HOLOTHYRIDS AND TICKS: NEW INSIGHTS FROM LARVAL MORPHOLOGY AND DNA SEQUENCING, WITH THE DESCRIPTION OF A NEW SPECIES OF *DIPLOTHYRUS* (PARASITIFORMES: NEOTHYRIDAE)

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ABSTRACT — The hypothesis of a close relationship between the suborders Holothyrida and Ixodida is re-examined based on new morphological and molecular data. Description of the larva of a new species of Neothyridae (Holothyrida), the first formal description of this instar for the suborder, does not provide additional morphological data supporting the relationship. However, because the larva is holotrichous, it does allow the first direct comparison of setation patterns among immatures of all four parasitiform suborders (Holothyrida, Ixodida, Opiliocarida and Mesostigmata). Analysis of sequence data for three loci (18S rRNA, 28S rRNA, and Elongation Factor 1-α), with representation of all families of Holothyrida, provided strong evidence for (1) the monophyly of Holothyrida, (2) a close relationship between Holothyrida and Ixodida, and (3) a grouping of the "large bodied" Parasitiformes (Opilioacarida, Holothyrida, and Ixodida), with the exclusion of Mesostigmata.

KEYWORDS — Parasitiformes; Holothyrida; molecular systematics; larva

INTRODUCTION

The mite suborder Holothyrida is small, currently consisting of 13 genera arranged into 3 families, the Allothyridae, Holothyridae, and Neothyridae (Lehtinen 1995, 1999; Kontschan and Mahunka 2004), although a considerably larger species-level diversity is suspected, at least for Allothyridae (Walter and Proctor 1998; Walter 2009). The distribution of the families is non-overlapping, with Allothyridae occurring in mainland Australia, Tasmania, and New Zealand, Neothyridae in the Caribbean and Northern South America, and Holothyridae on a string of islands in the Indian and Pacific Oceans ranging from Mauritius and the Seychelles to Sri Lanka, New Guinea, New

Caledonia, and Lord Howe Island (Lehtinen 1991, 1995). Adult Holothyrida are heavily sclerotized, but known nymphs of Allothyridae show a more leathery cuticle, reminiscent of that in Argasidae (Walter 2009).

Very little is known about their biology, other than the observation that they are generally not very active, and that they appear to be scavengers rather than predators (Travé 1982; Walter and Proctor 1998). Their main claims to fame are that the group includes some of the largest mites known outside of ticks (7 mm for some Holothyridae; Lehtinen 1995), and the apocryphal tale that eating holothyrids may cause death in chickens (Hampson and Green 1892; Mègnin 1897). The latter claim

needs to be confirmed, as it seems unlikely that the defensive gland secretions of these mites have sufficient potency for such an effect.

Yet, this small and poorly studied group may hold the key to answering questions on the origin of parasitism in ticks, suborder Ixodida, one of the most important lineages of arthropod vectors. Ticks are highly specialized parasites of terrestrial vertebrates, especially mammals, but also birds, squamates, and turtles. A few instars in a few species (e.g., males in some *Ixodes*, adults in *Carios* "Antricola group") are non-feeding, but all other instars feed exclusively on blood, disallowing inferences on the origin of blood-feeding based on within-group studies. The current study aims at clarifying sistergroup relationships of ticks, thus hopefully providing additional insights in the ecology of "proto-ticks".

The primary question in this context concerns relationships among the four suborders of Parasitiformes *sensu* Lindquist *et al.* (2009) (= Anactinotrichida sensu Van der Hammen (1968)): Opilioacarida, Holothyrida, Ixodida, and Mesostigmata. However, this is no easy task. The extreme specialization of many tick body parts, especially the mouthparts, and the generally high degree of divergence of body parts in Opilioacarida, make establishing hypotheses of homology with similar structures in other mite taxa quite difficult. As a result, overall progress based on morphological data has been slow, a fact reflected in the diversity of hypotheses concerning their relationships (Figures 1a-c). Holothyrida have been presented as the sistergroup to Opilioacarida (Baker and Wharton 1952), to Mesostigmata (Norton *et al.* 1993), and to Ixodida (Lehtinen 1991). Of these three hypotheses, Lehtinen's is the only one based on a phylogenetic analysis. Building on observations by Van der Hammen (1983), Lehtinen proposed a sistergroup relationship between Holothyrida and Ixodida based on two unique character states: the shared presence of a Haller's organ, and of muscle attachments on a subcapitular apodeme. The first of these may be more general, as Opilioacaridae and Ricinulei share the presence of a sensillum in a pit on tarsus I with Holothyrida and Ixodida. However, detailed

analyses of tarsus I sensillar patterns across Parasitiformes support the Lehtinen hypothesis (Moraza 2005). Lehtinen also discussed relationships within Holothyrida, hypothesizing that Neothyridae plus Holothyridae formed the sistergroup to Allothyridae (Lehtinen 1991).

Clearly new data sets are needed to properly address these questions. In this study, new information from two unrelated data sets will be explored: immatures, specifically larvae, and DNA sequence data. While homologies among adult Parasitiformes are very difficult to establish, larvae may allow more extensive comparisons, e.g. for palpal, leg, and idiosomal setation patterns (Klompen *et al.* 1997; Klompen 2000b). Setal patterns for this instar are often holotrichous, in other words, all setae can be individually designated. Patterns in post-larval instars, especially in adults, are usually hypertrichous, with numerous setae obscuring any pattern and disallowing individual setal designations. Detailed descriptions of larvae of Mesostigmata, Ixodida, and Opilioacarida have been published, but data on holothyrid larvae have been lacking. Some data on larval Allothyridae have been published (Klompen 1992; Moraza 2005) but without a complete description. Collection of the larva of a new species of *Diplothyrus* (Neothyridae) allows a full description of a larval holothyrid, and thus more extensive comparisons among larvae in all parasitiform suborders.

Second, molecular data, specifically DNA sequence data, are slowly becoming available. Results of published molecular phylogenetic studies have generally been consistent with Lehtinen's hypothesis (Dobson and Barker 1999; Klompen *et al.* 2000; Murrell *et al.* 2005; Klompen *et al.* 2007), but sampling of Holothyrida in these studies has been weak, with one or two exemplars, all of the family Allothyridae. These molecular data did generate a new hypothesis (Figure 1d) suggesting that Opilioacarida might be the sistergroup to a lineage of Holothyrida and Ixodida (Murrell *et al.* 2005).

The goal of the current study is to re-examine Lehtinen's hypothesis of a sistergroup relationship between Holothyrida and Ixodida using (1) morphological data from larvae in all suborders, and

(2) a phylogenetic analysis of sequence data from three different loci (18S rRNA, 28S rRNA, and Elongation Factor 1-α) including representatives of all three holothyrid families. The newly discovered neothyrid larva belongs to a new species, and is described for the larva and adults.

MATERIALS AND METHODS

Morphology. Initial imaging was based on whole organisms: adults using AutoMontage software (Syncroscopy, Frederick, MD) on a dissecting microscope (in alcohol), immatures in lactic acid in cavity slides using a Zeiss Axioskop® compound microscope. The larval and female specimens were cleared, dissected (in the case of females) and slide-mounted. Pencil drawings were prepared using a drawing tube attached to the compound microscope. Resulting images were scanned and redrawn in Adobe Illustrator® (Adobe Systems Inc., San Jose). Palpal chaetotaxy follows Evans (1963b), leg chaetotaxy Evans (1963a, 1969), and idiosomal chaetotaxy Lindquist and Evans (1965).

