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Evolution: disjunct degeneration of immunological determinants

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Abstract

The dissolution of calcified invertebrate skeletons releases an elaborate mixture of proteins, glycoproteins and polysaccharides. These 'skeletal matrix' macromolecules are thought to play a major role in calcification and were widely used for phylogenetical studies. We tested the reactivity of water-soluble macromolecules from a wide range of invertebrate skeletons with two antisera raised against the shell matrix of the bivalve, *Pinna nobilis*. Projections of our results on the phylogenetical tree of Starobogatov (1992) show for the first time that, during evolution, antigenic determinants may degenerate in some stocks while they remain intact in others. The phylogenetic implications of these patterns of disjunct degeneration are discussed.

Introduction

Immunological properties of biological macromolecules are often used for phylogenetic reconstructions (Muyzer et al. 1984, Lowenstein & Ryder 1985, Collins et al. 1988, Lowenstein & Scheuenstuhl 1991, Harte 1992, Robbins et al. 1993, Endo et al. 1994). The methodology exploits the ability of the mammalian immune system to produce antibodies to foreign macromolecules. Antibodies are a family of proteins capable of recognizing with exquisite specificity antigenic determinants on these macromolecules, i.e., small domains, which in the case of proteins typically contain 6–15 amino acids (Harlow & Lane 1988). Usually, the serum of an animal in which antibodies are raised by injecting one or several foreign macromolecules, contains a collection of several antibodies, directed against the various determinants to which the immune system was exposed. Such antisera are used as analytical reagents, whereby the presence of determinants in an unknown sample can be easily detected.

In general, an antiserum will strongly react with the macromolecules against which it has been raised, whereas weaker reactivities will be obtained with the homologous macromolecules of a different species. The reason is that evolutionary changes have affected the structure of the pertinent determinants. This principle is employed in measures of 'immunological distance' (Sarich & Wilson 1966, Prager & Wilson 1971, Olsen-Stojkovich et al. 1986). Thus, the major attraction of the method, although it does not provide structural information on the studied macromolecules by itself, is that an informative picture of the degree of structural similarity between macromolecules from different sources may be obtained with relatively little effort.

We present in this contribution the results of a study on the phylogenetic distribution of sets of determinants in water-soluble organic matrices extracted from invertebrate calcified tissues. Our data strongly suggest that, after their first appearance, the determinants may be widely dispersed through daughter lineages. Subsequently, they tend to disintegrate in some lineages while remaining intact in others. To the best of our knowledge, this is the first demonstration of disjunct degeneration of determinants during evolution. We suggest that, in principle, our finding may have

interesting implications for understanding the evolution of shell organic matrices. On the other hand, they put into question the uncritical use of immunology for phylogenetic reconstructions.

Materials and methods

When the mineral phase of calcified invertebrate skeletons is dissolved, complex amalgamates of proteins, glycoproteins, polysaccharides and other organic materials are obtained (Lowenstam & Weiner 1989). These 'organic matrices' play a regulatory role in calcification (Weiner & Hood 1975, Wheeler et al. 1988), but their macromolecular structure is difficult to elucidate (Weiner et al. 1983). Two major components may be distinguished in the organic matrix: a 'water-insoluble' fraction, thought to occupy an intercrystalline position, and an acidic 'water-soluble' fraction, tightly bound to the mineral (Crenshaw 1972). As a result of this close association, the latter fraction is well protected against microbial degradation and may remain intact for many years after the death of the animal (Weiner et al. 1979, Muyzer & Westbroek 1988; Collins et al. 1988, 1991a, b, Endo et al. 1995).

Using antisera against the soluble matrix of the nacreous layer of the bivalve *Pinna nobilis*, we conducted a comparative study of twenty bivalve genera (representing fifteen superfamilies), four gastropods, two cephalopods, three brachiopods and three scleractinian corals. An additional and more specific antibody preparation was obtained by adsorption of the anti-*Pinna* antibody with etched shell material of the cross-reacting bivalve, *Mercenaria mercenaria*. The data obtained with these two antisera (Figure 1, top) were compared with older data (Muyzer et al. 1984) of a similar experiment performed with an antiserum against the soluble matrix of *Mercenaria mercenaria* (Figure 1, bottom).

