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Phylogenetic relationships in the louse genus *Penenirmus* based on nuclear (EF-1α) and mitochondrial (COI) DNA sequences

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Abstract. Ischnoceran lice in genus *Penenirmus* are parasites of birds in orders Piciformes and Passeriformes. No comprehensive revision of this genus has been published, but a few host-based revisions have been done. Here we present a phylogenetic analysis of *Penenirmus* based on nuclear (elongation factor-1 alpha) and mitochondrial (cytochrome oxidase I) gene sequences. Sequences from portions of both these genes provide a well resolved tree that is relatively stable across methods of analysis and to bootstrap resampling. Some aspects of the *Penenirmus* phylogeny reflect the phylogeny of their avian hosts. We identified monophyly of a group of *Penenirmus* species occurring on Passeriformes as well as monophyly of a group containing species sometimes placed in a genus *Picophilopterus*. Species of *Penenirmus* occurring on Old World barbets fall in several positions at the base of the tree, suggesting that other lineages of *Penenirmus* may be derived from those occurring on Old World barbets.

Introduction

Lice (Insecta: Phthiraptera) are becoming model systems for the study of co-phylogenetic history between hosts and parasites. Lice are permanent parasites of both birds and mammals. Several studies have found considerable congruence between louse and host phylogenies (Hafner & Nadler, 1988, 1990; Hafner *et al*., 1994; Page *et al*., 1998; Paterson *et al*., 2000; Johnson & Clayton, 2001), whereas other louse groups show little evidence of co-speciation (Barker, 1991; Clayton *et al*., 2001). One major group of avian lice is family Philopteridae within suborder Ischnocera. This group of chewing lice specializes in eating feathers of their hosts. Philopteridae contains a diverse assemblage of avian lice whose phylogenetic relationships have been assessed only recently (Cruickshank *et al*., 2001; Smith, 2001). Past difficulties in separating phylogenetically informative morphological characters from convergent characters have inhibited phylogenetic work in this group in the past (Clay, 1949). Morphological similarity in some taxa is at odds with recent molecular studies and suggests convergence in body form between some groups of lice on the same host taxa (Johnson *et al*., 2001). Another hindrance to taxonomic and phylogenetic work is that several genera within avian Ischnocera contain large numbers of species and monophyly of these genera has been questioned (Clay, 1949). Often these genera are defined more on the basis of host associations, rather than on the basis of any real synapomorphic characters.

The goal of the present study is to assess phylogenetic relationships of one of these diverse ischnoceran genera: *Penenirmus* Clay & Meinertzhagen. This genus, containing forty-nine described species, is widespread on birds in orders Piciformes and Passeriformes (Hopkins & Clay, 1952). Within Piciformes, it is common on woodpeckers (Picidae) and barbets (Lybiidae, Megalaimidae and Capitonidae), but is conspicuously absent on toucans (Ramphastidae). Dalgleish (1972) revised the species of *Penenirmus* parasitizing woodpeckers and recognized eight species. Two of those species, *Penenirmus pici* Fabricius and *P. auritus* Scopoli, have a very wide host distribution, each occurring on more than ten host species within Picidae. This study demonstrated overlap of morphological variation among populations of *P. pici* and *P. auritus*, making further species distinction unwarranted. The *Penenirmus* of barbets have not undergone comprehensive revision, but eight species have been described (Tendeiro, 1961; Dalgleish, 1967). One species is known from honeyguides (Indicatoridae). In addition to a general abundance on

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Piciformes, species of *Penenirmus* are also found on many songbirds of order Passeriformes. The species of *Penenirmus* on passerines (thirty-two described species) are poorly known and there has been no comprehensive revision of this group. An understanding of the phylogenetic relationships among species of *Penenirmus* would aid in further revision of this genus by uncovering whether the relationships of these parasites reflect host taxonomy.

To evaluate the phylogenetic relationships within louse genus *Penenirmus*, we obtained samples of individuals in this genus from fifteen species of hosts, including woodpeckers, barbets and songbirds. In many cases our collections produced new host records, and these lice could not be assigned to currently described species on the basis of morphology. Thus, we have left these unnamed (indicating them informally with capital letters), awaiting further revision. For all samples, portions of both the nuclear elongation factor-1 alpha (EF-1α) and mitochondrial cytochrome oxidase I (COI) genes were sequenced. We used these sequences to construct a phylogeny for the species in this group, and compared the groups in this phylogeny to host phylogeny and biogeography.

