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Non-monophyly of the "cydnoid" complex within Pentatomoidea (Hemiptera: Heteroptera) revealed by Bayesian phylogenetic analysis of nuclear rDNA sequences

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Abstract

The "cydnoid" complex of pentatomoid families, including Cydnidae, Parastrachiidae, Thaumastellidae, and Thyreocoridae, is morphologically defined by the presence of an array of more or less flattened stout setae (called coxal combs), situated on the distal margin of coxae. These structures, suggested to prevent the coxal-trochanteral articulation from injuries caused by particles of soil, sand or dust, by their nature and function are unknown elsewhere in the Heteroptera. As such, coxal combs were regarded as a synapomorphy of this group of families, and enabled the definition of it as a monophylum. In this study, the monophyly of the "cydnoid" complex of families is tested for the first time, based on the combined analysis of nuclear ribosomal DNA sequences (28S rDNA D3 region, and 18S rDNA). Combined analyses of both genes are performed using Bayesian methods with the covarion option in MrBayes 3.2.0. Non-monophyly of the entire "cydnoid" complex of families, and independent origins of their coxal combs is suggested. The family Thaumastellidae is demonstrated not to be part of this complex as previously proposed. Challenging the existing classification system, the use of the name "cydnoid" complex is indicated as unwarranted, and therefore it should no longer be applied to this group of families.

Key words

Cydnidae *sensu lato*, Parastrachiidae, Thaumastellidae, Thyreocoridae, molecular phylogeny, ribosomal DNA, coxal combs, Bayesian estimation.

1. Introduction

The Cydnidae (colloquially known as "burrower bugs" or "burrowing bugs") is a family within the superfamily Pentatomoidea and comprises more than 750 species known from temperate, warm and tropical parts of the world (Lis 1994, 1999, 2006, 2013; Lis et al. 2000; Lis & Lis 2014, 2015; Cassis & Gross 2002; Schwertner & Nardi 2015). They are mostly soil-diggers that feed on plant roots, though some are above ground plant-feeders and may also be mycetophagous or feed on seeds (for review, see: Schaefer 1988; Lis 1994; Lis et al. 2000; Schwertner & Nardi 2015). However, some are cavernicolous (Linnavuori 1993; Kłys & Lis 2013; Lis & Lis 2016), and several are associated with ants (Froeschner 1975; Lis 2015).

Cydnidae have generally been considered of little economic importance, but to date, almost 30 species have been reported as pests, mainly in the Neotropics and Oriental region (Lis et al. 2000; Schwertner & Nardi 2015). At present, the family is divided into six subfamilies (Pluot-Sigwalt & Lis 2008; Lis 2010a; Schwertner & Nardi

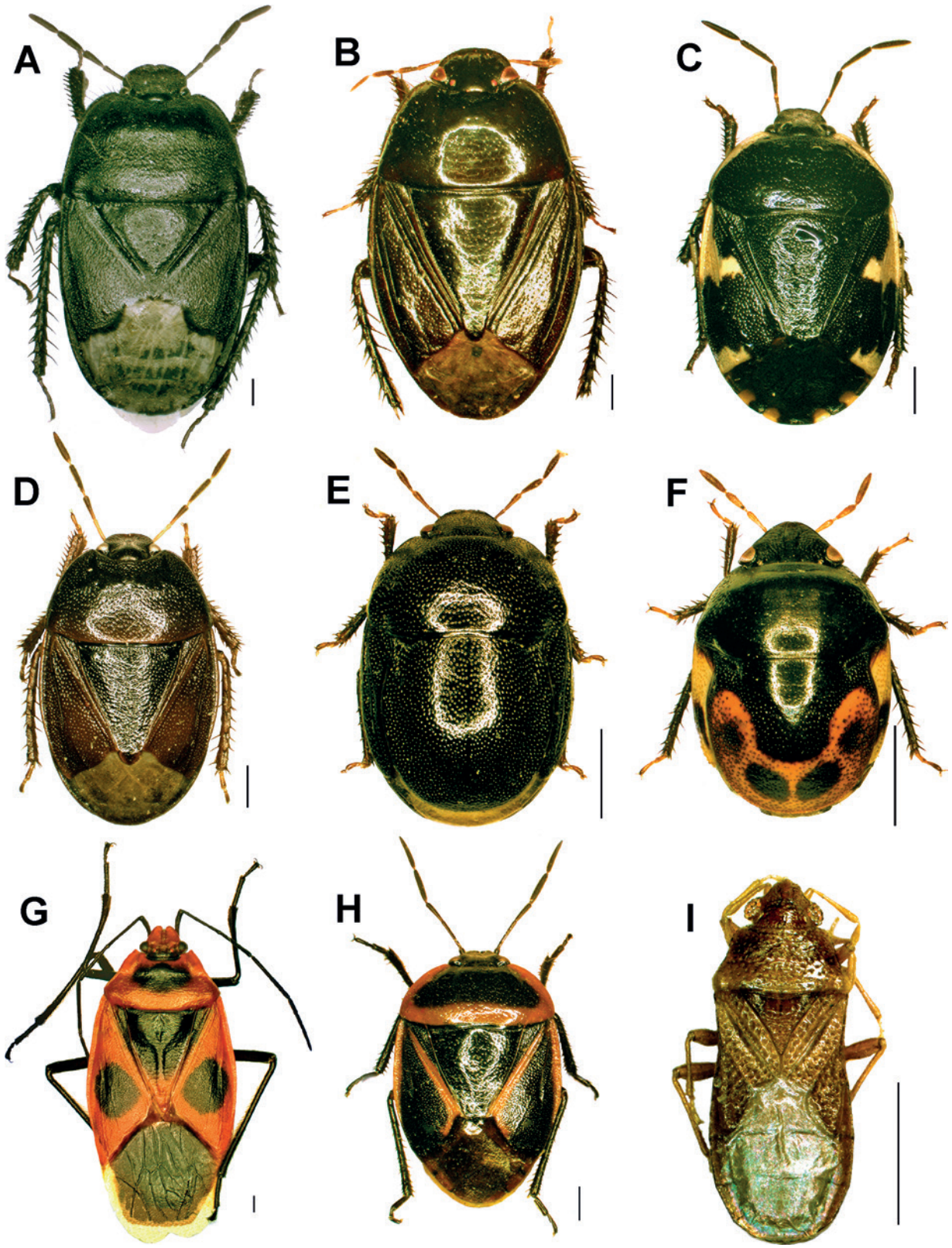

Fig. 1. Representatives of the "cydnoid" complex. **A–D**: Cydnidae: (A) *Cydnus aterrimus*, (B) *Macroscytus brunneus*, (C) *Tritomegas sexmaculatus*, (D) *Sehirus luctuosus*; **E–F**: Thyreocoridae: (E) Thyreocorinae, *Thyreocoris scarabaeoides*; (F) Corimelaeninae, *Galgupha vinculata*; **G–H**: Parastrachiidae: (G) *Parastrachia japonensis*, (H) *Dismegistus fimbriatus*; **I**: Thaumastellidae: *Thaumastella aradoides*. Scale bar = 1 mm.

2015), i.e., Amnestinae, Amaurocorinae, Cephalocteinae (with two tribes, Cephalocteini and Scaptocorini), Cydninae (with two tribes, Cydnini and Geotomini *sensu lato*), Garsauriinae, and Sehirinae (with a single tribe Sehirini *sensu lato*). Since the family was never thoroughly phylogenetically studied and, importantly, its monophyly was

questioned, a more appropriate name "Cydnidae *sensu lato*" was suggested for this family (Grazia et al. 2008; Pluot-Sigwalt & Lis 2008; Lis 2010a).

Apart from that, three other pentatomoid families, i.e. Parastrachiidae, Thaumastellidae, and Thyreocoridae (with two subfamilies, Thyreocorinae and Corimelaeninae) were often suggested to be closely allied to Cydnidae *sensu lato*, and were sometimes treated as its subfamilies in the past (e.g., Dolling 1981; Schuh & Slater 1995; Schaefer et al. 1988).

