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Ephemeroptera Egg Chorion Characters: A Test of Their Importance in Assessing Phylogenetic Relationships

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ABSTRACT The egg chorion ultrastructures of the *Hermanella–Traverella* (Insecta: Ephemeroptera) species complex were studied from a comparative point of view and used for the first time in a cladistic analysis. Egg characters, along with other nymphal and adult morphological characters, were used to assess the phylogenetic relationships of the species complex. In order to test the value of egg characters, analyses were performed on three matrices: 1) egg characters alone, 2) adult and nymphal characters, and 3) adult, nymphal, and egg characters. The computer program Pee-Wee was used to carry out the analysis. The cladistic analysis confirmed the value and potential of egg chorionic characters in assessing the phylogenetic relationships among ephemeropteran species. Egg characters, when added to the nymphal and adult character matrix, provided extra support to the monophyletic nature of the *Hermanella–Traverella* complex. Previously weakly defined clades were also resolved based on the new evidence. In the species studied the egg chorionic structures as well as their shape did not change after oviposition or water immersion, remaining constant through the different maturation stages of each species (mature nymph, subimago, and imago). For this reason, the eggs are a valuable source of information to unambiguously identify and associate a nymph to its correspondent adult stage when rearing is not possible. J. Morphol. 253:148–165, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: egg chorion; ultrastructure; Ephemeroptera; Leptophlebiidae; cladistics

Due to the short life of the adults, ephemeropteran eggs are already developed in the nymph before its emergence from water. As the eggs fill a great part of the insect body (all the abdomen and most of the thorax of the adult), taxonomists have tried to use them as a source of characters.

The first detailed studies on Ephemeroptera eggs were carried out by Burmeister (1848). Bengtsson (1913) in Europe and Morgan (1913) in the United States made significant contributions on comparative studies among various species inhabiting different areas. Degrange (1956, 1960) found and described for the first time the micropylar apparatus. However, comparative studies on egg chorionic structures as a basis for phylogenetic analysis of different groups only began with the studies of Koss (1968, 1970) and especially Koss and Edmunds (1974). These studies were very complete, but these authors used only light microscopy and applied their analysis mainly to family-level relationships.

From this starting point, the use of scanning electron microscopy allowed a more detailed observation of the external structures, elucidating the fine structure of the egg chorionic pattern in eggs of different Ephemeroptera families: Heptageniidae (Flowers, 1980; Alba Tercedor and Sowa, 1987; Gaino and Mazzini, 1987, 1988; Gaino et al., 1989; Klonowska, 1997), Leptophlebiidae (Gaino and Mazzini, 1984, 1989; Mazzini and Gaino, 1985), Ephemerellidae (Gaino and Bongiovanni, 1992a; Studeman et al., 1995; Studeman and Landolt, 1997), Baetidae (Gaino and Bongiovanni, 1992b), Palingeniidae (Gaino and Bongiovanni, 1993), and Siphlonuridae (Studeman et al., 1988). Also, a number of mainly taxonomic studies often included brief descriptions of chorionic structures or micrographs showing the eggs of the treated species (e.g., Pescador and Peters, 1982, 1985, 1987, 1990; Dominguez and Flowers, 1989; Domínguez, 1995).

Although several of the ultrastructural studies on mayfly eggs have pointed out the importance of the

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chorionic structures as a source of characters with phylogenetic potential, they have never been used in a cladistic analysis. Furthermore, it was stated that for some families, like Ephemerellidae, the chorionic pattern could not be used for taxonomic purposes at the generic level (Studeman and Landolt, 1997). One of the problems in using egg characters for cladistic analysis is the lack of cladistic hypotheses among the taxa available for testing. For this reason, as a cladistic hypothesis of the relationships of the *Hermanella* complex (Leptophlebiidae: Atalophlebiinae) using nymphal and imaginal characters became available (Flowers and Domínguez, 1991), we felt it would be an opportunity to check the applicability of the egg chorionic characters as an independent test, and also their possible use as complementary characters along with traditional characters in cladistic analyses.

Another reason for using the *Hermanella* complex was the peculiar shape of the eggs of their species. There is some incomplete information in the literature concerning the different shapes and sizes of eggs in relation to the biology of the different groups. For instance, although the eggs extracted from nymphs, subimagoes, and imagoes are identical in Ephemerellidae (Studeman and Landolt, 1997), in others (*Ameletus* and *Paraleptophlebia)* the final shape and size is attained during oviposition (Soldán, 1979; Kang and Yang, 1994). The reasons for these changes have been discussed by Soldán (1979, p. 269) using two possible hypothesis: "1) The eggs lose water during the subimaginal stage so that the weight of the future imago would be lower and flight abilities better. 2) The eggs are deformed only in a passive way so that they can arrange themselves in the oviduct economically so that each egg occupies a minimal area. The fact that the eggs acquire regular shape immediately after leaving the oviduct and after egg laying gives evidence for this hypothesis."

In this study we intend to: 1) contribute new information on the morphology of the egg chorion and associated structures of the species of the *Hermanella* complex from the South American Atalophlebiinae (Leptophlebiidae); 2) test the potential and importance of egg chorion morphology in Atalophlebiinae in producing phylogenetic hypotheses using cladistic methodology; 3) test the relationships of the species belonging to the *Hermanella* complex using egg chorionic characters alone and together with other morphological characters; and 4) test Soldán's (1979) hypothesis on the shape change of eggs before and after oviposition.

Background Information

According to Koss and Edmunds (1974), three main morphological features are useful for taxonomic purposes: 1) attachment structures, 2) micropyles, and 3) chorionic sculpturing. These main features were also subdivided into different categories. As all of the different structures described are not present in Leptophlebiidae, we will only include those that were found in our study.

Functionally, the attachment structures prevent most eggs from being washed downstream, or being carried to an environment unsuitable for development (Koss, 1968).

The micropyle is the structure that allows sperm to enter the egg and, according to Koss and Edmunds (1974), it is composed of a sperm guide and a micropylar canal. In Leptophlebiidae the micropylar canal is always funnelform and, according to these authors, is the most plesiomorphic type.

Chorionic sculptures are variable in some groups but can be constant in others. Koss (1968) stressed the importance of chorionic structures as a resource of characters for association and identification of immature and adult female mayflies to species level without males.

MATERIALS AND METHODS

Eggs were removed from adult females or mature nymphs of 14 species of Atalophlebiinae (Appendix A). When live specimens were available, eggs were fixed in Karnovsky's fixative (Karnovsky, 1965) at 4°C and rinsed in 0.1 M phosphate buffer (pH 7.2) for 2 h. When live specimens were not available, eggs were extracted from the abdomens of alcohol-preserved mature nymphs and adults.

After removal from the abdomen, the eggs were cleaned in an ultrasonic cleaner for no longer than 10 sec, dehydrated in a graded ethanol series, and dried by the critical-point method using CO_2 in a Bomar apparatus. Some samples were also prepared for scanning view without previous critical-point treatment, but the results were not satisfactory. Eggs were then mounted with double-sided tape on SEM stubs and sputter-coated with gold. They were observed with a JEOL 35CF Scanning Electron Microscope at 25 kV. All measurements were taken on 10 randomly selected eggs from each species.

In some cases our data were complemented with published descriptions or micrographs of species included in the analysis (see Domínguez and Flowers, 1989). Terminology used in the present study follows Koss and Edmunds (1974).

In order to test if there were morphological differences among the eggs from different maturation stages of the same species, eggs from nymphs, subimagoes, and imagoes were observed with SEM each time the different stages were available. To test if the prismatic egg shape, typical of the *Hermanella* complex species, was an artifact of fixation, female imagoes of *Traverella (Zonda) calingastensis* and *Hydrosmilodon saltensis* were collected alive, forced to oviposit in still water, and the eggs observed under a microscope at intervals for a period of 24 h.

A hypothesis of phylogenetic relationships of the taxa analyzed were obtained using the computer

program Pee-Wee (Goloboff, 1993a). Pee-Wee is a program for parsimony analysis under implied weights. It searches for trees that maximize fit across characters (Goloboff, 1993a,b). Trees with maximum fit resolve character conflicts in favor of the characters that have less homoplasy on the trees and imply that the average weight for the characters is as high as possible. To facilitate the diagnosis of characters as well as drawing trees, WinClada (Nixon, 1999) was used.

RESULTS

Systematic Descriptions

Hermanella (Hermanella) thelma Needham and Murphy (Fig. 1a–d). Egg size: 140–200 μm in length, 100 μm in width. General shape prismatic, hexagonal in cross section. One polar region strongly convex, the other flat or slightly convex (Fig. 1a). All surfaces with granules regularly arranged forming tenuous hexagonal ridges. Several knob-terminated coiled threads (KCT) concentrated only on the flat polar region, absent from the remaining chorion surface (Fig. 1b). KCT composed of a long, coiled thread ending in a rounded knob of about 4.5 μm in diameter. At rest, the knob sits on top of the coiled thread. The thread projects from the chorion, arising from the middle of a circular area of 9–11 μm diameter, outlined by a thin ridge (KCT collar), which is evident only when the thread is uncoiled (Fig. 1c). Surface of both thread and knob wrinkled. One micropyle supraequatorially located (Fig. 1d). Micropyle opening outlined by one regular ring.

Hermanella (Guayakia) grandis Domínguez and Flowers (Fig. 1e,f). Egg size: 160–170 μm in length, 100 μm in width. General shape prismatic, hexagonal in cross section. Both polar regions flat or slightly convex. Chorionic surface smooth or with small granules. KCTs scattered in all surfaces. One supraequatorial micropyle surrounded by a sieved area (Fig. 1e). Outline of the micropyle opening irregular (Fig. 1f).

Hermanella (Hermanella) guttata Domínguez and Flowers (Fig. 2a–d). Egg size: 130–140 μm in length, 100 μm in width. General shape (Fig. 2a) prismatic, hexagonal in cross section (Fig. 2b). Both polar regions flat. All surfaces with small, scattered, and irregularly distributed granules. KCTs regularly distributed in chorionic surface, both polar and lateral zones of the egg. KCT structure as in *H. thelma* except for the knob, which is smaller in diameter (3 μm). Circular area of about 6 μm in diameter (Fig. 2c), delimited by a thicker KCT collar. One supraequatorial micropyle present; micropyle surrounded by a sieved area (Fig. 2d), opening outlined by an irregular ring.

