has allowed the automation of the PCR process (Saiki *et al.* 1988). One problem with Taq is that it appears to lack the normal proof reading ability of most DNA polymerases. As a result, it can not correct errors in the synthesis of DNA, Saiki *et al.* (1988) have shown that it has an error rate of 0.25%. However, this should not prove a problem in this study. In the sequencing of the PCR products (see below), for every error there should be many molecules with the correct sequence. However, one thing to keep in mind is that if the PCR was attempted on a sample containing only a few copies of the target template, this error rate could present a serious problem.

Two genes were targeted for the study: Cytochrome Oxidase (COI) and 12SrRNA. Specific primers for the 12SrRNA (Sai and Sbi) and the COI (L6625 and H700S) genes were used (Hafner *et al*. 1994) to make up separate mixtures for each PCR mixture of reactants. The primers have been developed specifically for these genes in lice, because of the greater amount of evolutionary change in lice DNA, generally used insect primers for these genes would not be effective (ref). The PCR mix for each gene was made as shown below in Table 1, in these proportions for each sample The initial amount of DNA template used for each sample was 2µl. In some cases however, this was later increased (as explained below), and then the proportions of mix ingredients remained the same except for the water, which was variable in volume to make up 25µl of the mix in total. The mix was made up in a 1.5ml Eppendorf tube, excluding only the DNA template. Additional procedures when making up the PCR mix were to vortex the $MgCl_2$ before use, and to add the Taq last (it will degrade quickly at room temperature). Once the mix was made up, the tube was vortexed and then centrifuged for a short time to remove any liquid from the lid. At this stage the PCR mix was transferred in to PCR tubes and the extracted DNA was added last.

Table 1. The ingredients of the PCR mixture

PCR reagent	Amount (µl)
Buffer (Mg free)	2.5
$MgCl_2$	3.5
dNTPs	2.5
Taq	0.125
Primer 1	2.0
Primer 2	2.0
H_2O	10.375
DNA template	2.0

By premixing the PCR reagents, the number of sample transfers was minimised and therefore also the chances of sporadic contamination. The PCR negative contained the correct amount of PCR mix with 2µl of water instead of DNA template. This was pipetted last so that it reflected the total reagent handled. A PCR positives was also included; containing the mixture plus DNA that had been successfully put through the PCR reaction on a previous occasion. The tubes were then put into the PCR machine, which was programmed as follows:

1. 94°C for 1 minute.
2. 92°C for 30 seconds.
3. 45°C for 40 seconds.
4. The temperature is rapidly increased to 65°C and held there for 1 minute and 30 seconds.
5. The steps 1-4 are repeated 40 times.
6. 72°C for 10 minutes.
7. The cycle is finished and held at 10°C.

If the PCR was not successful, or elicited only a small amount of product, the process was repeated, increasing the amount of DNA in the PCR mix. Appendix 3 shows the concentrations of DNA template used for each PCR for every sample and the outcome. If a sample failed to elicit any PCR product even on increasing the amount of DNA used, DNA extraction and PCR was repeated for another louse in the sample. If this was still not successful, where possible, another sample of the same species was tried. This is documented in Appendix 2, showing the outcome of these trials.

Gel Electrophoresis

When PCR was complete, the samples were run on a gel to check for PCR product. The gel was made up as follows: 1g of agrose was measured into a 250ml conical flask.

50ml of 1x TAE (made from Tris Base, glacial acetic acid and EDTA) was added to this, and the flask mixed by shaking. This was then carefully boiled in the microwave until the agrose had completely dissolved. The jar was then taken to the fume cupboard where 2µl of Ethidium Bromide was added. This compound stains the DNA so that it fluoresces under ultra-violet (UV) light. Once the gel mixture had cooled slightly, it was then poured onto the casting plate. Any bubbles in the gel were removed. The gel was left for 30 minutes to harden. After this, the casting gates, and well-making combs were taken off, and the gel placed in the electrophoresis tank. 1x TAE was poured into the gel tank until the gel was covered. Before loading the wells, 5µl of 6x loading buffer was pipetted into each sample tube, this dyed the samples and they could subsequently be seen when loading them onto the well and when they were moving up the gel. 5µl of 100base pair (bp) ladder (this gives a band on the gel at every 100 bp, with a darker band at 500 bp) was then pipetted into the first well in each column on the gel. This ladder allowed for estimation of the size of bands on the gel in comparison. Once the PCR product was loaded into the wells, a charge of 70v was passed through the gel for 30 minutes. The gel was then removed from the casting plates and examined under UV light, and photographed. Successful bands of PCR product (at 400bp) were cut out from the gel using a scalpel and stored in 1.5ml Eppendorf tubes.

