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Evolution of Parental Care and Ovulation Behavior in Oysters

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Approximately half of all living oysters brood offspring in the inhalant chamber of their mantle cavities; the remainder are broadcast spawners which do not engage in parental care of young. Ostreid ovulation involves a complex behavioral sequence that results in the countercurrent passage of newly spawned eggs through the gills (ctenidia) and into the inhalant chamber. We constructed molecular and combined-evidence phylogenetic trees to test hypotheses concerning the directionality of parental care evolution, and the evolutionary significance of the trans-ctenidial ovulation pathway, in the Ostreidae. Representatives of all three ostreid subfamilies, together with gryphaeid and nonostreoidean pterioid outgroups, were sequenced for a 941-nucleotide fragment of the 28S ribosomal gene. Our phylogenetic analyses indicate that (1) the Ostreidae are robustly monophyletic, (2) broadcast spawning and larval planktotrophy are ancestral ostreid traits, (3) trans-ctenidial ovulation predates the evolution of parental care in ostreid lineages, and (4) brooding originated once in the common ancestor of the Ostreinae/Lophinae, involved a modification of the final behavioral step in the ancestral ovulation pathway, and has been retained in all descendent lineages. Our data permit an independent test of fossil-based ostreid phylogenetic hypotheses and provide novel insights into oyster evolution and systematics.

Key Words: **Mollusca; Ostreidae; 28S rDNA; reproduction; development; phylogeny.**

INTRODUCTION

In this paper, we construct molecular phylogenetic trees to test hypotheses concerning the directionality of parental care evolution, and the evolutionary significance of an atypical ovulation pathway, in a family of oysters. Parental care is a major facet of animal life histories and has long attracted the attention of evolutionary biologists due to its direct effect on fitness (Clutton-Brock, 1991). Marine invertebrates represent a particularly rich source of comparative material for evolutionary studies of life history traits because their buoyant, nondesiccating environment has permitted the evolution of a wide range of reproductive behaviors and developmental modes (Strathmann, 1990; McHugh and Rouse, 1998). Many marine taxa dispense completely with the cares of parenthood and reproduce by broadcasting enormous numbers of unprotected sperm and eggs directly into the water column (Levitan and Petersen, 1995). In contrast, smaller members of numerous benthic taxa localize fertilization at brood sites on/in the bodies of females and retain their young to intermediate or advanced stages of early development (Strathmann and Strathmann, 1982; Olive, 1985). Closely related marine invertebrate lineages often differ markedly in the extent of their parental behavior, e.g., oysters show a gradation of parental care from broadcast spawners to brooding species that release their young either as early planktotrophic larval stages or as advanced larvae capable of immediate metamorphosis (Buroker, 1985).

Phylogenetic studies of reproductively heterogeneous clades may provide insights into the pattern and tempo of parental care evolution and recent studies have revealed multiple parallel changes of reproductive/developmental patterns in diverse marine invertebrate taxa (Emlet, 1990; Reid, 1990; Lieberman *et al.*, 1993; Rouse and Fitzhugh, 1994; Hadfield *et al.*, 1995; Ó Foighil and Smith, 1995; Reid *et al.*, 1996; Wray, 1996; Arndt *et al.*, 1996; Hart *et al.*, 1997; McHugh and Rouse, 1998). Broadcast spawning is generally considered to be the ancestral condition in most reproductively heterogeneous marine invertebrate clades (Franzén, 1956, 1977; Jägersten, 1972) and this interpretation is supported by phylogenetic analyses of some taxa (Emlet, 1990; Lieberman *et al.*, 1993; Wray, 1996; Hart *et al.*, 1997; Ponder and Lindberg, 1997) but not others (Rouse and Fitzhugh, 1994). Strathmann and Eernisse (1994) emphasized the limitations of phylogenetic techniques for inferring ancestral larval traits, especially in cases of significant reductive loss (Strathmann, 1978; Wray and Raff, 1991) in which directional biases may exist in evolutionary transitions (see also Cunningham,

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1999). This is a potentially confounding factor for evolutionary studies of parental care in clades in which presence/absence of this reproductive behavior may be respectively linked with loss/gain of complex larval structures associated with planktivory. However, this factor is not necessarily a major concern in taxa such as oysters, in which an obligate feeding pelagic larval development predominates irrespective of parental care status (Buroker, 1985; Harry, 1985).

The approximately 30 species of the Ostreidae, which make up the bulk of living oysters (Harry, 1985), are sequential hermaphrodites and contain both broadcast spawners (Crassostreinae) and brooders (Lophinae, Ostreinae). Ostreid reproduction is characterized by their aberrant trans-ctenidial (gill) ovulation pathway and by the utilization of the mantle cavity inhalent chamber as a broodsite in species with parental care (Figs. 1a and 1b). Whether spawning sperm or eggs, ostreids release their gametes via gonopores into the exhalent chamber of the mantle cavity. Sperm (spermatozeugmata in brooders [Ó Foighil, 1989], individual cells in nonbrooders) are carried out of the mantle cavity in the exhalent stream. However, eggs are forced, by highly characteristic adductor muscle spawning contractions, in a counter-current direction through dilated ctenidial ostia into the inhalent chamber (Galtsoff, 1932, 1938, 1961, 1964; Nelson and Allison, 1940; Yonge, 1960; Stenzel, 1971; Andrews, 1979). This complex behavior, here termed "trans-ctenidial ovulation" (TCO), involves the coordinated activity of the mantle margins, gills, and adductor muscle and is expressed only while the animals are spawning as females. Broadcast spawners open a small spawning window between inhalent chamber mantle margins during TCO, through which eggs are expelled to the external environment by adductor muscle contractions (Fig. 1a). Brooders retain their eggs in the inhalent chamber in which they are fertilized and held (Fig. 1b) prior to release.

