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Multiple origins of parasitism in lice

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A major fraction of the diversity of insects is parasitic, as herbivores, parasitoids or vertebrate ectoparasites. Understanding this diversity requires information on the origin of parasitism in various insect groups. Parasitic lice (Phthiraptera) are the only major group of insects in which all members are permanent parasites of birds or mammals. Lice are classified into a single order but are thought to be closely related to, or derived from, book lice and bark lice (Psocoptera). Here, we use sequences of the nuclear 18S rDNA gene to investigate the relationships among Phthiraptera and Psocoptera and to identify the origins of parasitism in this group (termed Psocodea). Maximum-likelihood (ML), Bayesian ML and parsimony analyses of these data indicate that lice are embedded within the psocopteran infraorder Nanopsocetae, making the order Psocoptera paraphyletic (i.e. does not contain all descendants of a single common ancestor). Furthermore, one family of Psocoptera, Liposcelididae, is identified as the sister taxon to the louse suborder Amblycera, making parasitic lice (Phthiraptera) a polyphyletic order (i.e. descended from two separate ancestors). We infer from these results that parasitism of vertebrates arose twice independently within Psocodea, once in the common ancestor of Amblycera and once in the common ancestor of all other parasitic lice.

Keywords: parasites; Phthiraptera; Psocoptera; molecular phylogenetics

1. INTRODUCTION

Parasites make up approximately half the diversity of all life on Earth (Price 1980). Many groups of insects contain parasites of some form, ranging from herbivores and parasitoids of other insects to parasites of vertebrates. The parasitic habit is thought to be one of the key innovations allowing for the tremendous radiation of insects (Farrell 1998; Whitfield 1998). Understanding this diversity requires information on the origin of parasitism in various insect groups. Throughout the evolutionary history of insects, several groups have specialized as parasites of vertebrates, feeding on blood or other tissues of their vertebrate hosts. These groups range from lice (Phthiraptera) and fleas (Siphonaptera) to some groups of earwigs (Dermaptera), bugs (Hemiptera) and flies (Diptera). Among these groups, lice are unique in that they are permanent parasites of vertebrates, spending their entire life cycle on the body of the host. For this reason, lice have become a model system for the study of cophylogenetic relationships between hosts and parasites (Hafner *et al.* 1994; Page 1994; Huelsenbeck *et al.* 2000; Johnson *et al.* 2001*a*). However, the origins of parasitism in lice are only poorly understood.

Parasitism of vertebrate hosts requires a number of specialized morphological, physiological and behavioural adaptations, which have evolved for remaining attached to the host, escaping host defences and feeding on the host (Clayton *et al.* 2003). For example, lice glue their eggs to the feathers or hairs of the host and have specialized tarsal claws for hanging on to the host. These adaptations can result in the extreme specialization of parasites to particular host species and host microhabitats. For example, species of parasitic lice are often restricted to a single host species or subspecies (Price *et al.* 2003). It is generally assumed that these specializations to the parasitic habit are so significant that parasitism within this insect order could have evolved only once. However, this hypothesis has not been rigorously tested.

Identifying the origin of these specialized characteristics in relation to the origin of parasitism requires knowledge of the closest relatives of parasitic lice. These relatives are believed to be among the insect order Psocoptera (book lice and bark lice), and together these two insect orders are placed in the group Psocodea. The notion that these two groups of insects share a common ancestor is supported by several morphological characters (e.g. a unique water-vapour uptake system; Lyal 1985) and by limited molecular data (Whiting *et al.* 1997; Yoshizawa & Johnson 2003). Psocoptera are free-living insects, which most often feed on fungal spores (Mockford 1993). However, there are many records of various species of Psocoptera in the plumage of birds and the pelage of mammals, as well as in their nests (Hicks 1959; Pearlman 1960; Mockford 1967). This association is believed to be a short-term commensal (non-parasitic) relationship, which may have given rise to a more parasitic and permanent association (Hopkins 1949).

Precisely identifying the closest relatives of lice has proved difficult. Lice have a very simplified body form as a result of their parasitic habits, being wingless and dorsoventrally flattened. Consequently, it is difficult to identify morphological similarities between lice and potential relatives among the Psocoptera. Kim & Ludwig (1982) suggested that lice were derived from extinct ancestors of Psocoptera in the Carboniferous or Permian period. By contrast, Lyal (1985) provided morphological evidence that lice were derived from within the Psocoptera, possibly

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as recently as the Cretaceous, being the closest relative of a single family, Liposcelididae.