Skeletons of recent species, carefully selected to exclude contaminated or corroded specimens, were taken from the collections of the Laboratoire de Paléontologie (Université Paris XI, Orsay). Prof. E. Gittenberger of the National Museum of Natural History (Naturalis) in Leiden (the Netherlands), kindly provided additional materials.

The shells were mechanically cleaned, fragmented and the fragments soaked in 5% NaOCl for 24 h to remove contaminants. After thorough rincing in double distilled water, the fragments were dried and powdered. The powder of the individual shells (50 mg)

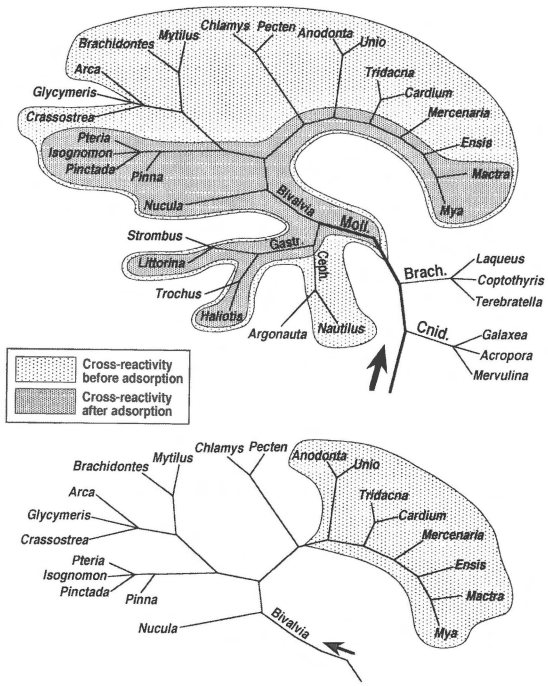

Figure 1. Superimposition of the immunological data obtained in Figure 2 on the phylogenetical tree of bivalves (top), proposed by Starobogatov (1992). The length of the branches in our representation does not have any significance on the phylogenetical distances between taxa. The unpurified antiserum gives a broad spectrum of reactivities since it reacts with most of the bivalvian soluble matrices (with the exception of *Glycymeris*), and also, with those extracted from *Haliotis*, *Littorina* (both gastropods) and the cephalopod, *Nautilus*. The adsorption of this antiserum on *Mercenaria mercenaria* shell powder restricts the pattern of reactivities, but, unexpectedly, not according to the taxonomic position of the tested samples. It seems that some 'ancestral' antigenic determinants, common to the whole phylum of molluscs, were lost independently by many lineages ('disjunct degeneration'), and were retained by others. The bottom figure illustrates results obtained previously by Muyzer et al. (1984), which are coherent with most of the classifications of bivalves, except those by Cope (1996) and by Morton (1996) Cnid = Cnidaria; Brach = Brachiopoda; Moll = Mollusca; Ceph = Cephalopoda; Gastr = Gastropoda.

was dissolved overnight in 3 ml of 20% EDTA, pH 8. After the complete dissolution of the shell, the solutions were centrifuged (5 minutes, 5000 rpm), and the supernatants containing the soluble matrix were directly used in Enzyme Linked Immuno Sorbent Assays (ELISA: see Clark & Adams 1977). The reader is referred to Muyzer et al. (1984) and Collins et al. (1988) for further details of the experimental procedure. The ELISA microplates were read with a Titertek multiscan spectrophotometer at 405 nm. All the tests were done in duplicate.