Around thirty years ago, Schaefer (1981, 1988) proposed gathering the four aforementioned families into a group of "primitive" pentatomoids. Additionally, he suggested Megarididae, Canopidae, Cyrtocoridae, Plataspidae, and Lestoniidae to be included on the basis of several morphological characters, including a metathoracic wing stridulitrum, stout bristles and setae on tibiae, and coxal combs (an array of more or less flattened stout setae, situated on the distal margin of coxae and adpressed to the surface of the trochanters, unknown elsewhere in the Heteroptera). Because those families (Fig. 1) showed cydnid affinities, this group was subsequently named the "cydnoid" complex (Lis 1994).

Nevertheless, two of those diagnostic characters, i.e. a metathoracic wing stridulitrum and stout bristles on tibiae, were then regarded as improper to define this group of families (e.g., Schaefer et al. 1988; Lis & Heyna 2001; Lis & Schaefer 2005; Lis 2010a), and, the presence of the coxal combs (Fig. 2) remained a single character that might be considered as its synapomorphy (e.g., Grazia et al. 2008; Lis 2010a). This crucial character, however, can only support some families representing the original "cydnoid" complex, and therefore a definition of this group was subsequently more or less narrowed (Jacobs 1989; Ahmad & McPherson 1990; Schaefer 1993; Schuh & Slater 1995; Packauskas & Schaefer 1998; Lis 2010a; Lis & Ziaja 2010). Thus, at present, the "cydnoid" complex includes only four families, i.e. Cydnidae, Parastrachiidae, Thaumastellidae, and Thyreocoridae (Grazia et al. 2008; Pluot-Sigwalt & Lis 2008; Lis 2010a; Yao et al. 2012).

To date, no thorough phylogenetic analysis testing the monophyly of this complex has been conducted. However, its non-monophyletic origin has already been suggested by Grazia et al. (2008) during studies on the phylogenetic relationships of family groups in Pentatomoidea based on molecular and morphological data, as well as by Pluot-Sigwalt & Lis (2008) during studies on morphology of the spermathecae in Cydnidae.

Unlike results of those two aforementioned studies, Yao et al. (2012), when analyzing the phylogeny of the infraorder Pentatomomorpha based on fossil and extant morphology, identified the Cydnidae *sensu lato* of Dolling (= "cydnoid" complex of Lis 1994), a morphologically well-supported clade.

However, the results of the phylogenetic analyses of Grazia et al. (2008) and Yao et al. (2012) were based only on a limited number of taxa representing the "cydnoid" complex.

Yao et al. (2012) included only four extant species, i.e. *Thaumastella elizabethae* Jacobs, 1989 (Thaumastellidae), *Thyreocoris scarabaeoides* (Linnaeus, 1758) (Thyreocoridae: Thyreocorinae), *Parastrachia japonensis* (Scott, 1880) (Parastrachiidae), *Sehirus cinctus* (Palisot de Beauvois, 1805) (Cydnidae: Sehirinae), and a single fossil *Cilicydnus robustispinus* Yao, Cai and Ren, 2007 (Cydnidae: Amnestinae). No species of the subfamily Corimelaeninae (Thyreocoridae), nor the subfamily Cydninae (Cydnidae) were incorporated into the analyses.

Though the phylogenetic analyses in Grazia et al. (2008) were based on molecular and morphological data, the "cydnoid" complex was represented by only a few sequences for a limited number of taxa, i.e. a single unidentified species of *Allocoris* McAtee & Malloch (Thyreocoridae: Corimelaeninae), two species of Parastrachiidae, *Parastrachia japonensis* and *Dismegistus sanguineus* (DeGeer, 1778), two species of Thaumastellidae (*Thaumastella elizabethae* Jacobs and *T. namaquensis* Schaefer & Wilcox, 1971), and three unidentified taxa of the subfamily Cydninae (Cydnidae).

Importantly, in a morphological sense, families of the "cydnoid" complex were always identified to form a monophylum (Gapud 1991; Grazia et al. 2008; Yao et al. 2012), with only the coxal combs and spinose tibiae used as defining characters for such a clade. As mentioned above, spinose tibiae are found in many heteropteran families in addition to those of the "cydnoid" complex, and therefore only the presence of coxal combs remains as a potential synapomorphic character for the "cydnoid" complex of families (Lis 2010a).

The aim of our molecular study was to test the monophyly of the "cydnoid" complex of families using more extensive material, and, for the first time, verify whether the presence of the coxal combs, considered the only synapomorphy for this group, are really homologous in all "cydnoid" families or may have evolved independently.

2. Material and methods

2.1. Taxa

In this study, a total of 46 terminal taxa were selected for analyses, with 40 taxa in the ingroup and 6 taxa in the outgroup. The ingroup contained 21 taxa representing the "cydnoid" complex with representatives of all its families (Cydnidae, Parastrachiidae, Thaumastellidae, and Thyreocoridae), and 19 taxa of other Pentatomoidea. Six species of the superfamily Coreoidea (i.e. a part of the Eutrichophora, the sister group of the superfamily Pentatomoidea; cf. Xie et al. 2005; Hua et al. 2008) were selected as outgroup representatives. Species names, their geographic origin, collectors' names, Opole University sample numbers (if applicable), and accession numbers for sequences deposited by us in GenBank, and of those

