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AN ELECTROPHORETICAL AND IMMUNOLOGICAL STUDY OF PYCNOGONIDA, WITH PHYLOGENETIC CONSIDERATIONS

by

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ABSTRACT

An electrophoretical and immunological study is made of nine species of pycnogonids, representing seven families, from the Catalan coast.

An electrophoretogram of each species is given and the antigenic properties of its protein bands are determined. Taking as comparative basis the serological affinities of the different species with *Ammothella longipes* and by morphological considerations as well, five major evolutionary lines have been established. The Nymphonidae are considered the more primitive. The Ammotheidae — Tanystylidae are considered a central group by their greater number of protein bands and their greater capacity of morphological diversification. The Phoxichilidiidae-Endeidae are considered intermediate between the former and the Callipallenidae and Pycnogonidae, the latter two lines are considered the most evolved.

RÉSUMÉ

Neuf espèces de Pycnogonides des côtes catalanes, représentant sept familles, sont étudiées sous l'aspect électrophorétique et immunologique.

On donne pour chaque espèce un électrophorétogramme et on détermine les propriétés antigéniques de ses bandes protéiques. Cinq lignées évolutives majeures sont reconnus, en prenant comme base des comparaisons les affinités sérologiques des différentes espèces avec *Ammothella longipes*, mais aussi en tenant compte de considérations morphologiques. On considère les Nymphonidae comme étant le groupe le plus primitif. Les Ammotheidae-Tanystylidae sont considérés comme occupant une position centrale, à cause de leur nombre plus élevé de bandes protéiques et de leur capacité supérieure de diversification morphologique. Les Phoxichilidiidae-Endeidae occupent une position intermédiaire entre les familles mentionnées et les Callipallenidae et Pycnogonidae (ces deux dernières lignées étant considérées comme étant les plus évoluées).

1. INTRODUCTION

The systematic arrangement of the 73 extant pycnogonid genera (Fry, 1978) presents important problems due to their particular morphological characters, which not always show clear indications to group them in clearly defined families. Hedgpeth (1947), recognizes 8 families: Nymphonidae, Pallenidae, Phoxichilidiidae, Endeidae, Ammotheidae, Tanystylidae, Colossendeidae and Pycnogonidae.

In previous publications (De Haro, 1967, 1969), one of us has taken the phyletic relationships with other groups into consideration. In the frame of a Ph.D. Thesis (Munilla, 1978), an electrophoretical and immunological study has been done on 8 genera, representing 7 families of the 8 considered by Hedgpeth, leaving out the Colossendeidae because no specimens were available from the Catalan coast.

Fry (1978), conscious of the obscure relationships, and searching new viewpoints, has applied techniques of multivariate analysis, in relation with numerical taxonomy, to pairs of genera, using the General Similarity Coefficient of Gower.

In the present work we try to cast some light on the phylogenesis of the pycnogonid families on morphological and serological grounds.

2. MATERIAL AND METHODS

(a) *Species studied*

The species studied have been collected on the Catalan coast (Spain) and are the following: *Nymphon gracile* Leach, 1814 (Nymphonidae); *Achelia echinata* Hodge, 1864 (Ammotheidae); *Ammothella longipes* (Hodge, 1864) (Ammotheidae); *Ammothella uniunguiculata* (Dohrn, 1881) (Ammotheidae); *Tanystylum orbiculare* Wilson, 1878 (Tanystylidae); *Anoplodactylus virescens* (Hodge, 1864) (Phoxichilidiidae); *Endeis spinosa* (Montagu, 1808) (Endeidae); *Callipallene emaciata emaciata* (Dohrn, 1881) (Callipallenidae); *Pycnogonum nodulosum* (Dohrn, 1881) (Pycnogonidae).

(b) *Electrophoretical techniques*

Disc electrophoresis has been utilized (Ornstein, 1964; Davis, 1964), with copolimer of acrilamide and bisacrilamide as support (Arias, 1973), with electrophoretical migration, tinction and decoloration (Munilla & Matallanas, 1979).

