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.
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1. Introduction

The family Psocidae is the largest family of barklice (Psocodea: 'Psocoptera') containing over 20% (899 of 4408) of psocopteran species (Lienhard and 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 and 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

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Table 1
History of taxonomic system of Psocidae

Roesler (1944)
  Psocinae[a]
    Amphigerontiini
    Psocini
    Cerastipsocini
    Thyrsophorini

Badonnel (1951); Lienhard (1998)[b]
Psocidae
  Amphigerontinae
  Cerastipsocinae
  Psocinae
Thyrsophoridae

Smithers (1972)
Psocidae
  Amphigerontiinae
  Cerastipsocinae
    Cerastipsocini
    Metylophorini
    Cycetini
  Psocinae
Thyrsophoridae

Mockford (1993), Lienhard and Smithers (2002), New and Lienhard
  (2007)
Psocidae
  Amphigerontiinae
  Psocinae
    Cerastipsocini
    Cycetini
    Metylophorini
    Psocini
    Ptyctini
  Thyrsophorinae

Li (2002)[c]
Psocidae
Amphigerontiinae
  Amphigerontiini
  Blastini
  Stylatopsocini
Psocinae
  Oreopsocini
  Psocini
  Ptyctini
  Trichadenotecnini
Cerastipsocinae
  Cerastipsocini
  Metylophorini
Sigmatoneurinae

Present study
Psocidae
  'Amphigerontiinae'
    Kaindipsocini **new tribe**
    Amphigerontini
    Blastini
    Stylatopsocini[d]
  Psocinae
    'Ptyctini'
    Psocini
    Atrichadenotecnini **new tribe**
    Sigmatoneurini
    Metylophorini
    Thyrsophorini
    Cycetini[d]

[a] Corresponds Psocidae in the present sense.
[b] The latter treats Thyrsophoridae as a subfamily of Psocidae.
[c] Chinese taxa only.
[d] Not analyzed in this study.

for some genera. For example, New (in New and Lienhard, 2007) proposed some different assignments for some genera from that proposed in Lienhard and 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 and 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.

2. 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 Collec-

tion, Japan and Illinois Natural History Survey Insect Collection, USA. Samples were selected from all subfamilies and tribes of Psocidae (Lienhard and 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 and Egan (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*, *Podopterocus* 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 (1000 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 and 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 = 03223; 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 3000 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 3000 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 3000 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. 2. 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 and 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

