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Phylogeny of the Arachnid Order Opiliones (Arthropoda) Inferred from a Combined Approach of Complete 18S and Partial 28S Ribosomal DNA Sequences and Morphology

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The phylogenetic relationships among the main evolutionary lines of the arachnid order Opiliones were investigated by means of molecular (complete 18S rDNA and the D3 region of the 28S rDNA genes) and morphological data sets. Equally and differentially weighted parsimony analyses of independent and combined data sets provide evidence for the monophyly of the Opiliones. In all the analyses, the internal relationships of the group coincide in the monophyly of the following main groups: Cyphophthalmi, Eupnoi Palpatores, Dyspnoi Palpatores, and Laniatores. The Cyphophthalmi are monophyletic and sister to a clade that includes all the remaining opilionid taxa (=Phalangida). Within the Phalangida the most supported hypothesis suggests that Palpatores are paraphyletic, as follows: (Eupnoi (Dyspnoi + Laniatores)), but the alternative hypothesis (Laniatores (Eupnoi + Dyspnoi)) is more parsimonious in some molecular data analyses. Relationships within the four main clades are also addressed. Evolution of some morphological characters is discussed, and plesiomorphic states of these characters are evaluated using molecular data outgroup polarization. Finally, Martens' hypothesis of opilionid evolution is assessed in relation to our results. © 1999 Academic Press

INTRODUCTION

The order Opiliones, commonly known as harvestmen, is a cosmopolitan group of arachnids. Opiliones are often found in disturbed habitats as well as in forests, under stones, in caves, on the trunks of trees, on the soil, in forest litter, in soil crevices, and sometimes rather deep in the soil. About 90 species are strictly cavernicolous (Rambla and Juberthie, 1994).

Three suborders of Opiliones are widely accepted: Cyphophthalmi, Laniatores, and Palpatores. Approximately 5000 species of Opiliones have been described. Cyphophthalmi (with about 100 species) have very discontinuous distributions, occurring mainly in tropical, subtropical, and temperate regions (Juberthie, 1988). Laniatores is the dominant group of Opiliones in southern latitudes, found mainly in America, tropical Asia, southern Africa, and Australia. Both Cyphophthalmi and Laniatores are poorly represented in Europe. Palpatores have fewer species than Laniatores; however, they are distributed worldwide and include the most commonly known Opiliones in the northern hemisphere. Fossil species from the Carboniferous are known and several genera are present in the Baltic amber fossil beds, as well as in other fossil beds.

Background in Opilionid Systematics

The first studies on the systematics of the Opiliones were developed in the last century. Sørensen (1873) divided the order Opiliones into two families named Gonyleptidae and Opilionidae. Thorell (1876) changed these families into taxonomic suborders, naming them SO. Laniatores and SO. Palpatores. Simon (1879) renamed these two suborders Mecostethi (=Laniatores) and Plagiostethi (=Palpatores) and described the new suborder Cyphophthalmi. Later, Hansen and Sørensen (1904) divided Palpatores into two tribes: Dyspnoi and Eupnoi. This grouping of Opiliones into three suborders and two tribes of Palpatores was perpetuated by Roewer (1923; and successive supplements) in his monumental *Compendium of the World Opilionid Fauna*.

Subsequently, Silhavý (1961) raised the Dyspnoi and Eupnoi to subordinal rank and also split Laniatores into two more suborders, Oncopodomorphi and Gonyleptomorphi, leading to the division of the order Opil-

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iones into five suborders. This proposal has not been widely followed.

The only author who considered the Opiliones paraphyletic was Savory (1977), who divided them into two different orders; the Cyphophthalmi and the Phalangida, the latter including Laniatores and Palpatores. This proposal by Savory was rebutted by Shear (1980), who did not agree with the cyphophthalmid "uniqueness" listed by Savory.

Despite the existence of these alternative phylogenetic hypotheses, the relationships among the main opilionid groups have received relatively little examination using modern systematic methods until Martens and co-workers presented their analyses derived from genitalic characters (Martens, 1976, 1980, 1986; Martens *et al.*, 1981), basically from the anatomy of the ovipositor. These characters led Martens to consider Palpatores a paraphyletic group, the representatives of which had successively split at different times from the precursors of the modern Cyphophthalmi. Consequently, he proposed to unite the Cyphophthalmi and the resulting paraphyletic Palpatores in a new clade named "Cyphopalpatores," based on their long, segmented ovipositor with a bifurcate tip (Fig. 1).

Shear (1980) discussed the presence of a second important character shared by Cyphophthalmi and Palpatores, the number of midgut diverticula (Dumitrescu, 1975a,b). The classification system of Martens was also adopted by some modern authors working on Opiliones taxonomy (Crawford, 1992; Cokendolpher and Lee, 1993).

Within the suborder Palpatores, Shear (1975) proposed the use of superfamily names to replace the old names "Eupnoi" and "Dyspnoi," and so Eupnoi was split into Caddoidea and Phalangioidea. The Dyspnoi was not rearranged, although it was renamed Troguloidea (including both the currently accepted Troguloidea and the Ischyropsalidoidea). One year later, Martens (1976) presented an opilionid cladogram, in which the tribe "Dyspnoi," the most problematic group of Palpatores, was divided into two taxa at the superfamily level: the Troguloidea and the Ischyropsalidoidea, which together were not considered a monophyletic clade. Thus, the former Palpatores became a paraphyletic clade, whose members were arranged in four superfamilies: Caddoidea, Phalangioidea, Troguloidea, and Ischyropsalidoidea. Further studies along these lines have been conducted by several authors (Gruber, 1978; Martens, 1980, 1986; Martens *et al.*, 1981; Shear and Gruber, 1983).

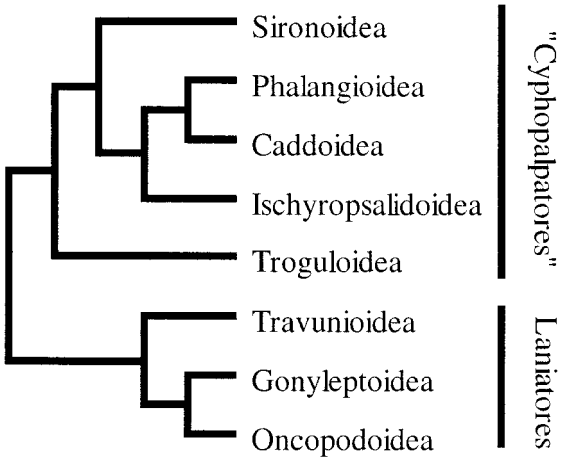

FIG. 1. Cladogram proposed by Martens (1976, 1980, 1986) and Martens *et al.* (1981) for the phylogenetic relationships within the order Opiliones.

