RE-INTERPRETATION OF TEREBRATULIDE PHYLOGENY BASED ON IMMUNOLOGICAL DATA

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ABSTRACT. Shell intracrystalline proteinaceous macromolecules isolated from forty four Recent terebratulide brachiopod species, covering all living superfamilies and two thirds of living families, have been compared using immunological techniques. Immunological data indicate that the examined species belong to one of the following four groups, which are also morphologically distinct: (1) Cancellothyridoidea, (2) 'Terebratelloidea' (Dallinidae, Terebratellidae, Laqueidae), (3) Terebratuloidea, and (4) a newly identified category (Kraussinidae, Megathyrididae, Macandreviidae, Ecnomiosidae). Immunological data clearly indicate that groups (3) and (4) form a coherent cluster, and that this cluster has a trichotomous relationship with the remaining two groups. This pattern was not predicted by traditional taxonomies, but reinforced previous immunological studies. The discovery that *Ecnomiosa* groups with kraussinids, along with megathyridids and *Macandrevia*, allows the presentation of a revised interpretation of terebratulide phylogeny, in which the extinct zeilleriids and kingenids are considered as a possible link between the Terebratuloidea and the newly recognized group which includes the Kraussinidae.

THE Terebratulida, the largest extant order of brachiopods, is characterized by the presence of a calcareous loop-shaped brachidium or loop, which functions as a support for the lophophore. The loop is not only of diagnostic value of the order, but is also of prime taxonomic importance at almost every rank within the order, because of its highly diversified form and intricate mode of development. In fact, the loop morphology has been regarded as so important among known characters, that terebratulide evolution and loop evolution have often been considered as almost synonymous to each other (Williams and Hurst 1977).

Collins *et al.* (1988), using immunological methods, demonstrated that the macromolecules embedded within the microcrystalline calcitic biocrystals of living brachiopod shells contained significant taxonomic information, and provided a new independent set of characters to reconstruct terebratulide phylogeny. Subsequent serotaxonomic studies (Collins *et al.* 1991*a*; Curry *et al.* 1991*a*) indicated that the short-looped superfamily Cancellothyridoidea (Cooper 1973*a*; '-OIDEA' is added to the superfamily stem as the preferred suffix according to the ICZN recommendation, Ride *et al.* 1985), the short-looped Terebratuloidea (Cooper 1983), and the long-looped Terebratelloidea (Muir-Wood *et al.* 1965) were almost equidistantly related to each other, and that a subset of the Terebratelloidea was more closely related to the Terebratuloidea than to the rest of the Terebratelloidea. In effect, this implied that forms with a long loop evolved at least twice independently.

These first studies of brachiopod serotaxonomy highlighted the problems of existing higher-level classifications of the Terebratulida, which had been determined only using a few morphological characters, such as the relative length of the loop and presence or absence of the support for the loop from the valve floor (Muir-Wood *et al.* 1965). A considerable revision of traditional classifications was demanded, as the serotaxonomic data appeared fairly robust, and consistent with the fossil record (Collins *et al.* 1991*a*; Curry *et al.* 1991*a*); but a problem remained. From a morphological standpoint, the relationships suggested by these serotaxonomic studies contained highly improbable phylogenetic patterns, the prime example being the derivation of the long-looped kraussinids and *Macandrevia* from the short-looped Terebratuloidea. This anomaly could not readily be explained

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TABLE 1. Samples used in this study. Abbreviations in the 'Status' column: L, living when collected and preserved wet; D, dead shells, preserved dry. *'*Frenulina*' from off Shimoda, Japan could possibly be a new species of a new genus. It much resembles *Frenulina* externally, but the loop is different from that of *Frenulina* (D. MacKinnon, pers. comm.).

Species	Locality	Status
Rhynchonellida		
Hemithyridae		
Notosaria nigricans (Sowerby)	Christchurch, New Zealand	L
Terebratulida		
Terebratulidae		
Liothyrella neozelanica (Thomson)	Foveaux Strait, New Zealand	L
L. uva notorcadensis (Broderip)	Ross Island, Antarctica	L
Tichosina floridensis Cooper	off Florida, Gulf of Mexico	D
Gryphus vitreus (Born)	off Corsica, Mediterranean	L
Dyscoliidae		
Abyssothyris parva Cooper	off Jacksonville, Florida, USA	D
Cancellothyrididae		
Cancellothyris australis Thomson	off Melbourne, Australia	D
Terebratulina retusa (Linnaeus)	Firth of Lorn, Scotland	L
T. septentrionalis (Couthouy)	Bay of Fundy, Canada	L
T. unguicula (Carpenter)	Friday Harbor, USA	D
T. unguicula rotundata Cooper	off Otsuchi, Japan	L
T. japonica (Sowerby)	Izu Islands, Japan	D
T. peculiaris Hatai	Izu Islands, Japan	D
T. pacifica Hatai	Kii Strait, Japan	D
T. crossei Davidson	Otsuchi Bay, Japan	L
T. reevei Dall	Sibuyan Sea, Philippines	D
T. abyssicola Adams & Reeve	off Inhambane, Mozambique	D
T. latifrons Dall	off Key West, Florida, USA	D
T. cailleti Crosse	off Pelican Island, Caribbean Sea	D
T. kiiensis Dall & Pilsbry	off Valparaiso, Chile	D
Chlidonophoridae		
Chlidonophora incerta (Davidson)	off Venezuela, Caribbean Sea	D
Dallinidae		
Dallina septigera (Loven)	Hebridges Shelf, Scotland	L
Campages basilanica Dall	Izu Islands, Japan	D
Terebratellidae		
Terebratella dorsata (Gmelin)	Strait of Magellan, Argentina	D
T. sanguinea (Leach)	New Zealand	L
Waltonia inconspicua (Sowerby)	Christchurch, New Zealand	L
Magellania macquariensis Thomson	South Pacific	D
Gyrothyris mawsoni antipodensis Foster	South Pacific	D
Neothyris lenticularis (Deshayes)	Foveaux Strait, New Zealand	L
Laqueidae		
Terebratalia coreaneca (Adams & Reeve)	Japan	D
Coptothyris grayi (Davidson)	Japan	L
Dallinella occidentalis (Dall)	off San Diego, California, USA	D
Jolonica nipponica Yabe and Hatai	Izu Islands, Japan	D
'*Frenulin*' sp.*	off Shimoda, Japan	D
Laqueus rubellus (Sowerby)	Sagami Bay, Japan	L
L. blanfordi (Dunker)	Otsuchi Bay, Japan	D
L. quadratus Yabe & Hatai	Kii Strait, Japan	D
Pictothyris picta (Dillwyn)	Sagami Bay, Japan	L

TABLE 1. (*cont.*)

Species	Locality	Status
Macandreviidae		
Macandrevia cranium (Muller)	Hebrides Shelf, Scotland	D
M. africana Cooper	off Angola, South Atlantic	D
Ecnomiosidae		
Ecnomiosa sp.	Izu Islands, Japan	D
Kraussinidae		
Kraussina rubra (Pallas)	Southern Tip, South Africa	D
Megerlia truncata (Gmelin)	off Corsica, Mediterranean	D
Megathyrididae		
Megathiris detruncata (Gmelin)	off Corsica, Mediterranean	D
Argyrotheca barretiana (Davidson)	Jamaica, Caribbean Sea	D

in terms of comparative morphology, and prompted misgivings about the immunological approach (e.g. Brunton and Hiller 1990).

Congruence between molecular and morphological data would provide a strong case for a reliable interpretation of phylogeny having been established, and hence should be one of the main aims of any taxonomic study (Curry and Endo 1991). In practice this is particularly desirable for brachiopod taxonomy, as most species are represented, and often only known, as fossils. In effect, therefore, suitable morphological explanations are required to justify phylogenetic inferences from molecular data when there is an apparent discrepancy between molecular and traditional morphological data. Such explanations are all the more important when there is a risk that the contrasting interpretations are portrayed as being contradictory and irreconcilable. The reality is that the majority of relationships suggested by serotaxonomy are entirely consistent with the widely-accepted classification of brachiopods, and it is the instances where the data are apparently incompatible that are the focus of attention because they are likely to be critical to the solving of some of the most puzzling attributes of brachiopod evolutionary history.

The main purpose of this paper, therefore, is to re-examine the contradiction between the traditional and serotaxonomic views of terebratulide relationships using an enlarged and much more comprehensive immunological dataset than has previously been available. An attempt is then made to provide an alternative new phylogenetic interpretation, which may solve the apparent discrepancy between the serotaxonomic and traditional schemes of phylogenetic interpretations.

MATERIALS AND METHODS

Samples of a total of forty four terebratulide species from world-wide locations were available for this study (Table 1) along with a rhynchonellide species as a control in the immunological assays. Out of this collection, samples of fifteen different species, representing twelve genera of wide taxonomic distribution, were available in sufficient abundance to allow the preparation of polyclonal antisera against shell macromolecules (Table 2). Previous serotaxonomic studies (Collins *et al.* 1988; Collins *et al.* 1991*a*; Curry *et al.* 1991*a*) utilized sub-sets of the antisera used in this study. The isolation of secondary shell fibres, extraction of intracrystalline macromolecules, immunization, and immunoassay (ELISA; enzyme-linked immunosorbent assay) procedures follow the methods described in Collins *et al.* (1991*a*), except that, in ELISA, the final detection of the bound antibodies was performed using a fluorescent substrate [0·2 mM MUP (4-methylumbelliferyl phosphate dilithium salt; Boehringer Mannheim) in 10 mM diethanolamine buffer containing 2 mM $MgCl_2$ (pH 9·8)], and the fluorescence was read after thirty minutes with a Dynatech Microfluor plate reader.

TABLE 2. Antisera used in this study. Abbreviations for the type of antigens: F, extracts from the fibrous secondary layer; W, extracts from the whole shell; P, semi-purified protein.

Serum ID No.	Species from which antigen originated	Type of antigen	Titre (1/x)
K5038	*Notosaria nigricans* (Sowerby)	F	3000
802	*Liothyrella neozelandica* (Thomson)	F	2000
K5010	*L. uva notocardensis* (Broderip)	F	3000
803	*Gryphus vitreus* (Born)	W	250
K4962	*Terebratulina retusa* (Linnaeus)	W	40000
173	*T. septentrionalis* (Couthouy)	F	3000
174	*T. unguicula* (Carpenter)	F	3000
171	*T. crossei* Davidson	F	400
K5007	*Dallina septigera* (Loven)	F	10000
K5040	*Waltonia inconspicua* (Sowerby)	F	20000
427	*Neothyris lenticularis* (Deshayes)	P	100
1191	*Laqueus rubellus* (Sowerby)	F	20000
1192	*Pictothyris picta* (Dillwyn)	F	5000
801	*Kraussina rubra* (Pallas)	F	20000
K5053	*Megerlia truncata* (Gmelin)	F	8000

Immunological reactivity of antisera was determined for all combinations of antisera and intracrystalline macromolecules extracted from each species, at an appropriate fixed dilution of each antiserum. In a series of antiserum dilution assays using the homologous antigen (the antigen against which the antiserum was prepared), the minimum concentration which gave a 90–100 per cent reading of the maximum reaction was taken for normal use ('titre' in Table 2), while the concentration which gave 50–70 per cent of the maximum reaction ('limiting' concentration) was used in the inhibition ELISA (see below).

