IMMUNO-TAXONOMY AND THE RECONSTRUCTION OF BRACHIOPOD PHYLOGENY

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ABSTRACT. The proposed use of immuno-taxonomy of brachiopod shells for the reconstruction of brachiopod phylogeny is analysed for its possible contribution to genealogical systematics and for the validity of its principal conceptual and methodological foundations. It is concluded that brachiopod immuno-taxonomy, as so far described, departs from important norms of immuno-taxonomic and scientific procedures and can be of only limited utility in systematics.

IN response to an earlier critique (Cohen 1992), immuno-taxonomy has been defended as providing a timely and practical approach to brachiopod systematics (Curry *et al.* 1993). Although the concept of applying this methodology to the brachiopod shell is novel and worthwhile, and it has potential importance for palaeobiology, the alternative analysis given here suggests that its practical utility remains highly uncertain because of conceptual and methodological limitations. These limitations cast doubt on, but do not necessarily disprove, systematic relationships inferred from the immuno-taxonomic data (Collins *et al.* 1991*c*; Curry *et al.* 1991).

Genealogical classification and the role of immuno-taxonomy

As Darwin (1859) presciently noted, the aim of taxonomists should be to create classifications that are 'as far as they can be so made, genealogies'. This target is now in sight, using methods by which phylogenetically useful information may be retrieved from genomes and rigorously interpreted (Hillis and Moritz 1990). Work in progress by Cohen, Gawthrop and Cavalier-Smith and by Stark, Thayer and Cohen has shown that such DNA sequence data will indeed provide a genealogical framework for the classification of living brachiopods, serving either to confirm classical morpho-taxonomy (Williams *et al. in* Moore 1965) or to provide novel insights. By contrast, and for reasons detailed below (and see Cohen 1992), immuno-taxonomy as currently practised cannot create a genealogical (i.e. phylogenetic) classification. Instead, the most important role for immuno-taxonomy is to provide the only known method by which the emerging genealogical classification of living brachiopods may, perhaps, be indirectly extended to at least some relatively recent fossils (Collins *et al.* 1991*a*, 1991*b*) or to Recent brachiopods from which DNA is unlikely to be extracted (e.g. empty shells of dead animals).

For immuno-taxonomy to fulfil this useful role, without danger of creating mis-information, it is essential that there should be prior demonstration of a secure correlation between immuno-taxonomic results and those of the DNA-based, genealogical approach. Although it was claimed that a high degree of congruence between the two approaches has already been established (Curry *et al.* 1993), this was based upon DNA divergence estimation between only one pair of species (Cohen *et al.* 1991) using two relatively insensitive methods of analysis (restriction fragment length polymorphism of mitochondrial DNA and allozyme electrophoresis) both of which revealed so much divergence as to be beyond their known regions of reliability (Nei 1987) and one of which is known to have been upwardly biased (Cohen *et al.* 1993). These data cannot validate the proposed relationship.

Nevertheless, given that a potentially useful role for the immuno-taxonomy of brachiopods can be envisaged, and even if this role is rejected in favour of it serving merely as a phenetic character that reflects the dubious entity 'gross molecular similarity' (Curry *et al.* 1993), the methodology should be applied and presented in a way consistent with normal practice and with known limitations (Friday 1980; Maxson and Maxson *in* Hillis and Moritz 1990). The discussion which follows highlights some areas in which brachiopod immuno-taxonomy appears to depart both conceptually and methodologically from normal practice.

Independent verifiability

Independent confirmation of the immunological results requires microtitre plate-reading equipment and immunochemical techniques that are not commonly available to taxonomists, together with the successful preparation of suitable antibodies that, as biological reagents, are inherently unpredictable. Also, the requirement for reciprocal antigen–antibody reactions (antibody anti-A versus antigen B and antibody anti-B versus antigen A) means that data-collection cannot be approached piecemeal. For each taxon to be investigated an antibody must be raised by the immunization, with suitable antigen, of a group of experimental animals of the same genetic constitution. The cross-reactivity of each antigen and antibody must then be measured in every possible pairwise combination of antigen–antibody reaction, both homologous and heterologous. Therefore, a complete collection of shells must be amassed, antigen prepared from them all, and the immunizations carried out more-or-less simultaneously. Thus, independent verification of brachiopod immuno-taxonomy is subject to practical barriers.

Nature of the antigens and the concept of Immunological Distance

The brachiopod shell antigens are said to be predominantly carbohydrate and, as soluble extracts of shells (Collins *et al.* 1991*b*), they must be complex mixtures (with one exception, see below). But normal immuno-taxonomy requires protein antigens since carbohydrate epitopes lack phylogenetic significance (Zuckerkandl and Pauling 1965; Cohen 1992). Moreover, the antigens should be homologous and available as purified preparations, as in the widely-exploited cases of serum albumin and lysozyme (Maxson and Maxson *in* Hillis and Moritz 1990). If complex mixtures of antigens are used to raise antisera, analysis becomes uncertain unless, fortuitously, one is immunodominant. Furthermore, in reporting immuno-taxonomic results, the logarithmic transformation of antibody titres to Immunological Distances (ID) is justified only by the empirical correlation between number of aminoacid replacements and ID in specific proteins and may not be appropriate for other types of antigen (Friday 1980; Maxson and Maxson *in* Hillis and Moritz 1990). Therefore both the nature of the antigens and the index used to report the quantitative results involve significant departures from immuno-taxonomic norms. Moreover, because of the chemistry of the brachiopod antigens, the results may even be misleading (Zuckerkandl and Pauling 1965; Cohen 1992).