The coloured bands of the gels were read with a Vitatron Densitometer, model MPS, of the Instituto de Investigaciones Pesqueras de Barcelona.

The spikes of the densitometric curves have been homologized in the different experiments with each species. Percentages corresponding to each protein band have been calculated by planimetry in the curves of the different species.

(c) *Obtaining electrophoretical samples and total proteins*

Adult specimens have been utilized, males and females, kept in a refrigerator at 7° C for some days. Crushing has been done with 15% saccharose and centrifugation at 4000 r.p.m. during 30 minutes. We have taken as standard concentration for the electrophoretical samples 10 mg wet weight by 0.1 ml of 15% saccharose.

In table I the wet weight of one specimen is shown, the number of specimens crushed to obtain the concentration desired to obtain optic density readings at 280 nm, and the corresponding total protein concentration calculated according to Neremberg (1968). A Beckman model DV-2 spectrophotometer was used for each reading, 1 μl of each specific sample was diluted in 100 μl of distilled water.

TABLE I

Number of specimens necessary to obtain 10 mg of wet weight, with the corresponding optic densities and total protein concentration.

	Wet weight 1 specimen (mg)	No. of specimens required	Optic density	Total protein (mg/ml)
Nymphon gracile	3.4	3	0.091	6.37
Ammothella longipes	0.48	21	0.090	6.3
A. uniunguiculata	1.37	7	0.066	4.62
Achelia echinata	0.68	15	0.120	8.4
Tanystylum orbiculare	0.36	28	0.046	3.22
Anoplodactylus virescens	1.8	5	0.070	4.9
Callipallene e. emaciata	0.46	22	0.044	3.08
Endeis spinosa	3.5	3	0.137	9.59
Pycnogonum nodulosum	3	3	0.080	5.6

(d) *Immunological techniques*

The test of double immunodiffusion (D.I.D.) of Ouchterlony (1948), in 2% agarose in physiological serum, was used.

After some experiments with cobayas and rabbits, using *Achelia echinata* and *Ammothella longipes* as antigens, we have preferred *A. longipes* and rabbits for producing antibodies.

We have used 6.09 mg/ml of total proteins and all injections, except the intravenous, have been done mixing this quantity with an equal volume of complete coadjuvant of Freud; so, the concentration of each injection was 3.05 mg/ml. In axilas and groins the injections were given in the lymphatic system. In the legs the injections have been intramuscular and subcutaneous in the back. In the ears they were intravenous (see table II).

TABLE II

Immunisation in rabbit against *Ammothella longipes*.

No. of injection	Day	Vol. (ml)	Via	Total ml injected
1	1	0.75	axilas	1.5
2	8	0.5	groins	1
3	15	1	post. hind leg	1
4	22	1	post. right leg	1
5	29	1	back	1
6	36	1	back	1
7	43	1	axilas	2
8	50	1	groins	2
9	57	1	ear	1

The blood extracted was maintained at 37° C during 1 hour, the centrifugation of the serum was done twice at 3000 r.p.m. and preservation at —20° C with sodium azide. The serum obtained at the 9th injection has offered the best results, with 5 lines of precipitation against homologous antigens in the D.I.D. test.

In order to determine the optimal concentrations of homologous precipitation, we have put pure antigen with 6.09 mg/ml of total proteins in one recipient and decreasing concentrations of antibodies diluted to the half in physiologic serum in others. The optimal concentration is without dilution.

This optimal concentration of antibodies of *Ammothella longipes* was utilized for carrying out cross experiments against the antigens of the different species (concentrations in table I). The affinities with the antibodies are shown by the common bands of precipitation.

2. RESULTS

(a) *Electrophoresis*

The electrophoretograms represent the protein density in decreasing sense, from black to hatched and dotted. The numbers of protein fractions are given in table III.

Densitometric profiles and electrophoretograms of the species studied are given in figs. 1 to 9.

