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Phylogenetic Corroboration of Superfamily Lycosoidae Spiders (Araneae) as Inferred from Partial Mitochondrial 12S and 16S Ribosomal DNA Sequences

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Kang Fang, Chih-Chieh Yang, Bor-Wei Lue, Shyh-Hwang Chen and Kuang-Yang Lue (2000) Phylogenetic corroboration of superfamily Lycosoidae spiders (Araneae) as inferred from partial mitochondrial 12S and 16S ribosomal DNA sequences. *Zoological Studies* **39**(2): 107-113. The genetic variations of two cribellate spider families, the Psechridae and Uloboridae, plus ecribellate spider families within the Araneomorphae, were examined by analyzing their partial mitochondrial 12S and 16S rDNA sequences, based on which phylogenetic closeness was tested. Using the Hexathelidae (*Macrothele holsti*) as an outgroup, the phylogenetic relationships of Lycosoidea superfamily spiders were investigated. The results indicate that cribellate spiders are polyphyletic. The reconstructed tree based on both maximum parsimony and neighbor joining analyses clearly demonstrates that the cribellate spider, Psechridae, is a member of the Lycosoidea clade that is comprised of the Lycosidae, Pisauridae, and Oxyopidae. The study also reaffirms that the cribellate funnel weavers, Psechridae, and orb weavers, Uloboridae, are two distinct lineages. However, the topology within the Lycosoidea clade from our analysis differs from that as inferred by morphological, ecological, and behavioral characteristics.

Key words: Psechridae, Mitochondrial rDNA, Phylogeny.

The plesiomorphic character of the anterior median spinneret has historically served as the basis for a fundamental taxonomic dichotomy in distinguishing monothetic cribellate and ecribellate lineages for labidognath spiders (Araneomorphae) (Lehtinen 1967). The cribellum, a flat plate spinneret homologue, is the source of densely packed spigots where silk is produced (Foelix 1996). Many arachnologists classify spiders based on the appearance of the cribellum, a primitive structure and which has been lost in some species during evolution (Lehtinen 1967, Eberhard 1988, Coddington and Levi 1991, Foelix 1996).

A fourth of all spider families have been grouped as cribellate (Lehtinen 1967). The appearance of the cribellum was considered as a crucial character in categorizing the funnel weavers, Psechridae (psechrid spiders), and orb weavers, Uloboridae, as cribellate (Lehtinen 1967, Foelix 1996). With characteristic funnel webs more than 1 m wide and body lengths up to 2 cm, the psechrid spiders are the biggest among cribellate spiders (Kayashima 1962, Lee 1964). The Psechridae are distributed in Southeast Asia including India, Burma, Borneo, and Indonesia, and in Australia. There are 4 genera with 13 species in the family, i.e., *Psechrus, Fecenia, Haurokoa*, and *Poaka* (Forster 1973, Levi 1982). *Psechrus* can be located at up to 2300 m in elevation throughout Taiwan (Yoshida 1978, Chen 1996a, b). A V-shaped marking on the sternum and a white median stripe on the venter of the abdomen are characteristic for *Psechrus* (Levi 1982). Similar to the ecribellate spiders, Pisauridae, the female *Psechrus* carries its egg-sac in the chelicerae. Unlike cribellate spiders, however, *Psechrus* are born with brood care behavior, eyes with a grate-shaped tapetum plus a vestigial structure, and a colulus with no apparent function, which are characteristic of ecribellate spiders (Levi 1982,

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Foelix 1996). Besides, the ambiguous genital structures of young spiders, plus the difficulties in collecting mature specimens, have made classification of the psechrid spiders unclear (Lehtinen 1967, Platnick 1977). Furthermore, the small epigynum and sclerotized sculpturing in the female genital tract that distorts the cribellum make identification even more complicated. Because of the structural and behavioral ambiguities distinct from those of the Uloboridae, the taxonomy of the Psechridae has remained inconclusive. Lehtinen (1967) suggested that the established cribellate and ecribellate dichotomy in spider systematics be dropped. However, this was intensely refuted by Platnick (1977).