Hermanella (Guayakia) maculipennis Domínguez and Flowers (Fig. 2e,f). Egg size: 150–160 μm in length, 100–110 μm in width. General shape prismatic, hexagonal in cross section. Both polar regions flat or slightly concave (Fig. 2e). Chorionic surface with small bumps and fine granules. The bumps are arranged in hexagonal areas where the KCTs are located; between these areas bumps are irregularly distributed. KCTs scattered on all chorion surfaces, polar and lateral regions, regularly distributed. KCT collars narrow and continuous. Both thread and knob surface wrinkled. Circular area delimited by KCT collar of about 5 μm in diameter. One supraequatorial micropyle (Fig. 2f), lacking surrounding sieved area. Micropyle outlined by one regular ring.

Hermanella (Guayakia) froehlichi Ferreira and Domínguez (Fig. 3a–d). Egg size: 110–160 μm in length, 100 μm in width. General shape (Fig. 3a) prismatic, quadrangular to hexagonal in cross section. One polar region convex, the other concave. Chorionic surface smooth or with low elevated small granules (Fig. 3b). KCTs restricted to the concave polar region. KCT structure as in *H. thelma,* knob 5 μm in diameter. Circular area of about 7.5 μm in diameter delimited by a narrow, continuous, irregular KCT collar. Two funnelform micropyles with micropylar canal perpendicular to the chorion (Fig. 3c), close to the concave pole. Internal diameter of the micropyle between 2–2.5 μm. Undulating thin rings surround micropyle opening (3d).

Needhamella ehrhardti (Ulmer) (Figs. 3e,f, 4a,b). Egg size: 175–190 μm in length, 80–108 μm in width. General shape prismatic (Fig. 3e), quadrate to hexagonal in cross section. Both polar regions flat. Chorionic surface wrinkled (Fig. 3f). KCTs regularly distributed on all chorionic surfaces, both in polar and lateral regions. Terminal knob round, smooth 3.8–4 μm in diameter, elevated above coiled thread. Coiled thread externally wrinkled. KCT collar narrow, not continuous, granulated (Fig. 4a). One equatorial micropyle not surrounded by a sieved area. Micropyle opening irregular not outlined by a ring (Fig. 4b).

Hydrosmilodon saltensis Flowers and Domínguez (Fig. 4c–f). Egg size: 125 μm in length, 80–90 μm in width. General shape prismatic (Fig. 4c), quadrate in cross section. Both polar regions flat. Chorionic surface with granulated bumps and small granules scattered between the bumps (Fig. 4d). KCTs on all chorionic surface regularly distributed. KCT's terminal knob round, 6 μm in diameter, elevated above coiled thread (Fig. 4e). KCT collar narrow and continuous, with a small uplifted bulge (Fig. 4f). One supraequatorial micropyle. Opening of the micropyle irregular with three triangular bumps surrounding it.

Traverella (Zonda) calingastensis Domínguez (Fig. 5a,b). Egg size: 130–140 μm in length, 80 μm in width. General shape prismatic (Fig. 5a). Both polar regions flat. Chorionic surface smooth to granular. KCTs regularly distributed on all chorionic surfaces. KCT's terminal knob round, about 7–10 μm in diameter, elevated above coiled thread. KCT collar

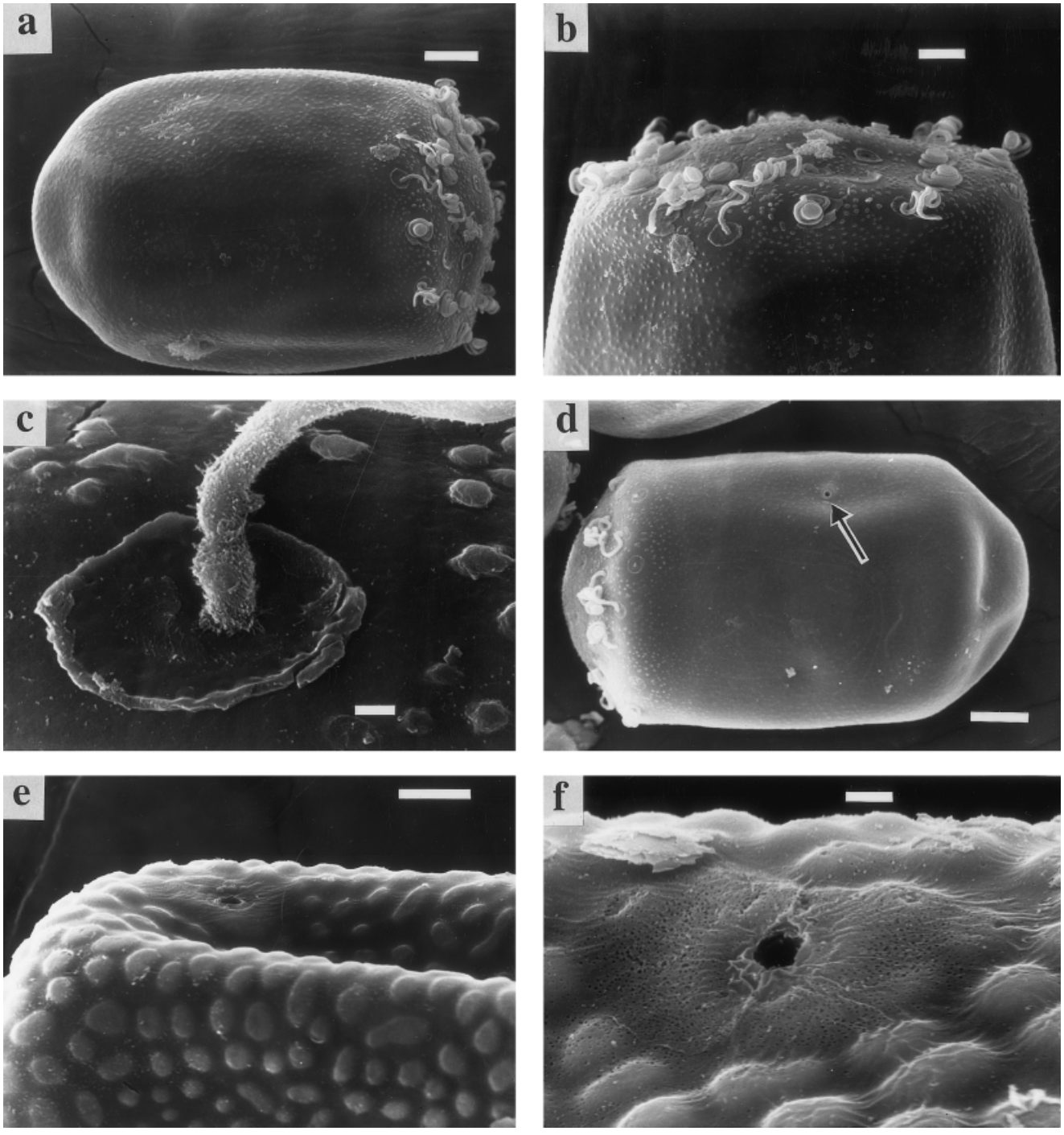

Fig. 1. *Hermanella (H.) thelma.* **a**: General shape of egg, character 49(1). Scale bar = 20 μm. **b**: Polar region with concentration of KCT attachment structures (character 51(1)). Note the chorion sculpture with fine granules arranged in hexagonal figures. Scale bar = 10 μm. **c**: KCT collar with base of thread arising from the middle of circular area. Scale bar = 1 μm. **d**: Egg with a supraequatorial micropyle outlined by a ring (arrow) (characters 54(1); 56(1)). Scale bar = 20 μm. *Hermanella (G.) grandis.* **e**: Position of micropyle, character 54(1). Scale bar = 10 μm. **f**: Detail of micropyle. Scale bar = 2 μm.

granular, not continuous, delimiting a round area of 11–14 μm in diameter (Fig. 5b). One supraequatorial micropyle. Micropyle opening irregular without a surrounding sieved area.

Traverella bradleyi (Needham and Murphy) (Fig. 5c–f). Egg size: 110–130 μm in length, 80 μm in width. General shape prismatic (Fig. 5c), quadrate in cross section (Fig. 5d). Both polar regions flat.