TABLE III

Number of protein fractions in the different species studied.

Species	No.
Nymphon gracile	5
Ammothella longipes	15
Ammothella uniunguiculata	14
Achelia echinata	16
Tanystylum orbiculare	13
Anoplodactylus virescens	11
Endeis spinosa	11
Callipallene e. emaciata	7
Pycnogonum nodulosum	9

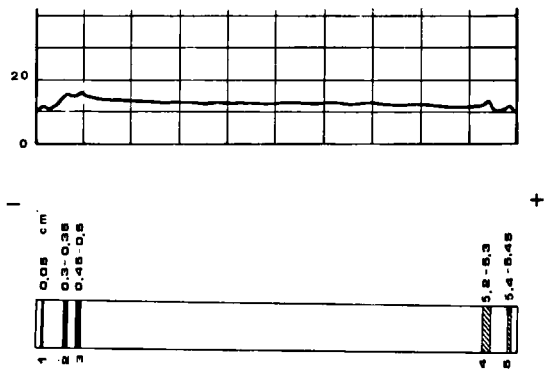

Fig. 1. Densitometric profile and electrophoretogram of *Nymphon gracile* Leach.

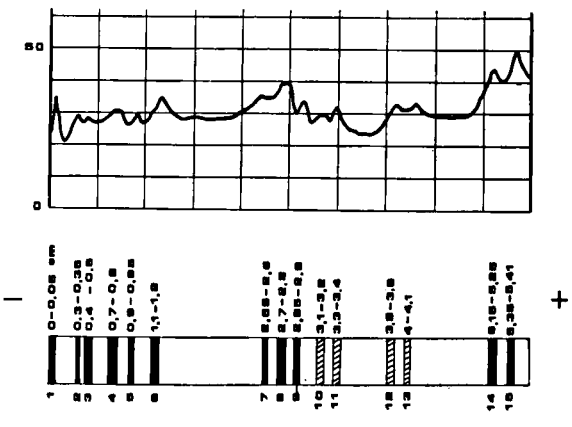

Fig. 2. Densitometric profile and electrophoretogram of *Ammothella longipes* (Hodge).

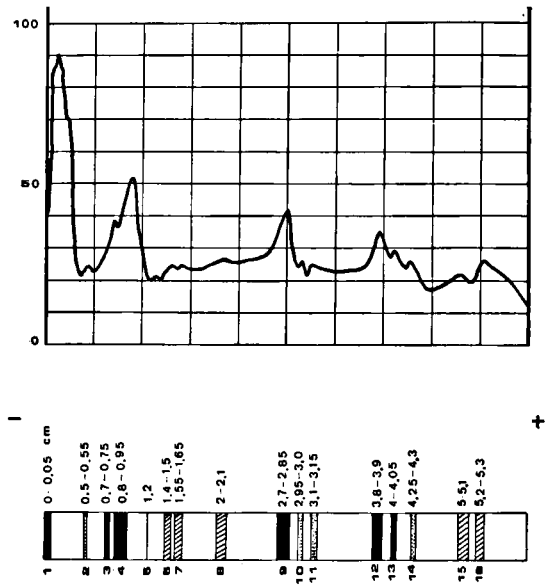

Fig. 3. Densitometric profile and electrophoretogram of *Achelia echinata* Hodge.

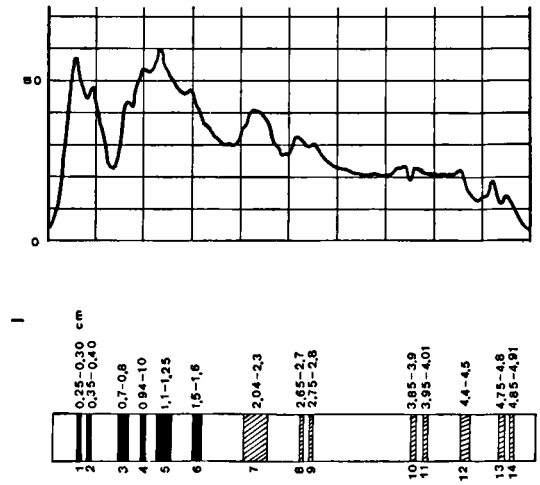

Fig. 4. Densitometric profile and electrophoretogram of *Ammothella uniunguiculata* (Dohrn).