Character homology

Characters in both phenetic and phylogenetic taxonomy should invariably be homologous. Since, with one exception, shell secondary-layer fibre or whole shell powder extracts served as antigen source (Collins *et al.* 1988, 1991*c*), homology depends on the presence in these diverse shells of an immuno-dominant antigen that is functionally and evolutionarily conserved. However, direct evidence against full homology of shell proteins amongst the taxa analysed exists; a specific chromoprotein (Cusack *et al.* 1992) is present in red brachiopod shells and, by implication, absent from others. Biochemical evidence of antigen homology might have been obtained by immuno-electrophoresis or immuno-blotting, which can be used to characterize antibody and antigen complexity and specificity, but such results were not reported. Thus, brachiopod shell antigen homology remains only an assumption.

Data heterogeneity

For one brachiopod species the antigen was a purified protein, but for all other species it was a whole-shell or shell-fibre extract (Collins *et al.* 1988, 1991*c*; Curry *et al.* 1991). Yet the results obtained with both types of resultant antibody (and both types of antigen) have been combined in a single distance matrix and related cluster analyses as if they were strictly comparable data (Collins *et al.* 1991*c*). This appears to be a substantial departure from normal practice.

Independent variation of antigen concentration and cross-reactivity

Normally, to measure differences in antigen cross-reactivity, a standardized quantity of antigen is added to each test. In the brachiopod cross-reactivity tests, antigen quantity was standardized by the mass of shell from which the tested volume of extract was derived, not by actual antigen quantity, there being no independent assay method. Thus, variation in the concentration of antigen-in-shell and variation in cross-reactivity may both contribute to the immuno-distance estimates. Depending on technique, the initial, antigen-coating stage of the ELISA reactions may provide an escape from this difficulty, but no details were given. Alternatively, it could be assumed that the concentration of antigen in the various samples is reasonably constant, but since one of the antigen samples was a purified protein whose concentration was standardized by a different criterion (Collins *et al.* 1991*c*), this assumption cannot apply. Thus, as well as combining heterogeneous data, the reported ID values confound two potentially independent variables.

Repeatability and reciprocity

It is customary, before basing new taxonomic work on novel quantitative data, to demonstrate that the measurements have desirable statistical properties such as repeatability and, in the case of reciprocal immuno-distance, acceptably small deviations from reciprocity (typically about 2 ID units – Maxson and Maxson *in* Hillis and Moritz 1990). In brachiopod immuno-taxonomy, standard deviations for non-reciprocal assays with three antibodies have been reported (Collins *et al.* 1988), but these relate to data expressed by a different index of cross-reactivity and cannot be applied to ID estimates. In the only reference to reciprocity differences of ID values it is stated that those observed would disrupt only more specific conclusions than were presented (Curry *et al.* 1991). Thus, for the principal brachiopod immuno-taxonomic data (Collins *et al.* 1991*c*; Curry *et al.* 1991), neither summary statistics nor the normal assessment of non-reciprocity (per cent standard deviation – Maxson and Maxson *in* Hillis and Moritz 1990; Hass and Maxson 1992) have been published.

In the absence of relevant statistical analysis two estimates of immuno-distance repeatability may be extracted from the published ID matrix, as follows.

(1) Divergence estimates between a distant outgroup and each member of an ingroup set may be treated as replicates because they effectively measure the same evolutionary relationship. Two independent sets of such comparisons are given (Curry *et al.* 1991, table 1) and summary statistics calculated from these data are: *Mercenaria* – ingroups mean ID = 342·27, SD = 84·84; *Notosaria* – ingroups mean ID = 281·20, SD = 81·65. A very large range of ID values lie within ± 2 SD around these means, suggesting either that repeatability is low or that immuno-distance is not closely related to evolutionary divergence. However, this calculation may be inappropriate if experimental errors of log-transformed immuno-distances are not normally distributed.

(2) When the IDs between a distant outgroup and two or more *closely-related* ingroups are determined, the outgroup–ingroup distances should more certainly be identical within experimental error. For the remote rhynchonellid outgroup *Notosaria* and the closely-related terebratulid ingroups *Gryphus* and *Liothyrella*, IDs are given as *Notosaria–Gryphus* 188, *Notosaria–Liothyrella* 324 (Curry *et al.* 1991, table 1). These very different values do fall within the large ± 2 SD overlap

noted above, but a more appropriate statistical test might separate them. Either way, they raise questions about reliability of the method.