This controversy can potentially be resolved using independent molecular data. Previous molecular work with limited taxon sampling used mitochondrial 12S and 16S rDNA sequences to evaluate the phylogenetic position of lice (Yoshizawa & Johnson 2003). Although this study did support a close relationship between Liposcelididae and lice, rendering Psocoptera paraphyletic, there was little resolution among the major groups of lice or with regard to the monophyly of parasitic lice. One limitation of these data is that 12S and 16S mitochondrial genes generally evolve at a substantially elevated rate in lice and Liposcelididae (Johnson *et al.* 2003; Yoshizawa & Johnson 2003) and have a highly variable secondary structure (Page *et al.* 2002) making phylogenetic reconstruction at deep taxonomic levels extremely difficult. More slowly evolving nuclear genes have more potential to resolve these deep phylogenetic relationships.

The goal of the present study was to reconstruct the phylogenetic relationships among the major lineages of Psocodea using nuclear 18S rDNA sequences. This gene has been shown to have phylogenetic utility for recovering deep phylogenetic relationships within and among orders of insects (Campbell *et al.* 1995; Whiting *et al.* 1997). We sampled all major groups within Psocoptera and Phthiraptera and included 145 species in our study. Using trees derived from phylogenetic analysis of these data, we address three main questions: (i) is paraphyly of Psocoptera also supported by a more slowly evolving nuclear gene; (ii) what are the closest living relatives to parasitic lice; and (iii) how many times has parasitism evolved within Psocodea (i.e. do parasitic lice form a monophyletic group)?

2. MATERIAL AND METHODS

In this study, we sampled 21 species of parasitic lice (Phthiraptera) and 113 species of book lice and bark lice (Psocoptera) for the ingroup (see electronic Appendix A). For parasitic lice we sampled each of the four suborders (Lyal 1985): chewing lice in the suborders Amblycera (nine species), Ischnocera (six species) and Rhynchophthirina (one species) and sucking lice in the suborder Anoplura (five species). We sampled multiple representatives of each of the three psocopteran suborders (Lienhard & Smithers 2002) in rough proportion to their family-level diversity: Trogiomorpha (11 species), Troctomorpha (19 species) and Psocomorpha (83 species). The group Psocodea is placed within a group of hemimetabolous insects, the hemipteroid assemblage (Paraneoptera) (Yoshizawa & Saigusa 2001). Thus, for outgroup sampling we included representatives of the two other orders in this group: Hemiptera (true bugs and allies) and Thysanoptera (thrips). This group of insects is thought to be the sister taxon of holometabolous insects (Whiting *et al.* 1997), so we used members of this group as a more distant outgroup (see electronic Appendix A).

We extracted DNA from individual specimens and prepared vouchers as previously described for these insects (Johnson *et al.* 2001*b*; Johnson & Mockford 2003). PCR was used to amplify the 18S rDNA gene in three fragments using the primer combinations Ns1–Ns2a (Barker *et al.* 2003), 18Sai–18Sbi (Whiting *et al.* 1997) and Ns5a–Ns8 (Barker *et al.* 2003). In some cases, we redesigned primers for specific groups of Psocoptera, and these primers were substituted as follows: Ns8P for Psocoptera except Nanopsocetae (5′-TACTTCCTCTAAACGATCAAG-3′), Ns5aP2 for Psocomorpha (5′-TGAAACTTAAAGGAATTGACGGAAA-3′) and Ns2P for Peripsocidae and Epipsocidae (5′-CGCGGCTGCTG GCACCAGACTTTTCC-3′). PCR products were purified and sequenced as described by Johnson *et al.* (2001*b*). Sequences for some species of lice had been previously published (Johnson & Whiting 2002; Barker *et al.* 2003) and were obtained from GenBank along with sequences for many of the outgroup taxa (see electronic Appendix A).