Materials and methods

Sampling and host records

Lice were removed from bird hosts using the ethyl acetate fumigation method described by Clayton *et al.* (1992). Samples of *Penenirmus* were included from fifteen host species. Nine outgroup species were used to help root the tree and to test the monophyly of *Penenirmus* (Table 1). Outgroup genera were chosen on the basis of a previous molecular study (Cruickshank *et al.*, 2001), focusing on those taxa with potentially close relationships to *Penenirmus*.

Sequencing

Specimens were stored either dry at –70°C or in 95% ethanol at –20 °C. DNA was extracted from individual lice by removing the head from the body using a pair of jeweller's forceps. The head and body of the louse were placed in digestion buffer from a Qiagen DNA tissue extraction kit. Digestion proceeded for 56 h at 55°C. After digestion, the head and the body of the louse were removed from the buffer and mounted together in Canada balsam on a microslide as a voucher and for species identification. Voucher specimens were deposited in the Price Institute for Phthirapteran Research, University of Utah. The DNA extraction procedure was completed using manufacturer's protocols (Qiagen, Valencia, California).

The DNA extracts were used in PCR amplifications of both the EF-1α and COI genes. The primers EF1-For3 and EF1-Cho10 were used for EF-1α (Danforth & Ji, 1998) and the primers L6625 and H7005 were used for COI (Hafner *et al.*, 1994). PCR amplification protocols and sequencing followed Johnson & Clayton (2000). Sequences were aligned using Sequencher 3.0 (GeneCodes) and alignment was unambiguous because both regions code for proteins (GenBank accession numbers AF356700–AF356747).

Phylogenetic analyses

Phylogenetic analyses were conducted using PAUP* (Swofford, 2000). The relative substitution rates of COI and EF-1α were explored by plotting percent sequence divergence from pairwise comparisons for COI against those for EF-1α. Differences in substitution rates between gene regions can potentially result in conflicting signals if one gene is essentially saturated (Bull *et al.*, 1993; Chippendale & Wiens, 1994). Thus, the partition homogeneity test (Farris *et al.*, 1994, 1995; Swofford, 2000) was used to evaluate whether COI and EF-1α contained significantly different phylogenetic signal. This test indicated no significant incongruence between gene regions ($P = 0.36$, see Results), so they were combined in subsequent phylogenetic analyses.

Several methods were used to estimate the phylogeny of *Penenirmus*. First, unordered parsimony searches (ten random addition replicates) were conducted with the combined data. Non-parametric bootstrapping (Felsenstein, 1985), with full heuristic searches (1000 replicates), was used to evaluate the relative support for nodes in the most parsimonious tree.

Maximum likelihood searches were used to test the sensitivity of the tree topology to method of analysis. The best-fit model that could not be rejected in favour of a more complicated (parameter rich) model was estimated using the general framework of likelihood ratio tests described by Huelsenbeck & Crandall (1997). The unordered parsimony tree was used to estimate the model parameters. These likelihood ratio tests indicated that a model incorporating six substitution types (general time reversible), unequal base frequencies and rate heterogeneity according to a gamma distribution (eight rate categories) was better than simpler models. The parameters estimated in these analyses were used in likelihood searches (twenty random addition replicates with TBR branch swapping). Full heuristic bootstrap analysis was also performed using the likelihood model (100 replicates, NJ starting trees, NNI branch swapping). For comparison of the parasite phylogeny to relationships among host taxa, a host phylogeny for the avian families, as derived from DNA–DNA hybridization data by Sibley & Ahlquist (1990), was used. These major relationships have also been confirmed to some extent by mitochondrial DNA sequence data (Lanyon & Hall, 1994; Barker & Lanyon, 2000).

Results

In making the collections for this study, we documented several new host records for *Penenirmus* (Table 1). *Penenirmus* species have not been reported previously on New World barbets or African woodpeckers and thus our records from *Eubucco* and *Dendropicos* document association

Table 1. Specimens used in the study.