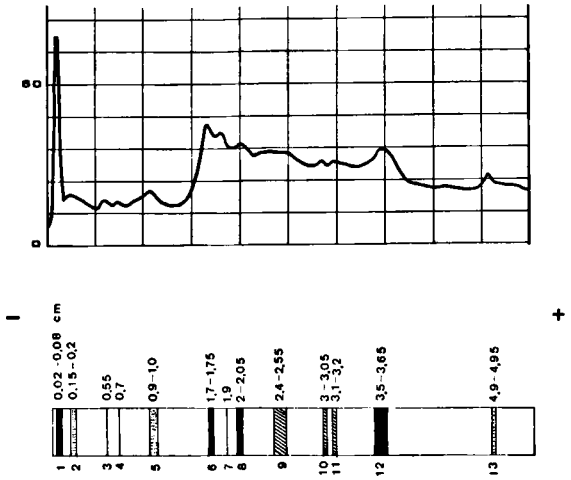

Fig. 5. Densitometric profile and electrophoretogram of *Tanystylum orbiculare* Wilson.

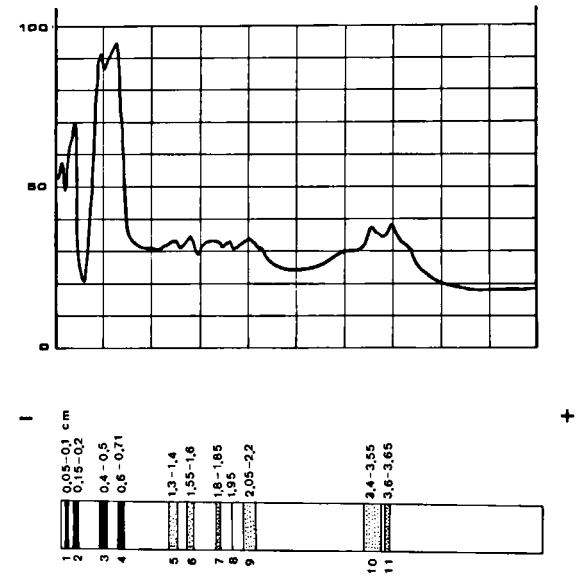

Fig. 6. Densitometric profile and electrophoretogram of *Anoplodactylus virescens* (Hodge).

(b) *Double immunodiffusion* (D.I.D.)

In fig. 10 the precipitation lines obtained in the species studied are shown, with anti-*Ammothella longipes*-serum, and the number of homologous lines obtained. The exact identification of the correspondence of the precipitation lines has been obtained visually, putting the plate in a photographic amplifier in the dark room.

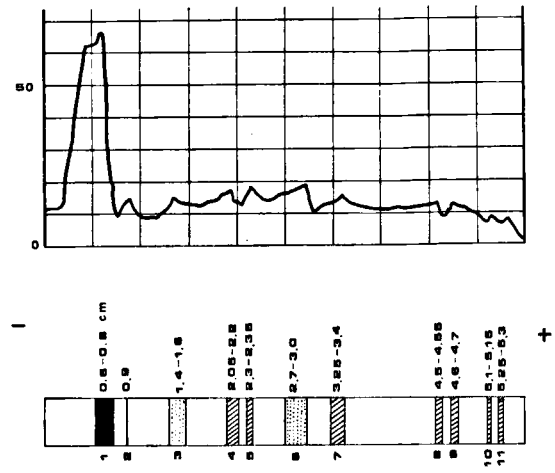

Fig. 7. Densitometric profile and electrophoretogram of *Endeis spinosa* (Montagu).