Yonge (1960) and Stenzel (1971) proposed that broadcasting spawning represents the ancestral condition in the Ostreidae, based on the prevalence of this condition in the Bivalvia and on its relative simplicity. According to Yonge (1960), TCO initially evolved as a broadcast-spawning adaptation, maximizing fertilization success by promoting suspension of unfertilized eggs in the water column. Conversely, Andrews (1979) hypothesized that TCO evolved as a brooding adaptation, that parental care represents the ancestral condition in the Ostreidae, and, unlike the generally assumed paradigm (Franzén, 1956, 1977; Jägersten, 1972), that parental care was secondarily lost in the Crassostreinae. Prevalence of parental care in many marine invertebrate taxa, including oysters (Buroker, 1985), is negatively correlated with body size, possibly due to allometric constraints (Strathmann and Strathmann, 1982; Hess, 1993). Crassostreinid oysters are significantly larger than brooding confamilials (Buroker, 1985) and Andrew's (1979) hypothesis implies an association between the proposed evolutionary loss of parental care and increasing ancestral body size.

Although somewhat limited, the available evidence is generally consistent with Andrews' (1979) hypothesis. Brooders occupy a basal position relative to nonbrooding ostreids in a 13-character morphological cladogram (Littlewood, 1994). The single brooder included in Littlewood's (1994) molecular phylogeny of the Crassostreinae is also basal, although this result is necessarily qualified by the use of the distantly related blue mussel *Mytilus edulis* as the sole outgroup. The fossil record is equivocal in that it has been variously interpreted as implying that (1) brooding and broadcast spawning Ostreidae are diphyletic (Hudson and Palmer, 1976), (2) the Ostreidae are monophyletic and brooders (Lophinae) are basal (Stenzel, 1971), and (3) the Ostreidae are monophyletic and nonbrooders are basal (Malchus, 1990, 1995, 1998).

In addition to Littlewood (1994), a number of molecular studies have provided valuable new insights into oyster evolutionary relationships (Banks *et al.*, 1993, 1994; Anderson and Adlard, 1994; Jozefowicz and Ó Foighil, 1998; Ó Foighil *et al.*, 1998, 1999; Boudry *et al.*, 1998). None of these have sampled all three ostreid subfamilies. The aim of this present study was to test competing hypotheses (Yonge, 1960; Stenzel, 1971; Andrews, 1979) for the evolution of parental care in the Ostreidae by mapping reproductive and developmental traits onto phylogenetic tree topologies (Wiley, 1980). Our dataset incorporated representatives of all three ostreid subfamilies together with gryphaeid and nonostreoidean pterioid outgroups (Adamkewicz *et al.*, 1997; Campbell, 1998; Steiner, 1999). We used the same region of domains D1, D2, and D3 of the large-subunit nuclear rRNA gene (28S) employed for Littlewood's (1994) study of the Crassostreinae. Our results do not support Andrews' (1979) hypothesis and provide novel insights into ostreoidean evolution.

MATERIALS AND METHODS

With two exceptions, original sequences were generated for all of the study taxa and these have been deposited in GenBank (AF137032–52). We experienced difficulty in obtaining reliably identified specimens of *Saccostrea* and used previously published (Littlewood, 1994) 28S sequences for *S. commercialis* and *S. cucullata*. Details of sampling locality data and voucher specimen information for the brooding taxa [Lophinae (four species) and Ostreinae (seven species)] are available in Jozefowicz and Ó Foighil (1998). For the remaining study taxa, this information is presented in Table 1.

Apart from two commercially produced samples, all specimens were received from sampling sources fixed in 95% ethanol. Species identification was initially determined by the respective collector and confirmed upon