Across this diverse group of insects there are many regions of insertions and deletions within the secondary structure of the 18S rRNA gene. We used two approaches to align these sequences. First, CLUSTALX (Thompson *et al.* 1997) was used with a gap : gap–extension cost of 10 : 1. To test the sensitivity of the analysis to the method of alignment, we manually constructed a second alignment based on rRNA secondary structure. A secondary-structure model for *Drosophila melanogaster* (Cannone *et al.* 2002) was used as a guide for the initial alignment and as a benchmark for determining the degree of secondary-structure variation. Both the CLUSTAL and secondary-structure alignments with secondary-structure annotation following Wuyts *et al.* (2000), are available electronically from http://darwin.zoology.gla.ac.uk/~vsmith/papers/psocodea/. For the analysis of each alignment, exclusion sets were generated, which differed in their level of sequence conservation. For the CLUSTAL alignment, only conserved sequence blocks were included, and these generally correspond to stem regions. For the secondary-structure alignment, the most conservative alignment preserved stem regions, but very little loop sequence. We also generated a second, less conservative, exclusion set that preserved a greater proportion of the loop regions that were reasonably aligned.

For each alignment and exclusion set we used several different analytical techniques for reconstructing phylogenetic relationships. First, we used unordered equally weighted parsimony in heuristic searches (maxtrees unconstrained) with tree bisection–reconnection (TBR) branch swapping in PAUP* (Swofford 2001). The number of trees found by this search for the CLUSTAL alignment exceeded computational memory capacity, so we ended the search when this limit was reached (426 531 trees). We also bootstrapped these datasets and searched under a parsimony criterion using 100 replicates (maxtrees of 1000). As a second analytical technique, we used maximum likelihood (ML). To determine the simplest model that could not be rejected in favour of a more complex model, we used a series of nested likelihood ratio tests as implemented in MODELTEST (Posada & Crandall 1998). We used the model identified by MODELTEST in a heuristic ML search with TBR branch swapping and a neighbour-joining tree as a starting tree using PAUP*, and performed bootstrapping with 100 replicates and nearest-neighbour interchange branch swapping. To assess more fully support measures for each node, we conducted an ML analysis using a Bayesian optimality criterion with a Markov chain Monte Carlo (MCMC) search strategy using MRBAYES v. 3.0 (Huelsenbeck & Ronquist 2001). For each alignment and exclusion set, we ran four chains for 10 000 000 generations and sampled every 1000 generations. For these analyses, the ML score of the trees was generally stable after 200 000 generations, so we discarded the first 200 trees as burn-in. We computed a 50% majority rule consensus of the remaining 9800 trees to estimate the posterior probability for nodes in the tree. Because not all parameters in a MCMC Bayesian search burn in at the same time (Huelsenbeck *et al.* 2002), we also examined the 2000 sampled trees from the final 2 000 000 generations

to evaluate the stability of posterior probability values for nodes of interest. Datasets and trees are deposited in TreeBASE (http://www.treebase.org/).

To evaluate whether there is support for the monophyly of parasitic lice (Phthiraptera), we used parametric bootstrapping as implemented by the SOWH test (Swofford *et al.* 1996; Goldman *et al.* 2000). The SOWH test simulates sequence evolution over the optimal ML tree and over the most likely tree recovered with a particular node constrained (in this case louse monophyly). These simulated datasets are then analysed using full ML searches, and the distribution of differences in likelihood scores between the constrained and unconstrained trees is computed. The difference in these scores using the real data is compared with this simulated distribution to calculate a *p*-value. Non-parametric tests of monophyly, such as the Kishino–Hasegawa (Kishino & Hasegawa 1989) and Shimodaira–Hasegawa (SH) (Shimodaira & Hasegawa 1999) tests, have been shown to have serious biases (Shimodaira 2002) and thus do not perform well as tests of clade monophyly (Goldman *et al.* 2000). Unfortunately the computational complexity of the SOWH test, involving a full ML analysis of simulated datasets, is too high to conduct this test using the entire set of 145 species. Therefore, we subsampled taxa within the clades of interest (one species of Rhynchophthirina and two species from each other louse suborder, plus various members of the Psocoptera; see figure 1 and electronic Appendix A). We used the program Seq-gen (Rambaut & Grassly 1997) to generate the sequence data and PAUP* to perform the likelihood searches (see description by Goldman *et al.* (2000)). For comparison with the SOWH test, we also used the program Consel (Shimodaira & Hasegawa 2001) to perform the relatively conservative SH and approximately unbiased (AU) tests (Shimodaira 2002) on the same taxon set.

3. RESULTS

As previously reported for lice (Barker *et al.* 2003), the 18S gene has a large hypervariable insertion region ranging from 59–808 bp, corresponding to section V4, *sensu* Wuyts *et al.* (2000). This and other insertion regions were difficult to align and were therefore excluded from our analyses. Despite these problems, the insect model of secondary structure for the 18S gene (Wuyts *et al.* 2000) was identifiable in all sequences, and all sequences could be readily aligned according to this model.