Homann (1971) clustered several families together as the Lycosoidea clade that includes the Psechridae based on their common grate-shaped tapetum, oval calamistrum, and tibial crack. This taxonomic hypothesis was strongly supported by Griswold (1993). In both Homann and Griswold's work, the Psechridae were included within Lycosoidea, which also consists of Lycosidae, Pisauridae, Oxyopidae, and Ctenidae (Levi 1982). Since previous taxonomy of psechrid spiders mainly relied on their morphological and behavioral features (Coddington and Levi 1991), different criteria have to be developed to test the validity of the tree.

To examine whether the Psechridae is included in the Lycosoidea superfamily, we have analyzed their partial mitochondrial (mt) 12S and 16S ribosomal DNA (rDNA) sequences using taxa samples within the clade. The results corroborate that cribellate Psechridae is indeed clustered together with the ecribellate Lycosidae, and with Pisauridae and Oxyopidae within the superfamily Lycosoidea clade as previously hypothesized, and that the cribellate funnel weavers, Psechridae, and orb weavers, Uloboridae, are two distinct lineages.

MATERIALS AND METHODS

Study sites and materials collection

The spiders used for the 12S and 16S rDNA sequence determination were from various localities in Taiwan as listed in table 1. Prior to sacrificing, the spider specimens were maintained in a plastic box

Table 1. Locality citations and their corresponding localities in latitude and longitude of spider species investigated for mitochondrial 12S and 16S rDNA sequence analyses

Spider classification	Locality
Mygalomorphae	
Hexathelidae	
Macrothele holsti (Pocock, 1901)	121°39'E, 25°00'N
Araneomorphae	
Araneoidea	
Agelenidae	
Agelena limbata (Phorell, 1897)	121°06'E, 24°30'N
Uloboridae	
Zosis geniculatus (Olivier, 1879)	121°08'E, 24°55'N
Tetragnathidae	
Nephila maculata (Fabricius, 1793)	121°02'E, 24°02'N
Lycosoidea	
Lycosidae	
Lycosa coelestis (L. Koch, 1877)	121°39'E, 25°00'N
Pardosa takahashii (Saito, 1936)	121°39'E, 25°00'N
Pisauridae	
Dolomedes raptor (Boes and Str., 1906)	121°44'E, 25°06'N
Oxyopidae	
Oxyopes sertatus (L. Koch, 1877)	121°39'E, 25°00'N
Psechridae	
Psechrus sp. GLH	121°21'E, 24°50'N
Psechrus sp. HS	121°02'E, 24°02'N
Psechrus sp. ALS	120°47'E, 23°32'N
Psechrus sp. PL	121°42'E, 24°56'N
Psechrus sp. SS	120°15'E, 22°39'N

with a piece of leaf to keep proper humidity for no more than 4 d. The sacrificed samples were either preserved in alcohol at room temperature or frozen in a –78 °C freezer before DNA extraction.

DNA extraction

DNA from fresh samples was exracted following modified protocols of Huber et al. (1993). For bigger spiders, muscle or leg tissue was dissected out of the femora; for smaller ones, the complete prosoma and legs were taken for DNA extraction. The tissue was digested in a solution containing 70 mM NaCl, 10 mM Tris-HCl, pH 7.4, 25 mM EDTA, 0.9% SDS and 6 μg/ml protease K (Promega; Madison, WI). The mixture was incubated for 12 h at 50 °C and extracted with a phenol/chloroform solution (1:3; v/v). The top supernatant was precipitated with buffer of 0.5 M potassium acetate, pH 5.2 and ethanol. The precipitate was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and digested with RNase A (125 μg/ml) followed by phenol/chloroform extractions. The precipitated DNA from 60% isopropanol (v/v) was washed with 70% ethanol, air-dried, and suspended in 50 μl of TE buffer. The purified DNA was stored at –20 °C.

