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3 **Changes in Base Composition Bias of Nuclear and Mitochondrial**
4 **Genes in Lice (Insecta: Psocodea)**

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Abstract While it is well known that changes in the general processes of molecular evolution have occurred on a variety of timescales, the mechanisms underlying these changes are less well understood. Parasitic lice (“Phthiraptera”) and their close relatives (infraorder Nanopsocetae of the insect order Psocodea) are a group of insects well known for their unusual features of molecular evolution. We examined changes in base composition across parasitic lice and bark lice. We identified substantial differences in percent GC content between the clade comprising parasitic lice plus closely related bark lice (= Nanopsocetae) versus all other bark lice. These changes occurred for both nuclear and mitochondrial protein coding and ribosomal RNA genes, often in the same direction. To evaluate whether correlations in base composition change also occurred within lineages, we used phylogenetically controlled comparisons, and in this case few significant correlations were identified. Examining more constrained sites (first/second codon positions and rRNA) revealed that, in comparison to the other bark lice, the GC content of parasitic lice and close relatives tended towards 50% either up from less than 50% GC or down from greater than 50% GC. In contrast, less constrained sites (third codon positions) in both nuclear and mitochondrial genes showed less of a consistent change of base composition in parasitic lice and very close relatives. We conclude that relaxed selection on this group of insects is a potential explanation of the change in base composition for both mitochondrial and nuclear genes, which could lead to nucleotide frequencies closer to random expectation (i.e., 50% GC) in the absence of any mutation bias. Evidence suggests this relaxed selection arose once in the non-parasitic common ancestor of Phthiraptera + Nanopsocetae and is not directly related to the evolution of the parasitism in lice.

Keywords: molecular evolution; GC content; relaxed selection; slightly deleterious mutation.

Introduction

Understanding variation in the process of DNA substitution across lineages is fundamental to understanding the factors that influence molecular evolution. For strictly neutral mutations, the DNA substitution rate is predicted to be directly proportional to mutation rate, independent of population size (Kimura 1962). However, not all mutations are strictly neutral, and many DNA mutations can be deleterious or beneficial in which the rate of substitution can be affected by population size (Ohta 1973, 1992). Therefore, it is expected that patterns of molecular evolution will vary across groups of organisms. Identifying whether differences in substitution rates are a result of direct selection or a result of underlying differences in mutation (e.g., non-selective effects such as drift on mutation repair mechanisms) is important for understanding this variation. Increases in DNA substitution rates in small populations might be expected when mutations are slightly deleterious (Ohta, 1973, 1992). For example, more non-synonymous substitutions occurred in island birds compared to mainland ones (Johnson and Seger 2001), providing evidence that slightly deleterious mutations might substitute more rapidly in small populations than in large populations. In contrast, differences in the rate of substitution in neutral mutations between lineages is more likely to be due to differences between them in the underlying mutation rate.

Variation in the process of molecular evolution across lineages is also known to confound phylogenetic analyses. Variation in substitution rates among lineages is a widely studied phylogenetic problem (e.g., Cameron and Crespi 1995; Huelsenbeck 1997). Such variation can lead to the problem of long branch attraction (Felsenstein 1978). Another feature of molecular evolution that can reduce the accuracy of phylogenetic estimation is variation in base composition (Galtier and Gouy 1995; Sheffield et al. 2009). In this case, taxa might be united based on similar base composition rather than shared evolutionary history. While there are methods being developed to deal with these problems, base composition bias continues to remain a difficulty rarely accounted for in phylogenetic studies (Sheffield et al. 2009). Finally, variation among lineages in RNA secondary structure makes aligning ribosomal DNA sequences difficult, which also potentially reduces accuracy of phylogenetic estimation (Rosenberg 2009).

All three of these phenomena occur in parasitic lice and their non-parasitic relatives (=Nanopsocetae of the insect order Psocodea). Parasitic lice and Nanopsocetae both have a dramatically elevated rate of nucleotide substitution in comparison to other insects (Rosenberg 2009; Page et al. 1998; Johnson et al. 2003; Yoshizawa and Johnson 2003; Johnson et al. 2004), substantial variation in

mitochondrial ribosomal secondary structure (Page et al. 2002; Yoshizawa and Johnson 2003), and strong base composition biases of mitochondrial genes (Yoshizawa and Johnson 2003). Potentially related phenomena in the louse genome include rearrangements of mitochondrial gene order (Shao et al. 2001; Cameron et al. 2007), the mitochondrial genome being separated into mini-chromosomes (Shao et al. 2009; Cameron et al. 2011; Wei et al. 2012), and very small nuclear genome size which is almost devoid of introns in each gene (Kirkness et al. 2010). Currently, these unusual evolutionary trends are known only from a limited sample of genes in lice and free-living close relatives (mostly mitochondrial genomes), and it is not known whether these trends also occur in nuclear genes (except for the accelerated substitution in 18S rDNA: Johnson et al. 2004; Yoshizawa & Johnson 2010) and, if so, how they might be correlated with patterns observed in mitochondrial genes.

