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Molecular systematics of the barklouse family Psocidae (Insecta: Psocodea: 'Psocoptera') and implications for morphological and behavioral evolution

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Abstract

We evaluated the higher level classification within the family Psocidae (Insecta: Psocodea: 'Psocoptera') based on combined analyses of nuclear 18S, Histone 3, wingless and mitochondrial 12S, 16S and COI gene sequences. Various analyses (inclusion/exclusion of incomplete taxa and/or rapidly evolving genes, data partitioning, and analytical method selection) all provided similar results, which were generally concordant with relationships inferred using morphological observations. Based on the phylogenetic trees estimated for Psocidae, we propose a revised higher level classification of this family, although uncertainty still exists regarding some aspects of this classification. This classification includes a basal division into two subfamilies, 'Amphigerontiinae' (possibly paraphyletic) and Psocinae. The Amphigerontiinae is divided into the tribes Kaindipsocini (new tribe), Blastini, Amphigerontini, and Stylatopsocini. Psocinae is divided into the tribes 'Ptyctini' (probably paraphyletic), Psocini, Atrichadenotecnini (new tribe), Sigmatoneurini, Metylophorini, and Thyrsophorini (the latter includes the taxon previously recognized as Cerastipsocini). We examined the evolution of symmetric/asymmetric male genitalia over this tree and found this character to be quite homoplasious.

Key words: Insecta; Psocodea; Psocoptera; Psocidae; phylogeny; evolution

INTRODUCTION

The family Psocidae is the largest family of barklice (Psocodea: 'Psocoptera') containing over 20% (899 of 4408) of psocopteran species (Lienhard & Smithers, 2002). Traditionally, this family had been divided into four subfamilies (Amphigerontiinae, Cerastipsocinae, Psocinae and Thyrsophorinae) and two tribes (Cerastipsocini and Metylophorini within Cerastipsocinae) (Table 1). This classification scheme was largely based on some easy-to-observe external morphological characters such as wing venation, length of antenna, and the shape of maxillary palpus. More recently, Mockford (1993) re-classified the family into three subfamilies, Amphigerontiinae, Thyrsophorinae, and Psocinae. He also subdivided Psocinae into five tribes: Cerastipsocini, Cycetini, Metylophorini, Psocini and Ptyctini. This classification relies on what are considered more useful phylogenetic characters (such as male and female terminalia), and thus has been widely accepted (e.g., Yoshizawa, 1998, 2001; Lienhard & Smithers, 2002). However, some group diagnoses include apparent plesiomorphies. For example, in the definition of Ptyctini, Mockford (1993) noted that 'male clunial-epiproctal interface was either straight [= plesiomorphic] or epiproct overlapping clunium [= apomorphic]' (interpretations noted in [] are from Yoshizawa, 2002, 2005). This definition includes a plesiomorphic character state and thus could be diagnosing a paraphyletic group. There are also some uncertainties in the subfamilial or tribal assignments for some genera. For example, New (in New & Lienhard, 2007) proposed some different assignments for some genera from that proposed in Lienhard & Smithers (2002) (e.g., *Kaindipsocus* in Ptyctini of Psocinae,

not Amphigerontinae; *Psocidus* s.str. in Psocini, not Ptyctini). A completely new higher classification system was proposed recently (Li, 2002) (Table 1), increasing the confusion regarding the higher classification of Psocidae (Lienhard, 2003).

The difficulties in providing a stable classification for Psocidae are, in part, due to the extremely high diversity of morphology within the family, especially in male genitalic structures. Although male genitalia are the most important systematic characters for Psocidae, frequent parallelisms and reversals have also been identified. For example, Mockford (1993) placed heavy importance on the clunium-epiproct interface for the subdivision of Psocinae. However, apparent homoplasy of the dorsal flap of the clunium (apomorphic and characteristic for Psocini, Metylophorini, Cerastipsocini and Thyrsophorinae) has also been identified in the monophyletic genus *Trichadenotecnum* of the tribe Ptyctini (Yoshizawa & Lienhard, 2004). If this character is highly homoplasious in the other tribes as well, the higher level classification proposed by Mockford (1993) might be in need of re-evaluation.

In addition to the systematic and morphological problems, establishing a stable higher classification for Psocidae is important to understand the evolutionary history of an interesting behavior observed in the family, i.e., aggregation of nymphs. Aggregation of nymphs is known for Metylophorini and Cerastipsocini, as well as the closely related family Myopsocidae. However, the phylogenetic relationships of these taxa are presently unclear, and origin of this nymphal behavior is completely unknown to date.

A robust morphology-independent phylogenetic hypothesis is required for establishing a stable higher level classification of Psocidae and also for uncovering evolutionary changes of systematically relevant morphological characters and

interesting behavioral traits. In the present paper, we estimate a molecular phylogenetic tree for this family using partial sequences of the mitochondrial COI, 12S and 16S rDNA genes and the nuclear wingless, histone 3, and 18S rDNA genes. Based on the tree recovered by phylogenetic analyses, we propose a new higher classification of Psocidae and also the inferred evolutionary history of relevant morphological and behavioral characters.

MATERIALS AND METHODS

In general, recently collected specimens stored in 99.5% ethanol (original concentration) were used for DNA extraction. However, fresh material was not available for a few specimens of important taxa, and in these cases specimens stored in 80% ethanol for up to 20 years were also used. Total genomic DNA was extracted from a whole body or separated abdomen following the methods described by Cruickshank *et al.* (2001). Voucher specimens are preserved in the Hokkaido University Insect Collection, Japan and Illinois Natural History Survey Insect Collection, USA. Samples were selected from all subfamilies and tribes of Psocidae (Lienhard & Smithers, 2002) except for the tribe Cycetini. A total of 29 genera and 45 species of Psocidae were used in the analyses, and the other families of the infraorder Psocetae (Hemipsocidae, Myopsocidae, and Psilopsocidae), to which Psocidae belongs, were used as outgroups.

We sequenced six gene fragments: nearly complete sequences of nuclear 18S rDNA and Histone 3 and partial sequences of mitochondrial 12S rDNA, 16S rDNA, and COI and the nuclear wingless genes. Primers and PCR procedures followed Johnson *et al.* (2004) (18S), Yoshizawa (2004) (12S, 16S, COI), Colgan *et al.* (1998) (Histone 3) and Brower *et al.* (1997) (wingless). However, the following new primers

were designed because of primer mismatch or for amplifying shorter fragments from the older specimens: 18S bba (AAG AAT TTC ACC TCT AAC GTC GC) and 18S aab (TAC CTT GAA CAA ATT TGA GTG C) to amplify shorter fragments of 18S by combining with 18S ai and 18S bi, respectively; 16S bba (CTG TTA TCC CTA AGG TAA TTT) and 16S bar (GGG ACG AGA AGA CCC TAT AGA TCT T) to amplify shorter fragments of 16S by combining with 16S ai and 16S bi, respectively; Wg1P (ACW ACM TGY TGG ATG MGG YTN CC) for substitution of LepWg1; Wg2P (RCA CCA TRG GAA TGT RCA BDT RCA CC) and Wg4 (CCR CAR CAC ATD ATT GCA CAH CC) for substitutions of LepWg2; COIL6631 (GRT TYT TYG GNC AYC CHG AAG T) + H7005 for second PCR of weak initial PCR products.

Alignment of Histone 3, COI, and the wingless gene was straightforward based on amino acid sequences. Alignment of rDNA was done by eye based on RNA secondary structure estimated by Kjer (2004) (18S), Page et al. (2002) (12S) and Buckley et al. (2000) (16S). Some poorly aligned loop regions of mt rDNA sequences were excluded from the analyses. NEXUS files of the aligned sequences are available from the URL at <<http://data.psocodea.org>> and the journal's website (Supplementary appendix).

