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Intergeneric distances between *Ostrea*, *Crassostrea*, and *Saccostrea*, studied by means of crossed immuno-electrophoresis

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ABSTRACT: Polyspecific antisera were obtained from rabbits for each of the following oyster species: *Ostrea edulis* (Linné) and *O. lurida* (Carpenter), *Crassostrea gigas* (Thurnberg) and *C. virginica* (Gmelin), and *Saccostrea commercialis* (Iredale & Roughley) and *S. echinata* (Quoy & Gaimard). By means of crossed immuno-electrophoresis (CIE), genus-specific and genus-non-specific antigen-antibody reactions were recorded, in all 28 for *Ostrea*, 28 for *Crassostrea*, and 27 for *Saccostrea*. Genetic distances, defined by 1 – (number of genus-specific antigen-antibody reactions) ÷ (number of genus-specific and genus-non-specific reactions), were 0.25 to 0.29 between *Ostrea* and *Crassostrea*, 0.32 to 0.33 between *Ostrea* and *Saccostrea*, and 0.22 to 0.26 between *Crassostrea* and *Saccostrea*. The results accord with the current distinction between *Ostrea* and the 2 other taxa and support the latters' division into the 2 genera *Crassostrea* and *Saccostrea*.

INTRODUCTION

In 1939 Iredale wrote the following, which describes a continuing problem:

> 'Though beloved by gourmets from earliest times, oysters have never been a delight to systematic conchologists.'

Much controversy exists concerning the definition of genera within the pelecypod family, Ostreidae (Rafinesque, 1815). Dall (1898) separated Ostreidae into 2 groups, the monoecious (hermaphrodite) *Ostrea* (Linné, 1758), and the dioecious (separate sexes) *Crassostrea* (Sacco, 1897), and later Orton (1928) used the same criterion for defining his 2 genera *'Monoeciostrea'* and *'Dioeciostrea'*.

Lamy (1929) recognized only one genus, *Ostrea*, which he subdivided into 12 subgenera. Vialov (1936) recognized 116 fossil and live genera and subgenera. Iredale (1939) recognised 4 genera in his work on Australian oysters: *Ostrea, Lopha* (Roding, 1798), *Saxostrea* (Iredale, 1936), and *Dendostrea* (Swainson, 1835); his *Saxostrea* did not, however, include all the species comprising *Saccostrea* (see below). While Stenzel (1947) recognized 12 genera, Ranson (1948) arranged the Ostreidae species into 3 genera, *Pycnodonte* (Fisher de Waldheim, 1807), *Gryphae* (Lamarck, 1801), and *Ostrea*. Three years later, Gunter (1951) recognized 3 recent genera, *Ostrea, Crassostrea* (= Ranson's *Gryphae*), and *Pycnodonte*, a user-friendly taxonomy employed by e.g. Thomson (1954), Younge (1960), Galtsoff (1964) and Ahmed (1971). Carreon (1969) also recognized these 3 genera. He distinguished *Ostrea* from the other 2 by its relatively large gill ostia, its larviparity, and by absence of the promyal chamber (a passage situated on the right side of the visceral mass, between the adductor muscle and the hinge). Further, *Pycnodonte* was distinguished from *Crassostrea* by having a rectum which penetrates through the heart ventricle.

Stenzel's nomenclature (1947) was revised in 1971. He subdivided Ostreidae [which had been split apart from Gryphaeidae (Vialov 1936) including the subfamily Pycnodonteinae (Stenzel 1959)] into the 2 subfamilies, Ostreinae (Rafinesque 1815) and Lophinae (Vialow 1936). He recognized the following 4 genera of recent species as belonging to Ostreinae: *Crassostrea, Saccostrea* (Dollfus & Dautzenberg 1920), *Striostrea* (Vialow 1936) and *Ostrea*. The arguments for splitting Dall's *Crassostrea* into 2 genera were that *'Saccostrea* differs from *Crassostrea* in its deeper umbonal cavity, strong chromata, and tendency to conical rudistiform or cornucopia-like shapes' and 'eggs of *S. cucullata* have no effect in stimulating ejaculation of sperm from ripe

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males of *C. virginica* and species of these 2 genera cannot be made to crossfertilize each other' (Galtsoff & Smith 1932, Menzel 1968).