Mitochondrial DNA PCR amplification

DNA fragments from both mt 12S and 16S rDNA were amplified using modified primer sequences following the published protocols, and the sizes of the products were approximately 430 and 350 bp, respectively. In the PCR assay, a total of 250 ng DNA was mixed in a 25-μl buffer containing 100 μM dNTP in the presence of 0.5 unit *Taq* DNA polymerase and 400 nM of each primer (16S-sense primer: CGC CTG TTT ATC AAA ACA T; antisense primer: GTC TGA ACT CAG ATC ACG T; Cunningham et al. 1992). (12S-sense primer: GGG ATT AGA TAC CCC ACT AT, antisense primer: TTC AGC ATT GTT CCA TTC ACA TG; Kocher et al. 1989). The thermal profile was as follows: (1) DNA denaturation at 94 °C for 1.5 min, (2) annealing for 2 min, ranging from 54 to 38 °C depending on the species used; and (3) extension for 2 min at 72 °C (Masumoto 1997). The products were resolved on a 0.8% agarose gel, and the fragments that appeared at nearly 430 and 350 bp for 12S and 16S rDNA, respectively, were excised, eluted, and cloned into the pGem-T vector (Promega; Madison, WI), respectively. The selected ampicillin-resistant clones of PCR products were sequenced by a Pharmacia autosequencer with a dideoxy chain terminating sequencing kit (Pharmacia; Uppsala, Sweden). The sequences were also determined by direct sequencing in both directions by an ABI PRISM 377 DNA autosequencer. In all cases studied, at least 2 individuals of each species or different parts of the same individual were examined and the differences were less than 1%. The consensus sequence was taken from the same source of DNA.

DNA sequence analysis

Partial sequences of 12S and 16S rDNA were aligned, based on which phylogeny was constructed. The sequence for the Hexathelidae (*Macrothele holsti*) was used as an outgroup. Pairwise alignment of the DNA sequences and calculation of percentage differences were done by the PILEUP program of the GCG software package (Devereux et al. 1991). Gaps were treated as missing data and counted as penalties in phylogenetic reconstruction. The numbers of variable and potentially phylogenetically informative sites were calculated. The aligned DNA sequences were analyzed using the MEGA program (Kumar et al. 1993). Phylogenetic analyses were performed using MEGA (Kumar et al. 1993) by neighbor joining (Saitou and Nei 1987) with Kimura's (1980) 2-parameter method corrected for transition/transversion bias plus base composition bias (Tamura 1992). Maximum parsimony analysis was conducted by the heuristic search using PAUP 3.1.1 (Swofford 1993). The robustness of each clade was tested by bootstrapping with 500 replicates (Felsenstein 1985).

RESULTS

Amplified partial mitochondrial 12S and 16S rDNA sequences

The appropriate annealing temperature for the reproducible PCR reaction was found to range between 54 and 38 °C depending on the species used. In addition, a rather high concentration of magnesium ion ranging between 1.5 and 5.8 mM was required for PCR amplification. The eluted amplified fragments were either sequenced directly or cloned into the pGem-T vector.

Composition and analysis of the sequenced rDNA

Among all the sequences, *M. holsti* has the shortest size fragment (414 and 354 bp for 12S and

16S rDNA, respectively) and the lowest A-T content (Table 2). The alignment indicates that there are 5 and 7 conserved blocks in 12S and 16S rDNA, respectively. The result is consistent with those in the sequences of vertebrates (Kjer 1995), ctenid spiders (Huber et al. 1993), and *Escherichia coli* (Boros et al. 1979). The FASTA program, a function implemented in GCG, identified the DNA sequences as 12S and 16S rDNA.

The sequences from the PCR products of mt 12S and 16S rDNA from the cribellate and ecribellate spiders were combined together for analysis. For 16S rDNA fragments, there are 449 aligned sites, of which 323 are variables and 184 informative. For 12S rDNA sequences, 276 variable and 190 informative sites were detected in 381 aligned sites. Five populations of psechrid spiders from different locations in northern, central, and southern Taiwan were analyzed altogether, and their distance was estimated to be 1.1% to 14.4% for 12S and 1.2% to 5.0% for 16S rDNA. Meanwhile, interfamily divergences among the genera, *Psechrus, Lycosa, Pardosa, Oxyopes,* and *Dolomedes* varied from 11.1% to 22.4% for 16S and 12.1% to 25.9% for 12S rDNA. On the other hand, the sequence divergence between *Psechrus* and *Zosis* (Uloboridae) ranged from 10.9% to12.0% for 16S and 25.7% to 27.9% for 12S rDNA. The result signifies a greater genetic distance between Psechridae and Uloboridae compared to those within the Lycosoidea. The distance between *Zosis* and *Agelena* is 0.17 for 16S and 0.38 for 12S rDNA, indicating a close affinity between the two taxa.