Molecular data

Taxon selection. Holothyrida is represented by 6 exemplars (2 Allothyridae, 3 Neothyridae, and 1 Holothyridae), Ixodida by 7 Ixodidae and 5 Argasidae (including representatives of all major lineages), Opilioacaridae by 3 exemplars from 3 different genera, and Mesostigmata by 7 exemplars, representing Trigynaspida (2), Uropodina s. l. (2), Sejina s. l. (1) and Gamasina s. l. (2) (following results of Klompen et al. 2007).

Marker selection. Criteria for marker selection were: (a) easy amplification and well established protocols; (b) informative at this taxonomic level (mitochondrial markers generally evolve too fast). Markers selected included 18S rRNA ($\approx$1800 bp), the most commonly used marker at this taxonomic level (Black et al. 1997; Dobson and Barker 1999; Klompen et al. 2007), two segments of 28S rRNA representing the D3-D5 and D9-D10 variable regions (total $\approx$1200 bp) (Klompen et al. 2007), and partial sequences of Elongation factor 1-α (EF-1α; $\approx$1100bp) (Klompen 2000a; Lekveishvili and Klompen 2004). 18S sequences are available for all of
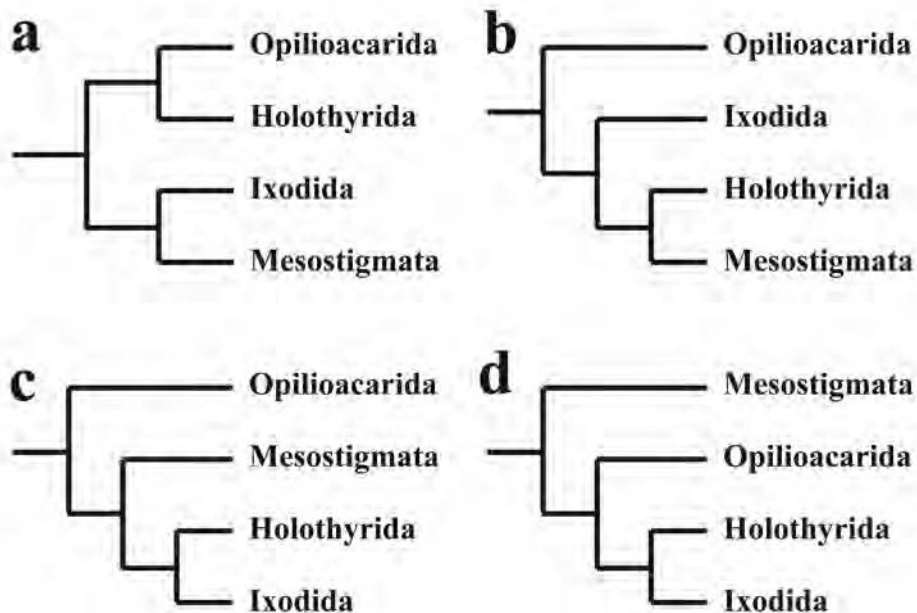

FIGURE 1: Previous hypotheses of subordinal relationships in the Parasitiformes. (a) – Baker and Wharton (1952); (b) – Norton et al. (1993); (c) – Lehtinen (1991); (d) – Murrell et al. (2005)

these taxa, but the data set is far from complete for the two other loci (28S, EF-1α). They are still included to allow a preliminary test of whether results from other loci are compatible with those for 18S. The total number of ingroup taxa is 28. A single acariform mite, *Terpnacarus cf. gibbosus* (Terpnacaridae), was used as outgroup.

Extraction, amplification, and sequencing, followed procedures listed in Lekveishvili and Klompen (2004) and Klompen *et al.* (2007). Alignment for EF-1α is relatively straightforward, although the segment considered included an intron of variable length. The latter was excluded from the analyses. Alignment for the rRNA segments was adjusted by hand, following procedures outlined in Klompen *et al.* (2007). Some regions are too variable for this approach, and are excluded. Regions excluded are: for 18S, the core of terminal loop H184b (part of variable region V2) and for 28S, two sections of variable region D3 (terminal loops of stems D3-1 and D3-2) and a section of variable region D10 (terminal loop of stem D10b) (total of 288 positions). Of the remaining 4960 positions, 1114 are parsimony informative. The aligned matrix has been deposited at Treebase (study accession number S2599; matrix accession number M4965).

Vouchering. When dealing with very large mites (e.g., Holothyrida, Ixodida), extractions are based on parts of a single specimen, using the remainder as a primary voucher. However, for the smaller Mesostigmata and Opilioacarida, the entire individual was used. In those cases, the remains of the specimens used in the actual extraction (if successfully recovered) are designated "primary" vouchers (Johnson *et al.* 2001). A second set of vouchers ("secondary" vouchers) was drawn from the same series of specimens from which material to be extracted was selected (Klompen *et al.* 2007) (all specimen series are drawn from single collections). A combination of primary and secondary vouchers is used for final identification. Voucher numbers for new extractions are listed with the collection data in Table 1.

Analysis. Data were analyzed using both parsimony and Bayesian inference. Parsimony analyses were conducted in PAUP*4.0, using heuristic searches with 100 random taxon addition sequences to avoid local optima. Lineage support is measured by calculating Bremer Support (BS) (Bremer 1988) and by jackknife (JS) analysis (Lanyon 1985). Jackknife analyses were executed using the settings: 37% deletion, emulate "JAC" resampling, 1,000 replications, "random addition sequences"= 1, and "hold trees"= 2 (Freudenstein *et al.* 2004).

For Bayesian analyses, a general time reversible (GTR) model of nucleotide substitution with a proportion of invariant sites (I) and a gamma distribution (G) of among-site rate heterogeneity was selected. This model provided the best fit as judged by both the Akaike (AIC) and Bayesian (BIC) Information Criteria as implemented in Modeltest 3.7 (Posada and Crandall 1998). The GTR+I+G model was implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with the command nst=6 rates=invgamma. The number of categories used to approximate the gamma distribution was set at 4. Average percentage of invariant sites was 30%, and the average gamma value 0.50. Four Markov chains were run for 1,000,000 generations. Stabilization of model parameters (burn-in) occurred around 25,000 generations. Every 10.000th tree after stabilization (burn-in) was sampled to calculate a majority consensus tree. Values on this tree are interpreted as the posterior probability values (PB) of the nodes.

Following Mallatt *et al.* (2004) posterior probabilities (PB) equal or over 95% in Bayesian trees, and jackknife values equal or over 60%, are interpreted as significant support.

DESCRIPTION

Diplothyrus lecorrei Klompen

(Figures 2-6)

Diagnosis

The new species shares with the type species of the genus *Diplothyrus*, *D. schubarti* Lehtinen, the presence of what appear to be 2 pairs of dorsolateral orifices (Thon's organ system) connected by a distinct strip of cuticle, an epiandrum that is hardly

TABLE 1: Taxa examined, voucher- and GenBank accession numbers.