The present sample contains some taxa with missing data (Table 2). Taxa with missing data can reduce the accuracy of phylogenetic estimation (Platnick et al., 1991; Novacek, 1992). In a Bayesian analyses including all taxa and gene partitions, these taxa with missing data can be classified into three categories as follows: (1) the position of the taxon is highly unstable (*Atlantopsocus personatus*); (2) the position of the taxon is relatively stable, but position of the inclusive clade is unstable (*Blastopsocus* sp., *Blaste quieta*, *Camelopsocus monticolus*, Genus sp. and *Oreopsocus buholzei*); (3) the

position of the taxon itself and the inclusive clade are both stable (*Metylophorus purus*, *Sigmatoneura kolbei*, *Podopteroicus* sp. and *Thyrsophorus* sp.). The effects of incomplete taxa category 3 for phylogenetic reconstruction is likely to be minor, but categories 1 and 2 can reduce the accuracy of phylogenetic estimation. Therefore, we prepared three data sets: (a) all complete and incomplete taxa; (b) excluding category 1 only; and (c) excluding both categories 1 and 2.

To compare pairwise homogeneity of each gene region, the partition homogeneity test (1,000 replicates) (Farris *et al.*, 1994, 1995) was performed using PAUP*. We also compared the phylogenetic signal in each gene partition by comparing 50% MP bootstrap consensus trees estimated separately for each gene. These analyses were performed using the 38 species with no missing data.

For each data set, we performed maximum parsimony (MP) and maximum likelihood (ML) analyses using portable version of PAUP* 4b10 (Swofford, 2002) and Bayesian MCMC using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). For MP analysis, all data were weighted equally, and TBR branch swapping was performed with 100 random-addition replicates. For ML analyses, TBR branch swapping was performed with neighbor-joining tree, the MP and Bayesian consensus trees were used as starting trees. Parameters for ML analysis were estimated using Modeltest 3.7 (Posada and Crandall, 1998) on the basis of Akaike information criterion (AIC: Akaike, 1974). As a result of Modeltest, the GTR+G+I model was selected (unequal base frequencies: A = 0.3126, C = 0.1659, G = 0.1992 T = 0.3223; six substitution categories: A-C = 1.2816, A-G = 4.8207, A-T = 2.8105, C-G = 1.0967, C-T = 6.5358, G-T = 1; gamma distributions shape parameter = 0.5633 based on four rate categories; proportion of invariant sites = 0.5540). Bootstrap supports for branches in the trees

were calculated using 100 replicates with TBR branch swapping, but TBR rearrangement was limited to 3,000 for ML bootstrapping because full TBR rearrangements were unacceptably time consuming and, in all of our ML analyses (different starting trees and different data sets), trees obtained by 3,000 TBR rearrangements (best ML tree in some cases) were always better than trees obtained by full SPR or NNI rearrangements. After this first ML bootstrapping, we also applied a constraints strategy to expand tree search space. Constraints were given for any clade receiving 100% support from the previous ML bootstrapping. Full TBR was still too time consuming and a 3,000 rearrangements limit was enforced. Support values for some branches were significantly changed by this strategy and the constrained ML bootstrapping values are indicated in parentheses in Fig. 1. Modeltest-estimated parameters were also adopted for ML bootstrapping. Confidence for some clades of interest (i.e., where conflicts between the molecular tree and the morphological classification were evident) was estimated with the approximately unbiased test (AU test: Shimodaira, 2002) using CONSEL 0.1h (Shimodaira & Hasegawa, 2001) with the default settings. For Bayesian analyses, we ran two analyses each with four chains for 2,000,000 generations, and a tree was sampled every 1000 generations. The first 200 trees were excluded as burnin, and we compared a 50 % majority consensus tree of the remaining trees to estimate posterior probabilities of branches in the tree. Two different Bayesian analyses were performed, with data set unpartitioned and partitioned into the following 8 categories: 18S, 12S+16S, and 1st, 2nd and 3rd codons of nuclear protein-coding genes and 1/2/3 codons of mitochondrial COI. Substitution models for Bayesian MCMC were estimated using MrModeltest 2.2 (Nylander, 2004). GTR+G+I model was selected on the basis of AIC criterion for the unpartitioned data set and all

categories of partitioned data set except for 2nd codons of nuclear (SYM+G+I) and mitochondrial (HKY+I) protein-coding genes.

Based on the resulting trees, transformation series of some systematically and evolutionary relevant morphological and behavioral characters were examined using MacClade (Maddison & Maddison, 2001). For some genera, a parthenogenetic species was used in the molecular analyses. When the evolution of male genital structure was examined, information from a closely related bisexual species was adopted for those taxa (i.e., *T. circularoides* cf. *T. gonzalezi*: Mockford, 1991, *T. sp.* cf. *T. alexanderae*: Betz, 1983; Yoshizawa, 2004, *H. morio* cf. *H. gradinii*: Lienhard, 1998). We scored the male hypandrium as symmetric or asymmetric. When significant features were only observed on one side of the hypandrium and/or the hypandrium itself was strongly skewed to one side, the character was coded as asymmetric. Ciliation or slight differences in numbers of processes were ignored. For example, lateral margins of the median strap of the hypandrium is fringed with numerous spines in *Atlantopsocus personatus*, and the exact numbers of spines might differ between each side of the strap. However, such differences were ignored and the character was coded as symmetric.

The taxonomic system and names presented in Lienhard & Smithers (2002) were used unless specified below. A new classification based on the present phylogenetic analyses is proposed in Table 1, left margin of Fig. 2, and Taxonomic treatments section in the Discussion.

RESULTS

Data evaluation

The aligned data set consists of 1834 bp of 18S, 330 bp of Histone 3, 391 bp of

Wingless, 501 bp of 16S (with 11 excluded characters), 372 bp of 12S (with 31 excluded characters) and 388 bp of COI. Plots of uncorrected pairwise distances of COI against those for 18S (the least divergent gene) reveal considerable multiple substitution in the COI gene. In these plots, COI divergences leveled off at around 15%. In contrast, such multiple substitution was not as prevalent for other gene regions, and divergence of these gene regions continued to increase with increasing 18S divergence, although the slopes slightly decrease at the far right of the graph (Fig. 1). The nuclear protein coding genes appeared to be similar in the rate of accumulation of substitutions, while 16S tended to have a lower divergence (per 18S divergence) than did 12S (Fig. 1).

The partitioned homogeneity test detected significant heterogeneity between gene regions in all comparisons performed (among nuclear genes; between nuclear genes and mitochondrial genes). Significant heterogeneity was also detected when 12S and 16S were compared ($P = 0.032$) even though they are both linked mitochondrial genes expected to share the same phylogenetic history (Fig. 1). When MP bootstrap trees estimated from separate data sets were compared, all trees (except for the almost completely unresolved COI tree) were concordant in some shallower clades (e.g., the clade composed of Cerastipsocini + Thyrsophorinae and branching pattern within the clade, the close relationship between *Ptycta* and *Copostigma*), although resolution of deeper divergence was extremely poor in all separated analyses (trees not shown). These results indicate that the significant heterogeneity detected by the partitioned heterogeneity test was probably not due to different phylogenetic background, but rather differences in substitution rates (Barker and Lutzoni, 2002; Darlu and Lecointre, 2002). Therefore, in the following analyses, we combined all data sets into a single

matrix and analyzed it simultaneously.

