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CYTOGENETIC STUDY OF *OSTREA CONCHAPHILA* (MOLLUSCA: BIVALVIA) AND COMPARATIVE KARYOLOGICAL ANALYSIS WITHIN OSTREINAE

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ABSTRACT Chromosome preparations of the Olympia oyster *Ostrea conchaphila* Carpenter were studied using conventional Giemsa, silver staining, and C-banding techniques. The karyotype consists of six metacentric (1, 2, 4, 6, 8, and 10) and four submetacentric (3, 5, 7, 9) chromosome pairs. The silver-stained nucleolus organizer regions (Ag-NORs) were terminally located on the short arms of the submetacentric pair 5 (56% of cases) and on the long arms of submetacentric pair 7 (6% of cases). Constitutive heterochromatin was observed as telomeric C-bands on the short arm of the NOR-bearing chromosome pair 5 and as centromeric blocks of several chromosome pairs. Comparative analysis of patterns of karyotype, Ag-NORs, and C-bands of this species and of five other flat oysters, *Ostrea angasi, O. chilensis, O. denselamellosa, O. edulis,* and *O. puelchana,* for which data have been previously published, were performed, allowing the inference of cytotaxonomic relationships within Ostreinae.

KEY WORDS: *Ostrea conchaphila,* cytogenetics, cytotaxonomy, Ostreinae

INTRODUCTION

Studies on oyster cytogenetics have been performed so far on 26 species of Ostreacea (see Nakamura 1985, Ieyama 1990, Thiriot-Quiévreux 2002). The first data only concerned chromosome number and gross morphology (Ahmed & Sparks 1967, Menzel 1968). Later, morphometric measurements of chromosomes enabled the comparison among karyotypes at the interspecific and intraspecific level (e.g., Ladron de Guevara et al. 1996, Li & Havenhand 1997). During the last decade, the development of banding techniques has allowed the fine characterization of individual chromosomes (e.g., Leitão et al. 1999a).

According to the morphologically based classification of Harry (1985), which is currently used, the family Ostreidae includes three subfamilies, that is, Lophinae, Ostreinae, and Crassostreinae. These oysters are sequential hermaphrodites and contain both broadcast spawners (Crassostreinae) and brooders (Lophinae and Ostreinae). Recent techniques such as molecular phylogenetic analysis provided novel insights into oyster evolution and systematics (Littlewood 1994, Jozefowicz & Ó Foighil 1998, Ó Foighil & Taylor 2000). Karyological analysis among cupped oysters, the Crassostreinae (Leitão et al. 1999b), has proven complementary to these approaches and has provided additional evolutionary inferences.

Among the flat brooding oyster species, the Ostreinae, five species have been previously karyologically investigated: *Ostrea edulis* (Linné) (Thiriot-Quiévreux 1984), *O. denselamellosa* (Lischke) (Insua & Thiriot-Quiévreux 1991), *O. puelchana* (Orbigny) (Insua & Thiriot-Quiévreux 1993), *O. chilensis* (Philippi) (Ladron de Guevara et al. 1994), and *O. angasi* (Sowerby) (Li & Havenhand 1997).

The Olympia oyster, *O. conchaphila* (Carpenter 1857), previously known as *O. lurida* (Carpenter 1864), has been studied by Ahmed and Sparks (1967) and Ahmed (1973) using squash techniques and tentative grouping of chromosomes. *Ostrea conchaphila,* native to the western United States and Canada, ranges from the southeast Alaska to Baja California (in tidal channels, estuaries, bays, and sounds). Commercially important in the late 19th century, this species was cultured in the state of Washington until near-collapse of the industry in the 1950s (Baker 1995).

In the present work, the karyotype, nucleolus organizer regions (NORs), and constitutive heterochromatin distribution were studied in *Ostrea conchaphila* (Carpenter 1857) and a comparison with previously published karyological data on the five other flat oyster species mentioned above was performed to analyze cytotaxonomical relationships within Ostreinae.

MATERIALS AND METHODS

Specimens of the Californian Olympia oyster *Ostrea conchaphila* (G0) were imported from the Pacific Institute (Olympia, WA). Oysters were strictly confined to the quarantine facilities of the IFREMER hatchery of La Tremblade, Charente-Maritime, France, according to international recommendations. After reproduction, the progeny (G1) used in this experiment was reared in the same quarantine facilities for at least 5 mo before sampling.