Taxon name	Voucher number (OSAL) (a)	18S	28S D3-D5	28S D9-D10	EF-1α
Terpnacarus nr. *gibbosus*		AY620904*	AY626622*	AY626585*	AF256521*
Neocarus texanus		AF124935*	AF124963*	AF120302*	AF240849
Caribeacarus armasi	070463	GU392113	---	---	---
Opilioacaridae, Australia		AF287235*	---	---	---
Allothyrus australasiae gr.		AY620910*	AY626628*	AY626589*	---
Allothyrus constrictus gr.	004761	AY620911*	AY626629*	AY626590*	GU392107
Sternothyrus braueri	070487	AY620912	---	AY626591	GU392108
Neothyridae, Venezuela	000284	GU392114	---	---	---
Neothyridae, Panama	070464	GU392115	GU392118	GU392120	GU392109
Diplothyrus lecorrei	084990	GU392116	---	---	---
Argas persicus		L76353*	---	AF289194*	---
Argas lahorensis		L76354*	---	---	---
Carios puertoricensis		L76357*	---	---	---
Ornithodoros turicata		AF213370*	---	AF120298*	---
Otobius megnini		L76356*	---	AF120297*	---
Ixodes affinis		L76350*	---	AF120288*	CD052493*(b)
Ixodes kopsteini		L76352*	---	AF120293*	---
Ixodes tasmani		AF115368*	---	AF120295*	---
Ixodes uriae	048647	AF115369*	---	AF120296*	GU392110
Amblyomma americanum		AF291874*	AF291874*	AF291874*	AF240836*(c)
Dermacentor andersoni		L76340*	---	AF120311*	---
Haemaphysalis inermis		L76338*	---	AF120309*	---
Euzercon latus		AY620915*	---	AY626596*	AY624008*
Megisthanus floridanus		L76341*	AY626635*	AF120300*	AY624009*
Microgynium incisum		AY620919*	AY626638*	AY626600*	---
Polyaspis lamellipes	044488	AY620923	AY626642*	AY626604*	GU392111
Sejus carolinensis		AY665724*	---	DQ144652*	AF240856
Epicrius mollis.	000458	GU392117	GU392119	---	GU392112
Gamasiphis pulchellus		AF115374*	AY626656*	AL120301*	---

* sequence drawn from GenBank; --- no sequence available

(a): numbers refer to new sequences only; (b): sequence of *Ixodes scapularis*; (c): sequence of *Amblyomma* sp.

depressed, and a palp tibial comb consisting of 5-6 thick setae. The species differs from *D. schubarti* primarily by the absence of a modified seta on the palp genu, and by a different structure of Thon's organ. In *D. schubarti* both orifices of Thon's organ are positioned at equal distance to the dorsal shield margin, in *D. lecorrei* the posterior orifice is positioned substantially closer to that margin, and the connecting strip in fact ends at the margin of the dorsal shield.

Larva

Chelicera (Figure 2a) poorly developed: tips of both fixed and movable digit with a broad mass of small cuticular outgrowths, but without distinct teeth. Dorsal seta and one lyrifissure present, second lyrifissure not observed.

Palp (Figure 2b) relatively well developed, with 5 distinct segments, although tibia and tarsus appear immovably attached. Trochanter without setae, femur with 4 (*al*, *pd1*, *pd2*, *pl*), genu with 5 (*al*, *ad1*, *ad2*, *pd1*, *pl*), tibia with 9, and tarsus with 14 sensilla ("sensilla" as used here includes mechanoreceptors (setae s.s.) as well as chemoreceptors etc.). Three tarsal sensilla terminating in a small round structure (Figure 2c). Femoral setae *pd1* and *pd2*, genual setae *ad1* and *pd1*, and 1 tarsal seta barbed, all other palpal sensilla smooth, setiform. Pretarsus/apotele 3-tined.

Subcapitulum (Figure 2d) with 2 pairs of hypostomal setae and small horn-like corniculi (inserted dorsally). Lateral lips poorly developed, labrum not
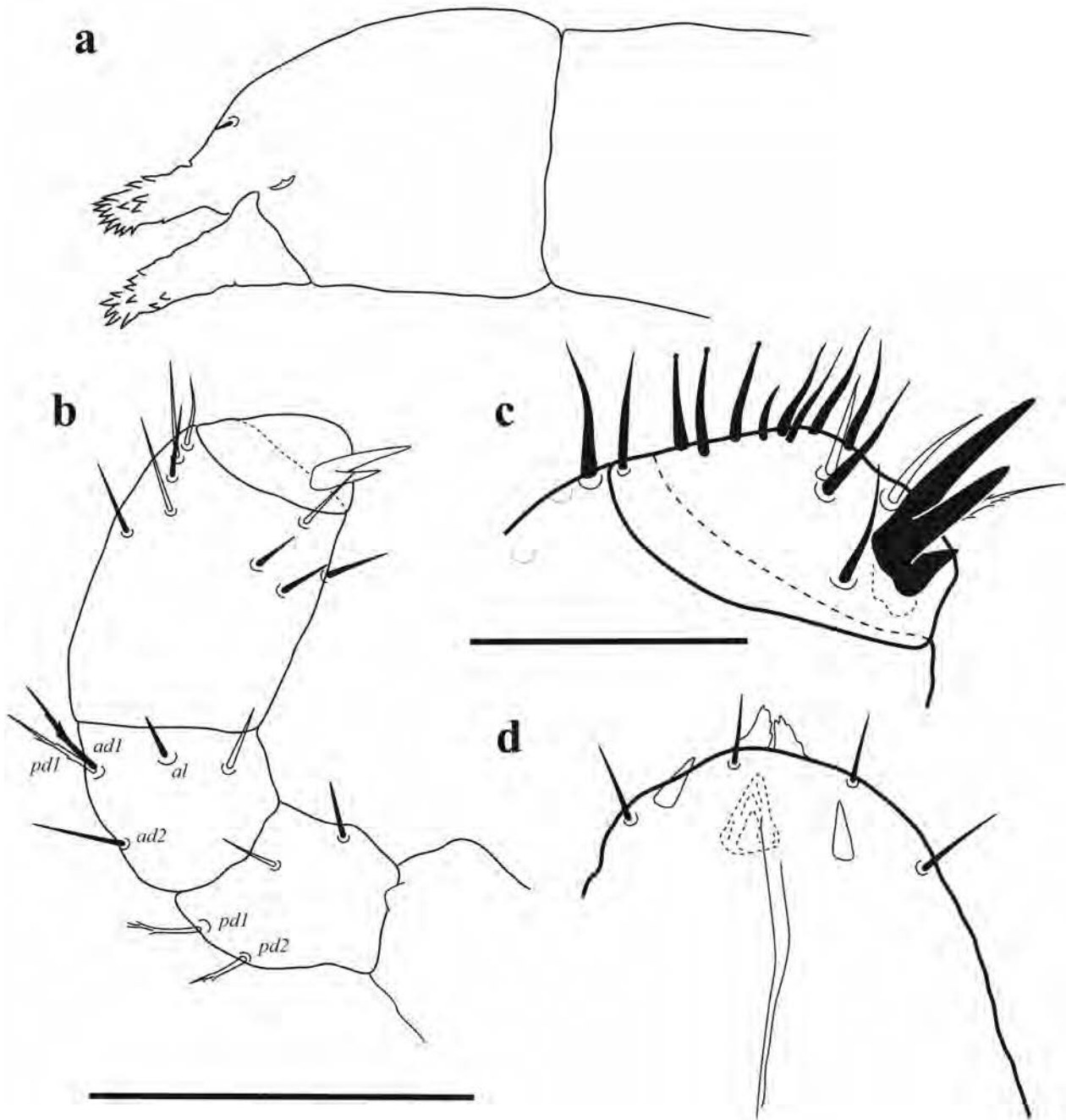
FIGURE 2: *Diplothyrus lecorrei* n. sp. Larva, gnathosoma. (a) – chelicera; (b) – palp, anterolateral view; (c) – palp tarsus, detail, anterolateral view; (d) – subcapitulum. Scale for a, b, d identical, scale bar = 100 μm; scale bar for c = 50 μm.

observed. Deutosternal groove very weakly developed. Based on the structure of the subcapitulum and chelicera it seems unlikely that this instar feeds.