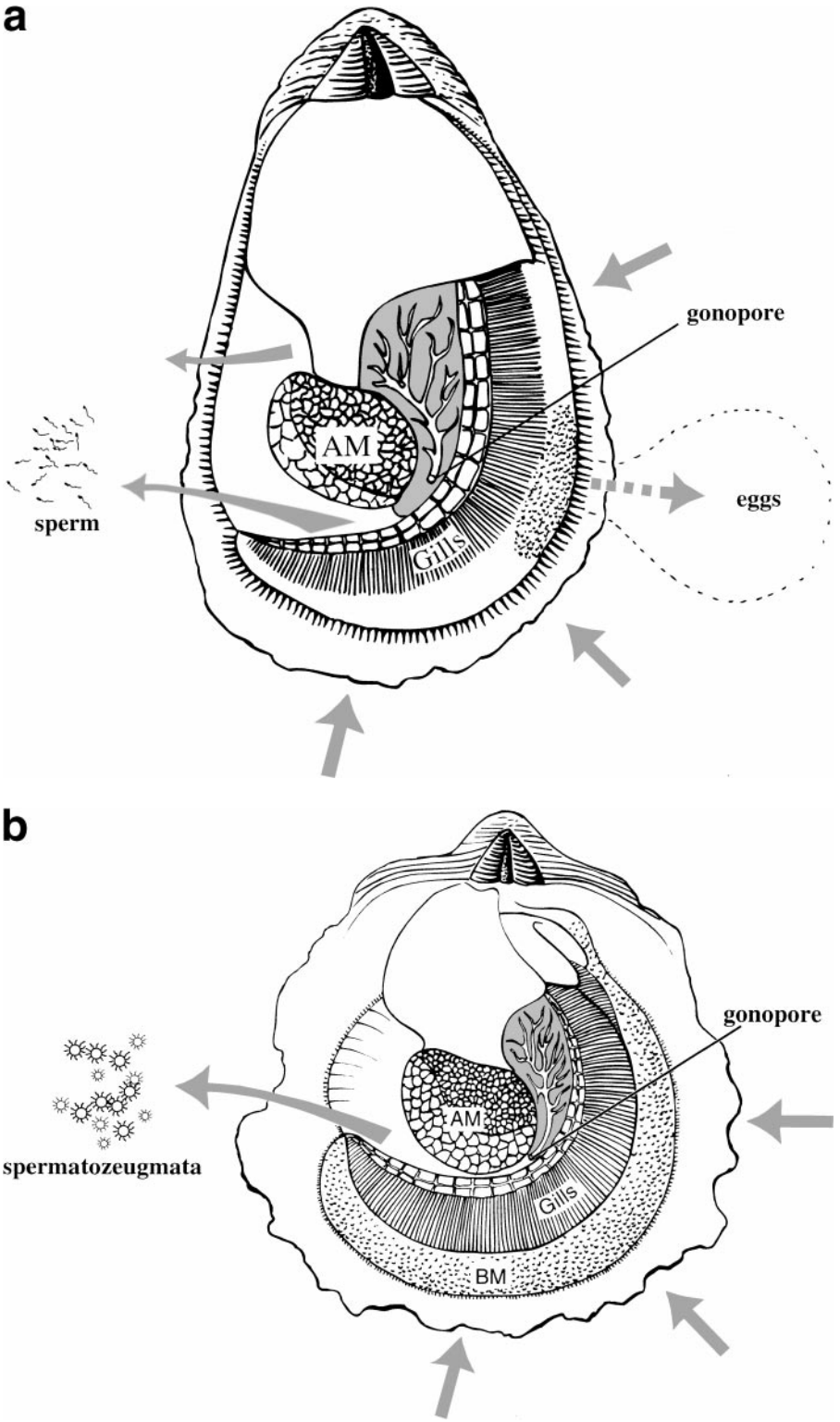

FIG. 1. Summary diagram showing spawning pathways for sperm and eggs in nonbrooding (a) and brooding (b) ostreids depicted with the right valve and mantle fold excised (after Yonge, 1960). Water flow through the mantle cavity is indicated by the unbroken arrows and sperm (not to scale) exit in the exhalent flow. Eggs are passaged through the gills by adductor muscle (AM) contractions and into the inhalent chamber. They are expelled to the exterior in nonbrooders (broken arrow; a) but retained to form a brood mass (BM; b) in species with parental care.

TABLE 1

Locality Data and Voucher Specimen Information (Mollusc Division Catalog Number, University of Michigan Museum of Zoology) for the Nonbrooding Taxa Sequenced for This Study

Species	Collection locality	Collector	UMMZ catalog no.
Superfamily Ostreoidea			
Family Gryphaeidae			
Subfamily Pycnodonteinae			
Hyotissa hyotis (Linné, 1758)	Guam	G. Paulay	265995
Parahyotissa numisma (Lamarck, 1819)	Guam	G. Paulay	265996
Neopycnodonte cochlear (Poli, 1795)	Maui, HI, USA	G. Paulay	265997
Family Ostreidae			
Subfamily Crassostreinae			
Crassostrea ariakensis (Fujita, 1913)	Hatchery stock, Haskin Shellfish Research Lab., NJ, USA	S. Allen	265991
Crassostrea gigas (Thunberg, 1793)	WA commercial product, USA	—	—
Crassostrea rhizophorae (Guilding, 1827)	Twin Cays, Belize	E. Duffy	265992
	Chengue Bay, Colombia	F. Borrero	265993
Crassostrea virginica (Gmelin, 1791)	P.E.I. commercial product, Canada	—	—
	Chesapeake Bay DL, USA	P. Gaffney	—
Striostrea margaritacea (Lamarck, 1819)	Port Alfred, South Africa	A. Hodgson	265994
Superfamily Pterioidea			
Pinctada imbricata Roeding, 1798	Lower Florida Keys, USA	D. O'Foighil	265998
Isognomon alata (Gmelin, 1791)	Lower Florida Keys, USA	D. O'Foighil	265999

Note. See Jozefowicz and Ó Foighil (1998) for the equivalent information for the brooding ostreid taxa: Lophinae (*Dendrostrea frons, D. folium, Lopha cristagalli, Alectryonella plicatula*); Ostreinae (*Ostrea edulis, O. angasi, O. chilensis, O. denselamellosa, O. conchaphila, O. puelchana, O. algoensis*).

receipt by the first author. See Park and Ó Foighil (2000) for details of DNA extraction, PCR conditions, and the primers used to amplify (D1F and D6R) and to sequence (D1F, D23F, D4CF, D6R, D4RB, and D24R) the target 28S gene fragment and their respective annealing sites. A negative control (no template) was included in each amplification run. Double-stranded products were isolated on 1% agarose gels, excised under long-wavelength UV light, and extracted using a GeneClean (Bio 101) NaI/glass powder kit. Both strands of the amplified fragments were directly cycle-sequenced using an ABI Big Dye kit and sequencing reaction products were electrophoresed on an ABI 377 automated DNA sequencer.

Initial alignments were constructed with the default parameters in Clustal W (Thompson *et al.,* 1994) and then manually adjusted to minimize mismatches. Best trees were assessed with three optimality criteria in PAUP* 4.0b2: maximum-parsimony (MP), genetic distance, and maximum-likelihood (ML). We used the branch-and-bound algorithm to search for optimal trees under unweighted MP. Inferred sequence gaps were either considered to be additional character states or removed from the analyses. Branch support levels were estimated using nonparametric bootstrapping (500 heuristic iterations with random stepwise addition with 10 replications) and Bremer support (decay index) values (Bremer, 1995). The TreeRot program (Sorenson, 1996) was used to establish a constraint statement for each node in the strict consensus tree. Support indices were calculated by subtracting the number of steps in the shortest unconstrained tree from the number of steps found in each of the constrained searches. Alternative topological hypotheses were tested using the Kishino–Hasegawa test. In model fitting for the ML analysis, we minimized arbitrariness and the inclusion of superfluous parameters by conducting a series of likelihood ratio tests (LRTs) of nested models (Huelsenbeck and Rannala, 1997). This approach aims to include only those parameters that significantly increase the likelihood of the resulting tree. Rate heterogeneity parameters were added to the model both before and after changing the number of substitutional classes from two to six (Cunningham *et al.,* 1998). PAUP* 4.0b2a was also employed to perform heuristic distance analyses using Kimura two-parameter-corrected distance matrices.

