

# Phylogeny of pholcid spiders (Araneae: Pholcidae): Combined analysis using morphology and molecules

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## Abstract

The spider family Pholcidae comprises a large number of mainly tropical, web-weaving spiders, and is among the most diverse and dominant spider groups in the world. The phylogeny of this family has so far been investigated exclusively using morphological data. Here, we present the first molecular data for the family analyzed in a phylogenetic context. Four different gene regions (12S rRNA, 16S rRNA, cytochrome *c* oxidase subunit I, 28S rRNA) and 45 morphological characters were scored for 31 pholcid and three outgroup taxa. The data were analyzed both for individual genes, combined molecular data, and molecular plus morphological data, using parsimony, maximum likelihood, and Bayesian methods. Some of the phylogenetic hypotheses obtained previously using morphology alone were also supported by our results, like the monophyly of pholcines and of the New World clade. On the other hand, some of the previous hypotheses could be discarded with some confidence (monophyly of holocnemines, the position of *Priscula*), and still others need further investigation (the position of holocnemines, ninetines, and *Metagonia*). The data obtained provide an excellent basis for future investigations of phylogenetic patterns both within the family and among spider families.

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## 1. Introduction

Among spiders, pholcids stand out with respect to a variety of aspects. First, a series of publications about the functional morphology of the copulatory organs has produced a data set that is unequalled not only in spiders, but probably in arthropods in general (Huber, 1994, 1995, 1996a,b, 1997a,b, 1998, 2002; Huber and Eberhard, 1997; Uhl et al., 1995). Second, pholcids contain an unusually high number of synanthropic species that have been exploited successfully for in-depth, single-species studies in many parts of the world (e.g., USA: Blanchong et al., 1995; Jakob, 1991, 1994; Kaster and Jakob, 1997; Porter and

Jakob, 1990; Central America: Eberhard, 1992; Eberhard et al., 1993; Huber, 1996b, 1997a, 1998; Huber and Eberhard, 1997; Australia: Jackson, 1990, 1992; Jackson and Rowe, 1992; Jackson et al., 1990, 1992; Europe: Bartos, 1998; Fürst and Blandenier, 1993; Huber, 1994, 1995; Schaefer and Uhl, 2003; Schäfer and Uhl, 2002; Uhl, 1993a,b, 1994a,b, 1996, 1998; Uhl et al., 2004; Yoward, 1998; Asia: Strickman et al., 1997). Third, there is evidence that pholcids are among the commonest, if under-appreciated, web-builders in the tropics, with an immense, yet mostly unknown diversity (Huber, 2000, 2003b).

Evolutionary interpretations of the existing results, as well as the design of future research into this group will largely hinge on a sound phylogeny of the family. Considerable advances have been made by the first cladistic analysis of the family (Huber, 2000), replacing the previous, more than a century old non-phylogenetic classification by a cladistic hypothesis on pholcid relationships. However, these results, as well as subsequent minor

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modifications (Huber, 2001, 2003a,b,c), are preliminary, for being based on morphological characters only. As a result, several parts of the cladograms remain unresolved or ambiguous. Ambiguities concern even basic, subfamily level groups. Some of these groups appear well supported by morphology, e.g., pholcines, the New World clade, and ninetines. However, even in these groups, open questions remain. For example, is the large New World genus *Metagonia* really part of the otherwise almost exclusively Old World pholcines? Is the Andean genus *Priscula* part of the New World clade or is it more closely related to the overall more similar (but absent in South America) ‘holocnemines’ (Huber, 2000)? Are ninetines monophyletic or is the clade based on convergences due to constraints related to small size and life in interstices (Huber, 2003d; Huber and Brescovit, 2003)? Other groups appear weakly supported, as for example the subfamily Holocneminae. Thus, the existing cladograms of the group do not yet fulfill some of the main purposes of phylogenetic statements: to provide a stable framework for the reconstruction of character evolution, for the evolutionary interpretation of descriptive data, for guiding systematic research, and for the prediction of character distribution.

Molecular sequence data have been published so far in pholcids only from *Pholcus phalangioides* (Fuesslin) (Chenuil et al., 1997; Sommer et al., 1992). These gene regions, however, are not routinely used for phylogenetic inference. In the present paper, we provide the first phylogenetic analysis of pholcids based on molecular data. Our aim is three-fold: first, to evaluate the usefulness in pholcids of several markers routinely used for phylogenetic inference in other groups; second, to improve on the existing phylogenetic hypotheses based exclusively in morphological data; and third, to provide a basis for future studies at this and other hierarchical levels.

## 2. Materials and methods

### 2.1. Taxon sampling

The species used in this study, together with localities and other details, are listed in Table 1. We studied a total of 31 pholcid species that were selected to represent all of the four currently recognized major clades—New World clade, pholcines, holocnemines, and ninetines (Huber, 2000). The outgroup comprised representatives of Diguettidae and Plectreuridae (putative sister group of Pholcidae (Coddington and Levi, 1991)), and of Filistatidae.

### 2.2. Morphological data

Specimens for all species were evaluated for the following 45 morphological characters (see matrix in Table 2). Most characters are described in detail in Huber (2000); references are given for characters not scored in Huber (2000) or for those where there have been changes in character scoring.

- Character 1*, Eyes: (0) eight (1) six  
*Character 2*, PME-ALE: (0) <0,55× PME diameter (1) >0,55× PME diameter  
*Character 3*, Carapace sculpture: (0) no indentation (1) median groove (2) median pit  
*Character 4*, Clypeus height: (0) shorter than chelicerae (1) as long as or longer than chelicerae  
*Character 5*, Clypeus modification: (0) absent (1) present  
*Character 6*, Sternum width: (0) wider than long (1) longer than wide (Huber, 2003b)  
*Character 7*, Humps on sternum: (0) absent (1) present  
*Character 8*, Epiandrous spigots: (0) absent (1) present  
*Character 9*, Spigots on PLS: (0) present (1) absent  
*Character 10*, Spigots on ALS: (0) about seven present (1) only two present  
*Character 11*, Female internal genitalia: (0) symmetric (1) asymmetric (corresponds to character 16 in Huber, 2000)  
*Character 12*, Trochanter cuneal notch: (0) absent (1) present  
*Character 13*, Enlarged femora walking legs: (0) absent (1) present  
*Character 14*, Spines on walking leg femora in one or two rows: (0) absent (1) present  
*Character 15*, Tibia 1/tibia 4: (0) about 1 (1) >1.15  
*Character 16*, Trichobothria on tibiae: (0) >3 (1) 3  
*Character 17*, Prolateral trichobothrium on tibia 1: (0) present (1) absent (Huber, 2003b)  
*Character 18*, Retrolateral trichobothrium: (0) distal (after 45% tibia length) (1) proximal (before 45% tibia length)  
*Character 19*, Tibia 1/carapace width: (0) up to 2,5 (1) >2,5  
*Character 20*, Pseudosegments: (0) absent (1) present  
*Character 21*, Number pseudosegments: (0) up to 10 (1) >10  
*Character 22*, Regularity pseudosegments: (0) regular (1) irregular  
*Character 23*, Chelicerae sexual dimorphism: (0) absent (1) present  
*Character 24*, Stridulatory ridges chelicerae: (0) absent (1) present  
*Character 25*, Cheliceral lamina: (0) regular (1) strongly pointed  
*Character 26*, Proximolateral apophyses male chelicerae: (0) absent (1) present  
*Character 27*, Long modified hairs male chelicerae: (0) absent (1) present (Huber, 2003b)  
*Character 28*, Apophysis in ‘knee’ of palpal coxa: (0) absent (1) present (Huber, 2003b)  
*Character 29*, Palpal trochanter apophysis: (0) absent (1) present (Huber, 2003b)  
*Character 30*, Procursus: (0) absent (1) present  
*Character 31*, Hinged process on procurus: (0) absent (1) present  
*Character 32*, Procursus: ventral pocket and dorsal apophysis: (0) absent (1) present