Idiosoma (Figure 3). Length 377 μm, width 339 μm (measurements based on a cleared specimen in a cavity slide). Dorsal shield(s) very weakly developed, margins unclear. With very distinct muscle scars mid-dorsal, and distinct shieldlets lateral (see Figure 3a). Dorsal setation pattern slightly hypotri-
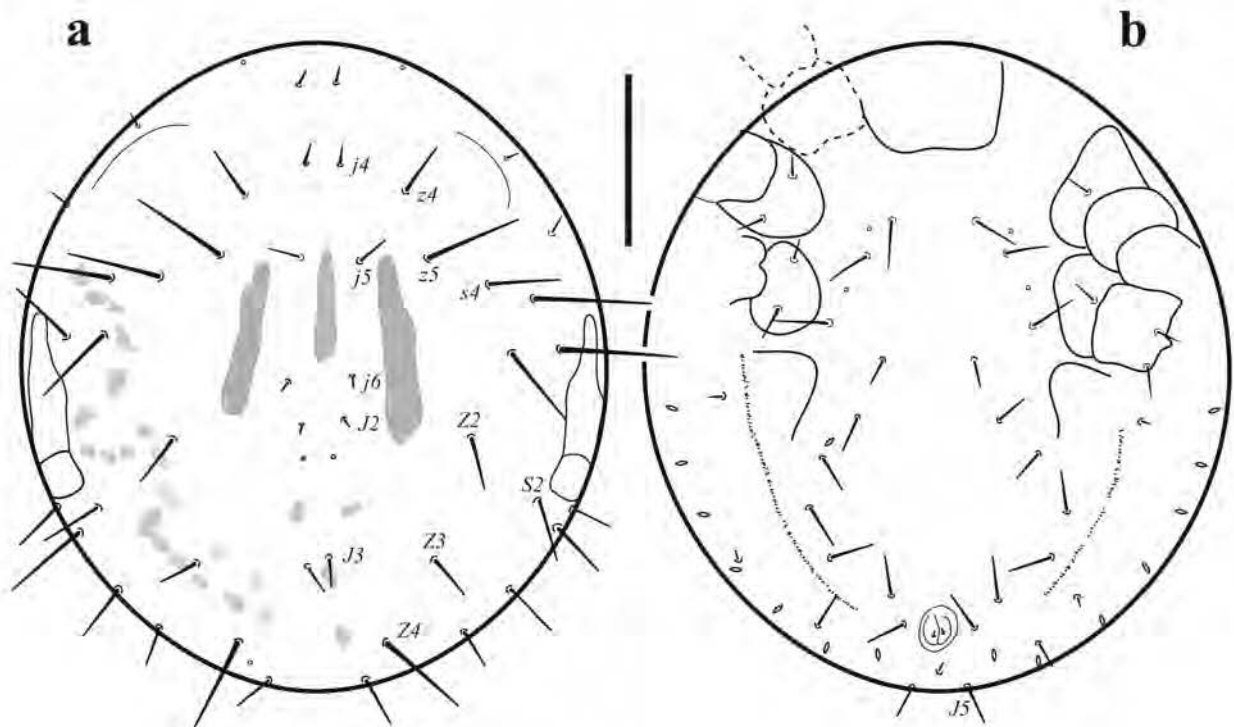
FIGURE 3: *Diplothyrus lecorrei* n. sp. Larva, idiosoma, reconstructed. (a) – dorsal view; (b) – ventral view. Scale bar = 100 μm.

chous. Anterior half of the dorsum with 12 pairs of setae, posterior half with another 12 pairs. Setal length varies from 70 ($z5$) to 7 ($J2$) μm. Venter with distinct remnants of legs IV (as in larval Opilioacaridae and Allothyridae). Sternal region without obvious shields, with 3 pairs of sternal setae and 2 pairs of pores (exact nature unclear). Opisthogaster with a distinct pattern of cuticular modifications forming a V-shaped pattern originating lateral to legs IV and joining around the anus. Lateral to this "V" a series of lyrifissures. Anal plates with a single small seta each. Anus flanked by a pair of paranal setae (at mid-level of anal plates). A small, unpaired postanal seta present. Cribrum not observed. With a variety of setae both median and lateral to the "V". Tentatively all median setae are considered part of the *Jv* and *Zv* series, all lateral ones part of the *Sv* series.

Legs (Figure 4). Length: 360, 300, and 310 μm, respectively. Leg I with an indistinct acrotarsus, and an indistinct basifemur; legs II-III with well-developed basitarsi and indistinct basifemora. Se-

tation (legs I-III by segment): trochanters: 4, 5, 4; femora: 9 (2 2/1 2/0 2), 7 (1 2/1 2/0 1), 6 (1 1/2 1/1 0); genua: 8 (1 2/1 2/1 1), 8 (1 2/1 2/1 1), 8 (1 2/1 2/1 1); tibiae: 7 (1 1/1 2/1 1), 7 (1 1/1 2/1 1), 7 (1 1/1 2/1 1). Tarsus I: telotarsus: 19; acrotarsus: 16 "normal" and 5 modified sensory sensilla (Figure 4b). Pretarsus: 0 setae. Tarsi II-III: basitarsi: 6 (1 2/0 2/0 1), 6 (1 2/0 2/0 1); telotarsi: 14 (2 2/3 2/3 2), 14 (2 2/3 2/3 2); pretarsi: 2. With the exception of some sensory setae on acrotarsus I, all leg setae are thin and setiform. All segments for which the chaetotaxy could be scored with whorls of 6 setae.

Nymphs

The collection included two nymphs of quite different size. Their overall color, as for the larva, is whitish. Unlike nymphal Allothyridae (Walter 2009), these nymphs have distinct dorsal and ventral shields, but these do not cover the entire idiosoma. On the dorsum they carry a narrow antero-marginal shield, an extensive dorsal shield (more similar to the dorsal shield in larval Argasidae than to dorsal shields in any other parasitiform taxon),
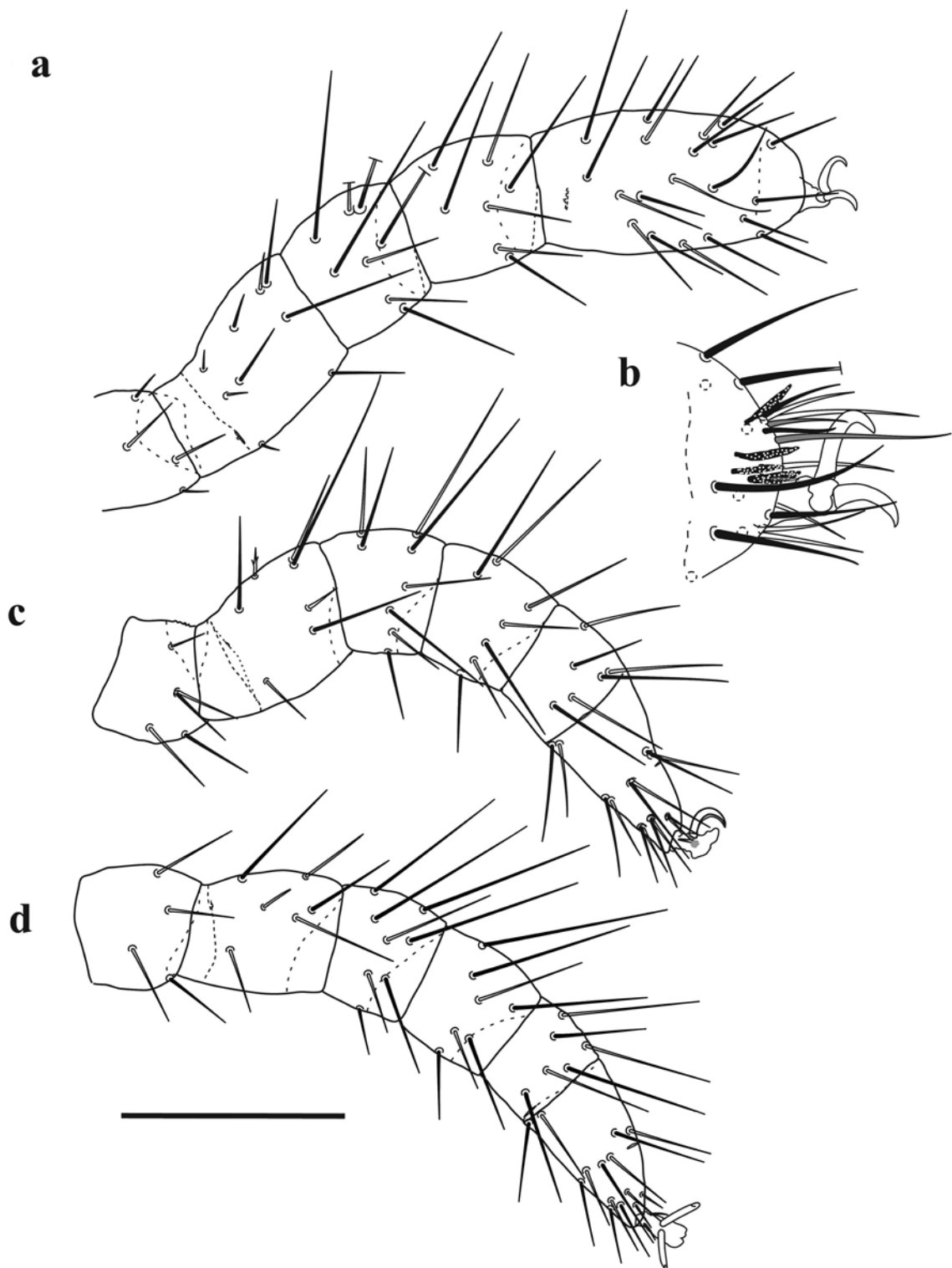
FIGURE 4: *Diplothyrus lecorrei* n. sp. Larva, legs. (a) – leg I; (b) – detail tarsus I; (c) – leg II; (d) – leg III. Scale bar for a, c, d = 100 μm.