Louse species	Host species	Host family	Locality
Ingroup			
Penenirmus auritus 1	*Colaptes campestris*	Picidae	Bolivia
Penenirmus auritus 2*	*Dendropicos goertae*	Picidae	Ghana
Penenirmus auritus 3*	*Eubucco bourcierii*	Capitonidae	Peru
Penenirmus auritus 4*	*Melanerpes candidus*	Picidae	Bolivia
Penenirmus auritus 5*	*Picumnus aurifrons*	Picidae	Brazil
Penenirmus auritus 6*	*Piculus flavigula*	Picidae	Brazil
Penenirmus pici 1	*Picus mentalis*	Picidae	Borneo
Penenirmus pici 2*	*Blythipicus rubiginosus*	Picidae	Borneo
Penenirmus zumpti	*Lybius torquatus*	Lybiidae	South Africa
Penenirmus sp. A*	*Myrmecocichla formicivora*	Turdidae	South Africa
Penenirmus sp. B*	*Serinus atrogularis*	Fringillidae	South Africa
Penenirmus sp. C*	*Psaltriparus minimus*	Aegithalidae	Utah
Penenirmus sp. D*	*Gymnobucco calvus*	Lybiidae	Ghana
Penenirmus guineensis	*Lybius dubius*	Lybiidae	Ghana
Penenirmus sp. E*	*Megalaima monticola*	Megalaimidae	Borneo
Outgroup			
Philopterus sp. A	*Batis pririt*	Muscicapidae	South Africa
Philopterus sp. B	*Momotus momota*	Momotidae	Mexico
Rallicola columbiana	*Dendrocolaptes certhia*	Dendrocolaptidae	Mexico
Rallicola fuliginosa	*Dendrocincla anabatina*	Dendrocolaptidae	Mexico
Formicaricola analoides	*Formicarius moniliger*	Formicariidae	Mexico
Fomicaphagus sp.	*Thamnophilus doliatus*	Formicariidae	Mexico
Brueelia sp.	*Parus niger*	Paridae	South Africa
Brueelia marginella	*Momotus momota*	Momotidae	Mexico
Nyctibicola longirostris	*Nyctibius jamaicensis*	Nyctibidae	Mexico

*Indicates a new host record for *Penenirmus*.

with these host groups. All three of the *Penenirmus* species recovered from passerines represent new host records.

We sequenced two individuals of *Penenirmus guineensis* Tendeiro from an individual of *Lybius dubius* for the COI gene. These sequences were identical and are consistent with a general pattern in lice that individuals of the same louse species, from the same host species, differ little in mitochondrial sequence (Johnson & Clayton, 2001). In contrast, individuals of the same louse species from different host species showed remarkable levels of divergence in COI sequences (7.6% in *P. pici* and up to 23.7% in *P. auritus*). This high sequence divergence of lice on different host species also accords with patterns observed in several other louse genera: *Columbicola*, *Physconelloides* (Johnson & Clayton, 2001) and *Anaticola* (Johnson, unpublished data). Within *Penenirmus*, uncorrected COI sequence divergences ranged from 7.6% to 28.7% (Fig. 1).

Sequence divergences for the EF-1α gene were considerably less than for COI (Fig. 1). However, again individual lice of the same species from different hosts showed appreciable divergence in the EF-1α gene sequences (between 0.3% and 1.9% within *P. auritus*). Within *Penenirmus*, uncorrected EF-1α sequences divergences ranged from 0.0% to 12.5% (Fig. 1).

