Phylogeny of the lice (Insecta, Phthiraptera) inferred from small subunit rRNA

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Accepted: 28 August 2002

Barker, S. C. Whiting, M., Johnson, K. P. & Murrell, A. (2003). Phylogeny of the lice (Insecta: Phthiraptera) inferred from small subunit rRNA. — *Zoologica Scripta*, *32*, 407–414.

There has been much argument about the phylogenetic relationships of the four suborders of lice (Insecta: Phthiraptera). Lyal's study of the morphology of lice indicated that chewing/biting lice (Mallophaga) are paraphyletic with respect to sucking lice (Anoplura). To test this hypothesis we inferred the phylogeny of 33 species of lice from small subunit (SSU) rRNA sequences (18S rRNA). *Liposcelis* sp. from the Liposcelididae (Psocoptera) was used for outgroup reference. Phylogenetic relationships among the four suborders of lice inferred from these sequences were the same as those inferred from morphology. The Amblycera is apparently the sister-group to all other lice whereas the Rhynchophthirina is apparently sister to the Anoplura; these two suborders are sister to the Ischnocera, i.e. (Amblycera (Ischnocera (Anoplura, Rhynchophthirina))). Thus, the Mallophaga (Amblycera, Ischnocera, Rhynchophthirina) is apparently paraphyletic with respect to the Anoplura. Our analyses also provide evidence that: (i) each of the three suborders of lice that are well represented in our study (the Amblycera, Ischnocera, and Anoplura) are monophyletic; (ii) the Boopiidae is monophyletic; (iii) the genera *Heterodoxus* and *Latumcephalum* (Boopiidae) are more closely related to one another than either is to the genus *Boopia* (also Boopiidae); (iv) the Ricinidae and Laemobothridae may be sister-taxa; (v) the Philopteridae may be paraphyletic with respect to the Trichodectidae; (vi) the genera *Pediculus* and *Pthirus* are more closely related to each other than either is to the genus *Pedicinus*; and (vii) in contrast to published data for mitochondrial genes, the rates of nucleotide substitution in the SSU rRNA of lice are not higher than those of other insects, nor do substitution rates in the suborders differ substantially from one another.

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Introduction

There are 4900 or so species of lice, which infest birds and mammals. Lice tend to be reasonably host-specific, but this is not always the case. Indeed, host-switching has had a substantial effect on the evolution of host–louse associations (e.g. Barker 1991; Johnson *et al*. 2002).

The phylogeny of the lice has been contentious for well over 100 years. However, most modern louse systematists agree that the Phthiraptera comprises four suborders: the sucking lice (Anoplura) and so-called chewing or biting lice (Rhynchophthirina, Ischnocera and Amblycera). The Anoplura parasitize eutherian mammals exclusively. The Rhynchophthirina (with only one family and one genus, *Haematomyzus*) parasitize African and Asian elephants, warthogs and bush pigs. The Ischnocera and Amblycera parasitize both birds and mammals.

It is almost universally accepted that the phthiraptera comprises the four groups listed above but there has been much argument over the phylogeny and classification of these four groups. The argument stems back to the taxonomic division of lice into two groups — the sucking lice (Anoplura) and the chewing lice or Mallophaga (Rhynchophthirina, Ischnocera and Amblycera). This classification was convenient but it may not reflect the phylogenetic relationships of the suborders because chewing/biting may be a plesiomorphy rather than a synapomorphy.

Harrison (1928) appears to have been the first to argue that the Ischnocera was more closely related to the Anoplura than to the Amblycera, and thus that the Mallophaga was paraphyletic. Subsequently, Webb (1946), Hopkins (1949), Clay (1970), Haub (1980), Lyal (1985), Johnson & Whiting (2002)