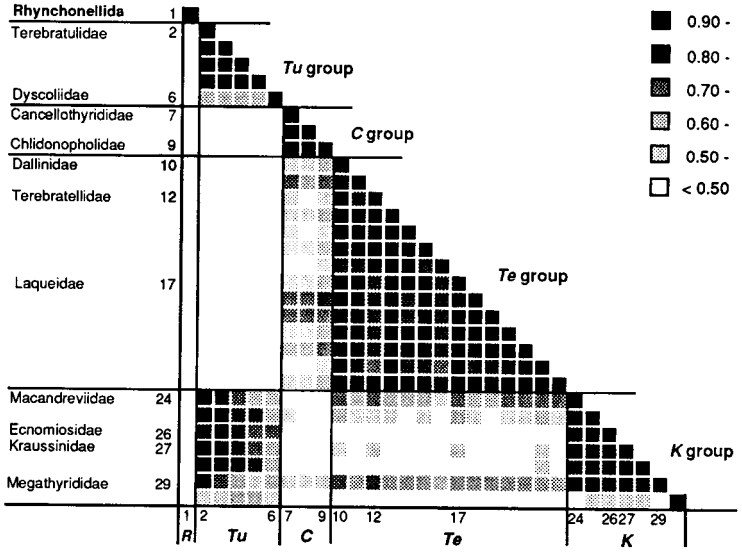
Thirty species, representing all the available genera, were assayed using antisera against twelve different genera by ELISA to assess the framework of relationships. Cancellothyridid and chlidonophorid species, a total of fifteen species, were separately assayed by normal ELISA using four different *Terebratulina* antisera.

In order to examine in more detail the relationships within each taxonomic group, attempts were made to increase the specificity of the brachiopod antisera, by mixing, or 'preabsorbing', each antiserum with antigens (shell extracts from species of interest) to remove the antibody reactivity to that particular antigen (a procedure known as inhibition ELISA; Johnstone and Thorpe 1987). Preabsorptions were performed either against one and the same antigen for each antiserum simply to remove common non-informative activities of each antiserum, or against a panel of antigens extracted from every species within each group to examine detailed inhibition patterns produced by preabsorptions with different antigens. One part of antigen [in 20 per cent w/v EDTA (ethylenediamine tetraacetate) solution, pH 8·0] was mixed with nine parts antiserum [in 10 mM Tris buffered saline (TBS, pH 7·4), 0·02 per cent (v/v) Tween 20, 0·2 per cent (w/v) gelatin solution] with the appropriate 'limiting' concentration of antiserum. Preparations with the homologous antigen and blank EDTA (20 per cent w/v, pH 8·0) solutions were also included as controls, and were expected to give 100 per cent and 0 per cent inhibitions respectively. The preparations were thoroughly mixed, and incubated overnight at 4 °C. The resulting precipitations were removed by centrifugation at 4000 *g* for twenty minutes at 4 °C immediately prior to the supernatant being added to the prepared ELISA plates.

ELISA and inhibition ELISA were performed at least in duplicate. For normal ELISA and inhibition ELISA using antisera preabsorbed against one and the same antigen, the reactivity of an

TABLE 3. Immunological reactivity scores between thirty brachiopod antigens and twelve antisera. Reactions with homologous antigen are listed as **100**, and reactions with negative control (bivalve *Codakia* sp.) are listed as **0**. Negative readings are listed as 0. Data represents the average of duplicate experiments. Figures with an asterisk contained 10–20 per cent variations between the duplicate readings, other figures contained less than 10 per cent variations. For antigen identities see Table 1, antiserum identities see Table 2; *1, *L. neozelanica*; *2, *L. uva notocardensis*; *3, *T. septentrionalis*; *4, *T. dorsata*; *5, *L. rubellus*; *6, *M. cranium*; *7, *M. africana*.

Antigens \ Antisera	5038 Not	803 Lio	5010 Lio	802 Gry	173 Ter	5007 Dal	5040 Wal	427 Neo	1191 Laq	1192 Pic	801 Kra	5053 Mer
Rhynchonellida												
1 *Notosaria*	**100**	**0**	3	5	*0	15	8	1	**0**	1	11	1
Terebratulida												
						Terebratuloidea						
Terebratulidae												
2 *Liothyrella**1	**0**	**100**	96	134	19	71	16	1	10	41	39	86
3 *Liothyrella**2	**0**	102	**100**	136	**0**	64	9	**0**	10	7	49	89
4 *Tichosina*	**0**	92	94	119	**0**	**0**	8	1	7	0	42	68
5 *Gryphus*	**0**	82	80	**100**	2	**0**	2	1	6	0	22	40
Dyscoliidae												
6 *Abyssothyris*	**0**	17	47	**0**	**0**	1	9	1	9	12	11	11
						Cancellothyridoidea						
Cancellothyrididae												
7 *Cancellothyris*	**0**	48	9	**0**	102	104	17	**0**	7	40	13	8
8 *Terebratulina**3	**0**	**0**	5	**0**	**100**	116	9	**0**	7	20	10	5
Chlidonophoridae												
9 *Chlidonophora*	**0**	4	3	**0**	90	103	17	**0**	6	*41	12	7
						'Terebratelloidea'						
Dallinidae												
10 *Dallina*	**0**	**0**	7	22	1	**100**	92	1	80	97	16	44
11 *Campages*	**0**	3	6	**0**	41	108	96	1	75	98	*19	7
Terebratellidae												
12 *Terebratella**4	**0**	8	7	**0**	**0**	106	98	70	78	**100**	22	63
13 *Waltonia*	**0**	**0**	7	11	**0**	105	**100**	17	88	103	10	7
14 *Magellania*	**0**	**0**	6	**0**	**0**	**100**	95	73	81	101	10	14
15 *Gyrothyris*	**0**	5	7	**0**	**0**	104	**100**	2	85	102	12	8
16 *Neothyris*	**0**	3	6	**0**	**0**	103	96	**100**	84	103	13	5
Laqueidae												
17 *Terebratalia*	**0**	**0**	7	43	**0**	113	82	1	78	99	10	9
18 *Coptothyris*	**0**	**0**	6	11	67	111	87	**0**	85	102	12	8
19 *Dallinella*	**0**	**0**	3	**0**	61	108	94	1	77	105	12	7
20 *Jolonica*	**0**	8	8	34	**0**	112	93	**0**	86	101	11	7
21 '*Frenulina*'	**0**	5	7	27	41	112	92	1	85	**100**	12	8
22 *Laqueus**5	**0**	*4	7	104	**0**	104	90	1	**100**	101	12	5
23 *Pictothyris*	**0**	8	7	21	**0**	108	95	**0**	88	**100**	12	8
						Newly identified category						
Macandreviidae												
24 *Macandrevia**6	**0**	31	79	105	**0**	81	58	1	74	86	92	**100**
25 *Macandrevia**7	**0**	66	74	*79	**0**	86	20	1	19	37	*51	74
Ecnomiosidae												
26 *Ecnomiosa*	**0**	60	89	53	**0**	86	9	1	10	**0**	65	85
Kraussinidae												
27 *Kraussina*	**0**	67	70	94	**0**	78	27	1	9	27	**100**	88
	0	73	82	124	**0**	74	9	1	9	4	73	**100**
28 Megerlia												
Megathyrididae												
29 *Megathiris*	**0**	33	49	30	**0**	*84	*10	1	29	57	50	79
30 *Argyrotheca*	**0**	47	6	**0**	**0**	1	6	1	11	**0**	13	7



TEXT-FIG. 1. *Q*-mode bivariate comparisons of immunological reactivity data. Similarity coefficients of cosine θ measure (cf. Lespérance 1990) between each pair of antigens (rows in Table 3) were calculated, and their values indicated as the interval to which each value belonged. High values indicate high similarities. Figures along *x*- and *y*-axes correspond with the species (antigen) identity numbers in Table 1. Four major groups (*Tu*, *C*, *Te*, *K*) have been recognized within the Terebratulida, demonstrating high similarities within each group and between the groups *Tu* and *K*.

antiserum to an unknown antigen was given as a percentage of the fluorescence reading of the sample to that of the positive control (i.e. reaction with homologous antigen). In both cases the negative control reading (i.e. reaction with shell extracts from the bivalve *Codakia* sp.) was subtracted before the calculation. In inhibition ELISA using antisera preabsorbed against a panel of different antigens, the reactivity of an antigen to each preabsorbed antiserum was also expressed as a percentage, where the reaction with the antiserum preabsorbed by the homologous antigen, and the reaction with the non-preabsorbed antiserum ('preabsorbed' with EDTA) were taken as 0 and 100 per cent, respectively.

The resulting immunological reactivity data were transformed to distance matrices, expressing the degree of similarity both among antigens (*Q*-mode) and antisera (*R*-mode), using the similarity coefficients of cosine θ measure and Euclidean distance (cf. Lespérance 1990), to produce dendrograms by the single linkage clustering methods (cf. Sneath and Sokal 1973). Data analyses were performed with the aid of an Apple Macintosh computer using the Odesta Corporation package 'Datadesk Professional version 2.0'.

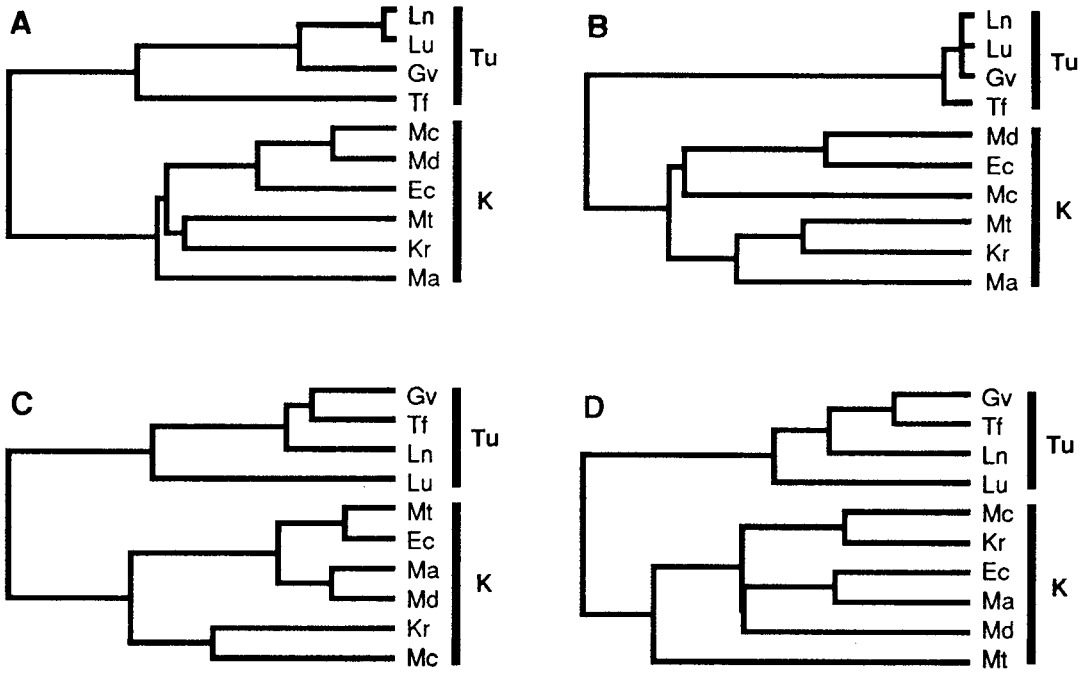
RESULTS

Framework relationships of the Terebratulida

The calculated reactivity scores for thirty antigens of the examined genera against twelve brachiopod antisera are summarized in Table 3. The rhynchonellide species, included as a control to check the specificity of the antisera, is clearly discriminated from the terebratulides. The antiserum (laboratory number K5038) prepared against the rhynchonellide *Notosaria* reacted only with *Notosaria* antigen and did not react with any others. The *Notosaria* antigen (i.e. the macromolecules extracted from the shell of *Notosaria*) showed no significant reactions with any of the antisera, except with K5038 itself.