Whole juvenile animals (ca. 2.5 cm length) were incubated for 7–9 h in a 0.005% solution of colchicine in seawater. The gills were then removed by dissection and treated for 30 min in 0.9% sodium citrate in distilled water. The material was fixed in a freshly prepared mixture of absolute alcohol and acetic acid (3:1) with three changes of 20 min each. Fixed pieces of gill from each individual were dissociated in 50% acetic acid with distilled water solution. The suspension was dropped onto heated slides at 44°C and air-dried (Thiriot-Quiévreux & Ayraud 1982).

For conventional karyotypes, gill preparations were stained with Giemsa (4%, pH 6.8) for 10 min. The silver-staining method for NORs was performed on unstained slide preparations according to the procedure of Howell and Black (1980). This method only detects those NORs that were active at the precedent interphase (Miller et al. 1976). Chromosomal Ag-NORs can serve as characters for inferring phylogenetic relationship (e.g., Amemiya

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& Gold, 1990). Constitutive heterochromatin regions (C-bands) were revealed using the method of Sumner (1972) with the counterstain propidium iodide. The evolutionary significance of the heterochromatin has previously been discussed in vertebrates (e.g., Hsu & Arrighi 1971, Saffery et al. 1999, Chaves et al. 2000).

Images of Giemsa-stained metaphases and C-banding were acquired with a CCD camera (Axioplan, ZEISS) coupled to a ZEISS Axioplan microscope. Digital images were processed using Adobe Photoshop 5.0 (Windows) using functions affecting the whole of the image only. Microphotographs of Giemsa stained metaphases and C-banding were taken with a ZEISS Axioplan microscope. Digital images were processed using Adobe photoshop 5.0 (Windows). Microphotographs of suitable NOR-stained metaphases were taken with a ZEISS III photomicroscope.

After karyotyping, chromosome measurements of 10 suitable metaphases were made with a digitizer table (Summa Sketch II) interfaced with a Macintosh. Data analysis was performed with an Excel macro-program. Relative length was expressed as 100 times the absolute chromosome length (in μm) divided by the total length of the haploid complement. Centromeric index was calculated by dividing 100 times the length of the short arm by the total chromosome length. The arm ratio was determined (length of short arm divided by length of long arm). Both centromeric index and arm ratio are given because each expresses centromere position and allows comparison with other karyological studies. Terminology relating to centromere position (m: metacentric, sm: submetacentric) follows that of Levan et al. (1964).

To elucidate similarities between Ostreinae species, a hierarchical agglomerative flexible clustering program was used (Lance & Williams 1966). Both NOR and centromeric index information of *O. conchaphila* and five previously studied Ostreinae species were used to cluster species. The Manhattan metric was used to discriminate and then to associate individual species. Manhattan distance appears appropriate to this kind of combination of quantitative (centromeric index values) and qualitative (NORs positions) data and to measure an association between individual objects (species) (Legendre & Legendre 1998).

RESULTS

Analysis of 60 mitotic metaphase spreads from 15 individuals of *O. conchaphila* confirmed the diploid chromosome number of $2n = 20$, scored by Ahmed and Sparks (1967). For karyotyping, the chromosomes of 21 well-spread metaphases were paired on the basis of chromosome size and centromere position. From these, the 10 best spreads were used for chromosome measurements and classification (Table 1). The karyotype (Fig. 1A) consists of ten chromosome pairs. Pairs 1, 2, 4, 6, 8, and 10 were metacentric. Pairs 3, 5, 7, and 9 were submetacentric.

The Ag-NORs were examined in another 122 metaphases from 10 animals. A variable number of one to three Ag-NOR chromosomes were identified (Fig. 1B). The NOR site was located terminally on the short arms of the submetacentric pair 5 and on the long arms of the submetacentric chromosome pair 7. The most frequent case (56% of observed silver-stained metaphases) was one active silver-stained NOR chromosome in pair 5. The Ag-NORs located on pair 7 occurred in few cases (6%).