Table 1  
Material, collection details, and GenBank Accession Numbers\*

Taxon	Voucher No.	Locality	GenBank Accession No.			
			28S	12S	16S	COI
<b>Ingroup</b>						
<i>Artema atlanta</i> Walckenaer	BB01	USA: Florida		AY560685	AY560663	AY560771
<i>Carapoia paraguaensis</i> González-Sponga	BB02	VENEZUELA: Bolivar		AY560723	AY560680	
<i>Ciboneya antraia</i> Huber and Pérez	BB03	CUBA: Pinar del Rio	AY560732	AY560687	AY560665	AY560794
<i>Coryssocnemis simla</i> Huber	BB04	TRINIDAD: Arima-Blanchisseuse Rd.		AY560688		AY560773
<i>Crossopriza lyoni</i> (Blackwall)	BB05	SOUTH AFRICA: Kwazulu Natal	AY560762	AY560689	AY560666	AY560774
<i>Holocnemus plucheii</i> (Scopoli)	BB06	AUSTRIA: Vienna	AY560734	AY560691		AY560776
<i>Kaliana yuruani</i> Huber	BB07	VENEZUELA: Bolivar		AY560694	AY560668	
<i>Mecolaesthus longissimus</i> Simon	BB08	VENEZUELA: Aragua	AY560736	AY560696		
<i>Mesabolivar aurantiacus</i>	BB09	TRINIDAD: Arena Forest	AY560735	AY560695	AY560669	AY560778
<i>Mesabolivar brasiliensis</i> (Moenkhaus)	BB10	BRAZIL: São Paulo	AY560738	AY560698		AY560780
<i>Mesabolivar cyaneotaeniatus</i> (Keyserling)	BB11	BRAZIL: Rio de Janeiro	AY560739	AY560699	AY560671	AY560781
<i>Psilochorus itaguyrussu</i> Huber, Rheims and Brescovit	BB12	BRAZIL: São Paulo	AY560740	AY560701		AY560782
<i>Metagonia</i> sp. 1	BB13	BRAZIL: São Paulo	AY560741	AY560702		AY560783
<i>Metagonia</i> sp. 2	BB14	BRAZIL: Iporanga	AY560742	AY560703	AY560673	AY560784
<i>Micropholcus fauroti</i> (Simon)	BB15	CUBA: La Habana	AY560743	AY560704		AY560785
<i>Ninetis subtilissima</i> Simon	BB16	JEMEN: Ja'ar	AY560744	AY560705		
<i>Pholcus opilionoides</i> (Schrank)	BB17	AUSTRIA: Upper Austria	AY560745	AY560707	AY560674	
<i>Pholcus phalangioides</i> (Fuesslin)	BB18	AUSTRIA: Vienna	AY560746	AY560708	AY560675	
<i>Pholcus</i> sp. 1	BB19	USA: Pennsylvania	AY560749	AY560706		AY560786
<i>Physocyclus dugesi</i> Simon	BB20	COSTA RICA: San Pedro de Montes de Oca	AY560750	AY560711		AY560787
<i>Physocyclus globosus</i> (Taczanowski)	BB21	CUBA: La Habana	AY560751	AY560712		AY560788
<i>Priscula</i> sp. 1	BB22	VENEZUELA: Lara	AY560752	AY560713	AY560678	
<i>Psilochorus simoni</i> (Berland)	BB23	GERMANY: Bonn	AY560753	AY560714		AY560789
<i>Psilochorus</i> sp. 1	BB24	USA: Colorado	AY560754	AY560715		AY560790
<i>Quamtana bonamanzi</i> Huber	BB25	SOUTH AFRICA: Kwazulu Natal	AY560757	AY560719		
<i>Quamtana embuleni</i> Huber	BB26	SOUTH AFRICA: Mpumalanga	AY560759	AY560721		AY560793
<i>Quamtana vidal</i> Huber	BB27	SOUTH AFRICA: Kwazulu Natal	AY560758	AY560720		AY560792
<i>Smeringopus natalensis</i> Lawrence	BB28	SOUTH AFRICA: Mpumalanga	AY560755	AY560717		
<i>Spermophora senoculata</i> (Dugès)	BB29	USA: New York	AY560756	AY560718		AY560791
<i>Stenosfemuraia</i> sp. 1	BB30	VENEZUELA: Aragua	AY560760			
<i>Systemita prasina</i> Simon	BB31	VENEZUELA: Aragua	AY560761	AY560722		
<i>Trichocycclus</i> sp.	BB32	AUSTRALIA: Western Australia	AY560733	AY560686	AY560664	AY560772
<b>Outgroup</b>						
<i>Kukulcania hibernalis</i> (Hentz) (Filistatidae)	BB33	COSTA RICA: San Antonio de Escazú	AY560764		AY560684	AY560796
<i>Plectreurys tristis</i> Simon (Plectreuridae)	BB34	USA: Arizona	AY560768		AY560682	
<i>Diguertia</i> sp. (Diguertiae)	BB35	USA: Arizona	AY560766	AY560724		AY560795

\* Vouchers of all species are deposited at the Zoological Research Institute and Museum Alexander Koenig, Bonn.