Phylogenetic analysis

Totally 830 combined sites were used for analysis, of which 599 were variables and 374 informative. With *M. holsti* designated as an outgroup, a single maximum parsimony tree with a length of 613 steps (consistency index, 0.750; retention index, 0.699) (Kluge and Farris 1969, Farris 1989) was identified. *Nephila maculata* appeared to be the most distant lineage relative to Psechridae, followed by *Zosis geniculatus* and *Agelena limbata* based on both parsimony and neighbor joining analyses. Two genera, *Pardosa takahashii* and *Lycosa coelestis*, were included in the Lycosidae. The Lycosidae-Pisauridae pair formed a sister group to the clade of Oxyopidae and Psechridae. Thus, the reconstructed tree clearly demonstrates that Psechridae, Oxyopidae, Pisauridae, and Lycosidae are grouped together as the Lycosoidea clade (Fig. 1). The amaurobiodes, Agelenidae and Uloboridae, were previously placed in the RTA (retrolateral tibial apophysis) and Orbiculariae clades, respectively (Coddington and Levi 1991). In contrast, they were clustered together according to molecular similarity in this work. The great distance between the Psechridae and Ulobridae also indicates the polyphyly of cribellate spiders.

DISCUSSION

Because of maternal inheritance, mitochondrial DNA sequences have been proven to be a convenient tool in studying genetic structure and phylogenetic relationships of arthropods (Moritz et al. 1987). The nucleotide compositions in the spider mt DNA sequences studied are rich in adenine and thymine. The proportions of thymine and adenine in DNA sequences of the Uloboridae and the families studied within infra-order Araneomorphae are close, but

Table 2. Nucleotide composition, length, and GenBank accession number of the sequenced 12S and 16S rDNA of Mygalomorphae and Araneamorphae (A = adenosine, C = cytidine, G = guanosine, T = thymine)

Species	12S rDNA						16S rDNA					
	A	C	G	T	Length (bp)	Accession number	A	C	G	T	Length (bp)	Accession number
	(%)						(%)					
M. holst	39.0	16.4	12.1	32.5	354	AF145036	33.1	18.8	16.4	31.7	414	AF145270
N. maculata	39.8	10.1	13.3	36.8	367	AF145035	35.6	14.2	13.7	36.5	444	AF145269
Z. geniculatus	41.4	10.5	9.7	38.4	362	AF145034	39.2	11.7	11.0	38.1	428	AF145268
L. coelestis	42.5	10.4	10.9	36.2	367	AF145030	39.6	11.6	12.1	36.6	429	AF145264
D. raptor	42.7	10.8	10.5	36.0	370	AF145031	40.0	11.1	11.3	37.6	433	AF145265
P. takahashii	43.6	10.4	10.7	35.3	365	AF145032	41.5	11.3	11.1	36.1	432	AF145266
O. sertatus	43.7	10.7	10.2	35.4	364	AF145033	41.0	11.0	12.8	35.2	429	AF145267
A. limbata	43.6	12.3	11.0	33.1	365	AF145037	38.3	11.8	11.1	38.3	433	AF145271
P. sp. PL	40.8	8.2	11.0	40.0	365	AF144650	40.4	11.0	10.8	37.8	426	AF145263

lower for the Hexathelidae (Mygalomorphae). A high adenine-thymine bias has been found in16S rDNA of invertebrates, including ctenid spiders (77%, Huber et al. 1993), king crabs (73%, Cunningham et al. 1992), *Drosophila* (76%, Clary and Wolstenholme 1985), planthoppers (71%-74%, Yeh et al. 1998), and mosquitoes (76%, HsuChen et al. 1984). The same trend has been observed in12S rDNA of honeybees (85%, Crozier and Crozier 1993). An increase of the adenine and thymine content of mt DNA seems to be a general tendency in the evolution of arthropods (Zehethofer and Sturmbauer 1998), which reflects that sequences have not been subjected to functional constraints or mutational pressure for a long time (Li 1997). The nucleotide sequence divergence is distinct between *Psechrus* sp. and *Zosis* compared to those among families in the Lycosoidea clade, thereby resolving the Psechridae and Uloboridae relationship.