TABLE 4. Inhibition ELISA on *Tu* and *K* groups (Terebratulidae, Macandreviidae, Ecnomiosidae, Kraussinidae and Megathyrididae). Each of four different antisera (803, 802, 801, K5053) was preabsorbed with each of ten different antigens (columns) from species of *Tu* and *K* groups, and assayed with the ten antigens (rows). Reaction with the non-preabsorbed antiserum are listed as **100**, while those with the antiserum preabsorbed by the homologous antigen are listed as **0**. Data represent the average of duplicate experiments.

	Antisera (preabsorbed against)									
Antigens	-Ln	-Lu	-Gv	-Tf	-Md	-Mc	-Ma	-Ec	-Kr	-Mt
Anti-Liothyrella (803)										
Liothyrella neozelanica (Ln)	**0**	**0**	21	19	**100**	91	89	**100**	85	88
Liothyrella uva (Lu)	**0**	**0**	**0**	94	**100**	**100**	87	**100**	87	45
Gryphus vitreus (Gv)	**0**	**0**	**0**	3	92	97	22	74	79	88
Trichosina floridensis (Tf)	**0**	**0**	16	19	91	90	80	91	70	86
Megathiris detruncata (Md)	**0**	**0**	2	**0**	3	**0**	3	**0**	1	**0**
Macandrevia cranium (Mc)	**0**	**0**	7	**0**	10	**0**	10	**0**	8	**0**
Macandrevia africana (Ma)	**0**	**0**	1	**0**	71	56	7	82	11	55
Ecnomiosa sp. (Ec)	**0**	**0**	1	**0**	6	5	6	21	2	**0**
Kraussina rubra (Kr)	**0**	**0**	**0**	**0**	61	56	40	83	1	25
Megerlia truncata (Mt)	**0**	**0**	**0**	**0**	36	56	43	69	9	6
Anti-Gryphus (802)										
Liothyrella neozelanica	**0**	**0**	**0**	**0**	**100**	98	49	**100**	62	58
Liothyrella uva	**0**	**0**	**0**	**0**	96	81	55	95	50	55
Gryphus vitreus	**0**	**0**	**0**	8	89	90	**0**	**0**	66	76
Tichosina floridensis	**0**	**0**	**0**	**0**	89	97	70	**100**	52	78
Megathiris detruncata	**0**	**0**	**0**	**0**	63	53	27	79	3	5
Macandrevia cranium	**0**	**0**	**0**	**0**	59	**0**	37	64	26	**0**
Macandrevia africana	**0**	**0**	**0**	**0**	81	58	42	77	19	6
Ecnomiosa sp.	**0**	**0**	**0**	**0**	70	52	39	73	7	2
Kraussina rubra	**0**	**0**	**0**	**0**	**100**	69	68	**100**	29	36
Megerlia truncata	**0**	**0**	**0**	**0**	83	23	47	93	33	**0**
Anti-Kraussina (801)										
Liothyrella neozelanica	2	**0**	2	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Liothyrella uva	14	**0**	5	**0**	9	**0**	8	3	**0**	**0**
Gryphus vitreus	3	**0**	2	**0**	3	**0**	**0**	**0**	**0**	**0**
Tichosina floridensis	2	**0**	3	1	5	1	4	3	**0**	**0**
Megathiris detruncata	1	1	**0**	**0**	4	2	1	3	**0**	3
Macandrevia cranium	75	41	60	51	**0**	**0**	**0**	**0**	**0**	**0**
Macandrevia africana	18	8	15	28	**0**	**0**	**0**	2	**0**	**0**
Ecnomiosa sp.	20	20	31	23	2	1	2	1	**0**	1
Kraussina rubra	83	93	94	91	56	22	63	79	**0**	79
Megerlia truncata	41	26	49	56	6	**0**	4	6	**0**	**0**
Anti-Megerlia (K5053)										
Liothyrella neozelanica	9	21	48	46	36	51	54	68	39	**0**
Liothyrella uva	31	**0**	38	15	31	**0**	32	22	11	**0**
Gryphus vitreus	**0**	**0**	5	6	6	**0**	**0**	**0**	**0**	**0**
Tichosina floridensis	10	**0**	15	23	24	11	35	45	**0**	**0**
Megathiris detruncata	56	58	63	72	2	13	37	21	3	**0**
Macandrevia cranium	69	46	70	76	13	**0**	15	18	**0**	**0**
Macandrevia africana	69	54	86	82	43	**0**	24	41	11	**0**
Ecnomiosa sp.	79	67	84	79	20	9	36	31	**0**	**0**
Kraussina rubra	80	69	83	85	37	26	57	46	7	**0**
Megerlia truncata	85	80	90	89	61	67	72	79	58	**0**



TEXT-FIG. 2. *R*-mode cluster analyses on inhibition data on *Tu* and *K* groups (Table 4), demonstrating a clear discrimination of the two groups. Euclidean distance was used as the similarity coefficient (cosine θ measure could not be calculated because of the 0 values in the data). A, data from assays using anti-*Liothyrella* (803) serum; B, using anti-*Gryphus* (802); C, using anti-*Kraussina* (801); D, using anti-*Megerlia* (K5053). For species identity, see Table 4.

Antisera prepared against *Liothyrella*, *Gryphus*, *Kraussina*, and *Megerlia* (803, K5010, 802, 801, K5053) all showed very similar patterns of reactivity, with strong reactions with the terebratulids, kraussinids, megathyridids, *Macandrevia*, and *Ecnomiosa*, and little reaction with other species. The dyscoliid *Abyssothyris* and the megathyridid *Argyrotheca* were among the least reactive terebratulide antigens to most of the antisera, but reacted moderately with one of the two antisera prepared against *Liothyrella* (803, K5010).

Antiserum prepared against *Terebratulina septentrionalis* (173) (the antisera are hereafter described as 'anti-*Terebratulina septentrionalis*', etc.) reacted strongly with cancellothyridids and *Chlidonophora*, and showed weaker reactions with only a few dallind and laqueid species. Cancellothyridid and chlidonophorid antigens reacted strongly with anti-*Terebratulina* (173) and anti-*Dallina* (K5007), and moderately with anti-*Pictothyris* (1192).

Antisera prepared against *Waltonia*, *Laqueus*, and *Pictothyris* (K5040, 1191, 1192) again had very similar reaction profiles, showing strongest reactions with terebratellids, dallinids and laqueids, moderately strong reactions with one of the *Macandrevia* species (*M. cranium*), and sporadically weaker reactions with other terebratulides. Anti-*Dallina* (K5007) was the least specific antiserum, but it barely reacted with the rhynchonellide, and only weakly with terebratulids, *Abyssothyris*, kraussinids, megathyridids, *Macandrevia*, or *Ecnomiosa*. Anti-*Neothyris* (427) serum, on the contrary, reacted very specifically only with the terebratellid genera, *Neothyris*, *Magellania*, *Terebratella*, and *Waltonia*. These patterns of reactions, as described above, were visualized by a pair-wise comparison of the antigens (Text-fig. 1).

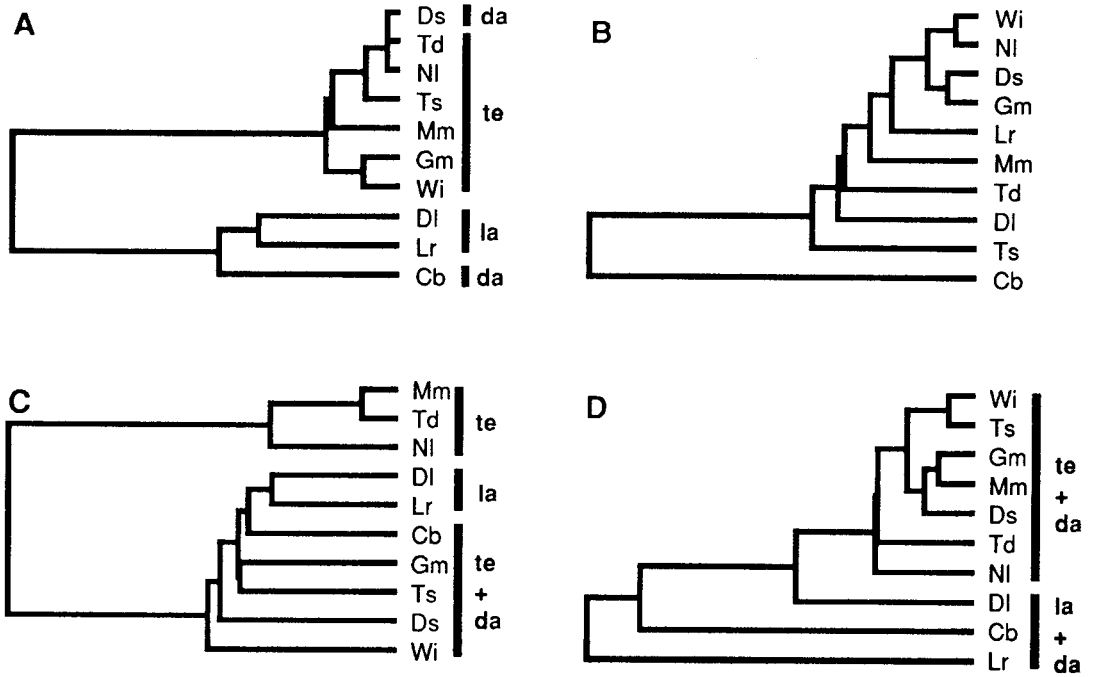
These data reinforce the three-fold division of living terebratulides proposed by previous immunological investigations (Collins *et al.* 1991*a*; Curry *et al.* 1991*a*), and assign the examined taxa into the following three divisions, involving four major taxonomic units.

TABLE 5. Inhibition ELISA on *Te* group (Terebratellidae, Dallinidae, and Laqueidae). Each of four different antisera (K5007, K5040, 427, 1191) was preabsorbed with each of ten different antigens (columns) from species of *Te* group, and assayed with the ten antigens (rows). Reactions with the non-preabsorbed antiserum are listed as **100**, while those with the antiserum preabsorbed by the homologous antigen are listed as **0**. Data represent the average of duplicate experiments.