Analyses based on various taxon sets (including and excluding incomplete taxa) resulted in very similar trees. The only major differences involved the branching pattern of some deep and poorly supported clades within Ptyctini, indicating limited effect of missing data in the present analyses (Wiens, 2003). The following discussions are based on the results obtained from the data set including all taxa (Fig. 2).

Phylogenetic analyses

All analyses with various taxon sets and data combinations produced very similar results. No significant difference in topology and branch support could be detected between the partitioned and unpartitioned Bayesian analyses. However, results from the MP analyses were highly unstable and usually not concordant with ML and Bayesian trees (trees not shown). In particular, the position of *Kaindipsocus* was highly unstable and sometimes clustered with *Symbiopsocus*. However, when the conservative rDNA data only was analyzed, a MP tree concordant with the ML and Bayesian trees was obtained. Therefore, the instability of the MP analyses is probably due to frequent homoplasies included in the rapidly evolving genes. In fact, 14 of 22 (ca. 64%) potential synapomorphies of *Kaindipsocus* and *Symbiopsocus* are from more rapidly evolving protein coding genes (Fig. 1) even though these region composed less than 30% of the data set. Figure 2 shows the tree obtained from the ML analysis of the combined data set, together with branch supports obtained from Bayesian PP (not partitioned), ML and MP bootstrapping (and constrained ML bootstrapping).

Monophyly of the family Psocidae was recovered, and received strong support by all analyses. Monophyly of the subfamily Amphigerontiinae was not recovered, and

the genus *Kaindipsocus* was always separated from the other genera of the subfamily, in contrast to the morphology-based classification scheme (Lienhard & Smithers, 2002). Separation of *Kaindipsocus* from the other genera received strong support by Bayesian PP, but was weakly supported by ML and MP bootstrapping (lower than 50%). Using the AU test, monophyly of Amphigerontiinae could not be rejected (P=0.203; 95% confidence intervals CI=20.0).

Monophyly of the subfamily Psocinae was never recovered by any analyses, and the subfamily Thyrsophorinae was always imbedded within Psocinae. Placement of Thyrsophorinae as sister to the genus *Cerastipsocus* (Psocinae: Cerastipsocini) received very strong support by all analyses. Strict monophyly of Psocinae was rejected by the AU test (P<0.001, CI=269.2). However, monophyly of the clade Psocinae + Thyrsophorinae was always recovered, except for the MP tree in which *Kaindipsocus* was clustered with *Symbiopsocus*, but this basal branch was extremely short and was very poorly supported.

Within the Psocinae-Thyrsophorinae clade, the tribe Ptyctini composed a basal 'grade'. Monophyly of Ptyctini was not recovered by any analysis and was also rejected by the AU test (P=0.025, CI=48.3). Some clades were recognized within Ptyctini but, except for the *Copostigma-Ptycta* complex (100% by all analyses: Bess & Yoshizawa, 2007) and *Indiopsocus-Psocidus* (s.str.) clade (100% PP, 55-60% bootstrap), deep inter-generic relationships within Ptyctini were poorly supported.

The tribes Psocini, Cerastipsocini and Metylophorini always composed a monophyletic group, together with the subfamily Thyrsophorinae. This clade received strong support by Bayesian PP (100%) and was moderately well supported by constrained ML bootstrapping (58%) (but lower than 50% by unconstrained ML/MP

bootstrapping). Within this clade, the tribe Psocini composed a basal 'grade', and the genus *Atrichadenotecnum* was always separated from the other genera of the tribe. The placement of *Atrichadenotecnum* away from the other genera of Psocini received strong support by Bayesian PP (100%) and was moderately well supported by constrained ML bootstrapping (58%). However, this relationship was only weakly supported by ML/MP bootstrapping (lower than 50%). Monophyly of Psocini could not be rejected by the AU test ($P=0.254$, $CI=34.0$).

The tribes Cerastipsocini and Metylophorini, together with the subfamily Thysophorinae, composed a monophyletic group, and this clade received strong support by all analyses. The genus *Podopteroocus* was recently synonymized with *Sigmatoneura*, and *Sigmatoneura* was recognized as a member of Metylophorini by Yoshizawa et al. (2004). This synonymy, proposed on the basis of morphological characters, was also supported by the molecular data, but the *Sigmatoneura-Podopteroocus* clade was always separated from the genus *Metylophorus* in the present analyses. The branch uniting *Metylophorus*, Cerastipsocini, and Thysophorinae was well supported by Bayesian PP (99%) and constrained ML bootstrapping (100%), but received poor support by ML/MP bootstrapping (lower than 50%). Monophyly of Metylophorini could not be rejected by the AU test ($P=0.427$, $CI=31.8$).

As mentioned above, the subfamily Thysophorinae was always placed as the sister group of *Cerastipsocus*, and this placement was very stable throughout the analyses. Monophyly of Cerastipsocini + Thysophorinae received strong supports by all analyses.

DISCUSSION

Phylogenetic relationships

The phylogenetic trees based on sequences from six genes are in general agreement with the widely accepted taxonomic classification of Psocidae (e.g., Lienhard & Smithers, 2002). However, some incongruence can be detected. Most importantly, monophyly of two of three subfamilies presently recognized in Psocidae was not recovered by any analyses.

Monophyly of Amphigerontiinae was not supported by any analyses, although it could not be rejected by AU test. Monophyly of Amphigerontiinae has been suggested by a number of unique male terminalia features (e.g., widely sclerotized 8th sternum, laterally directed phallosomal sclerites), and these character states can also be observed in the enigmatic genus *Kaindipsocus* (Lienhard, pers. comm.; Yoshizawa, pers. obs.). In the ML and Bayesian analyses, *Kaindipsocus* is sister to all other included taxa of Psocidae and then the rest of Amphigerontiinae is sister to the Psocinae + Thyrsochorinae clade. Therefore, it is possible that the character states observed in Amphigerontiinae represent the plesiomorphic condition of the family Psocidae. The homology of genital structures between Psocinae and Amphigerontiinae and also between Psocidae and related families is not well established (Yoshizawa, 2005). These homology assessments should be carefully performed to understand evolutionary changes in the male genitalia of Psocidae. Wide sclerotization of 8th sternum is also observed in the distantly related genus *Trichadenotecnum* (Yoshizawa, 2001). The sclerotized portions of the 8th sternum support retractor muscles of the phallosome (Yoshizawa, pers. obs.) and thus functional convergence of the sclerotized 8th sternum might be frequent. None of the present analyses supported the systematic

placement of *Kaindipsocus* within the tribe Ptyctini of the subfamily Psocinae, as proposed by New (in New & Lienhard, 2007). Monophyly of Amphigerontiinae excluding *Kaindipsocus* was stable and strongly supported. This clade can be subdivided into two clades which are concordant with the tribes Amphigerontini and Blastini proposed by Li (2002). According to Li (2002), Amphigerontini is characterized by the elongated discoidal cell and Rs-M crossvein in forewing, whereas Blastini is characterized by the normal discoidal cell and Rs+M fusion. The latter character states are plesiomorphic (Yoshizawa, 2005). Therefore, examination of further taxa may prove the latter tribe paraphyletic.

Monophyly of Psocinae was also not supported because the subfamily Thyrsophorinae was always imbedded within Psocinae and further within Cerastipsocini. This placement was stable throughout all analyses. Independent subfamilial or familial status of Thyrsophorinae has been suggested based on unique forewing venation (i.e., fusion of R4+5 and M veins and pterostigma broadest at basal part: New, 1978). However, these forewing character states are autapomorphic for this group and thus have no phylogenetic information, except as an evidence of monophyly of Thyrsophorinae. The relationship between Thyrsophorinae and Cerastipsocini has already been pointed out by previous authors based on the male and female genitalic characters (e.g., New, 1978). Therefore, our result based on molecular data is also concordant with the morphological evidence.