Table 2
Taxa analyzed in this study

Taxa	Locality	GenBank Accession Nos.					
		18S rDNA	12S rDNA	16S rDNA	Histone 3	Wingless	COI
Hemipsocidae							
Hemipsocus sp. 196	Malaysia	AY630543	EF662229	EF662100	EF662139	EF662184	Missing
Hemipsocus sp. 228	Malaysia	AY630544	Missing	DQ104765	DQ104792	EF662185	EF662063
Psilopsocidae							
Psilopsocus malayanus	Malaysia	AY630541	EF662230	EF662101	EF662140	EF662186	EF662064
Myopsocidae							
Myopsocus sp.	USA	EF662265	EF662231	EF662102	EF662141	EF662187	EF662065
Lichenomima sp.	Japan	AY630540	EF662232	EF662103	EF662142	EF662188	EF662066
Psocidae							
Amphigerontinae							
Amphigerontia jezoensis	Japan	AY630546	EF662233	EF662104	EF662143	EF662189	EF662067
*A.*sp.	USA	EF662266	EF662234	EF662105	EF662144	EF662190	EF662068
Blaste quieta	USA	AY630547	Missing	EF662106	EF662145	Missing	EF662069
Blaste sp. (cf. *longipennis*)	USA	EF662267	EF662235	EF662107	EF662146	EF662191	EF662070
Blastopsocus lithinus	USA	AY630548	AY275313	AY275363	EF662147	EF662192	AY275288
Blasto. sp.	USA	EF662268,9[a]	Missing	EF662108	EF662148	EF662193	EF662071
Kaindipsocus sp.	Vietnam	EF662270	EF662236	EF662109	EF662149	EF662194	EF662072
Psocinae							
Cerastipsocini							
Cerastipsocus trifasciatus	USA	EF662271	EF662237	EF662110	EF662150	EF662195	EF662073
Clematoscenea sp.	Malaysia	AY630560	EF662238	EF662111	EF662151	EF662196	EF662074
Longivalvus nubilus	Japan	AY630559	AY139905	AY139952	EF662152	EF662197	EF662075
Podopterocus sp.[b]	Malaysia	AY630557	EF662239	EF662112	Missing	EF662198	EF662076
Psococerastis sp.	PNG	EF662272	EF662240	EF662113	EF662153	EF662199	EF662077
Metylophorini							
Metylophorus novascotiae	USA	AY630558	AY275311	AY275361	EF662154	Missing	AY275286
M. purus	USA	EF662273	EF662241	EF662114	EF662155	EF662200	Missing
Sigmatoneura kolbei	Japan	AY630556	EF662242	EF662115	Missing	EF662201	EF662078
Psocini							
Atrichadenotecnum sp.	Malaysia	EF662274	EF662243	EF662116	EF662156	EF662202	EF662079
A. quadripunctatum	Japan	AY630551	AY374622	AY374572	EF662157	EF662203	AY374555
Atropsocus atratus	USA	EF662275	EF662244	EF662117	EF662158	EF662204	EF662080
Hyalopsocus morio	Switzerland	EF662276	EF662245	EF662118	EF662159	EF662205	EF662081
H. floridanus	USA	EF662277	EF662246	EF662119	EF662160	EF662206	EF662082
H. sp.	USA	EF662278	EF662247	EF662120	EF662161	EF662207	EF662083
Psocus bipunctatus	Japan	AY630555	EF662248	EF662121	EF662162	EF662208	EF662084
P. crosbyi	USA	EF662279	EF662219	EF662122	EF662163	EF662209	EF662085
Ptyctini							
Atlantopsocus personatus	Italy	EF662280	EF662250	EF662123	EF662164	Missing	Missing
Camelopsocus monticolus	USA	EF662281	Missing	EF662124	EF662165	EF662210	EF662086
Copostigma sp.	Fiji	EF662282	EF662251	EF662125	EF662166	EF662211	EF662089
Indiopsocus bisignatus	USA	EF662283	EF662252	EF662126	EF662167	EF662212	EF662087
I. sp. (cf. *infumatus*)	USA	EF662284	EF662253	EF662127	EF662168	EF662213	EF662088
Loensia moesta	USA	AY630550	AY275310	AY275360	EF662169	EF662214	AY275285
L. variegata	France	AY630549	AY139906	AY139953	EF662170	EF662215	AY374556
L. conspersa	USA	EF662285	EF662254	EF662128	EF662171	EF662216	EF662090
Oreopsocus buholzeri	Switzerland	EF662286	EF662255	EF662129	EF662172	Missing	Missing
Psocidus (s.str.) sp.	Japan	EF662287	EF662256	EF662130	EF662173	EF662217	EF662091
Psocomestes sp.	Chile	EF662288	EF662257	EF662131	EF662174	EF662218	EF662092
Ptycta johnsoni	Japan	AY630553	AY139907	AY139954	EF662175	EF662219	EF662093
P. sp.	Hawaii	EF662289,90[a]	EF662258	EF662132	Missing	EF662220	EF662094
Steleops elegans	USA	EF662291	EF662259	EF662133	EF662176	EF662221	EF662095
S. sp.	USA	EF662292	EF662260	EF662134	EF662177	EF662222	EF662096
Symbiopsocus hastatus[c]	Japan	AY630552	AY374625	AY374575	EF662178	EF662223	AY374559
Genus undet.	UAE	EF662293	EF662261	EF662135	EF662179	EF662224	EF662097
Trichadenotecnum circularoides	Australia	EF662294,5[a]	AY374623	AY374573	EF662180	EF662225	AY374557
T. sp. (cf. *alexanderae*)	USA	AY630554	AY275312	AY275362	Missing	Missing	AY275287
T. quaesitum	USA	EF662296	EF662262	EF662136	EF662181	EF662226	EF662098

Table 2 (*continued*)

Taxa	Locality	GenBank Accession Nos.					
		18S rDNA	12S rDNA	16S rDNA	Histone 3	Wingless	COI
T. desolatum	USA	EF662297	EF662263	EF662137	EF662182	EF662227	EF662099
Thyrsophorinae							
Thyrsophorus sp.	Costa Rica	EF662298	EF662264	EF662138	EF662183	EF662228	Missing

Taxonomic system presented in Lienhard and Smithers (2002) was adopted.

[a] Partly missing.