	Antisera (preabsorbed against)									
Antigens	-Nl	-Td	-Ts	-Wi	-Mm	-Gm	-Ds	-Cb	-Lr	-Do
Anti-Dallina (K5007)										
Neothyris lenticularis (Nl)	**0**	**0**	**0**	16	8	10	**0**	66	60	49
Terebratella dorsata (Td)	**0**	**0**	5	27	10	21	**0**	70	64	59
Terebratella sanguinea (Ts)	**0**	**0**	**0**	**0**	8	3	**0**	69	**0**	53
Waltonia inconspicua (Wi)	**0**	**0**	**0**	6	6	5	**0**	69	59	53
Magellania macquariensis (Mm)	**0**	**0**	**0**	14	6	15	**0**	64	62	49
Gyrothyris mawsoni (Gm)	**0**	**0**	**0**	6	7	10	**0**	63	58	50
Dallina septigera (Ds)	**0**	**0**	7	25	10	26	**0**	69	69	56
Campages basilanica (Cb)	**0**	**0**	**0**	10	4	9	**0**	60	53	37
Laqueus rubellus (Lr)	**0**	**0**	**0**	**0**	4	7	**0**	62	33	27
Dallinella occidentalis (Do)	**0**	**0**	**0**	**0**	8	4	**0**	64	43	34
Anti-Waltonia (K5040)										
Neothyris lenticularis	**0**	20	21	**0**	9	**0**	**0**	41	3	**0**
Terebratella dorsata	**0**	8	2	**0**	13	10	7	41	14	27
Terebratella sanguinea	**0**	29	48	**0**	11	**0**	**0**	57	**0**	29
Waltonia inconspicua	**0**	24	8	**0**	18	8	13	55	20	26
Magellania macquariensis	**0**	14	6	**0**	9	**0**	5	47	6	12
Gyrothyris mawsoni	4	30	12	**0**	19	**0**	**0**	36	12	24
Dallina septigera	**0**	9	28	**0**	5	**0**	**0**	56	14	18
Campages basilanica	2	1	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Laqueus rubellus	**0**	21	9	**0**	12	**0**	**0**	39	**0**	**0**
Dallinella occidentalis	**0**	21	1	**0**	17	**0**	**0**	28	**0**	**0**
Anti-Neothyris (427)										
Neothyris lenticularis	**0**	33	83	75	32	58	65	82	**100**	90
Terebratella dorsata	**0**	**0**	68	75	**0**	79	45	76	93	98
Terebratella sanguinea	**0**	**0**	**0**	93	**0**	22	1	39	65	50
Waltonia inconspicua	**0**	7	44	11	13	54	50	64	57	32
Magellania macquariensis	**0**	7	69	77	**0**	73	48	76	87	72
Gyrothyris mawsoni	**0**	1	**0**	**0**	4	1	2	15	28	17
Dallina septigera	**0**	**0**	21	53	**0**	40	4	57	62	69
Campages basilanica	**0**	4	**0**	**0**	**0**	**0**	**0**	**0**	26	7
Laqueus rubellus	**0**	**0**	**0**	**0**	**0**	6	**0**	1	**0**	8
Dallinella occidentalis	**0**	**0**	15	**0**	**0**	29	23	9	16	5
Anti-Laqueus (1191)										
Neothyris lenticularis	**0**	9	**0**	**0**	7	10	8	37	**0**	17
Terebratella dorsata	**0**	2	**0**	**0**	4	5	6	35	**0**	15
Terebratella sanguinea	**0**	22	**0**	**0**	4	2	2	34	**0**	21
Waltonia inconspicua	**0**	10	**0**	**0**	6	6	4	41	**0**	23
Magellania macquariensis	**0**	**0**	**0**	**0**	**0**	2	3	31	**0**	11
Gyrothyris mawsoni	**0**	**0**	1	**0**	1	2	2	27	**0**	11
Dallina septigera	**0**	1	**0**	**0**	5	4	3	32	**0**	16
Campages basilanica	**0**	**0**	**0**	**0**	**0**	**0**	1	4	**0**	**0**
Laqueus rubellus	59	77	78	80	79	75	81	81	**0**	69
Dallinella occidentalis	34	38	38	41	40	44	49	53	**0**	23

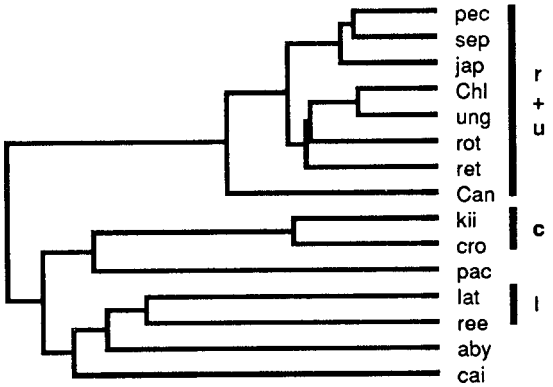
TABLE 6. Inhibition ELISA on *Te* group using antisera preabsorbed with *Macandrevia* antigen. Each of five different antisera (K5007, K5040, 427, 1191, 1192) was preabsorbed with antigens from *Macandrevia cranium* (columns), and assayed with the 15 antigens from *Te* group species (rows). Reactions with the homologous antigen are listed as **100**, while reactions with the negative control (bivalve *Codakia* sp.) are listed as **0**. Data represent the average of duplicate experiments.

Antigens	Antisera	K5007 (Dal)	K5040 (Wal)	427 (Neo)	1191 (Laq)	1192 (Pic)
Dallina	(Ds)	**100**	**43**	35	75	98
Campages	(Cb)	115	88	18	76	72
Gyrothyris	(Gm)	144	101	17	79	75
Waltonia	(Wi)	138	**100**	26	77	101
Terebratella dorsata	(Td)	109	77	87	76	85
Neothyris	(Nl)	96	96	**100**	73	97
Jolonica	(Jn)	**0**	96	**0**	72	97
'*Frenulina*'	(Fr)	35	116	**0**	76	111
Terebratalia	(Tc)	51	110	**0**	103	101
Coptothyris	(Cg)	57	108	**0**	92	110
Dallinella	(Do)	68	110	**0**	85	104
Laqueus rubellus	(Lr)	17	96	**0**	**100**	84
L. blanfordi	(Lb)	81	90	**0**	94	94
L. quadratus	(Lq)	53	77	**0**	86	85
Pictothyris	(Pp)	10	118	**0**	51	**100**



TEXT-FIG. 3. *R*-mode cluster analyses on inhibition data on *Te* groups (Table 5), demonstrating a general separation of terebratellids and laqueids. Euclidean distance was used as the similarity coefficient. A, data from assays using anti-*Dallina* (K5007) serum; B, using anti-*Waltonia* (K5040); C, using anti-*Neothyris* (427); D, using anti-*Laqueus* (1191). For species identity, see Table 5.

TEXT-FIG. 4. *Q*-mode cluster analysis on inhibition data on *Te* group (Table 6), demonstrating a clear discrimination between terebratellids and laqueids. Euclidean distance was used as the similarity coefficient. For species identity, see Table 6.



TEXT-FIG. 5. *Q*-mode cluster analysis on immunological reactivity data on *C* group (Table 7). Antisera were not preabsorbed. Euclidean distance was used as the similarity coefficient. For species identity, see Table 7.

(1) Short-looped Terebratuloidea Cooper, 1983, denoted as *Tu* group here, plus a group of long-looped families Kraussinidae, Megathyrididae, Macandreviidae Cooper, 1973*b*, and Ecnomiosidae Cooper, 1977 (collectively referred to here as the *K* group).

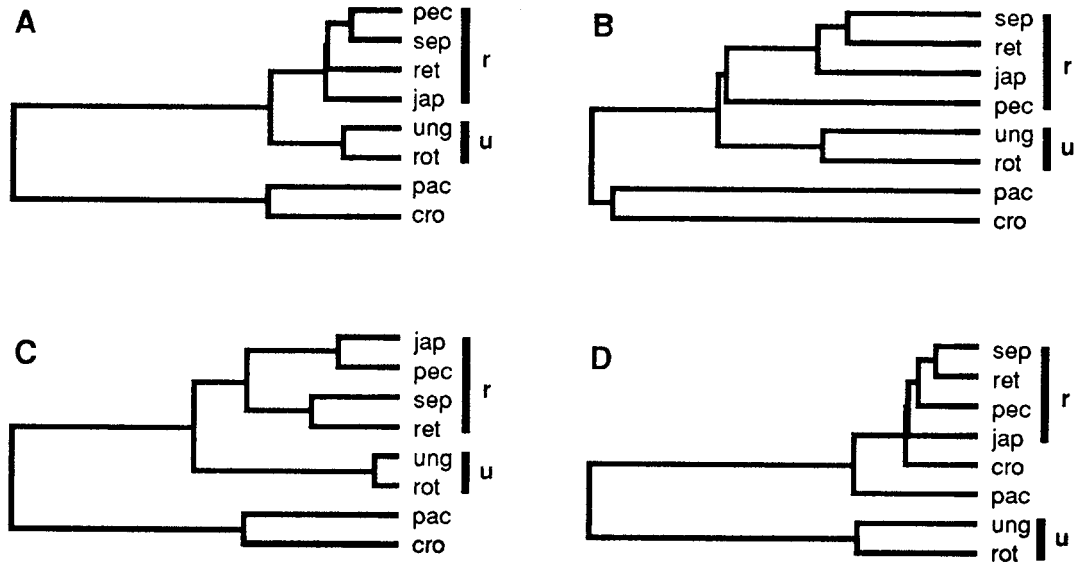
(2) Short-looped Cancellothyridoidea Cooper, 1973*a* – the *C* group.

(3) A group of long-looped families Dallinidae, Laqueidae (Richardson 1975; except for *Macandrevia*), and Terebratellidae – the *Te* group (Text-fig. 1).

Strong and consistent correlations between *Tu* and *K* groups were demonstrated, while no affinity between the short-looped *Tu* and *C* groups, and only weak affinities between the long-looped *Te* and *K* groups were observed (Text-fig. 1), confirming the critical disagreements with traditional classifications suggested by the original immunological study (Collins *et al.* 1988).

Relationships within major taxonomic groups

These results, based entirely on assays carried out using crude antisera, clearly separated the three major groups, but the data were generally insufficient to resolve relationships within each group as many of the reactions were oversaturated (Table 3). Separation between the morphologically distinctive *Tu* and *K* groups was also unclear (Text-fig. 1). However the more specific inhibition ELISA on the species belonging to *Tu* and *K* groups clearly discriminated between these two groups (Table 4; Text-fig. 2). Coherence of the genus *Liothyrella* and family Kraussinidae was also suggested (Text-fig. 2A), but generally the data were still too noisy to allow further elucidation of relationships. Assays on species of the *Te* group using the preabsorbed antisera separated the 'laqueids' of the northern hemisphere and the terebratellids of the southern hemisphere, but failed to detect the 'dallinids' (*Dallina* and *Campages*) as a coherent group (Tables 5–6; Text figs 3–4). More work needs to be carried out to determine the relationships of dallinids to the other two



TEXT-FIG. 6. *R*-mode cluster analyses on inhibition data on *C* group (Table 8), demonstrating a discrimination between *r* and *u* subgroups, and separation of these subgroups from *T. crossei* and *T. pacifica*. Euclidean distance was used as the similarity coefficient. A, data from assays using anti-*Terebratulina retusa* (K4962) serum; B, using anti-*T. septentrionalis* (173); C, using anti-*T. unguicula* (174); D, using anti-*T. crossei* (171). For species identity, see Table 8.

TABLE 7. Immunological reactions among *C* group species (Cancellothyrididae and Chlidonophoridae). Reactions with the homologous antigen are listed as **100**, while reactions with the negative control (bivalve *Codakia* sp.) are listed as **0**. For details of the antisera and antigens, see Tables 1 and 2. Antisera were not preabsorbed with antigens.