To evaluate trends in the molecular evolution of a variety of genes, we sequenced both protein coding and ribosomal RNA genes from both the mitochondrial and nuclear genomes of lice and relatives. While also used to estimate phylogenetic relationships (Yoshizawa and Johnson 2010), this data set allows us to examine patterns of molecular evolution across different genes. Here, we examine changes of nucleotide base composition in parasitic lice (Phthiraptera) and their non-parasitic relatives (Nanopsocetae) between nuclear and mitochondrial genes and compare this to other free-living non-parasitic bark lice (order Psocodea). In particular, the mitochondrial genes of lice are less AT biased (Yoshizawa and Johnson, 2003) than other insects, which have highly AT biased mitochondrial genomes (Jermini et al. 1994). Nucleotide base composition bias is a long recognized and long studied biological phenomenon; however, to our knowledge there has not been a study of base composition in both nuclear and mitochondrial genes simultaneously. Here we examine the base composition of both nuclear and mitochondrial protein coding and ribosomal RNA coding genes (nuclear 18S rDNA and Histone 3 and mitochondrial 16S rDNA and COI) and examine both heavily constrained and more lightly constrained portions of each gene. In the previous studies, nuclear and mitochondrial genomes have largely been analyzed independently for base composition biases (e.g., Page et al. 1998, 2002; Johnson et al. 2003; Cameron et al. 2011). However, if there is a cell wide bias in the availability of nucleotides, we might expect a correlation between nuclear and mitochondrial base composition biases. The present data set gives us an opportunity to explore the nature of any correlated changes between nuclear and mitochondrial base composition.

Materials and Methods

101 To compare changes in base composition among parasitic lice and bark lice, we used the data and
102 maximum likelihood tree from a previous study (Fig. 1) (Yoshizawa and Johnson 2010). The data matrix
103 included sequences from four genes (nuclear 18S rDNA and Histone 3 (H3) and mitochondrial 16S
104 rDNA and COI) obtained from 69 taxa representing all the infraorders of Psocodea (parasitic lice and
105 bark lice). These genes included both ribosomal and protein-coding genes from nuclear and mitochondrial
106 genomes, and there was no missing data in the matrix.

107 For ribosomal DNA, we prepared a data set excluding all gapped regions. The gapped regions
108 were excluded because the rDNA data set included a considerable portion of some sequences that could
109 not be compared between groups, and the base composition of gapped regions for sequences with gaps in
110 the alignment is undefined. For protein-coding genes, each gene was divided into two data sets by codon
111 position to represent more and less constrained positions: 1st+2nd codon positions (H3(1st+2nd) and
112 COI(1st+2nd)) and 3rd position (H3(3rd) and COI(3rd)).

113 The base composition of different gene partitions was calculated from the terminal taxa using
114 MacClade 4.08 (Maddison and Maddison 2005) (Fig. 1). Because of phylogenetic non-independence, we
115 did not analyze these data statistically. Each data set was separated into two categories *a priori*,
116 Nanopsocetae (non-parasitic infraorder including Sphaeropsocidae, Pachytroctidae and Liposcelididae) +
117 Phthiraptera (parasitic lice) (NP) versus other Psocodea (OP). NP taxa were the groups identified in
118 previous studies that appear to have the greatest difference in molecular evolutionary trends compare to
119 OP taxa, including substitution rate, mitochondrial base composition bias, and rRNA secondary structure
120 (Page et al. 1998, 2002; Johnson et al. 2003, 2004; Yoshizawa and Johnson 2003).

121 To explore whether correlations of changes in base composition between genes occurred along
122 branches within each group, we controlled for potential phylogenetic correlation in the data by
123 constructing phylogenetically independent comparisons using PDAP package (Midford et al. 2010) for
124 Mesquite (Maddison and Maddison 2010). This software package implements the phylogenetically
125 independent contrast method (PIC) (Felsenstein 1985). We also compared the independent contrast
126 obtained from between the NP clade and its sister taxon (asterisk in Fig. 1) with all other contrasts to see
127 if the change in the ancestor of the NP clade is an outlier (which would indicate a large change in base
128 composition on the ancestral branch of this clade). Statistical tests were performed using JMP v. 8 (SAS
129 2009).