Within the Psocinae-Thyrsophorinae clade, the tribe Ptyctini composed a basal 'grade'. The genus *Psocidus* s.str. was also imbedded in this grade, and this is concordant with the system presented in Lienhard & Smithers (2002). The systematic placement of this genus under Psocini as proposed by New (in New & Lienhard, 2007)

cannot be justified. Although the deep branches connecting ptyctini genera are short and poorly supported, monophyly of the tribe was also rejected by the AU test. The original definition of the tribe Ptyctini includes a plesiomorphic character state (straight clunium-epiproct interface) which could easily lead to the recognition of a paraphyletic taxon. Therefore, monophyly of Ptyctini cannot be justified from either morphological or molecular evidence. In contrast, the 'chair-shaped epiproct', which is also included in the original definition of Ptyctini, appears to be apomorphic (Yoshizawa, 2005) and thus the tribe might be maintained as a monophyletic group based on this character state. However, in all trees estimated in the present analyses, taxa with chair-shaped epiproct never compose a monophyletic group (i.e., the genera *Indiopsocus* and *Psocidus* s.str. have flat clunium-epiproct interface, and the clade is clustered with taxa having a chair-shaped epiproct). Furthermore, the chair-shaped epiproct is also observed in some taxa of Amphigerontiinae, and thus it possibly represents the plesiomorphic condition of Psocidae. Some clades can be recognized within Ptyctini (Fig. 2); however, except for the strongly supported *Copostigma-Ptycta* complex (Yoshizawa & Smithers, 2006; Bess & Yoshizawa, 2007) and the fairly well supported *Psocidus-Indiopsocus* clade, inter-generic relationships of ptyctini genera are nearly unresolved. Because dense taxon sampling is critical for the accuracy of phylogenetic estimation (Graybeal, 1998), inclusion of additional genera or additional species of some poorly sampled genera (e.g., *Camelopsocus* and *Atlantopsocus*) may improve the resolution of deep branches. Li (2002) proposed two tribes for ptyctini genera: Oreopsocini (*Oreopsocus* and *Symbiopsocus*) and Trichadenotecnini (*Trichadenotecnum* and *Loensia*). However, establishment of these taxa cannot be justified based on the present results.

A major clade consistently recovered by our analyses comprises Psocini,

Metylophorini, Cerastipsocini, and Thyrsophorinae. Although this clade received strong support by Bayesian PP and fair support by constrained ML bootstrapping, ML/MP bootstrap values for this clade were lower than 50%. Morphologically, this clade can be supported by the extension of the clunial shelf over the epiproct in males. This character is absent in the genus *Hyalopsocus* and is also observed in a species of *Trichadenotecnum* (*T. archiforme*: Ptyctini). However, the most parsimonious reconstruction of this character based on the ML tree would identify these character states as secondary reductions (*Hyalopsocus*) and homoplasious (*Trichadenotecnum*). In many taxa included in this clade, a shoulder or lobes are observed on the male paraproct lateral to the distal process, and this character may provide further support for this clade. However, absence of this character in this clade is also frequent (e.g., *Sigmatoneura* and *Cerastipsocus*).

The tribe Psocini comprises the basal 'grade' of the Psocini-Metylophorini-Cerastipsocini-Thyrsophorinae clade, and *Atrichadenotecnum* was always separated from the other genera of Psocini. Monophyly of Psocini cannot be rejected by the AU test. *Atrichadenotecnum* was first considered to be the sister group of *Psocus* based on a morphological character state (left skewed male phallosome: Yoshizawa, 1998), but this supposition was rejected by the AU test. Other morphological characters supporting the placement of *Atrichadenotecnum* within Psocini are also observed in Metylophorini and Cerastipsocini (e.g., transversal crest on the distal part of the paraproct) and thus systematic placement of *Atrichadenotecnum* within Psocini is not well supported by either morphological or molecular data.

Monophyly of the clade composed of Metylophorini, Cerastipsocini, and Thyrsophorinae was recovered and received strong support by all analyses.

Morphological apomorphies observed throughout this clade are large body size (forewing > 4 mm) and absence of glandular setae in nymphs. This result also strongly suggests that the aggregation behavior of nymphs has evolved in the common ancestor of this clade, although nymphal biology of the very scarce species of Thyrsophorinae is unknown to date (Mockford, 1992). Nymphal aggregation is also observed in some groups of Myopsocidae. Because Metylophorini-Cerastipsocini-Thyrsophorinae clade is distant from the base of Psocidae, this result also demonstrates that evolution of the nymphal aggregation behavior has evolved independently in Myopsocidae and Psocidae.

Monophyly of Metylophorini was not recovered by any analyses, and *Sigmatoneura* (including a recently synonymized genus *Podopteroacus*: Yoshizawa et al., 2005) was always separated from *Metylophorus*, although monophyly of the tribe could not be rejected by the AU test. Among the diagnostic morphological characters of Metylophorini, *Sigmatoneura* lacks some important male genitalic characters (i.e., asymmetrical hypandrium, sense cushion of male paraproct, etc.: Mockford, 1993; Yoshizawa et al., 2005). The systematic placement of *Sigmatoneura* under Metylophorini was justified based only on one apomorphy (absence of the distal process of the dorsal valve of gonapophyses: Yoshizawa et al., 2005), but this state can also be observed in some Cerastipsocini taxa and thus is highly homoplasious. The deep divergence of *Sigmatoneura* from *Metylophorus* and heterogeneity of the former genus within Metylophorini is evident both from morphological and molecular data. For *Sigmatoneura*, the independent tribal status proposed by Li (2002) can be justified by our molecular analyses.

Monophyly of Cerastipsocini + Thyrsophorinae is stable throughout the

analyses. As already discussed above, their close relationship has already been suggested based on male and female genitalic morphology (New, 1978). In addition, the short 4th segment of the maxillary palpus is also apomorphic and is observed throughout Cerastipsocini + Thyrsophorinae. The present analyses further indicate that Thyrsophorinae is imbedded within the Cerastipsocini and thus the latter is paraphyletic in the present sense.

Asymmetry of male genitalia

A great diversity of male genitalia in Psocidae has been noticed by previous researchers (e.g., Pearman, 1932; Thornton, 1961; Mockford, 1984; Yoshizawa, 2005). As discussed above, this great divergence has led to difficulties in assessing phylogenetic relationships within the family, but such diversity might provide an excellent model case for the study of evolution of genitalia (Eberhard, 1985; Hosken & Stockley, 2004). The greatest diversity of the form of the male genitalia in Psocidae is observed on the hypandrium (9th sternum), which is ornamented with processes and lobes of various shapes and numbers. However, homology of the hypandrial ornamentations between different genera is extremely difficult to identify, and more detailed morphological examinations throughout the family are needed to discuss their evolution. In contrast, left-right asymmetry can be coded as a binary character and is easily compared throughout the family and outgroups. In particular, asymmetry is most frequently observed in Psocidae (with only a few exceptions in other psocopteran families - Ectopsocidae: *Ectopsocopsis* and Lachesillidae: *Lachesilla*). Symmetric and asymmetric forms are observed in nearly all subfamilies and tribes (except for Psocini, which contains the asymmetric form only), and thus independent origins or reversals of

symmetric/asymmetric forms have already been predicted by morphological assessments (Yoshizawa et al. submitted). Although some uncertainties regarding deeper relationships within Psocidae exist, the molecular phylogenetic hypothesis provides the first opportunity to test independent origins/reversals of symmetric/asymmetric forms in Psocidae based on a morphology-independent data set.