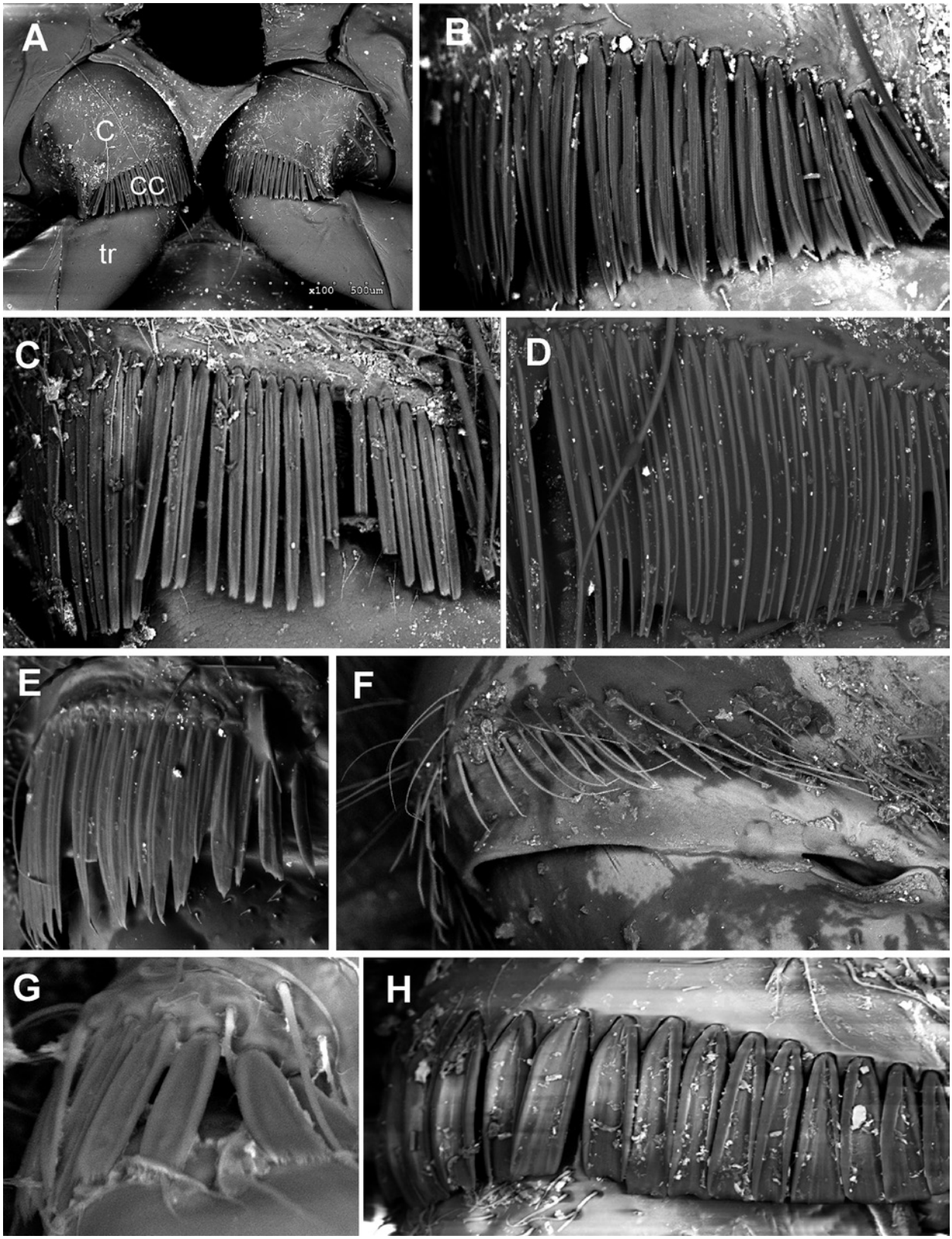

Fig. 2. Coxal combs in different representatives of the "cydnoid" complex. **A–E**: Cydnidae: (A,B) *Macroscytus brunneus*, (C) *Microporus nigrita*, (D) *Cydnus aterrimus*, (E) *Tritomegas sexmaculatus*; **F**: Parastrachiidae: *Parastrachia japonensis*; **G**: Thaumastellidae: *Thaumastella aradoides*; **H**: Thyreocoridae: *Galgupha vinculata*. — ***Abbreviations*** in Fig. 2A: c – coxa, cc – coxal combs, tr – trochanter.

published previously and obtained directly from GenBank are provided in Table 1. The *Chilocoris assmuthi* 28S rDNA sequence (KY886256) was combined with the *Chilocoris confusus* 18S rDNA sequence (KY911201), and the *Megymenum* sp. 18S rDNA sequence (KJ535879) was combined with the *Megymenum brevicorne* 28S

Table 1. List of specimens used in the phylogenetic analyses with GenBank accession numbers. Other information about the specimens is provided in Supplement File 2.

Family	Species	GenBank accesion numbers for 18S rDNA	GenBank accesion numbers for 28S rDNA
Acanthosomatidae	*Elasmostethus interstinctus*	KY911197	KY886252
	Elasmucha laeviventris	KJ535865	KJ535865
	Stauralia chloracantha	AY252268	AY252512
Canopidae	*Canopus* sp.	AY252229	AY252472
Cydnidae	*Adomerus biguttatus*	KY911198	KY886253
	Adrisa magna	KY911199	KY886254
	Canthophorus niveimarginatus	KY911200	KY886255
	Chilocoris confusus	KY911201	—
	Chilocoris assmuthi	—	KY886256
	Cydnus aterrimus	KY911202	KY886257
	Fromundus pygmaeus	KJ535871	KJ535871
	Geotomus convexus	KY911203	KY886258
	Macroscytus brunneus	KY911204	KY886259
	Microporus nigrita	KY911205	KY886260
	Ochetostethomorpha secunda	KY911206	KY886261
	Pangaeus bilineatus	KY911207	KY886262
	Rhytidoporus indentatus	KY911208	KY886263
	Sehirus luctuosus	KY911209	KY886264
	Tritomegas sexmaculatus	KY911210	KY886265
Dinidoridae	*Cyclopelta obscura*	KJ522641	KJ522642
	Megymenum sp.	KJ535879	—
	Megymenum brevicorne	—	KY886256
Lestoniidae	*Lestonia haustorifera*	KT188471	KT188472
Parastrachiidae	*Dismegistus sanguineus*	EF641203	EF641183
	Parastrachia japonensis	EF641204	EF641184
Pentatomidae	*Eurydema maracandica*	KJ535867	KJ535867
	Graphosoma lineatum	KY911211	KY886267
	Oechalia schellenbergii	EF641205	EF641185
	Rhaphigaster nebulosa	X89495	EU426880
Plataspidae	*Coptosoma bifarium*	KJ461259	KJ461239
	Coptosoma scutellatum	KY911212	KY886268
Scutelleridae	*Cantao ocellatus*	KJ461182	KJ461230
	Coleotichus costatus	EF641219	EF641194
Tessaratomidae	*Eurostus validus*	KJ461181	KJ461181
Thaumastellidae	*Thaumastella elizabethae*	EF641221	EF641195
	Thaumastella namaquensis	EF641222	EF641196
Thyreocoridae	*Allocoris* sp.	AY252323	AY252562
	Galgupha australis	KY911213	KY886269
	Thyreocoris scarabaeoides	KY911214	KY886270
Urostylididae	*Tessaromerus licenti*	KJ535883	KJ535883
	Urochela luteovaria	KJ461205	KJ461306
	Urostylis chinai	KJ535886	KJ535886
Alydidae (outgroup)	*Leptocorisa acuta*	AY627322	AY252462
	Riptortus pedestris	AB725684	AB725684
Coreidae (outgroup)	*Cletus punctiger*	KJ461173	KJ461219
	Aulacosternum nigrorubrum	AY252258	AY252500
Rhopalidae (outgroup)	*Stictopleurus punctatonervosus*	KJ461217	KJ461286
Stenocephalidae (outgroup)	*Dicranocephalus alticolus*	KJ461228	KJ461267

rDNA sequence (KY886266). The classification of the family Cydnidae follows Pluot-Sigwalt & Lis (2008).

2.2. Photographic documentation

Dorsal view images of specimens representing the "cydnoid" complex were captured with a Moticam 1000 digital camera mounted on an Olympus SZX10 microscope, using Images Plus 2.0 software (Motic Asia, Hong Kong). Multiple focal planes were merged using Helicon Focus 4.50.3 software (Helicon Soft Ltd.). The scanning electron microscopy of the coxal combs was carried out using the Hitachi S-3000N microscope to produce all micrographs.

Table 2. Primers used for PCR amplification and sequencing of the nuclear 18S and 28S genes.

Gene fragment	Primers	Sequence (5′ → 3′)	Source
28S	28Sa	GAC CCG TCT TGA AAC ACG GA	Whiting et al. (1997)
	28Sb	TCG GAA GGA ACC AGC TAC TA	Whiting et al. (1997)
18S	1F	TAC CTG GTT GAT CCT GCC AGT AG	Giribet et al. (1996)
	5R	CTT GGC AAA TGC TTT CGC	Giribet et al. (1996)
	3F	GTT CGA TTC CGG AGA GGG A	Giribet et al. (1996)
	18Sbi	GAG TCT CGT TCG TTA TCG GA	Whiting et al. (1997)
	5F	GCG AAA GCA TTT GCC AAG AA	Giribet et al. (1996)
	9R	GAT CCT TCC GCA GGT TCA CCT AC	Giribet et al. (1996)

2.3. DNA subunits

Because the oldest fossil records of Cydnidae, and the presence of coxal combs, are known from the Late Jurassic to Early Cretaceous Yixian Formation of China (Yao et al. 2007, 2010), we decided to use two nuclear markers, i.e. 28S and 18S ribosomal DNA, in our analyses, which were useful for resolving problems related to such old evolutionary events (Hillis & Dixon 1991). In Heteroptera, mainly the D3 region of 28S rDNA was sequenced, analyzed and deposited in GenBank, and therefore only this region was comparatively analyzed, whereas 18S rDNA was sequenced and analyzed in full.