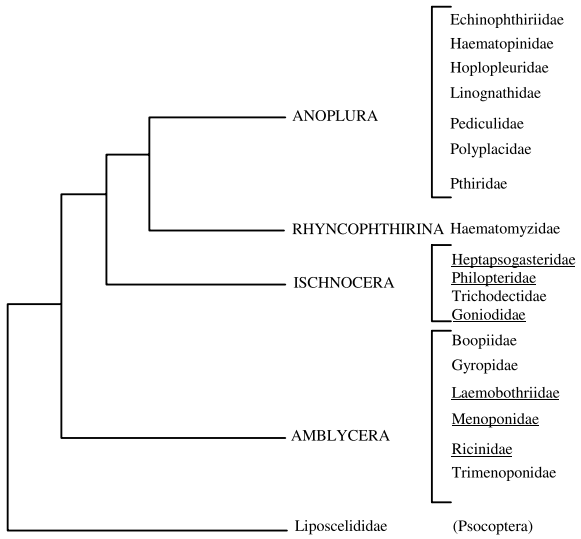

Fig. 1 Phylogeny of Phthiraptera after Lyal (1985). Liposcelididae = hypothezised sister-group of the Phthiraptera. Underscored families contain species that infest birds; the remainder are more or less restricted to mammals. The families are those recognized by Clay (1970) for the Amblycera (except for the Goniodidae which is now recognized by most workers), by Hopkins & Clay (1952) for the Ischnocera and by Kim, Pratt & Stojanovich (1986) for the Anoplura. We have shown only 7 of the 15 families of Anoplura that are currently recognized; see Durden & Musser (1994) for a complete list.

and others have presented opinions and/or evidence to indicate that the Mallophaga is paraphyletic. Indeed, only Kim & Ludwig (1982) concluded that the Mallophaga was monophyletic. Although most taxonomists now agree that it is probably paraphyletic, a range of opinions remains on how the four groups of lice should be classified and whether the apparently paraphyletic group, Mallophaga, should be retained (see Kim & Ludwig 1982; Lyal 1985). By far the simplest scheme, proposed by Königsmann (1960, fig. 2) and Clay (1970) and later advocated by Lyal (1985) and Calaby & Murray (1991), is four taxa (suborders) of equal hierarchical rank: Anoplura, Rhynchophthirina, Ischnocera and Amblycera.

There have been a number of recent phylogenetic studies of lice (Paterson *et al*. 2000; Smith 2000, 2001; Johnson *et al*. 2001a, b; Marshall, submitted). In particular, the phylogenetic relationships of the Anoplura (Kim & Ludwig 1978), Amblycera (Clay 1970) and Ischnocera (Johnson & Whiting 2002) have been studied. However, there have been only three analyses of the phylogeny of the entire order Phthiraptera. Lyal (1985) analysed cladistically features of the morphology and life-history of lice and the Psocoptera. Lyal concluded that the order Phthiraptera was monophyletic and proposed that the four suborders of lice were related in the following way: (Amblycera (Ischnocera (Anoplura, Rhynchophthirina))) (Fig. 1). He also proposed that the sister-group of the lice was the family Liposcelididae (Order Psocoptera). Cruickshank *et al*. (2001) used 347 bp of the elongation factor 1-alpha (EF1-α) gene to examine relationships within the Phthiraptera. Cruickshank *et al*., (2001) found strong support for some shallow level relationships (e.g. for the genera *Chelopistes* and *Oxylipeurus*), but multiple substitutions and the short sequences precluded tests of hypotheses about deep level relationships. Johnson & Whiting (2002) used three genes to test the monophyly of the suborder Ischnocera, finding evidence for monophyly of each of the suborders; however, they were not able to test relationships among them.

In the present study we sequenced and analysed all or most of the small subunit rRNA (SSU rRNA) for 33 species of lice (from all four suborders) and from one outgroup (*Liposcelis* sp., Psocoptera). Our analyses provide the first molecular test of Lyal's morphological hypothesis of the phylogeny of the four suborders of lice.

Materials and Methods

Lice studied

The species are listed in Table 1. *Liposcelis* sp. was used for outgroup reference because Lyal's (1985) cladistic analysis indicated that the Liposcelididae (Psocoptera) is the sister-group of the lice. Incidentally, if this is true then the Psocoptera is paraphyletic.

DNA methods

SSU rRNA sequences were generated by the authors working in two teams: (1) SCB & AM and (2) KPJ & MW. The methods of the latter are described in Johnson & Whiting (2002).