As a summary of the systematics of the Opiliones, the existence of three principal groups (Cyphophthalmi, Laniatores, and Palpatores) has been commonly accepted, except by Martens and co-workers. The evident difficulties emerge when establishing their phylogenetic relationships.

The present work is a phylogenetic approach to the arachnid order Opiliones, based on cladistic analyses of molecular and morphological data. The molecular data matrix has complete 18S rDNA sequences and partial 28S rDNA sequences (D3 region) from 15 species of Opiliones, representing the three suborders: 3 species of Cyphophthalmi; 6 species of Laniatores representing the three main lines, Gonyleptoidea, Oncopodoidea, and Travunioidea; and 6 species of Palpatores representing the Dyspnoi (Ischyropsalidoidea and Troguloidea) and the Eupnoi (Phalangioidea and Caddoidea) (see Table 1). Sequences from other arachnids were used to test the monophyly of the Opiliones, and two Merostomata (*Limulus polyphemus* and *Carcinoscorpius rotundicaudatus*) were used as outgroup for all the Arachnids. A data matrix of 45 morphological and ecological characters was developed for the 15 opilionid species.

The aim of this work was to test higher categories used in opilionid systematics and to solve the internal relationships of the order, testing the classical morphological classifications with the topologies obtained from molecular data. Furthermore, the cladistic analysis based on total evidence (combined morphological + molecular data) was used to provide answers about the plesiomorphic/synapomorphic state of some of the morphological characters used by different authors, with the aim of finding the most coherent solution for these characters according to present-day knowledge of the Opiliones.

MATERIALS AND METHODS

Biological Samples

Fifteen opilionid species were collected and frozen at −80°C or preserved in absolute ethanol for the molecular study. The material used for the morphological study was preserved in 70% ethanol. An effort was made in the taxonomic sampling to analyze the maximum morphological diversity inside each clade, which is also assumed to reflect the maximum genetic diversity. A checklist of the sampled species and their

TABLE 1

Taxa Used in this Study with the Accession Codes from GenBank

	Species name	18S	28S
Class Chelicerata			
Subclass Arachnida			
Order Opiliones			
Cyphophthalmi			
Superfamily Sironoidea			
F. Sironidae			
	Siro rubens	U36998	U91494
	Parasiro coiffaiti	U36999	U91495
F. Stylocellidae			
	Stylocellus sp.	U91485	U91496
'Palpatores'			
Eupnoi			
Superfamily Phalangioidea			
F. Phalangiidae			
	Odiellus troguloides	X81441	U91500
F. Leiobunidae			
	Nelima sylvatica	U91486	U91501
Superfamily Caddoidea			
F. Caddidae			
	Caddo agilis	U91487	U91502
Dyspnoi			
Superfamily Ischyropsalidoidea			
F. Ischyropsalidae			
	Ischyropsalis luteipes	U37000	U91497
Superfamily Troguloidea			
F. Dicranolasmatidae			
	Dicranolasma soerenseni	U37001	U91498
F. Nemastomatidae			
	Centetostoma dubium	U37002	U91499
Laniatores			
Superfamily Travunioidea			
F. Triaenonychidae			
	Equitius doriae	U37003	U91503
Superfamily Oncopodoidea			
F. Oncopodidae			
	Oncopus cf. *alticeps*	U91488	U91504
Superfamily Gonyleptoidea			
F. Phalangodidae			
	Maiorerus randoi	U37004	U91505
	Scotolemon lespesi	U37005	U91506
F. Cosmetidae			
	Gnidia holnbergii	U37006	U91507
F. Gonyleptidae			
	Pachyloides thorellii	U37007	U91508
Order Solifugae	*Eusimonia wunderlichi*	U29492	
Order Ricinulei	*Pseudocellus pearsei*	U91489	
Order Scorpionida	*Androctonus australis*	X77908	
Subclass Merostomata			
Order Xiphosura	*Limulus polyphemus*	U91490	U91492
	Carcinoscorpius rotundicaudatus	U91491	U91493

systematic positions is given in Table 1. Detailed information on collecting localities can be provided upon request, and voucher specimens are deposited at the Departament de Biologia Animal (Universitat de Barcelona).

Sample Preparation for the Molecular Study

Genomic DNA samples were obtained from fresh, frozen, or ethanol-preserved tissue, homogenized in a solution of guanidinium isothiocyanate following a modified protocol for RNA extraction (Chirgwin *et al.*, 1979). The 18S rDNA was PCR-amplified in two or three overlapping fragments of about 950, 900, and 850 bp each, using primer pairs 1F-5R, 3F-18Sbi, and 5F-9R, respectively. Primers used in amplification and sequencing are described in Giribet *et al.* (1996), except for forward primer 18Sa2.0 (5′-ATGGTTGCAAAGCTGAAAC-3′) and reverse primer 18Sbi (5′-GAGTCTCGTTCGTTATCGGA-3′). The 28S rDNA fragment, of about 400 bp, was amplified using primer pair 28Sa (5′-GACCCGTCTTGAAACACGGA-3′) and 28Sb (5′-TCGGAAGGAACCAGCTACTA-3′). Amplification was carried out in a 100-μL volume reaction, with 0.6 units of DynaZyme polymerase, 100 μM dNTPs, and 0.5 μM each primer. The PCR program consisted of a first denaturing step of 5 min at 95°C and 35 amplification cycles (94°C for 45 s, 49°C for 45, 72°C for 1 min).

Some samples were purified and ligated into pUC 18 *Sma* I/BAP dephosphorylated vector using the Sure-Clone Ligation Kit (Pharmacia P-L Biochemicals) as described in Giribet *et al.* (1996), but competent JM 105 or DH5 strains of *Escherichia coli* cells were used. Sequencing was performed by the dideoxy termination method (Sanger *et al.*, 1977) using T7 DNA polymerase (^{T7}Sequencing Kit from Pharmacia Biotech).