2.4. DNA extraction

For genomic DNA extraction, ethanol-preserved specimens collected by the authors or other researchers (see: the Acknowledgements section) were mostly used. However, since there is evidence of successful PCR on early 20th century dry museum Pentatomoidea specimens (Lis et al. 2011a, 2012), we also attempted to sequence the nuclear ribosomal DNA from dried museum specimens (for results, see Tabe 1). For each species (regardless of form of specimen preservation), total genomic DNA was extracted from thorax muscle tissues using a DNeasy Tissue Kit (QIAGEN Inc., Santa Clara, California) following the manufacturer's protocol. Once DNA was extracted, the remains of specimens were inserted in tubes with 96% ethanol and lodged in a deep freezer at the Department of Biosystematics, Opole University, Poland (for the Opole University sample numbers, see Table 1).

2.5. PCR amplification, purification and sequencing

The PCR reactions for 28S were conducted in an Eppendorf Master Thermocycler using 0.02 U/µl of HiFi Taq® DNA Polymerase in a 25 µl reaction mixture containing 0.4 µl each primer, 200 µM dNTPs and 1 µl genomic DNA template. The thermal cycling profile consisted of initial denaturation for 2 min at 92°C, followed by 34 cycles of 30 sec at 92°C, 30 sec at 56°C and 20 sec at 72°C. The final elongation step was 5 min at 72°C. The 28S rDNA was amplified using the primer pair 28Sa and 28Sb (Whiting et al. 1997; for primer pair sequences, see Table 2).

The PCR amplification for 18S was performed in a 25 µl reaction volume containing 1 µl DNA template, 1 × reaction buffer, 0.5 µl each primer, 200 µM dNTPs, and 0.02 U/µl of HiFi Taq® DNA Polymerase. The 18S rDNA target segments were too long to be amplified in one step; therefore, three overlapping fragments were amplified using the following primer pairs: 1F-5R (950 bp), 3F-18Sbi (900 bp), and 5F-9R (850 bp) (Giribet et al. 1996; Whiting et al. 1997), which are listed in Table 2. PCR reactions were conducted in an Eppendorf Master Thermocycler and run for 36 cycles consisting of 1 min denaturation at 93°C, 1 min annealing at 59°C and 40 sec extension at 72°C, with an initial denaturation step of 93°C for 2 min and a final extension step of 72°C for 5 min. The quality of PCR products were evaluated by 1% agarose gel electrophoresis. The successful samples were purified using a QIAquick PCR Purification Kit (QIAGEN Inc., Santa Clara, California) and eluted in 30 µl elution buffer.

All experimental PCR runs were performed alongside negative controls (without template DNA). Any PCR runs that showed a band in the negative control were discarded in their entirety. Purified amplicons were sequenced in the Health Care Center GENOMED (Warsaw, Poland) with appropriate sequencing primers. To ensure our results were not contaminated, the obtained sequences were compared to databases using BLAST searches, which showed high similarities to sequences of other species of the superfamily Pentatomoidea already deposited GenBank (the utility of this procedure, especially for dried pentatomoid museum specimens was confirmed by Lis et al. 2011a).

2.6. Phylogenetic analyses

2.6.1. Sequence alignments and analyses

Sequences were aligned using ClustalW (with default parameters) in MEGA7 software (Kumar et al. 2016), and then truncated at both ends to avoid the influence of missing data resulting from incomplete sequences. As the secondary structure of ribosomal sequences can have an impact on the sequence alignment and tree reconstruction (Kjer 1995; Letsch & Kjer 2011; Letsch et al. 2010), the original alignments were adjusted manually using sec-

ondary structure models for 28S rDNA of *Eurydema maracandica* (Pentatomoidea: Pentatomidae) and *Lestonia haustorifera* (Pentatomoidea: Lestoniidae) as references (Yu et al. 2013; Wu et al. 2016), and secondary structures models for 18S rDNA of *E. maracandica* (Pentatomoidea: Pentatomidae) (Yu et al. 2013), *L. haustorifera* (Pentatomoidea: Lestoniidae) (Wu et al. 2016), *Fromundus pygmaeus*, *Macroscytus brunneus* and *Microporus nigrita* (all representing Pentatomoidea: Cydnidae) (Supplement File 1) as references. The analyses were performed with the covarion option (Galtier 2001) using MrBayes v.3.2.0 (Ronquist et al. 2012). Gene partitions were unlinked, and were allowed to evolve under different evolutionary rates.

2.6.2. Analysis of the phylogenetic signal

Multiple substitutions during the evolution of genes can significantly obscure the final phylogenetic information contained in the analyzed sequences, and can lead to misinterpretations in true phylogenetic relationships among analyzed taxa (Xia & Lemey 2009). Therefore, homoplasy due to multiple substitutions during the evolution of genes was tested by plotting numbers of transitions and transversions against Kimura-2-parameter distance (K2P) using DAMBE ver. 6.4.29 (Xia 2013). The substitution saturation analyses were performed for each gene separately and for the combined sequence data, first on all sites of the sequence alignments, and then on fully resolved sites only. The aligned sequences were regarded as phylogenetically informative if the observed substitution saturation index (*Iss*) was significantly lower than the critical value of *Iss* for both symmetrical and asymmetrical topologies (*Iss. cSym*, and *Iss. cAsym*), and the P value was lower than 0.0001 (Xia et al. 2003; Xia & Lemey 2009).

2.6.3. Substitution model selection

In order to avoid the problem of an "*a priori selection*" of only one scheme of nucleotide substitution types versus an "*a posteriori selection*" of the most appropriate model (Alfaro & Huelsenbeck 2006), we employed two strategies to identify the best-fitting substitution models for each partition analysis. First, we identified the best-fitting *a priori* model under the Bayesian Information Criterion (BIC; Schwartz 1978), and the Akaike Information Criterion corrected (AICc; Hurvich & Tsai 1989, 1991; Posada & Buckley 2004), as implemented in MEGA 7.0 (Kumar et al. 2016) for each gene. Then, we used the procedure known as reversible jump MCMC to sample across the substitution model space in the Bayesian MCMC analysis itself (Huelsenbeck et al. 2004) using the command "*lset nst=mixed rates=gamma*" in MrBayes v.3.2.0 (Ronquist et al. 2012).

2.6.4. MCMC settings

Two independent runs with three heated and one cold Markov chains per analysis were performed simultaneously; each run lasted for the number of generations needed for the chains to converge, which means the average standard deviation of split frequencies fell below the default stop value (0.01). However, in order to avoid the ambiguous situation that suggested chain convergence when the analyses became trapped on local optima, each analysis was diagnosed with 100,000 replications with Tracer v. 1.6. (Rambaut et al. 2014) to test for effective sampling size (ESS) and convergence of parameters. If necessary, the analysis was prolonged for the next 100,000 replications. Then, the final average standard deviation of split frequencies for all parameters were verified whether they had achieved stationarity, confirmed by the value of the potential scale reduction factor PSRF+ (Gelman & Rubin 1992), which should be close to 1,000 (Ronquist et al. 2012). The starting temperature values of the heated chains were lowered from the default (0.20) to 0.10. Trees were sampled and stored every 100 generations. The burn-in percentage was set to default, discarding the first 25% of samples from the cold chain. Tree topologies, their branch-length information and posterior probabilities for nodes were gathered from all post burn-in sampled trees.

2.6.5. Tree topology and reliability of clades

While testing the hypothesis of monophyly of the "cydnoid" complex of taxa, we were aware that poorly supported clades can be unreliable due to many factors (see, for instance: Erixon et al. 2003; Huelsenbeck et al. 2002; Huelsenbeck & Rannala 2004; Zander 2001, 2004). Therefore, only clades with posterior probability values (pp) of equal to or greater than 91% (0.91–1.0) were accepted as strongly supported monophyla and regarded as taxonomically significant.

2.6.6. Tree editing

The consensus tree for each analysis was edited with Mesquite v.3.10 (Maddison & Maddison 2016); the posterior probability values for nodes were calculated using the appropriate tree files. The final trees were saved as TIF files and then prepared for publication in Adobe Photoshop Elements 10 and CorelDraw X8.