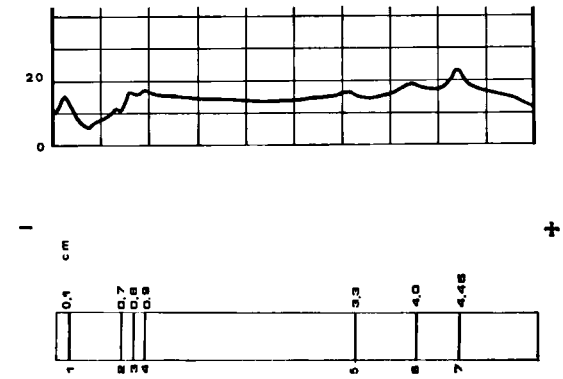

Fig. 8. Densitometric profile and electrophoretogram of *Callipallene e. emaciata* (Dohrn).

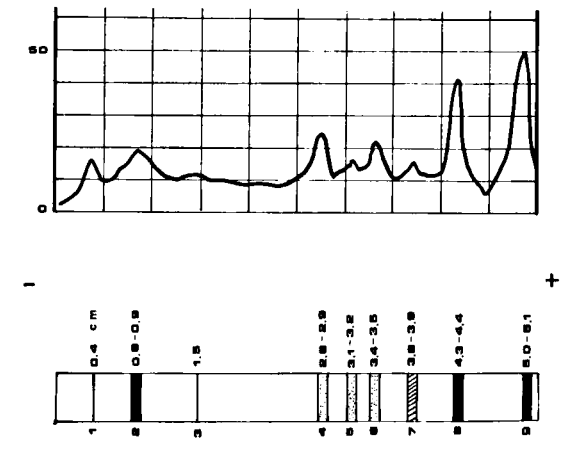

Fig. 9. Densitometric profile and electrophoretogram of *Pycnogonum nodulosum* (Dohrn).