130 In addition, to assess whether the differences in base composition might be explained by
131 differences in amino acid substitution rates, we also compared the non-synonymous to synonymous

substitution ratio (dN/dS ratio) of the protein-coding genes between two taxon categories using the TestBranchDNDS batch file implemented in HyPhy v2.0 (Kosakovsky et al. 2005). The default setting was selected except for the selection of the complete model for site-by-site variation. Original data (Nexus file of sequence data: Supplementary Data 1; CSV file of GC% table: Supplementary Data 2) are available from the journal web site, or <http://kazu.psocodea.org/data/gc/>.

Results

Strong differences in base composition between Nanopsocetae + Phthiraptera (NP) and other Psocodea (OP) were evident for 18S, H3(1st+2nd), 16S, and COI(1st+2nd), but base composition was less different between the two groups of taxa for third positions of protein coding genes (H3 and COI; Fig. 2, Table 1). GC content of 18S, 16S and COI(1st+2nd) in the NP taxa was almost consistently higher than that of OP taxa (7-10 of top 10 taxa were NP taxa), whereas that of H3(1st+2nd) was almost consistently lower in NP than OP taxa (9 of lowest 10 were NP) (Fig. 2).

Several genes and partitions, i.e., 18S, H3(1st+2nd), 16S, and COI(1st+2nd), of the OP taxa exhibited a relatively constant range of GC% for that gene (Table 1, Fig. 2 white bars). In contrast, in the variability of GC% of third positions for H3 and COI was large, even among the OP taxa (Table 1, Fig. 2 white bars). The variability in base composition of the Nanopsocetae + Phthiraptera (NP) taxa showed similar trends, but results from the Bartlett's test showed that the variability in base composition of 16S and COI(1st+2nd) were significantly higher compared to OP taxa (Table 1). Except for COI(3rd), the standard deviations of base composition is always higher in NP taxa than that of OP. The variability in base compositions of 18S and H3(1st+2nd) in the NP taxa was low across these taxa as well as the OP taxa, while third position base composition of H3 and COI for the NP taxa was highly variable, similar to the OP taxa (Table 1).

We used phylogenetically independent contrasts to determine whether the changes in base composition were correlated between genes along branches across the phylogeny and also within each of the major groups. There were no correlations among closely related taxa in the increase or decrease of GC content between different data sets (Table 2). In a few comparisons, significant correlations were identified (asterisks in Table 2) but none of them were significant when accounting for multiple testing. When only the branches in the NP clade were analyzed, a correlation was identified only among mitochondrial data sets, and this was not significant after accounting for multiple testing. Comparisons of phylogenetically independent contrast absolute values revealed that the value for the ancestor of the NP

clade is higher for 16S dataset (1/68) but not for any other gene or partition (Table 3). In other comparisons, the phylogenetically independent contrast value for the ancestor of the NP clade was generally higher than average (8-17/68), but the contrast for the third positions of COI was even lower than the average (41/68) (Table 3).

Comparisons of dN/dS ratios between the NP and OP taxa revealed no significant difference for both H3 ($P=0.66448$) and COI ($P=0.96373$).

Discussion

The Nanopsocetae + Phthiraptera clade of parasitic lice and non-parasitic bark lice (Insecta: Psocodea) shows a different pattern of base composition than other members of Psocodea in all the genes examined, including nuclear and mitochondrial genes, both protein-coding and ribosomal RNA (Fig. 2). Analyses that accounted for phylogenetic correlation using independent contrasts, however, only showed this correlation for a limited number of comparisons, and none of these are significant after accounting for multiple testing (Table 2). That is, the overall changes nuclear and mitochondrial base composition in the ancestor of NP clade was not reflected in any additional correlated changes within this group. Such a correlation might be expected, for example, if there is a cell wide bias in the availability of nucleotides. Thus, these results suggest that the major change in base composition across genes between NP and OP taxa occurred once in the evolutionary history of this group and directional change in base composition is not continuing. However, the absolute value of the contrast for the ancestor of the NP clade was only significantly larger than all other contrasts for the 16S gene, and this is also not significant after accounting for multiple testing (Table 3).