3. Results

3.1. Sequence alignments

The final 28S (D3) rDNA and 18S rDNA alignments contained 381 and 1865 sites, respectively, and 2246 sites for combined 28S+18S alignment. The number of conserved sites and variable sites was 236 and 119 for 28S alignment, 1340 and 498 for 18S alignment, and 1578 and 612 for combined 28S+18S alignment, respectively. There were 84 sites in the 28S alignment, 234 sites in the

Table 3. Data statistics for the substitution saturation analyses on separate and combined sequences.

Sequences analysed	Sites performance	*Iss* value	*Iss.c* values		*P* values	
			symmetrical topology	asymmetrical topology	symmetrical topology	asymmetrical topology
28S	on all sites	0.063	0.682	0.682	0.0000	0.0000
	on fully resolved sites only	0.063	0.682	0.682	0.0000	0.0000
18S	on all sites	0.028	0.780	0.780	0.0000	0.0000
	on fully resolved sites only	0.029	0.780	0.780	0.0000	0.0000
28S+18S	on all sites	0.034	0.789	0.789	0.0000	0.0000
	on fully resolved sites only	0.033	0.789	0.789	0.0000	0.0000

Table 4. Substitution models, number of generations needed to reach convergence, the average standard deviation of split frequency values under each substitution criterion for the combined 28S+18S rDNA dataset.

Substitution criterion	Substitution model		Number of generations	Average standard deviation
	28S	18S		
Bayesian Information Criterion (BIC)	K2+G	K2+G+I	5 100 000	0.003854
Akaike Information Criterion corrected (AICc)	GTR+G	GTR+G+I	11 400 000	0.004089
reversible jump MCMC (rjMCM)	[121341]		15 000 000	0.006506

18S alignment, and 316 sites in combined alignment that were parsimony-informative.

3.2. Analysis of the phylogenetic signal

In both analyzed genes alone, as well as their combined sequences, the observed values of *Iss* in the saturation tests (performed on all sites, and on fully resolved sites only) were smaller than the *Iss.c* values for both symmetrical and asymmetrical topologies (Table 3). However, the results indicate that the combined data were more suitable for further phylogenetic analysis than the separate data for each gene (Table 3; Fig. 3).

3.3. Substitution model comparison

When the Bayesian Information Criterion was used, the Kimura two-parameter model (Kimura 1980) was identified as the best-fitting *a priori* substitution model for both 28S (D3) rDNA and 18S rDNA sequences, with a discrete Γ-distribution of the variable sites (K2+G) for the former, and a discrete Γ-distribution of the variable sites and a proportion of invariant sites (K2+G+I) for the latter. Maximum-likelihood (ML) model search under the Akaike Information Criterion determined the General Time Reversible model with a discrete Γ-distribution of the variable sites (GTR+G) for the28S rDNA sequences, and the General Time Reversible model with a discrete Γ-distribution of the variable sites and a proportion of invariant sites (GTR+G+I) for the 18S rDNA sequences. The reversible jump MCMC procedure indicated the M_{136} model (gtrsubmodel [121341]) as the most appropriate for the combined 28S+18S rDNA alignments (pp = 0.303). The number of generations needed to reach convergence and the average standard deviation of split frequencies values under each substitution model are provided in Table 4.

3.4. Tree topology

Two analyses (i.e. under the Akaike Information Criterion, and under the reversible jump MCMC criterion) resulted in trees with almost the same topology, where differences in the clade placements and their posterior probability values were only minimal (Figs. 4, 5). However, the third tree, based on the K2+G/K2+G+I model under the Bayesian information Criterion showed only a single dissimilarity to the two aforementioned trees, i.e. the position regarding the clade including *Adrisa magna* and *Chilocoris assmuthi* / *confusus* (Fig. 6).

Importantly, all trees indicated the family Thaumastellidae as a clade outside the "cydnoid" complex, and as sister to all other Pentatomoidea (with the maximum pp = 1.0). Additionally, all analyses allied species of the subfamily Sehirinae with species of the family Parastrachiidae, and hypothesized them as a natural group, giving this clade maximum support (pp = 1.0). Conversely, the subfamily Cydninae and its tribes (the Cydnini and the Geotomini *sensu lato*) were identified in all analyses as polyphyletic. The family Thyreocoridae, including species of the subfamily Thyreocorinae and Corimelaeninae, was identified as a monophyletic taxon, but its grouping was not strongly supported (posterior probability values from 0.60 in the M_{136} model under the reversible jump MCMC criterion, to 0.88 in K2+G/K2+G+I model under Bayesian Information Criterion).

4. Discussion

4.1. Molecular phylogeny and monophyly of the "cydnoid" complex

Our molecular phylogeny does not support previous morphology-based groupings of the families (Cydnidae, Para-

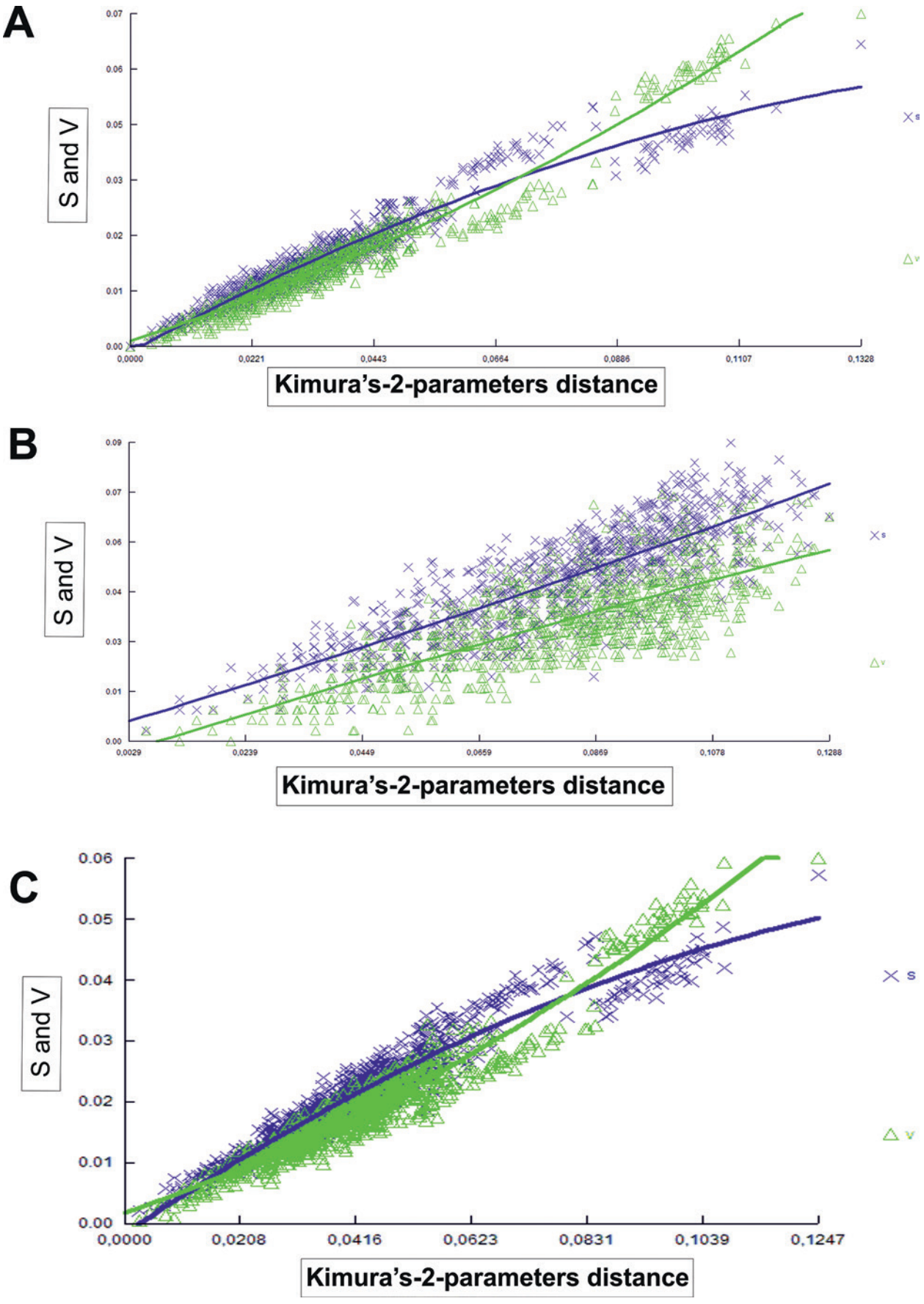

Fig. 3. Substitution patterns of 18S rDNA, 28S rDNA, and 28S + 18S rDNA sequences. The number of transitions (s) and transversions (v) was plotted against Kimura-2-parameter (K2P) distance considering all sites. **A**: 18S rDNA saturation plot. **B**: 28S rDNA saturation plot. **C**: Combined sequences saturation plot.