Constitutive heterochromatin was observed in 31 karyotypes made from well-spread C-banded metaphases from 13 animals. Telomeric C-bands were always observed on the short arm of the NOR-bearing chromosome pair 5. In addition, centromeric blocks were also found in chromosome pair 2 in 84% of observed metaphases, pairs 1, 4, and 5 in 68%, pairs 6 and 8 in 58% of the C-banded karyotypes and in fewer cases in pairs 3, 7, and 9 (35%), and in pair 10 (26%) (Fig. 1C).

To compare the karyological data from *O. conchaphila* and from the other five flat oyster species previously studied, ideograms (Fig. 2) were constructed from relative length and centromeric index values of *O. conchaphila* (see Table 1), *O. edulis* (after Leitão 2000, French population of La Tremblade hatchery, Charentes Maritimes, France), *O. angasi* (after Li & Havenhand 1997), *O. chilensis* (after Ladron de Guevara et al. 1994), *O. denselamellosa* (after Insua & Thiriot-Quiévreux 1991), and *O. puelchana* (after Insua & Thiriot-Quiévreux 1993). The location of Ag-NORs was also included because chromosomal NOR have been used as characters for inferring hypothesis of cytotaxonomic relationships (e.g., Amemiya & Gold 1990, Leitão et al. 1999 b).

The comparison of the relative length and centromeric index of the 10 chromosomes pairs of the studied species showed that pair 1 was similar among all species, pair 2 was also similar except for *O. puelchana*, pair 3 and 4 were similar except for *O. conchaphila*, but taking into account the close relative length and the standard deviation of pair 3 and 4 of *O. conchaphila*, they may be inverted. Pair 5 was variable among species, pairs 6 and 7 were identical except for *O. denselamellosa*, but in this case, the pairs 6 and 7 cannot be inverted because of their different relative length and the

TABLE 1.

Chromosome measurements and classification in 10 cells of *Ostrea conchaphila*.

Chromosome Pair No.	Relative Length		Arm Ratio		Centromeric Index		Classification
	Mean	SD	Mean	SD	Mean	SD	
1	12.77	0.99	2.39	0.21	42.12	1.78	m
2	11.60	0.55	2.54	0.18	43.87	1.89	m
3	10.64	0.57	1.26	0.20	27.76	2.86	sm
4	10.54	0.50	2.37	0.27	41.94	2.81	m
5	10.47	0.87	1.72	0.16	34.05	2.28	sm
6	9.88	0.78	2.48	0.25	42.81	2.67	m
7	9.46	0.38	1.26	0.16	27.56	2.59	sm
8	9.30	0.58	2.53	0.39	43.59	3.92	m
9	8.79	0.63	1.52	0.25	31.27	3.37	sm
10	6.56	0.65	2.43	0.26	42.18	2.60	m