Conclusion

Immuno-taxonomy of brachiopod shell antigens has a potentially important role in the extension of genealogical classification to fossils and empty shells, but in its current state departs in several important respects from immuno-taxonomic norms. Whilst departures from normal practice may reflect useful innovations, that is hardly so in this case. In consequence, the practical utility of the approach will remain in doubt until it (a) can be conducted with purified, homologous antigens, preferably with aminoacid epitopes, (b) has been calibrated against genomic divergence, (c) has been demonstrated to be satisfactorily repeatable and reciprocal, (d) has been shown to be unconfounded by variation in the concentration of antigen-in-shell, and (e) has been independently confirmed. Thus, to advocate it as a practical approach and basis for new palaeontological work (Curry *et al.* 1993) appears premature.

Acknowledgements. I am grateful to Professor A. C. Wardlaw, Department of Microbiology, University of Glasgow, for helpful discussions. Research support has been received from the University of Glasgow, the UK Natural Environment Research Council, the Carnegie Trust for the Universities of Scotland, the Royal Societies of London and New Zealand and the Science and Engineering Research Council of Canada.

REFERENCES

COHEN, B. L. 1992. Utility of molecular phylogenetic methods: a critique of immunotaxonomy. *Lethaia*, **25**, 441–442.

—— BALFE, P., COHEN, M. and CURRY, G. B. 1991. Molecular evolution and morphological speciation in North Atlantic brachiopods (*Terebratulina* spp.). *Canadian Journal of Zoology*, **69**, 2903–2911.

—— —— —— —— 1993. Molecular and morphometric variation in European populations of the articulate brachiopod *Terebratulina retusa*. *Marine Biology*, **115**, 105–111, 694.

COLLINS, M. J., CURRY, G. B., QUINN, R., MUYZER, G., ZOMERDIJK, T. and WESTBROEK, P. 1988. Sero-taxonomy of skeletal macromolecules in living terebratulid brachiopods. *Historical Biology*, **1**, 207–224.

—— MUYZER, G., WESTBROEK, P., CURRY, G. B., SANDBERG, P. A., XU, S., QUINN, R. and McKINNON, D. 1991*a*. Preservation of fossil biopolymeric structures: conclusive immunological evidence. *Geochimica et Cosmochimica Acta*, **55**, 2253–2257.

—— —— CURRY, G. B., SANDBERG, P. A. and WESTBROEK, P. 1991*b*. Macromolecules in brachiopod shells: characterization and diagenesis. *Lethaia*, **24**, 387–397.

—— CURRY, F. B., MUYZER, G., QUINN, R., XU, S., WESTBROEK, P. and EWING, S. 1991*c*. Immunological investigations of relationships within the terebratulid brachiopods. *Palaeontology*, **34**, 785–796.

CURRY, G. B., QUINN, R., COLLINS, M. J., ENDO, K., EWING, S., MUYZER, G. and WESTBROEK, P. 1991. Immunological responses from brachiopod skeletal macromolecules; a new technique for assessing taxonomic relationships using shells. *Lethaia*, **24**, 399–407.

—— —— —— —— —— —— —— 1993. Molecules and morphology – the practical approach. *Lethaia*, **26**, 5–6.

CUSACK, M., CURRY, G., CLEGG, H. and ABBOTT, G. 1992. An intracrystalline chromoprotein from red brachiopod shells: implications for the process of biomineralization. *Comparative Biochemistry and Physiology*, **102B**, 93–95.

DARWIN, C. 1859. *On the origin of species*. John Murray, London, 502 pp.

FRIDAY, A. E. 1980. The status of immunological distance data in the construction of phylogenetic classifications: a critique. 289–304. *In* BISBY, F. A., VAUGHAN, J. G. and WRIGHT, C. A. (eds). *Chemosystematics: principles and practice. Special Volume of the Systematics Association*, **16**, 1–299.

HASS, C. A. and MAXSON, L. R. 1992. Nonreciprocity in immunological studies: a reply. *Systematic Biology*, **42**, 210–215.

HILLIS, D. M. and MORITZ, C. (eds) 1990. *Molecular systematics*. Sinauer, Sunderland, Mass., 588 pp.

MOORE, R. C. (ed.) 1965. *Treatise on invertebrate paleontology, Part H, Brachiopoda*. Geological Society of America and University of Kansas Press, Boulder, Colorado and Lawrence, Kansas, 927 pp.

NEI, M. 1987. *Molecular evolutionary genetics*. Columbia University Press, New York, 512 pp.
ZUCKERKANDL, E. and PAULING, L. 1965. Molecules as documents of evolutionary history. *Journal of Theoretical Biology*, **8**, 357–366.

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Typescript received 2 February 1994
Revised typescript received 12 April 1994

Note added in proof.
Some of the issues raised here have been mentioned in a further account of immuno-taxonomic data which appeared whilst this paper was in press (Endo *et al.*, 1994).

REFERENCE

ENDO, K., CURRY, G. B., QUINN, R., COLLINS, M. J., MUYZER, G. and WESTBROEK, P. 1994. Re-interpretation of terebratulide phylogeny based on immunological data. *Palaeontology*, **37**, 349–373.