Table 2  
Morphological character and state data matrix

Taxon	Characters				
	1	111111112	222222223	333333334	44444
	1234567890	1234567890	1234567890	1234567890	12345
<i>Artema atlanta</i>	0021000110	0100110111	1110?00001	0111000001	00000
<i>Crossopriza lyoni</i>	0021000111	0101111111	1111100001	0001000001	00000
<i>Holocnemus pluchei</i>	0021000111	0101110111	1111100001	0001000001	00000
<i>Physocyclus globosus</i>	0011000010	0100110111	1111100001	0111000001	00000
<i>Physocyclus dugesi</i>	0011000010	0100110111	1111100001	0111000001	00000
<i>Priscula</i> sp. 1	0111000010	0100110111	1110100001	0010?00001	00010
<i>Smeringopus natalensis</i>	0021000110	0100110111	1110100001	0001000001	00000
<i>Trichocyclus</i> sp. 1	0021000010	0100110111	1011100001	0111?00001	00000
<i>Carapoia paraguanaensis</i>	0111000011	0100110111	1010000101	0000?00001	00000
<i>Ciboneia antraia</i>	0011000011	0100011111	1010000101	0000?00001	00001
<i>Coryssocnemis simla</i>	0111000011	0111110111	1010000101	0000?00001	00000
<i>Kaliana yuruani</i>	0111100011	0100110111	1010000101	0000?00001	10000
<i>Mecolaesthus longissimus</i>	0111000011	0100110111	1010000101	0000?00011	00000
<i>Psilochorus itaguyrussu</i>	012100001?	0100110111	10?0000101	0000?00001	00100
<i>Mesabolivar brasiliensis</i>	0111000011	0100110111	1010000101	0000?00001	10000
<i>Mesabolivar cyaneotaeniatus</i>	0111000011	011011?111	1010000101	0000?00001	11000
<i>Psilochorus simoni</i>	0011000011	0110110111	1010000101	0000?00001	00101
<i>Psilochorus</i> sp. 1	0011000011	0100110111	1010000101	0000?00001	00101
<i>Stenosfemuraia</i> sp. 1	0111000?11	0100110111	1010000101	0000?00001	00100
<i>Systemita prasina</i>	1111000011	0100110111	1010000101	0000?00001	00000
<i>Metagonia</i> sp. 1	1001100110	1100111111	1010000001	1001000101	00000
<i>Metagonia</i> sp. 2	1001100110	1100111111	1010000001	1001000101	00000
<i>Micropholcus fauroti</i>	0001000110	0100111111	1010010011	0001001001	00000
<i>Pholcus opilionoides</i>	0001000110	0100111111	1010010011	0001011001	00000
<i>Pholcus phalangioides</i>	0001000110	0100111111	1010010011	0001011001	00000
<i>Pholcus</i> sp. 1	00010001?0	010011?111	1010010011	0001011001	00000
<i>Spermophora senoculata</i>	1001000110	0100111111	1010010011	1001010101	00000
<i>Quamtana bonamanzi</i>	0001000110	0100111111	1010011011	0001001001	00000
<i>Quamtana vidal</i>	1001000110	0100111111	1010011011	0001001001	00000
<i>Quamtana embuleni</i>	0001000110	0100111111	1010011011	0001001001	00000
<i>Ninetis subtilissima</i>	0001001110	0100011001	0011000001	0001100001	00000
<i>Kukulkanian hibernalis</i>	0110000100	000010??10	??00?00000	???1000?00	01000
<i>Digueta</i> sp. 1	1?00010000	010000?000	??00000000	???1000?01	00000
<i>Plectreurys tristis</i>	0100010010	010000?000	?010000000	???0?00?01	00001

*Character 33*, Pseudotrachia distally on procurus: (0) absent/few (1) many

*Character 34*, Palpal tarsal organ shape: (0) exposed (1) capsulate

*Character 35*, Tarsal organ orifice: (0) >35% diameter (1) <35% diameter

*Character 36*, Serrate apophysis on bulb: (0) absent (1) present (Huber, 2003b)

*Character 37*, Proximal bulbal sclerite: (0) absent (1) present (Huber, 2003c)

*Character 38*, Attachment site of bulb: (0) prolateral (1) dorsal (Huber, 2003c)

*Character 39*, Male carapace inflation: (0) absent (1) present

*Character 40*, Cribellum: (0) absent (1) present

*Character 41*, Epigynum median groove or pocket: (0) absent (1) present

*Character 42*, Spines on male metatarsi: (0) absent (1) present

*Character 43*, 'Pup'-apophysis on male palpal femur: (0) absent (1) present

*Character 44*, Position of tarsal organ: (0) not elevated (1) on stalk

*Character 45*, Vertical hairs on male tibiae: (0) absent (1) present

### 2.3. DNA extraction, amplification, and sequencing

Specimens were preserved in 96% ethanol at  $-20^{\circ}\text{C}$ . Prior to DNA extractions specimens were washed in sterile distilled water. Depending on the body size, total genomic DNA was isolated from legs, prosoma with legs, or from the whole individual spider; the Qiagen DNeasy Tissue Kit was used for all DNA extractions.

DNA fragments corresponding to: approximately 1100 bp of D1–D3 regions of the 28S rRNA gene, app. 350 bp of 12S rRNA gene (between positions 14233 and 14588 of the *Drosophila yakuba* mitochondrial genome Clary and Wolstenholme, 1985), app. 500 bp of 16S rRNA gene (between positions 12887 and 13398 of the *D. yakuba* mitochondrial genome), and app. 650 bp of the cytochrome c oxidase subunit I (COI hereafter) gene (between positions

Table 3  
Primers used for PCR

Gene	Primer name	Primer sequence
28S rDNA	28S-B1	5' GACCGATAGCAAACAAGTACCG 3'
	28S-B2	5' GATTAGTCTTTTCGCCCTATA 3'
12S rDNA	12S-ai <sup>a</sup>	5'AAACTAGGATTAGATACCCTATTAT 3'
	12S-bi <sup>a</sup>	5'AAGAGCGACGGGCGATGTGT 3'
16S rDNA	LRN-B <sup>a</sup>	5'CACCTGTTTATCAAAAACAT 3'
	LRJ-B <sup>a</sup>	5'CTCCGGTTTGAAGTACAGATCAT 3'
COI	HCO <sup>b</sup>	5'TAAACTTCAGGGTGACCAAAAAATCA 3'
	LCO <sup>b</sup>	5'GGTCAACAAATCATAAAGATATTGG 3'

<sup>a</sup> From Simon et al., 1994.

<sup>b</sup> From Folmer et al., 1994.

1539 and 2172 of the *D. yakuba* mitochondrial genome) were amplified using the primers shown in Table 3. Amplification was carried out in a Biometra thermal cycler, in a total volume of 20 µl containing 1 µl DNA (app. 10–50 ng), 10 pmol primers, 0.2 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, and 1 U BioTaq Red DNA polymerase (Bioline). PCR conditions were as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of 60 s denaturation at 94 °C, 60 s annealing at 48–60 °C, 60 s elongation at 72 °C, and a final extension for 10 min at 72 °C. The annealing conditions were optimized depending on the particular combination of taxa and primers. PCR products were checked for length and purity on 1.5% agarose gels and purified directly from the PCR mixture using the PeqLab EZNA Cycle-Pure kit or from agarose gels when additional bands were present.

Sequencing of both strands of purified PCR products was performed at MWG Biotech AG sequencing service (Germany), with the same primers used in PCR amplifications. Sequence data are deposited in GenBank under Accession Numbers AY560685–AY560723 for 12S rRNA, AY560663–AY560680 for 16S rRNA, AY560723–AY560762 for 28S rRNA and AY560771–AY560794 for COI (Table 1).