Parsimony analysis of the Clustal alignment (1696 included characters) produced 426 531 most parsimonious trees (length of 3727; retention index of 0.747). The consensus of these trees was generally similar to those produced from the most conservative (1949 included characters; 4172 trees; length of 6429; retention index of 0.704) and least conservative (2045 included characters; 2104 trees; length of 6911; retention index of 0.699) inclusion sets of the secondary-structure-based alignment. Neither Psocoptera nor Phthiraptera were monophyletic in these trees (see also figure 1), and in all cases the psocopteran family Liposcelididae was sister to the phthirapteran suborder Amblycera.

Likelihood ratio tests indicated that a model incorporating two transition rates and a single transversion rate, unequal base frequencies, invariant sites and rate heterogeneity according to a gamma distribution (TrN + I + G) had a significantly higher likelihood than simpler models. ML analysis of the Clustal alignment produced a single tree (figure 1). This tree also recovered a sister relationship between Liposcelididae and Amblycera, with bootstrap support of 82%. In most respects this tree was very similar to that generated by parsimony analyses. Other clades corresponding to traditional classification received high support. The monophylies of Psocodea, Psocomorpha, Trogiomorpha, Amblycera and Anoplura were all recovered in more than 90% of bootstrap replicates, indicating that the 18S gene contains a substantial phylogenetic signal for deep relationships within the Psocodea. Bayesian analysis of this alignment produced a consensus tree nearly identical to that produced by ML analysis (figure 1). The sister relationship between Amblycera and Liposcelididae was recovered in 100% of trees from the Bayesian search. In fact, a tree with louse monophyly was not among the 9800 trees sampled from the final 9 800 000 generations of the Bayesian chain. Support for various weakly supported nodes in the Bayesian tree generally increased substantially from a burn-in of 200 trees (200 000 generations) to 8000 trees (8 000 000 generations), indicating that basing a burn-in on only the likelihood score may not be adequate for large datasets (Huelsenbeck *et al.* 2002). For example, the Bayesian posterior probability for the monophyly of Ischnocera increased from 87% with a 200 000 generation burn-in to 98% with an 8 000 000 generation burn-in. Similarly, monophyly of a clade containing all Nanopsocetae (Liposcelididae, Pachytroctidae and Sphaeropsocidae) plus Phthiraptera increased from 76% to 99% when only the 2000 trees from the final 2 000 000 generations were included in the consensus.

The results of likelihood and Bayesian analyses of the secondary-structure-based alignments were also similar to the Clustal alignments. None of these analyses supported the monophyly of Phthiraptera or Psocoptera. In the Bayesian analyses of the less conservative alignment, *Pachytroctes*, a member of Pachytroctidae, came near the outgroup Hemiptera, whereas all other analyses put it as sister to *Peritroctes*, another member of the subfamily Pachytroctinae. *Pachytroctes* contained several regions of insertions within loops, so it is likely that these are poorly aligned and their inclusion in the less conservative dataset was problematic for the analyses.

For the reduced taxon set (see figure 1 and electronic Appendix A), the difference in likelihood score between the monophyly-of-Phthiraptera constrained and unconstrained was 12.2. This difference was highly significant using the parametric bootstrapping method of the SOWH test ($p < 0.01$). The relatively conservative SH and AU tests were also significant ($p = 0.046$ and $p = 0.041$, respectively). These results indicated that monophyly of Phthiraptera could be rejected as significantly less likely with these data.

4. DISCUSSION

Comprehensive analyses of nuclear 18S rDNA sequences for 21 species of parasitic lice (Phthiraptera) and 113 species of book and bark lice (Psocoptera) do not support monophyly of either group. Although the paraphyly of Psocoptera has been suggested previously (Lyal 1985; Yoshizawa & Johnson 2003), the polyphyly of Phthiraptera has not. These phylogenetic results indicate that parasitism evolved twice independently in Psocodea, once in the common ancestor of Amblycera and once in the common