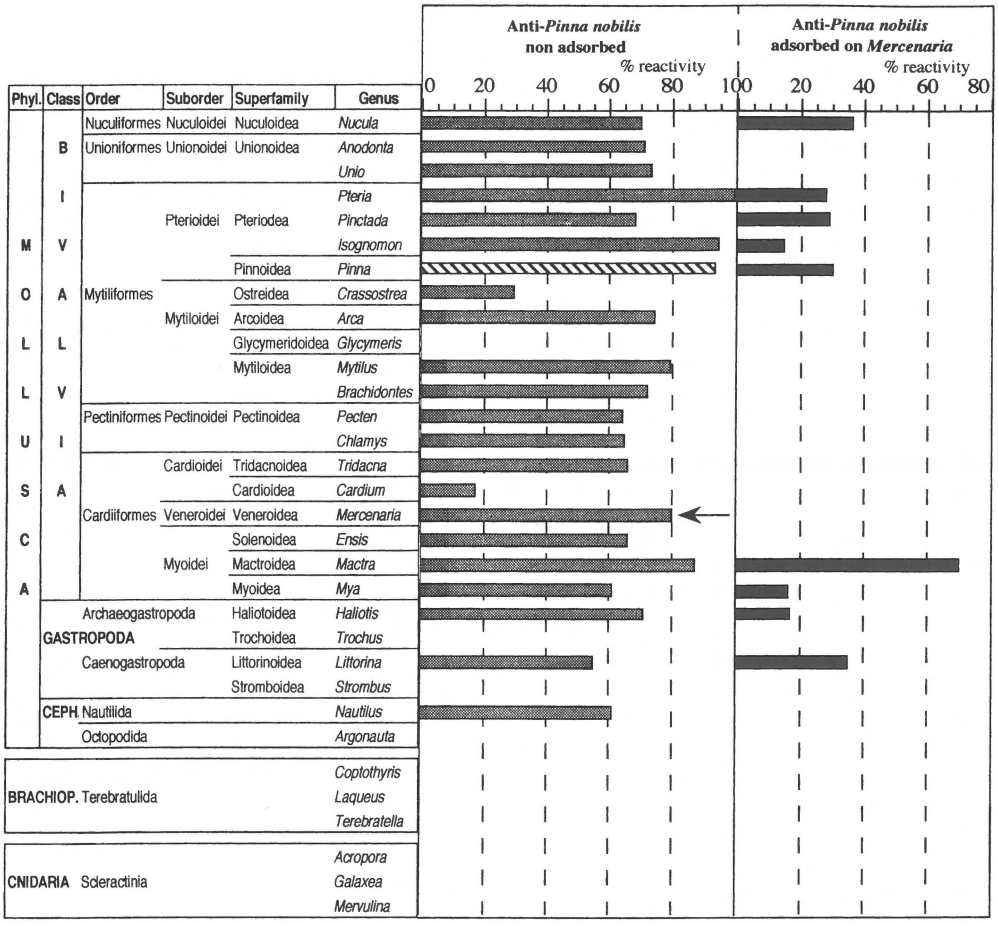

Figure 2. Immunological reactivity pattern obtained with a polyclonal antibody raised against the soluble matrix of the nacreous layer of the bivalve, *Pinna nobilis* (hatched bar). The presentation of the data for the class of Bivalvia follows the classification of Starobogatov (1992), selected for giving the highest consistency with our results. The antiserum was used in non-purified form (grey histogram) and after adsorption against EDTA-etched *Mercenaria mercenaria* shell powder (black histogram). Against each of the EDTA-soluble matrices, the two antisera were tested with four different dilutions ranging from 500 to 25000. Ceph = Cephalopoda; Brachiop = Brachiopoda.