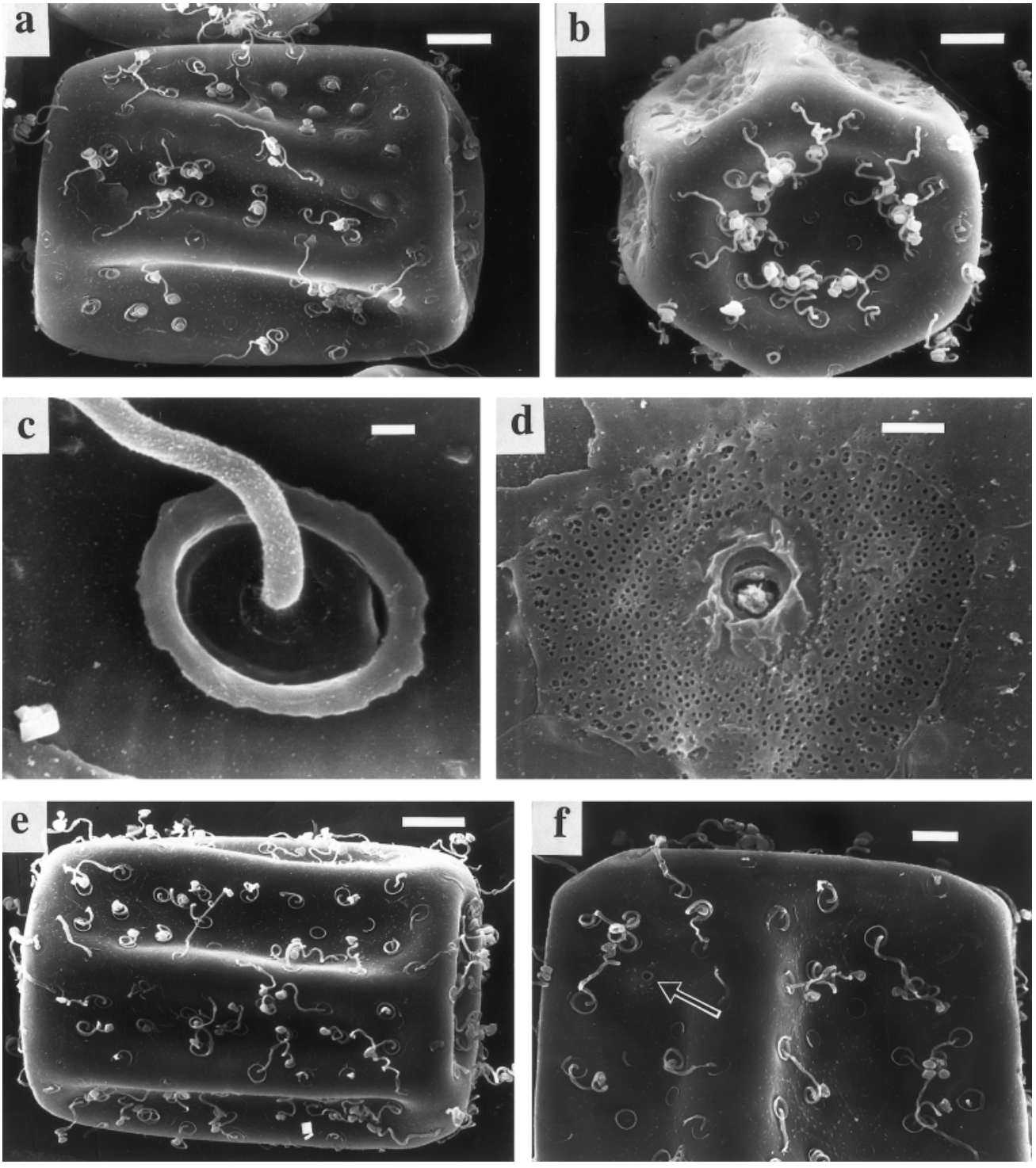

Fig. 2. *Hermanella (H.) guttata*. **a**: General shape of egg, with polar regions flat or slightly concave (character 50(2)). Scale bar = 20 μm. **b**: Hexagonal cross section of egg (character 49(1)). Scale bar = 20 μm. **c**: KCT collar narrow and continuous (character 53(2)). Scale bar = 1 μm. **d**: Detail of irregularly outline micropyle, character 56(0). Scale bar = 2 μm. *Hermanella (G.) maculipennis*. **e**: General shape of egg, characters 49(1), 50(2). Scale bar = 20 μm. **f**: Supraequatorial micropyle (arrow), character 54(1). Scale bar = 10 μm.

Chorionic surface granular. KCTs regularly distributed on all chorionic surface. KCT's terminal knob 7.5 μm in diameter, elevated above coiled thread (Fig. 5e). KCT collar absent. One supraequatorial micropyle present, not surrounded by a sieved area (Fig. 5f). Micropylar opening irregular.

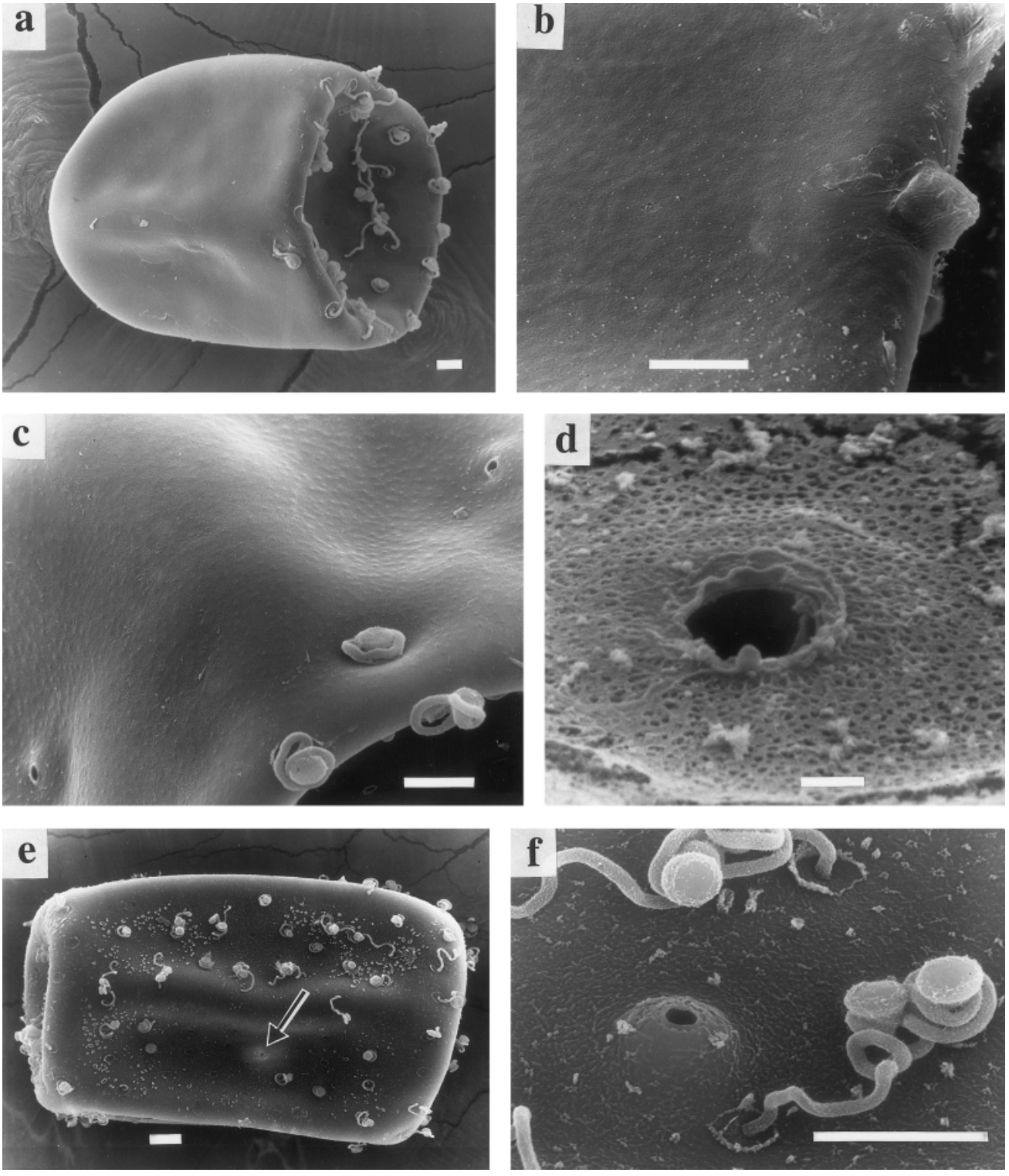

Fig. 3. *Hermanella (G.) froehlichi*. **a**: General shape of egg, character 49(1). Scale bar = 10 μm. **b**: Chorionic surface smooth or with very fine small granules, character 48(0). Scale bar = 10 μm. **c**: Position of the two micropyles present in this species. Scale bar = 10 μm. **d**: Detail of one of the micropyles. Note the undulating thin rings surrounding the micropyle opening and surrounding sieve area (character 55(1)). Scale bar = 1 μm. *Needhamella ehrhardti*. **e**: General shape of egg; arrow indicates the position of the micropyle. Scale bar = 10 μm. **f**: View of an area of the wrinkled chorionic sculpture (character 48(2)) with micropyle surrounded by KCTs. Scale bar = 10 μm.

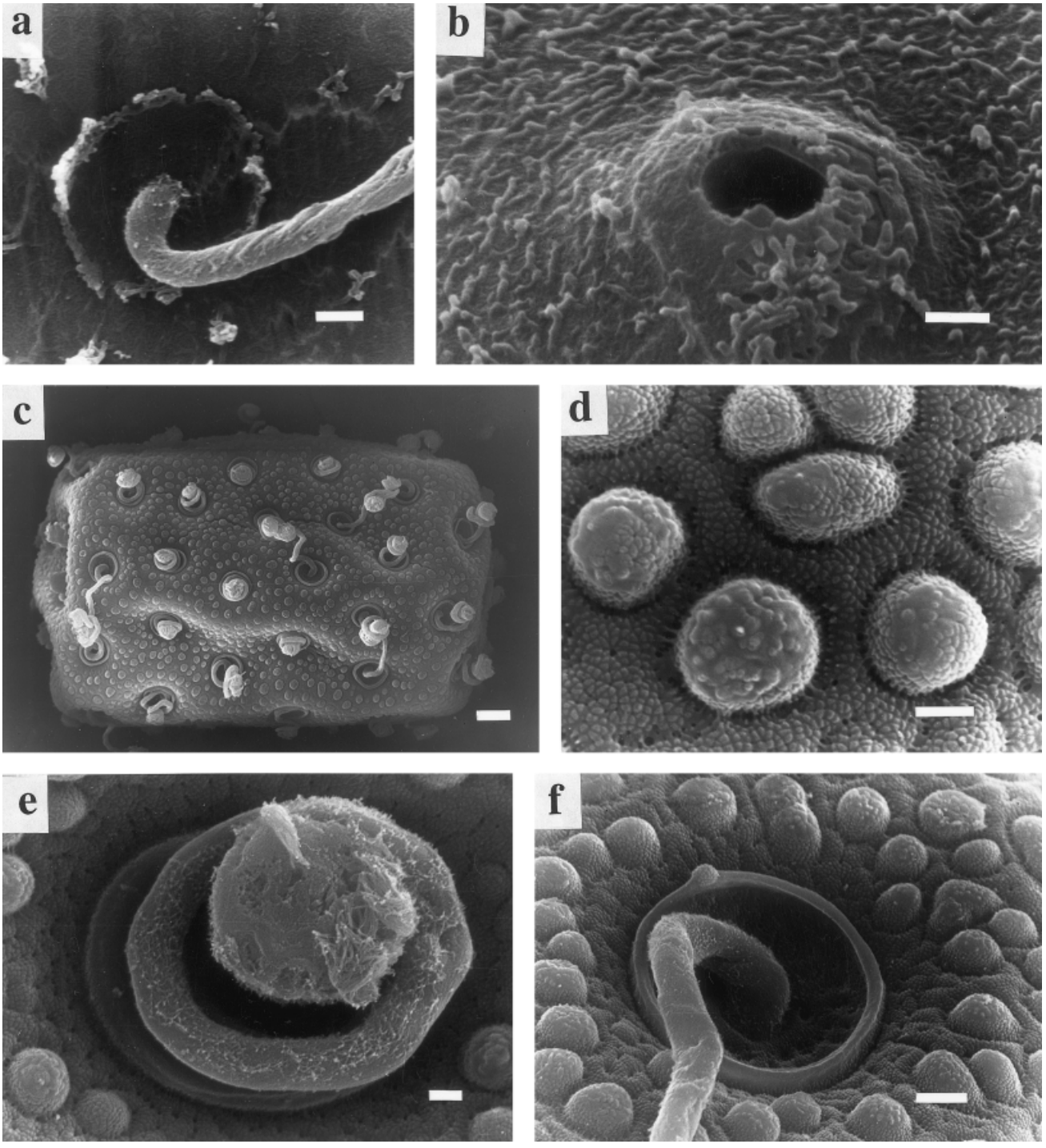

Fig. 4. *Needhamella ehrhardti.* **a**: KCT collar narrow, not continuous, granulated, character 53(1). Scale bar = 1 μm. **b**: Detail of the micropyle opening which is irregular in outline, character 56(1). Scale bar = 1 μm. *Hydrosmilodon saltensis.* **c**: General shape of eggs. Scale bar = 10 μm. **d**: Chorion sculpture showing the presence of numerous big round bumps (character 47(2)); remaining egg surface with granules (Character 48(1)). Scale bar = 1 μm. **e:** Morphology of KCT with round terminal knob elevated above coiled thread (character 52(1)). Note that the knob only partially covers the coiled thread (character 57(1)). Scale bar = 1 μm. **f**: KCT collar narrow with small dorsal knob. Scale bar = 1 μm.