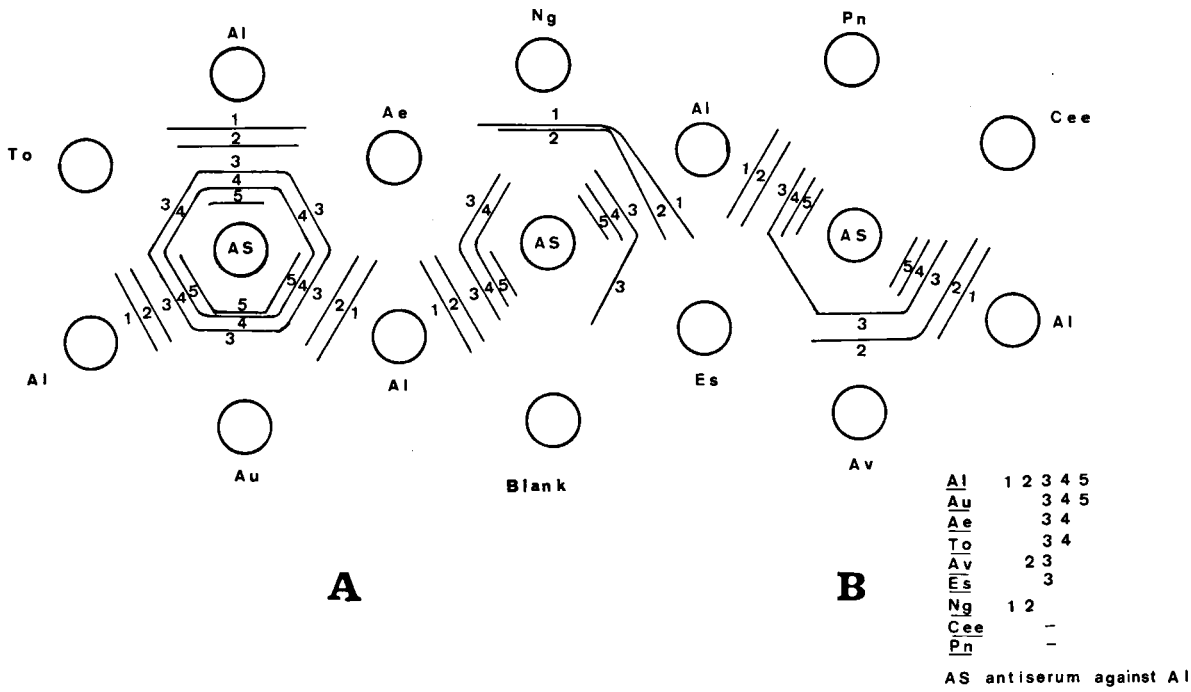

Fig. 10. A, Precipitation lines in cross experiments by D.I.D. with anti-*Ammothella longipes*-serum obtained in rabbit. B, Number of homologous lines of precipitation obtained.

Fig. 11 shows the comparative electrophoretograms of the species, considering bands 2, 3, 4, 5, and 6 of *Ammothella longipes* as the antigenic proteins producing the precipitates in the rabbit serum.

By the position they occupy, these bands resemble immunoglobulins, being lipoprotein in band 1, and we homologize these bands with the protein fractions of the species, in accordance with the electrophoretic and immunodiffusion methods.

3. PHYLOGENETIC CONSIDERATIONS

Considering the number of protein fractions obtained in the electrophoretograms, the families Ammotheidae and Tanystylidae are presenting the greater number of bands, followed by the Phoxichilidiidae and Endeidae.

The Nymphonidae present the lowest number of bands, followed by the Callipallenidae and Pycnogonidae (table III).

It results from the immunodiffusion experiments that the Pycnogonidae and Callipallenidae are far remote from *Ammothella longipes*, not presenting any homologous protein bands with it.

Notwithstanding, *Nymphon* presents two precipitation bands in common with *Ammothella longipes*, viz. numbers 1 and 2 (figs. 10 and 11).

On morphological grounds, and in agreement with Hedgpeth (1947), we consider the Nymphonidae the more primitive, by its well developed palps and chelifores, and by the presence of ovigers in both sexes. Considering these characters, the Pycnogonidae are highly evolved (Hedgpeth, 1947). Both families represent the two extremes of pycnogonid evolution.

The Nymphonidae are serologically nearer to the Ammotheidae than the Pycnogonidae and Callipallenidae. The difference in the number of protein fractions could be produced by protein diversification from the Nymphonidae, showing a greater morphological diversification in the Ammotheidae (Hedgpeth, 1947), with great capacity of ecological penetration (De Haro, 1978).

The reduction of protein fractions in Callipallenidae and Pycnogonidae could be produced by protein specialization.