Using parsimony reconstruction of the character states, at least 9 steps are required to explain the evolution of symmetric/asymmetric forms (Fig. 3a). The ancestral condition for Psocidae is reconstructed as symmetric, and two independent origins of asymmetrical forms are required, i.e., in *Blastopsocus* of the Amphigerontiinae and in the common ancestor of the Psocinae-Thyrsophorinae clade. Interestingly, seven independent reversals from the asymmetric to symmetric forms were also identified. The deep branching pattern within Ptyctini is nearly unresolved and thus five independent reversals within the tribe might be an overestimation, but two independent reversals within the Metylophorini-Cerastipsocini-Thyrsophorinae clade and also an independent reversal within the genus *Trichadenotecnum* would be recovered no matter the arrangement of the uncertain clades. The other hypothesis, i.e., independent origins of asymmetric forms with no reversals, requires at least 13 steps (4 more steps than the most parsimonious reconstruction) to explain the evolution of symmetric/asymmetric forms (Fig. 3b).

According to Huber (2004), two major causes for asymmetric genitalia can be identified: (1) female internal asymmetry owing to space constraints or (2) an asymmetric copulatory position (Ludwig, 1932). In most insects, asymmetry of male genitalic structure is thought to evolve through the latter scenario and, in spiders, the former case is regarded as plausible (Huber, 2004). As far as is known, female internal

genitalia occur in a symmetric form in all Psocidae. Although slightly asymmetric female external genitalia are known for some species with highly asymmetric male genitalia (e.g., *Trichadenotecnum incognitum*), symmetric female genitalia are more frequent even for the species with highly asymmetric male genitalia (e.g., *T. majus*) (Lienhard, 1998; Yoshizawa et al., submitted). Copulation of psocids is not well studied, but some species of *Trichadenotecnum* are known to copulate in a completely symmetric position, even though their male genitalic structure is highly asymmetric (*T. sexpunctatum* studied by Klier, 1956; *T. incognitum* studied by Yoshizawa, 1999). Therefore, neither scenario mentioned by Huber (2004) is likely in the case in Psocidae. Further studies are needed, but genitalia evolution in Psocidae may provide an additional interesting example of possible causes for asymmetry of male genitalia.

Conclusion and taxonomic treatments

Our phylogenetic analyses based on DNA sequences from six genes helped to refine the higher level classification of Psocidae and provided some new insights regarding the homology and transformation series of systematically relevant characters. In contrast, perhaps surprisingly, a combined analysis of six genes was not enough to resolve deep and short branches of the possibly paraphyletic tribe Ptyctini. Further molecular and morphological data are required to establish a stable taxonomic system of Psocidae but, based on the most highly supported and stable branches in our trees, we propose a revised higher level classification of Psocidae (Table 1, Fig. 2) and new nomenclatural acts as follows.

Cerastipsocini Roesler, 1940, a new junior synonym of Thyrsophorini Kolbe, 1882

Type genus of Cerastipsocini: *Cerastipsocus* Kolbe, 1884.

The subfamily Thyrsophorinae was characterized and separated from the other subfamilies of Psocidae on the basis of unique forewing venation (see above). By putting heavier importance on this unique character state, this subfamily was once even treated as an independent family, Thyrsophoridae (Table 1). However, our molecular analyses showed that *Thyrsophorus*, the type genus of Thyrsophorinae, is closely related to *Cerastipsocus* and thus the subfamily is imbedded within the tribe Cerastipsocini. This supposition is also supported morphologically (New, 1978). An independent subfamilial status for Thyrsophorinae, making the tribe Cerastipsocini and subfamily Psocinae paraphyletic, was strongly rejected by AU tests. The family-group name, Cerastipsocini Roesler, 1940, is younger than Thyrsophorini Kolbe, 1882. Therefore, here we regard the tribe Cerastipsocini (-idae, -inae etc.) as a junior synonym of Thyrsophorinae (-idae, -ini etc.). Thyrsophorini is here treated as a tribe of the subfamily Psocinae.

Oreopsocini Li, 2002, a new junior synonym of Ptyctini Mockford, 1993

Type genus of Oreopsocini: *Oreopsocus* Roesler, 1939.

As clearly shown in the present analyses, two oreopsocini genera *Oreopsocus* and *Symbiopsocus* are not closely related to each other. Furthermore, a close relationship between *Oreopsocus* and *Loensia* is fairly well supported, and the latter genus was classified under the different tribe Trichadenotecnini by Li (2002). Therefore, Li's proposed group Oreopsocini cannot be justified. In the present analyses, monophyly of Ptyctini is rejected and thus the tribe should be divided into several monophyletic groups. However, because many of the branches within Ptyctini do not

receive strong support, this treatment should await analysis of a wider range of genera within Ptyctini with additional data. At present, establishment of additional independent tribes for the genera now classified under Ptyctini would make taxonomy of Psocidae even more complicated. Therefore, although the tribe Oreopsocini might be revived by future study, here the tribe is treated as a junior synonym of Ptyctini.

Trichadenotecnini, Li, 2002, a new junior synonym of Ptyctini Mockford, 1993

Type genus of Trichadenotecnini: *Trichadenotecnum* Enderlein, 1909.

As clearly shown in the present analyses, two trichadenotecnini genera *Trichadenotecnum* and *Loensia* are not closely related to each other. Therefore, Li's group Trichadenotecnini cannot be justified. The other genera included in the tribe, *Trichadenopsocus* and *Conothoracalis*, have already been synonymized with *Trichadenotecnum* based on some stable morphological data (Mockford, 1993; Yoshizawa et al., 2007; Yoshizawa et al., submitted). *Trichadenotecnum*, the type genus of Trichadenotecnini, forms well supported clade which is deeply divergent from the other genera. Therefore, an independent tribal status for a single genus may be justified. However, as also mentioned under Oreopsocini, establishment of new tribes within Ptyctini should await analysis of a wider variety of ptyctini genera.

Kaindipsocini, a new tribe of the subfamily Amphigerontiinae

Type genus: *Kaindipsocus* Smithers & Thornton, 1981.

Included genus: *Kaindipsocus*.

Diagnosis. As generic diagnosis of the type genus (Smithers & Thornton, 1981).

The genus *Kaindipsocus* was first assigned to the subfamily Amphigerontiinae by Lienhard & Smithers (2002) according to unpublished data by E. L. Mockford. Although none of the present analyses supported this placement, the AU test cannot reject it. According to the present results, the deep divergence of the genus from the other members of Amphigerontiinae is apparent. In addition, some tribes have been proposed within Amphigerontiinae by Li (2002), in which *Kaindipsocus* cannot be assigned. Therefore, establishment of a new tribe for the genus *Kaindipsocus* is justified.

Atrichadenotecnini, a new tribe of the subfamily Psocinae

Type genus: *Atrichadenotecnum* Yoshizawa, 1998.

Included genus: *Atrichadenotecnum*.

Diagnosis. As generic diagnosis of the type genus (Yoshizawa, 1998).