DNA was extracted from whole lice following Crampton *et al*. (1996). In most cases the entire ssq rDNA was amplified in three pieces with the published primers Ns1 (5′ GTA GTC ATA TGC TTG TCT C 3′), Ns2a (5′ CGC GGC TGC TGG CAC CAG ACT TGC 3′), Ns5a (5′ TGA AAC TTA AAG GAA TTG ACG GAA G 3′) and Ns8 (5′ TCC GCA GGT TCA CCT ACG GA 3′) (Black *et al*. 1997). Primers designed by SCB & AM included Ns10 (5′ AGG CTC TGC AAT CGG AAT G 3′) and Ns11 (5′ GTC AAA TTA AGC CGC AAG C 3′). These, together with 13+a (5′ TTT CAA ATG TCT GAC TTA TCA ACT 3′), 13-a (5′ AGT TGA TAA GTC AGA CAT TTG A 3′), 35+a (5′ ATA GGG ACA GGC GGG GCA TTA GT 3′) and 35-a (5′ CGA CGA TCC AAG AAT TTC ACC TCT 3′) (also designed by SCB & AM) and 58-2a (5′ ATC GGT AGT AGC GAC GGG CGG TGT G 3′) and 58+2a (5′ AAT TCC GAT AAC GAA CGA GAC TC 3′) (designed by Black *et al*. 1997), were used for sequencing. The PCR protocol was 95 °C for 30 s, 40 °C for 1 min, 72 °C for 1 min for 10 cycles, 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min for 25 cycles, then 72 °C for 5 min. The 25 μL recipe included 25 ng DNA, 2.25 mM $MgCl_2$, 0.1 μM each primer, 0.2 mM each dNTP, 2.5 μL 10X buffer

Table 1 Species studied, taxonomy, hosts and GenBank accession numbers. Sequences generated by SCB & AM have identification numbers starting with B: the other sequences were from the lab of M.W.

Species, taxonomy, host	GenBank accession numbers
Order PHTHIRAPTERA	
Suborder AMBLYCERA	
Family Boopiidae	
Heterodoxus calabyi B796 ex captive *Macropus parryi*	AY077759
Latumcephalum sp. B955 ex *Macropus agilis*	AY077760
Boopia sp. cf. *uncinata* B560 ex *Dasyurus hallucatus*	AY077761
Family Gyropidae	
Gliricola porcelli B836 ex *Cavia porcellus*	AY077762
Family Menoponidae	
Colimenopon urocolius ex *Urocolius indicus*	AF385070(1)
Machaerilaemus sp. ex *Hirundo abyssinica*	AF385068(9)
Dennyus hirundinis ex *Apus apus*	AF385064(5)
Trinoton querquedulae ex *Anas platyrhynchos*	AF385074(5)
Family Ricinidae	
Ricinus sp. ex *Cyanocompsa parellina*	AF385072(3)
Family Laemobothridae	
Laemobothrion atrum ex *Fulica americana*	AF385076(7)
Suborder ISCHNOCERA	
Family Philopteridae	
Anatoecus icterodes ex *Anas platyrhynchos*	AF385056(7)
Cirrophthirius testudinarius ex *Recurvirostra americana*	AF385050(1)
Columbicola columbae ex *Columba livia*	AF385044(5)
Neophilopterus incompletus B1110 ex *Ciconia ciconia*	AY077763
Craspedorrhynchus platystomus B1106 ex *Buteo buteo*	AY077764(5)
Degeeriella fulva B1105 ex *Buteo bueto*	AY077766
Pectinopygus sulae B769 ex *Sula sula*	AY077768
Fulicoffula longipila ex *Fulica americana*	AF385042(3)
Colilipeurus colius ex *Urocolius indicus*	AF385046(7)
Austrophilopterus subsimilis ex *Ramphastos sulphuratus*	AF385052(3)
Family Goniodidae	
Campanulotes compar ex *Columba livia*	AF385036(7)
Goniodes dissimilis/giga B927 ex *Gallus gallus*	AY077767
Family Trichodectidae	
Bovicola ovis B801 ex *Ovis aries*	AY077769
Felicola subrostratus B888 ex *Felis catus*	AY077770
Suborder ANOPLURA	
Family Haematopinidae	
Haematopinus pacochoeri ex *Phacochoerus aethiopicus*	AF385058(9)
Family Polyplacidae	
Neohaematopinus sciuri ex *Sciurus carolinensis*	AF385060(1)
Family Echinophthiridae	
Lepidophthirus macrorihini B933 ex *Mirounga leonia*	AY077771(2)
Family Hoplopleuridae	
Hoplopleura pacifica B1182 ex *Rattus rattus*	AY077773
Family Linognathidae	
Linognathus vituli B802 ex *Bos taurus*	AY077774
Family Pediculidae	
Pediculus humanus capitis B761 ex *Homo sapiens*	AY077775
Family Pthiridae	
Pthirus pubis B931 ex *Homo sapiens*	AY077776
Family Pedicinidae	
Pedicinus sp. B1371 ex *Trachypothecus pharyrei*	AY077777
Suborder RHYNCHOPHTHIRINA	
Family Haematomyzidae	
Haematomyzus elephantus B1191 ex *Elephas maximus*	AY077778
Order PSCOPTERA	
Suborder TROCTOMORPHA	
Family Liposcelididae	
Liposcelis sp. B1093 (from Sydney, Australia)	AY077779