A more detailed examination of these differences revealed some other interesting patterns, and may provide a more general mechanism for changes in both nuclear and mitochondrial base composition in this group of insects. First, for more constrained sites, such as first and second codon positions and ribosomal DNA, the GC% is relatively invariant throughout OP taxa compared to NP taxa (Fig. 2, Table 1). First and second positions of H3 are GC biased and first and second positions of COI and rDNA are AT biased. In theory, if mutation and substitution are random, over time an equal frequency of all four nucleotides will result (Li 1997). Therefore, the relatively biased base composition (i.e. away from 50% GC) in the OP taxa suggests either mutational bias (from environmental or cellular constraints) or that selection is operating to maintain base composition in relation to secondary structures of rRNA and mRNA, amino-acid composition, and 3D structure of protein (Foster et al. 1997; Chiusano et al. 1999). In

three out of four data sets of constrained sites, the nucleotide composition of the NP taxa is more GC biased compared to those of the OP taxa (Fig. 2, Table 1). One exception is that nucleotide composition of H3(1st+2nd) in the NP taxa are more AT biased compared to those of OP taxa, showing an opposite trend from other genes. However, in all cases the change in base composition in the NP taxa is in the direction of 50% GC, either up towards 50% for the ribosomal RNA genes and first and second positions of COI, or down towards 50% in the case of first and second positions for H3 (Fig. 2).

For less constrained third codon positions, the base composition is more highly variable even in the OP taxa, and this is the case for both the nuclear H3 and mitochondrial COI genes (Fig. 2, Table 1). Such variability in third position GC content has also been previously detected in *Drosophila* (Matsuo 2003). The differences of GC content between the NP and OP taxa are also much less striking for these third positions compared to more constrained rDNA or 1st and 2nd codon positions (Fig. 2, Table 1). Selection is much weaker on third codon positions regardless of whether proteins are under different selection pressures in different taxa. Therefore, the much weaker difference in GC% for third positions of H3 and COI between the OP and NP taxa may be explained by lower selection pressure on 3rd codon positions for both groups of taxa. Even so, there does appear to be some differences in the GC content of third positions for COI between the OP and NP taxa (Fig. 2). While the GC% for H3 fluctuates above and below 50%, the third codon position of the COI gene is strongly AT biased (Fig. 2), while also being highly variable. The mitochondrial genomes of insects are known to be strongly AT biased, which may be maintained by selective pressure (Jermiin et al. 1994). In the present case, the most strongly biased species contains only 3.5% of GC at the 3rd codon position of COI gene, and it seems likely that selection may be operating to maintain such a strong bias (Jermiin et al. 1994). Therefore, the nucleotide composition for third codon positions of mitochondrial genes is probably subject to selection, and the different GC% (closer to 50%) in third positions of COI for the NP taxa may also indicate a change in selective pressure.

Given the nature of all the biases observed, it seems the best explanation for the changes in base composition across these taxa may be relaxed selection. In particular, the changes in base composition in the NP taxa are in different directions for H3 (1st+2nd) (GC% lowered) compared to other genes (GC% increased). However, these changes converge towards 50% GC in all cases. This result suggests that there is not an overall change in base composition in one direction in both nuclear and mitochondrial genomes. Rather, in the absence of mutation biases, accumulation of random substitutions will tend to lead to a more equal frequency of nucleotide composition (Li 1997). Reduction of base composition bias is also

reported from *Drosophila* when selection is relaxed (Shields et al. 1988; Sharp and Li 1989; Moriyama and Gojobori 1992). Therefore, less pronounced base composition bias in NP taxa can be explained by relaxed selection and accumulation of more random substitution.

Significantly higher variance of the GC% for rDNA and 1st+2nd codon positions in the NP clade compared to OP taxa also supports the hypothesis of relaxed selection against base composition bias (Fig. 2, Table 1). Given that the NP taxa are more closely related to each other than the OP taxa, it is even more unlikely that the NP taxa have higher variance by chance alone. An exception is the GC% of third codon positions (i.e., H3(3rd) and COI(3rd)), which showed high variance in both the OP taxa and NP taxa (Fig. 2, Table 1). The third codon positions of protein-coding genes are largely silent sites. In effect these sites are already under relaxed selection, which would lead to high variance in base composition in both OP and NP taxa. In particular, the GC% of third positions of H3 fluctuates with similar variance around 50% GC for both NP and OP taxa (Table 1), suggesting these positions may be under equally relaxed selection in both groups. Thus, the high variance of base composition at third sites in the OP taxa is consistent with our conclusions about relaxed selection at more constrained sites. In contrast to the nuclear H3 gene, third codon positions of the mitochondrial COI are highly AT biased, particularly in the OP taxa, which cannot be explained by consistently relaxed selection at these sites. However, it is widely known that the insect mitochondrial genome is highly AT biased probably either due to mutation bias or selection favoring this bias (Jermini et al. 1994). In our analysis, the GC% of COI(3rd) in the NP taxa is less AT biased (Fig. 2, Table 1), which might also be indicative of relaxed selection at these sites, if selection maintains the strong AT bias in most insects.