The genus *Atrichadenotecnum* was first considered to be the sister taxon of the genus *Psocus* and thus was placed in the tribe Psocini. A putative synapomorphy between *Atrichadenotecnum* and *Psocus* was the asymmetrical, left skewed phallosome (Yoshizawa, 1998). Other morphological characters on which the systematic placement of *Atrichadenotecnum* was based (e.g., posterodorsal extension of the clunium) are also observed in Metylophorini and Cerastipsocini. Although monophyly of Psocini including *Atrichadenotecnum* could not be rejected, a sister group relationship between *Atrichadenotecnum* and *Psocus* was rejected ($P=0.008$, $CI=52.7$) by the AU test. Therefore, the left skewed phallosome should be considered to be a homoplasy, independently evolved in these genera. An asymmetrical phallosome is also observed in some other genera, such as *Trichadenotecnum*,

Indiopsocus (Ptyctini) and *Hyalopsocus* (Psocini). Therefore, there is no consistent morphological support for the systematic placement of *Atrichadenotecnum* into Psocini. In contrast, although monophyly of Psocini including *Atrichadenotecnum* cannot be rejected statistically, all analyses supported independence of *Atrichadenotecnum* from the remaining genera of Psocini, and their deep divergence is also evident from the phylogram. Here we propose an independent tribal status for *Atrichadenotecnum*.

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Figure Captions

Fig. 1. Plot of uncorrected pair wise distances in nuclear Histone 3 and wingless vs 18S (top-left), 12S and 16S vs 18S (top-right) and COI vs 18S (bottom). Incomplete taxa (taxa with missing data) are not included.

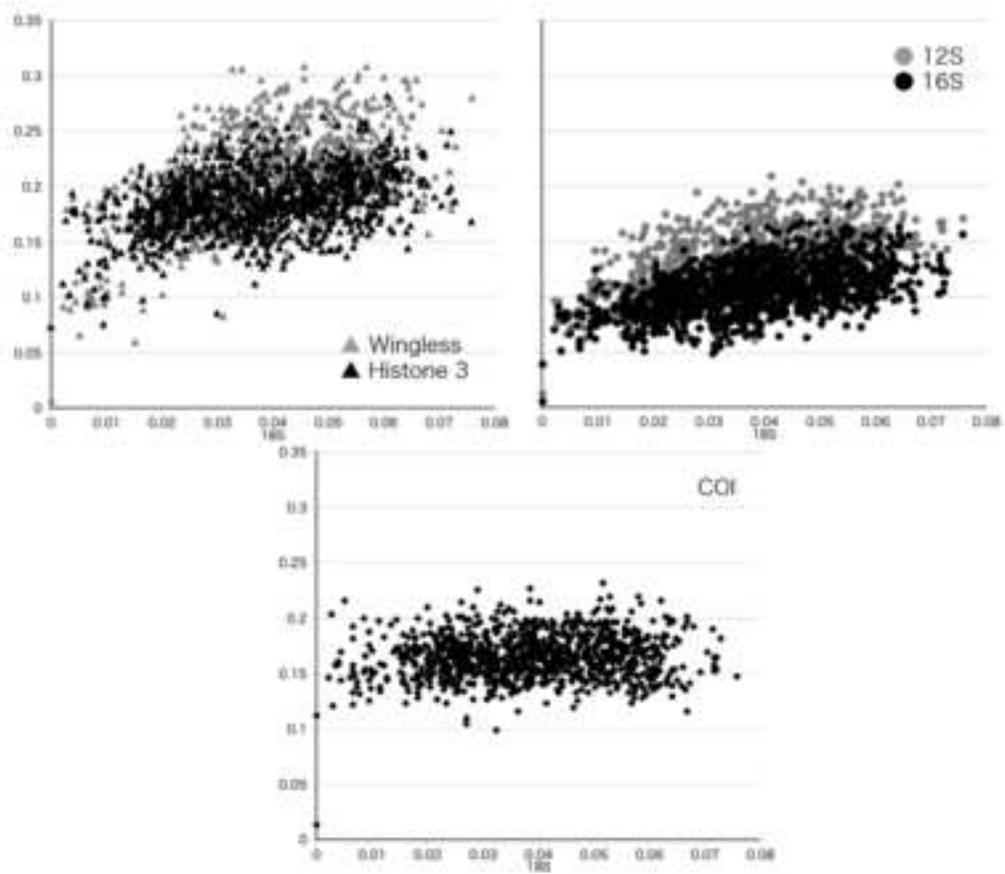
Fig. 2. The ML tree ($-Ln = 38410.025$) estimated from the dataset including all incomplete taxa. Branch lengths are proportional to ML estimated branch lengths. The numbers associated the branches are Bayesian posterior probability/ ML bootstrap/ MP bootstrap supports and (constrained ML bootstrap support). (con) indicate the constrained branches (see Materials and Methods).

Fig. 3. Most parsimonious reconstruction of symmetric/asymmetric male hypandrium. Character states are reconstructed as unordered (left) and irreversible (right).

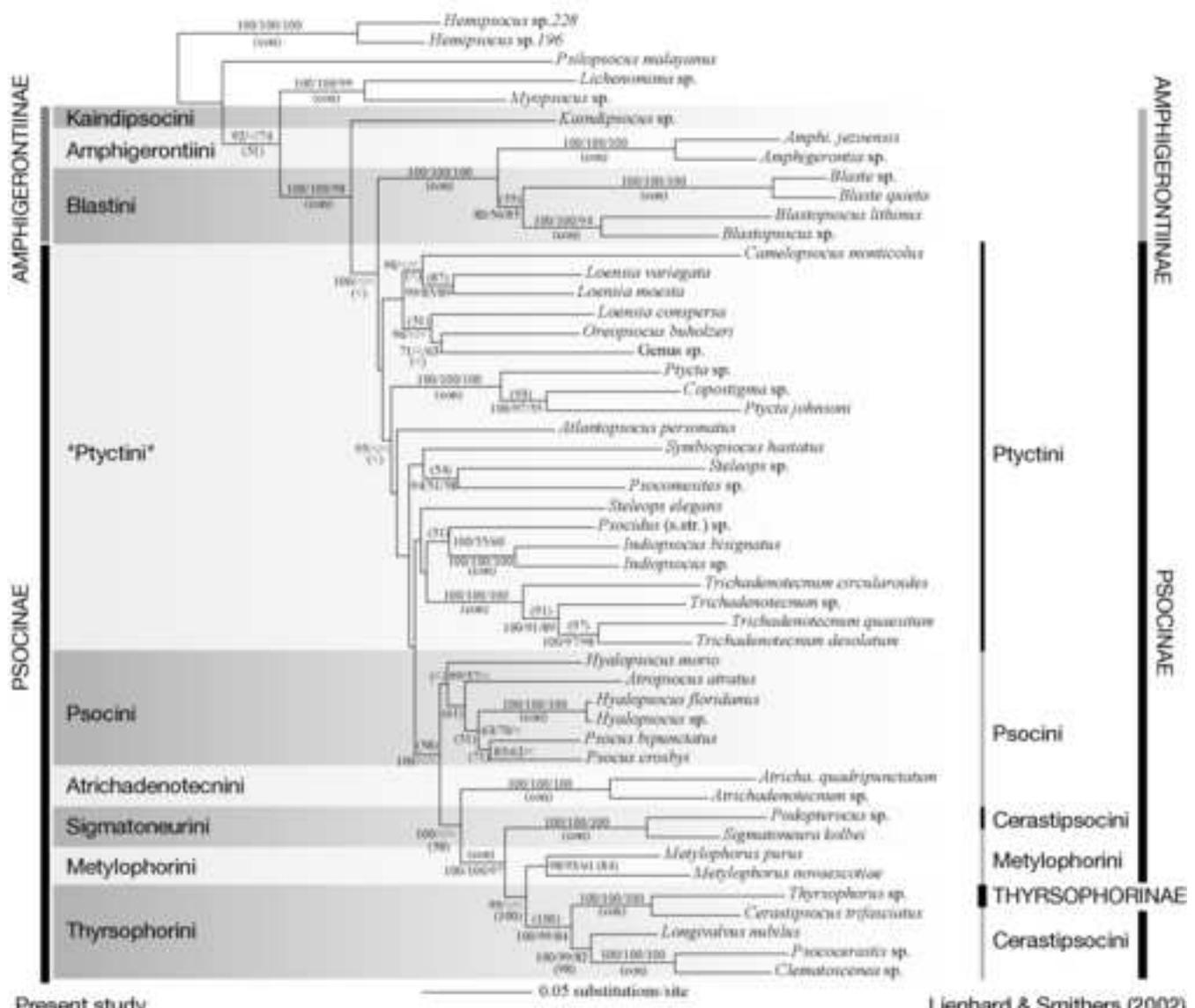
Online supplementary data: Aligned data set in NEXUS format