and 0.5 U Redhot TAQ polymerase (Advanced Biotechnologies). Negative controls (no template) were always run simultaneously with our PCR experiments; all reaction mixtures were discarded when any DNA appeared in the negative control. QIAGEN Purification columns were used to purify 50–150 μL of PCR product according to the manufacturer's instructions. Nucleotide sequences were obtained by direct sequencing of PCR products with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit; ~50 ηg of purified PCR product was used for each reaction and extension products were purified by ethanol precipitation prior to electrophoresis in an ABI 377 sequencer.

Phylogenetic analyses

Consensus sequences were compiled from both forward and reverse sequences. Sequences were initially aligned with ClustalW (Thompson *et al*. 1994) then adjusted by eye. The multiple sequence alignment was 3247 bp long; 1936 bp were excluded because they could not be aligned reliably or because the 'middle' part of the gene could not be amplified (the 14 sequences of KPJ & MW). Phylogenetic analyses with PAUP 4.0b8 (Swofford 2001) employed maximum parsimony (MP) and genetic distance neighbour-joining (NJ) (Saitou & Nei 1987) – distance HKY85 – (Hasegawa *et al*. 1985). MP searches were with the heuristic algorithm; gaps were alternately treated as a 'fifth base' or as 'missing' in MP analyses. One hundred random addition replicates were used to find the shortest MP trees. Consistency indices, retention indices and tree lengths were calculated after parsimony-uninformative characters were excluded. Five hundred cycles of bootstrap resampling (Felsenstein 1985) were used to test support for branches in phylogenetic trees inferred by MP (heuristic search) and genetic distance (NJ). Markov Chain Monte Carlo (MCMC) analysis of maximum likelihood (ML) was executed with MRBAYES 2.01 (Huelsenbeck & Ronquist 2001). The number of substitution types was set at six and the substitution rate was according to a gamma distribution (shape parameter = 0.4269). These values, based on the ML model that best fit the data, were calculated in MODELTEST 3.06 (Posada & Crandall 1998). Two hundred thousand MCMC generations or 2000 trees were executed with MRBAYES; the first 75 trees were excluded from the consensus tree (these are the 'burn-in', as the ln likelihood sum converges on a stable value — see Huelsenbeck & Ronquist 2001). The percentage representation of each branch was then calculated; this is called the posterior probability. Bremer support values for branches (Bremer 1988, 1994) were calculated with RADCON 1.1.2 (Thorley & Page 2000) and PAUP 4.0b8. Bremer support is the number of extra steps required to lose a branch from an MP tree. Nucleotide substitution rates were evaluated with RRTree (Robinson-Rechavi & Huchon 2000). The nucleotide substitution rate in the Phthiraptera (represented by *Pedicinus* sp. (Anoplura), *Heterodoxus calabyi* (Amblycera) and *Neophiloterus incompletus* (Ischnocera)) was compared with that of *Drosophila melanogaster*. Substitution rates in the suborders Anoplura, Ischnocera and Amblycera were also compared with each other (all 33 species were included in this analysis).

Results

The mean nucleotide content of the SSU rRNA sequences was as follows: 25% A (range 24.7–25.8%), 24% C (23.3–24.9%), 28% G (26.9–28.5%) and 23% T (21.9–23.9). After ambiguously aligned regions were removed, the multiple sequence alignment was 1311 bp long (complete alignment available at ftp://ftp.ebi.ac.uk/pub/databases/embl/align; accession = ALIGN_000289). These 'trimmed' SSU sequences were 1265–1292 bp long. Most of the full-length sequences were about 1950–2200 bp long but in some species they were much larger, e.g. 2329 bp for *Linognathus vituli*, 2419 bp for *Pthirus pubis*, 2661 bp for *Gliricola porcelli* and 2758 bp for *Pedicinus* sp. The nucleotide substitution rate of SSU rRNA in the Phthiraptera was not significantly greater than that of *D. melanogaster*, nor was the substitution rate of the outgroup species, *Liposcelis* sp. The nucleotide substitution rates in the suborders of lice were not significantly different from one another: Ischnocera vs. Amblycera ($P = 0.48$), Anoplura vs. Amblycera ($P = 0.89$) and Anoplura vs. Ischnocera ($P = 0.11$).