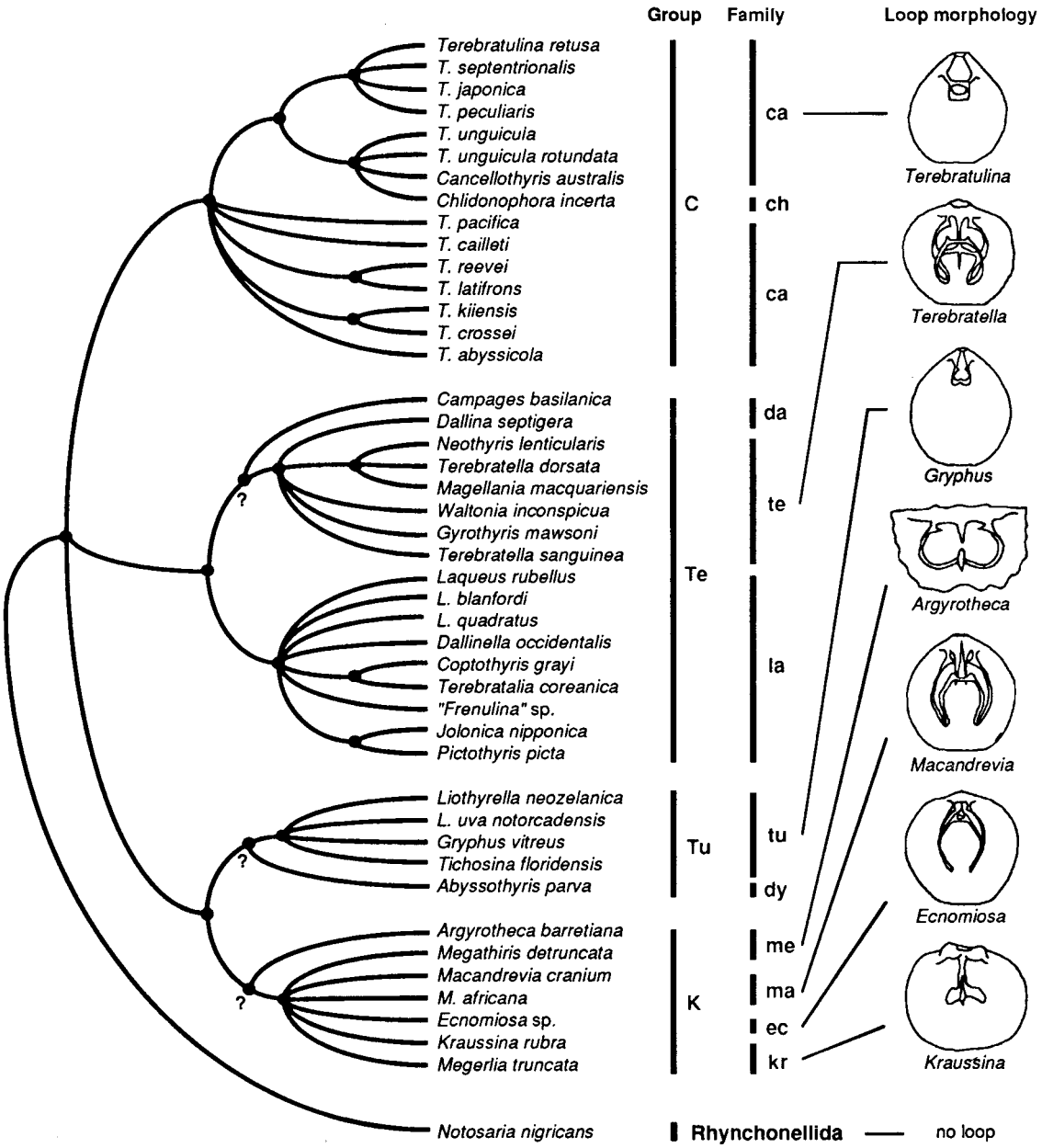
Antigens	Antisera	K4962 (ret)	173 (sep)	174 (ung)	171 (cro)
Terebratulina retusa	(ret)	**100**	90	87	83
T. septentrionalis	(sep)	102	**100**	97	105
T. unguicula	(ung)	93	87	**100**	77
T. unguicula rotundata	(rot)	93	90	102	61
T. japonica	(jap)	106	89	93	95
T. peculiaris	(pec)	107	95	98	99
T. pacifica	(pac)	56	81	78	61
T. crossei	(cro)	59	75	66	**100**
T. reevei	(ree)	56	29	72	51
T. abyssicola	(aby)	75	44	76	11
T. latifrons	(lat)	73	36	92	67
T. cailleti	(cai)	92	63	93	29
T. kiiensis	(kii)	46	66	77	96
Chlidonophora incerta	(Chl)	99	86	102	73
Cancellothyris australis	(Can)	113	94	105	60

families. The 'laqueids' group comprises *Laqueus*, *Pictothyris*, *Jolonica*, *Terebratalia*, *Coptothyris*, and *Dallinella* (Text-fig. 4), and hence the immunological data can be considered as supporting the assignment of these genera to the Laqueidae (Richardson 1975).

TABLE 8. Inhibition ELISA on *Terebratulina* species. Each of four different antisera (K4962, 173, 174, 171) was preabsorbed with each of eight different antigens (columns) from *Terebratulina* species (*C* group), and assayed with the eight antigens (rows). Reactions with the non-preabsorbed antiserum are listed as **100**, while those with the antiserum preabsorbed by the homologous antigen are listed as **0**. Data represent the average of duplicate experiments.

	Antisera (preabsorbed against)							
Antigens	-re	-se	-un	-ro	-ja	-pe	-pa	-cr
Anti-T. retusa (K4962)								
Terebratulina retusa (re)	**0**	7	22	25	16	**0**	33	47
T. septentrionalis (se)	**0**	**0**	14	22	1	**0**	15	18
T. unguicula (un)	**0**	4	**0**	**0**	5	4	40	50
T. unguicula rotundata (ro)	**0**	5	**0**	**0**	1	2	39	24
T. japonica (ja)	**0**	7	10	5	**0**	6	36	44
T. peculiaris (pe)	**0**	13	19	18	3	11	42	53
T. pacifica (pa)	**0**	**0**	11	12	6	3	**0**	11
T. crossei (cr)	**0**	**0**	**0**	3	**0**	2	**0**	**0**
Anti-T. septentrionalis (173)								
Terebratulina retusa	5	**0**	32	29	14	40	57	60
T. septentrionalis	**0**	**0**	27	37	19	26	52	69
T. unguicula	8	**0**	20	34	22	35	64	83
T. unguicula rotundata	8	**0**	24	31	12	31	56	78
T. japonica	10	**0**	40	48	10	33	59	73
T. peculiaris	**0**	**0**	38	38	12	13	65	84
T. pacifica	10	**0**	33	35	3	8	19	50
T. crossei	11	**0**	21	35	**0**	**0**	16	38
Anti-T. unguicula (174)								
Terebratulina retusa	20	31	**0**	1	5	9	76	95
T. septentrionalis	37	27	**0**	1	2	19	75	79
T. unguicula	45	45	**0**	**0**	47	40	70	86
T. unguicula rotundata	45	43	**0**	3	42	48	80	87
T. japonica	50	41	**0**	2	13	15	78	92
T. peculiaris	32	31	**0**	**0**	13	8	77	81
T. pacifica	17	7	**0**	1	**0**	1	27	59
T. crossei	21	5	**0**	2	13	15	43	14
Anti-T. crossei (171)								
Terebratulina retusa	**0**	**0**	87	97	**0**	**0**	**0**	**0**
T. septentrionalis	**0**	**0**	50	80	4	**0**	19	**0**
T. unguicula	23	12	8	41	18	43	76	**0**
T. unguicula rotundata	9	**0**	**0**	21	25	15	46	**0**
T. japonica	**0**	**0**	62	70	**0**	**0**	**0**	**0**
T. peculiaris	**0**	**0**	85	76	19	**0**	4	**0**
T. pacifica	**0**	**0**	70	92	**0**	**0**	**0**	**0**
T. crossei	32	27	72	88	47	19	31	**0**

Antisera prepared against four *Terebratulina* species, even without preabsorption treatment, detected a considerable amount of molecular variation among *C* group species (Table 7). The variability between species of *Terebratulina* was even greater than that detected between the families of *Te* group. *C* group species were subdivided into two major groups; one comprised *T. retusa*, *T. septentrionalis*, *T. japonica*, *T. peculiaris*, *T. unguicula*, *T. unguicula rotundata*, *Cancellothyris australis*, and *Chlidonophora incerta*, the other group comprised *T. pacifica*, *T. reevei*,



TEXT-FIG. 7. Summary of immunological view of terebratulide relationships, with stylized loop morphology (after Davidson 1886, except for the original *Ecnomiosa*) of selected genera. Polychotomous branchings indicate unresolved relationships. Horizontal and vertical axes are not proportional to any similarity values. Abbreviations for families: ca, Cancellothyrididae; ch. Chlidonophoridae; da, Dallinidae; te, Terebratellidae; la, Laqueidae; tu, Terebratulidae; dy, Dyscoliidae; me, Megathyrididae; ma, Macandreviidae; ec, Ecnomiosidae; kr, Kraussinidae.

T. crossei, *T. kiiensis*, *T. latifrons*, *T. cailleti*, and *T. abyssicola* (Text-fig. 5). The data further suggest the separations of the following subgroups: *T. crossei* and *T. kiiensis* (subgroup *c*); and *T. latifrons* and *T. reevei* (subgroup *l*) (Text-fig. 5). Assays with preabsorbed antisera confirmed the separation between subgroup *r* (*T. retusa*, *T. septentrionalis*, *T. japonica*, and *T. peculiaris*) and subgroup *u* (*T. unguicula*, *T. unguicula rotundata*) (Table 8; Text-fig. 6). The apparent affinity of both *Cancellothyris* and *Chlidonophora* to *T. unguicula* and *T. unguicula rotundata* (Table 7; Text-fig. 5) suggests that the diversification of the ancestors of the living *Terebratulina* occurred before the divergence of *Chlidonophora* and *Cancellothyris* from *Terebratulina* stocks.

The relationships among the other cluster of *Terebratulina* species were poorly characterized because anti-*T. crossei* was the only antiserum directed to a species belonging to this cluster. This antiserum indicated that *T. crossei* was very distantly related to *T. abyssicola* and *T. cailleti* (Table 7; Text-fig. 5). The assays with preabsorbed antisera demonstrated a large molecular variation between *T. crossei* and *T. pacifica* (Table 8). These facts suggested an involvement of a number of deeply branched lineages in this second major group of *Terebratulina*.

A schematic summary of the immunological results concerning the relationships among examined terebratulide species is given in Text-figure 7.