#### 2.4. DNA sequence alignment

Sequences of ribosomal RNA genes were aligned using the default gap opening-gap extension parameters (15.0–6.66) in ClustalW 1.7 (Thompson et al., 1994). To down-weight numerous large gaps present in ribosomal genes, we applied the “simple method” for recoding gaps described in Simmons and Ochoterena (2000) and Simmons et al. (2001) using the program GapCoder (Young and Healy, 2002). The matrices obtained this way were then used in both maximum parsimony and Bayesian searches. Alignments of the COI gene were trivial, and no insertions or deletions needed to be postulated. BioEdit 5.09 (Hall, 1999) was used for manual inspection and editing of alignments.

#### 2.5. Phylogenetic analyses

Phylogenetic relationships were estimated by parsimony (MP) and maximum likelihood (ML) in PAUP v4.0b10 (Swofford, 2000), and by Bayesian analysis with Markov

Chain Monte Carlo sampling in MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). A partition-homogeneity test (ILD-test) was used to assess congruence between partitions (28S, 12S, 16S, COI, and morphological dataset). MP and Bayesian analyses were applied for each separate dataset as well as for the combined dataset, while ML analyses comprised only separate 28S, 12S, 16S, and COI datasets.

MP analyses were performed with a heuristic search option (1000 random taxon-addition replicates) with tree-bisection-reconnection (TBR) branch swapping, all characters equally weighted. Bootstrap values were calculated from 1000 replicates in parsimony analyses. Bremer support and partitioned Bremer support values (Baker and DeSalle, 1997) were calculated based on the strict consensus MP tree using the program TreeRot.v2 (Sorenson, 1999).

The hierarchical likelihood ratio test criterion (hLRT) implemented in Modeltest version 3.06 (Posada and Crandall, 1998) was used to select the best-fit maximum likelihood model and parameters for each gene. The estimated parameters were used for the ML heuristic searches (100 random addition replicates) with TBR branch-swapping.

Bayesian MCMC analyses with four parallel Markov chains were run for 1,500,000 generations with a sampling frequency of one in every hundred trees. Likelihood parameters in the analyses of the separate genes were the same as those used in the ML heuristic searches, while for the combined analysis we applied a GTR+I+G model. Burn-in time was determined by the time of convergence of likelihood scores; the consensus tree (with posterior probabilities for each node) was constructed based on the trees sampled after burn-in from several independent runs.

The matrices used in the analyses are available online at the MPE website.

### 3. Results

#### 3.1. Morphology

Forty-five morphological characters were scored in 31 pholcid taxa and three outgroup taxa (Table 2). Parsimony analysis in PAUP resulted in a total of 60 equally most parsimonious trees of length 81 (CI=0.575, RI=0.820), the strict consensus of which is shown in Fig. 1. Although support for most nodes is weak (and several are unresolved), three main clades within Pholcidae are evident in the consensus tree. The first one consists of mostly Old World pholcines together with ninetines (i.e., *Ninetis subtilissima* as the only representative of this clade) being sister to them, the second is the New World clade, and the third one includes all holocnemines. Within the clades, the resolution is fairly complete in the pholcines, while the New World clade remains for a large part unresolved and holocnemines are poorly resolved. Bayesian analysis also resulted in a poorly resolved consensus tree (results not shown), with monophyletic pholcines and the members of the New World clade, and paraphyletic holocnemines. Numerous

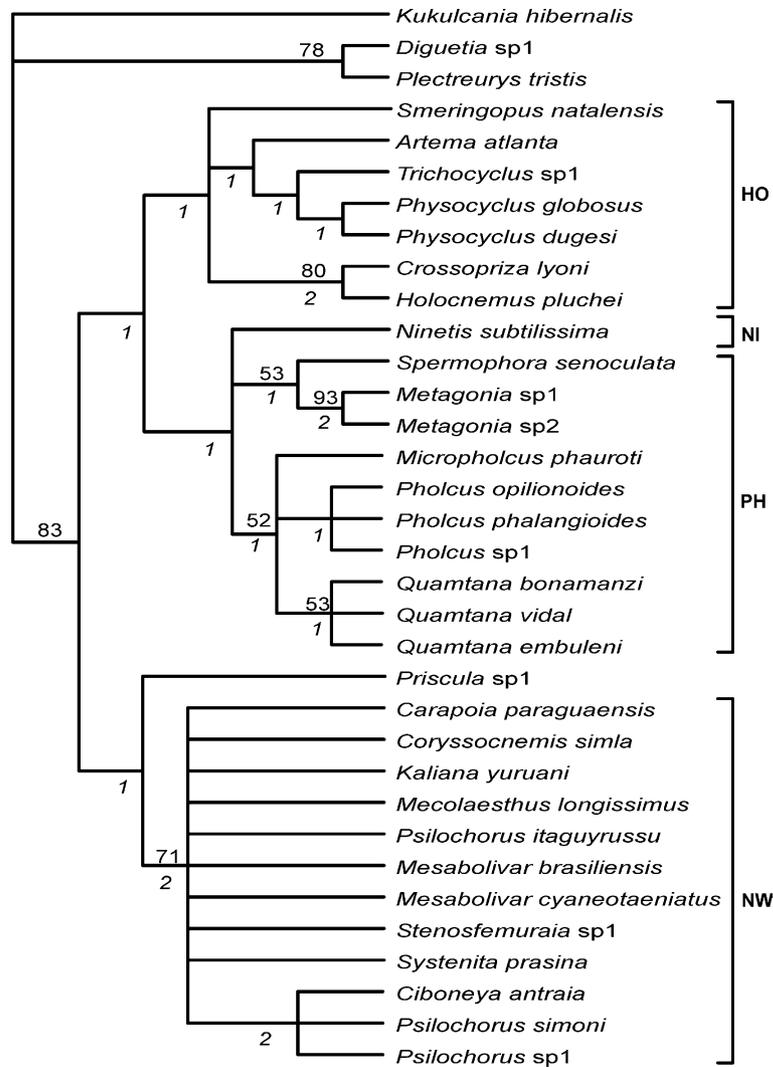


Fig. 1. Phylogenetic relationships within Pholcidae based on morphological data: Strict consensus of 60 equally most parsimonious trees. Numbers above nodes are bootstrap values (above 50%), and below nodes the Bremer support values. Currently recognized higher clades are indicated by vertical bars and acronyms: NW: New World clade; PH: pholcines; HO: holocnemines; NI: ninetines.

negative PBS values for different characters (characters were divided into groups of somatic/genitalic, i.e., according to body parts) on most of the nodes in the MP tree (results not shown) suggest that the reason for such low resolution is substantial character conflict, but also the lack of data for some of the nodes.