There was one MP shortest tree (length = 1140 steps, CI = 0.459, RI = 0.742, HI = 0.541) (Fig. 2). The MCMC consensus tree (not shown) was similar to the MP tree as was the NJ tree (also not shown). There were some differences in the trees produced by the three methods (MP, NJ, MCMC). As expected, these differences corresponded to branches of the trees that had low bootstrap or Bremer support. Several clades were the same for all three methods and had greater than 70% bootstrap support for two of the three phylogenetic methods used. These well supported relationships were: (i) monophyly of the three suborders of lice that were represented by more than one species, Amblycera (100% support, 38 steps of Bremer suppport), Anoplura (99–100%, 16) and Ischnocera (58–100%, 9); (ii) the suborder Rhynchophthirina, represented by one species, was the sister-group of the suborder Anoplura (79–100%, 5); (iii) the Ischnocera was the sister-group of the Anoplura plus Rhynchophthirina (96–100%, 15); (iv) the Amblycera was the sister-group to the Anoplura, Rhynchophthirina and Ischnocera in all trees generated; (v) the family Boopiidae (the three species from three genera studied) was monophyletic (96–100%, 8); and (vi) the family Trichodectidae (two species from the two genera studied) was monophyletic (100%, 19).

Discussion

Systematics of the four suborders

Monophyly of the suborders. Our analyses do not allow us to reject the hypothesis that each of the four suborders is