For the preparation of the crude anti-*Pinna* serum, we refer to Marin et al. (1994). The more specific 'adsorbed' antibody preparation (anti-*Pinna* serum – *Mercenaria*) was obtained by reacting aliquots of dilute (0,01) anti-*Pinna* serum with powders of *Mercenaria* shell that had been EDTA-etched to expose the matrix macromolecules. After three consecutive adsorptions with excess shell powder, ELISA showed no reaction between the final adsorbed antibody preparation and the soluble matrix of *Mercenaria*. This proved that all antibodies reacting with *Mercenaria* determinants had been removed effectively from the original serum. The specificity of the adsorption was also controlled by adding to the antiserum, prior to the adsorption, another antiserum that did not react with any of the shell antigens (anti-*Artemia* elongation factor). After adsorption, the titer of the latter antiserum did not show any decrease when tested against its own target antigen (*Artemia* elongation factor). The addition of the anti-*Artemia* antiserum did not modify the titer of the anti-*Pinna* antiserum.

Results and discussion

The results are shown in Figure 2. The optical density (OD) measures were translated in percentage of immunological reactivity, with the highest reactivity representing 100% (with *Pteria*) and the background

OD being 0%. Note that reactivities below 10% of the maximum are not shown, because they could not be distinguished unambiguously from the background.

Three types of reactions were distinguished: negative before adsorption, positive before, but not after adsorption, and positive both before and after adsorption. We plotted our results on phylogenetical trees of the class Bivalvia, which have been proposed in the last six years (Starobogatov 1992, Cope 1996, Morton 1996, Salvini-Plawen & Steiner 1996, Waller 1997, Adamkewicz et al. 1997). To arrive at Figure 1, we selected the tree of Starobogatov (1992), because its outline showed the highest consistency with our results. We superimposed on one tree the data for anti-*Pinna* and for the adsorbed antiserum. Note again that the determinants reacting with the absorbed serum were a subset of those recognized by the crude anti-*Pinna* serum. These plots suggest from which ancestral line the recognized determinants originate, although an earlier origin remains possible. The possibility cannot be excluded that non-homologous determinants are recognized in the different stocks.

The reactivity patterns for the various antibody preparations in Figure 1 differ significantly. Thus, although overlap may well occur, different sets of determinants were detected by the three antibody preparations. These preparations reacted with matrices of widely different sources. Particularly interesting are the very strong reactions between the two anti-*Pinna* sera with the organic matrices of the gastropods *Haliotis* and *Littorina*, whereas the gastropods *Trochus* and *Strombus* and even the bivalve *Glycymeris* were not recognized. The reactivity between the non-adsorbed anti-*Pinna* and the cephalopod *Nautilus* was also considerable. From the cross-reactivities with genera belonging to different molluscan classes, we conclude that some of the determinants predate the molluscan radiation at the begining of the Cambrian. Only the anti-*Mercenaria* pattern was fully consistent with the phylogeny eastablished by Starobogatov (1992; Figure 1, bottom), while both other antibody preparations gave distinct departures from the standard tree (Figure 1, top).

We conclude that antigenic determinants that are shared by a wide range of ancient calcifying invertebrates were lost indiscriminately during evolution. Recurring, disjunct degeneration of the original immunological signal, detected at the levels of genera, families, orders and subclasses, is the most significant observation to be derived from Figure 1.

Several considerations might argue against the significance of these results:

1. as immunology does not reveal the chemical nature of antigenic determinants, we cannot exclude the possibility of false reactivities based on similar, but not homologous chemical structures (Faye & Chrispeels 1988), but we believe, in view of the generally high specificity of the immune response, that false reactions cannot have affected the patterns significantly;

2. different types of in vivo structural alterations may have similar effects on the reactivity of the matrix preparations, so that a mutation at the DNA level cannot be distinguished from a conformational change owing to post-translational modification;

3. bacterial infection may lead to a rapid degradation of the water-insoluble fraction of the matrix (Simon et al. 1990), whereas chemical hydrolysis and polymerisation may cause partial diagenesis of the soluble fraction (Gaffey 1988, Collins et al. 1991b, 1992). On the other hand, the soluble matrix may be protected for long periods of time from contamination and degradation at ambient temperatures, owing to its close association with the mineral phase (Crenshaw 1972, Muyzer & Westbroek 1988).