Traverella albertana (McDunnough) (Fig. 6a,b). Egg size: 240–250 μm in length, 145–160 μm in width. General shape cylindrical (Fig. 6a). Both polar regions flat. Chorionic surface granular. KCTs regularly distributed in all chorionic surface. KCT's terminal knob round, 7.6–9 μm in diameter, elevated above coiled thread (Fig. 6b); knob partially overlapped by coiled thread. KCT collar thin and continuous with external

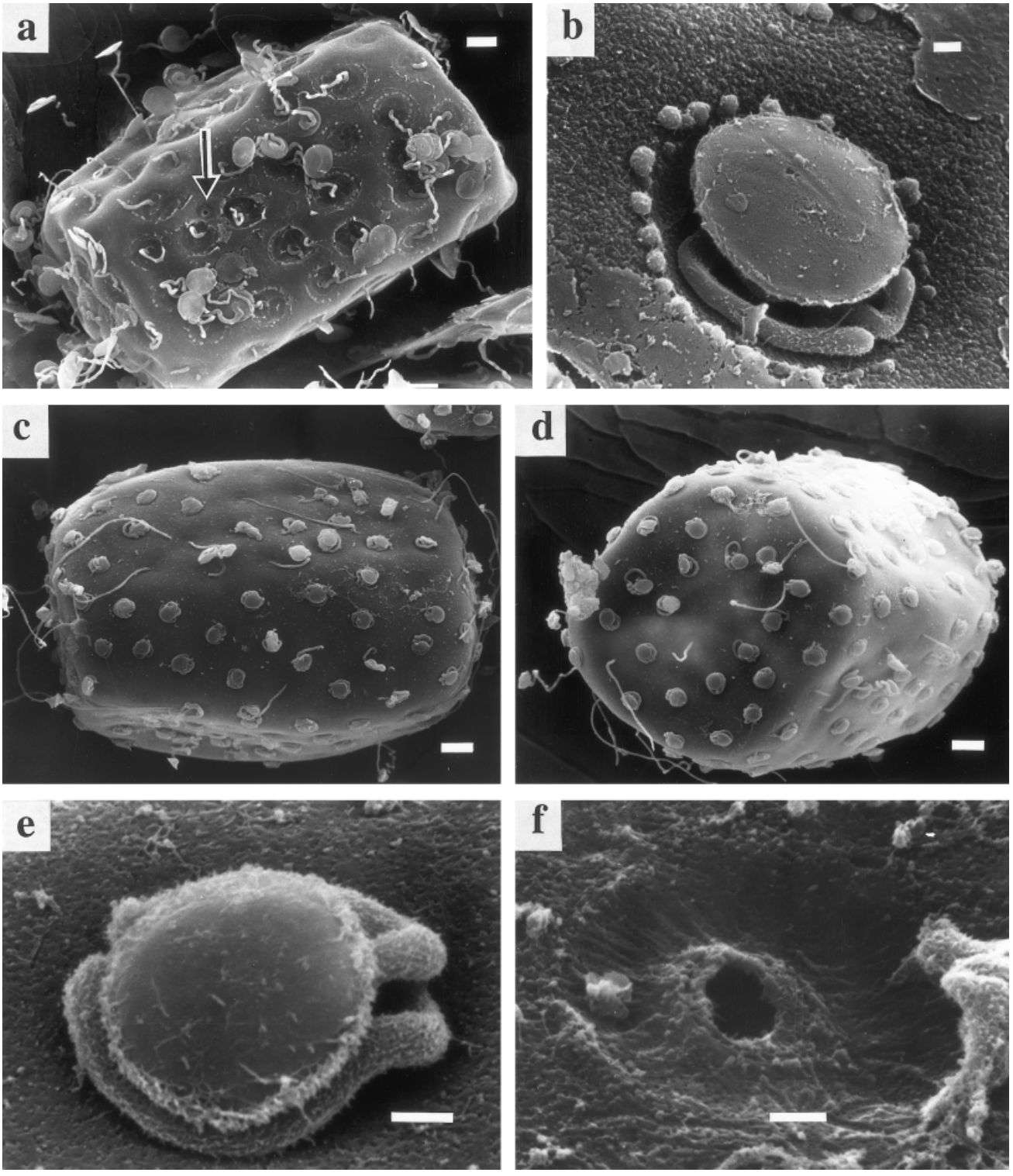

Fig. 5. *Traverella (Z.) calingastensis.* **a**: General shape of egg. Note the supraequatorial position of micropyle (arrow), character 54(1). Scale bar = 10 μm. **b**: KCT with typical big round knob (character 53(1)) and a thin discontinuous collar, character 53(1). Scale bar = 1 μm. *Taverella bradleyi.* **c**: General shape of egg. Note the supraequatorial position of the micropyle. Scale bar = 10 μm. **d**: View of one of the polar regions with quadrate cross section. Note that KCT collars are absent (character 53(3)). Scale bar = 10 μm. **e**: KCT with knob elevated above coiled thread, character 52(1). Scale bar = 1 μm. **f**: Detail of micropyle without sieve area surrounding it (character 55(0)). Scale bar = 1 μm.

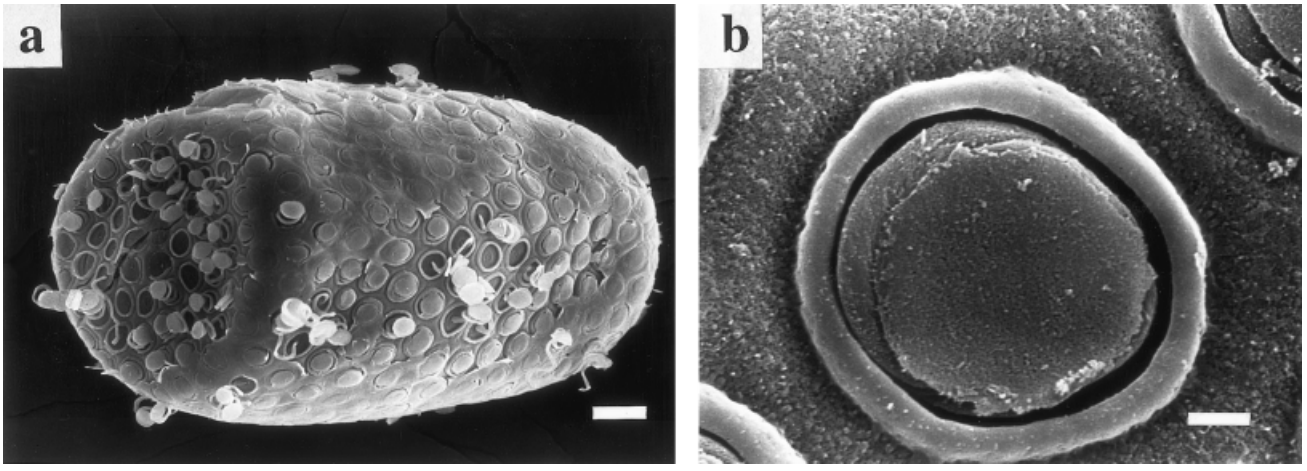

Fig. 6. *Traverella albertana*. **a**: Cylindric shape of egg, character 49(2). Note that knob of KCT covers all coiled thread (character 57(0)). Scale bar = 10 μm. **b**: KCT with narrow continuos collar, character 53(2). Scale bar = 1 μm.

even border, surface of collar smooth. One supraequatorial micropyle present, not surrounded by sieved area. The micropyle located between two KCTs, its opening irregular.

Nousia delicata Navás (Fig. 7a,b). Egg size: 200 μm in length, 100–115 μm in width. General shape oval (Fig. 7a). Both polar regions convex. Chorionic surface with small to medium-sized granules (Fig. 7b). KCTs scattered over entire egg surface. KCT collar smooth, thin, and continuous, KCT knob round about 4 μm in diameter. Threads not as long as in *Ulmeritus carbonelli* (q.v.), coiled inside the area delimited by collar but not overlapping collar. Knob not elevated above coiled thread. One micropyle present in equatorial position.

Ulmeritus carbonelli Traver (Fig. 7c–e). Egg size: 142–148 μm in length, 90–100 μm in width. General shape oval (Fig. 7c). Both polar regions convex. Chorionic surface smooth. KCTs regularly distributed and overlapping all chorionic surfaces: when threads are completely coiled no chorionic surface shows among the KCTs. KCT's coiled thread long, completely covering the KCT collar. Collar smooth with hexagonal outer border; internally, the area delimited by the collar is round (Fig. 7d). The thread is coiled around the knob so that the knob is not elevated above the thread. One micropyle present, located in the equatorial area. The micropyle, located among three KCTs, can only be distinguished when the KCT threads are uncoiled (Fig 7e).

The eggs of the following species were not in good shape and for this reason only a few characters were observable.

Hagenulus caligatus Eaton (Fig. 7f). Egg size: 140–150 μm in length, 80–90 μm in width. General shape oval (Fig. 7f). Both polar regions convex. KCTs regularly distributed. KCT's knob not elevated above the thread. One micropyle present in equatorial position.

Hylister plaumanni Domínguez and Flowers. Egg size: 80–100 μm in length, 50–60 μm in width. General shape quadrate in cross-section. Both polar regions flat. KCTs restricted to both polar regions.