and a pair of small, lateral shieldlets with mammillate patterning, similar in position to the shieldlets of the larva or the mammillate zones near Thon's organ of the adults. Both shields and soft cuticle carry numerous medium long setae. Venter with a small sternal shield adjoining a large expanded ventral shield. Anus not incorporated into the ventral shield. Thon's organ(s) present in same position and general shape as in adult (closely associated with posterior edge of shieldlets).

Adults

Gnathosoma. Observations and measurements based on a single dissected female.

Chelicera (Figure 5a) well-developed. They appear to consist of 4 parts, movable and fixed digit, and 2 basal segments. Total length 1410 μm; basal-1 415 μm, basal-2 500 μm, fixed digit 495 μm, movable digit 260 μm. Movable digit with two large, and numerous very small, teeth; fixed digit with a single, median large tooth, a subterminal spine-like structure, and numerous small teeth in between (latter as for movable digit). With a single dorsal seta (17 μm) on the fixed digit, no other cheliceral setae observed. One lyrifissure (*id*?) dorsal near the base of the fixed digit, lyrifissure *iα* not observed. Chelicera with a single, complex branched outgrowth (120 μm), inserted antiaxial on the fixed digit at the articulation with the movable digit. Basal segments without setae. The fixed digit includes what appears to be the next instars' chelicera, suggesting adults in this species molt. The possibility that the most basal segment may represent the basal segment of the "new" chelicera (squeezed out during slide mounting) cannot be ruled out completely.

Palps (Figure 5b). Total length 1290 μm, individual segments 180, 410, 250, 390, and 70 μm, respectively. Trochanter with 2 spinose, pointed setae, one lightly barbed. Femur with 15-16 setae; *av* and 3-4 *al* setae spinose finely barbed; distal *av* seta with a blunt tip, all others pointed. Genu with 17-18 setae, *av* and 1 *al* seta spinose, finely barbed; *av* seta with blunt tip and dense, fine barbs. Long specialized seta listed by Lehtinen (1999) not observed. Tibia with approximately 70 setae; 5 *av* and 1 *al* setae spinose with blunt tips and dense, fine barbs (the *av* setae make up the "palpal comb"); 5-6 other ven-

tral (*v*) setae strong, smooth, pointed spines. Tarsus distinct, but with limited independent movement; with numerous sensilla (most not figured), including a long sensillum with a expanded tip (figured). Pretarsus/apotele well-developed, 3-tined.

Subcapitulum (Figure 5c). Width 1030 μm, height (hypostome to base) 630 μm. Deutosternum poorly developed, with no visible teeth. Cuticular patterning limited to mammillate zones on each side of the deutosternum. Subcapitulum with about 9 relatively short setae, hypostomal lobes each with 3 longer (160-190 μm) setae. Cornicula (140 μm) horn-like, inserted dorsally. Lateral lips well developed, with numerous small, short projections; labrum appears poorly developed (but could be damaged in the dissected specimen). Gnathotectum very poorly developed or absent (unclear in dissected specimen).

Idiosoma. Generally well-sclerotized, light brown in color. Length (including gnathosoma) and width in female 2060 x 1530 μm, in male 1980 x 1520 μm, suggesting no major size difference between the sexes.