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

[c] This species was previously mentioned as Ptyctini Gen. sp. by Yoshizawa (2004) and Johnson et al. (2004).
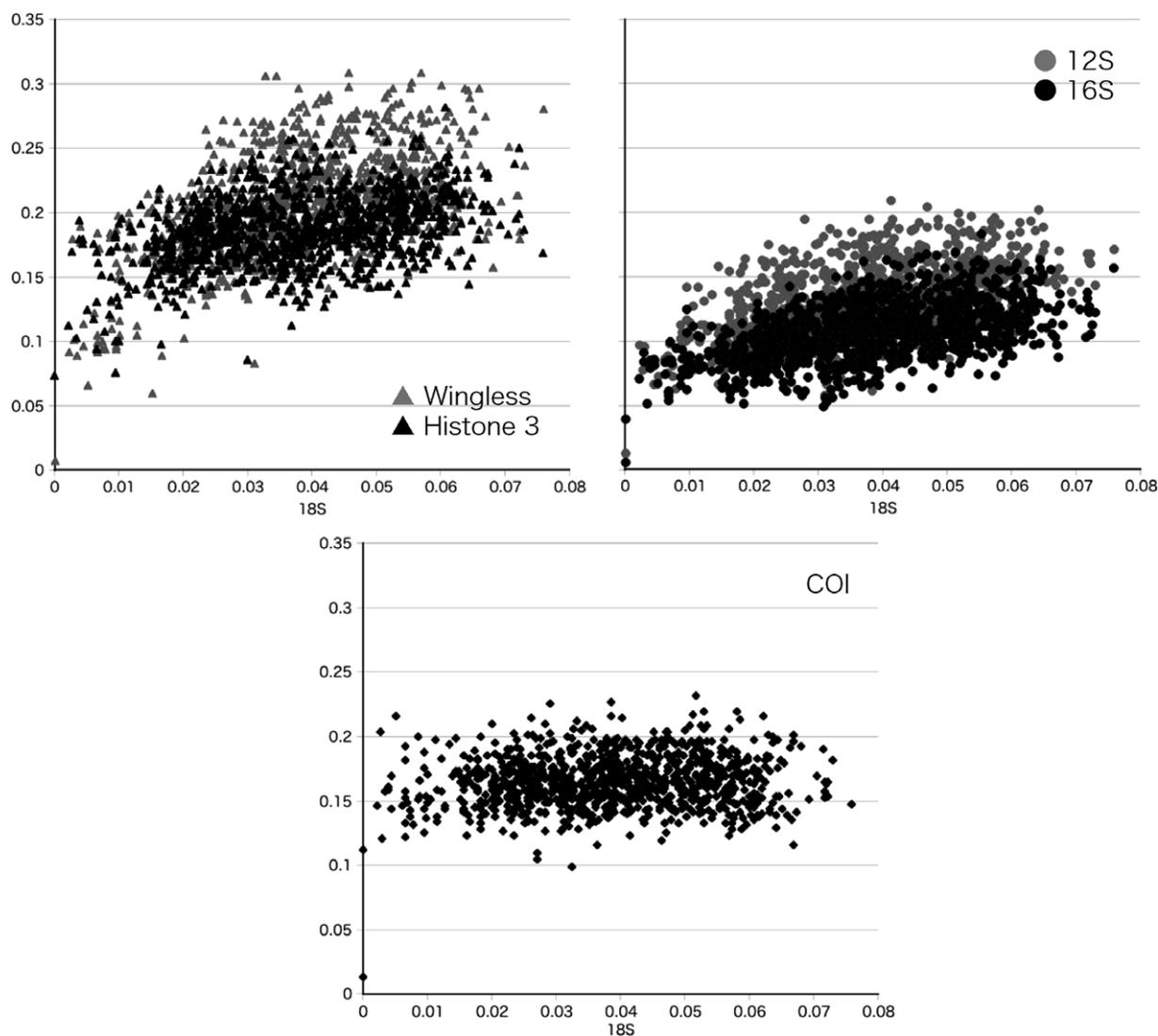
Fig. 1. Plot of uncorrected pair wise distances in nuclear Histone 3 and wingless vs 18S (top-left), 12S and 16S vs 18S (top-right) and COI vs 18S (bottom). Incomplete taxa (taxa with missing data) are not included.

and partitioned into the following eight 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 Mac Clade (Maddison and 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 and 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 in Section 4.