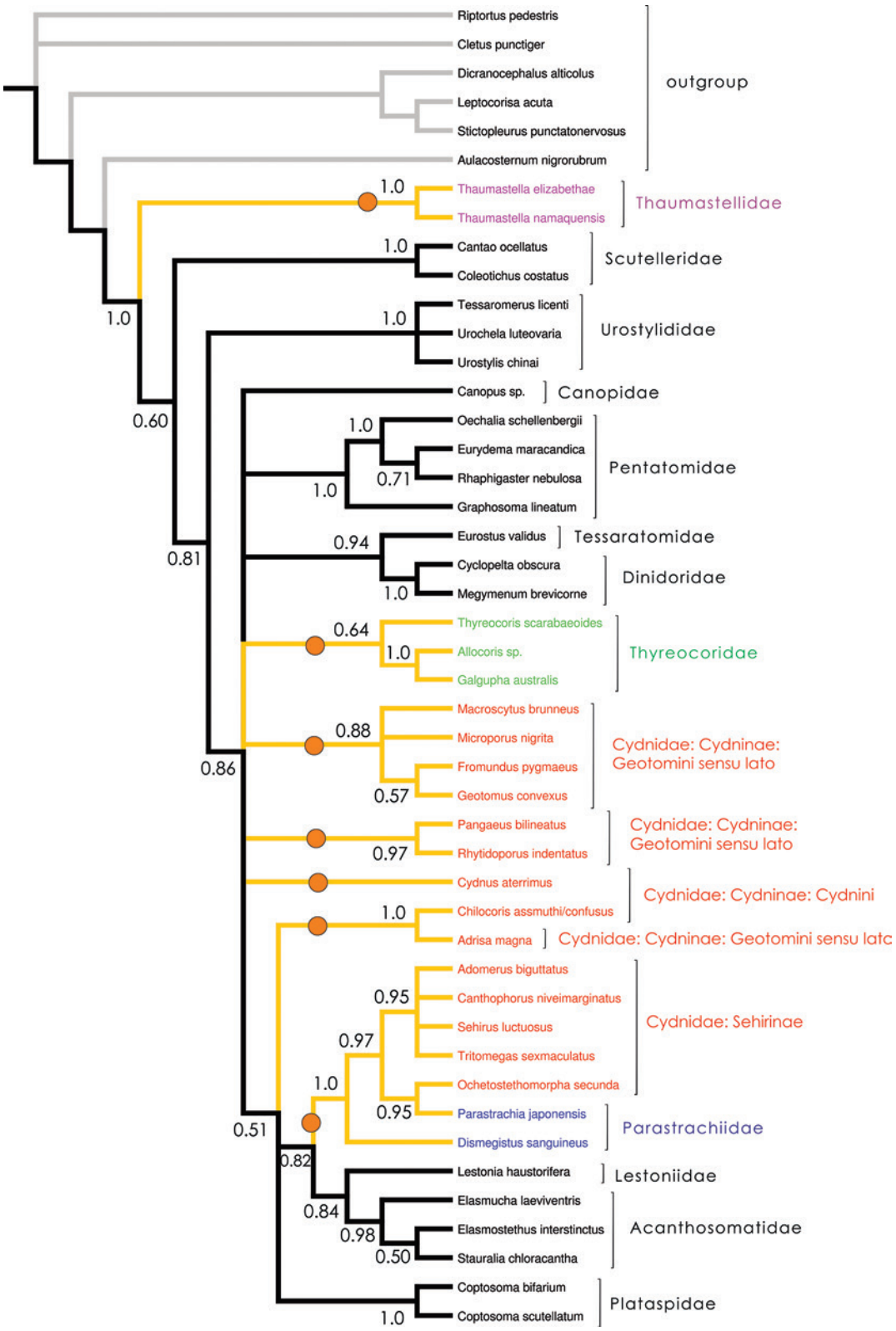

Fig. 4. Bayesian analysis tree of the combined 28S+18S rDNA dataset using the GTR+G/GTR+G+I substitution model recovered under the Akaike Information Criterion. — ***Representation***: Numbers indicate posterior probability values. Branches in grey – outgroup; branches in orange – representatives of the "cydnoid-complex"; branches in black – other Pentatomoidea. Red circles denote clades in which coxal combs are present.

strachiidae, Thaumastellidae, and Thyreocoridae), which are characterized by the presence of coxal combs (Dolling 1981; Jacobs 1986; Schaefer et al. 1988; Gapud 1991; Schuh & Slater 1995; Grazia et al. 2008; Lis 2010a; Yao et al. 2012). This attribute, regarded until now as a unique autapomorphy of the "cydnoid" com-