Based on comparisons of the pairwise sequence divergences for COI against those for EF-1α, it appears that COI might be

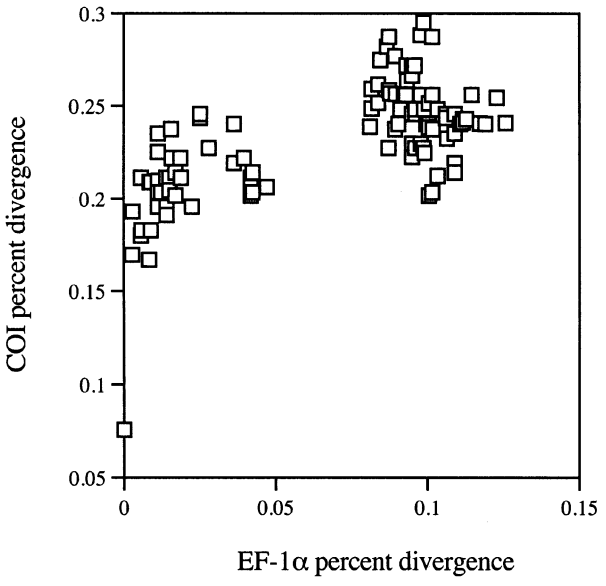

Fig. 1. Plot of sequence divergence from pairwise comparisons within *Penenirmus* only for the COI gene (379 bp) against those for the EF-1α gene (347 bp).

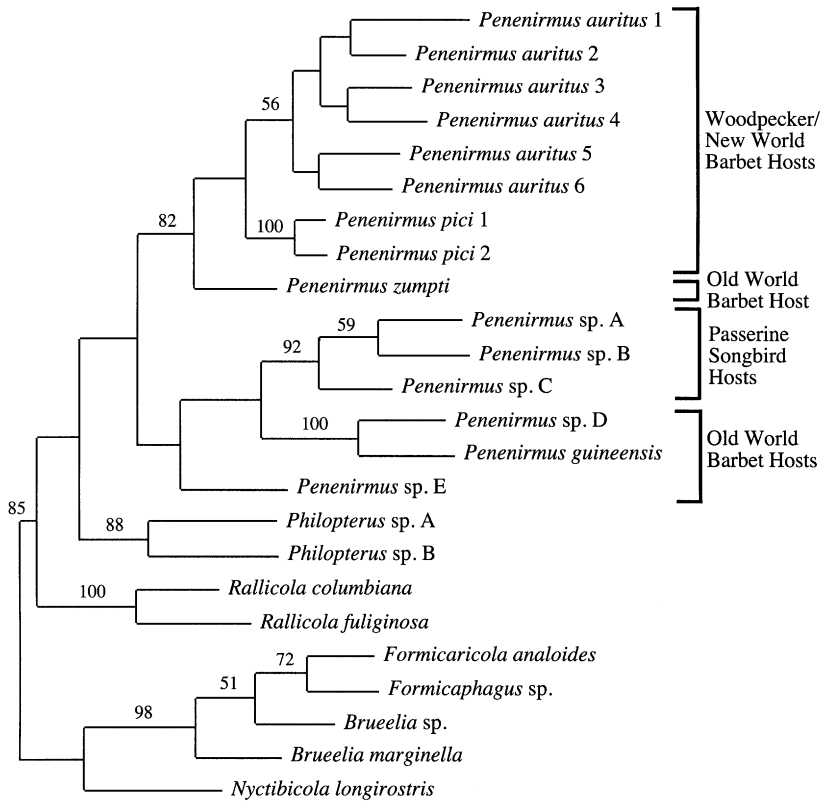

Fig. 2. Single most parsimonious tree from unordered parsimony analysis of combined COI and EF-1α sequences (length = 1543, RC = 0.151). Numbers above branches indicate support from 1000 bootstrap replicates. Unnumbered nodes received less than 50% bootstrap support. Branch lengths are proportional to the reconstructed number of changes.

more subject to multiple substitutions at these divergences (Fig. 1). Thus, methods that take into account rate differences, such as weighted parsimony or maximum likelihood, should provide a better estimate of phylogenetic relationships (Huelsenbeck & Hillis, 1993; Johnson & Sorenson, 1998). Despite the dramatic rate differences between gene regions, the partition homogeneity test (Farris *et al*., 1994, 1995; Swofford, 2000) detected no significant conflict between them over the phylogeny, even in unordered parsimony comparisons ($P = 0.36$).