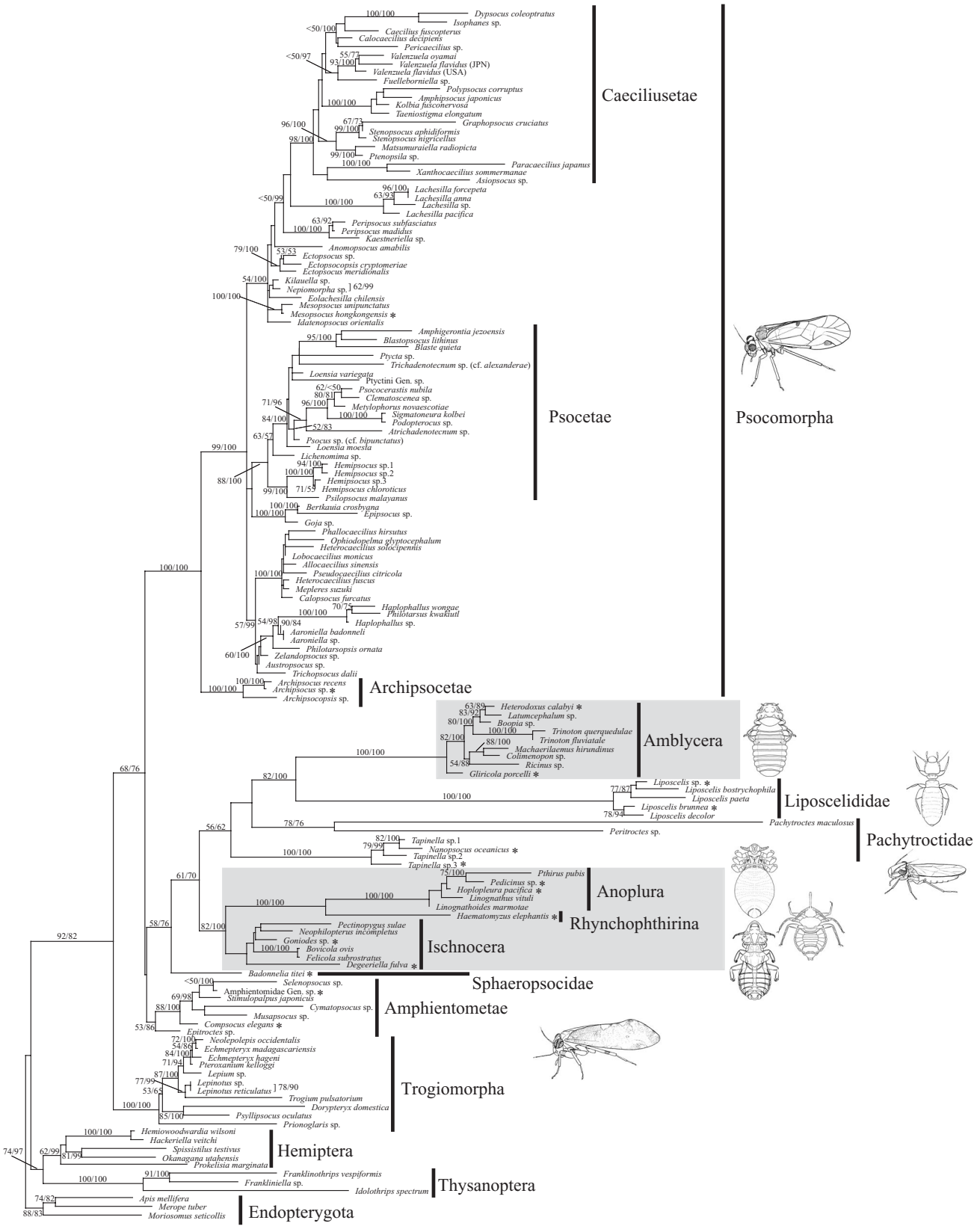

Figure 1. The ML tree from a heuristic search of the CLUSTAL alignment under the TrN + I + G model (transversion rate, 1; A–G, 2.78; C–T, 3.92; A, 0.308; C, 0.205; G, 0.206; T, 0.281; I, 0.368; alpha, 0.561). The tree is rooted on Endopterygota. Branch lengths are proportional to ML estimated branch lengths. The numbers associated with the nodes are ML bootstrap or Bayesian posterior probabilities for any nodes that had higher than 50% bootstrap support and/or 90% Bayesian posterior probability. Major groups are indicated with bold bars. Parasitic lice ('Phthiraptera') are highlighted with shading. Asterisks indicate species used in the SOWH test.

ancestor of all other lice (figure 1). This scenario is more parsimonious than a single origin and multiple losses (three) of parasitism, and it is also more plausible because multiple losses of parasitism would require the re-evolution of flight within the Pachytroctidae and Liposcelididae (although this might be possible in some insect groups; Whiting *et al.* 2003). In comparison with other major groups of vertebrate ectoparasites, parasitism evolved only once in fleas (Whiting 2002), whereas there are several origins of parasitism in mites (Proctor & Owens 2000). The two origins of parasitism in lice indicate that the morphological characteristics associated with parasitism are convergent.