Although *Ostrea* and *Crassostrea* have been used, *Saccostrea* seems now to be generally accepted as the genus name for the farmed Sydney rock oyster *Saccostrea commercialis* (Iredale & Roughley) (e.g. Wisely et al. 1979, Braley 1984, Nell & Dunkley 1984) and the New Zealand rock oyster *S. glomerata* (Gould) (Dinamani & Lenz 1977), however see Dayton et al. (1989). Ahmed (1975) also recognized the genus *Saccostrea*, stressing that all tuberculated species of *Crassostrea* should in the future be assigned to *Saccostrea*, thus also the species *S. echinata* (Quoy & Gaimard).

In the present investigation, genetic distances between the genera *Ostrea, Crassostrea*, and *Saccostrea* were studied by crossed immuno-electrophoresis using polyspecific rabbit-antibodies against *O. edulis, O. luridae, C. gigas, C. virginica, S. echinata*, and *S. commercialis*.

MATERIALS AND METHODS

Collection and storage of oyster material. Samples of 40 to 70 individuals were transported live to the laboratory. The following species were studied: *Ostrea edulis* (L.) from Northern Europe, (Limfjord, Denmark); *O. lurida* (Carpenter) and *Crassostrea gigas* (Thurnberg) from the East Pacific (Oregon, USA); *C. virginica* (Gmelin) from the West Atlantic (New York, USA); *Saccostrea commercialis* (Iredale & Roughley) from the West Pacific (New South Wales, Australia); and *S. echinata* (Quoy & Gaimard), from the West Pacific (Queensland, Australia). Individual samples of hepatopancreas were pooled for each species, frozen at −80 °C, and stored at −40 °C. Shells of type specimens are kept at the Division of Invertebrate Zoology, Australian Museum, Sydney (*S. commercialis*: C. 163743 and *S. echinata*: C. 163744), and by the author.

Antigen preparation. Antigen samples were obtained from pooled hepatopancreas tissue homogenates from about 50 individuals of each of the species after starvation for 3 to 6 d. Tissue samples were homogenized with physiological saline solution and diluted to obtain a protein concentration of about 15 mg, which was determined according to Lowry et al. (1951). All procedures were carried out at 0 °C.

Antibody formation. Formation of polyspecific rabbit antibodies was carried out for each of the 6 species. For each species, 2 rabbits were immunized by intramuscular injections with the antigen solution: the first and second times using 1 mg antigen in Freund's complete adjuvant (700 µl) and phosphate buffer (750 µl) at 2 wk intervals; thereafter, 4 injections at 1 wk intervals with the same amount of antigen and buffer using Freund's incomplete adjuvant. One month after the last intramuscular injection, the rabbits immunized with *Ostrea* and *Crassostrea* antigens received an intravenal booster injection of 0.2 mg antigen in physiological saline solution prior to total bleeding 2 wk later; the rabbits immunized with the *Saccostrea* antigens were bled 2 wk after the last muscular immunization. The booster injections had no evident effect upon the antibody concentrations. After removal of blood cells by centrifugation, NaN (0.1 %) was added to the sera. Immunoglobulins were precipited with $(NH_4)_2SO_4$ (25 g per 100 ml antiserum), dialyzed with frequent shifts of acetate buffer (pH = 5) to precipitate lipoproteins, and diluted to original concentrations. Presence of immunoglobulins in the purified solution was stated by cellulose acetate electrophoresis, and immunoglobulin concentrations were determined by densiometric scanning. All preparations were carried out at 0 °C.

Titer determination and crossed immuno-electrophoresis (CIE). Antibody titres were assayed by immuno-electrophoresis using 5 µl of each antigen solution and different amounts of the corresponding rabbit antibodies in an electrophoresis gel. The antibody solutions from each pair of rabbits immunized with the same antigens contained similar antibody titres and were mixed. These species-related antibody solutions were thereafter used in the study.

CIE was carried out as described earlier (Brock 1987). Antigen-antibody precipitates were identified on the gels which were stained lightly to identify pronounced precipitates, and then heavily to visualize weaker precipitates. Genus-specific reactions were substantiated by CIE by precipitation of genus-nonspecific reactions in an intermediary gel containing antibodies against one of the other genera and precipitation of genus-specific reactions in a receiver gel containing antibodies against the tested antigens (Brock 1987).