### 3.2. DNA data analyses

#### 3.2.1. 28S ribosomal RNA gene

Approximately 1100 bp of the 28S gene (spanning the D1–D3 regions) were sequenced in 26 pholcid taxa and three outgroup taxa. Total length of the alignment used in phylogenetic analysis was 1323 bp; scoring of gaps in Gap-Coder resulted in additional 303 characters included in analyses.

An unweighted parsimony heuristic search resulted in two equally most parsimonious trees of 4280 steps (CI=0.505, RI=0.521). Bootstrap analysis (Fig. 2A)

resulted in two supported clades (of the four currently recognized higher groupings), the pholcines, and the New World clade, while holocnemines appear paraphyletic (*Trichocyclus* plus *Physocyclus* being sister group to all other pholcids). *Metagonia* appears as sister group of the New World clade, and *Priscula* is grouped within it. The position of *Spermophora* within the New World clade contradicts morphology. Ninetines are positioned as sister group of other pholcids except for *Trichocyclus* plus *Physocyclus*. The resolution within the clades is almost complete, with the exception of several nodes with lower BPs (mostly within the New World clade, indicating possible rapid divergence within that clade). However, the relationships between the main groups could not be resolved with high support.

Maximum likelihood search under the GTR+G model of evolution (selected by hLRT as the best-fit model of evolution) resulted in one tree (-Ln likelihood=19251.26869), shown in Fig. 2B. The overall topology of the ML tree is

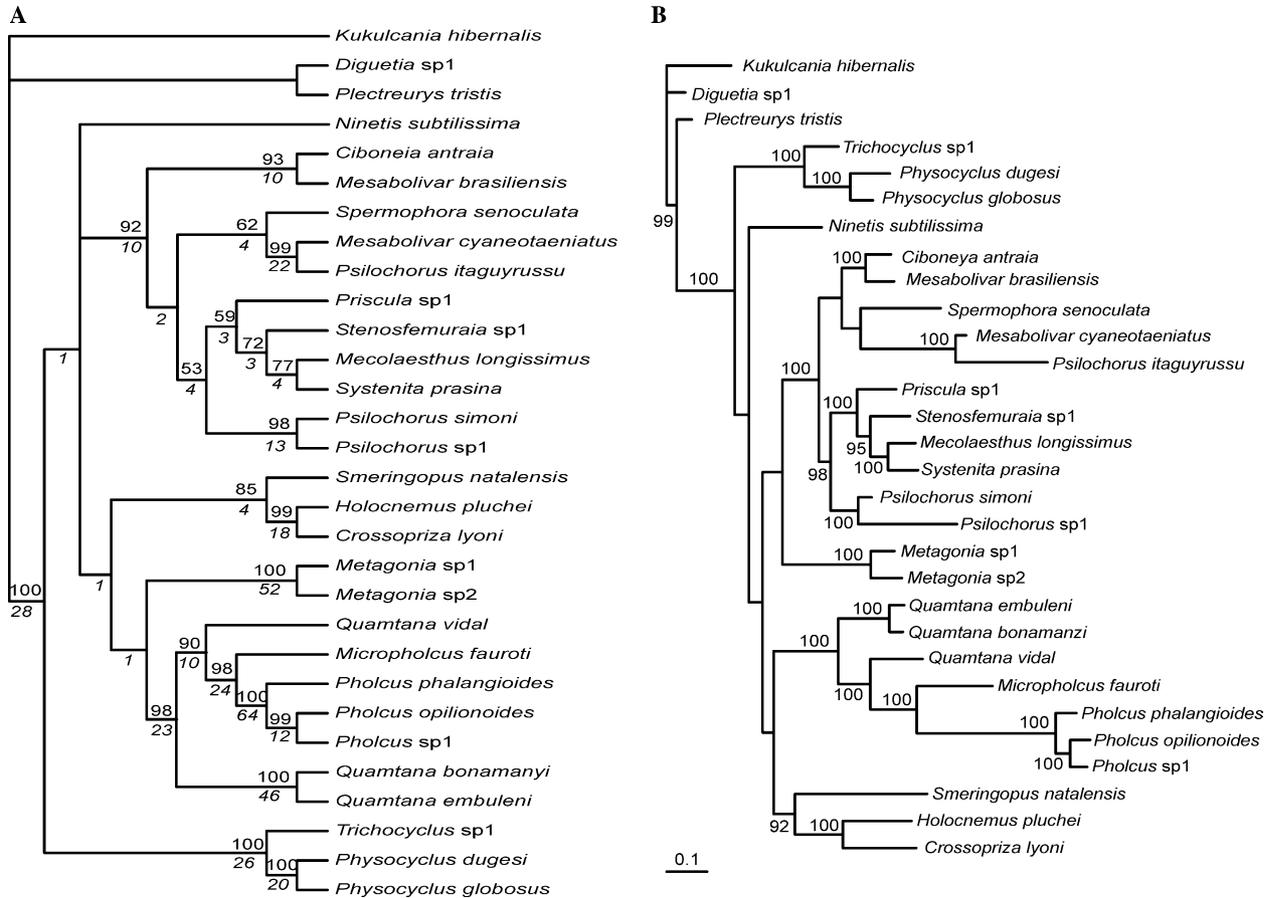


Fig. 2. Phylogenetic relationships within Pholcidae based on 28S rRNA sequence data: (A) Strict consensus of two equally most parsimonious trees. Numbers above nodes are bootstrap values (above 50%) and below nodes Bremer support values; (B) ML tree inferred using the GTR+G model. Numbers on the nodes represent posterior probability values from Bayesian MCMC analysis (above 90). Branch lengths are proportional to amount of evolutionary change.

fairly consistent with the MP tree. Bayesian analysis under the same substitution model resulted in a fully resolved consensus tree with high posterior probability values for most of the nodes that were also highly supported in the MP bootstrap tree. The topology of the Bayesian tree is completely congruent with the ML tree; therefore, we noted posterior probability values directly on the ML tree (Fig. 2B).

### 3.2.2. 12S ribosomal RNA gene

Thirty pholcid taxa were sequenced for a part of the 12S rRNA gene (approximately 350 bp of domain III), resulting in an alignment of 385 bp (plus 138 gap characters).

MP analysis resulted in one most parsimonious tree of 2090 steps (CI=0.388, RI=0.426). Support for most nodes (Fig. 3A) is low, and there are also some substantial differences with respect to the positions of several clades when compared to the 28S tree. *Ninetis* appears as sister to all other pholcids, pholcines are monophyletic, including *Metagonia*, and holocnemines are again paraphyletic. The New World clade appears polyphyletic.

Maximum likelihood search under the GTR+G+I model of evolution resulted in one tree (-Ln likelihood = 8537.87381424) (Fig. 3B) that differed from the MP tree in the position of several clades. In contrast to the MP analysis, the New World clade here appears monophyletic, and *Trichocyclus* plus *Physocyclus* are sister group to the New World clade. Bayesian analysis resulted in a tree that is compatible with the ML tree, and posterior probability values are shown on the ML tree.