Other samples were purified with Geneclean II kit (BIO 101 Inc.) and directly sequenced using an automated ABI Prism 377 DNA sequencer. Cycle sequencing with AmpliTaq DNA Polymerase FS using dye-labeled terminators (ABI PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit) is also based on the Sanger method and was performed in a Perkin–Elmer GeneAmp PCR system 9600 or 2400. Amplification was carried out in a 20-μl volume reaction: 8 μl of Terminator Ready Reaction Mix, 10–30 ng/ml of PCR product, 5 pmol of primer, and dH_2O to 20 μl. The cycle-sequencing program consisted of a previous step of 94°C for 3 min, 25 sequencing cycles (94°C for 10 s, 50°C for 5 s, 60°C for 4 min), and a rapid thermal ramp to 4°C and hold. The dye-labeled PCR products were ethanol-precipitated with 0.1 volumes of 3 M NaOAc, pH 5.2, and 2 volumes of 95% ethanol; 10 min on ice and 20 min centrifuging at 12,500 rpm. The pellet was cleaned with 50 μl of 70% ethanol and dried in a speed-vac at 60°C for 5 min.

All the new sequences were deposited in GenBank (see accession codes in Table 1).

Phylogenetic Analysis

DNA sequences were aligned using the GDE editor (Smith *et al.*, 1994) helped by the 18S rDNA secondary structure prediction of the spider *Eurypelma californica* (Hendriks *et al.*, 1988). Two small regions of the 18S rDNA locus (23 positions) and three of the 28S

rDNA locus (57 positions) corresponding to divergent loops that could not be aligned unambiguously were excluded from the data set (see details under Results).

The morphological characters of the 15 opilionid species were compiled initially from literature sources and from direct specimen examination. In total, 45 characters were analyzed, covering internal anatomy, external morphology, penis and ovipositor anatomy, as well as ecological and ethological characters (Appendix 2).

Parsimony analyses of the parsimony-informative positions were performed with PAUP 3.1.1 (Swofford, 1993). Heuristic searches were run for 100 random addition sequence replicates using TBR branch swapping. Tree lengths and statistical indexes are referred to informative sites only. Character optimization was done with CLADOS v. 1.49.5 (Nixon, 1995) using Fitch optimization favoring reversals.

Different parsimony approaches were tried with the original molecular data sets independently and in a combined analysis. First, equally weighted parsimony was used, with all characters treated as unordered. After performing this analysis, the transition/transversion ratio over the stored MPTs was estimated with the State Changes & Stasis option in MacClade 3.04 (Maddison and Maddison, 1992), and this average ratio was used in a step matrix for a differentially weighted parsimony analysis. In both cases, gaps were treated as missing data. Furthermore, a third analysis of equally weighted parsimony was performed using gaps as a fifth character state. The two merostomates have been used as the arachnid outgroup, as established in morphological cladistic analyses (Weygoldt and Paulus, 1979; Lauterbach, 1983; Shultz, 1990; Wheeler *et al.*, 1993; Wheeler, 1997) as well as in previous molecular analysis (Wheeler *et al.*, 1993; Wheeler, 1997).

Nonparametric bootstrapping (Felsenstein, 1985) and branch support (Bremer, 1988, 1994) were performed to evaluate branches in the phylogenetic estimates. Bremer support was calculated with PAUP by storing all trees from 1 to 11 steps longer than the MPT's, using 10 random addition replicates with TBR branch swapping. Nodes collapsed in the strict consensus tree of all these sets of trees ($N + 1, N + 2, \ldots, N + 11$) represent Bremer support values of 1, 2, . . ., 11.

Morphological characters were equally weighted. All characters (except 2, 3, 8, 13, 18, 19, and 20) were always considered unordered. No considerations of outgroup relationships were taken into account because of the difficulty of finding characters homologous with other arachnid groups that at the same time would be useful in solving internal phylogenetic relationships of the order Opiliones. The few characters that could be found in other arachnid groups were treated as ordered.

Molecular and morphological data sets were combined into a single matrix for a parsimony analysis. No weighting was applied to any character. Character congruence among molecular data sets and among molecular and morphological data sets was estimated using the Mickevich–Farris length incongruence index (Mickevich and Farris, 1981).

RESULTS

Sequence Data Analysis

The total alignment length of the complete 18S rDNA gene of 15 Opiliones plus 5 other chelicerates comprised 1826 sites. Positions 675–688 and 1382–1390 corresponding to the regions E21-1 and 41, respectively (Hendriks *et al.*, 1988), were removed from the analyses, due to their alignment ambiguity (see Discussion). The total number of characters after removal was 1803, of which 207 were parsimony informative (the numbers refer to the analyses that code gaps as missing data). Thus, parsimony-informative positions made up 11.48% of the total alignment. Uninformative positions were excluded in order to avoid overestimation of CI. Most of the phylogenetically informative positions of the alignment were found at the following sites (named after the secondary structure prediction of Hendriks *et al.*, 1988): 6, 8-9, E10-1, E10-2, 11, E-21, 41, 42, 44, and 47. The transition/transversion ratio estimated for the four MPTs obtained after the heuristic search in PAUP was 1.59 on average, 39% of changes being T ↔ C transitions.

The total alignment of the D3 region of the 28S rDNA gene of 15 Opiliones plus 2 merostomates comprised 373 sites. Positions 92–121, 136–155, and 176–182 were also removed from the analysis, giving a final alignment of 316 positions, of which 41 were parsimony informative. The rate of parsimony-informative positions was 12.97%, slightly higher than 18S data values. The transition/transversion ratio estimated for the six MPTs obtained after the heuristic search in PAUP was 1.60 on average.

Consequently, the combined molecular alignment comprised 2119 positions and 248 parsimony-informative sites, and the estimated transition/transversion ratio for the three MPTs obtained was 1.54.

Alignments in GDE format are accessible by anonymous ftp from porthos.bio.ub.es/pub/incoming/18Sphylogeny.

Phylogenetic Analysis of Molecular Data

For the equally weighted parsimony analysis of the 18S rDNA data set, a 100-replicate heuristic search was undertaken in PAUP. The number of MPTs obtained was four (497 steps; CI = 0.579; RI = 0.727). These trees differed in the internal branching pattern of the Laniatores and in the position of the Solifugae and Ricinulei, but did not affect interrelationships among the main opilionid clades.

The strict consensus tree of the 18S rDNA data set

showed the following branching pattern: (Merostomata ((Scorpionida + Ricinulei + Solifugae) + (Cyphophthalmi + (Palpatores t+ Laniatores)))). Opiliones were thus represented as a monophyletic clade, with the Cyphophthalmi as the sister taxon to the remaining opilionid taxa. The Palpatores were also monophyletic (as in Fig. 2A) and divided into two evolutionary lines, the Eupnoi and the Dyspnoi (*sensu* Hansen and Sørensen). The Laniatores were also monophyletic, but their internal resolution was not clear. The same results were found when using gaps as a fifth character state (four MPTs of 517 steps; CI = 0.592; RI = 0.732).