Cladistic Analysis

Characters and coding. A matrix of 57 characters (Table 1) was compiled, including 31 nymphal, 15 adult, and 11 egg characters. Most of the adult and nymphal characters and their coding follows Flowers and Domínguez (1991). Nevertheless, due to the different taxonomic coverage of the present study and in light of new information, it was necessary to redefine some characters. Some other characters used in Flowers and Domínguez (1991) are omitted as they are not informative for the smaller set of taxa in this analysis. Thirty-four characters are binary and 24 are multistate. Multistate characters were treated in two different ways: additive (11 characters) when it was possible to hypothesize their sequences of change, and the remaining 12 as nonadditive. Polymorphic taxa were coded in the matrix and they appear indicated by an asterisk. Characters with no information available were assigned a missing code "?." Character steps, extra steps, minimum and maximum possible steps, and character weights are reported in Table 2. A complete list of the characters used and their coding is compiled in Appendix B.

Taxa selection. The species included in this study belong to the monophyletic clade *Traverella–Hermanella* complex (Flowers and Domínguez, 1991). *Nousia delicata,* one of the basal South American genera of Atalophlebiinae, was used to root the network in the cladistic analysis. In order to test the monophyly of the ingroup *Hagenulus caligatus*, the sister group of the mentioned clade, and a more distantly related species, *Ulmeritus carbonelli,* were

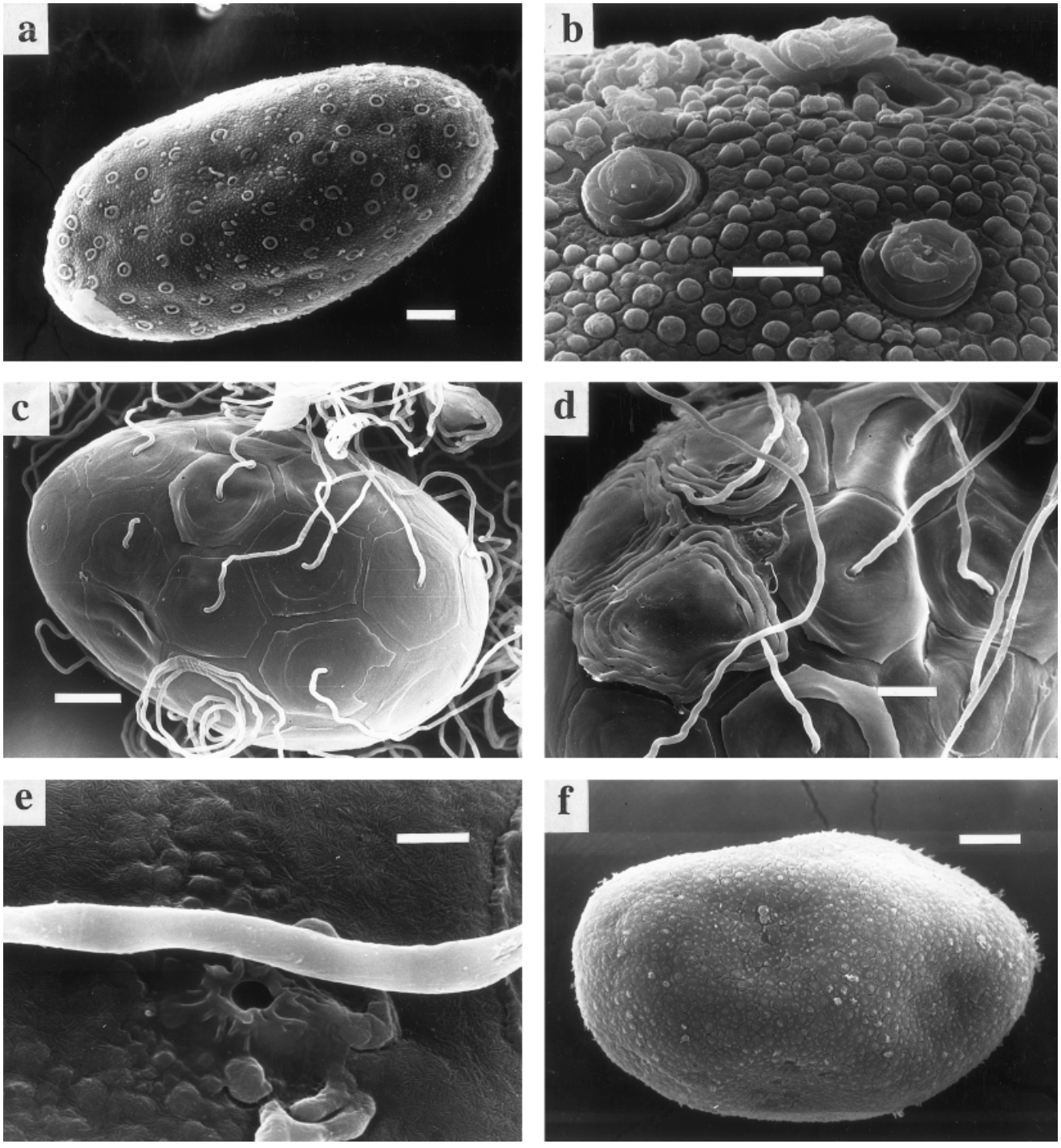

Fig. 7. *Nousia delicata.* **a**: Oval shape of egg, (character 49 (0)) with convex polar regions (character 50(0)). Scale bar = 20 μm. **b**: Chorion sculpture with bumps and small granules among the KCTs, character 47(1). Scale bar = 10 μm. *Ulmeritus carbonelli.* **c**: Egg with oval general shape and uncoiled KCTs. Scale bar = 20 μm. **d**: KCTs with knob not elevated above coiled thread but surrounded by the thread (character 52(0)). Note that the shape of the collar is wide and polyhedric (character 53(0)). Scale bar = 10 μm. **e**: Detail of micropyle. Scale bar = 1 μm. *Hagenulus caligatus.* **f**: General shape of egg. Scale bar = 20 μm.

included in the analysis. These "outgroups" were selected on the basis of the published cladistic hypothesis of Flowers and Domínguez (1991). The genera *Needhamella, Hylister* and *"T. bradleyi"* are monotypic and their characters are based on their type species. *Traverella s.s.* is represented by *T. albertana; Traverella (Zonda)* is based on its type species *(T. calingastensis*); and *Hydrosmilodon* is based on

TABLE 1. Data matrix of taxa and characters (1–57) used in this study

	Nymphs							Adults									Eggs					
	1	5	10	15	20	25	30	35	40	45	50	55										
Nousia	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	2	1	0	0	0	2	0	0	0	1
Ulmeritus	0	1	0	1	1	0	0	1	1	1	0	2	1	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	1	0	0	0	0	0	0	0	0	2
Hagenulus	2	1	1	1	0	0	0	0	1	1	0	0	1	2	1	0	0	0	0	2	0	1	2	1	1	1	1	0	0	0	1	0	0	1	?	1	0	0	0	?	0	?	0	?								
H. guttata	1	0	1	1	1	0	2	1	1	2	2	3	1	0	0	1	2	0	1	2	2	0	1	0	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	0	1	0	1	2	0	1	2	1	1	0	1
H. thelma	1	0	1	1	1	0	2	1	1	2	2	3	1	0	0	1	2	0	1	2	2	0	1	0	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	0	1	0	1	1	1	2	1	1			
H. froehlichi	1	0	1	1	1	0	2	1	1	2	2	3	1	0	0	1	2	0	1	2	2	0	1	0	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	0	0	0	1	1	1	2	1	1	1		
H. grandis	1	0	1	1	1	0	2	1	1	2	2	3	1	0	0	1	2	0	1	2	2	0	1	0	1	1	1	0	1	0	1	0	1	1	1	1	2	1	1	1	0	0	0	1	2	0	1	2	1	0	1
H. maculipennis	1	0	1	1	1	0	2	1	1	2	2	3	1	0	0	1	2	0	1	2	2	0	1	0	1	1	1	0	1	0	1	0	1	1	1	1	2	1	1	1	0	1	0	1	2	0	1	2	1	1	1
Hylister	1	0	1	1	1	0	2	1	1	2	2	2	1	0	0	1	2	0	1	2	2	0	1	2	1	1	1	0	1	1	0	1	1	0	1	1	2	1	1	1	0	?	?	1	2	2	?	?	?	?	?
Needhamella	2	0	1	1	0	1	2	1	1	2	2	2	1	0	0	1	2	0	1	2	2	1	1	0	1	1	1	1	2	1	1	1	1	0	0	2	1	2	0	0	1	2	0	1	2	0	0	1	2	0	0	0	1
Hydrosmylodon	1	0	1	1	0	0	2	1	1	2	2	2	0	0	0	1	2	0	1	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	?	2	1	1	2	0	0	1	1	0	0	1	
T. calingastensis	2	0	1	1	0	1	1	1	2	2	2	1	1	0	0	0	2	1	1	2	1	0	2	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	2	0	1	1	1	0	0	0			
T. bradleyi	1	0	1	1	0	0	2	1	1	2	2	2	1	1	0	0	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	?	1	1	2	0	1	3	1	0	0	1	
T. albertana	2	0	1	1	0	1	2	1	1	2	2	2	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	2	2	2	0	1	2	1	0	0	0			

* = polymorphic character; ? = unknown condition.

H. saltensis. The two species of *Hermanella s.s. (H. thelma* and *H. guttata*) and the three of *Hermanella (Guayakia)*: *H. froehlichi, H. grandis,* and *H. maculipennis* form the rest of the analyzed taxa. Several other taxa, not included in the matrix, were also studied to test the consistency and variability of the characters used. They were not included because only a few species of each genus were available and generic variability could not be assessed (e.g., two species of *Thraulodes* were studied out of several dozens described).