RESULTS

Littlewood (1994) published homologous 28S sequences obtained from cloned PCR-amplified products for seven crassostreinid and one ostreinid species and our data set incorporated his sequence for two crassostreinid taxa (*Saccostrea commercialis* and *S. cucullata*). Cloned PCR products generated by *Taq* polymerase typically contain a small fraction of errors (0.2–2×10^{-4} per bp per cycle) produced during amplification (Bracho *et al.,* 1998) and we directly sequenced the target gene fragment for five of Littlewood's (1994) taxa

which were available to us [*Crassostrea gigas, C. ariakensis* (=*C. rivularis*), *C. virginica, C. rhizophorae,* and *Ostrea edulis*] to confirm the published sequences. In three of these cases, only very minor discrepancies were found; however, replicate samples of the two northwestern Atlantic crassostreinid taxa (*C. virginica* and *C. rhizophorae*) from divergent parts of their respective ranges (Table 1) yielded species-specific genotypes that differed at >80 positions from the published sequences (Littlewood, 1994). Independent RFLP analyses of a 796-nt portion of the target 28S gene fragment for *C. virginica* and *C. rhizophorae* population samples produced multiple restriction profiles consistent with our sequences but not with the published data (P. Gaffney, University of Delaware, pers. comm.), indicating that our sequences for these taxa are likely to be accurate.

Of the 941 positions in the aligned data set (including inferred gaps), 542 are constant and 318 are informative under conditions of parsimony. A highly skewed distribution ($g_1 = -1.5178$ with gaps treated as missing and $g_1 = -1.50$ with all gapped sites removed) was obtained for 10^5 randomly sampled trees generated in PAUP, indicating that significant ($P < 0.01$) cladistic information exists in the data set (Hillis and Huelsenbeck, 1992). Likelihood mapping (Strimmer and von Haeseler, 1997) also provided an *a priori* indication of the signal in this alignment. This graphical tool plots likelihood quartets from a test set onto basins of attraction of which there are three types—star-like, net-like, and tree-like. Our data showed 86.8% in the tree-like areas and only 8% in the star-like areas, indicating a moderately strong signal and potential for tree resolution. The hierarchical model fitting analysis yielded a parameter-rich model (Table 2). The optimal model (−lnL = 5034.97) had six substitution types ($R_a = 0.6782$, $R_b = 1.248$, $R_c = 0.7964$, $R_d = 0.5671$, $R_e = 4.0647$) and among-site rate heterogeneity (invariable sites = 0.3455 and gamma approximation with four rate categories = 0.56). Adding among-site rate heterogeneity before adding the four extra substitution types (i.e., HKY + inv + Γ) gave an inferior model (−lnL = 5069.40).

Figure 2 shows the single most-parsimonious tree (923 steps, CI = 0.6901, RI = 0.7727) obtained by heuristic PAUP analyses of the 28S data set, including inferred gaps, and utilizing the pterioidean taxa *Pinctada* and *Isognomon* as outgroups. Robust support is evident for reciprocal monophyly of the pycnodonteinid Gryphaeidae and the Ostreidae and (bearing in mind the limited outgroup sampling) for monophyly of the superfamily Ostreoidea. The broadcast spawning Crassostreinae are paraphyletic and the northwestern Atlantic species (*C. virginica* and *C. rhizophorae*) occupy a weakly supported basal position within the Ostreidae. A clade is formed by the remaining crassostreinids within which the three Indo-Pacific taxa (*Striostrea margaritacea, Saccostrea cucullata,* and *Saccostrea commercialis*) co-cluster. Brooding oysters form a well-supported (Decay Index = 7) clade; however, the two constituent subfamilies are not reciprocally monophyletic: the South African ostreinid *Ostrea algoensis* is sister to the lophinid taxa. *Dendostrea frons* has a basal placement within the lophinid clade and the two *Dendostrea* species are not sister taxa. The only known oyster species lacking an extended pelagic feeding larval development, *Ostrea chilensis,* is nested among other ostreinids in a derived position and forms a well-supported clade with *O. edulis* and *O. angasi.*

In all of the phylogenetic analyses, the brooding taxa remained monophyletic and in a derived position within a well-supported ostreid clade. In contrast, the basal-branching orders recovered for the crassostreinid taxa were sensitive to the phylogenetic method employed and the only constant was the sister relationship of *Striostrea margaritacea* to the two *Saccostrea* species (all members of the Indo-Pacific tribe Striostreini). When inferred sequence gaps were coded as "missing" characters for unweighted maximum-parsimony analyses, a single most-parsimonious tree (795 steps,

TABLE 2

Model Fitting of Oyster 28S Sequences Using Likelihood Ratio Tests (Huelsenbeck and Crandall, 1997)

Null hypothesis	Models	$-\ln L_0$	$-\ln L_1$	$-2 \ln \lambda$	*df*	*P*
Equal base frequencies	H_0: JC69 H_1: F81	5518.15	5495.16	45.98	3	<0.000001
Equal ti/tv rates	H_0: F81 H_1: HKY85	5495.16	5411.30	167.72	1	<0.000001
Equal ti and equal tv rates	H_0: HKY85 H_1: GTR	5411.30	5356.73	109.14	3	<0.000001
Equal rates among sites	H_0: GTR H_1: GTR+ Γ	5356.73	5086.42	540.62	1	<0.000001
Proportion of invariable sites	H_0: GTR+ Γ H_1: GTR+ Γ + invar	5086.42	5034.97	102.90	1	<0.001402