Because no evidence for conflict between gene regions existed, we decided to combine genes in subsequent analyses. Unordered parsimony analyses of the combined gene regions produced a single tree (Fig. 2). This tree recovers monophyly of *Penenirmus*, but this result is not strongly supported by bootstrapping. The parsimony tree indicates monophyly for each of the species sampled from more than a single host. In addition, the species of *Penenirmus* sampled from passerines form a monophyletic group (bootstrap 92%). Species of *Penenirmus* from barbets generally fall at the base of the tree, sister to either those on passerines or woodpeckers. An individual of *P. auritus* from a New World barbet (*Eubucco bourcierii*) falls within other *P. auritus*, all on woodpeckers.

Maximum likelihood searches with the estimated model produced a single tree (Fig. 3). This tree indicated exactly the same relationships between the species of *Penenirmus* as the parsimony tree (Fig. 2). However, the relationships among the individuals of *P. auritus* were slightly rearranged. In all other respects, the branching patterns between the parsimony and likelihood trees were identical. Bootstrap support for various branches using likelihood analyses was generally increased over the parsimony analysis, perhaps indicating that when rate differences are taken into account, the combined dataset becomes more consistent. More specifically, the monophyly of *Penenirmus* received appreciable bootstrap support (76%).

Discussion

Combined analysis of sequences for portions of the mitochondrial COI and nuclear EF-1α genes for species in the louse genus *Penenirmus* produced well resolved trees, with reasonable bootstrap support. Parsimony and likelihood analyses produced nearly identical trees, the only differences involving rearrangements among individuals within a single species (*P. auritus*). Although sequence divergences within species on

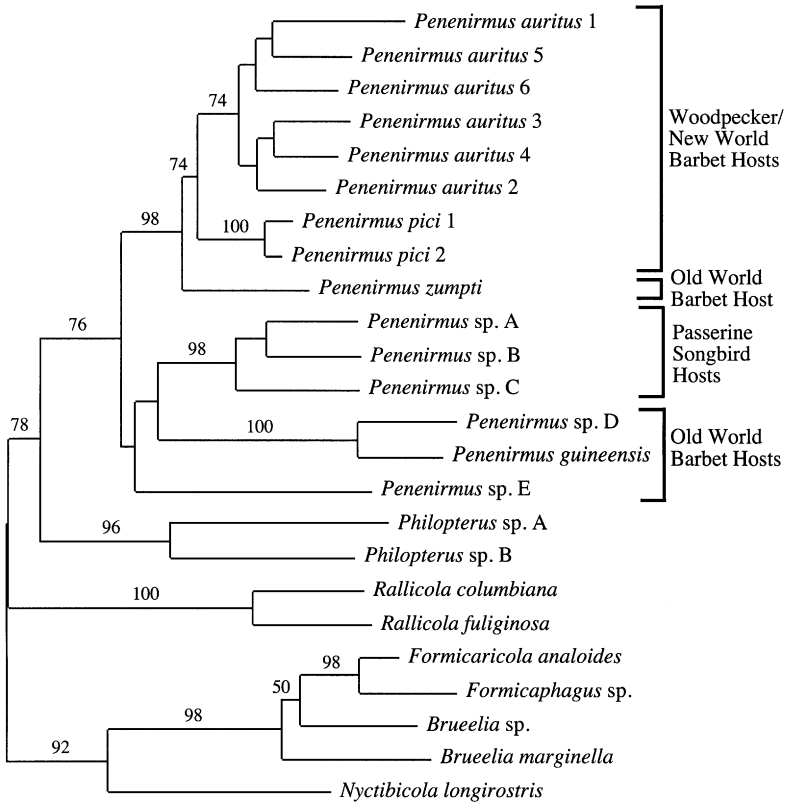

Fig. 3. Tree resulting from twenty maximum likelihood random addition replicate searches with TBR branch swapping (ln likelihood = −7025.89). Gamma shape parameter 0.176, substitution matrix: A-C = 0.797, A-G = 9.915, A-T = 3.562, C-G = 1.988, C-T = 11.606, G-T = 1. Numbers above branches indicate support from 100 maximum likelihood bootstrap replicates with NJ starting trees and NNI branch swapping. Unnumbered nodes received less than 50% bootstrap support. Branch lengths are proportional to the number of changes per site under the model.

multiple host species were large (up to 23.7% for COI), in such cases these species formed monophyletic groups. In addition, monophyly of *Penenirmus* was recovered in both analyses, suggesting that the validity of this genus will hold under further scrutiny. Assessing generic boundaries in lice has been a difficult task in the past (Clay, 1949, 1951; Eichler, 1963), but the recovery of monophyly for a relatively diverse genus, such as *Penenirmus*, bodes well for the state of generic level taxonomy in Phthiraptera.