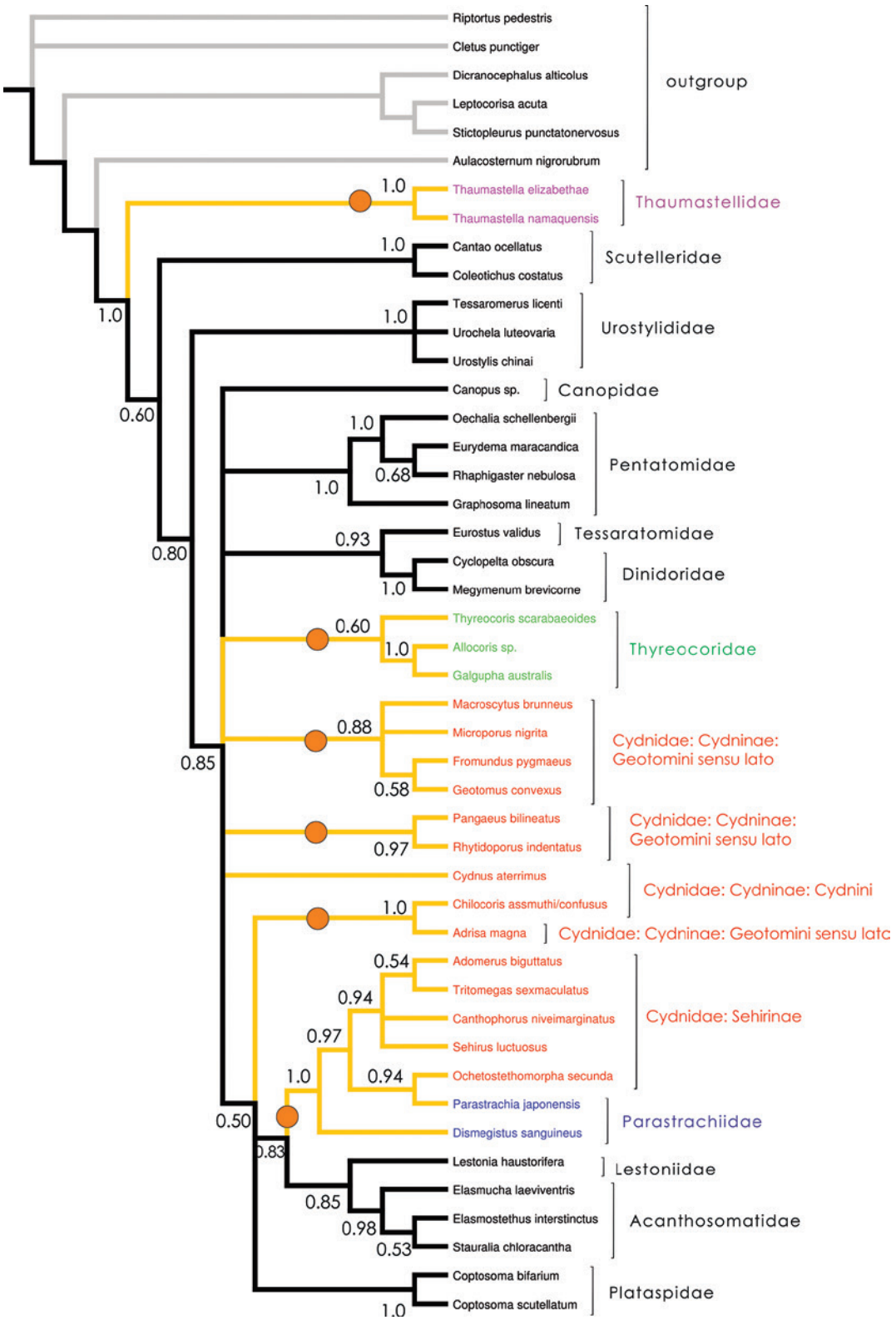

Fig. 5. Bayesian analysis tree of the combined 28S+18S rDNA dataset using the M_{136} substitution model recovered under the reversible jump MCMC criterion. — ***Representation***: as in Fig. 4.

plex of families (Grazia et al. 2008; Lis 2010a) we have now demonstrated to originate in Thaumastellidae, independently of three other "cydnoid" families (Cydnidae, Parastrachiidae, and Thyreocoridae). A similar kind of scale-like setae is also found at the apex of the first antennal segment (Jacobs 1989: fig. 13), and the apex of the first labial segment (Jacobs 1989: fig. 16), indicating that scale-like setae evolved on different body parts and making their convergent evolution on the coxae more plausible.

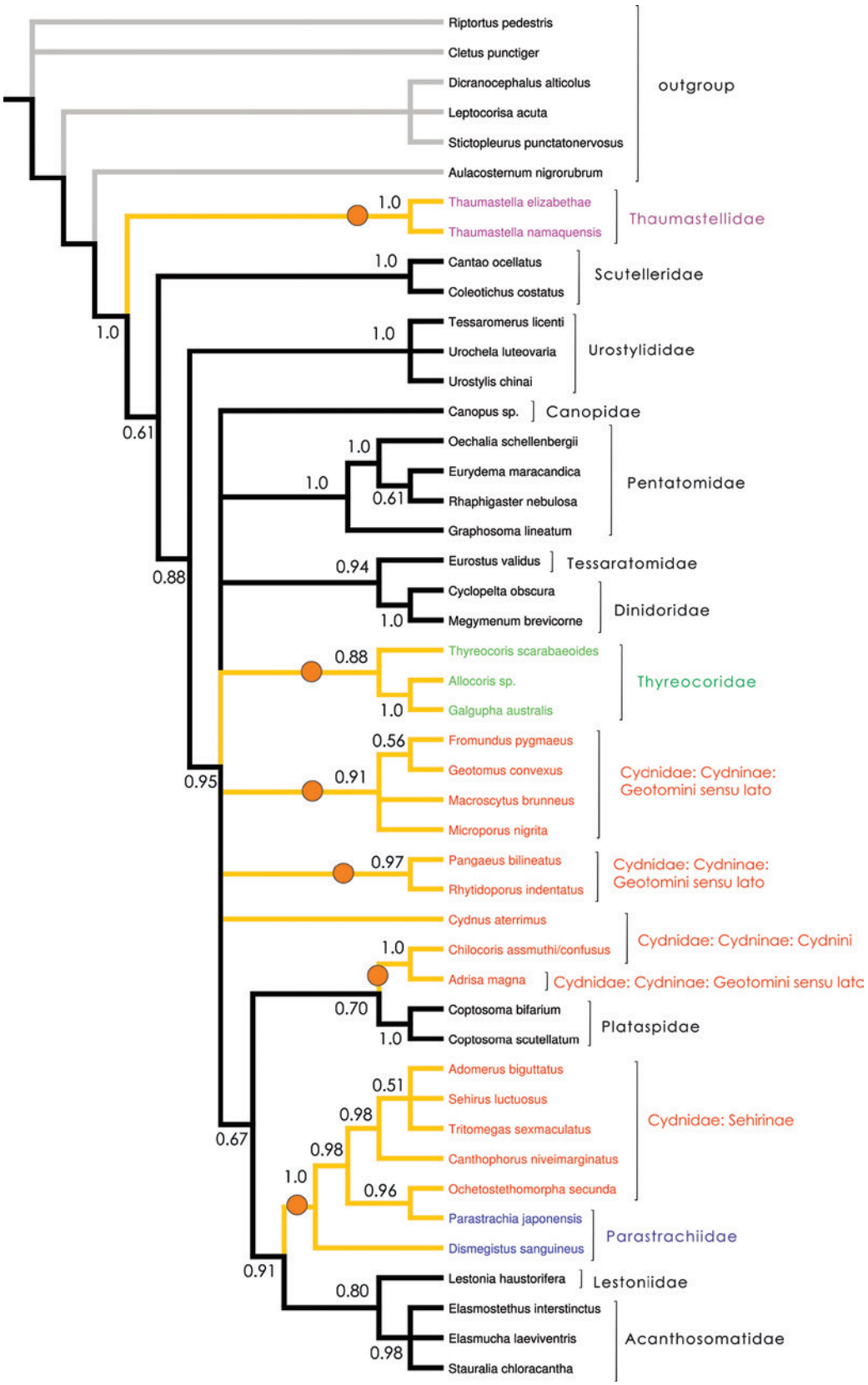

Fig. 6. Bayesian analysis tree of the combined 28S+18S rDNA dataset using the K2+G/K2+G+I substitution model recovered under the Bayesian Information Criterion. — ***Representation***: as in Fig. 4.

Therefore, we suggest their independent origin in Thaumastellidae and in all other "cydnoid" families. Within the latter, whether they evolved from the common ancestral state or originated independently is unknown. Moreover, our results indicate the polyphyly of the family Cydnidae (see in 4.4.), which may additionally sup-

port a hypothesis suggesting independent origin of the coxal combs.

4.2. Thaumastellidae, the sister clade to all other Pentatomoidea

Thaumastellidae, in a morphological sense, was almost always recognized as a taxon of considerable antiquity closely related to the Cydnidae (Schaefer & Wilcox 1971; Schaefer 1981; Henry 1997; Popov & Pinto 2000; Grazia et al. 2008; Pluot-Sigwalt & Lis 2008; Lis 2010a), or was included in the broadly conceived Cydnidae (Cydnidae *sensu lato*), usually as a subfamily (Dolling 1981; Jacobs 1989; Zrzavý 1990; Gapud 1991; Schuh & Slater 1995; Yao et al. 2012). However, Štys (1964), when proposing a new family for the genus *Thaumastella* Horváth, argued it to be an early offshoot of the main pentatomoid stock, which should therefore be regarded as a sister taxon to all remaining Pentatomoidea. Nevertheless, in the most comprehensive molecular analysis of this superfamily conducted by Grazia et al. (2008), Thaumastellidae was proposed as holding various positions within Pentatomoidea, depending on the analysis parameters, but was never identified as the taxon sister to all other pentatomoids. Conversely, our analyses indicate Thaumastellidae as the sister group to all remaining Pentatomoidea (and always with the maximum pp = 1.0) thus supporting the suggestions of Štys (1964). Importantly, the same also recently resulted from the analyses of the secondary structure of the nuclear rRNA sequences (Wu et al. 2016).