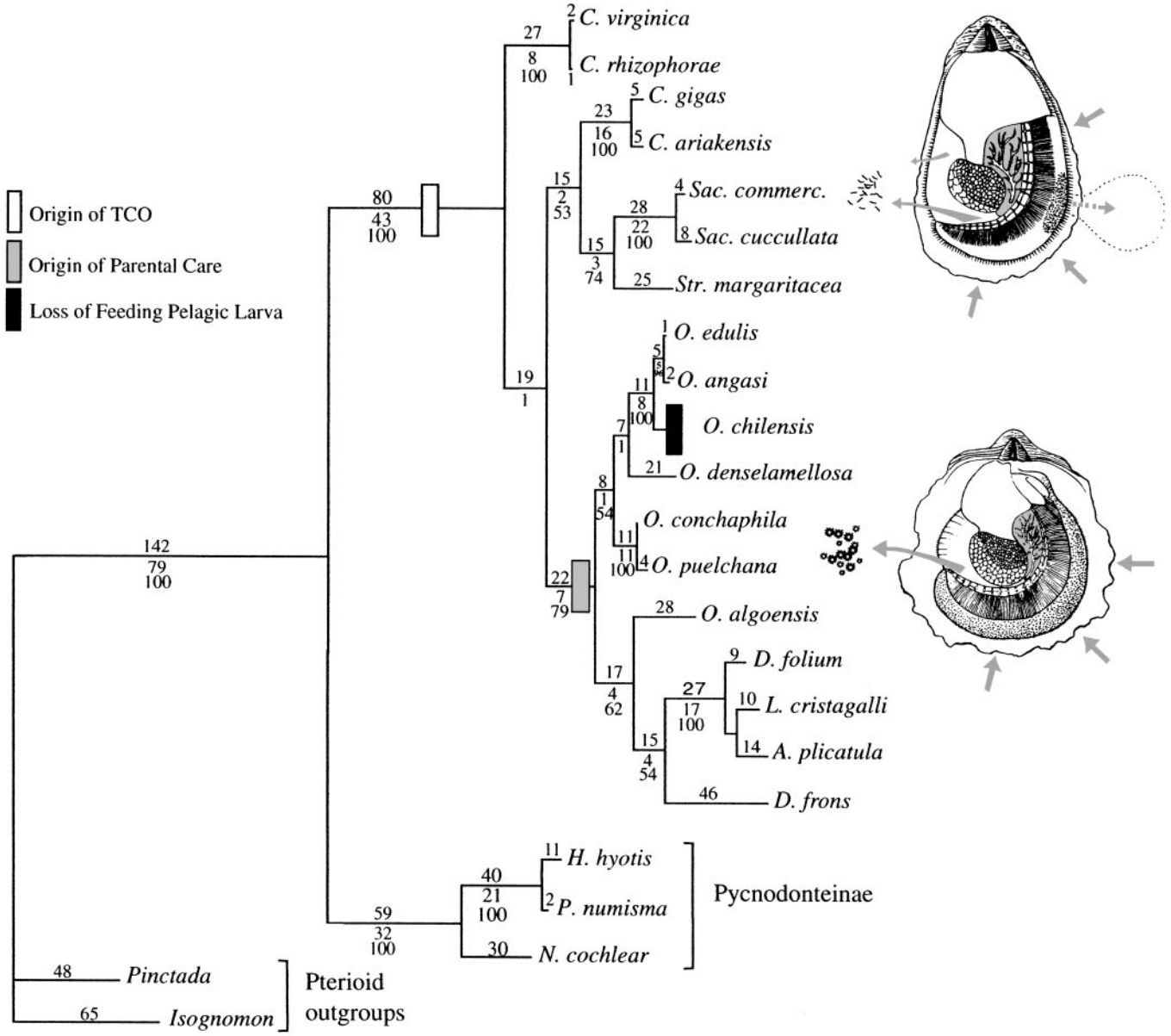

FIG. 2. The single most-parsimonious tree (923 steps, CI = 0.6901, RI = 0.7727) obtained by heuristic unweighted searches of the 28S genotypes (including inferred gaps) for the 21 oyster study taxa with the two pterioids, *Pinctada* and *Isognomon*, designated as outgroups. The pycnodonteinid gryphaeid oyster species sampled cluster separately from the three ostreid subfamilies. The crassostreinid taxa are aligned with the upper drawing showing a broadcast-spawning ostreid. A lower drawing of a brooding ostreid is placed adjacent to the parental care clade, which contains ostreinid (*O. edulis–O. algoensis*) and lophinid (*D. folium–D. frons*) taxa. The respective numbers of steps are indicated above each branch and the decay index and bootstrap values (if >50) supporting each node are presented below the branches. TCO, trans-ctenidial ovulation.

CI = 0.6717, RI = 0.7568) was obtained (not shown) in which the crassostreinid oysters now formed three paraphyletic clusters with the Indo-Pacific taxa being placed between the basal northwestern Atlantic species and the two Asian species. Heuristic genetic distance analysis (Fig. 3) yielded a crassostreinid topology congruent with that of Fig. 2, whereas maximum-likelihood analysis (Fig. 4) placed the Striostreini in a basal position and the four *Crassostrea* species formed a clade. When gryphaeid outgroups were used, the tree topologies generated were unchanged from those depicted in Figs. 2–4, except that the four *Crassostrea* species formed a clade (bootstrap value = 62) in the genetic distance analysis (not shown). The instability of basal crassostreinid branching patterns obtained under different models of phylogenetic analysis indicate that there is insufficient phylogenetic signal in the 28S dataset to robustly resolve the interrelationships of the