DISCUSSION

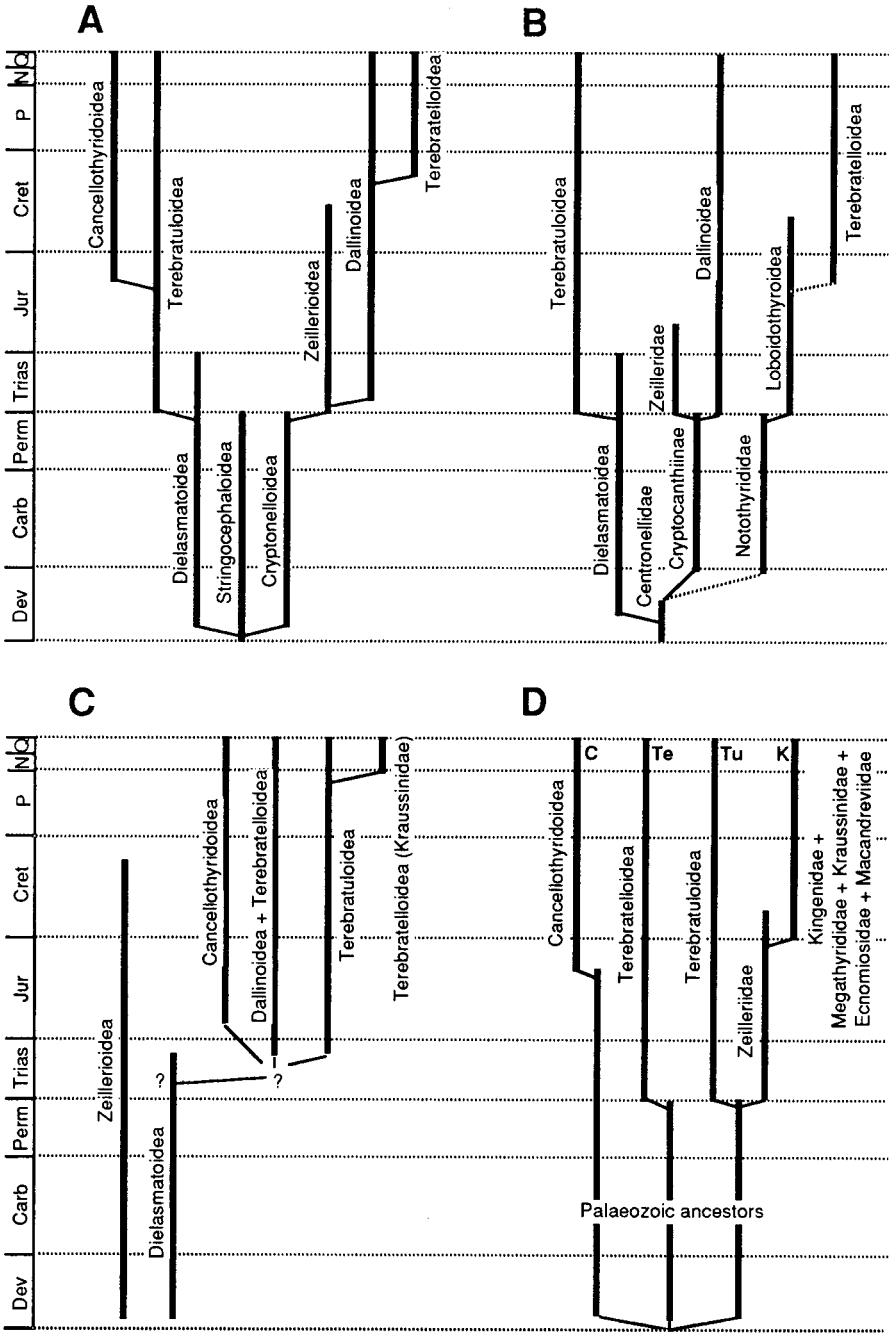
Nature of the immunological data

Immunological methods utilize the highly specific reaction between antigenic molecules, commonly proteins or polysaccharides, and antibodies. The production of the latter is elicited by the introduction of antigens into the body fluids of a susceptible animal (typically, a rabbit). The degree of reaction may vary depending on the structural similarity between the assayed molecules and those that originally elicited the antibodies. When applied to phylogenetic studies, immunological data therefore provide a measure of overall similarities between examined biomolecules; this then can be extrapolated to the degree of divergence between organisms that carry the molecules. The biomolecules thus compared in this study were the brachiopod intracrystalline macromolecules occluded in the secondary shell layer.

Compositional analysis has demonstrated that the brachiopod secondary shell fibrous calcites contain proteins, lipids (Curry *et al.* 1991*b*), and neutral carbohydrates (Collins *et al.* 1991*b*; Clegg and Moers, pers. comm.). The brachiopod intracrystalline proteins have been partially characterized by amino acid analysis, gel electrophoresis, liquid chromatography, and N-terminal amino acid sequencing (Curry *et al.* 1991*b*, 1991*c*; Collins *et al.* 1991*b*; Cusack *et al.* 1992). Curry *et al.* (1991*c*) reported that at least three different proteins of discrete sizes (47 kDa, 16 kDa, and 6·5 kDa) are present in the terebratellid *Neothyris lenticularis* (Deshayes) and at least one protein (30 kDa) in the cancellothyridid *Terebratulina retusa* (Linnaeus). The amino acid sequences of these proteins had no significant similarity with known proteins (Curry *et al.* 1991*c*). The functions of brachiopod intracrystalline proteins are poorly known, although one protein is believed to be responsible for shell colour (Curry *et al.* 1991*b*; Cusack *et al.* 1992). Our immunological data did not group taxa of a particular shell colour; therefore, it appears very unlikely that the colour-related proteins evolved convergently to produce the present patterns of immunological reactivities among terebratulide species.

The antibodies utilized in this study and previous studies (Collins *et al.* 1988; Collins *et al.* 1991*a*; Curry *et al.* 1991*a*) were prepared against crude extracts from the fibrous secondary layer of the shell or the whole shell powder, except for the anti-*Neothyris* serum which was prepared against a semi-purified protein (Collins *et al.* 1991*a*). Immunological assays using these sera on the liquid chromatography fractions of purified shell extracts indicated that these antisera were directed not only against proteins but also against carbohydrates (Collins *et al.* 1991*b*; Endo, unpublished data).

The nature and extent of glycosylation in the brachiopod intracrystalline proteins remains unclear. Heavy glycosylation was suggested by Collins *et al.* (1991*b*). The fact that the first twenty amino acids of the '10·5 kDa protein' and the first ten of the '47 kDa protein' could be sequenced



TEXT-FIG. 8. Interpretations of terebratulide phylogeny. Only relevant taxa are indicated. Upper panel: traditional interpretations. A, Muir-Wood *et al.* (1965) with modifications by Cooper (1973*a*, 1979); B, Smirnova (1984). Lower panel, interpretations based on immunological data. C, Collins *et al.* (1991*a*); D, the revised interpretation.

with the Edman degradation (Curry *et al.* 1991*c*) indicates that at least these residues have no covalently bound sugars. Curry *et al.* (1991*b*) reported no galactosamine or glucosamine from the brachiopod shell fibre extracts.

In general, the immunological data were most consistent and unequivocal at family-superfamily levels and above. The long-lived (Jurassic–Recent) genus *Terebratulina*, for which species level inferences were possible, was a remarkable exception. Indeterminate reaction patterns observed at levels lower than family rank may be due partly to the fact that antibodies were directed also against carbohydrate epitopes, which are usually considered as much less informative than protein epitopes (Cohen 1992). Another possible cause for spurious immunological reaction patterns is the variation in the antigen concentration per shell weight, since the amount of antigen was adjusted by the amount of dried shell fibres in this study. This factor may explain minor variations, for example, the slightly odd reaction patterns of *Gryphus vitreus* and *Macandrevia cranium* antigens, which gave systematically weaker and stronger reactions, respectively, compared with other antigens (Table 3).

In this study, we use common similarity coefficients and simple clustering algorithms to graphically present structures of the immunological data. Different tree-building methods may produce minor changes in the tree topologies, but the framework of relationships, the separation of *Tu*, *C*, *Te*, and *K* groups and their interrelationships, will not change, as the raw data set (Table 3) almost self-evidently demonstrates.

Traditional interpretations of terebratulide phylogeny

In Muir-Wood *et al.* (1965), post-Palaeozoic terebratulides were assigned in one of the two suborders, the short-looped Terebratulidina and the long-looped Terebratellidina. The former consisted of the single superfamily Terebratuloidea, and the latter embraced two superfamilies, namely the Terebratelloidea and the extinct Zeillerioidea. Dagys (1968, 1972) proposed a different subdivision of the long-looped terebratulides, erecting the new superfamily Dallinoidea, which embraced the Zeilleriidae, Dallinidae and Laqueidae on the basis of the presence or absence of dental plates and a cardinal process, in addition to loop characteristics. Dagys (1968, 1972) concluded that the other long-looped superfamily Terebratelloidea, consisting of the Terebratellidae, Megathyrididae, Platidiidae and Kraussinidae, was derived not from the Dallinoidea but from the newly erected short-looped superfamily Loboidothyroidea, casting doubt on the assumption implicit in Muir-Wood *et al.* (1965) interpretation, or the separation of the long- and short-looped forms at subordinal rank.

Among the short-looped Terebratuloidea, Cooper (1973*a*) recognized in the Cancellothyrididae a fundamental difference in the way the pedicle muscles attach to the dorsal shell. In particular he noticed that the muscles attach to the valve floor rather than to the hinge plate, which is unlike the situation in other terebratuloids. On the basis of this muscle attachment and the characteristic features of the cardinalia, Cooper (1973*a*) erected the superfamily Cancellothyridoidea. The long looped superfamily was also divided into two superfamilies, the Terebratelloidea and Dallinoidea (e.g. Cooper 1979), presumably after Dagys (1972), but the separation of the long- and short-looped suborders (Cooper 1981*a*, 1981*b*, 1982) and the separation of the superfamily Zeillerioidea from other long looped superfamilies (Cooper 1989), were retained (Text-fig. 8A). As to the relationships among long-looped terebratulides, Richardson (1975) regarded the following three families, Terebratellidae, Dallinidae, and Laqueidae as different from the rest of the then known long-looped forms (the Kraussinidae, Platidiidae, Megathyrididae, and Thaumatosiidae) in many respects, considering that the former three families constitute the typical stock of the superfamily Terebratelloidea.

In the latest account of the traditional classification of brachiopods, Smirnova (1984) followed Dagys (1968, 1972) as to both the separation of the Terebratelloidea and Dallinoidea, and the connection of the Terebratelloidea with the short-looped Loboidothyroidea, but with major amendments concerning the origins of these superfamilies (Text-fig. 8B). The familial separations of the Dallinidae, Laqueidae, Macandreviidae, Kingenidae and the new family Diestothyrididae were

recognized, and they were included in the superfamily Dallinoidea, distinguishing this superfamily from the other long-looped superfamily Terebratelloidea which embraced the Terebratellidae, Megathyrididae, Platidiidae, and Kraussinidae (Smirnova 1984).