psocidae-mol.nexus

Figure



Figure



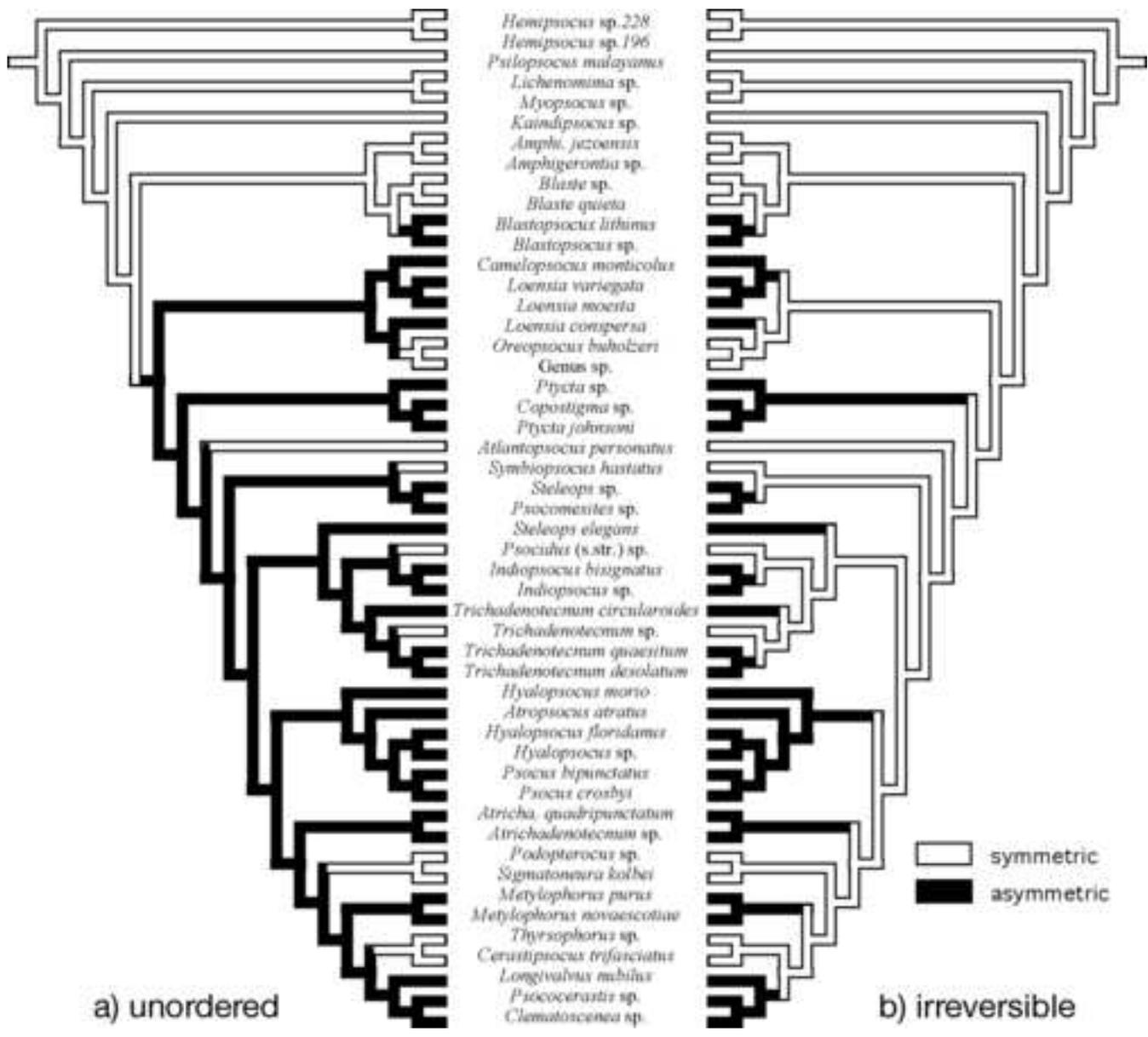


Table 1. History of taxonomic system of Psocidae.

Roesler (1944)

Psocinae*1
 Amphigerontiini
 Psocini
 Cerastipsocini
 Thyrsophorini

Badonnel (1951); Lienhard (1998)*2

Psocidae
 Amphigerontinae
 Cerastipsocinae
 Psocinae
Thyrsophoridae

Smithers (1972)

Psocidae
 Amphigerontiinae
 Cerastipsocinae
 Cerastipsocini
 Metylophorini
 Cycetini
 Psocinae
Thyrsophoridae

Mockford (1993), Lienhard & Smithers (2002), New & Lienhard (2007)

Psocidae
 Amphigerontiinae
 Psocinae
 Cerastipsocini
 Cycetini
 Metylophorini
 Psocini
 Ptyctini
 Thyrsophorinae

Li (2002)*3

Psocidae

Amphigerontiinae
 Amphigerontiini
 Blastini
 Stylatopsocini
Psocinae
 Oreopsocini
 Psocini
 Ptyctini
 Trichadenotecnini
Cerastipsocinae
 Cerastipsocini
 Metylophorini
Sigmatoneurinae

Present study

Psocidae

 'Amphigerontiinae'
 Kaindipsocini **new tribe**
 Amphigerontini
 Blastini
 Stylatopsocini*4
 Psocinae
 'Ptyctini'
 Psocini
 Atrichadenotecnini **new tribe**
 Sigmatoneurini
 Metylophorini
 Thyrsophorini
 Cycetini*4

*1 corresponds Psocidae in the present sense

*2 the latter treats Thyrsophoridae as a subfamily of Psocidae

*3 Chinese taxa only

*4 not analyzed in this study

Table 2. Taxa analyzed in this study. Taxonomic system presented in Lienhard & Smithers (2002) was adopted.