Other unusual evolutionary trends observed in genomes of parasitic lice and non-parasitic close relatives, such as accelerated substitution rates (Page et al. 1998; Johnson et al. 2003; Yoshizawa and Johnson 2003; Johnson et al. 2004), modifications of ribosomal RNA secondary structure (Yoshizawa and Johnson 2003; Page et al. 2002), and minicircularization of mitochondrial chromosome (Shao et al. 2009; Cameron et al. 2011; Wei et al. 2012) all involve what would normally be slightly deleterious mutations. Therefore, these phenomena can also be explained by relaxed selection at molecular level. The other explanations previously proposed for these phenomena in lice and their relatives are less applicable than relaxed selection hypothesis. For example, short generation time was proposed as a potential factor that could increase substitution rate (Hafner et al. 1994). However, this effect cannot explain other phenomena including nucleotide base composition biases. The presence of symbionts is also known to affect molecular evolutionary trends of organisms harboring them (Nigro and Prout 1990; Kambhampati

et al. 1992), and the highly AT-biased endosymbionts of parasitic lice (Sasaki-Fukatsu et al. 2006; Fukatsu et al. 2007) may have some relation to GC-bias of many genes in parasitic lice. However, this cannot explain the more AT-biased first and second positions of H3.

Relaxed selection at the molecular level has been identified in parasitic plants (Young and dePamphilis 2005), parasitic wasps (Dowton and Austin 1995; Dowton et al. 2009), and endosymbionts of insects (Clark et al. 1999; Woolfit and Bronham 2003). Thus, the parasitic lifestyle of lice may play a role in maintaining such relaxed selection in this group. One possibility is that relaxed selection is result of small effective population sizes in these insects (Ohta 1973, 1992), which provides a greater potential for the fixation of slightly deleterious mutations. It is generally argued that the effective population size of louse species is small due to highly structured populations with frequent bottlenecks (Page et al. 1998). However, our results indicate relaxed selection originated in the non-parasitic ancestor of the NP clade, and as such is not directly related to the origins of the parasitism in lice. It is currently unknown if such structured populations with frequent bottleneck events occur in Nanopsocetae. Furthermore, comparisons of dN/dS ratios revealed no significant differences between NP and OP taxa. If small effective population size is the major factor causing relaxed selection in NP taxa, we would expect increased accumulation of non-synonymous substitutions compared to synonymous ones, because synonymous substitutions are less likely to be deleterious and thus less affected by population size (Kimura 1962). However, the documented acceleration of substitution rates in parasitic lice is also evident at third codon positions (Johnson et al. 2003), which are mostly synonymous substitutions. No significant differences in dN/dS ratios were detected between NP and OP taxa, indicating a proportional increase in both synonymous and non-synonymous substitutions in NP taxa. Therefore, it seems unlikely that the changes in base composition occur as strictly the accumulation of slightly deleterious mutations in small populations.

Rather, relaxed selection may extend to the mutation repair machinery itself, because the strength of selection for mutation repair is directly proportional to the selection coefficient against deleterious mutations genome wide (Leigh 1970). In the case of parasites and endosymbionts, certain genes may lose function because of overall simplification in body form, in which case the number of genome wide potential deleterious mutations is reduced. This hypothesis predicts increased substitution rates of both neutral and selected sites and thus can account for both an accelerated substitution rate at third codon position (Johnson et al. 2003) and a lack of significant difference in dN/dS ratio between the NP and OP taxa. These unusual molecular evolutionary phenomena, including minicircularization of mitochondrial chromosome (Wei et al. 2012), are also observed in some non-parasitic Nanopsocetae and thus cannot be

attributed to the origins of parasitism in lice. Generally, members of Nanopsocetae have a smaller body size, simpler morphology with many reduced features (e.g., reduced size in eyes, reduction or absence of wings), and a more cryptic lifestyle (e.g. under bark) than other groups of free-living bark lice. Such a reduction in morphological and behavioral complexity may lead relaxed selection at many loci even for these non-parasitic bark lice. The possible features leading to relaxed selection in these insects deserve further study.