Analysis

Data were analyzed using the commands "Hold 1000" and "Mult*50" of the Pee-Wee Program. For additional information on the computer program, see Goloboff (1993a,b). Hold 1000 keeps in memory up to 1,000 trees for the analysis. Mult is a command for tree searching—it randomizes the order of the taxa, creates a weighted Wagner tree, and submits it to branch swapping using tree-bisection-reconnection. The "*50" repeats the process 50

TABLE 2. Character steps, extra steps, and weight implemented by the Pee-Wee maximum fit tree

Character	Steps	Extra steps	Min/ Max	Weight	Character	Steps	Extra steps	Min/ Max	Weight
1	4	2	2/6	6.0	30	2	1	1/2	7.5
2	1	0	1/3	10.0	31	1	0	1/3	10.0
3	1	0	1/2	10.0	32	1	0	1/3	10.0
4	1	0	1/1	10.0	33	2	1	1/2	7.5
5	1	0	1/1	10.0	34	4	2	2/5	6.0
6	1	0	1/6	10.0	35	2	0	2/3	10.0
7	2	1	1/3	7.5	36	4	3	1/5	5.0
8	3	1	2/5	7.5	37	1	0	1/2	10.0
9	1	0	1/3	10.0	38	2	1	1/3	7.5
10	2	1	1/2	7.5	39	1	0	1/2	10.0
11	3	1	2/6	7.5	40	1	0	1/1	10.0
12	1	0	1/3	10.0	41	6	4	2/11	4.2
13	1	0	1/3	10.0	42	2	1	1/2	7.5
14	1	0	1/2	10.0	43	2	0	2/3	10.0
15	2	0	2/4	10.0	44	1	0	1/2	10.0
16	2	0	2/3	10.0	45	1	0	1/3	10.0
17	3	0	3/8	10.0	46	2	1	1/3	7.5
18	3	2	1/3	6.0	47	6	4	2/7	4.2
19	1	0	1/3	10.0	48	4	2	2/6	6.0
20	2	0	2/3	10.0	49	2	0	2/3	10.0
21	2	1	1/5	7.5	50	2	0	2/5	10.0
22	4	2	2/7	6.0	51	2	0	2/3	10.0
23	3	1	2/4	7.5	52	1	0	1/3	10.0
24	1	0	1/2	10.0	53	4	1	3/4	7.5
25	3	1	2/7	7.5	54	2	1	1/4	7.5
26	3	1	2/8	7.5	55	2	1	1/4	7.5
27	2	1	1/4	7.5	56	1	0	1/3	10.0
28	1	0	1/5	10.0	57	2	0	2/3	10.0
29	4	2	2/4	6.0					

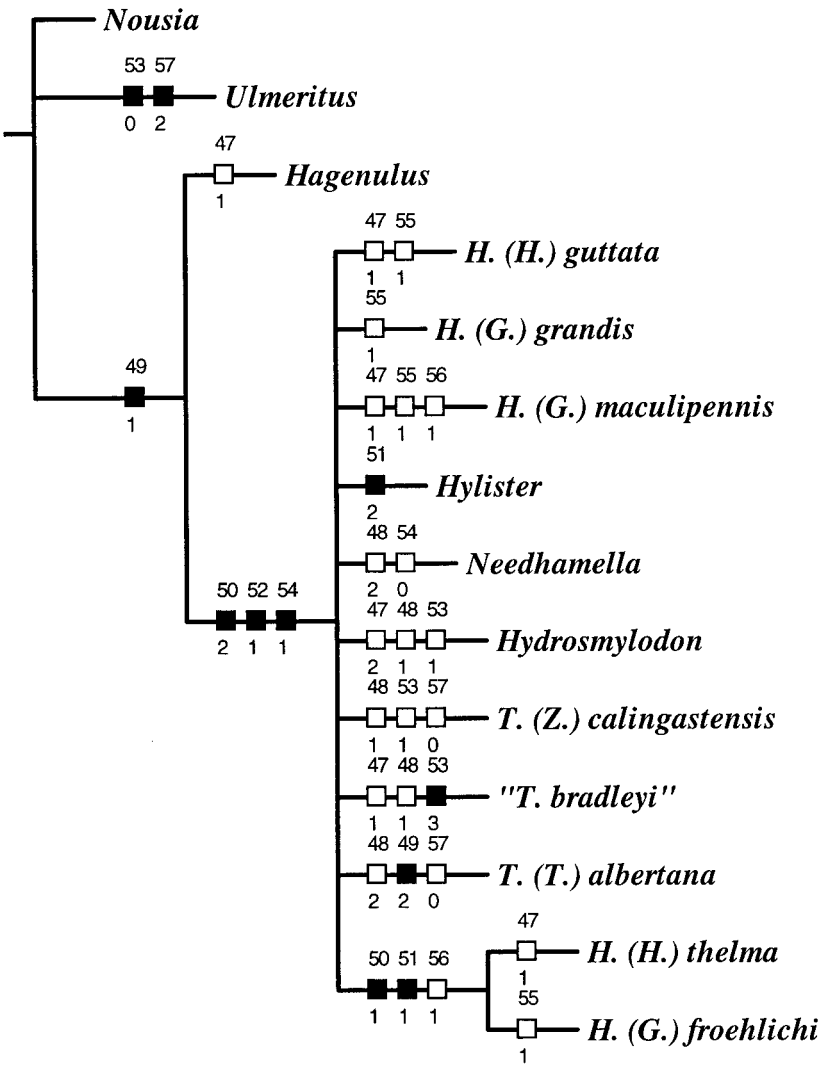

Fig. 8. Consensus tree with egg characters only. Solid squares = synapomorphies; open squares = homoplasies. Numbers above node are character numbers; those below nodes are character state numbers.

times. Some other commands for statistics of characters were used: ICC, reports fit for each character in the tree; STEPS, reports the number of steps and extra steps for each character in the tree. For evaluation of the information provided by the egg characters, three different analyses were performed: the first with the egg characters alone, the second with the adult and nymphal characters, and the third combining both sets of characters. It is important to stress that the first analysis (eggs only) was carried out to find what specific information can be provided by the egg ultrastructural morphology. However, the logical way to analyze the information is the one presented by the third analysis, which is based on all available characters. Attempting to make a phylogeny on egg characters alone would be a partial analysis, the same as using, for example, leg or wing characters alone.

The results from the analysis on the different sets of characters are as follows:

1) Egg characters alone (characters 47–57, Table 1). 80 trees were obtained, with a fit of 95.2. A strict consensus was obtained and is shown in Figure 8, with the characters illustrated.
2) Adult and nymphal characters (characters 1–46, Table 1). Only one tree was obtained, with a fit of 366.0. It is illustrated in Figure 9.
3) Adult, nymphal and egg characters together (characters 1–57, Table 1). Again, only one tree was obtained, with a fit of 456.9. This tree is illustrated in Figure 10.

Although the number of replications of "Mult*" were incremented up to 300, the results remained the same. To evaluate the branch support of the preferred hypothesis (Fig. 10), a Bremer support

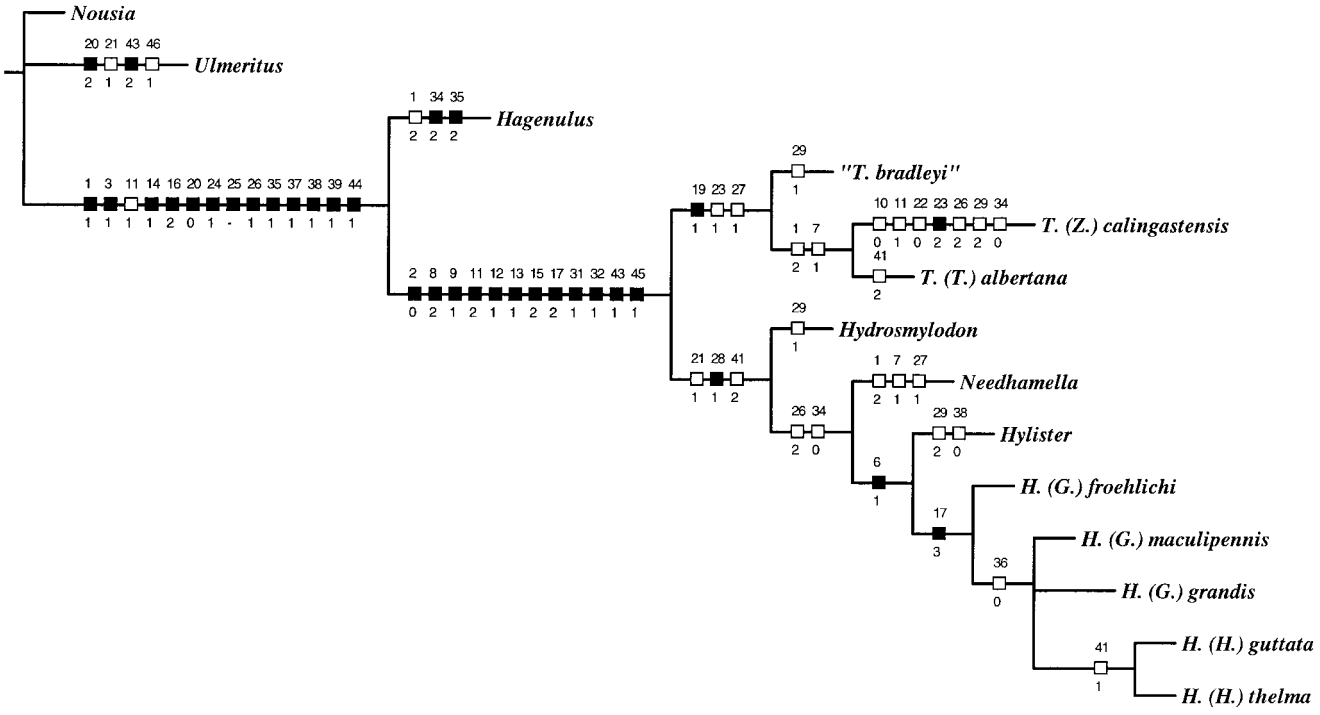

Fig. 9. Tree with maximum fit using nymph and adult characters only from matrix of Table 1.

(Bremer, 1988, 1994) was calculated with the commands implemented in Pee-Wee: "bs." Suboptimal trees, in this case, up to 30 steps less fit longer than the best tree were calculated to allow the estimation of Bremer values. The supports for each node are shown in Figure 11.