### 3.2.3. 16S ribosomal RNA and cytochrome c oxidase subunit I genes

Parts of the 16S rRNA and COI genes (approximately 400 and 650 bp, respectively) were successfully sequenced in significantly smaller numbers of taxa than the 28S and 12S rRNA genes (see Table 1). Nevertheless, we performed MP, ML, and Bayesian analyses for both of the genes (all characters treated equally in both datasets and additionally with weighting 1:1:0 and 2:3:1 for the first:second:third codon position in COI gene). All of these searches resulted in poorly resolved trees (not shown), especially at the base of the tree, but most of the relations corresponded to those revealed by 28S and/or 12S genes.

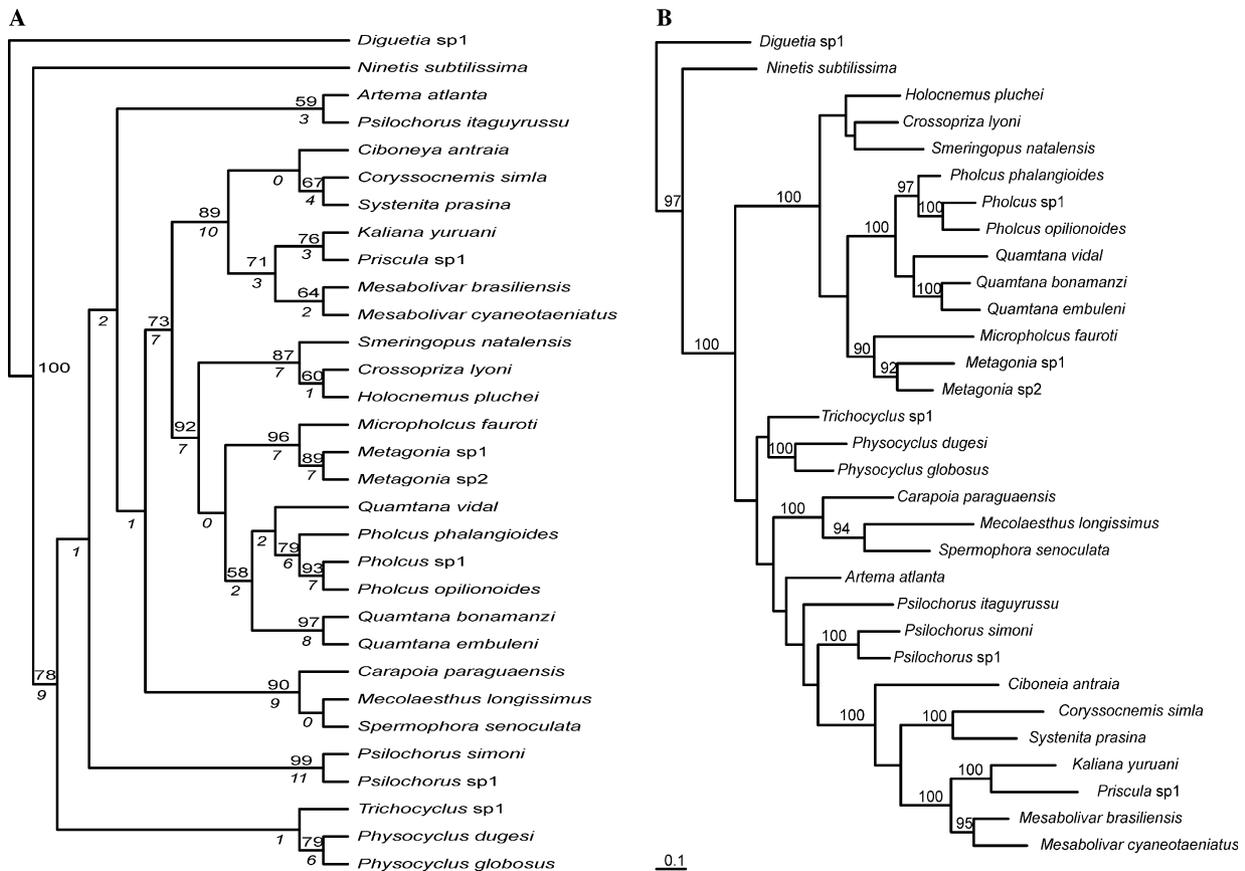


Fig. 3. Phylogenetic relationships within Pholcidae based on 12S rRNA sequence data: (A) MP tree. Numbers above nodes are bootstrap values (above 50%), below nodes Bremer support values; (B) ML tree inferred using the GTR+I+G model. Numbers on the nodes represent posterior probability values from Bayesian MCMC analysis (above 90).

### 3.3. Combined molecules and morphology

The ILD-test indicated that the separate gene partitions were not significantly incongruent with one another or with morphology ( $P=0.01$ ), so we combined all gene partitions and morphology in one dataset. Total length of the combined dataset (molecules + 45 morphological characters) was 3447 characters.

MP search resulted in six equally parsimonious trees of 9026 steps (CI = 0.463, RI = 0.417) (Fig. 4A). Few clades are highly supported, but the strict consensus tree is mostly resolved, with the exception of the relationships within the New World clade. *Ninetis* appears as sister to all other pholcids, *Priscula* is grouped within the New World clade, and the holocnemines are polyphyletic, with *Trichocyclus*–*Physocyclus*–*Artema* being sister group to the New World clade, and *Smeringopus*–*Crossopriza*–*Holocnemus* being positioned closer to pholcines. *Metagonia* is grouped with *Smeringopus*, *Crossopriza* and *Holocnemus*, with low support, while *Spermophora* sticks closer to pholcines as well. Partitioned Bremer support values (summarized in Table 4) indicate that the overall contribution is highest for the 28S rRNA data partition (51.8%), having the highest positive Bremer support values for most of the nodes, while 12S rRNA data conflict with several of the nodes, and COI and

16S rRNA contribute to even a much lesser extent. Morphology contributes to an overall 17.1% PBS and does not strongly conflict with any of the nodes found in the consensus tree.

The topology of the Bayesian consensus tree (Fig. 4B) is largely compatible with the MP tree—differences occur only within the New World clade, and with respect to *Metagonia*, here being positioned as sister group of pholcines.