Relationships demonstrated by the immunological data

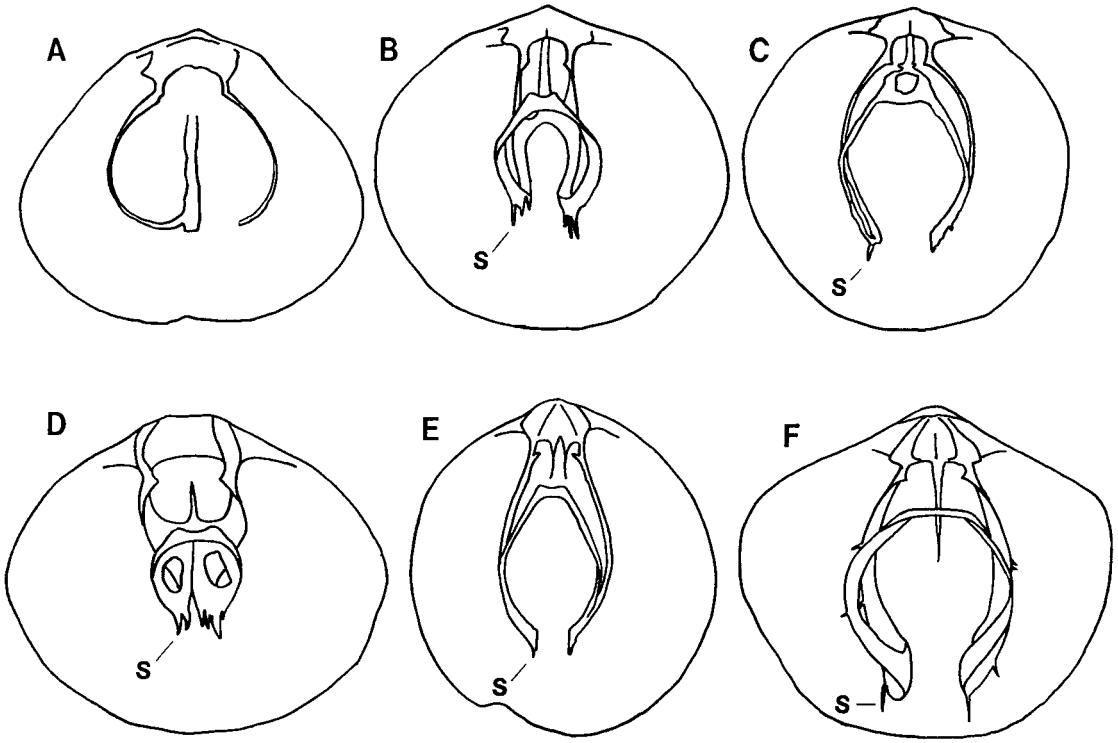
Immunological data clearly indicate that the long loop of the living terebratulides evolved at least twice (Text-fig. 7). This pattern of relationships contradicts not only the interpretations of Muir-Wood *et al.* and Cooper (Text-fig. 8A) but also the scheme of Dagys and Smirnova (Text-fig. 8B). This result was surprising, but may not be as radical a suggestion as it may at first appear. Significantly, perhaps, every one of the new long-looped terebratulide taxa recognized in this study – kraussinids, megathyridids, *Macandrevia*, and *Ecnomiosa* – has characteristic 'aberrant' morphological features which distinguish it from 'typical' long-looped terebratulides, such as the dallinids, terebratellids and laqueids. The kraussinids and megathyridids have a simpler structure of the lophophore and loop, which is believed to be neotenously derived from the typical long-looped stocks (Williams and Hurst 1977). The adult loop of *Macandrevia* is not particularly aberrant, but the cardinalia have very characteristic features which are reflected in the various assignments of this genus to the Dallinidae (Muir-Wood *et al.* 1965), Macandreviidae (Cooper 1973*b*), and Laqueidae (Richardson 1975). The adult loop of *Ecnomiosa*, on the other hand, is superficially also of standard long-looped type. However the mode of loop development is distinctive in this genus, and aspects of its cardinalia are unique. Cooper (1977, p. 129) considered that 'the loop of this brachiopod is so unusual as to set the genus apart from all others known', and erected the new family Ecnomiosidae for this genus, a family which was later assigned to the Dallinoidea (Cooper 1981*a*).

Another major finding of the earlier immunological investigations that was confirmed in this study was that the Cancellothyridoidea (Cooper 1973*a*; *C* group, Text-fig. 9) was almost equidistantly related to both a subset of the Terebratelloidea (Muir-Wood *et al.* 1965; *Te* group) and the Terebratuloidea (Cooper 1983; *Tu* group) to which another subset of the Terebratelloidea (*K* group) was linked. From the earliest fossil record of these three major superfamilies and the families to which the second long-looped species belong, Collins *et al.* (1991*a*) and Curry *et al.* (1991*a*) postulated that the last common ancestor of all living terebratulides diverged in the Triassic, and Collins *et al.* (1991*a*) further suggested that the second long-looped lineage diverged earliest in the Cretaceous from the terebratuloid stocks (Text-fig. 8C). These interpretations are not inconsistent with available information from the fossil record, at least in respect of the time of the first occurrence for each relevant taxon. But, considering the fact that the Terebratuloidea had already been morphologically established in the Cretaceous (Cooper 1983), the number of simultaneous mutations required to derive the megathyridids in the Cretaceous, or kraussinids in the Tertiary from terebratuloids, are very large, as Brunton and Hiller (1990) have pointed out; and the relationships among the second long-looped species were virtually unknown.

The questions are, therefore, how the species of the second long-looped terebratulides recognized in this study (*K* group) can be related to each other – the morphological affinities among the other long-looped group (*Te* group), dallinids, terebratellids, and laqueids, have been suggested by Richardson (1975) – and how the second long-looped lineage could be derived from the short-looped Terebratuloidea? The key seems to lie with the 'aberrant' loop and cardinalia of *Ecnomiosa* and *Macandrevia*.

Morphology of Ecnomiosa *and* Macandrevia

The specimen of *Ecnomiosa* used in the immunological assay was one of the two adult specimens collected from a locality in Japanese waters. It was a dead, though fresh, complete articulated shell (the other being alive when collected) which allowed observations of both the external features and the internal features, including the loop, and unequivocal identification of the genus. The size, the extent of sulcation, and the geographical distribution suggest that these individuals constitute a new



TEXT-FIG. 9. Dorsal valve interior morphology of *Ecnomiosa*, *Macandrevia*, and *Zeilleria*. A, *Ecnomiosa inexpectata*, a juvenile specimen with loop welded to valve floor, 4·3 mm in shell width (after Cooper 1981*a*); B, *Ecnomiosa inexpectata*, with a bilacunar loop, 12 mm in shell width (after Cooper 1981*a*); C, *Ecnomiosa gerda*, showing an adult loop connected to the median septum by a pair of medio-vertical connecting bands, 27·6 mm in shell width (after Cooper 1977); D, *Macandrevia cranium*, with a loop at the bilacunar stage, 4·5 mm in shell length (after Davidson 1886); E, *Macandrevia americana*, showing a teloform adult loop and peculiar cardinalia with a pair of hinge plates joining the valve floor, 25·0 mm in shell length (after Cooper 1982); F, *Zeilleria leckenbyi*, with a teloform loop, 12·2 mm in shell length (after Baker 1972). Note the development of spiny projections from the loop (labelled *s*) in these three genera.

species. The loop development and other morphological characters of two other species, from the Caribbean Sea and the Indian Ocean, have been illustrated and described by Cooper (1977, 1981*a*).

The adult loop pattern of *Ecnomiosa* is characterized by the presence of medio-vertical connecting bands and the absence of the lateral connecting bands (Text-fig. 9C; loop terminology as used by Richardson 1975). This is certainly an advanced phase of the bilacunar phase of the Laqueidae *sensu* Richardson (1975), as the loop of a younger stage retains the lateral connecting bands (the bilacunar phase; Text-fig. 9B; see Cooper, 1977, pl. 35, figs 13–14; 1981*a*, pl. 14, figs 7–11). Richardson (1975) did not list a genus with the adult loop pattern of *Ecnomiosa*, but listed the genera with the adult loop of the bilacunar pattern as *Aldingia*, *Jolonica*, *Kingena*, and *Paraldingia*. Of these, *Aldingia* and *Paraldingia* have strikingly similar internal and external morphologies to that of *Ecnomiosa*, such as shell outline, prominent concentric growth lines, tendencies to sulcation, cardinalia organization, and, of course, the loop pattern. Indeed, the only major morphological features distinguishing the former two genera from *Ecnomiosa* appears to be the presence or absence of the lateral connecting bands and the extent of calcifications in the cardinalia and the loop. *Aldingia* had been assigned in the Kraussinidae or the subfamily which used to embrace the kraussinid genera (Thomson 1927; Muir-Wood *et al.* 1965), until Richardson (1975) reassigned it to the Laqueidae. *Paraldingia* was erected by Richardson (1973) for the species which differ from *Aldingia* in possessing the dental

plates, more excavated and thinner cardinalia, and the thicker, broader, and spinous loop elements. The presence of the dental plates suggests that the affinity of *Ecnomiosa* lies more with *Paraldingia* than with *Aldingia*.

The prominent spinosity of the ascending and descending branches of the juvenile loop is another major feature of *Ecnomiosa* morphology (Text-fig. 9B; see Cooper 1981*a*, pl. 14, figs 7–14, 16–20). This is the very feature that is also observed in *Macandrevia* and almost completely lacking, except for the only slightly-developed spinous outgrowths from the septum (Richardson, 1975), in the species of the other long-looped terebratulide group (terebratellids, 'laqueids', and dallinids) recognized in this study. Although Elliott (1953) noted the spiny nature of many juvenile and adult dallinid loops, an observation which was cited by Atkins (1959) in the study of *Macandrevia*, it may be noteworthy that *Macandrevia* was then thought to be one of the most typical dallinids, and as far as the dallinid species (as then considered) used in this study except for *Macandrevia* are concerned (e.g. *Dallina* and *Dallinella* figured in Thomson 1927), the spinous outgrowths are distinguishably trivial compared with those of *Macandrevia* or *Ecnomiosa*.

Another unusual juvenile feature of *Ecnomiosa* which warrants mention is the welding of the descending lamellae to the valve floor during the early growth stages when only descending branches and the septal pillar are developed (Text-fig. 9A; Cooper 1981*a*, pl. 14, fig. 15). The meeting of the descending branches to the valve floor is also observed in the adults of *Argyrotheca*, *Megathiris*, and *Gwynia* (all megathyridids), and a Jurassic genus, *Zellania*, whose familial allocation in the Terebratelloidea is uncertain (Muir-Wood *et al.* 1965). No welding is observed in any other terebratulide species included in this study.

The loop development of *Macandrevia* is well documented (Friele 1877; Davidson 1886; Elliott 1953; Richardson 1975), and the basic features are certainly those of the typical long-looped species except for the spinous projections (Text-fig. 9D), and the very early abortion, for a dallinid species, of the connection between the loop and the septum (as noted by Smirnova 1984). The cardinalia, on the other hand, are unique, with the inner hinge plates sloping to join the valve floor separately (Text-fig. 9E). This feature is considered to reflect the unusual organization of the muscles of *Macandrevia*, in particular the dorsal adjustors (Cooper 1973*b*).

The mode of attachment of the dorsal adjustors to the dorsal valve could therefore be considered as a common, but not exclusive, motif for the *Ecnomiosa–Macandrevia*–kraussinids–megathyridids cluster, where the dorsal adjustor attachment sites appear to be confined to either the inner hinge plates, or the valve floor (or both for *Macandrevia*). By contrast, in the species of the terebratellid-'laqueid'-dallinid cluster, the dorsal adjustors never attach to the valve floor, but attach to parts of the cardinalia, notably the outer and inner hinge plates. The development of the outer hinge plates is known in *Ecnomiosa*, but they are narrow as described in Cooper (1977, p. 129), and their function as sites of muscle attachment is doubtful.

From the discussions above, it may be concluded that the two long-looped genera, *Ecnomiosa* and *Macandrevia* constitute a coherent group, and species of the other two families, the kraussinids and megathyridids, may well have been derived by a process of neoteny from this long-looped group, possibly independent of each other. This intepretation, however, is still inadequate in the light of the fossil record and the affinity of this group to the Terebratuloidea.

Megathyridids and kraussinids are known from the Upper Cretaceous and Miocene, respectively (Muir-Wood *et al.* 1965), while *Macandrevia* is known from the Miocene (Muir-Wood *et al.* 1965), and no fossil is known for *Ecnomiosa*. From the distribution on both sides of the Atlantic and both sides of the American continents in the present seas, Thomson (1927) suggested that the geological history of *Macandrevia* should extend back at least to the Eocene. Living *Macandrevia cranium* is known to occur in shallow cold waters, but most species, including *M. cranium*, range down to 1000–4000 metres (Cooper 1975). This abyssal distribution might explain the lack of a fossil record for this genus. The geographical distribution of *Ecnomiosa* also suggests a long history of this genus. Assuming a close relationship between *Ecnomiosa*, *Aldingia*, and *Paraldingia*, the record of this group extends back to the Upper Eocene, since *Aldingia* and *Paraldingia* are known from the Upper Eocene to Recent and the Upper Eocene to the Lower Miocene respectively (Richardson 1973).