Dorsum. Highly domed, with numerous relatively long (100-120 μm) setae. Almost completely encased in well sclerotized shields. Dorsal shield cuticle with light reticulate patterning interrupted by numerous shallow, round indentations. Lateral, especially between peritremes and Thon's organ, with patches of mammillate cuticle (Figure 6c, d) in the same position as the lateral shieldlets in the immatures. Peritremes well-developed, at lateral edge of dorsal shield; stigmata at level of coxae III; peritremes extending anterior from stigma beyond coxa I and posteriorly to the posterior edge of coxae IV. Two pairs of orifices dorsolateral on the shield, connected by a distinct cuticular strip running postero-ventral towards the edge of the shield (Figures 6c, d). The more posteroventral of the two orifices is probably the true Thon's organ. It is connected internally by a membranous funnel to grape-like structures, strongly resembling the structure of Thon's organ in *Sternothyrus braueri* (Thon) (Travé 1983). The nature of the second (more anterodorsal) structure in unknown. It shows a small dorsal rim, but no distinct internal structures.
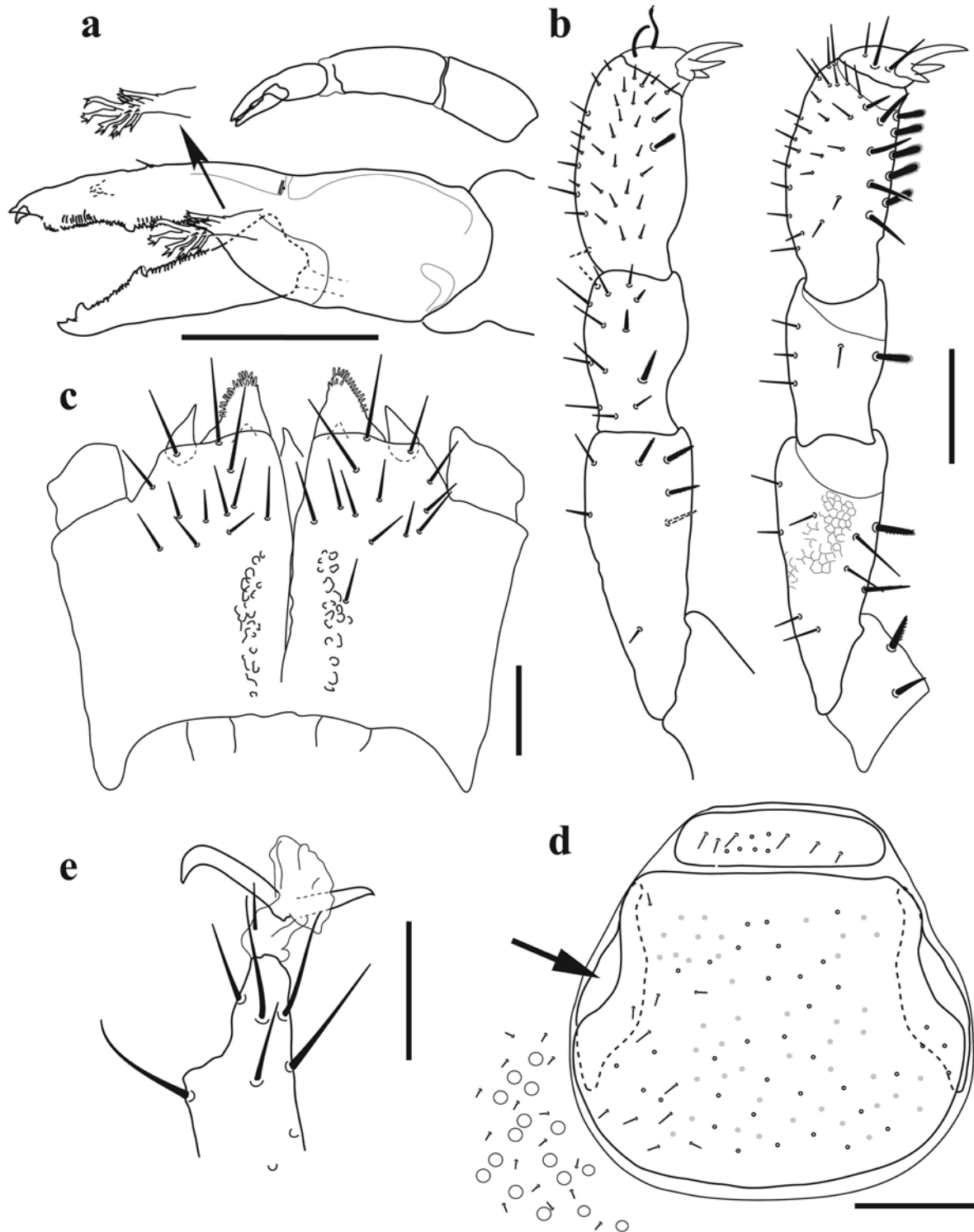
FIGURE 5: *Diplothyrus lecorrei* n. sp. Female. (a) – chelicera (details of whole chelicera and membranous outgrowth); (b) – palp, antero-lateral (left) and posterolateral (right) views (setae drawn in dashed lines not consistently present); (c) – subcapitulum; (d) – genital area (arrow indicates presumed latigynal shield), open circles: setal bases, filled grey circles: pores; (e) – pretarsus II. Scale bars = 200 μm.
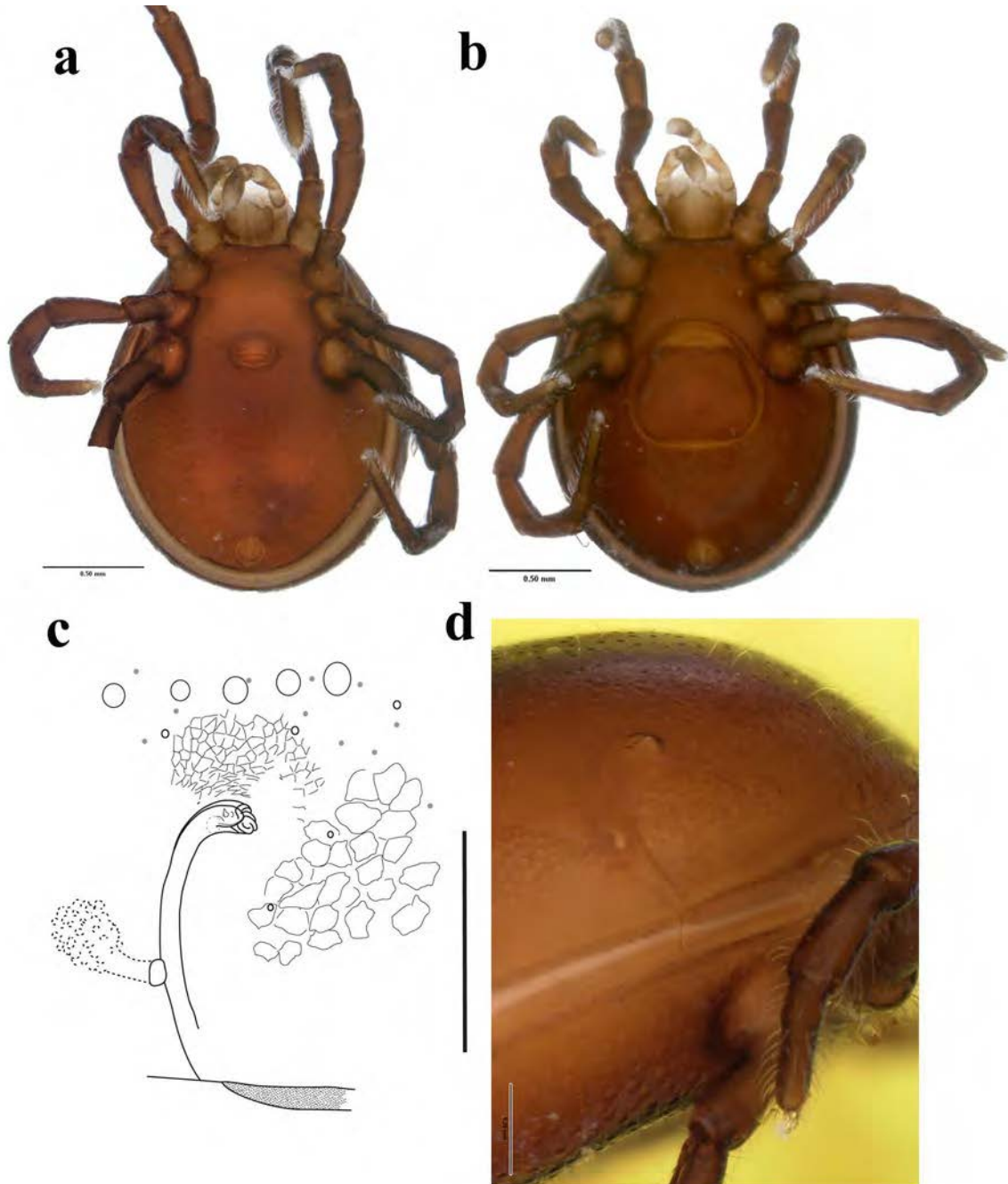
FIGURE 6: *Diplothyrus lecorrei* n. sp. Adult, idiosoma. (a) – male, ventral view; (b) – female, ventral view; (c) – male, Thon's organ; (d) – female, Thon's organ, detail view. Scale bars for a, b = 500 μm; scale bars for c, d = 200 μm.

Venter (Figures 6a, b) almost completely covered by a holoventral shield; ventral shield adjoining, but not connected to, dorsal shield. Shield surface finely granulate; with some indistinct reticulate patterning in posterior sternal region, and the round indentations noted for the dorsum. Otherwise without patterning. Sternal lyrifissures (if present) not observed. Entire ventral shield with many short setae. Anus relatively small (145 x 140 μm in female; 130 x 125 μm in male), with 3-4 setae on each valve. Female genital region (Figures 5d, 6b) with a very well developed mesogynal (480 x 630 μm), and a much smaller sternogynal shield. Possible remnants of latigynal shields (arrow) small, largely obscured by expanded mesogynal shield. Genital shields with numerous small setae and small pores (grey circles in Figure 5d), cuticle finely granulate, without distinct patterning of any kind. Male genital region (Figure 6a) consisting of two subequal sized plates (130 x 200 μm) positioned between coxae IV. Anterior plate with numerous setae, posterior one nude or with very few setae. Epiandrum indistinct.