3. Results

3.1. 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
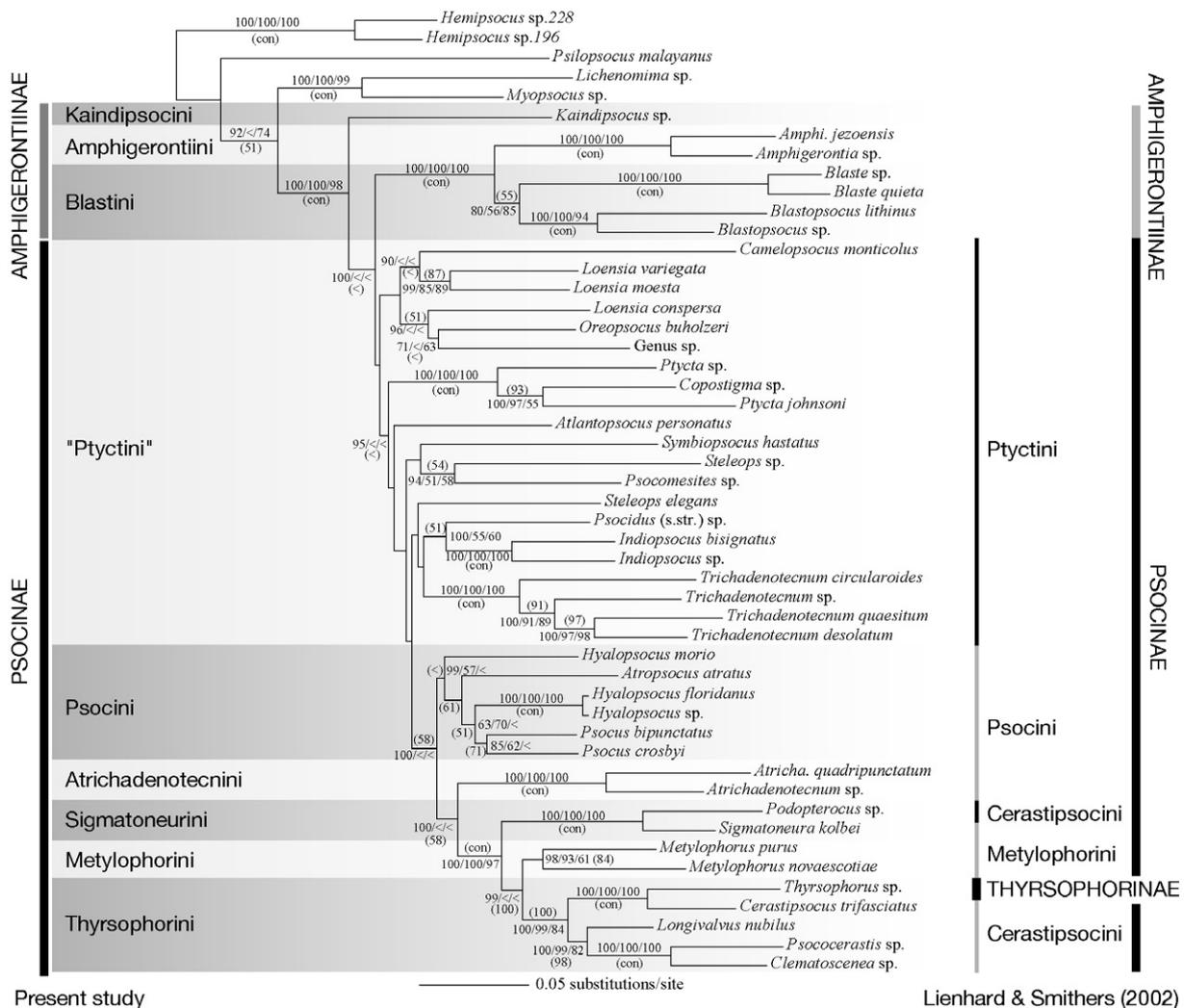

Fig. 2. The ML tree (−ln = 38,410.025) estimated from the dataset including all incomplete taxa. Branch lengths are proportional to ML estimated branch lengths. The numbers associated the branches are Bayesian posterior probability/ML bootstrap/MP bootstrap supports and (constrained ML bootstrap support). (con) indicate the constrained branches (see Section 2).

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 partition 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 partition 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).

3.2. 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. Fig. 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 and 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 and 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 unconstraint 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 Thyrsophorinae, composed a monophyletic group, and this clade received strong support by all analyses. The genus *Podopterocus* was recently synonymized with *Sigmatoneura*, and *Sigmatoneura* was recognized as a member of Metylophorini by Yoshizawa et al. (2005). This synonymy, proposed on the basis of morpho-

logical characters, was also supported by the molecular data, but the *Sigmatoneura-Podopterocus* clade was always separated from the genus *Metylophorus* in the present analyses. The branch uniting *Metylophorus*, Cerastipsocini, and Thyrsophorinae 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 Thyrsophorinae 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.

4. Discussion

4.1. 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 and 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 + Thyrsophorinae 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 and 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), Amphigerontiini 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 and Smithers (2002). The systematic placement of this genus under Psocini as proposed by New (in New and 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 and Smithers, 2006; Bess and 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 *Podopterocus*: 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 Cerastopsocini 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.