### 4. Discussion

Generating robust phylogenetic hypotheses often requires gathering different data partitions and analyzing them in a combined data set, especially in cases where separate genes or morphology fail to give reliable results, or give results that are at odds with each other. Combination of data sets with varying rates of evolution (e.g., mitochondrial and nuclear genes), can efficiently accommodate for variation in results of individual analyses (Flook et al., 1999). Here, we present 2.5 Kb of molecular data for the spider family Pholcidae, added to extensive morphological data modified from previous publications (Huber, 2000, 2003b,c), and use it in an effort to gain a better understanding of intergeneric and sub-familial relationships. We generated nucleotide sequence data from three mitochondrial genes (two ribosomal and one

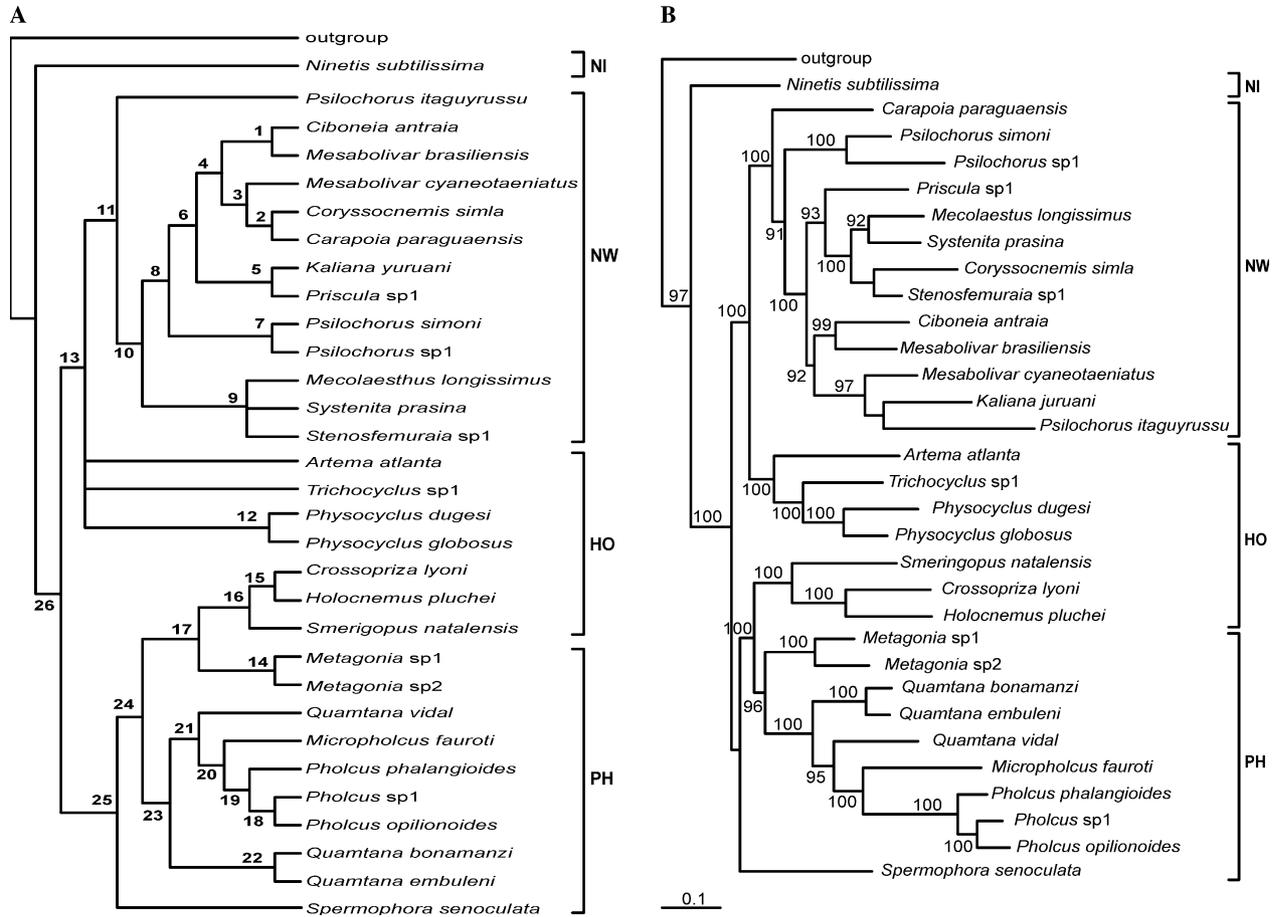


Fig. 4. Phylogenetic relationships within Pholcidae based on the combined dataset of 28S rRNA, 12S rRNA, 16S rRNA, cytochrome c oxidase subunit I and 45 morphological characters: (A) Strict consensus MP tree. Nodes are numbered, and the corresponding tree-statistics (bootstrap values, Bremer and partitioned Bremer support) are given in Table 4. (B) Majority-rule consensus tree resulting from Bayesian MCMC analysis. Numbers on the nodes represent posterior probability values (above 90). Branch lengths are proportional to number of changes. Currently recognized higher clades (according to morphology) are indicated by vertical bars and acronyms: NW, New World clade; PH, pholcines; HO, holocnemines; NI, ninetines.

protein-coding) and one nuclear ribosomal gene for which we assumed they might be informative within a given time-scale and range of divergence of pholcid taxa. This assumption was based on the fact that these genes were previously used with success in a range of phylogenetic analyses in spiders and other arthropod groups (e.g., Arnedo et al., 2004; Edgecombe et al., 2002; Fang et al., 2000; Hedin and Maddison, 2001; Maddison and Hedin, 2003; Prendini et al., 2003). We weighted all nucleotide positions of ribosomal genes equally no matter of their position in secondary structure, because a number of investigations and simulations have shown that substantial amount of phylogenetic information might be lost if we disregard any portion of structurally complex ribosomal genes (e.g., Dixon and Hillis, 1993). Furthermore, to minimize the effect that large gaps might have on the outcome of analyses if considered as fifth-state characters, we decided to score gaps according to the “simple” method of Simmons and Ochoterena (2000) as separate presence/absence characters. We assumed that this approach should be justifiable in the case of pholcid phylogeny because it was already successfully used in a number of recent phylogenetic studies dealing with similar problems (e.g., Arnedo

et al., 2004; Kawakita et al., 2003; Oliveira et al., 2003; Sánchez et al., 2003). It was shown that this method results in the increased resolution of the resulting trees in comparison to the ones obtained when treating the gaps as fifth-state characters (Simmons et al., 2001). There are also some other approaches to dealing with gaps in alignments (e.g., Giribet and Wheeler, 1999; Phillips et al., 2000; Wheeler, 1995), all showing the importance of including the gap information in phylogenetic analyses.