Determination of genetic distances. Genetic relations at intergeneric levels are expressed by numbers of shared (genus-non-specific) antigen-antibody reactions, related to numbers of possible (genus-non-specific + genus-specific) antigen-antibody reactions. Distances are calculated by a dissimilarity coefficient, $1 - X$ (Dino & Georgi 1982), where X = a non-weighted, simple matching coefficient obtained by dividing the number of shared antigen-antibody reactions by the total number of identified antigen-antibody reactions.

RESULTS

Numbers of recorded antigen-antibody reactions for each of the 3 oyster genera are listed in Table 1. Antigen-antibody reactions obtained with one or both of the 2 species of a genus are considered 'genus-

Table 1. *Ostrea, Crassostrea,* and *Saccostrea.* Genus-specific plus genus-non-specific (bold), and genus-non-specific antigen-antibody reactions (normal print) recorded by crossed immuno-electrophoresis. For example, antigens from *Crassostrea* give 20 detected reactions with antibodies against *Saccostrea,* while antigens from *Saccostrea* give 21 detected reactions with antibodies against *Crassostrea.* Oe: *Ostrea edulis*; Ol: *O. lurida*; Cg: *Crassostrea gigas*; Cv: *C. virginica*; Sc: *Saccostrea commercialis*; Se: *S. echinata*

Antigens from:	Antibodies against: Oe + Ol	Cg + Cv	Sc + Se
Oe + Ol	**28**	21	19
Cg + Cv	20	**28**	20
Sc + Se	18	21	**27**

characteristic' (not genus-specific) antigens. By counting the antigen-antibody reactions which are genus-specific, and those which are genus-characteristic but genus-non-specific, calculation of genetic distances between the genera is possible. The calculated genetic distance was found to be 0.29 between the genera *Ostrea* and *Crassostrea,* when *Crassostrea* antigens reacted with antibodies against *Crassostrea* (28 precipitates) and with antibodies against *Ostrea* (20 precipitates). When *Ostrea* antigens reacted with antibodies against *Ostrea* (28 precipitates) and antibodies against *Crassostrea* (21 precipitates), the same genetic distance was found to be 0.25. The genetic distance between *Ostrea* and *Saccostrea* was found to be 0.33 when *Saccostrea* antigens reacted with antibodies against *Saccostrea* (27 precipitates) and antibodies against *Ostrea* (18 precipitates) and 0.32 when *Ostrea* antigens reacted with antibodies against *Ostrea* (28 precipitates) and antibodies against *Saccostrea* (19 precipitates). The genetic distance between *Crassostrea* and *Saccostrea* was found to be 0.26 when *Crassostrea* antigens reacted with antibodies against *Crassostrea* (28 precipitates) and antibodies against *Saccostrea* (20 precipitates), and 0.22 when *Saccostrea* antigens reacted with antibodies against *Saccostrea* (27 precipitates) and antibodies against *Crassostrea* (21 precipitates). Table 2 shows the calculated genetic distances.

DISCUSSION

The genus *Crassostrea* was separated from *Ostrea* by biological ('natural') criteria such as egg size (Walne 1964), whether a species is dioecious or monoecious Orton (1928), and on cytological chromosome differences (Ahmed 1973). Most bivalves are dioecious, e.g. *Crassostrea* and *Saccostrea,* therefore the monoecious *Ostrea* was considered more specialized (Stenzel 1971). The idea that *Ostrea* is a recently evolved form has also been proposed by Ahmed (1975) who suggested that *Ostrea* evolved from a *Crassostrea*-like ancestor through tight chromosome coiling. His work shows that species from 3 different genera, *O. lurida, C. gigas,* and *S. commercialis,* each contain the same chromosome number (2N = 20); this constant number neither supports nor contradicts the assumption that *Ostrea* is a more recently evolved genus than *Crassostrea.* However, Ahmed's theory is consistent with the fossil records (Stenzel 1971) which show that *Crassostrea* is older than *Ostrea* (earlier Cretaceous versus Cretaceous). The recent fossils of *Saccostrea* (Miocene) together with the present results suggest that this genus may have evolved from a *Crassostrea* and not from an *Ostrea* form. (The evolution of Ostreiidae is currently under study using comparisons of repetitive DNA sequences; Brock & Christiansen 1989).