Legs. Length legs I-IV (female): 1800, 1400, 1440, and 2190 μm. All femora with a distinct basifemur. Tarsus I without acro- or basitarsus; tarsi II-IV with distinct basitarsi, each with 2-3 whorls of setae. Leg setation on all segments based on whorls of 8 or 9 setae each. Tarsus I with dense cluster of sensilla set in depression, but without internalized sensilla (as in the capsule of the tick Haller's organ). Pretarsus I with almost sessile claws; pretarsi II-IV (Figure 5e) with an ambulacral stalk carrying 1 pair of small setae, well developed claws, and a large empodium.

Deposition types

The holotype female (OSAL106890-106894; 5 slides, specimen dissected), and paratypes (1 male (OSAL84992), 2 nymphs (OSAL84991, 84990), 1 larva (OSAL106889) deposited at Ohio State University Acarology Laboratory (OSAL), Columbus, U.S.A.

Collection information

French Guyana: Kaw Mountain, Amazone Nature Lodge, "monkey site", elev. 306 m, 04°33′27"N 052°12′29"W, collector Klompen, H., 2 Aug 2008, ex litter in forest, moderately wet, coll. no. AL013589 (holotype female, nymphs and larva). Same general area, "buttress site", elev. 299 m, 04°33′46"N 052°12′13"W, coll. Klompen, H., 5 Aug 2008, ex litter near buttress tree, AL013606 (male)

Etymology

This species is named in honor of Fréderic LeCorre, our host at Amazone Nature Lodge, for his strong support during our stay.

Phylogenetic analysis

Nucleotide frequencies for the combined matrix averaged A 22.1%, C 27.6%, G 23.5%, T 26.8%. There is no indication of substantial overall bias in nucleotide frequencies (P= 0.00 in χ^2 test of homogeneity across all taxa; df= 84). Distinct nucleotide bias was also absent in matrices for individual loci, although the EF-1α matrix did show relative low frequencies of A (17%) and relatively high ones for G (37%).

Parsimony analyses [excluding uninformative characters] of the combined data generated a single most parsimonious tree (length 4099, CI= 0.50, RI= 0.52) (Figure 7). Results for Bayesian analyses were very similar, and can be discussed simultaneously. The only differences in topology concerned a grouping of *Carios* and *Ornithodoros* (to the exclusion of *Otobius*), and *Epicrius* and *Gamasiphis* (to the exclusion of *Sejus*) in the Bayesian analyses. Any set of relationships among groups of these taxa is poorly resolved and supported, and these differences can be ignored. Both analyses (parsimony and Bayesian) provided strong support for monophyly of all 4 suborders of Parasitiformes. Support for Ixodida was weakest (BS= 4; JS= 87%; PB= 99%), but only in comparison with support for the other suborders (Figure 7). In terms of relationships among the suborders of Parasitiformes, the analyses provide strong support for the grouping of Holothyrida and Ixodida (consistent with Lehtinen 1991), and for a sistergroup relationship of Opilioacarida with Holothyrida/Ixodida, to the exclusion of Mesostigmata (consistent with Murrell *et al.* 2005).

Single locus analyses for each of the three markers showed if not similar, than at least compatible, results. As expected given the fact that this was the most complete and largest data set, results based on 18S-only analyses were quite similar to those of the combined analysis. The only differences involved minor changes in topology within Metastriate ticks and in the arrangement of the main lineages of Mesostigmata, and generally lower support values (parsimony only). 28S-only analyses (24 taxa) failed to provide support for monophyly of either Ixodida or Holothyrida, but did support all individual families in those suborders. In addition they indicated support for the groupings of Holothyrida/Ixodida (JS= 68%), and Opilioacarida/Holothyrida/Ixodida (JS= 52%). Finally, EF-1α-only analyses (only 13 taxa) generated poorly resolved trees (no branches with JS > 50%), but the grouping of Opilioacarida/Holothyrida/Ixodida, to the exclusion of Mesostigmata, was supported in the most parsimonious trees. That means that each of the three markers independently supports the grouping of these three suborders.

Within Holothyrida, the combined analyses generated significant support for a sistergroup relationship between Holothyridae and Neothyridae. Allothyridae is clearly more basal within this suborder. Monophyly of Neothyridae and Allothyridae is also supported, but this result should be reanalyzed using a broader taxon sampling. For the other suborders, relationships inferred are, not surprisingly, quite similar to those found in previous analyses with similar data sets. Within Ixodida, the Ixodinae (genus *Ixodes*) is paraphyletic with respect to Metastriata, a result similar to one obtained in an earlier analysis of relationships within all Parasitiformes (Klompen *et al.* 2007). This may be an artifact of insufficient sampling or of the predominant use of rRNA, because studies using more extensive sampling (taxa and characters) within Ixodidae (Klompen *et al.* 2000) or different markers (Murrell *et al.* 2003; Shao *et al.* 2005) appear to support the traditional view of a monophyletic *Ixodes*. Little can be said about relationships in Opilioacarida. The data set for this group was relatively poor (good 18S, very weak for all other loci),

and taxon sampling is simply insufficient for detailed analyses. That being said, sequence differentiation levels within Opilioacarida are surprisingly low. Observed or "P" distances, the number of nucleotide differences per site as measured between two aligned sequences (18S only, to provide more broadly comparable results), varied from 0.01-0.03; this in contrast to similar measures in Holothyrida (0.01-0.07), Ixodida (0.01-0.09) and Mesostigmata (0.14-0.24). Within Mesostigmata, Trigynaspida, Uropodina s. l., and Gamasina/Sejina s. l. are monophyletic, but support for relationships among and within these lineages is relatively weak, a result consistent with previous analyses (Klompen *et al.* 2007).