The consensus tree of the 80 original trees obtained from the matrix based on egg characters alone supports the monophyly of the *Hermanella–Traverella* generic complex, as was proposed by Flowers and Domínguez (1991). This group is supported by three synapomorphies: the polar regions of the eggs are either flat or slightly concave [character 50(2)], as in Figures 2a,e, 4c, 5a,c, although this character changed to state (1): one convex, the other variable, in *H. thelma* and *H. froehlichi* (Figs. 1a,d,

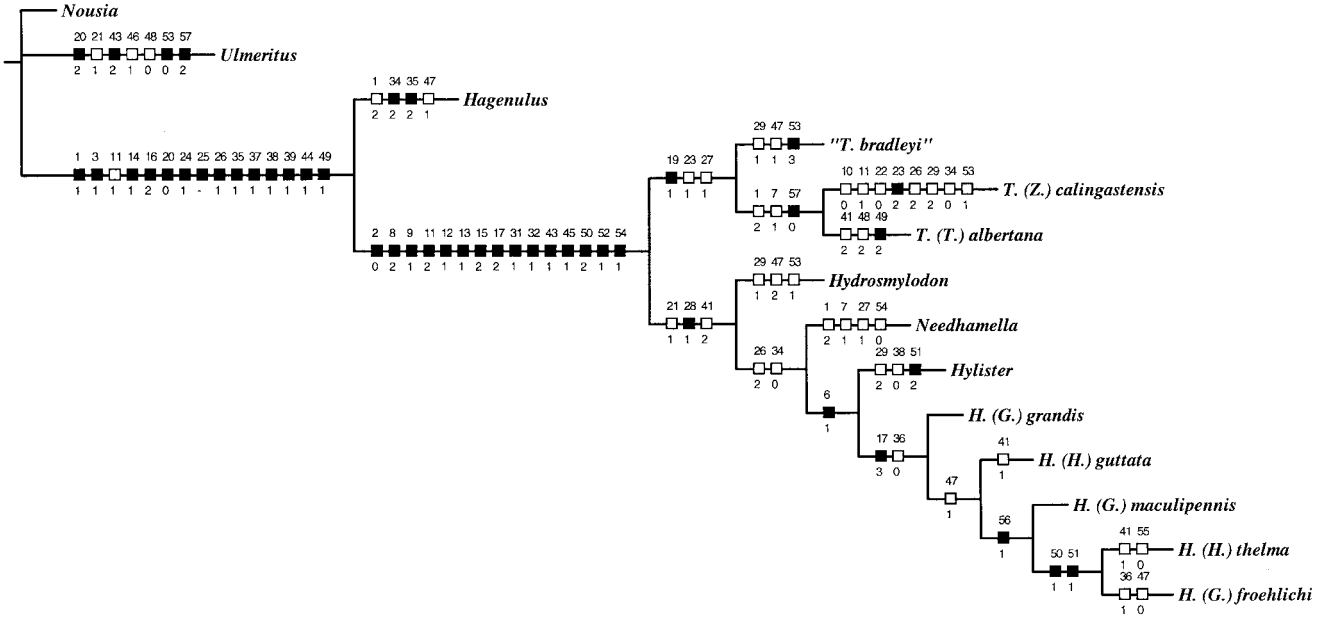

Fig. 10. Tree of maximum fit using nymph, adult, and eggs characters from matrix of Table 1.

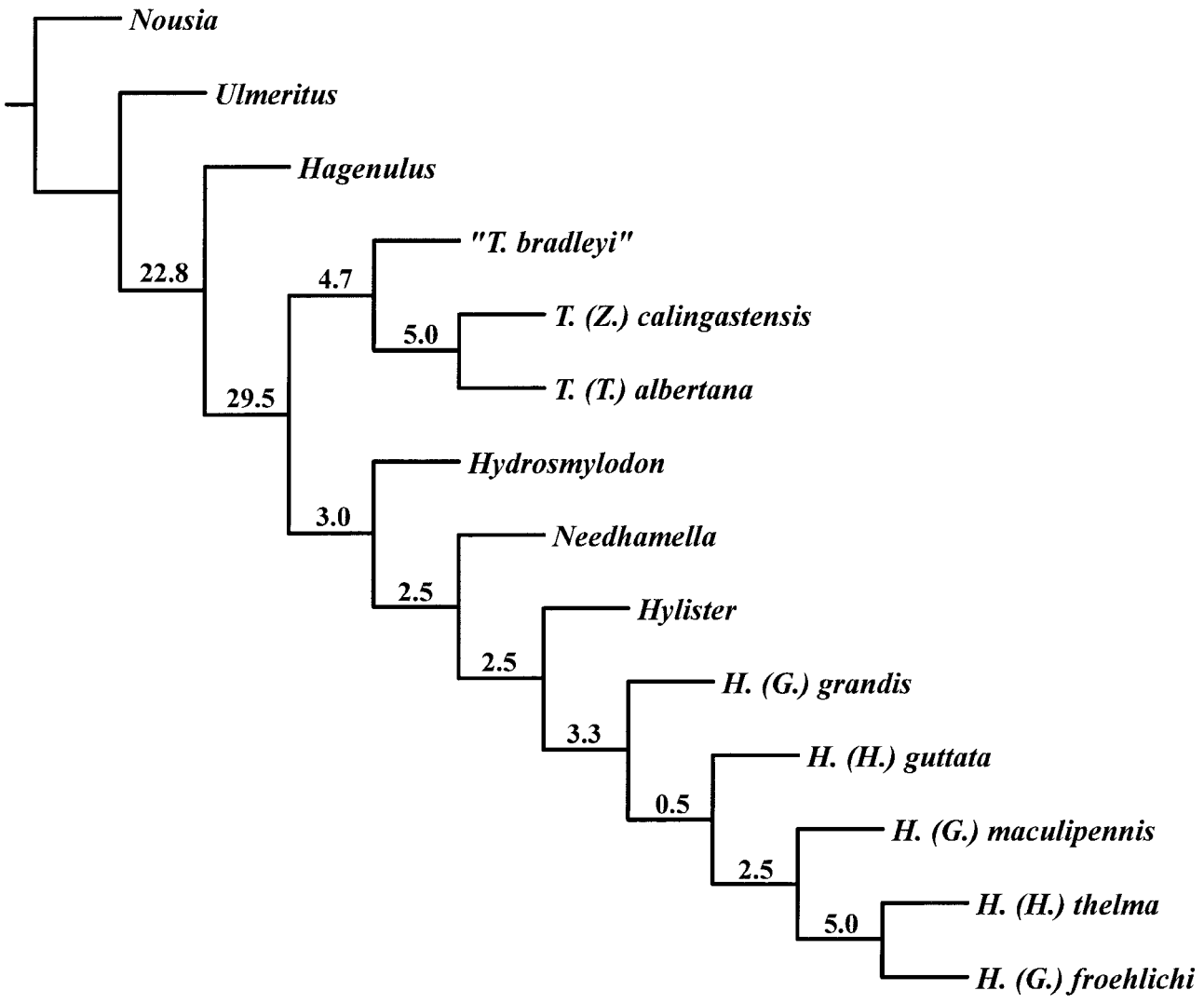

Fig. 11. Bremer support values for the preferred hypothesis illustrated in Figure 10.

3a); the terminal knobs of KCTs are always elevated above the coiled thread [character 52(1)] (Figs. 4e, 5b) and the micropyle is always supraequatorial [character 54(1)] (Figs. 1e, 2f, 3c). Within this complex, the only group defined is for *H. thelma* + *H. froehlichi*, by the character 50(1) mentioned above, and KCTs restricted to one polar region [character 51(1) in Fig. 1b]. It is interesting to note that these two species were described in different subgenera, based on morphological characters. Character 49(1) seems to be a synapomorphy of *Hagenulus* and the *Traverella–Hermanella* complex, but it must be kept in mind that there are many genera not included in the analysis. The remaining egg characters are either noninformative at this level (autapomorphic): 49(2), 51(2), and 53(3) or homoplastic: 47, 48, 53(1), 55, and 56.

The relationships of the genera present in the tree obtained from the analysis of the adult and nymphal characters are similar to the one presented by Flowers and Domínguez (1991). Nevertheless, in this case all the species of the genus *Hermanella* are included in the analysis, while in the 1991 article, only the subgenera were included, represented by their type species. From a taxonomic point of view, it is interesting to note that according to Figure 9 the subgenus *Hermanella (Hermanella)* is monophyletic *H. thelma* and *H. guttata* are in the same clade, while *Hermanella (Guayakia)* is not.

Finally, when analyzing all the characters together (Fig. 10) we can see the effect of the egg characters in the general context. The groups well supported obviously will not change, but in some cases will receive extra support. This is the situation for the clade of the whole *Traverella–Hermanella* complex, with synapomorphic egg characters 50, 52, and 54 (the highest Bremer support = 29.5). In others, not so well supported, as was the case of character 57(0), for example (see Fig. 9), the clade *T. calingastensis* + *T. albertana*, egg characters can

provide critical support, becoming the first synapomorphic character for the group (Bremer support = 5.0). It is interesting to note that when analyzing the egg matrix alone, character 57 appears as homoplastic but, when the whole matrix was analyzed, this character becomes a synapomorphy of this clade (compare this character in Figs. 8 and 10). The egg characters also produced some change in the topology of the tree, especially in clades poorly supported. For instance, the species of *Hermanella* in Figure 9 change radically in their relationships with the addition of three egg synapomorphic characters (50, 51, and 56). In Figure 10, both subgenera appear as paraphyletic.

DISCUSSION

It has been argued that in some groups the ultrastructural egg characters are not informative, due to their apparent random variability (Studeman and Landolt, 1997). In other groups, its possible phylogenetic values have been mentioned, but as far as we know no attempt to prove any of these hypotheses or to use egg characters for a cladistic analysis has been done. As a result of this study, we consider that egg characters behave as any other kind of morphological character: some are informative and some are not, depending on various factors such as taxonomic level of analysis or character selection and coding. In modern cladistics, characters are evaluated within the context of the analysis (Nixon and Carpenter, 1993). For this reason, we cannot consider a certain character "primitive," "plesiomorphic," or "apomorphic" a priori. Such designations are actually one of the results of the analysis within a context of global parsimony. One example of this is character 57.

In the present phylogenetic analysis some of the well-supported clades received extra support from the newly added egg characters. But more importantly, in some weakly supported clades the eggs provided the first synapomorphies, as is the case for the clade representing the genus *Traverella s.s*. (excluding *T. bradleyi*, considered a different genus). In the previously mentioned clades, the egg characters did not produce any change in the topology of the cladogram, corroborating the previous results. Inversely, in the *Hermanella* clade the addition of the egg characters produced a change in the tree topology. The cladogram based on the nymph and adult characters corroborated the monophyly of *Hermanella (Hermanella)* (see Fig. 9), while the addition of the egg characters produced a different topology with both subgenera [*H. (Hermanella)* and *H. (Guayakia)*] (see Fig. 10) appearing as paraphyletic. In this sense, the egg characters of the *Hermanella* group showed that they can provide critical phylogenetic information.