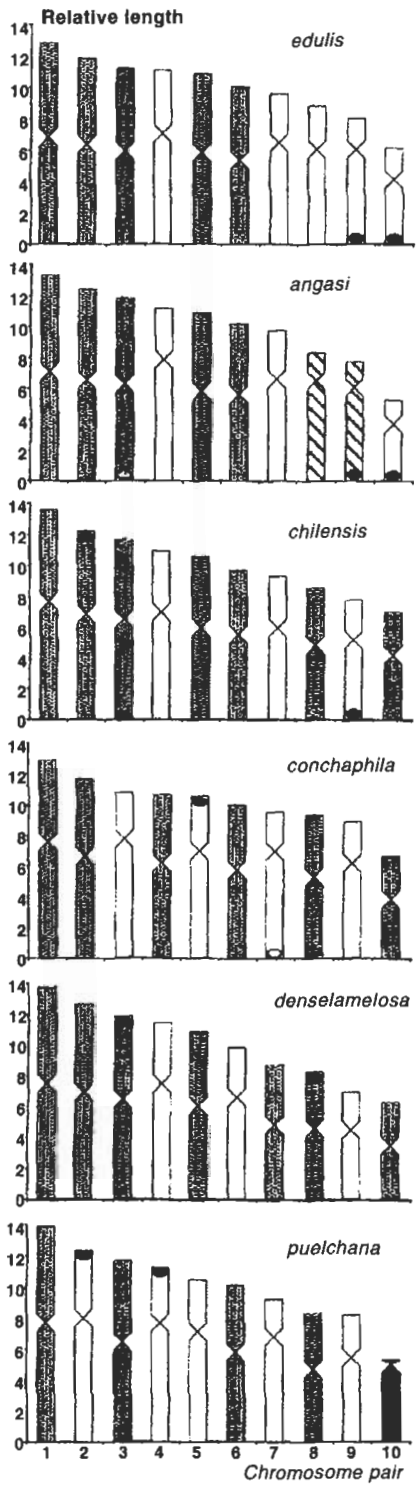

Figure 2. Ideograms of six flat oysters constructed from relative length and centromeric index values. Stippled chromosome: metacentric, white chromosome: submetacentric, striped chromosome: subtelocentric, black chromosome: telocentric. Circles indicate Ag-NORs, dark circles the most frequent case.

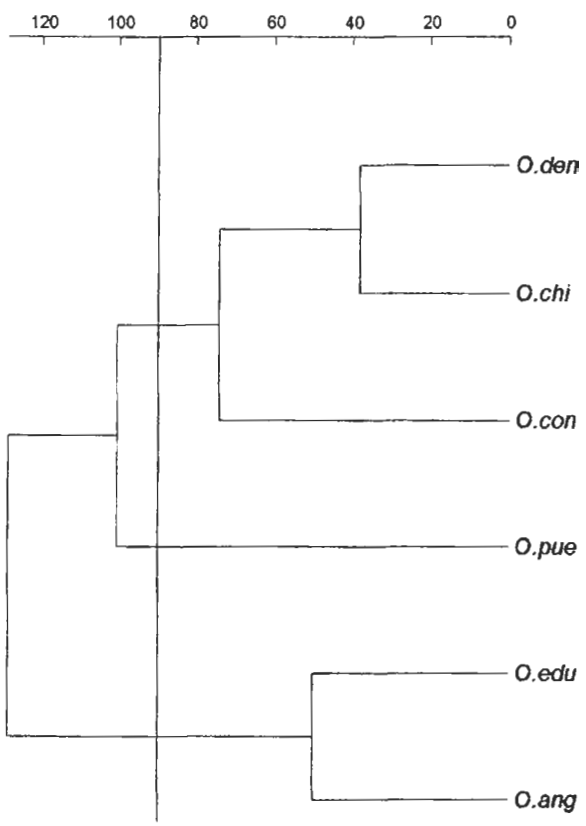

Figure 3. Hierarchical agglomerative flexible clustering of *Ostrea* spp. O. den: *Ostrea denselamellosa;* O. chi: *O. chilensis;* O. con: *O. conchaphila;* O. pue: *O. puelchana;* O. edu: *O. edulis;* O. ang: *O. angasi.*

including six metacentric and four submetacentric chromosome pairs, and the NOR and C-band distribution differ from the other ostreinid species studied. The comparison of the relative length and centromeric index of the 10 chromosome pairs of the studied species shows that, if one postulates that shared chromosome pairs with the same relative length and centromeric index may be considered as primitive, pairs 1, 3, and 4 are primitive and pairs 5, 8, and 10 the most derived. However, these chromosome homologies should be confirmed by other banding techniques.

The comparison of karyotypes and location of Ag-NORs among species highlighted first the chromosome similarity between the European species *O. edulis* and the Australian and New Zealand species *O. angasi,* already pointed out by Li and Havenhand (1997). Their karyotypes differ slightly (5m, 5 sm in *O. edulis* and 5m, 3 sm, 2 st in *O. angasi*), but the phenomenon of variation in the number of submetacentric and subtelocentric chromosomes have been reported in French populations (Thiriot-Quiévreux 1984). More striking is that the most frequent Ag-NOR patterns are similar in both species.

The isolated karyotype of *O. puelchana* is remarkable because of the single telocentric chromosome. The occurrence of telocentric chromosomes has been only seen in one other species of Ostreidae, *Dendrostrea folium* (Lophinae) (Ieyama 1990).