Differentially weighted parsimony (TS/TV = 1.5) analysis gave a single cladogram of 747 steps, which differs considerably from equally weighted parsimony analysis. In this latter case, the total internal resolution was fully compatible with that shown in morphological data analysis, with the following pattern: (Cyphophthalmi (Eupnoi (Dyspnoi + Laniatores))) (as in Fig. 2B). The internal resolution of each group was similar to that obtained in unweighted parsimony analysis and it only differed in the Laniatores: (*Equitius* (*Scotolemon* (*Oncopus* (*Maiorerus* (*Gnidia* + *Pachyloides*))))).

The same analyses were run for the 28S data set. In the equally weighted parsimony analysis, six MPTs of 90 steps were obtained (CI = 0.611; RI = 0.750). Palpatores always appeared to be a paraphyletic group. The Eupnoi sometimes grouped with the Cyphophthalmi and sometimes as the sister group of the clade (Dyspnoi + Laniatores). The grouping of Dyspnoi + Laniatores is well supported in all trees (100% bootstrap value). The internal phylogeny of the Cyphophthalmi, the Eupnoi, and the Dyspnoi was the same as in all other analyses. Within the Laniatores, the Travunioidea (*Equitius*) was the sister group of the remaining taxa. In addition, the two Phalangodids (*Scotolemon* and *Maiorerus*) were monophyletic, as were *Gnidia* + *Pachyloides*.

The differentially weighted parsimony (TS/TV = 1.5) analysis gave two MPTs compatible with the trees obtained in the equally weighted parsimony analysis of the 28S data set. The internal topologies differed slightly within the Laniatores, but the Travunioidea

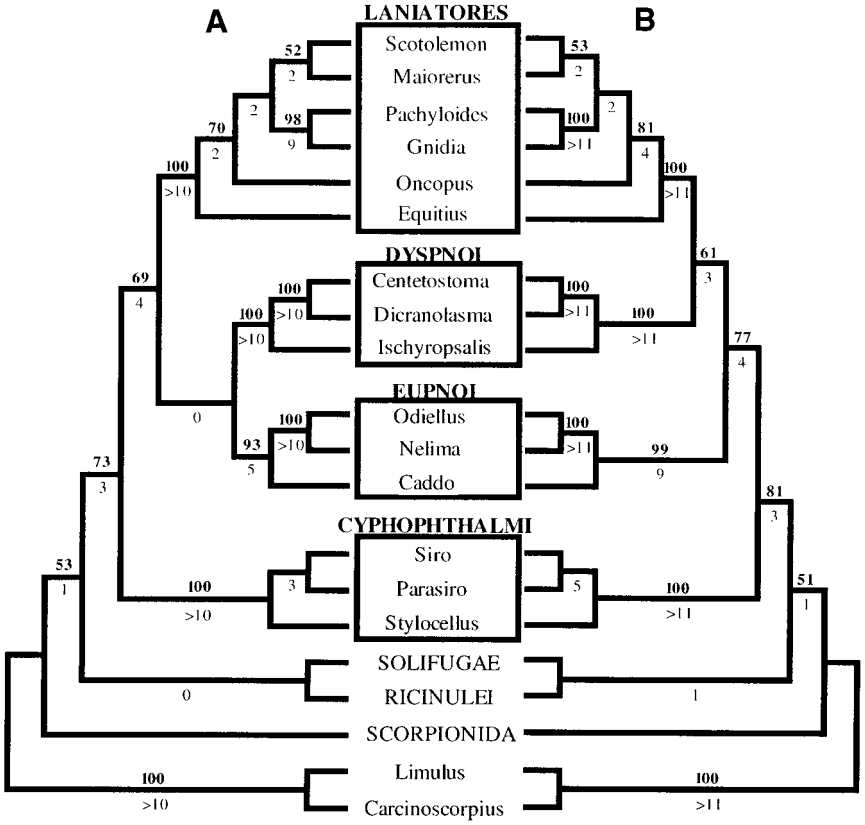

FIG. 2. Results of the relationships for the order Opiliones. Tree (A) represents one of the three MPTs (629 steps; CI = 0.604; RI = 0.725) obtained using equally weighted parsimony for the combined molecular data set (the other two MPT's are compatible with tree B). Tree (B) represents one of the two MPTs (655 steps; CI = 0.603; RI = 0.747) obtained using equally weighted parsimony for the total combined morphological and molecular approach. Tree B also represents the topology obtained using weighted parsimony for the combined molecular data set and for the combined morphological and molecular data set. Bold numbers above branches represent bootstrap values, while numbers under branches represent Bremer support values.

still are sister to the remaining Laniatores. A single character supported the (Cyphophthalmi + Eupnoi) clade.

In the equally weighted parsimony analysis of combined molecular data (18S + 28S), three MPTs of 592 steps (CI = 0.579; RI = 0.725) were obtained. Once more the Cyphophthalmi were sister taxa to the remaining opilionids. The Eupnoi could be the sister group of the Dyspnoi (tree from Fig. 2A) or the sister group of the (Dyspnoi + Laniatores) (two trees compatible with tree from Fig. 2B and thus not shown). The resolution within the Cyphophthalmi, Eupnoi, and Dyspnoi were the same as in former analyses. Within the Laniatores the following pattern is found: (Travunioidea (Oncopodoidea + Gonyleptoidea)). Within the Gonyleptoidea the two Phalangodids grouped together, as well as *Gnidia* + *Pachyloides*.

When differentially weighted parsimony was used, a single tree of 940 steps was obtained, which was identical to one of the three unweighted parsimony MPTs (as in Fig. 2B), as follows: (Cyphophthalmi (Eupnoi (Dyspnoi + Laniatores))).

Phylogenetic Analysis of Morphological Data

Parsimony analysis of the 45 morphological characters (3 uninformative characters were ignored in the analysis) coded for 15 species of Opiliones led to four networks of 67 steps (CI = 0.806; RI = 0.902). Considering that these networks are rooted between the Cyphophthalmi and the remaining Opiliones (as obtained in the molecular analyses) the resultant cladograms depict a monophyletic Cyphophthalmi, a monophyletic Laniatores, and a paraphyletic Palpatores split into two main groups: Eupnoi and Dyspnoi (*sensu* Hansen and Sørensen, 1904). The strict consensus of the four trees is shown in Fig. 3. Two additional steps (69 steps) are needed to force a cladogram with monophyletic Palpatores.