One more important point is that the changes in base composition in the parasitic lice and their close relatives may also affect the accuracy of molecular phylogenetic reconstruction. The parasitic lice have long been thought to have a single evolutionary origin and thus classified as a single order, Phthiraptera. However, a molecular phylogeny based on 18S rDNA gene sequences suggests that parasitic lice have evolved twice independently (Johnson et al. 2004). This hypothesis was tested by the data set also used in the current study (Yoshizawa and Johnson 2010), which provided tentative support for the polyphyly of lice hypothesis. However, significant base composition bias may have affected the results. A couple of recent studies, albeit with relatively poor taxon sampling, suggested monophyly of Phthiraptera (Wei et al. 2012; Johnson et al. 2013). Future tests of this hypothesis should adopt the methods that account for differences in base composition, though accurately doing this may be difficult (Sheffield et al. 2009).

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Figure Legends

Figure 1. ML tree estimated from all data sets combined (redrawn from Yoshizawa & Johnson, 2010). Relative branch lengths of the tree is shown at the top-left corner. GC% of terminal taxon is shown at the tip of branches (18S, H3(1st,2nd), H3(3rd), 16S, COI(1st,2nd), COI(3rd)) followed by Taxon ID# (See Fig. 2).

Figure 2. Base composition of each taxon for each gene. Black bar indicates samples from Nanopsocetae+parasitic lice (NP taxa), and white bar indicates other Psocodea samples (OP taxa). See Fig. 1 for Taxon ID#.

Supplementary Data

Supplementary data 1. Nexus format data matrix of the gene data analyzed in this paper.

Supplementary data 2. CSV format data file of the GC% of terminal taxa.

Tables

Table 1. Comparisons of GC% between the NP (Nanopsocetae + Phthiraptera) and OP (other Psocodea) taxa. P-value indicates the results from the Bartlett's test for homogeneity of variance. Except for COI(3rd), standard deviation (SD) of NP taxa is always higher than that of OP. Asterisks indicate significant at 1% (**) or 0.1% (***).

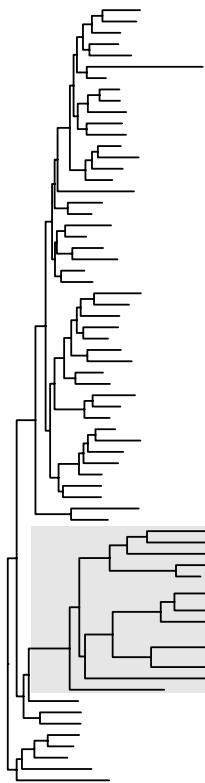
Gene Result	OP range	OP mean	OP SD	NP range	NP mean	NP SD	Homogeneity of Variances (Bartlett: NP vs OP)
18S	44.9-48.5	46.6	0.828	47.3-51.6	49.6	1.060	P = 0.2299
H3(1st+2nd)	51.8-55.9	54.2	0.794	50.5-53.6	51.8	0.947	P = 0.3986
H3(3rd)	34.5-84.5	53.6	10.735	47.2-79.4	59.2	11.450	P = 0.7615
16S	27.1-33.0	29.0	1.247	29.5-45.8	37.1	4.179	P < 0.0001***
COI(1st+2nd)	37.9-44.3	40.1	1.274	38.7-47.7	42.1	2.395	P = 0.0012**
COI(3rd)	3.5-43.1	14.3	7.050	12.8-40.7	23.3	7.017	P = 0.9827

Table 2. Correlations of the phylogenetically independent contrasts (PIC) between genes. P-values were calculated for analyses including all taxa (total) and the Nanopsocetae-Phthiraptera clade analyzed alone (NP). * indicate significant at 5% level, but none of these are significant after correcting for multiple testing.