Phylogenetic analysis of 12S or 16S rDNA data alone among the spiders analyzed produced similar trees with minor differences (data not shown). The differences may reflect noise in the disparate data or independent histories derived from the two genes. In the case of the former, resolution can be improved by analyzing the combined data of closely related genes (Hillis 1991). Both mitochondrial 12S and 16S rDNA genes are adjacent to that of valine-tRNA, and it is highly unlikely that they evolved independently (Kluge 1989, Hillis and Huelsenbeck 1992). The analysis of joining all informative sites between 2 adjacent mt 12S and 16S genes has been applied successfully in resolving the wide-ranging genus *Phoxinus* (Simons and Mayden 1998) and extant amphibian families (Hay et al. 1995, Fu 1998). Thus, to clearly resolve different topologies produced by either noise or an insufficient phylogenetic signal, all the informative sites from 12S and 16S rDNA sequences have been combined altogether for analysis. With *M. holsti* as an outgroup, a single parsimonious tree indicates that *Nephila maculata* is phylogenetically basal to the Lycosoidae, which is also strongly supported by the neighbor joining method, and agrees with the previously reported classification. Based on the tree organization, 4 spider families, i.e., Lycosidae, Pisauridae, Oxyopidae, and Psechridae, are clustered as the Lycosoidea clade. Contrary to previous findings, from taxa distribution, Oxyopidae is rather closely related to the Lycosidae-Pisauridae pair that merged with Pse-

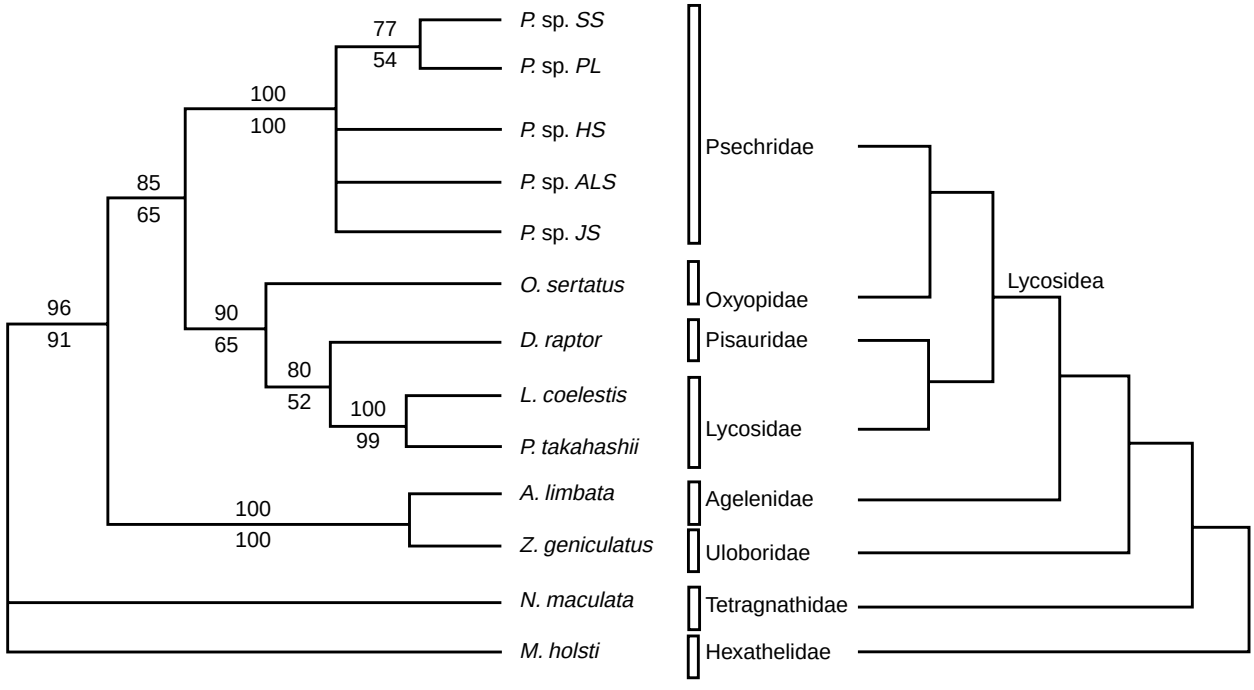

Fig. 1. Phylogenetic tree of spiders as inferred from mitochondrial 12S and 16S rDNA sequences analyzed by maximum parsimony and neighbor joining methods (left). In the reconstructed tree obtained, numbers on top of the branch are bootstrap values based on parsimony and those below from neighbor joining. The right panel is the cladistic tree for infra-order Araneomorphae and Mygalomorphae as hypothesized by Coddington and Levi (1991). Various species on the left panel are grouped together into families as represented by vertical block bars.