Within the Cyphophthalmi the two Sironids (*Siro* and *Parasiro*) group together. The Eupnoi groups the Caddoidea (*Caddo*) and the two Phalangioidea (*Odiellus* + *Nelima*); the Dyspnoi groups the Ischyropsalidoidea (*Ischyropsalis*) and the two Troguloidea (*Dicranolasma* + *Centetostoma*). There are no differences in these topologies between the four MPTs. Within the Laniatores, the Oncopodoidea (*Oncopus*) or the Travunioidea (*Equitius*) are represented as the sister group of the remaining taxa, which results in a politomy of five branches in the strict consensus tree, with the only resolved clade being *Gnidia* + *Pachyloides* (Fig. 3). The low resolution inside the Laniatores is likely due to characters used in the analysis which were not chosen to study internal Laniatorid phylogeny.

No differences were obtained when treating characters 2, 3, 8, 13, 19, and 20, as either ordered or unordered.

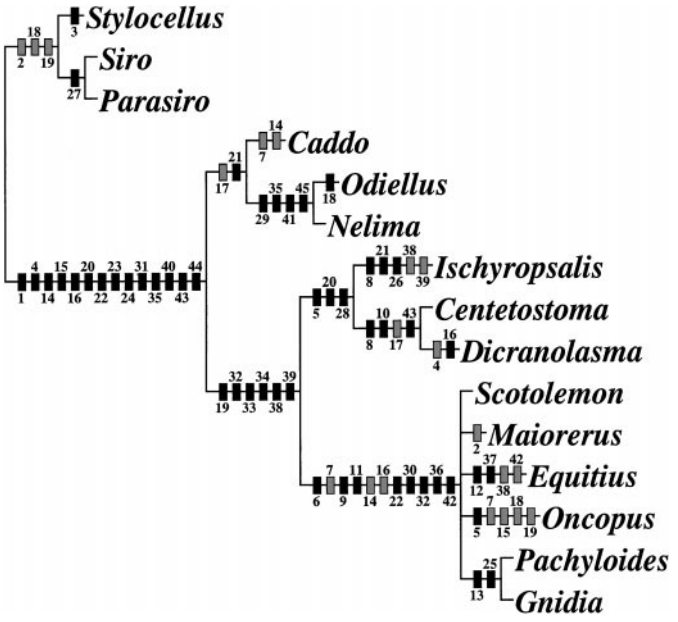

FIG. 3. Strict consensus of the four morphological MPTs (67 steps; CI = 0.806; RI = 0.902). Changes optimized using a modified ACCTRAN optimization (Nixon, 1995). Numbers next to the boxes refer to character numbers as given in Appendix 2. ■, Nonhomoplastic change; ▨, homoplastic change. This tree is rooted between the Cyphophtalmi (*Stylocellus*, *Siro*, and *Parasiro*) and the Phalangida (the remaining groups) as obtained in the molecular trees.

Combined Morphological and Molecular Data

The combined analysis of morphological and molecular data (2164 characters coded for 20 terminal taxa) gave two MPTs of 655 steps (CI = 0.603; RI = 0.747) that differ in outgroup topology, but not in ingroup relationships (Fig. 2B). The Cyphophthalmi are monophyletic and sister to a clade that includes all the remaining opilionid taxa. Laniatores were also monophyletic and their internal topology was the one obtained in combined molecular analyses. The Palpatores were a paraphyletic group with the Eupnoi as the sister group of (Dyspnoi + Laniatores). This topology was compatible with the morphological analysis, and now the plesiomorphic states of the morphological characters can be defined. Internal topologies were the same as in former analyses.

Bootstrap values of the combined data matrix (Fig. 2B) were near 100% in the four main clades (Cyphophthalmi, Eupnoi, Dyspnoi, and Laniatores) as well as at the family or the superfamily taxonomic level. These high bootstrap values coincide with high values of Bremer support. The values supporting the clade (Dyspnoi + Laniatores) were low (a bootstrap value of 61% and a Bremer support of 3). Nevertheless, the bootstrap value for the alternative topology (monophyly of Palpatores) was much lower (19%).

The Mickevich–Farris length incongruence difference for the combined molecular data sets produced

0.0065 extra homoplasy and 0.0060 for the combined molecular and morphological data sets, showing low levels of character incongruence between all sources of data.

DISCUSSION

The assumption of assigning an equal weight to each molecular character used in the phylogenetic analysis may not be correct, but was adopted because of the lack of consensus in judging whether stem mutations should be downweighted or upweighted. Arguments favoring each of the hypotheses have been put forward by several authors. Wheeler and Honeycutt (1988) argued for downweighting the stem mutations, because of the nonindependence resulting from compensatory mutations. Instead of this option, other authors preferred to upweight stem substitutions because mutations are mainly accumulated in loops, where saturation occurs first (Miyamoto *et al.*, 1994). In addition, stem mutations may be compensated immediately, compensated later in the phylogenetic history of the lineage, or not compensated at all (Kraus *et al.*, 1992; Dixon and Hillis, 1993; Gatesy *et al.*, 1994), as sometimes occurred in our data. Furthermore, secondary structure predictions may be distant from the correct structure, which is three-dimensional and unpredictable by present means and which would probably lead to the assignment of different weights to each single stem and to each single loop. Finally, alignments based on different secondary structure models can give different solutions (Winnepenninckx and Backeljau, 1996).

Another kind of weighting of molecular characters, which does not depend on the topological properties of the secondary structure predictions, is to assign different weights to those events that occur at different rates inside the molecule, as a two-parameter model assigning different weights to transitions and transversions. Furthermore, weighted parsimony has been shown to perform better than unweighted parsimony in most simulation studies and experimental phylogenies (Hillis *et al.*, 1994), and so we used it to test the results obtained with unweighted parsimony.

Both the methodology and the drawbacks of combining data sets have been thoroughly reviewed in recent literature (Kluge, 1989; Bull *et al.*, 1993; De Queiroz, 1993; Eernisse and Kluge, 1993; Kluge and Wolf, 1993; Chippindale and Wiens, 1994; De Queiroz *et al.*, 1995; Miyamoto and Fitch, 1995; Huelsenbeck *et al.*, 1996; Nixon and Carpenter, 1996; Page, 1996) and are not under discussion in the present paper. Recently, many authors have used the "total evidence" or "simultaneous analysis" approach of molecular and morphological data sets (i.e., Kluge, 1989; Eernisse and Kluge, 1993; Wheeler *et al.*, 1993; Chavarría and Carpenter, 1994; Bridge *et al.*, 1995; Wheeler, 1997). The arguments of Nixon and Carpenter (1994) about character independence statements, the possibility of emergence of secondary signals, and explanatory power seem to us reasons enough for using the total evidence approach.