28S rRNA and, to a lesser extent, 12S rRNA genes proved to be useful in separate as well as in combined analyses, despite the observed large sequence divergences among distantly related taxa. Maximum parsimony, maximum likelihood, and Bayesian searches gave fairly consistent results for each of these two genes, supporting their reliability. Similar results were obtained in recent studies of other arthropod groups (e.g., Edgecombe et al., 2002; Edgecombe and Giribet, 2004; Prendini et al., 2003). On the other hand, 16S and COI did not perform so well in separate analyses, presumably because of their fast diversification, but also because of substantial lack of data. 16S and COI are therefore supposed to be responsible for a certain

Table 4  
Nodal support for Fig. 4A

Node No.	Bootstrap support	Bremer support	Partitioned Bremer				
			28S	12S	16S	COI	Morph.
1	<50	3	15	-5.5	1	-10	2.5
2	<50	0	0	0	0	0	0
3	<50	0	0	0	0	0	0
4	<50	1	3	-9	5	0	2
5	54	4	-3.5	10	2	-5.5	1
6	<50	3	2	1	-2	-1	3
7	99	24	5	6	3	4	6
8	<50	3	3	3	1	-6	2
9	<50	4	1	2	3.7	-5	2.3
10	50	4	4	1	-2	-3	4
11	71	7	6	-3	-6	-1	11
12	90	4	4	2	1	0	3
13	55	6	4	-3	-1	0	6
14	100	71	33.4	21.3	4	4.4	7.9
15	100	28	8	7	2	5	6
16	95	15	5	8	1	-2	3
17	<50	0	0	0	0	0	0
18	99	19	6	13	0	0	0
19	100	84	54.4	20.3	7	0	2.3
20	99	23	12	4	3	1	3
21	79	10	10	-1	-1	2	0
22	100	56	25	22	2	5	2
23	99	36	21	10	1	3	1
24	<50	0	0	0	0	0	0
25	<50	0	0	0	0	0	0
26	87	16	3	6.2	1	1	4.8
	Total Bremer	427	221.3	115.3	25.7	-8.1	72.8
	Percentage (%)	100	51.8	27	6	-1.9	17.1

Note. Columns list bootstrap, Bremer, and partitioned Bremer support (the contribution of the specified gene to the total Bremer support at the indicated node) as calculated for the combined data partition phylogeny (Fig. 4A). Bootstrap support values result from 1000 bootstrap analysis replicates.

amount of “noise” in the combined data set, reflected by bootstrap values lower than those obtained in the analyses of separate 28S and 12S rRNA genes. Partitioned Bremer support values, being higher for 28S and 12S rRNA genes than for 16S rRNA and COI data partitions (the last one having negative overall partitioned Bremer supports), also support this assumption. Nevertheless, the overall resolution of the “combined” MP tree is satisfactory enough and can be used as a guide in evaluation of several hypotheses about pholcid relationships, based so far exclusively on morphology. Furthermore, the Bayesian approach using all available data in combined analysis, resulting in a consensus tree that is largely congruent with the MP tree, gave us additional confidence in the following conclusions.

In all of the analyses conducted here, the monophyly of the Old World *Pholcus*-group (sensu Huber, 1995) as well as of the New World clade (sensu Huber, 2000) was not seriously questioned (only the MP analysis of 12S rRNA did not result in a monophyletic NW clade). Apart from that, the molecular data cast new light on several contentious issues in pholcid systematics, and they highlight some topics particularly worthy of further research.

#### 4.1. Ninetines

In most of the analyses of molecular data as well as in combined analysis, ninetines (here represented by only one taxon) appear as sister group to all other pholcids with high support. This is in agreement with the original idea of Simon (1893) and with preliminary analysis of morphological data (Huber, 2000: 36). Future analyses will have to include additional species, to further test this hypothesis and to test the monophyly of ninetines (doubted e.g., by Huber, 2003d).

#### 4.2. *Priscula*

Cladistic analyses of morphological data have suggested a close relationship of this Andean genus with ‘holocnemines,’ a group that is otherwise not present in South America. However, the synapomorphies were considered not compelling and similarities with New World genera were noted, warranting further study (Huber, 2000: 128). Our new molecular data strongly support inclusion of the genus in the New World clade. Its position within this clade, however, could not be resolved. While the NW group is almost always monophyletic (see above), the overall resolution within this clade is low, possibly because of rapid diversification—a conclusion being supported by very short internal branches in ML searches compared to terminal ones.

#### 4.3. *Holocnemines*

Holocnemines continue to be a problem, even after transfer of *Priscula* to the NW clade. *Trichocycclus*, an exclusively Australian genus, and *Physocycclus*, a New World genus, group together with high support in most of the analyses (*Artema* being close to them). This relationship is also supported by a unique and complex morphological character, i.e., modifications of the male genitalia that lock the right and left palps together and result in an asymmetric insertion (Huber and Eberhard, 1997).

Another group within ‘holocnemines’ that is well supported both by morphological and molecular data comprises the genera *Crossopriza*, *Holocnemus*, and *Smeringopus*, genera with an Ethiopian–Palaeartic distribution. Our results suggest closer affinity of *Crossopriza*, *Holocnemus*, and *Smeringopus* to pholcines than to other groups of pholcids, but the relationship of these two supra-generic taxa, the question of mono/para/polyphyly of ‘holocnemines’, as well as the position of *Trichocycclus*, *Physocycclus*, and *Artema* with respect to the rest of pholcids could not be robustly solved.

#### 4.4. *Metagonia*

This genus is a New World endemic, but previous analyses have suggested closer affinities with the largely Old World pholcines than with other New World taxa. This hypothesis is largely supported by molecular data. In

most analyses, *Metagonia* appears closer to the Old World pholcines, with the exception of 28S searches where it appears as sister group to the New World clade with a low support. Further investigations are needed to clarify this question.

#### 4.5. *Spermophora*

The position of this genus within pholcines has never been seriously contested, and a current revision of the genus supports this assumption (Huber, 2005). It comes thus as a surprise that our only representative of this genus, the type species *Spermophora senoculata*, has an unstable position. We strongly suggest further investigation of this and related (congeneric) species to clarify this question.

#### 4.6. Neotropical genera

Eugene Simon's expedition to Venezuela in 1887–88 resulted in a fairly good sample of pholcids and these formed the basis for several new genera (Simon, 1893), including *Mecolaesthus*, *Systemita*, *Coryssocnemis*, and *Litoporus*. Since then, many species have been added in most of these genera, and the taxonomic limits of these genera that first appeared well defined have become blurred and difficult to support within the framework of phylogenetic systematics. The present analysis underlines this problem, especially with regard to *Mesabolivar*, *Mecolaesthus*, *Systemita*, and *Stenosfemuraia*. The large Neotropical genus *Mesabolivar* appears paraphyletic in all of the analyses. *Mecolaesthus longissimus*, *Systemita prasina*, and *Stenosfemuraia* sp. form a clade in most analyses, and they all come from a limited geographic area, within a radius of ca. 100 km in the Cordillera de la Costa in northern Venezuela. Both *Systemita* and *Stenosfemuraia* are monotypic genera, and might well turn out to be synonyms of *Mecolaesthus*.

The first molecular dataset for the spider family Pholcidae, presented here, exhibits substantial phylogenetic signal in two ribosomal genes, 28S rRNA and 12S rRNA, while the other two genes, mitochondrial 16S rRNA and COI, appear to contain less phylogenetic information for the given range of diversification of taxa. The combination of all four genes and morphology, however, allowed us to draw some conclusions about the phylogenetic relationships of pholcids and to test some of the hypotheses based until now entirely on morphology. The results presented here are a valuable basis for further studies including a greater taxon sampling to get a stable and more detailed idea of the phylogenetic relationships in this spider family.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2005.08.016.

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