chridae taxa, and Uloboridae is excluded from the Lycosoidea clade. The reconstructed tree based on both neighbor joining and parsimony analyses corroborates with a previous hypothesis (Homann 1971, Coddington and Levi 1991) that the superfamily ought to include Psechridae, and that Agelenidae and Uloboridae are 2 ancestral taxa of Araneomorphae. Since Hexathelidae is distant to other taxa of Araneomorphae, the phylogenetic structure was analyzed again by omitting *M. holsti* to detect possible homoplasies due to distal basal branching. Using *N. maculata* as an outgroup, an identical parsimonious tree of length 321 steps (consistency index, 0.690; retention index, 0.769) can also be built. The reconstructed phylogenetic structure and the branching orders within the tree remain consistent and variations in bootstrap values in both neighbor joining and parsimony are not altered significantly (less than 5%). Taken together, the vigorous and congruent trees from studying 2 genes strongly support that (1) Agelenidae and Uloboridae are 2 distal families relative to Psechridae; (2) cribellate Psechridae and Uloboridae are indeed 2 distinct lineages; and (3) Psechridae can be included within the superfamily Lycosoidea.

Both unequivocal cladistic and phenetic analyses imply that, within the variation detected, the limited numbers of *Psechrus* in Taiwan can be resolved based on the collection location. More study is needed to test the effectiveness and the extent for analysis in combining 2 slow-evolving genes for eliciting additional evolutionary history and taxonomy of ancient spider organisms. As of today, only a handful of papers addressing molecular phylogeny of spiders have appeared (Huber et al. 1993, Gillespie et al. 1994, Zehethofer and Sturmbauer 1998). In most works, a single gene, either 12S or 16S rDNA, was used. The conflicts among groups and clades produced suggested that more informative sites must be included to scale down diagnostic noise due to insufficient data based on the information from a single gene. Given the complicated synapomorphies of Araneae systematics, the analysis of contiguous mitochondrial genes altogether proved useful, and the potential levels of taxonomic resolution need to be examined.

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結合粒線體 12S 及 16S 核醣體 DNA 序列分析狼蜘總科的親緣關係

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褸網蜘蛛(Psechridae)在構造上與篩疣類蜘蛛相似，但是在雌蜘抱卵行為及其眼部構造又與無篩疣類蜘蛛相似，其分類地位未能完全被瞭解及定位。本文旨在使用分子生物學驗證其親緣關係。實驗設計以臺灣產褸網蜘屬(*Psechrus*)為主，放大並且分析粒線體部份 12S 及 16S 核醣體 RNA (rDNA)序列，與狼蜘總科 (Lycosidae) 間比較其類緣關係。實驗樣本包括臺灣產無篩疣類的草蜘科 (Agelenidae) 、長腳蜘蛛科 (Tetragnathidae) 與狼蜘總科的狼蜘科 (Lycosidae) 、跑蜘科 (Pisauridae) 、貓蜘科 (Oxyopidae) ，並且以篩疣類的陰蜘科 (Uloboridae) 和褸網蜘蛛科為內群種類，而以原疣亞目的赫思特上戶蜘蛛為外群。將定出粒線體 12S 及 16S rDNA 之核甘酸序列結合之後，合併一同比對，以瞭解各分類群之間親緣關係。由 maximum parsimony 及 neighbor joining 所得到的強烈支持共同關係樹顯示，屬於篩疣類蜘蛛但是結漏斗狀網的褸網蜘蛛應與無篩疣類狼蜘、跑蜘及貓蜘等有較親近的親緣關係，反而與同屬篩疣類但是結圓形網的陰蜘親緣關係較遠。這項結果支持 Homann (1971)及 Coddington and Levi (1991)所提出褸網蜘蛛應與狼蜘、跑蜘及貓蜘一併形成狼蜘總科的假說。這項以結合鄰近保守基因，共同分析方式，對釐清繁瑣複雜的新疣亞目蜘蛛(Araneae)的親緣關係，提供了一個不同的研究方向。

關鍵詞：褸網蜘蛛，粒線體核醣體 DNA ，親緣關係。

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