A note about egg characters: it has been maintained that in some cases the eggs extracted from nymphs, subimagoes, and imagoes are identical (Studeman and Landolt, 1997); in others, they change in size and form after oviposition (Soldán, 1979; Kang and Yang, 1994). Does this situation undermine the value of egg characters? We believe that as long as the characters are constant in one stage, they are usable. For example, the polar caps, present in eggs of certain mayfly families, are very regular, while in the abdomen of the female, and have been widely used. But when the eggs come in contact with water the polar caps swell and totally change form (see fig. 41 of Koss, 1968). In the same sense, if, for example, the eggs are consistently prismatic in the *Traverella–Hermanella* complex while in the nymphal and adult abdomen, this can be used as a character, no matter if they can potentially change in form when laid in water.

CONCLUSIONS

Based on this study, we conclude that:

1) The morphology of eggs is an important and valuable source of information for associating the different stages of maturation in each species. In Atalophlebiinae it was found that the different chorionic structures do not change in shape and size among the different stages. The KCTs, the attachment structures present in the Atalophlebiinae, are the most important source of information (four out of eleven egg characters in the matrix), plus some other characters not coded in the matrix but present in the descriptions. Neither the structure of the micropyle nor the chorion sculpture are very variable among the different species. However, it was found that the position of the micropyle in the *Hermanella–Traverella* complex represents a very important character to define the species complex since it is supraequatorial, while in the remaining of the studied Atalophlebiinae species it is equatorial. The prismatic egg shape in the generic complex analyzed is not a consequence of a fixation artifact. This shape is natural and represents a synapomorphy for the complex.
2) The egg ultrastructural characters can be valuable for phylogenetic analysis. As in any stage of living beings, some characters are variable, some are not. As systematists and morphologists our task is to find the ones that can provide the information we need at a determined level of analysis. We consider that there is already enough good descriptive information on the egg ultrastructure to complement traditionally used characters to elucidate the possible character homologies and phylogenetic relationships among Ephemeroptera taxa.
3) Egg characters were indeed found to be important in establishing the phylogenetic relationships in the *Hermanella–Traverella* generic complex.

4) The eggs in the studied Atalophlebiinae present the same shape before and after oviposition; in the case of the *Hermanella–Traverella* complex, the prismatic shape of the eggs is not a deformation from being packed in the oviduct. The prismatic shape is maintained after oviposition in water.
5) According to the present analysis, current subgenera in *Hermanella* are paraphyletic.

ACKNOWLEDGMENTS

R.W. Flowers and E. Gaino provided interesting comments and improved the English. We thank the staff of the Electron Microscopy Laboratory of Northwestern Argentina (LAMENOA) from the National University of Tucumán. Special thanks are extended to Ing. Alberto Andrada for his help with the scanning electron microscope. K. Nixon (Cornell University) generously provided the software Winclada.

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APPENDIX A: LIST OF THE SPECIES EXAMINED WITH SEM

The material used in this study belongs to the following institutions: Instituto-Fundación Miguel Lillo, Tucumán, Argentina (IFML); Florida A&M University, Tallahassee, FL, USA (FAMU); and National Museum of Natural History, Smithsonian Institution, Washington, DC, USA (NMNH).

Traverella (Zonda) calingastensis Domínguez: Argentina, San Juan Prov., Calingasta, Los Patos River, 28/I/82, Domínguez Col.

Traverella albertana (McDunnough): USA, Colorado, Mesa Co., Colorado River at Fruita. 28/VIII/1973. G.F. and H. Edmunds Col.

Traverella bradleyi Panama, Bocas del Toro Prov. Quebrada Cañaza at pipeline. 16/5/85. Flowers, R. Col.

Hermanella (Guayakia) froehlichi Ferreira and Domínguez: Paratype, Brasil, Sao Paulo, XI/89, Carrego Do Pedregulho, M. Ferreira and N. Froehlich Col.

Hermanella (Guayakia) grandis Dominguez and Flowers: Argentina, Misiones, San Vicente, 30/12/86. Domínguez, Col.

Hermanella (Guayakia) maculipennis (Ulmer): Argentina, Misiones, Arroyo Yaboti, 9/11/98. Domínguez Col.

Hermanella (Hermanella) thelma Needham and Murphy: Argentina, Misiones, Iguazu River, 23/11/82. Bechara Col.

Hermanella (Hermanella) guttata Dominguez and Flowers: Argentina, Misiones, Alegre, Piray Guayzu, 4/12/86. Domínguez Col.

Hylister plaumanni Domínguez and Flowers: Brasil, Paraná, Ipriranga River, 23/II/69, W.L. and J.G. Peters Col.

Needhamella ehrhardti (Ulmer): Argentina, Misiones, Alegre Piray-Guazú, 3-4/XII/86, Domínguez Col.

Hydrosmilodon saltensis Flowers and Domínguez: Argentina, Tucuman, El Boyero, Rio Salí, 830 mt. Domínguez and Cuezzo Col.

Hagenulus caligatus: Cuba, La Habana Prov. Guines, III-1966. P. Alayo. Cuba, Pinar del rio Soroa, 27/4/83, P.J. Spangler and I. Fernandez Col.

Nousia delicata Navas: Chile, Manle Prov., laguna del Manle, 2200 mts., 18/4/57.

Ulmeritus carbonelli Traver: Uruguay, Artigas, Arroyo de la Invernada. 21/II/1954, C.S. Carbonell Col.

APPENDIX B: CHARACTER LIST

Nymphal Characters

Labrum

1. Width of labrum \ width of clypeus; 0: <or=1.1; 1: 1.2-1.4; 2: =or>1.5 [additive].
2. Denticles on anteromedian emargination; 0: absent; 1: present.
3. Median hood; 0: absent; 1: present.
4. Dorsal row of setae; 0: apical; 1: medial.
5. Shape of dorsal row of setae; 0: entire; 1: divided.
6. Area anterior to dorsal row covered with long setae; 0: absent; 1: present.

Clypeus

7. Anteromedian projection; 0: absent; 1: present.
8. Lateral margins; 0: parallel; 1: divergent; 2: strongly concave [additive].

Maxillae

9. Subapical pectinate setae; 0: present; 1: absent.
10. Tusk on inner apical margin; 0: absent; 1: present.
11. Segment 2 / segment 1 of palpi; 0: subequal; 1: 1.1-2; 2: >2 [additive].
12. Ordered row of setae on segment 3 of palpi; 0: absent; 1: present.
13. Thick blunt setae on segment 1 of palpi; 0: absent; 1: present.
14. Setae on outer margin of stipes; 0: present; 1: absent.
15. Position of articulation of palpi; 0: on apical 2/3; 1: medial; 2: basal [additive].

Mandible

16. Shape of outer margin; 0: smoothly curved; 1: obtuse; 2: right angled [additive].
17. Setae on outer margin; 0: on 2/3 or more; 1: on 1/2; 2: on basal 1/4; 3: absent [nonadditive].
18. Denticles on outer incisor; 0: present; 1: absent.
19. Patch of long setae on venter; 0: absent; 1: present.

Labium

20. Spines on palpi; 0: absent; 1: on segment 3 only; 2: on 2 and 3 [additive].
21. Subapical seta row on paraglossae; 0: absent; 1: present.
22. Segment 1/segment 2 of palpi; 0: >1.1; 1: subequal, 1.1-0.9; 2: <0.9 [additive].
23. Segment 3/ segment 2 of palpi; 0: <0.8; 1: 0.8-1.2; 2: >1.2 [nonadditive].

24. Shape of palpi segment 2; 0: not elbowed; 1: elbowed.
25. Row of dorsal setae on palpi segment 2; 0: absent; 1: present, <4; 2: present, many [additive].

Legs

26. Dorsal spines or setae on fore tibia; 0: absent; 1: setae present; 2: spines present [additive].
27. Dense row of setae on outer margins of femora and tibiae; 0: absent; 1: present.
28. Denticles on tarsal claws; 0: subequal; 1: subapical larger.

Gills

29. Gills on abdominal segments; 0: 1-7; 1: 7th vestigial; 2: 1-6 [nonadditive].
30. Gill shape; 0: plate-like, no processes; 1: otherwise.

Abdomen

31. Posterolateral projections on segments; 0: 5 or 6 to 9; 1: 7 or 8 to 9.

Adult Characters

Fore Wings

32. Fork of MA; 0: symmetrical; 1: asymmetrical.
33. Slanting cross vein above MA fork; 0: absent; 1: present.
34. Attachment of ICu1; 0: free basally; 1: attached to CuA; 2: attached to CuP [nonadditive].

Hind Wings

35. Shape of costal projection; 0: obtuse; 1: acute; 2: very acute [additive].
36. Location of apex of costal projection; 0: in basal 1/2; 1: beyond basal 1/2.
37. Vein MP; 0: forked; 1: unforked.
38. Length of Sc; 0: >than 0.6 of wing length; 1: <than 0.6 of wing length.
39. Ending of Sc; 0: in wing margin; 1: in cross vein or costal projection.

Legs

40. Claws of a pair; 0: similar; 1: dissimilar.

Male Genitalia

41. Paired projections on subgenital plate; 0: absent; 1: broad; 2: narrow [additive].
42. Division of penis; 0: completely divided; 1: apical 1/2-1/4 separated.
43. Segment 1 of forceps; 0: broader in basal 1/3-1/4; 1: subcylindrical; 2: basal swelling [nonadditive].
44. Spines on penis; 0: absent; 1: present.
45. Base of penis abruptly swollen; 0: absent; 1: present.

Abdomen

46. 9th female sternite; 0: strongly cleft; 1: entire or shallowly cleft.

Eggs

47. Bumps; 0: absent; 1: small, scattered; 2; big, numerous [nonadditive].
48. Chorion surface; 0: smooth; 1: granulated; 2: wrinkled [nonadditive].
49. General shape; 0: oval, rounded in cross section; 1: prismatic, hexagonal or quadrate in cross section; 2: cylindrical [nonadditive].
50. Polar regions; 0: both convex; 1: one convex the other variable; 2: both flat or slightly concave.
51. KCTs; 0: regularly distributed in all surface; 1: restricted to one polar region; 2: restricted to both polar regions [nonadditive].
52. Terminal knob of KCTs; 0: not elevated, leveled with coiled thread; 1: elevated above coiled thread.
53. KCT collar; 0: wide, continuous, polyhedric; 1: narrow, not continuous; 2: narrow, continuous; 3: absent [nonadditive].
54. Position of micropyle; 0: equatorial; 1: supraequatorial.
55. Area surrounding micropyle; 0: not sieved; 1: sieved.
56. Micropyle opening; 0: irregular; 1: ring-like.
57. KCT Knob; 0: knob covering completely the coiled thread; 1: knob covering partially the coiled thread; 2: knob surrounded by thread coils [nonadditive].