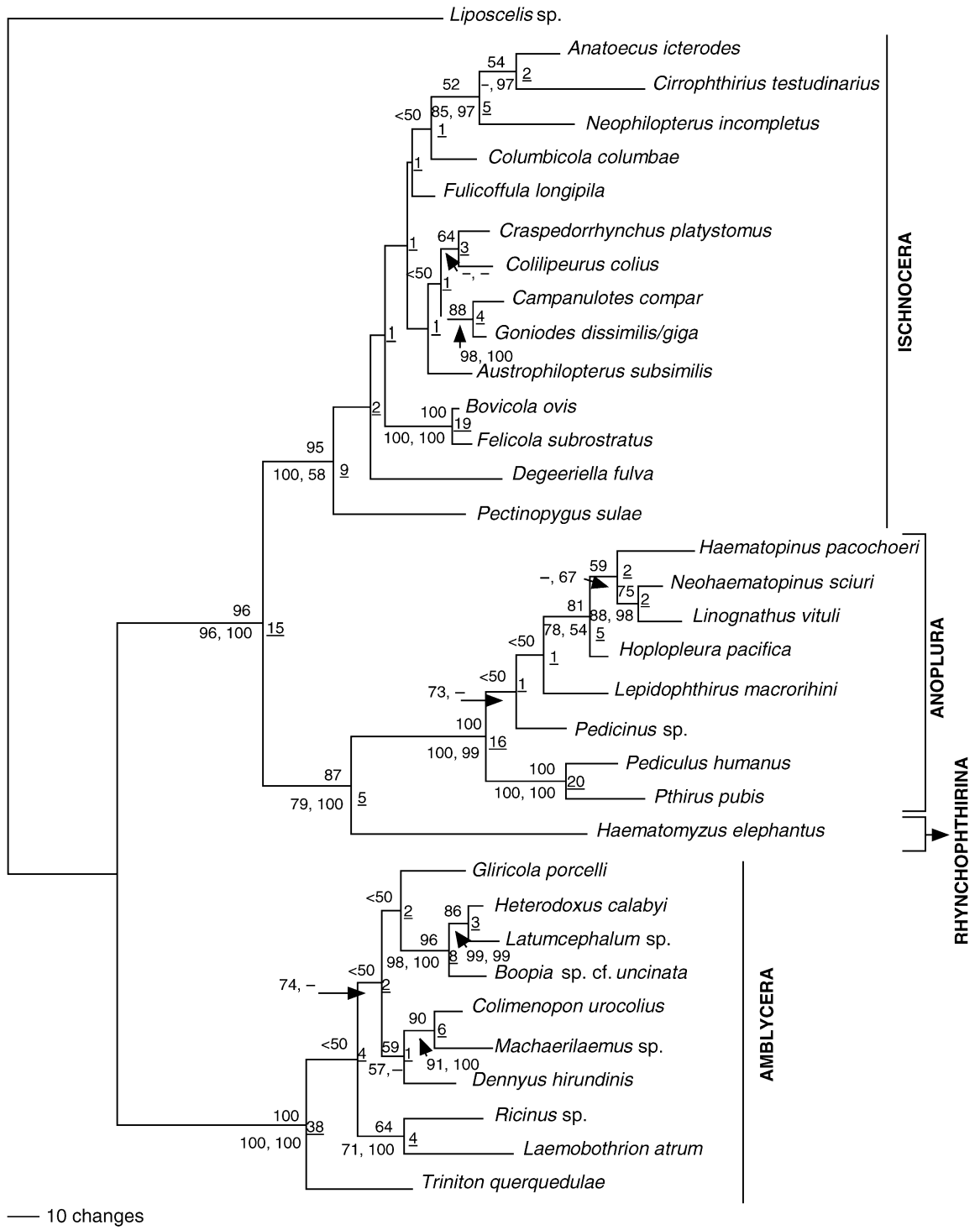

Fig. 2 The single shortest maximum parsimony (MP) tree. Length = 1140 steps; consistency index (CI) = 0.459; retention index (RI) = 0.742; homoplasy index = 0.541. Numbers show bootstrap support from 1000 cycles of bootstrap resampling from MP (above branches) and GD analyses (beneath branches, left hand side) and the posterior probability values for MCMC analyses (beneath branches, right hand side). Numbers to the right of each branch show Bremer support.

monophyletic. Indeed, the SSU gene provided strong support for monophyly of the three suborders which we sampled well: Amblycera (100% bootstrap support, Bremer 38), Ischnocera (58–100%, 9) and Anoplura (99–100%, 16).

Paraphyly of the Mallophaga. The traditional view of louse evolution, which is still widely held by many nonspecialists, places the lice with mouthparts adapted for chewing/biting (as opposed to piercing and sucking) in a natural group (≡ monophyletic lineage) called the Mallophaga (Ischnocera, Amblycera and Rhynchophthirina). Morphological (Lyal 1985) and now molecular studies (this study), allow us to reject the hypothesis of a monophyletic Mallophaga. The phylogenetic relationships of the suborders of lice suggest that mouthparts adapted for chewing/biting are plesiomorphic for the Ischnocera, Amblycera and Rhynchophthirina, whereas piercing and sucking (as in all Anoplura) is synapomorphic. Thus, we recommend that the term Mallophaga be abandoned. Unfortunately, it is entrenched in the veterinary literature so it may take time to disappear from common use. Nonetheless, we strongly advocate that the term Mallophaga be abandoned. Some people may say that this will cause unnecessary confusion and a proliferation of terms, but this is not true as the suborder Rhynchophthirina has only three species thus the word 'Rhynchophthirina' is rarely, if ever, encountered by many people. Therefore, in common usage the term Mallophaga would be 'replaced' by the suborder names Amblycera and Ischnocera. The names Amblycera and Ischnocera hve been used for well over 50 years and are well known to many workers.

Phylogenetic position of the Rhynchophthirina. The Rhynchophthirina comprises three known species in one genus. These lice infest the African and Indian elephants (infested by *Haematomyzus elephantus*), the warthog (infested by *H. hopkinsi*), and the bush pig of eastern Africa (infested by *H. porci*). Our analyses confirm those of Johnson & Whiting (2002) that the SSU rRNA indicates that the Rhynchophthirina is the sister-group to the Anoplura. However, note that both of the two taxonomic schemes proposed for the Anoplura and the Ryncopthirina are consistent with the apparent phylogeny of these lice i.e.: (i) the Anoplura and Rynchopthirina as separate suborders and (ii) the Rynchophthirina as part of the Anoplura.

Other phylogenetic insights

Boopiidae. The three species from this family, which are almost entirely restricted to Australian and New Guinean marsupials, formed a monophyletic lineage with strong support (96–100% bootstrap support, Bremer 8). Further, there was strong support for the species of *Heterodoxus* plus *Latumcephalum*, to the exclusion of the species of *Boopia* (86–99%, 3). This indicates that the SSU rRNA may be instructive at the level of genus in this family (there are six genera at present).