Data removal is controversial in phylogenetic reconstruction (see Gatesy *et al.*, 1993). In our case, two small divergent regions corresponding to 23 sites at loops E21-1 and 41 were removed from the 18S data set, and three regions corresponding to 57 positions in the variable D3 region were removed from the 28S gene fragment. The decision of removing these regions was based on the unreliability of the primary homology statements after the alignment.

We avoided study of low-level taxonomic resolvent morphological characters for Opiliones and based our study on those characters able to give resolution at a higher phylogenetic level. Our data matrix was mainly based on characters described in the literature. This provided unrooted cladograms that became rooted after molecular analyses and allowed discussion of previous hypotheses about opilionid evolution. But it must be emphasized that morphological polarization is difficult to do with morphological characters alone because of the lack of homologous characters useful for opilionid phylogeny in other arachnid groups.

Phylogenetic Inferences

On outgroups and the monophyly of the Opiliones. Focusing on the outgroups used in the analyses, we decided to employ merostomates because of the lack of consensus about sister-group relationships among Opiliones and other arachnid groups, as discussed above. In addition, some other arachnid groups were employed to test the monophyly of the Opiliones. The low rate of change observed in the entire study group when compared with other arthropod groups made the use of merostomates as an outgroup possible.

We also tried to include all the arachnid groups which could potentially affect the monophyly of the Opiliones, i.e., those mentioned in publications as sharing a clade with the Opiliones: Solifugae, Pseudoscorpionida, Ricinulei, and Acari (Weygoldt and Paulus, 1979) and Scorpionida, Pseudoscorpionida, and Solifugae (Shultz, 1990). The pseudoscorpion and mite sequences, although not affecting the monophyly of the Opiliones, were excluded from the final data set because they were very divergent in some regions, making them difficult to align reliably in zones where all other sequences were highly conserved.

The monophyly of the Opiliones has only been disputed by Savory (1977), who considered the Cyphophthalmi a different order, from which had evolved the "Phalangida" (Palpatores + Laniatores) and the Ricinulei. Since the Ricinulei (*Pseudocellus pearsei*) included in the 18S molecular analyses was never found within the Opiliones, this hypothesis can be rejected.

On ingroup relationships. The Cyphophthalmi clade is well supported in both morphological and molecular analyses as well as in the combined analysis, with bootstrap values of 100% and high Bremer support in all cases except for the 28S weighted parsimony analysis. The following morphological synapomorphies support the clade: presence of ozophores (1); absence of central eyes (2) (this character state also present in *Maiorerus*, a cavernicolous species); walking leg I the longest (14); walking legs tarsi with one to three segments (15); ninth abdominal tergite present and well developed (20); sternum absent or rudimentary (22); cephalotorax and abdomen both completely fused forming a *scutum completum* (23); gnathocoxae present in all coxae (24); gonostome without operculum or with a vestigial one (31); presence of a jointed (segmented) ovipositor with only the apical ring split (35); short penis, membranous and not divided into truncus and glans (40), with three muscle pairs (43). Cyphophthalmi is the sister group to the remaining opilionid groups and, contrary to the hypothesis of Martens and co-workers, is not grouped within the Palpatores.

The Laniatores is the other fully supported clade in all analyses (with bootstrap values of 100% in all cases), with the following synapomorphies: presence of robust palpi (6); prehensile palpal claws (9); walking leg claws III and IV differing from I and II (11); long and thin sternum (22); three pairs of midgut diverticula (30); gonostome with a fully jointed operculum (32); unjointed ovipositor radially symmetrical with cruciform vagina (36); absence of penile musculature (42).

The internal topology of this monophyletic group is resolved as (*Equitius* (*Oncopus* ((*Scotolemon* + *Maiorerus*) + (*Gnidia* + *Pachyloides*)))) in combined molecular analyses and in combined molecular + morphological analysis. *Scotolemon* and *Maiorerus* are members of the family Phalangodidae and so were expected to group together. The grouping of *Gnidia* and *Pachyloides* was also expected, because Cosmetidae and Gonyleptidae are very close families. These four species belong to the Gonyleptoidea, which is the sister group of the Oncopodoidea (*Oncopus*). The first branch of the Laniatores is the Travunioidea (*Equitius*). The global arrangement found in our data, (Travunioidea (Oncopodoidea + Gonyleptoidea)), is the same as that proposed in former phylogenetic analyses (Martens, 1976, 1980, 1986; Martens *et al.*, 1981) using only morphological data.

Within the Palpatores, the most problematic group of Opiliones, we have evidence concerning two main evolutionary groups, Eupnoi and Dyspnoi, first proposed by Hansen and Sørensen (1904). The Eupnoi comprises the two superfamilies Caddoidea + Phalangioidea (Shear, 1975), while the Dyspnoi groups the superfamilies Troguloidea + Ischyropsalidoidea. Both clades are well resolved at the superfamily taxonomic level, are clearly monophyletic, and are supported at near 100% bootstrap values in almost all analyses (in a few cases the Eupnoi has bootstrap values near 80%) and by high branch support. A paraphyletic Dyspnoi group, as suggested by Martens and co-workers, is never obtained.

Problems arise in considering the status of "Palpatores" as a whole. The monophyly of both (Eupnoi + Dyspnoi) is obtained in some of the analyses, but in most cases they appear to be paraphyletic, with the Laniatores included as the sister group of the Dyspnoi. This is also the most parsimonious solution for explaining the evolution of the ovipositor morphology on which Martens based his studies (Martens, 1976, 1980, 1986; Martens *et al.*, 1981). Thus, the segmented ovipositor can be considered as having plesiomorphic status in opilionid phylogeny (based on molecular polarization), contrary to the apomorphic status assigned by Martens and co-workers. The main criticism of Martens' hypothesis of opilionid evolution is his consideration of the unjointed ovipositor as plesiomorphic. But the opilionid ovipositor is unique among arachnids and thus cannot be homologous to any other arachnid structure. Consequently, *a priori* polarization of such a character could be spurious and could lead to wrong phylogenetic hypotheses.