Apart from the findings of our molecular analysis, the hypothesis of the sister relationship of Thaumastellidae to all other Pentatomoidea can also be supported by the presence of an m-chromosome, unknown elsewhere in this superfamily (Ueshima 1979; Jacobs 1989), the chemical composition of the scent gland secretions which are intermediate between Lygaeoidea and Pentatomoidea (Jacobs et al. 1989), and the structure of spermatheca which in Thaumastellidae is more lygaeoid or pyrrhocoroid than pentatomoid (Pluot-Sigwalt & Lis 2008). Some other morphological characters of Thaumastellidae in their relation to other Pentatomoidea were summarized in Schuh & Slater (1995) and Grazia et al. (2008), and are not repeated here.

4.3. Monophyly of Parastrachiidae and its position within the "cydnoid" complex

Results of all preceding molecular analyses, where both genera of the family Parastrachiidae (i.e. *Parastrachia* and *Dismegistus*) were included (Grazia et al. 2008; Lis et al. 2012, 2015; Wu et al. 2016), have always defined Parastrachiidae as a well-supported monophylum, which was surprisingly not recovered in the present analyses. Inclusion of the monophyletic Parastrachiidae (as a subfamily) into the Thyreocoridae (Grazia et al. 2008; Matesco et al. 2012), was also not confirmed by our results. Unlike results of all previous molecular studies, our analyses always kept *Parastrachia* separate from *Dismegistus*, and never identified them as a clade. Importantly, *Parastrachia japonensis* is a sister taxon to *Ochetostethomorpha secunda* (Sehirinae) in all analyses (pp = 0.94–0.96), and both always form the taxonomically significant sister clade (pp = 0.97–0.98) to the monophyletic group consisting of the remaining four species of Sehirinae.

4.4. Polyphyly of Cydnidae

Our study demonstrates the polyphyly of the family Cydnidae, thus confirming previous suggestions of its non-monophyletic origin (Grazia et al. 2008; Pluot-Sigwalt & Lis 2008; Lis et al. 2011b, 2015). This may also be supported by recent findings of Hosokawa et al. (2012), who indicated the polyphyly of burrower bug gut symbionts, suggesting their multiple evolutionary origins among the Cydnidae. Apart from that, taxonomic groups of lower rank, i.e. the subfamily Cydninae (and both its tribes, Cydnini and Geotomini *sensu lato*) and the subfamily Sehirinae (including a single tribe Sehirini *sensu lato*), are consistently identified as non-monophyletic in our analyses. With respect to the tribes Geotomini *sensu lato* and Sehirini *sensu lato*, our outcome is in congruence with results based on morphological characters provided by Pluot-Sigwalt & Lis (2008). However, in the case of the tribe Cydnini, which was considered by Pluot-Sigwalt & Lis (2008) as homogeneous with regard to the spermathecal structures, our results indicate the exact opposite, suggesting a non-monophyletic origin.

4.5. Monophyly of Sehirinae

The monophyly of the subfamily Sehirinae itself was not identified in our analyses, and, because of the discrete position *of Ochetostethomorpha secunda*, our results showed this subfamily as polyphyletic. Importantly, *Parastrachia japonensis* (Parastrachiidae) was a sister taxon to *O. secunda* (Sehirinae) in all analyses (pp = 0.94–0.96), and these taxa always formed a sister clade to the monophyletic group consisting of all remaining Sehirinae (pp = 0.97–0.98). The most striking result is the position of *Dismegistus sanguineus*, which was always basal to the clade including all species of Sehirinae + *P. japonensis*. The entire group *Dismegistus* + (Sehirinae + *Parastrachia*) is the clade with the maximum posterior probability values (pp = 1.0) in all our analyses.

Our findings are supported by Grazia et al. (2008), where results of some morphological analyses associated *Sehirus cinctus* (Palisot) (Sehirinae) with two species of Parastrachiidae, and by Lis et al. (2015), where *S. luctuosus* was a part of the clade that included also *Parastrachia* and *Dismegistus*. A close relationship of *P. japonensis*

and the *Ochetostethus* species (which are allied to *Ochetostethomorpha*, the subfamily Sehirinae) was also suggested during studies on spermathecal structures (Pluot-Sigwalt & Lis 2008). Additionally, the most essential result of the preceding studies (Tachikawa & Schaefer 1985; Schaefer et al. 1988; Sweet & Schaefer 2002; Lis & Heyna 2001; Lis & Schaefer 2005; Lis 2002, 2010b; Inadomi et al. 2014) suggested *Parastrachia* and *Dismegsistus* as being related to representatives of the subfamily Sehirinae, not only in their morphological characteristics, but also in their maternal care habits.

5. Conclusions

(1) This study is by far the most comprehensive molecular phylogenetic analysis of the "cydnoid" complex of pentatomoid families (Cydnidae, Parastrachiidae, Thaumastellidae, and Thyreocoridae), which was morphologically defined as a monophylum due to the presence of coxal combs.
(2) Results of the combined 28S+18S rDNA sequences analyses question the monophyly of this group of families, and exclude the Thaumastellidae from the "cydnoid" complex with strong support in all analyses, providing evidence for the independent origin of their coxal combs.
(3) The subfamily Cydninae of Cydnidae and both its tribes (Cydnini and Geotomini *sensu lato*) were recovered as being polyphyletic.
(4) The monophyly of the family Thyreocoridae was not very highly supported. The Thyreocoridae in the broadest sense (also including Parastrachiinae) was not confirmed by our results.
(5) The monophyly of the subfamily Sehirinae was questioned; however, we emphasize the monophyly of the group including Sehirinae and Parastrachiidae, which formed a very strongly supported clade in all analyses (with the posterior probability values always equal to 1.0).
(6) Our results improve the knowledge of the "cydnoid" families' relationships. Challenging the existing classification system, the use of the name "cydnoid" complex is indicated as unwarranted, and therefore it should no longer be applied to this group of families. Moreover, the name Cydnidae *sensu lato*, assigned to the group consisting of Cydnidae, Parastrachiidae and Thyreocoridae, should also no longer be used since the group was proved to be polyphyletic and of no taxonomic significance.

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Electronic Supplement Files

at http://www.senckenberg.de/arthropod-systematics

File 1: lis&al-cydnoidcomplex-asp2017-electronicsupplement-1.pdf – Secondary structure models of 18S rRNA of *Fromundus pygmaeus* (Pentatomoidea: Cydnidae) (GenBank accession number: KJ535871), *Macroscytus brunneus* (Pentatomoidea: Cydnidae) (GenBank accession number: KY911204), and *Microporus nigrita* (Pentatomoidea: Cydnidae) (GenBank accession number: KY911205). Base pairing is indicated as follows: standard canonical pairs by lines (C-G, G-C, A-U, U-A); wobble GU pairs by dots (G·U); AG and AC pairs by open circles (A○G, A○C); other non-canonical pairs by filled circles (e.g. U●U). Construction of the secondary structure model referred to the model of 18S rRNA of *Lestonia haustorifera* China, 1955 (Wu et al. 2016). The numbering system for LVRs followed the system used by Wu et al. (2016).

File 2: lis&al-cydnoidcomplex-asp2017-electronicsupplement-2.doc – List of specimens used in the phylogenetic analyses, their geographic origin, GenBank accession numbers, Opole University sample numbers for newly sequenced species, names of the persons who provided the specimens for analyses, and the sources for the sequences downloaded from GenBank. All newly sequenced specimens were identified to species by the first author (JAL). Acronyms for the persons who collected and/or provided the specimens for analyses are as follows: AW (Andrzej Wolski), BL (Barbara Lis), EH (Ernst Heiss), JAL (Jerzy A. Lis), PL (Paweł Lis), RD (Roland Dobosz), RSZ (Richard S. Zack), SK (Shin-ichi Kudo), WU (Wolfgang Ullrich).

File 3: lis&al-cydnoidcomplex-asp2017-electronicsupplement-3.nex – The alignment file of the combined 28S+18S rDNA dataset used for phylogenetic analysis.