In an immuno-absorbance assay, Numacki (1962) demonstrated the existence of 5 non-quantified genus-specific antigen-antibody reactions for the *Crassostrea* species *C. gigas, C. angulata, C. rivularis* and *C. virginica* as compared to *O. edulis,* whereas the *Crassostrea* types could not be separated from each other by the method used. In a comparison of the 2 intensely studied *Mytilus* forms, *M. edulis* and *M. galloprovincialis,* Brock (1985) could not separate them by means of CIE as the 2 forms shared all 27 identified antigen-antibody reactions, while the difference between the

Table 2. *Ostrea, Crassostrea,* and *Saccostrea.* Genetic distances between the 3 genera calculated from the results presented in Table 1 using the expression:

$$\text{Genetic distance} = 1 - \frac{\text{antigen-antibody reactions shared by the 2 genera}}{\text{total no. of antigen-antibody reactions for the 2 genera}}$$

Genetic distance between:	Antigens from: *Ostrea*	*Crassostrea*	*Saccostrea*
Ostrea and *Crassostrea*	0.25	0.29	
Ostrea and *Saccostrea*	0.32		0.33
Crassostrea and *Saccostrea*		0.26	0.22

Table 3. Methods suitable for measuring different forms of genetic variability and distances. Unless modified, methods for measuring smaller genetic distances are unproportionally resource-consuming when applied to larger genetic distances

Method	Intra-individual	Inter-individual	Inter-population	Inter-specific	Inter-generic
cDNA sequence analysis	×	×	×	×	×
Fingerprinting with probes	×	×	×	×	×
Protein/isozyme analyses	×	×	×	×	×
Immunology		×	×	×	×
Physiology		×	×	×	×
Morphology		×	×	×	×
Gamete hybridization				×	×

genera *Mytilus* and *Musculus* was found to be 0.30. In a similar study, the genetic distance between the genera *Acanthocardia* and *Cerastoderma* amounted to 0.63 (Brock 1989). This pronounced distance supports taxonomists in finding the 2 genera sufficiently different to be referred to distinct subfamilies. The genetic distance between the 2 genera *Musculus* and *Mytilus* is in good agreement with the distances between the 3 studied Ostreidae taxa found in this study and supports their separation into different genera. The classification by Harry (1985), according to which *Ostrea* is referred to the subfamily Ostreinae while *Crassostrea* and *Saccostrea* are referred to Crassostreinae is not confirmed, however it is not challenged by the present results since the distances between *Ostrea* and the 2 other genera are somewhat larger than the distance between them.

The visualized antigen-antibody reactions represent only soluble proteins which are large enough to act as antigens, and which occur in concentrations high enough to give rise to substantial antibody formation in rabbits. Thus only those proteins present in most of the oyster individuals, and not rare ones, will be detected. Also, the subtler differences between taxa are not registered by the method because the antigen-antibody reactions obtained are not unambiguously defined since different proteins may hold similar epitopes (antigen determinants) and therefore react with the same antibody. All this means that the immunological method used would probably be too coarse for measuring genetic distances at a subspecies level. For that purpose classical population comparisons based on isozyme studies (e.g. Buroker et al. 1979, Buroker 1983) and studies of DNA-variation are finer and hence more suitable tools (Brock 1989). However, as shown in Table 3, other immunological methods may be used for studies of inter-individual and inter-population distances, and intrageneric immunological differences and similarities are currently being studied with the inclusion of more species. For the present study, the method was purposely chosen for measurements of supraspecific distances since by ignoring the subtler inter-individual and inter-population details, the larger scale differences emerge.

In conclusion, independent immunological study of genetic distances between the oyster taxa *Ostrea, Crassostrea*, and *Saccostrea* strongly supports Orton's and Walne's separation of the genus *Ostrea* from Dall's *Crassostrea*. Further, it substantiates Stenzel's division of Dall's *Crassostrea* into the 2 distinct genera *Crassostrea* and *Saccostrea*.

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