Certainly, the Cyphopalpatores group was erected because some Palpatores (the Eupnoi) shared a similar ovipositor with the Cyphophthalmi, a trait on which Martens based his analysis. Even though the explanation for the evolution of a single character related to ovipositor morphology needed two steps in Martens' cladogram, only one step is required in our solution. Since many other characters related to ovipositor structure were used in their matrix, their final conclusion was necessarily far from the most parsimonious explanation. Nevertheless, despite our evidence for rejecting the Cyphopalpatores clade and for assessing Dyspnoi as monophyletic, contrary to the phylogenetic hypothesis of Martens, his enormous contribution must be recognized.

Choosing between one of these two solutions is the final task of this study, but beforehand we would like to summarize some points. Most aspects of Opiliones phylogeny have been addressed, like the primitive status of the Cyphophthalmi or the presence of two well-defined clades of Palpatores, whether they are monophyletic or not. The possibility of Palpatores being paraphyletic is supported by three synapomorphies uniting the Dyspnoi + Laniatores, two of these characters referring to the ovipositor and one to the penis morphology. Against this, the only unambiguous morphological character supporting the monophyly of Palpatores is the shape of the sternum. Whether somatic or genitalic characters are more important in phylogenetic reconstruction and the evolution of these characters among different members of the Opiliones is not fully understood because of the lack of homologous

organs in other arachnids. However, it seems that the best explanation for genitalic characters, once the cladogram is rooted near the Cyphophthalmi, is that Palpatores are paraphyletic (Fig. 4).

We also believe that the use of molecular characters to polarize such unique morphological characters in a combined approach throws light on the evolution of the group. They also help to test phylogenetic hypotheses and to expose homoplasy in morphological data. Combining different molecules can also contribute to a more robust phylogenetic hypothesis, as we observed when increasing resolution at a low taxonomic level, although the 28S rDNA data set on its own differs in many groups compared with the other analyses. This

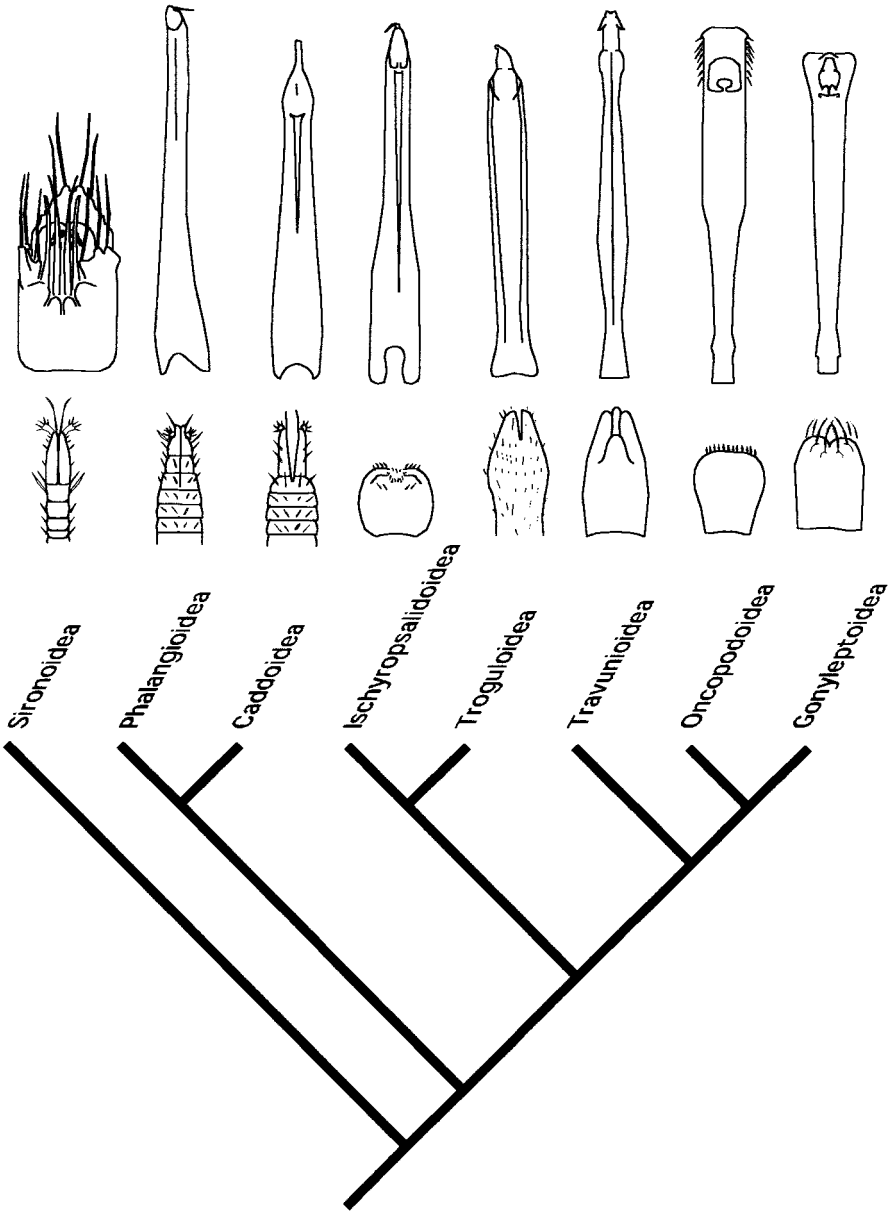

FIG. 4. Phylogenetic relationships of the superfamilies of Opiliones derived from the total evidence analysis here presented. Genitalic characters: penis (above) and ovipositor (below) are mapped. Penis morphology is divided into two main classes: short, membranous, and undivided (Sironoidea) or long, chitinous, and divided into trunk and glans (the remaining superfamilies). Ovipositor morphology is also divided into two types: jointed (Sironoidea, Phalangioidea, and Caddoidea) or unjointed (the remaining superfamilies). More details of each type of penis and ovipositors are given in Appendix 2 and in the text. Drawings are modified from Martens (1980) and Martens *et al.* (1981). In this hypothesis of relationships, the jointed ovipositor is considered a plesiomorphic state instead of a synapomorphy of the clade 'Cyphopalpatores' of Martens and co-workers. The explanation of this character requires no reversals in our hypothesis. On the other hand, whether the character of short, membranous, and undivided penis of the Cyphophthalmi is apomorphic or plesiomorphic cannot be determined, and both hypotheses require a single step.

fact could be due to the low number of informative positions used.