Although early diagenesis was found to cause smearing in the electrophoretic pattern of matrix molecules, the immunological reactivities remained largely intact (Collins et al. 1988, Marin unpublished data). Note that we took great care to use fresh shells and to remove contaminating materials with NaOCl.

Despite these limitations, our data strongly suggest that disjunct degeneration of immunological determinants is a major phenomenon in macromolecular evolution. This finding may have important implications for our understanding of calcification mechanisms, as it is generally believed that the constituent mineral microstructures of the shells are influenced by the chemistry of their matrices. The value of immunology for phylogenetical reconstructions was questioned earlier on theoretical grounds (Cohen 1992). The present contribution corroborates these considerations with experimental results. For example, our immunological data suggest a closer relationship of the bivalve *Mya* with the gastropod *Haliotis* than with the bivalve *Glycymeris*. A similar critical evaluation of other than immunological criteria for phylogenetical reconstructions is now in order.

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References

Adamkewicz, S.L., M.G. Harasewych, J. Blake, D. Saudek & C.J. Bult 1997 A molecular phylogeny of the bivalve mollusks – Mol. Biol. Evol. 14: 619–629

Clark, N.F. & A.N. Adams 1977 Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses – J. Gen. Virol. 34: 475–483

Cohen, B.L. 1992 Utility of molecular phylogenetic methods: a critique of immuno-taxonomy – Lethaia 25: 441–442

Collins, M.J., G.B. Curry, G. Muyzer, R. Quinn, T. Zomerdijk & P. Westbroek 1988 Sero-taxonomy of skeletal macromolecules in living terebratulid brachiopods – Histor. Biol. 1: 207–224

Collins, M.J., G. Muyzer, P. Westbroek, G.B. Curry, P.A. Sandberg, S.J. Xu, R. Quinn & D. MacKinnon 1991a Preservation of fossil biopolymeric structures: conclusive immunological evidence – Geochim. Cosmochim. Acta 55: 2253–2257

Collins, M.J., G. Muyzer, G.B. Curry, P. Sandberg & P. Westbroek 1991b Macromolecules in brachiopod shells: characterization and diagenesis – Lethaia 24: 387–397

Collins, M.J., P. Westbroek, G. Muyzer & J.W. de Leeuw 1992 Experimental evidence for condensation reactions between sugars and proteins in carbonate skeletons – Geochim. Cosmochim. Acta 56: 1539–1544

Cope, J.C.W. 1996 The early evolution of the Bivalvia. In: Taylor, J. (ed.) Origin and Evolutionary Radiation of the Mollusca – Oxford Univ. Press, Oxford: 361–370

Crenshaw, M.A. 1972 The soluble matrix from *Mercenaria mercenaria* shell – Biomineralization 6: 6–11

Endo, K., G.B. Curry, R. Quinn, M.J. Collins, G. Muyzer & P. Westbroek 1994 Re-interpretation of terebratulide phylogeny based on immunological data – Paleontology 37: 349–373

Endo, K., D. Walton, R.A. Reyment & G.B. Curry 1995 Fossil intra-crystalline biomolecules of brachiopod shells: diagenesis and preserved geo-biological information – Org. Geochem. 23: 661–673

Faye, L. & M.J. Chrispeels 1988 Common antigenic determinants in the glycoproteins of plants, molluscs and insects – Glycoconjugate J. 5: 245–256

Gaffey, S.J. 1988 Water in skeletal carbonates – J. Sedim. Petrol. 58: 397–414

Harlow, E. & D. Lane 1988 Antibodies, a Laboratory Manual – Cold Spring Harbor Lab. Publ., Cold Spring Harbor, NY

Harte, M.E. 1992 A new approach to the study of bivalve evolution – Amer. Malacol. Bull. 9: 199–206

Lowenstam, H.A. & S. Weiner 1989 On Biomineralization – Oxford Univ. Press, New York