| Taxa | Locality | GenBank accession numbers | | | | | |
|---|----------|---------------------------|----------|----------|----------|----------|----------|
| | | 18S rDNA | 12S rDNA | 16S rDNA | Histone3 | wingless | COI |
| HEMIPSOCIDAE | | | | | | | |
| <i>Hemipsocus</i> sp. 196 | Malaysia | AY630543 | EF662229 | EF662100 | EF662139 | EF662184 | missing |
| <i>Hemipsocus</i> sp. 228 | Malaysia | AY630544 | missing | DQ104765 | DQ104792 | EF662185 | EF662063 |
| PSILOPSOCIDAE | | | | | | | |
| <i>Psilopsocus malayanus</i> | Malaysia | AY630541 | EF662230 | EF662101 | EF662140 | EF662186 | EF662064 |
| MYOPSOCIDAE | | | | | | | |
| <i>Myopsocus</i> sp. | USA | EF662265 | EF662231 | EF662102 | EF662141 | EF662187 | EF662065 |
| <i>Lichenomima</i> sp. | Japan | AY630540 | EF662232 | EF662103 | EF662142 | EF662188 | EF662066 |
| PSOCIDAE | | | | | | | |
| Amphigerontinae | | | | | | | |
| <i>Amphigerontia jezoensis</i> | Japan | AY630546 | EF662233 | EF662104 | EF662143 | EF662189 | EF662067 |
| <i>A.</i> sp. | USA | EF662266 | EF662234 | EF662105 | EF662144 | EF662190 | EF662068 |
| <i>Blaste quieta</i> | USA | AY630547 | missing | EF662106 | EF662145 | missing | EF662069 |
| <i>Blaste</i> sp. (cf. <i>longipennis</i>) | USA | EF662267 | EF662235 | EF662107 | EF662146 | EF662191 | EF662070 |
| <i>Blastopsocus lithinus</i> | USA | AY630548 | AY275313 | AY275363 | EF662147 | EF662192 | AY275288 |
| <i>Blasto.</i> sp. | USA | EF662268,9* | missing | EF662108 | EF662148 | EF662193 | EF662071 |
| <i>Kaindipsocus</i> sp. | Vietnam | EF662270 | EF662236 | EF662109 | EF662149 | EF662194 | EF662072 |
| Psocinae | | | | | | | |
| Cerastipsocini | | | | | | | |
| <i>Cerastipsocus trifasciatus</i> | USA | EF662271 | EF662237 | EF662110 | EF662150 | EF662195 | EF662073 |
| <i>Clematoscenea</i> sp. | Malaysia | AY630560 | EF662238 | EF662111 | EF662151 | EF662196 | EF662074 |

| | | | | | | | |
|---------------------------------|-------------|--------------|----------|----------|----------|----------|----------|
| <i>Longivalvus nubilus</i> | Japan | AY630559 | AY139905 | AY139952 | EF662152 | EF662197 | EF662075 |
| <i>Podopterocus</i> sp.** | Malaysia | AY630557 | EF662239 | EF662112 | missing | EF662198 | EF662076 |
| <i>Psococerastis</i> sp. | PNG | EF662272 | EF662240 | EF662113 | EF662153 | EF662199 | EF662077 |
| Metylophorini | | | | | | | |
| <i>Metylophorus novascotiae</i> | USA | AY630558 | AY275311 | AY275361 | EF662154 | missing | AY275286 |
| <i>M. purus</i> | USA | EF662273 | EF662241 | EF662114 | EF662155 | EF662200 | missing |
| <i>Sigmatoneura kolbei</i> | Japan | AY630556 | EF662242 | EF662115 | missing | EF662201 | EF662078 |
| Psocini | | | | | | | |
| <i>Atrichadenotecnum</i> sp. | Malaysia | EF662274 | EF662243 | EF662116 | EF662156 | EF662202 | EF662079 |
| <i>A. quadripunctatum</i> | Japan | AY630551 | AY374622 | AY374572 | EF662157 | EF662203 | AY374555 |
| <i>Atropsocus atratus</i> | USA | EF662275 | EF662244 | EF662117 | EF662158 | EF662204 | EF662080 |
| <i>Hyalopsocus morio</i> | Switzerland | EF662276 | EF662245 | EF662118 | EF662159 | EF662205 | EF662081 |
| <i>H. floridanus</i> | USA | EF662277 | EF662246 | EF662119 | EF662160 | EF662206 | EF662082 |
| <i>H. sp.</i> | USA | EF662278 | EF662247 | EF662120 | EF662161 | EF662207 | EF662083 |
| <i>Psocus bipunctatus</i> | Japan | AY630555 | EF662248 | EF662121 | EF662162 | EF662208 | EF662084 |
| <i>P. crosbyi</i> | USA | EF662279 | EF662219 | EF662122 | EF662163 | EF662209 | EF662085 |
| Ptyctini | | | | | | | |
| <i>Atrantopsocus personatus</i> | Italy | EF662280 | EF662250 | EF662123 | EF662164 | missing | missing |
| <i>Camelopsocus monticolus</i> | USA | EF662281 | missing | EF662124 | EF662165 | EF662210 | EF662086 |
| <i>Copostigma</i> sp. | Fiji | EF662282 | EF662251 | EF662125 | EF662166 | EF662211 | EF662089 |
| <i>Indiopsocus bisignatus</i> | USA | EF662283 | EF662252 | EF662126 | EF662167 | EF662212 | EF662087 |
| <i>I. sp. (cf. infumatus)</i> | USA | EF662284 | EF662253 | EF662127 | EF662168 | EF662213 | EF662088 |
| <i>Loensia moesta</i> | USA | AY630550 | AY275310 | AY275360 | EF662169 | EF662214 | AY275285 |
| <i>L. variegata</i> | France | AY630549 | AY139906 | AY139953 | EF662170 | EF662215 | AY374556 |
| <i>L. conspersa</i> | USA | EF662285 | EF662254 | EF662128 | EF662171 | EF662216 | EF662090 |
| <i>Oreopsocus buholzeri</i> | Switzerland | EF662286 | EF662255 | EF662129 | EF662172 | missing | missing |
| <i>Psocidus</i> (s.str.) sp. | Japan | EF662287 | EF662256 | EF662130 | EF662173 | EF662217 | EF662091 |
| <i>Psocomestes</i> sp. | Chile | EF662288 | EF662257 | EF662131 | EF662174 | EF662218 | EF662092 |
| <i>Ptycta johnsoni</i> | Japan | AY630553 | AY139907 | AY139954 | EF662175 | EF662219 | EF662093 |
| <i>P. sp.</i> | Hawaii | EF662289,90* | EF662258 | EF662132 | missing | EF662220 | EF662094 |

| | | | | | | | |
|---------------------------------------|------------|-------------|----------|----------|----------|----------|----------|
| <i>Steleops elegans</i> | USA | EF662291 | EF662259 | EF662133 | EF662176 | EF662221 | EF662095 |
| <i>S. sp.</i> | USA | EF662292 | EF662260 | EF662134 | EF662177 | EF662222 | EF662096 |
| <i>Symbiopsocus hastatus</i> *** | Japan | AY630552 | AY374625 | AY374575 | EF662178 | EF662223 | AY374559 |
| Genus undet. | UAE | EF662293 | EF662261 | EF662135 | EF662179 | EF662224 | EF662097 |
| <i>Trichadenotecnum circularoides</i> | Australia | EF662294,5* | AY374623 | AY374573 | EF662180 | EF662225 | AY374557 |
| <i>T. sp. (cf. alexanderae)</i> | USA | AY630554 | AY275312 | AY275362 | missing | missing | AY275287 |
| <i>T. quaesitum</i> | USA | EF662296 | EF662262 | EF662136 | EF662181 | EF662226 | EF662098 |
| <i>T. desolatum</i> | USA | EF662297 | EF662263 | EF662137 | EF662182 | EF662227 | EF662099 |
| Thyrsophorinae | | | | | | | |
| <i>Thyrsophorus sp.</i> | Costa Rica | EF662298 | EF662264 | EF662138 | EF662183 | EF662228 | missing |

*partly missing

**The genus *Podopteroocus* was recently synonymized with *Sigmatoneura*, and the species was newly described as *Sigmatoneura kakisayap* Yoshizawa & Lienhard, 2005 in Yoshizawa et al. (2005). However, the species is labeled here as *Podopteroocus sp.* because molecular test of morphology-based systematic treatment is also an important subject of the present paper.

***This species was previously mentioned as *Ptyctini Gen. sp.* by Yoshizawa (2004) and Johnson et al. (2004).