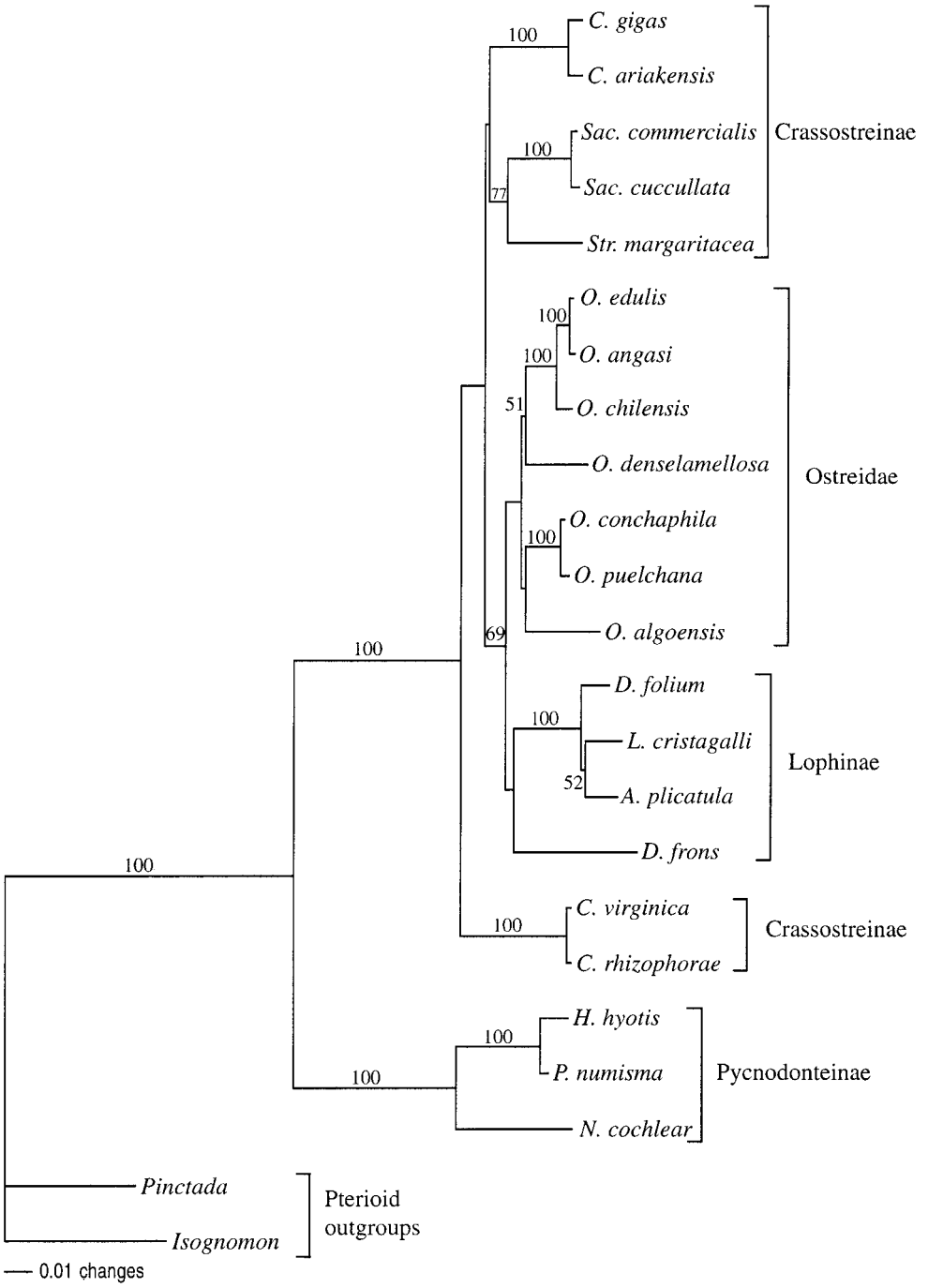

FIG. 3. Genetic distance tree obtained by a heuristic search of the oyster 28S (Kimura two-parameter-corrected) distance matrix with the two pterioids, *Pinctada* and *Isognomon*, designated as outgroups. Numbers above branches indicate percentage bootstrap values higher than 50%.

Asian Crassostreini, American Crassostreini, and Indo-Pacific Striostreini sampled for this study. Branching orders within the brooding clade were considerably less mutable and the only rearrangements of note occurred in genetic distance trees. In heuristic distance searches (Fig. 3), the two brooding subfamilies were reciprocally monophyletic [unlike parsimony and maximum-likelihood analyses (Figs. 2 and 4)], whereas in trees produced by neighbor-joining analyses (not shown), the lophinid *Dendostrea frons* was positioned basally (bootstrap value <50) within the brooding clade.

The MP searches were constrained to test whether historically proposed oyster relationships gave tree lengths that were significantly longer (Kishino–-