4.2. 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 and 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 inde-

pendent origins or reversals of symmetric/asymmetric forms have already been predicted by morphological assessments (Yoshizawa et al., in press). 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 (four 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
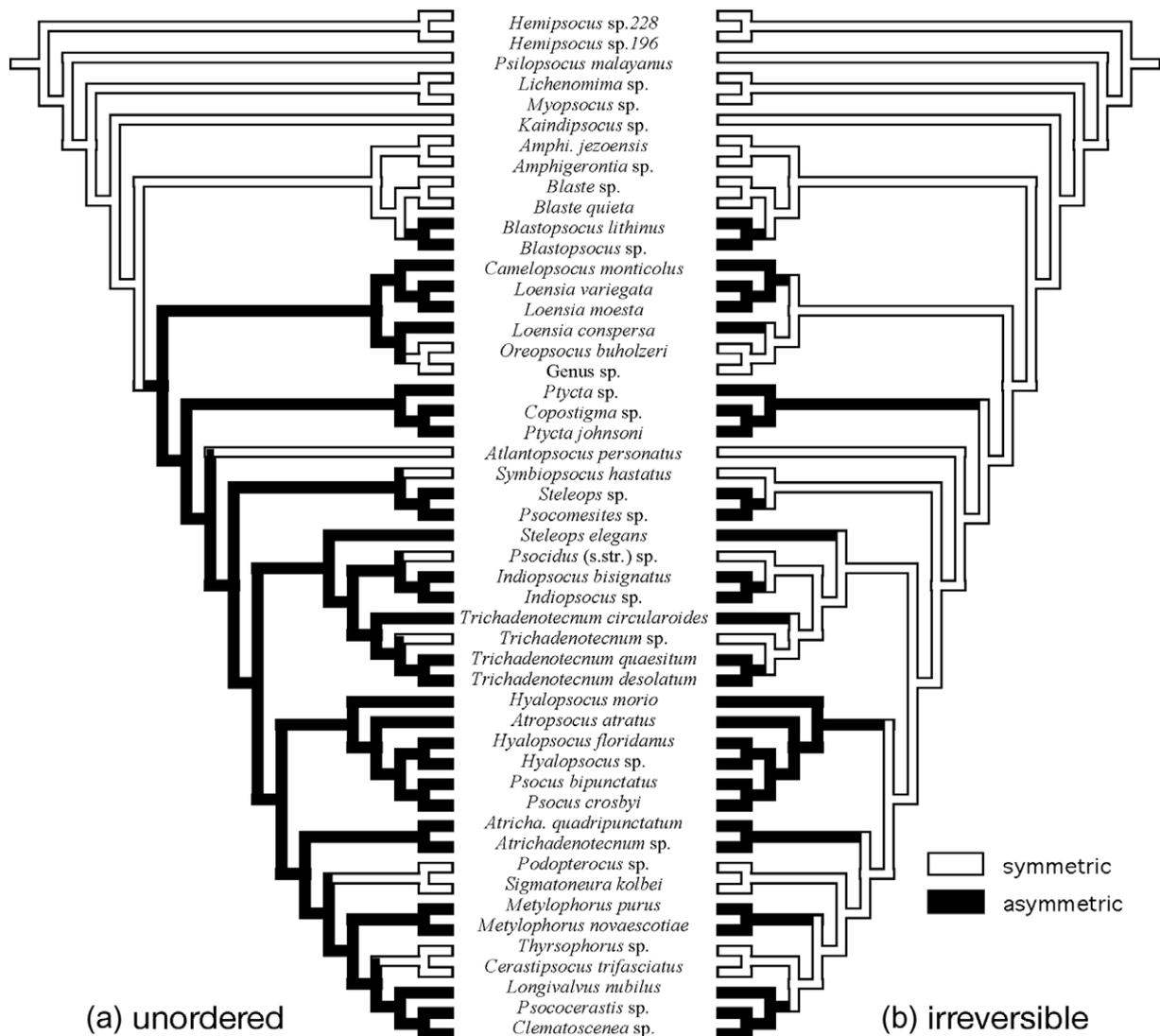

Fig. 3. Most parsimonious reconstruction of symmetric/asymmetric male hypandrium. Character states are reconstructed as unordered (left) and irreversible (right). Online supplementary data: aligned data set in NEXUS format psocidae-mol.nexus.

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., in press). 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.

4.3. 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 and 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., in press). *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 and Thornton, 1981.
Included genus: *Kaindipsocus*.
Diagnosis. As generic diagnosis of the type genus (Smithers and Thornton, 1981).

The genus *Kaindipsocus* was first assigned to the subfamily Amphigerontiinae by Lienhard and 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*.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.07.011.

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