The three other flat oysters bear high karyotype resemblance, that is, seven metacentric and three submetacentric pairs for *O. denselamelosa* and *O. chilensis* and six metacentric and four sub-

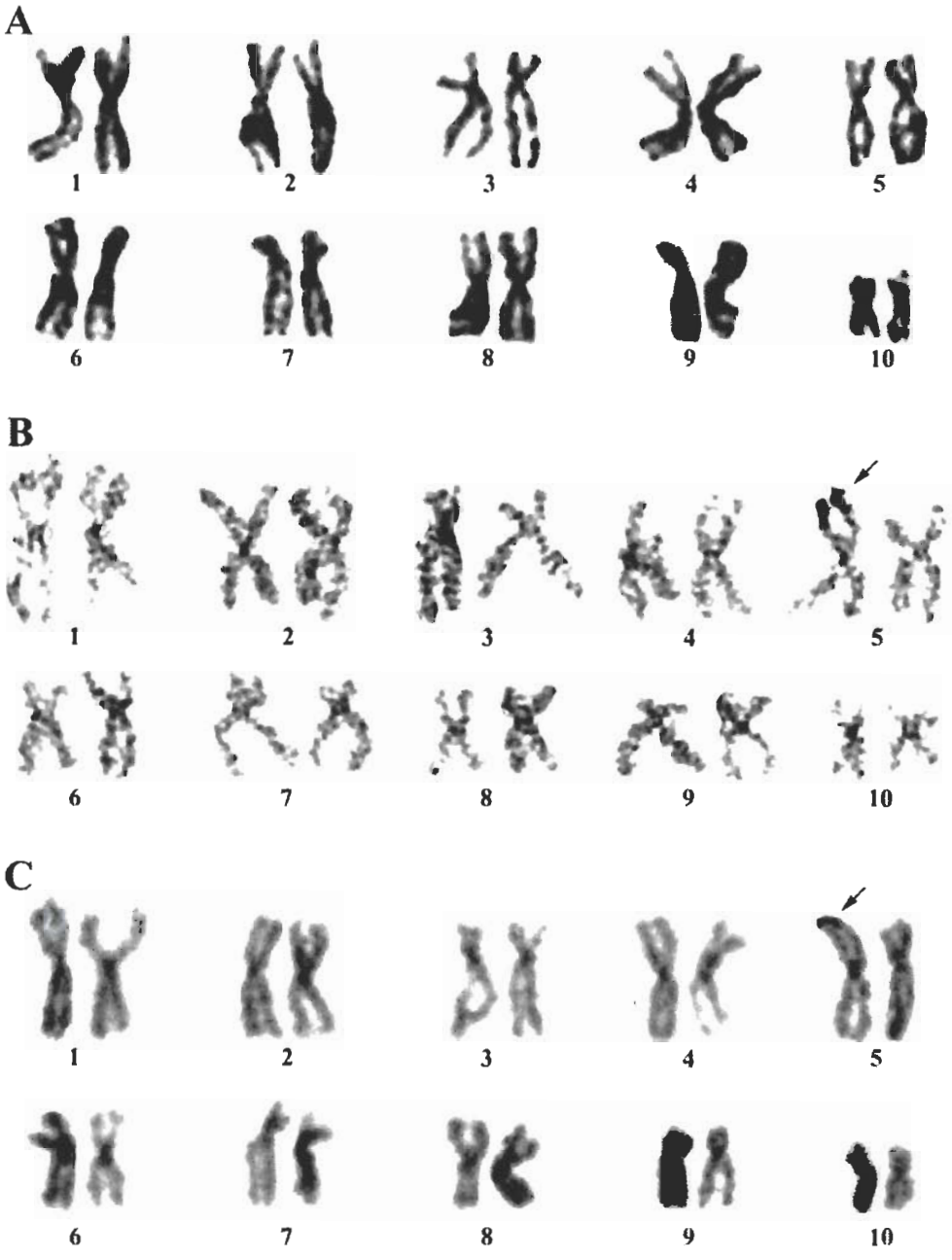

Figure 1. Karyotypes of *Ostrea conchaphila*. A, Conventional Giemsa staining; B, silver-stained nucleolus organizer regions (Ag-NORs); C, C-banding. Note the simultaneous presence of Ag-NOR and C-bands in a telomeric position on the short arms of pair 5 (arrows) and the centromeric heterochromatic blocks on chromosome pairs 1, 2, 4, 5, and 9. Scale bar = 5 µm.

C-banding of pair 6. Pair 8 was variable among species. Pair 9 was identical except for *O. angasi* and pair 10 was variable.