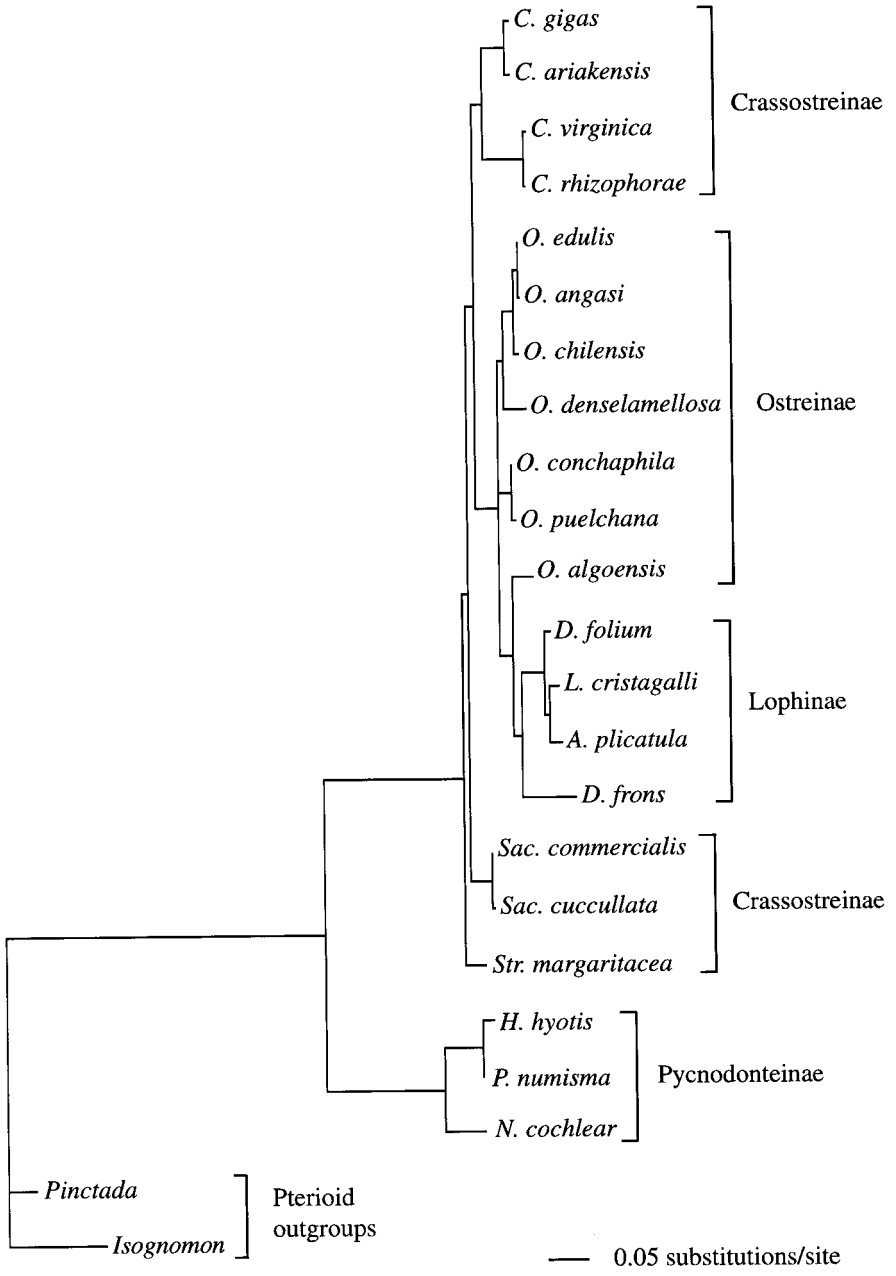

FIG. 4. Maximum-likelihood tree of oyster 28S rDNA sequences based on the GTR + invariant sites + Γ optimal model (lnL = −5007.68) obtained using the two pterioids, *Pinctada* and *Isognomon*, as designated outgroups.

Hasegawa tests) than those of the best tree. These tests are quite conservative (Halanych, 1998) and, although it entailed the addition of 7 extra steps, placing the nonbrooding crassostreinid taxa in a derived position (Andrews, 1979) within the clade of brooding Ostreidae [(((Ostreinae/Lophinae, (Crassostreinae)), Pycnodonteinae), pterioid outgroups)] did not yield a significantly longer tree (length = 930; difference = 7, $t = 1.15$, $P = 0.2500$). Forcing a sister relationship for the *Saccostrea* species and northwestern Atlantic *Crassostrea* species (Littlewood, 1994) did result in a significantly longer tree (length = 984; difference = 61, $t = 6.19$, $P < 0.0001$). Moving *Ostrea chilensis* to a basal position among the Ostreoidea (Chanley and Dinamani, 1980) also formed significantly longer trees (length = 998; difference = 75, $t = 8.43$, $P < 0.0001$). Placing the pycnodonteinid taxa sister to the cupped oysters (Hudson and Palmer, 1976) resulted in a tree-length that was 48 steps longer than the most-parsimonious unconstrained tree (length = 971; difference = 48, $t = 6.21$, $P < 0.0001$). Finally, constraining the ostreid clade in accordance with Stenzel's (1971)

interpretation of the appearance of subfamilies in the fossil record, ((Lophinae) ((Crassostreinae) (Ostreinae))), yielded two longer best trees of 936 steps (length = 936; difference = 13, $t = 1.99$, $P < 0.0474$ and length = 936; difference = 13, $t = 1.9$, $P = 0.0579$).

Littlewood (1994) coded 13 morphological characters, determined by Harry (1985), for oyster families and subfamilies. We added this morphological matrix to our ostreoidean molecular data and performed a combined unweighted maximum-parsimony analysis (953 characters of which 268 are parsimoniously informative) in which gaps were regarded as a "fifth base" and the gryphaeid taxa were employed as outgroups. This yielded the single most-parsimonious tree (693 steps, CI = 0.6753, RI 0.7805) depicted in Fig. 5. Topological relationships within the brooding clade were consistent with those produced by maximum-parsimony analysis of the molecular data alone (Fig. 2). Although basal nodes for the broadcast spawners were weakly supported (Decay Indices = 1), the combined analysis differed from that of the molecular data alone in that it yielded monophyletic Crassostreinae, Crassostreini (*Crassostrea* spp.), and Striostreini (*Saccostrea* and *Striostrea* spp.).

DISCUSSION

As depicted in Fig. 2, placement of reproductive/developmental characters on our molecular and combined analysis trees consistently indicates that a combination of trans-ctenidial ovulation, broadcast spawning, and planktotrophic larval development is the plesiomorphic condition in the Ostreidae. We therefore reject Andrew's (1979) hypothesis that parental care is an ancestral trait in this family and that TCO evolved as a brooding adaptation. A number of important caveats attend this conclusion. It hinges, of course, on outgroup comparisons and four of the five proposed nonostreid oyster subfamilies, together with the presumed stem ostreid subfamily Liostreinae (Malchus, 1990), are extinct and could not be included in the study. Although parental care does not occur in the pycnodonteinid Gryphaeidae (Stenzel, 1971; Galtsoff, 1964; Waller, 1998), their ovulation pathway(s) remain to be established so that the possibility that TCO is plesiomorphic for the Ostreoidea cannot be ruled out. Finally, our dataset is not exhaustive, although we sampled representatives of the widely recognized primary subgroupings among the extant Ostreoidea.

Despite these caveats, the available, phylogenetically tractable evidence indicates that parental care evolved once in the Ostreidae in the common ancestor of the Lophinae/Ostreinae and has been retained in all descendent lineages. A striking feature of ostreid parental care evolution is its apparent conservatism. Transctenidial ovulation is an ostreid fixed action pattern that differs among brooders and broadcasters only in the final step of the behavioral sequence: retention or expulsion of eggs from the mantle cavity. A relatively minor modification of this behavioral step should be sufficient to initiate a transition in parental care status. Yet, our data suggest that only one such transition has occurred during the evolutionary history of the extant Ostreidae, which stems at least from the Eocene (Malchus, 1990). This restrained evolutionary tempo contrasts markedly with those of other reproductively heterogeneous clades, such as asterinid seastars, in which multiple independent transitions in parental care status have occurred within the past 2 million years (Hart *et al.*, 1997).