Comparison PIC	P(total)	P(NP)
18S vs. H3(1st+2nd)	P=0.9557	P=0.6441
18S vs. H3(3rd)	P=0.0162*	P=0.1643
18S vs. 16S	P=0.8141	P=0.6404
18S vs. COI(1st+2nd)	P=0.3475	P=0.8386
18S vs. COI(3rd)	P=0.1779	P=0.4252
H3(1st+2nd) vs. H3(3rd)	P=0.0535	P=0.8735
H3(1st+2nd) vs. 16S	P=0.4859	P=0.0728
H3(1st+2nd) vs. COI(1st+2nd)	P=0.0571	P=0.9801
H3(1st+2nd) vs. COI(3rd)	P=0.1602	P=0.8863
H3(3rd) vs. 16S	P=0.8788	P=0.5843
H3(3rd) vs. COI(1st+2nd)	P=0.9713	P=0.5169
H3(3rd) vs. COI(3rd)	P=0.8623	P=0.7011
16S vs. COI(1st+2nd)	P=0.0145*	P=0.0313*
16S vs. COI(3rd)	P=0.0204*	P=0.0745
COI(1st+2nd) vs. COI(3rd)	P=0.0173*	P=0.0473*

Table 3. Comparisons of phylogenetically independent contrast absolute values for the ancestor of Nanopsocetae + Phthiraptera clade against all the other contrasts. *indicates significant at $P = 0.05$, but it is not significant after accounting for multiple testing.

Gene	Rank/Total Contrasts
18S	16/68
H3(1st+2nd)	8/68
H3(3rd)	17/68
16S	1/68*
COI(1st+2nd)	15/68
COI(3rd)	41/68