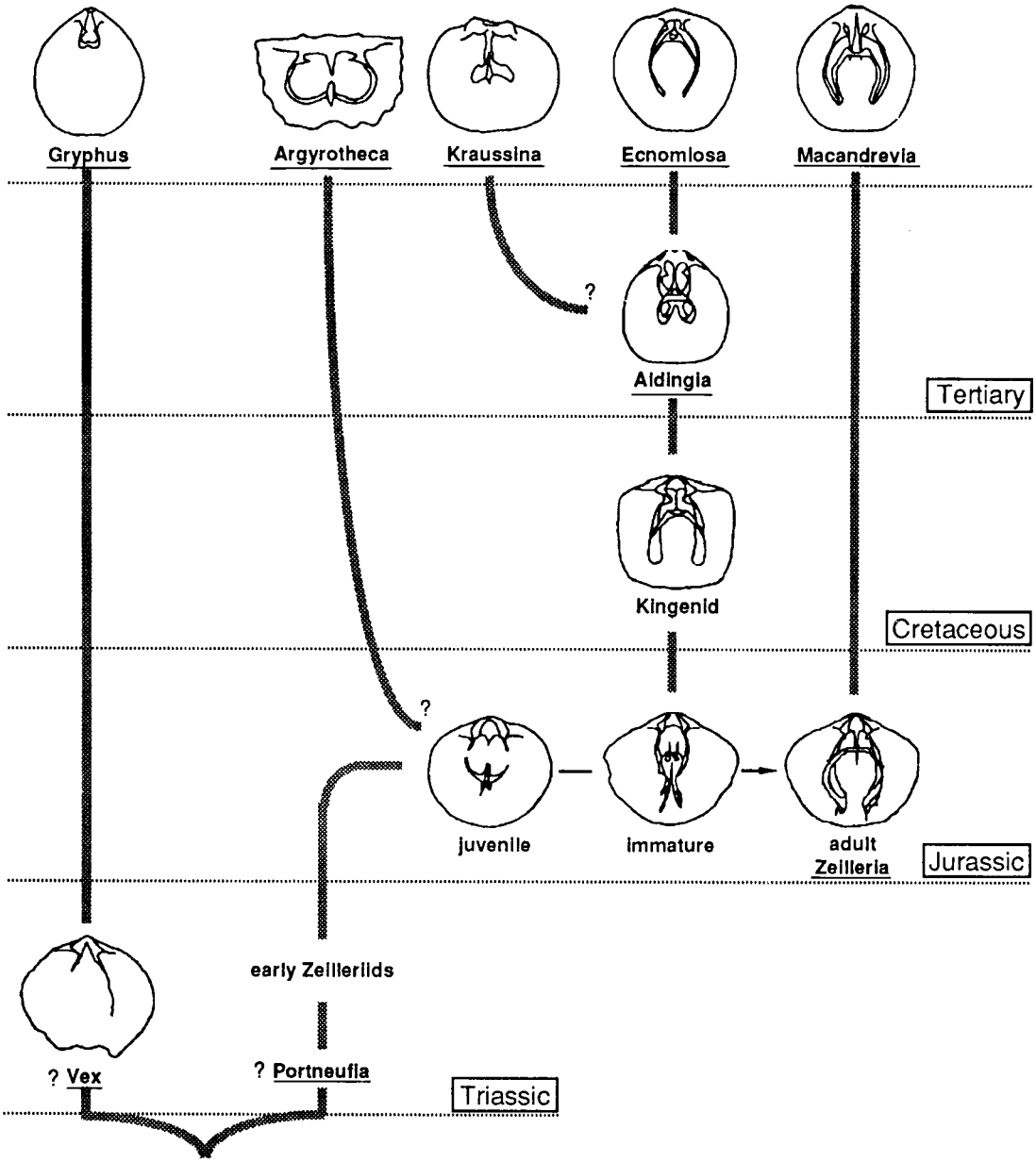
New interpretation of terebratulide phylogeny

From the pattern and the spinous nature of the loop, it is possible to link *Ecnomiosa* with *Kingena*, and by analogy, the Kingenidae, which is known from the Upper Jurassic to Cretaceous (Owen 1970), or to Recent in the view of Smirnova (1984) who included *Aldingia* and *Paraldingia* in the Kingenidae. Furthermore, from the spinous loop and the supposed general similarity in the way the dorsal adjustor muscles attach to the dorsal valve, it may be possible to speculate an association of these genera with the Zeilleriidae (cf. Text-fig. 9F). Morphological similarity between the Terebratelloidea and Zeilleriidae was suggested by Richardson (1975), and further specifically addressed by Elliott (1976). Baker (1972) demonstrated that the Jurassic *Zeilleria leckenbyi*, a 'typical' zeilleriid species, involved the septal pillar in its loop development, a character often attributed to the Terebratelloidea, and he insisted that the possession of spinose ascending and descending elements is of prime importance for the inference of the zeilleriid ancestry.

Indeed, this pattern appears to be the best palaeontological solution to the puzzling disagreements between the immunological and traditional schemes. The suggestion, therefore, is that the second long-looped lineage recognized in the immunological study (*K* group) was derived from the Terebratuloidea through the Zeilleriidae in Triassic times, when the Terebratuloidea and Zeilleriidae started to be established and had morphological plasticity. The fact that the Zeilleriidae includes two lineages, those which involve a septal pillar in the loop development, and those in which the loop development takes place without the pillar (Baker 1972; Elliott 1976), ties in well with the hypothesis of linking the long-looped *K* group species, which involve the septum in the loop development, and the short-looped terebratuloids (which do not). Morphological similarity between zeilleriids and *Kingena* has been noted by Muir-Wood *et al.* (1965). Smirnova (1984) pointed out that *Macandrevia* loses the connection between the loop and septum very early during ontogeny, unlike other 'dallinoids', a character which is suggestive of zeilleriid loop development. Baker (1972, p. 470) noted the 'resemblance between the early ascending lamellae of *Z. leckenbyi* and the two divergent plates which constitute the early development of the loop of *Kraussina*', an observation which fits perfectly with the interpretation that *Kraussina* originated from one of the descendants of the zeilleriids. Baker (1972) also reported the similarity of the juvenile loop pattern of *Zeilleria leckenbyi* with the adult loop pattern of *Kingena*, *Zittelina* (Kingenidae; Owen 1970), and *Trigonellina* (Dallinidae; Muir-Wood *et al.* 1965), although the possibility of neotenous evolution was denied on the basis of the presence or absence of the dental plate. Dagys (1972) and Smirnova (1984) also considered dental plates to be of high taxonomic value in terebratulide classification, and included kraussinids in the superfamily Terebratelloidea which generally lacks dental plates, and considered that this superfamily is distantly related to the other extant long-looped superfamily Dallinoidea, or to the extinct Zeilleriidae, both of which generally possess dental plates.

The dental plates appear to constitute a fairly invariable character at family level or even in higher classification. Although undoubtedly important, this character may not always be reliable at the higher classifications. Among the dallinids, juvenile *Dallina* has dental plates, but the adults have none, while in *Campages*, they are never present (Muir-Wood *et al.* 1965). The closely related genera, *Aldingia* and *Paraldingia*, can be distinguished by the presence or absence of the dental plates (Richardson 1973). Dental plates buttress teeth, so they can be interpreted as functioning to strengthen the articulation between the valves. These structures may be advantageous in nonstrophic brachiopods, such as most terebratulides, but in certain terebratulide taxa with a strophic tendency, such as megathyridids and kraussinids, dental plates may well have degenerated secondarily by the loss of functional constraints, since the posterior edges of the shell, being more or less parallel to the hinge line, would provide some support for articulation. These morphological changes could well have occurred independently in these taxa.

Cooper (1983, p. 51) noted that the early Triassic genus *Vex* had a 'loop, which suggests that of *Zeilleria*', but assigned it to the Terebratuloidea, rather than the Zeillerioidea, because of the lack of median septum and dental plates. Among the early Triassic terebratulides, Cooper (1983) also noted that *Portneufia*, first described as a short-looped dielasmatid genus (Hoover 1979), had a



TEXT-FIG. 10. A possible sequence of terebratulide phylogeny. See text for explanation. Brachiopod figures after Cooper (1983), Baker (1972), and Muir-Wood *et al.* (1965).

similar cardinalia morphology to *Vex*, in addition to a long loop, but discriminated it from *Vex* by the presence of dental plates (Cooper 1983). It is possible that these genera gave rise to the later terebratuloids and zeilleriids respectively, the latter further producing kingenids and other *K* group species, establishing the support for the loop from the median septum (Text-fig. 10).

Under this new interpretation based on immunological data, the radiation of the three major lineages recognized in this study, terebratuloids, cancellothyridoids, and 'terebratelloids', must

have occurred prior to the divergence between terebratuloids and zeilleriids in the Triassic. In the Palaeozoic, both the short-looped and long-looped lineages existed after early Devonian times (Muir-Wood *et al.* 1965). Therefore the time of radiation for the last common ancestors of existing terebratulides may be most parsimoniously assumed as the early Devonian (Text-fig. 8D), although questions about Palaeozoic ancestors of the three major lineages remained open. Among the most obscure is the origin of cancellothyridoids, as their ancestor is expected to have radiated at a similar time in the Devonian from the other two major lineages, but direct evidence is wanting, since the widely accepted earliest record of the cancellothyridoids is the Upper Jurassic (Muir-Wood *et al.* 1965). Taking *Pseudokingena* into consideration, which Cooper (1983) suggested as a member of cancellothyridoids, the earliest record would still only be in the Lower Jurassic. From the serotaxonomic viewpoint, cancellothyridoids are clearly separated not only from terebratelloids but also from terebratuloids, a fact which appears to be supported by the unusual morphology of the cancellothyridoids (cf. Cooper 1973*a*). Clearly, however, their origin remains enigmatic.

CONCLUSIONS

The serotaxonomic data suggest radical new ideas about the higher-order phylogeny within the Terebratulida. The immunological approach was also applied with reasonable success to the inference of lower-order relationships, down to species-level within the genus *Terebratulina*, using the more elaborate inhibition method. The serotaxonomic data have major implications for terebratulide classification, especially at subordinal and superfamilial arrangements. New taxonomic units and rearrangements are required for these ranks, but we are withholding the presentation of formal nomenclatorial recommendations, because of the absence in our study, due to the difficulty in obtaining specimens, of such important living taxa as Platidiidae, Thaumatosiidae, Phaneroporiidae, and Magadinae.

The immunological view does not flatly contradict traditional taxonomy, but urges further morphological investigations of the adult and juvenile stages of both Recent and fossil taxa, in particular those that lived during the late Palaeozoic and Mesozoic times. It is possible that the difficulty in observing loop development of fossil species, a procedure which requires a sample with a good ontogenetic sequence and often the use of serial sectioning, has been hindering the recognition of certain affinities among fossil taxa, especially of features which are only observed in juveniles. The apparent contradictions between the morphological and molecular data are only problematical if one or the other source of information is regarded as rigid and definitive; this would be entirely unrealistic, for brachiopods and all other organisms. The inferences from molecular data must never be assessed in isolation, but must instead be cross-checked with both geological information, morphology, and other molecular techniques, of which the sequencing of nucleic acids would be the logical next target in the hope of resolving some of the major classification problems in brachiopods.

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