The Ammotheidae, by serological and mor-

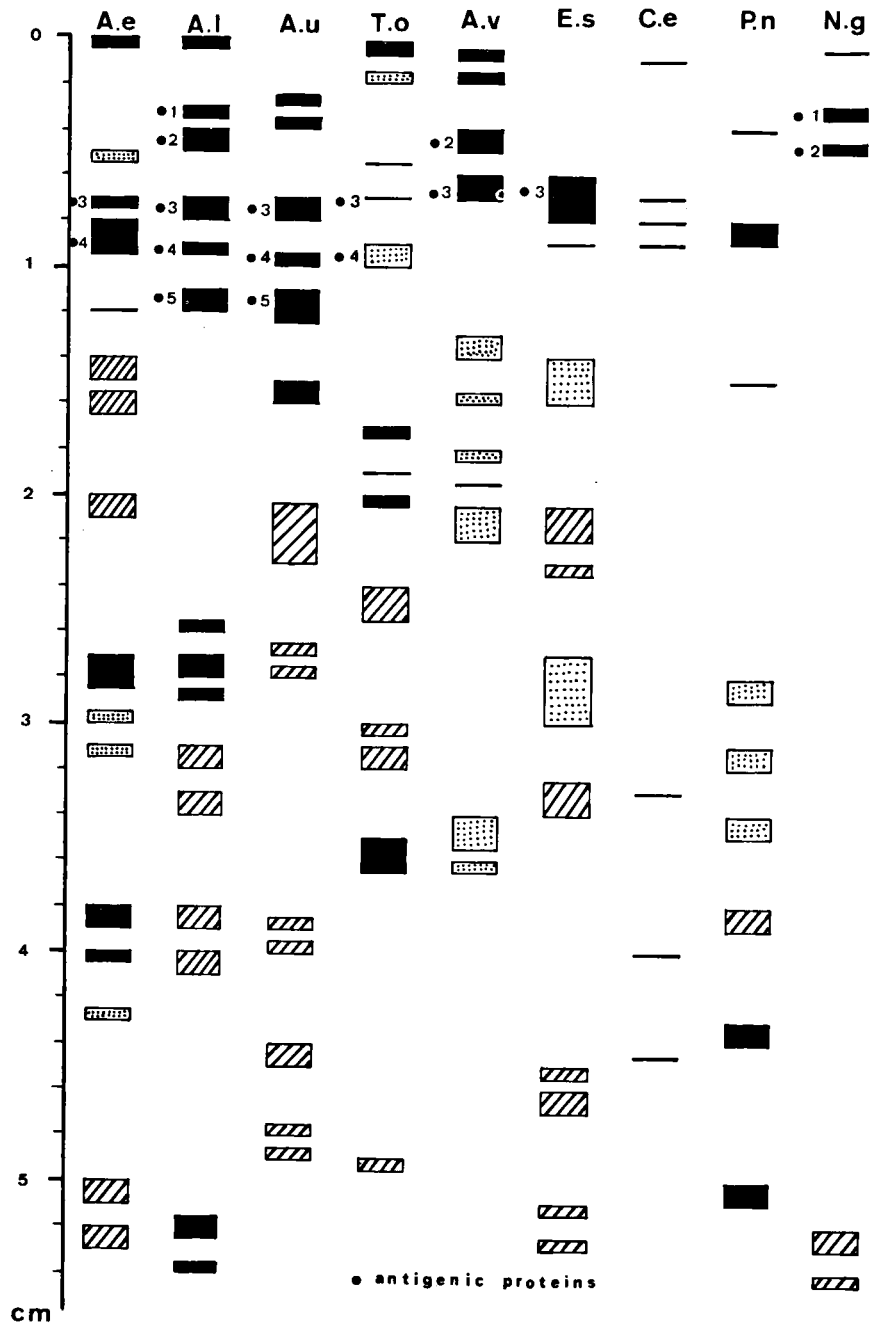

Fig. 11. Compared electrophoretograms of the species studied.

phological properties, present themselves as a central group, heavily linked with the Tanystylidae, within the evolution of the pycnogonids. This family is also linked with the Phoxichilidiidae and Endeidae since *Endeis* and *Anoplodactylus* share band 3 with it.

The Phoxichilidiidae-Endeidae occupy an intermediate position between the Ammotheidae and the Pycnogonidae, with protein bands reduced in number. We believe they are more evolved than the former.