Menoponidae. Three of the four Menoponidae species (*Colimenopon urocolius*, *Machaerilaemus* sp. and *Dennyus hirundinis*) grouped with the Gyropidae and Boopiidae to the exclusion of the other Menoponidae (*Trinoton querquedulae*), so the Menoponidae was not monophyletic in our trees. However, this relationship had low support. Thus, analyses of many more genera and other markers are needed to test the current hypothesis of a monophyletic Menoponidae.

Ricinidae and Laemobothridae. We sampled only one species from the sole genus of each family (*Ricinus* and *Laemobothrion*). Nonetheless, we note that a sister-group relationship between these families of bird lice was reasonably well supported (64–100%, 4).

Trichodectidae and Goniodidae. The two species of these mammal-infesting Ischnocera (*Bovicola ovis* and *Felicola subrostratus*) were strongly supported as monophyletic (100%, 19) as were the Goniodidae from birds (*Campanulotes compar* and *Goniodes dissimilis/giga*) (88–100%, 4).

Philopteridae. This large family of bird lice was paraphyletic in our MP tree due to 10 species of philopteridae grouping with the Trichodectidae and Goniodidae to the exclusion of the remaining two philopterid species (*Degeeriella fulva* and *Pectinopygus sulae*). However, there was little support for this relationship (Bremer support 1). Analyses of MCMC and NJ also had the Philopteridae as paraphyletic with respect to the Trichodectidae but the topologies differed slightly.

Lice of primates. Three genera of sucking lice infest primates: *Pediculus*, *Pthirus* and *Pedicinus*. There are two species of *Pthirus*: *Pt. pubis*, of humans and *Pt. gorillae*, of gorillas. Although many species of *Pediculus* have been described, only three or four are currently recognized: *P. schaeffi* ex chimpanzee and bonobo; *P. mjobergi* ex new world monkeys; and two species or subspecies of *Pediculus* that infest humans, clothing or body lice (*P. humanis* or *P. humanis humanis*) and head lice (*P. capitis* or *P. humanus capitis*). One hypothesis for the phylogeny of the lice of primates is that *Pediculus* is the sister-group to *Pthirus* to the exclusion of *Pedicinus* spp. We could not reject this hypothesis; indeed, we found strong support for *Pediculus* and *Pthirus* being sister-groups (100%, 20).

Phylogenetic utility and rates of nucleotide substitution in SSU rRNA of lice

Previous studies (Page *et al*. 1998; Johnson & Whiting 2002; Johnson *et al*. in press) found that nucleotide substitution rates were higher in lice than in other insects for some mitochondrial

genes. In contrast, we did not detect high substitution rates for the SSU rRNA. Furthermore, the nucleotide substitution rates of the suborders of lice did not differ significantly from one another, although the phylogenetic utility of SSU rDNA did: phylogenetic relationships within the Amblycera and Anoplura were better resolved than those within Ischnocera. Although the substitution rates in the lice were not greater than that of another insect, *D. melanogaster*, there were large expansion regions for the V4 domain (E23-4 to E23-7) in some species of lice compared to other lice and insects (Wuyts *et al*. 2000). Most insects have SSU rRNA sequences of approximately 2 kb but exceptions have been found (e.g. 3316 bp in *Xenos vesparum*; Chalwatzis *et al*. 1995). In some of the species of lice we studied, the SSU rRNA was over 2.6 kb long, e.g. *G. porcelli* at 2661 bp and *Pedicinus* sp. at 2758 bp.

Acknowledgements

We thank the following people who generously donated insects for this study: Ms. Bell, I. Beveridge, S. Cameron, Z. Doyle, M. Eldridge, A. Emmott, J. Fong, Kurniasih, E. Lonc, T. Nadler, M. Oakwood, R. Palma, L. Rozsa, R. Slade, C. Smithers, J. Wellington, L. Durden, R. Moyle, J. Weckstein, R. J. Adams, E. Osnas, S. Meagher, D. Clayton, and S. Al-tamimi. Jared Helm assisted MW with sequencing and R. J. Adams prepared slide mounts of voucher specimens for KPJ and assisted with identification. KPJ was supported by NSF grant DEB-0107891 and PEET DEB-0118794 during the writing of the manuscript. This study was also supported by an Australian Research Council grant to SCB.

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