In our phylogenetic analyses, lice were divided into two well-supported clades. The first comprises the suborder Amblycera, from which we sampled a diversity of families. The second clade comprises the suborders Ischnocera, Anoplura and Rhynchophthirina. The book louse family Liposcelididae is consistently recovered as the sister taxon of Amblycera and these taxa are further separated from other lice by members of the Pachytroctidae (figure 1). Pachytroctidae and Liposcelididae, together with Sphaeropsocidae, are members of the infraorder Nanopsocetae (suborder Troctomorpha), and this group consistently forms a clade with lice. Because members of both of the identified clades of lice parasitize both birds and mammals, it is currently not possible to identify the ancestral host of each clade (bird or mammal). However, more detailed sampling within each louse clade may allow the ancestral host to be reconstructed for each group.

Given the unexpected nature of these results, it is important to evaluate their robustness to the method of analysis and with measures of support. The polyphyly of lice was identified using two different alignment methods and three methods of analysis (parsimony, ML and Bayesian ML). In addition, the sister relationship of Amblycera and Liposcelididae received very strong support from the Bayesian analysis of the CLUSTAL alignment (100%), although support was weaker using other measures. We further tested the polyphyly of lice using the SOWH test and convincingly rejected louse monophyly using this parametric bootstrap technique. We recognize that the 18S gene constitutes a single linkage group and thus represents a gene tree. However, there is very high support for many traditionally recognized groups within Psocoptera and Phthiraptera, indicating that this gene has substantial signal for resolving phylogenetic relationships within these groups. For example, monophylies of the group Psocodea, suborders Trogiomorpha, Psocomorpha, Amblycera and Anoplura and infraorders Caeciliusetae and Psocetae all received ML bootstrap supports of between 90% and 100%. In addition, a sister relationship between Rhynchophthirina and Anoplura was supported in 100% of bootstrap replicates, and this relationship has been previously identified on the basis of morphology (Lyal 1985) and molecular analyses (Johnson & Whiting 2002; Barker *et al.* 2003). Given these observations, it seems unlikely that the consistently recovered polyphyly of Phthiraptera is a spurious result.

Given the novel result recovered by our analyses, an examination of the previous support for louse monophyly is warranted. Most morphological apomorphies (13 out of 19 character states) identified in support of the monophyly of lice are loss character states (e.g. reductions of the labial palpi, antennal flagellum and compound eye), and these are likely to be strongly linked to the parasitic lifestyle of lice (Lyal 1985). The six character states identified as gains potentially supporting the monophyly of lice (Lyal 1985) are all either (i) shared by at least Liposcelididae and Pachytroctidae (e.g. dorsoventral compression of the head), (ii) not consistent within lice (e.g. development of a lacinial gland), (iii) strongly linked to a parasitic lifestyle (e.g. egg-cement produced from the vagina), or (iv) not well studied in Psocoptera (spermatological and embryological characters). Taken together these characters are either strongly associated with a parasitic lifestyle or not particularly informative in this group of insects. An evaluation of other character systems, e.g. genitalic structures, is needed, and preliminary observations suggest similarities between Liposcelididae and Amblycera to the exclusion of other lice (K. Yoshizawa, personal observation), consistent with the results of the present study.

In summary, we have provided evidence from the 18S gene that the parasitic lice (Phthiraptera) do not form a monophyletic group. Rather, lice are polyphyletic, and parasitism of vertebrates has evolved twice independently within the Psocodea. To our knowledge, this is the first modern demonstration that an order of insects is polyphyletic. Further work using additional nuclear genes is needed to resolve further and in more detail the relationships among the major groups of Nanopsocetae and Phthiraptera. Furthermore, additional data could be used to estimate the timing of these multiple origins and potentially to identify the ancestral host (mammal or bird, or some ancestor; Wappler *et al.* 2004) for each lineage of parasitic lice.

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