In conclusion, we propose rejection of the "Cyphopalpatores" clade and the consideration of four main evolutionary lines in opilionid systematics, with a primitive Cyphophthalmi and three clades of Phalangida: Eupnoi, Dyspnoi, and Laniatores. Evidence for sister-group relationships among Dyspnoi + Laniatores is emerging, as shown by the total-evidence trees. However, our study cannot definitively support the monophyly of Dyspnoi + Laniatores, due to the existence of alternative solutions in molecular data analyses. We hope that future studies will focus on more detail within the Palpatores phylogeny as a means of understanding the uniqueness or otherwise of this still problematic group.

Appendix 1

[	10 20 30 40]
[	. . . .]
Siro	01100000000-0000022020000010000-010--010-0000
Parasiro	01100000000-0000022020000010000-010--010-0000
Stylocellus	01000000000-0000022020000000000?010--010-00??
Centetostoma	10111002-10-0112111122110001001110-0010110110
Dicranolasma	10101002-10-0111111122110001001110-0010110110
Ischyropsalis	10111001000-0112011112110101001110-0021110210
Odiellus	10110000000-0112100202110000010012--01100211
Nelima	10110000000-0112110202110000010012--01100211
Caddo	10110010000-0212110202110000010011--011102??
Pachyloides	10110110101112100112211110000011210-1010111-10
Gnidia	10110110101112100112211110000011210-1010111-10
Scotolemon	10110110101102100112211100000011210-1010111-10
Maiorerus	11110110101102100112211100000011210-1010111-10
Equitius	10110110101002100112211100000011210-1120110210
Oncopus	10112100101102000222211100000011210-1010111-??

Appendix 2

Opilionid Morphological Characters. All Character States are Scored as 0, 1, 2, ?, or —. All Characters Except the Ones Explicitly Indicated, Have Been Treated Always as Unordered

1—Ozophores: 0 = presence, 1 = absence.
2—Central eyes: 0 = presence, 1 = absence [ordered].
3—Lateral eyes: 0 = presence, 1 = absence [ordered].
4—Ocular prominence of central eyes: 0 = absent, 1 = present.
5—Chelicerae chela: 0 = toothed, 1 = combed, with diaphanous teeth.
6—Palpus: 0 = thin, 1 = robust.
7—Palpus: 0 = not thorny, 1 = thorny with spine-bearing tubercles.
8—Palpal claw: 0 = well developed, 1 = rudimentary, 2 = absent [ordered: the presence of palpal claw is considered to be plesiomorphic].
9—Palpal claw: 0 = not prehensile, 1 = prehensile.
10—Claviform glandular hairs on palpi: 0 = absent, 1 = present.
11—Walking leg claws: 0 = all similar, 1 = III and IV differing from I and II.
12—Different claws from legs III and IV: 0 = a single triramous or multiramous claw, 1 = two claws, − = inapplicable.

Appendix 2—*Continued*

13—Pseudoniquium: 0 = absence, 1 = presence [ordered].
14—The longest walking legs: 0 = leg I, 1 = leg II, 2 = leg IV.
15—Walking legs tarsi: 0 = 1 to 3 segments, 1 = multiarticulated (more than 3 segments).
16—Coxae of walking legs: 0 = pair I and sometimes pair II free (tending to fuse with pairs III and IV), 1 = the four pairs fused, 2 = the four pairs free.
17—Supracheliceral lamellae: 0 = absent, 1 = present.
18—Fusion of abdominal tergites in males: 0 = all tergites free, 1 = fusion of tergites 1st to 5th, 2 = fusion of tergites 1st to 8th [ordered: the presence of free tergites is the plesiomorphic condition in arachnids, and we consider the fusion of a few tergites as an intermediate state towards the fusion of more abdominal tergites].
19—Fusion of abdominal tergites in females: 0 = all tergites free, 1 = fusion of tergites 1st to 5th, 2 = fusion of tergites 1st to 8th [ordered: as in character 18].
20—Ninth abdominal tergite: 0 = present, 1 = rudimentary, 2 = absent [ordered: the presence of a tergite is plesiomorphic versus the reduction].
21—Cephalothorax: 0 = two thoracic tergites visible, 1 = only the last thoracic tergite visible, 2 = all tergites fused.
22—Sternum: 0 = absent or rudimentary, 1 = long and thin, 2 = short and wide.
23—Fusion of cephalothorax and abdomen: 0 = both completely fused forming the *scutum completum*, 1 = not fused or with just some abdominal tergites free.
24—Gnathocoxae: 0 = present in all coxae, 1 = present only in pair I and/or II.
25—Gnathocoxae II: 0 = present, 1 = absent or rudimentary.
26—Metapeltidial sensory cones: 0 = absent, 1 = present.
27—Male exocrine glands at the anal region: 0 = absent, 1 = present.
28—Male cheliceral glands: 0 = absent, 1 = present.
29—Tracheal system with accessorial stigmas in the tibia of the walking legs: 0 = absent, 1 = present.
30—Number of midgut diverticula: 0 = 4 pairs, 1 = 3 pairs.
31—Gonostome: 0 = without or with vestigial operculum, 1 = with well developed operculum.
32—Operculum: 0 = never jointed, 1 = partially jointed, 2 = always fully jointed, − = inapplicable, ? = unknown.
33—Ovipositor morphology: 0 = long and segmented (jointed type), 1 = unsegmented (unjointed type).
34—Special sense organs at the tip of the ovipositor: 0 = absent, 1 = present.
35—Jointed ovipositor: 0 = only the apical ring split, 1 = two apical rings split, 2 = three apical rings split, − = inapplicable.
36—Unjointed ovipositor: 0 = bilaterally symmetrical, with non-cruciform vagina, 1 = radially symmetrical with cruciform vagina, − = inapplicable.
37—Unjointed ovipositor: 0 = with two apical lobes, 1 = with four apical lobes, − = inapplicable.
38—Internal longitudinal musculature of ovipositor: 0 = present, 1 = reduced, 2 = absent.
39—Circular musculature of ovipositor: 0 = present, 1 = absent.
40—Penis morphology: 0 = short, membranous, and undivided, 1 = long, chitinous, and divided into trunk and glans.
41—Glans vs trunk: 0 = bent 90°, 1 = not bent, − = inapplicable.
42—Penis musculature: 0 = present, 1 = absent.
43—Number of penis muscles: 0 = three muscle pairs, 1 = two muscle pairs, 2 = one muscle pair, − = inapplicable.
44—Fecundation: 0 = through spermatic balls, 1 = through direct insemination, ? = unknown.
45—Mucous layer in the setting: 0 = present, 1 = absent, ? = unknown.

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