Monophyly of a *Penenirmus* clade occurring on woodpeckers and New World barbets was evident in both parsimony (Fig. 2) and likelihood analyses (Fig. 3, bootstrap 74%). The monophyly of *Penenirmus* occurring on woodpeckers is also supported by morphological evidence including 'absences of a postantennal suture, the presence of anterior median notches on tergites II-III, and basal sclerites on the penis' (Dalgleish, 1972). Some authors have recommended the recognition of genus *Picophilopterus* for this group of lice (Carriker, 1963), represented by *Penenirmus pici* and *P. auritus* in the present study. However, recognition of such a genus would result in paraphyly for *Penenirmus*, as members of '*Picophilopterus*' are imbedded within *Penenirmus*. Once a more comprehensive understanding of the phylogeny of groups of lice within *Penenirmus* are available, perhaps *Picophilopterus* will merit recognition as a subgenus. The occurrence of *P. auritus* 3 on *Eubucco*, a New World barbet, has not been reported previously. More sampling is needed to determine if other New World barbets also harbour lice in this '*Picophilopterus*' group.

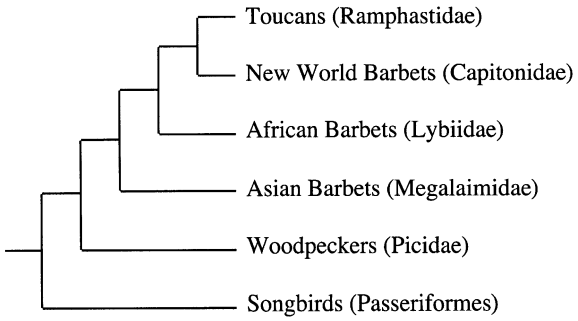

Fig. 4. Phylogeny for major relevant host groups. Compiled from Sibley & Ahlquist (1990), Lanyon & Hall (1994) and Barker & Lanyon (2000).

The phylogenetic relationships among species of *Penenirmus* reflect host phylogeny (Fig. 4) to some extent. One well supported similarity to host phylogeny is that all the

species from passerine songbirds form a monophyletic group. If the monophyly of this group holds upon the addition of more *Penenirmus* species from Passeriformes, then this group either represents an early colonization of Passeriformes or a remnant of the divergence between Passeriformes and Piciformes. A second strongly supported result mirroring host phylogeny is that all the species from woodpeckers (Picidae) fall in a single group.

Despite similarities of the *Penenirmus* phylogeny to host phylogeny, there are also some important differences that receive strong support. First, species from African barbets (Lybiidae) are not monophyletic. The hosts representing Lybiidae in this study almost certainly form a monophyletic group (Sibley & Ahlquist, 1990; Barker & Lanyon, 2000). In fact, two unrelated species, *P. guineensis* and *P. zumpti* Tendeiro, parasitize the same host genus (*Lybius*). A second strongly supported difference from the host phylogeny is that an individual of *P. auritus* sampled from a New World barbet (Capitonidae) falls within lice from woodpeckers, also of the species *P. auritus*. This incongruence appears to perhaps represent a host switch from woodpeckers to New World barbets.

The fact that *Penenirmus* species from Old World barbets fall in several positions at the base of the tree (in one case sister to *Penenirmus* from passerines and in the other case sister to *Penenirmus* from woodpeckers) suggests that *Penenirmus* perhaps first radiated on barbets and then switched to other host groups. More details on the relative timing of host and parasite diversification are needed to assess this hypothesis. In summary, general congruence of the *Penenirmus* phylogeny with host relationships makes *Penenirmus* a promising group for co-phylogenetic study. However, a more comprehensive phylogeny for avian hosts, revisionary work within *Penenirmus*, and more complete taxon sampling are needed to facilitate such a comparison.

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