Gel Extraction

Gel extraction was carried out using the QIAquick Gel Extraction kit and protocol (Qiagen, 2000). To each tube containing gel and PCR product, 3 volumes of buffer QG were added to 1 volume of gel. The tubes were then vortexed briefly and incubated in a 50°C waterbath for 10 minutes or until all gel had melted. 1x the weight of the band of Isopropenol was then added to the tube and again it was vortexed briefly. 700µl was pipetted from each tube into filter column (as provided by the QIAquick Gel Extraction kit) and then spun in the centrifuge for 1 minute at 13000rpm. The flow-through was discarded and the filters (containing the DNA) placed back in the same tube. This step was repeated until all DNA in the original tubes had been collected on the filter column. Then 500µl of QG buffer was added to the filter column, it was spun in the centrifuge at 13000rpm for 1 minute and the flow-through was discarded. Following this, 750µl of PE buffer was added, it was left to stand for 2 minutes and then spun again for 1 minute at 13000rpm and the flow-through discarded. The spin step was repeated and flow-through discarded once more. The filters were then placed in 1.5ml Eppendorf tubes,

25µl of water was pipetted onto each filter and they were left to stand for 2 minutes. They were then spun in the centrifuge for 1 minute at 13000rpm, after which the filters were thrown out and the tubes containing the DNA kept.

5µl of the DNA was then run on a small check gel. The gel was made as before (see above) but contained only 0.6g of agrose, 1.2µl of Ethidium Bromide and 30ml of TAE. Nescofilm was used to add 1µl of 6x loading buffer to 5µl of DNA and this was then pipetted into the wells of the gel, with 5µl of 100bp ladder in separate wells.

The gel was run as before (for 30 minutes on 70v) and examined under UV light and photographed.

DNA Sequencing

If the check gel was successful, the DNA was sent to the Molecular Biology Support Unit (MBSU) sequencing service. ABI 373 stretch and ABI 377 DNA sequencers are used by the MBSU to detect DNA molecules labelled with fluorescent dyes. Each dideoxynucleotide contains a specific fluorescent dye that can be excited by a laser. The MBSU uses specific software to produce a coloured electropherogram (each base type is coded by a different colour).

Appendix 2: PCR and sequencing of louse COI and 12S rRNA genes

Results of the laboratory work, showing success of PCR, for each gene, at different concentrations of DNA template. Sequencing reaction success is also indicated for each sample. ✓ indicates PCR product/sequencing was successfully obtained, × indicates failure. Host species the samples were taken from are also shown.

SAMPLE			Success of PCR at different DNA concentrations							
			COI				12S rRNA			
LOUSE BASE code/trial no.	Louse species	Host species	Amount of DNA template /µl			Gene Sequenced?	Amount of DNA template /µl			Gene Sequenced?
			2	4	6		2	4	6	
GLA 487/01	*Docophoroides harrisoni*	*Diomedea bulleri*	✓			✓	✓	✓		✓
GLA 501/01	*Docophoroides brevis*	*Diomedea gibsoni*	✓			✓	✓	✓		✓
MP 43/01	*Docophoroides sp.*	*Diomedea melanophris*	×			×	×			×
GLA 550/01	*Docophoroides harrisoni*	*Diomedea cauta*	✓	✓	✓	×	✓	✓		×
NZ 71/01	*Docophoroides levequei*	*Diomedea irrorata*	✓			✓	✓	✓	×	×
GLA 505/01	*Harrisoniella hopkinsi*	*Diomedea antipodensis*	✓			✓	✓			✓
FD 10/01	*Paraclisis diomedea*	*Diomedea melanophris*	✓			✓	✓			✓
GLA 529/01	*Paraclisis diomedea*	*Diomedea cauta*	✓		✓	✓	✓	✓		✓
NZ 7/01	*Paraclisis obscura*	*Macronectes giganteus*	×			×	×			×
NZ 7/02	*Paraclisis obscura*	*Macronectes giganteus*	×			×	×			×
NZ 7/03	*Paraclisis obscura*	*Macronectes giganteus*	×	×		×	×			×
NZ 47/01	*Paraclisis obscura*	*Macronectes giganteus*	×	×		×	×			×

Appendix 2 continued. PCR and sequencing of louse COI and 12S rRNA genes

Results of the laboratory work, showing success of PCR, for each gene, at different concentrations of DNA template. Sequencing reaction success is also indicated for each sample. ✓ indicates PCR product/sequencing was successfully obtained, × indicates failure. Host species the samples were taken from are also shown.

SAMPLE			Success of PCR at different DNA concentrations							
			COI				12S rRNA			
LOUSE BASE code/trial no.	Louse species	Host species	Amount of DNA template /µl			Gene Sequenced?	Amount of DNA template /µl			Gene Sequenced?
			2	4	6		2	4	6	
RF 18/01	*Haliperus sp.*	*Bulweria bulwerii*	×	×		×	×	✓	✓	×
NZ 6/01	*Haliperus procellariae*	*Procellaria aequinoctialis*	×			×	×			×
NZ 6/02	*Haliperus procellariae*	*Procellaria aequinoctialis*	×	×		×	×			×
NZ 8/01	*Pelmatocendra enderleini*	*Pelecanoides georgicus*	×			×	×			×
NZ 8/02	*Pelmatocendra enderleini*	*Pelecanoides georgicus*	×	×		×	×			×
NZ 52/01	*Pelmatocendra enderleini*	*Pelecanoides georgicus*	×	×		×	×			×
NZ 53/01	*Pseudonirmus gurlti*	*Daption capense*	×			×	×			×
NZ 54/01	*Pseudonirmus lugubris*	*Thalassocia antarctica*	×			×	×			×
NZ 54/02	*Pseudonirmus lugubris*	*Thalassocia antarctica*	×	×		×	×			×