A statistical analysis based on Ci and NORs (Fig. 3) highlighted the clustering of ***O. edulis*** and ***O. angasi*** and of *O. denselamellosa* and *O. chilensis* with *O. conchaphila* placed near this cluster. *O. puelchana* is separated from the other species by the highest dissimilarity.

DISCUSSION

This is the first report on karyotype after chromosome measurements and NORs and C-banding patterns of the Olympia oyster. The diploid chromosome number $2n = 20$ observed is characteristic of the genus *Ostrea* and is common throughout the Ostreacea (Nakamura 1985, Thiriot-Quiévreux 2002). The karyotype,

metacentric pairs for *O. conchaphila*. Their NOR chromosomal location revealed that there is a higher resemblance between the NOR patterns of *O. chilensis* and *O. conchaphila* than between these two species and *O. denselamellosa*. *O. chilensis* and *O. conchaphila* showed terminally located NORs on the short arms of one chromosome pair and on the long arms of another chromosome pair. On the contrary, in *O. denselamelosa*, Ag-NORs were always terminally located on the short arms of chromosome pairs.

Data on constitutive heterochromatin distribution only concerned three species, *O. denselamellosa* (Insua & Thiriot-Quiévreux 1991), *O. angasi* (Li & Havenhand 1997), and *O. conchaphila* (this study). Centromeric C-bands were observed in chromosome pairs 3, 6, 8, 9, and 10 in *O. angasi* and in pairs 6, 8, 9, and 10 in *O. denselamellosa*. Occasional C-bands were seen on the centromere of pairs 4 and 7 in *O. angasi* and on telomeres of pairs 3, 5, 6, 8, 9, and 10 in *O. denselamellosa*. A substantial proportion of the eukaryote genome consists of constitutive heterochromatin. This genomic fraction includes, among other repetitive sequences, satellite DNA. Sequence analysis of these repeats suggests that the sequences are rapidly evolving, and hence they are valuable as evolutionary markers; consequently, constitutive heterochromatin analysis can give insights about the phylogeny relationships of related species (Saffery et al. 1999, Chaves et al. 2000). The observation in *O. denselamellosa* and *O. conchaphila* of the simultaneous presence of Ag-NORs and C-bands on telomeric position in the same chromosome pair, that is, pairs 3 and 8 in *O. denselamellosa* and pair 5 in *O. conchaphila*, might corroborate the close karyological relationship between these two species noted above.

The cytotaxonomic relationships pointed out here are incongruent with the morphologically based classification of Harry (1985), who stated that *O. chilensis* and *O. angasi* were junior synonymous of *O. puelchana* in the subgenus *Eostrea* of the genus *Ostrea* and that *O. edulis* and *O. denselamellosa* were included in the subgenus *Ostrea* ss. The species *O. lurida* was considered as a junior synonymous of *O. conchaphila* in the genus *Ostreola*. Li and Havenhand (1997) have also previously disagreed with Harry (1985), placing *O. angasi* as a separate species, very close to *O. edulis*.

Our results show greater congruence with molecular phylogenetic analyses of the Ostreinae, based on partial mitochondrial 16S rDNA (Jozefowicz & Ó Foighil 1998) and nuclear 28S rDNA (Ó Foighil & Taylor 2000) datasets. This is most evident for *O. edulis* and *O. angasi*, where a sister species relationship for these European and Australian flat oysters is strongly supported by both karyological and gene tree data. The ostreinid mitochondrial gene trees place the six karyologically-characterized flat oysters into two clades: one containing (among other taxa) *O. puelchana*, *O. conchaphila*, and *O. denselamellosa*, the other composed of *O. edulis*, *O. angasi*, and *O. chilensis*. With the exception of positioning of *O. chilensis*, which in our study is closer to *O. denselamellosa*, these results are in broad agreement with the topology generated by our statistical analysis based on Ci and NORs.

All Ostreinae species are of the brooding type with an extended planktotrophic larval development with the exception of *O. chilensis*, which shows a greatly abbreviated pelagic phase (Walne 1963). This peculiarity is not reflected at the karyological level. However, *O. puelchana* is the only brooding oyster with a distinct dwarf male and it shows a unique phenomenon of settling the larvae on an expansion of the anterior shell margin (Pascual et al. 1989). These unique morphologic features could be related to the karyological isolation of *O. puelchana*.

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