Conclusions

Morphology. The larva of *D. lecorrei* is very similar to previously described larvae of especially Mesostigmata, and can easily be described in terms used for that group. It differs from larval Allothyridae by the absence of idiosomal hypertrichy (shared with most larval Parasitiformes), the leg chaetotaxy (whorls of 6 setae as in most Parasitiformes; adult *Diplothyrus* and all instars in Allothyridae carry whorls of 8 setae on most leg segments), and a strong suggestion of lack of feeding. The latter was also observed for Opilioacarid larvae, but the larva of Allothyridae has well-developed chelicera and may feed. The nymphs of *Diplothyrus* differ from those of Allothyridae by the presence of well-defined dorsal and ventral shields. These characters can be added to the series of differences among families based on adults (Lehtinen 1981). While they clearly establish differentiation within Holothyrida, none of these characteristics are shared exclusively with Ixodida. In terms of shared morphological characters of the suborders Ixodida, Holothyrida, and Opilioacarida (tentatively designated as the "large bodied" Parasitiformes), larvae in both Opilioacarida and Holothyrida retain vestigial legs IV, but these structures are absent in larval Ixodida, and most probably represent a primitive character state. Adult molting, first noted for Opilioacarida (Coineau and Legendre 1975), may also occur in Neothyridae, but again, this characteris-
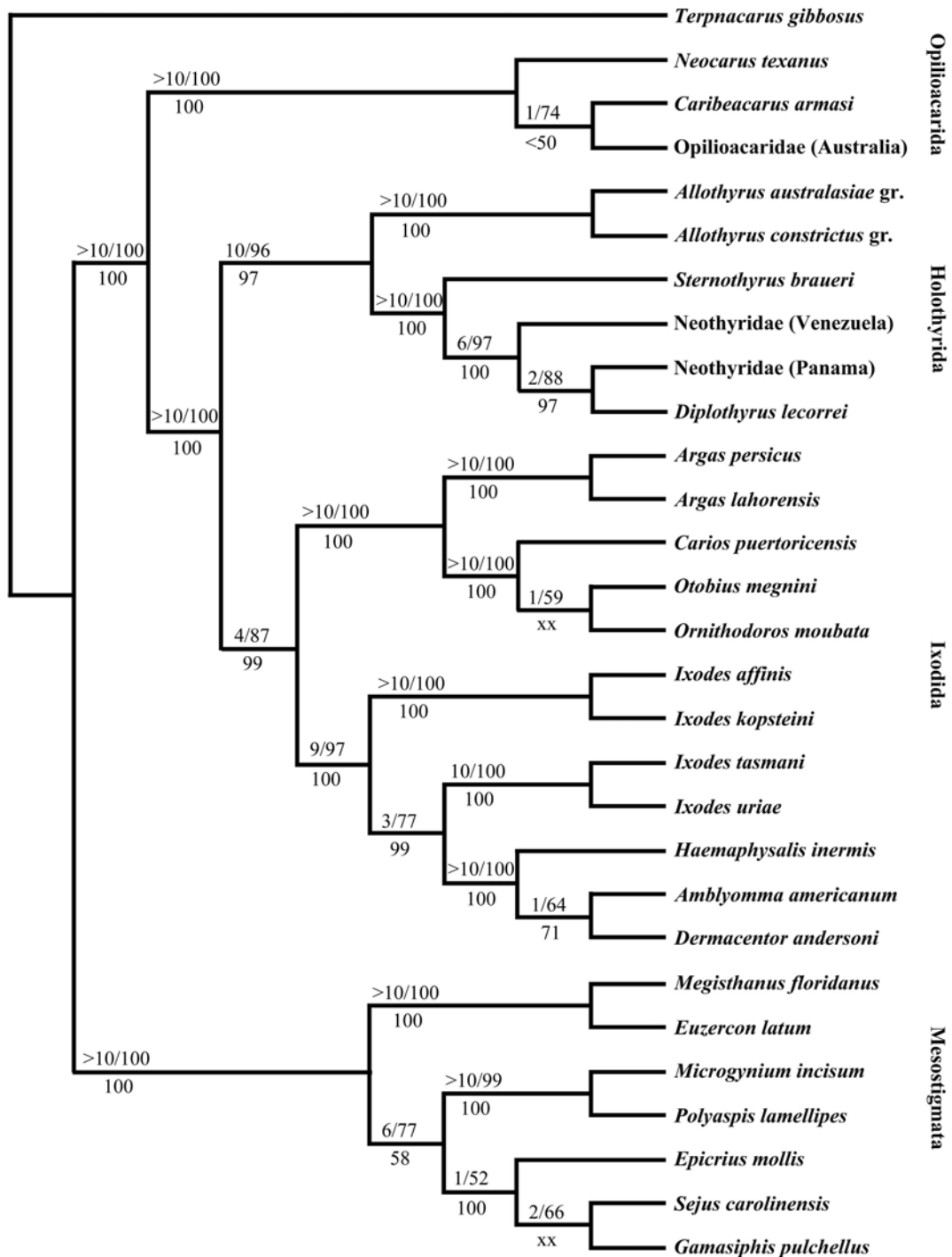
FIGURE 7: Single most parsimonious tree using a combination of DNA sequence data for three genes (18S rRNA, 28S rRNA and EF-1α). Numbers above branches: Bremer support/jackknife support (parsimony analysis); below branches: posterior probability (Bayesian analysis). xx: branch not supported in Bayesian analysis.

tic has not been observed in Ixodida. In summary, while the description of the larva of Neothyridae allows across suborder comparisons of characteristics such as chaetotaxy, it does not provide unambiguous added evidence for a close relationship of Holothyrida with Ixodida.

Molecular analysis. The current analyses provide significant support (>95% JS and PB) for monophyly of Holothyrida. This result is somewhat surprising, as the number of morphological characters supporting Holothyrida is actually very small. Lehtinen (1991) identified only one derived character, the presence of Thon's organ, a large secretory organ positioned posterior to the stigma and coxa IV (Thon 1906; Travé 1982). Other characters mentioned are either primitive or characterize only subgroups within Holothyrida (e.g., the presence of a palpal comb; Lehtinen 1991). The presence of whorls of 8, rather than 6 setae on the leg segments of at least post-larval instars (also larva Allothyridae) may represent another derived character for Holothyrida (it is absent in Mesostigmata, Opilioacarida, and Ixodida). It is interesting to note that the most poorly supported suborder in the molecular analysis is Ixodida. Ixodida is, of course, well supported by morphology, the exact opposite of the situation for Holothyrida, which has strong molecular and weak morphology based support.

In terms of the primary question for this study, the relationships among the parasitiform suborders, the sistergroup relationship between Holothyrida and Ixodida (Lehtinen 1991; Murrell *et al.* 2005; Klompen *et al.* 2007) is very strongly supported. While this result supports existing views, the second major result, the proposed relationship between Opilioacarida, Holothyrida, and Ixodida, excluding Mesostigmata, is likely to be more controversial. It is consistent with results by Murrell *et al.* (2005), based on a smaller taxon sampling and a single gene (18S), but differs considerably from traditional, morphology-based, views suggesting Opilioacarida as sistergroup to Parasitiformes s. s. (= Anactinotrichida *sensu* Grandjean 1969). Other molecular-based studies, e.g. Klompen *et al.* (2007), either supported the traditional view (Bayesian analyses) or an association of

Opilioacarida and Mesostigmata (parsimony analyses). Given the improved sampling for this analysis, the strong support for a grouping of all "large bodied" Parasitiformes cannot be dismissed out of hand. Lehtinen (1991) lists three characters as uniting Holothyrida, Ixodida, and Mesostigmata: (1) reduction of the number of lyrifissures, (2) male genital orifice rounded and sternal, and (3) the presence of a sclerotized abdomen. The validity of character (1) is unclear. The number of lyrifissures in some adult Holothyrida is quite high (Travé 1983) and may be comparable to that in Opilioacarida, while lyrifissure patterns in adult Ixodida simply have not been studied. The genital orifice (character 2) in male Opilioacarida is mid-sternal, as it is in Ixodida, Holothyrida, and most basal Mesostigmata. Which leaves abdominal sclerotization. Ignoring the fact that such sclerotization is absent in immature Allothyridae, all instars of Argasidae, all known instars of Nuttalliellidae (Ixodida), as well as in immature and female Ixodidae, it would be worthwhile to at least consider the possibility that the absence of sclerotized shields in immature and adult Opilioacaridae is derived. Lehtinen (1991) already noted the possibility that the presence of multiple stigmata, and the presence of "With's organ" (the second rutellum) may also be derived for Opilioacarida. Under this hypothesis, Opilioacarida is not necessarily a primitive group forming a bridge between Parasitiformes s. s. and Acariformes, but a derived lineage within Parasitiformes s. l.

The available molecular data cannot differentiate between these hypotheses, but it is worth noting the unexpectedly low variability levels in Opilioacarida (with taxa from Australia and the U.S.A. sampled). This observation fits easier with the idea that (extant) Opilioacarida are relatively young, than with the traditional view of Opilioacaridae as an ancient branch of Acari. These questions cannot be answered until relationships of Parasitiformes with other mites and non-mite arachnid groups have been resolved, an issue beyond the scope of this study.

Evolution of parasitism. The traditional hypothesis that Ixodida may have acquired a parasitic life style through intermediaries of (nest) predators

(Oliver 1989) is inconsistent with current results. Neither Holothyrida not Opilioacarida include any members that are known nest associates. Moreover, neither group appears to be predaceous. Instead published evidence (Travé 1982; Walter and Proctor 1998) suggests that both groups are scavengers, leaving the rather unsettling result that a lineage including some of the most successful obligate blood-feeders (Ixodida) arose from within a lineage of scavenging litter inhabitants. One can only hope that future studies of feeding behavior for both Holothyrida and Opilioacarida will help provide a better understanding of this transition.

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