Lowenstein, J.M. & O.A. Ryder 1985 Immunological systematics of the extinct quagga (Equidae) – Experientia 41: 1192–1193

Lowenstein, J.M. & G. Scheuenstuhl 1991 Immunological methods in molecular palaeontology – Phil. Trans. R. Soc. Lond. B 333: 375–380

Marin, F., G. Muyzer & Y. Dauphin 1994 Caractérisation électrophorétique et immunologique des matrices organiques solubles des tests de deux bivalves ptériomorphes actuels, *Pinna nobilis* L. et *Pinctada margaritifera* (L.) – C. R. Acad. Sci. Paris (sér. II) 318: 1653–1659

Morton, B. 1996 The evolutionary history of the Bivalvia. In: Taylor, J. (ed.) Origin and Evolutionary Radiation of the Mollusca. Oxford Univ. Press, Oxford: 337–359

Muyzer, G., P. Westbroek, J.P.M. de Vrind, J. Tanke, T. Vrijheid, E.W. de Jong, J.W. Bruning & J.F. Wehmiller 1984 Immunology and organic geochemistry – Org. Geochem. 6: 847–855

Muyzer, G. & P. Westbroek 1988 Phylogenetic implications and diagenetic stability of macromolecules from Pleistocene and recent shells of *Mercenaria mercenaria* (Mollusca, Bivalvia) – Histor. Biol. 1: 135–144

Olsen-Stojkovich, J., J.A. West & J.M. Lowenstein 1986 Phylogenetics and biogeography in the Cladophorales complex (Chlorophyta): some insights from immunological distance data – Botan. Marina 29: 239–249

Prager, E.M. & A.C. Wilson 1971 The dependence of immunological cross-reactivity upon sequence resemblance among lyzozymes – J. Biol. Chem. 246: 5978–5989

Robbins, L.L., G. Muyzer & K. Brew 1993 Macromolecules from living and fossil biominerals. Implications for the establishment of molecular phylogenies. In: Engel, M.H. & S.A. Macko (eds) Organic Geochemistry. Plenum Press, New York: 799–816

Salvini-Plawen, L. & G. Steiner 1996 Synapomorphies and plesiomorphies in higher classification of Mollusca. In: Taylor, J. (ed.) Origin and Evolutionary Radiation of the Mollusca. Oxford Univ. Press, Oxford: 29–51

Sarich, V.M. & A.C. Wilson 1966 Quantitative immunochemistry and the evolution of primate albumins: microcomplement fixation – Science 154: 1563–1566

Simon, A., M. Poulicek, R. Machiroux & J. Thorez 1990 Biodégradation anaérobique des structures squelettiques en milieu marin: II approche chimique – Cahiers Biol. Mar. 31: 365–384

Starobogatov, Y.I. 1992 Morphological basis for phylogeny and classification of Bivalvia – Ruthenica 2: 1–25

Waller, T.R. 1997 Origin of the molluscan class Bivalvia and a phylogeny of major groups. In: Johnston, P.A. & J.W. Haggart (eds) Bivalves: An Eon of Evolution, Univ. Calgary Press: 1–45

Weiner, S., L. Hood 1975 Soluble protein of the organic matrix of mollusc shells: a potential template for shell formation – Science 190: 987–989

Weiner, S., H.A. Lowenstam, B. Taborek & L. Hood, L. 1979 Fossil mollusk shell organic matrix components preserved for 80 million years – Paleobiology 5: 144–150

Weiner, S., W. Traub & H.A. Lowenstam 1983 Organic matrices in calcified exoskeletons. In: Westbroek, P. & E.W. de Jong (eds) Biomineralization and biological metal accumulation – D. Reidel Publ. Co., Dordrecht: 205–224

Wheeler, A.P., K.W. Rusenko, D.M. Swift & C.S. Sikes 1988 Regulation of *in vitro* and *in vivo* $CaCO_3$ crystallization by fractions of oyster shell organic matrix – Mar. Biol. 98: 71–80