The number of electrophoretic and immuno-

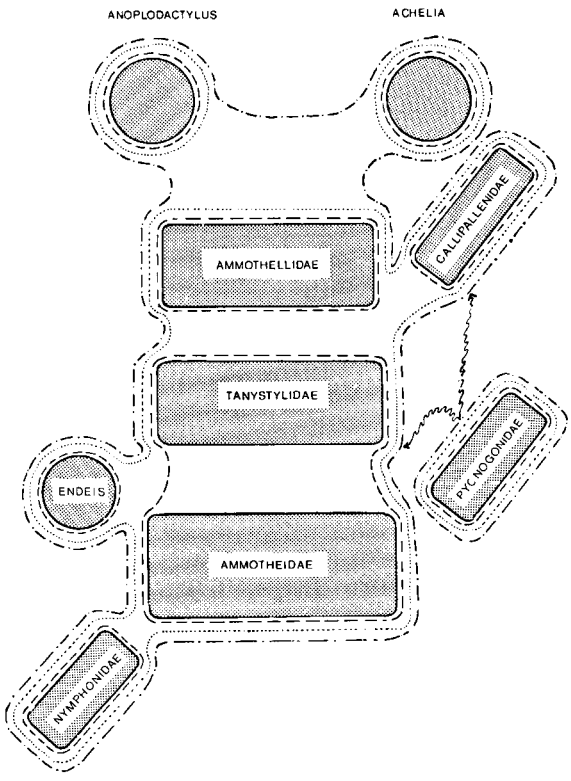

Fig. 12. Clusters of genera within families following the General Similarity Coefficient of Gower (cf. Fry, 1978, diagrammatic).

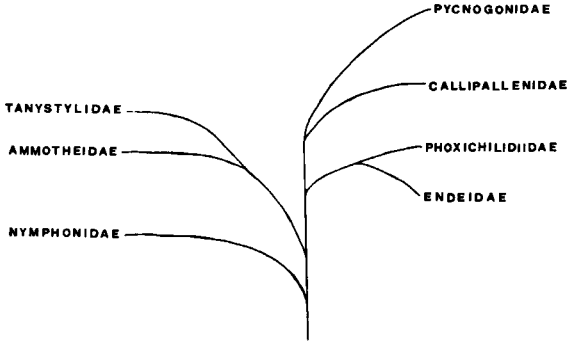

Fig. 13. Phylogenetic relations between families of pycnogonids following morphological and serological criteria.

diffusion bands allow the separation of 5 groups of families: (1) Nymphonidae; (2) Ammotheidae-Tanystylidae; (3) Phoxichilidiidae-Endeidae; (4) Callipallenidae; and (5) Pycnogonidae. In fig. 12 a phylogram of these groups is presented.

This clustering agrees basically with that obtained by Fry (1978), with methods of numerical taxonomy (fig. 13). This author shows the Pycnogonidae and Callipallenidae as lateral groups, far from the Nymphonidae. There is a central clustering of the Ammotheidae and Tanystylidae.

However, our results do not justify the separation of the Ammothellidae. *Achelia* seems serologically related to *Ammothella* and also to *Tanystylum*. Stock (1968) considers *Tanystylum* as a member of the Ammotheidae.

The position of *Anoplodactylus* (Phoxichilidiidae) in Fry (1978), agrees with our results.

Endeis seems serologically related with *Anoplodactylus*. Stock (1968) includes *Endeis* within the Phoxichilidiidae.

On the whole, we must stress the striking basic agreement of our results with those of Fry. To explain certain discrepancies surely more work is necessary, but we have by now obtained an insight about the phylogenesis of the pycnogonid families.

4. CONCLUSIONS

Electrophoretical and immunological studies with 9 species of Pycnogonida, belonging to seven families after Hedgpeth (1947), together with morphological considerations, allowed us to differentiate 5 evolutionary lines. Colossendeidae were not available for our studies. Of these lines, in agreement with Hedgpeth (1947), we consider the Nymphonidae the more primitive, with the lower number of protein fractions.

The line Ammotheidae-Tanystylidae with the greater morphological diversification and great capacity of ecological penetration, has also the greater number of protein fractions, probably by differentiation of proteins from the Nymphonidae.

The line Phoxichilidiidae-Endeidae, possessing antigenic bands in common with the last-named line, is considered by morphological and serological characters intermediate between the Ammotheidae and Pycnogonidae; in the latter protein bands are more reduced in number than in the former, probably by specialization of proteins.

The Callipallenidae line, having no serological affinities with the Ammotheidae, is considered more highly evolved than the latter, while the

Pycnogonidae line, which is more evolved by its morphological characters (Hedgpeth, 1947), has no serological affinities with the Ammotheidae either.

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