45.1	54.5	45.9	28.8	39.7	11.6	1	<i>Dypsocus coleoptratus</i>
45.4	54.1	54.5	29.5	38.5	5.8	2	<i>Isophanes sp.230</i>
46.4	54.1	41.8	28.8	38.5	14	3	<i>Fuelleborniella sp.</i>
46.6	54.5	53.6	28.5	39.1	15.1	4	<i>Calocaecilius decipiens</i>
46.2	54.1	51.8	29.2	41.4	11.6	5	<i>Pericaecilius sp.239</i>
44.9	53.6	50.9	31.9	39.3	9.3	6	<i>Asiopsocus sp.</i>
47.2	54.5	69.7	27.4	39.7	14	7	<i>Valenzuela flavidus (USA)</i>
46.4	53.6	45.7	27.8	40.8	17.4	8	<i>Stenopsocus aphidiformis</i>
46.6	54.1	50.5	28.1	40.2	17.4	9	<i>Stenopsocus nigricellus</i>
46.2	54.1	56.4	29.2	39.7	12.8	10	<i>Graphopsocus cruciatus</i>
46.8	53.2	53.2	28.1	40.8	16.3	11	<i>Matsumuraiella radiopicta</i>
47	54.6	54.8	28.8	40.2	23.3	12	<i>Xanthocaecilius sommermanae</i>
47	52.3	58.2	28.1	38.5	7	13	<i>Kolbia fusconervosa</i>
46.4	52.7	50.9	28.1	39.7	12.8	14	<i>Polypsocus corruptus</i>
46.1	55	52.7	27.4	39.7	7	15	<i>Amphipsocus japonicus</i>
47.4	53.6	50.9	27.1	37.9	12.8	16	<i>Taeniosigma elongatum</i>
47.2	53.2	53.3	32.6	39.1	18.6	17	<i>Lachesilla forcepeta</i>
46.8	54.3	44.5	29.2	42.5	16.3	18	<i>Kaestneriella sp.</i>
46.6	55	58.2	28.6	39.1	10.5	19	<i>Peripsocus subfasciatus</i>
45.4	53.6	50.9	29.9	42.5	14	20	<i>Anomopsocus amabilis</i>
47.2	55	50.9	29.5	39.7	9.3	21	<i>Eolachesilla chilensis</i>
46.8	54.5	46.8	30.6	40.8	17.4	22	<i>Kilauella sp.</i>
47.2	55	45.5	30.2	40.2	32.6	23	<i>Mesopsocus hongkongensis</i>
47	54.5	61.1	29.2	39.1	11.6	24	<i>Ectopsocus meridionalis</i>
47.2	55	46.4	28.8	39.1	11.6	25	<i>Ectopsocopsis cryptomeriae</i>
46.1	53.6	47.3	29.5	40.8	29.1	26	<i>Amphigerontia jezoensis</i>
45.4	54.1	42.7	29.5	41.4	17.4	27	<i>Ptycta johnsoni</i>
46.4	54.1	44.5	29.2	42	7	28	<i>Atrichadenotecnum sp.MY</i>
47.2	53.6	56.4	29.7	41.4	10.5	29	<i>Loensia variegata</i>
47	54.1	41.8	28.8	40.2	10.5	30	<i>Psocus sp.225</i>
46.2	54.1	43.6	28.5	39.7	9.3	31	<i>Metylphorus novaescotiae</i>
45.6	54.1	39.4	29.5	40.8	15.1	32	<i>Sigmatoneura kakisayap</i>
46.6	54.5	41.8	28.8	38.5	8.1	33	<i>Hemipsocus sp.196</i>
46.2	54.5	50	29.9	39.7	12.8	34	<i>Psilopsocus malayanus</i>
46.2	54.1	55.5	29.2	39.1	18.6	35	<i>Bertkauia crosbyana</i>
45.9	53.6	41.8	27.4	40.2	12.8	36	<i>Goja sp.</i>
46.1	53.2	48.2	28.8	39.7	18.6	37	<i>Epipsocus sp.MY</i>
46.4	55.5	68.2	27.4	39.7	3.5	38	<i>Allocaecilius sinensis</i>
44.9	53.6	55.5	28.5	40.2	12.8	39	<i>Pseudocaecilius citricola</i>
46.6	54.1	58.2	28.5	40.8	19.8	40	<i>Calopsocus furcatus</i>
46.6	55	60	28.8	40.2	17.4	41	<i>Heterocaecilius fuscus</i>
46.2	55.9	79.1	28.8	40.2	9.3	42	<i>Phallocaecilius hirsutus</i>
46.6	54.5	51.8	29.2	38.5	7	43	<i>Aaroniella badonneli</i>
45.6	55.9	44.5	27.9	39.1	3.5	44	<i>Philotarsus kwakiutl</i>
46.6	54.1	34.5	31.6	39.1	20.9	45	<i>Archipsocus sp.209</i>
47	54.5	51.8	33	41.4	10.5	46	<i>Archipsocus sp.226</i>
47.3	51.4	53.6	42.1	43.1	17.4	47	<i>Haematomyzus elephantis</i>
48.9	51.8	56.4	38.2	46	20.9	48	<i>Pediculus humanus</i>
49.1	53	42.7	45.8	47.7	40.7	49	<i>Damalinia sika</i>
50.3	52.7	79.1	38.7	42.5	18.6	50	<i>Campanulotes compar</i>
50.1	52.7	79.4	38.5	43.1	25.6	51	<i>Physconelloides eurysema</i>
49.3	51.8	70.4	38.9	41.1	29.1	52	<i>Menacanthus sp.272</i>
49.1	52.3	59.6	34.7	38.7	17.4	53	<i>Menopon gallinae</i>
48.5	50.5	59.4	29.5	40.2	15.1	54	<i>Heterodoxus calabyi</i>
49.7	51.8	71.8	37.5	40.8	23.3	55	<i>Laemobothrion cubense</i>
50.3	50.9	45	37.5	43.6	24.7	56	<i>Liposcelis brunnea</i>
51.1	50.9	45.4	37.5	42.4	27.1	57	<i>Liposcelis sp.KY2003</i>
50.3	50.9	56.7	34.4	40	30.8	58	<i>Liposcelis bostrychophila</i>
51.6	50.5	57.1	31.9	40.8	23.3	59	<i>Embiopsocus sp.400</i>
49.5	51.6	57.3	31.6	40.2	23.3	60	<i>Tapinella sp.192</i>
49.2	53.6	53.8	39.6	40.8	12.8	61	<i>Badonnelia titei</i>
48.5	54.5	46.3	28.8	39.7	14	62	<i>Stimulopalpus japonicus</i>
48	55.5	78	28.8	38.5	16.3	63	<i>Musapsocus sp.</i>
48	54.5	84.5	28.1	42.5	8.1	64	<i>Compsoecus elegans</i>
48.3	53.2	66.1	27.8	42	23.3	65	<i>Echmepteryx hageni</i>
48.3	53.6	63.6	27.8	40.2	12.8	66	<i>Neolepalepis occidentalis</i>
47.6	54.1	70.9	30.9	40.2	22.1	67	<i>Lepium sp.</i>
47	51.8	49.1	30.2	42	8.1	68	<i>Trogium pulsatorium</i>
47.8	53.6	78.2	28.2	44.3	43.1	69	<i>Psyllipsocus oculatus</i>

Psocomorpha

NP taxa

Parasitic Lice
“Phthiraptera”

Nanopsocetae

Amphientometae

Trogiomorpha

18 H3(12) H3(3) 16 COI(12) COI(3) ID#
terminal GC%

GC%