Apart from gaining parental care in the common ancestor of the Ostreinae/Lophinae, the only other major reproductive/developmental novelty among extant brooding lineages has been the loss of an obligate pelagic feeding larval phase in *Ostrea chilensis*. This ontogenetic change is associated with a greatly modified larval shell morphology (Chanley and Dinamani, 1980) and a loss of postoral velar ciliature (Chaparro *et al.*, 1999). Chanley and Dinamani (1980) proposed that the "nonostreid" larval shell morphology of *O. chilensis* results from a retention of primitive larval characters. However, our 28S data concur with mitochondrial gene trees (Jozefowicz and Ó Foighil, 1998) that place *O. chilensis* sister to *O. edulis* and *O. angasi* and indicate that loss of an extended feeding larval phase is a derived condition among brooding oysters.

Extinct fossil oyster lineages form a significant component of ostreoidean phylogenetic reconstructions (Stenzel, 1971; Hudson and Palmer, 1976; Torigoe, 1981; Freneix, 1982; Malchus, 1990, 1995, 1998; Carter, 1990; Lawrence, 1995; Waller, 1998), although Cleevley and Morris (1987) advised that they are "probably the most difficult single group of macrofossils to classify." Our phylogenetic trees are necessarily restricted to extant taxa and lack the extensive pteriomorph sampling required to address the outstanding issue of ostreoidean monophyly (Waller, 1998). Nevertheless, our trees have relevance to some of these paleontological studies. For instance, Hudson and Palmer (1976) inferred from a middle Jurassic fossil taxon that the Ostreidae are diphyletic and that the nonbrooding Crassostreinae are descendants of gryphaeid lineages. Our ostreid clade is robustly monophyletic and, given the necessary sampling constraints (only pycnodonteinid gryphaeids are extant), this result is consistent with reciprocal monophyly of the Gryphaeidae and the Ostreidae (Stenzel, 1971; Malchus, 1990, 1995; Waller, 1998).

Stenzel (1971) and Malchus (1990, 1995, 1998) expressed divergent views on the phylogeny and composition of the brooding subfamily Lophinae. According to Stenzel (1971), the Lophinae first appeared in the Upper Triassic, much earlier than the other two extant ostreid subfamilies. Malchus (1990) identified novel

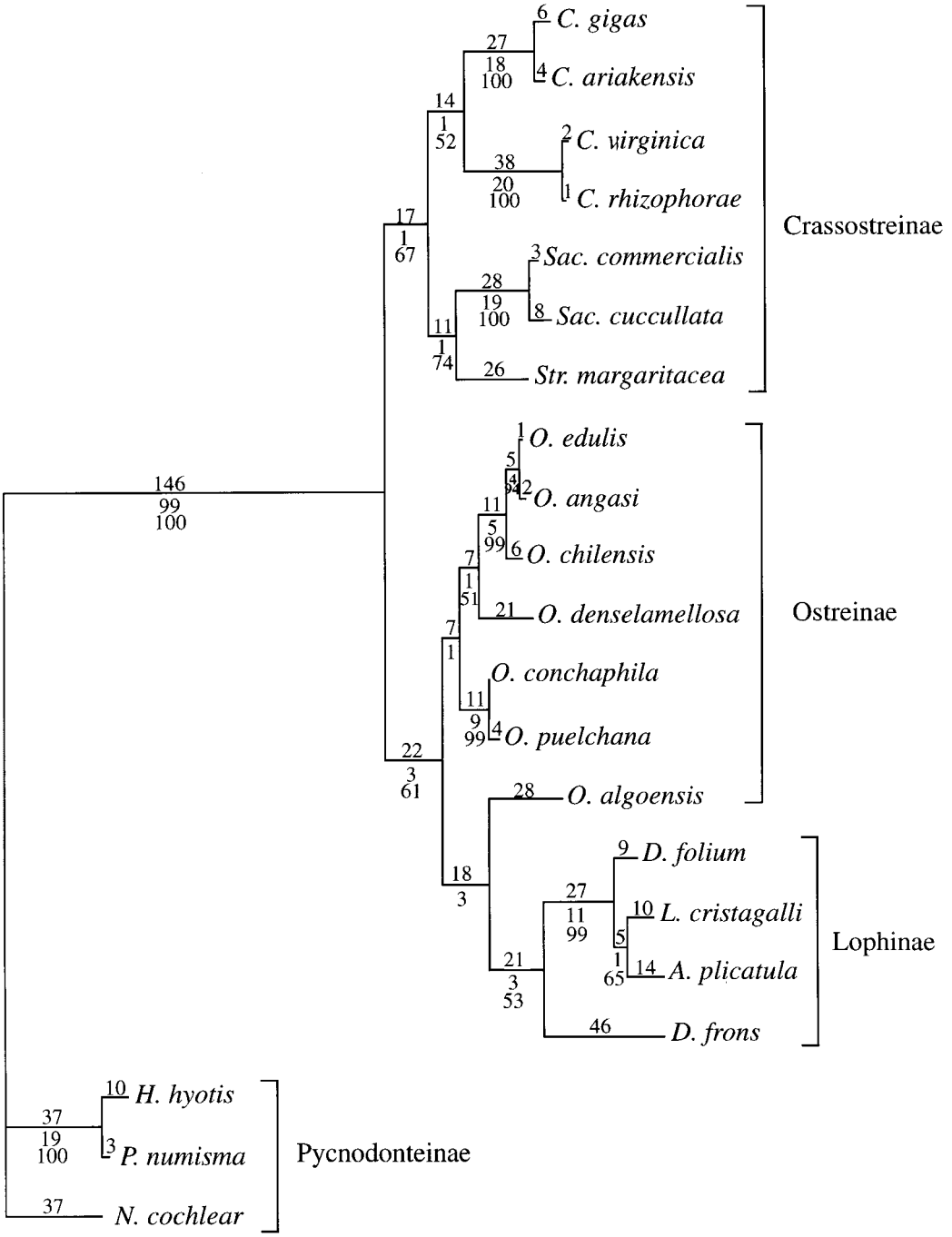

FIG. 5. The single most-parsimonious tree (693 steps, CI = 0.6753, RI 0.7805) obtained by a combined evidence heuristic analysis of 21 oyster 28S genotypes (Fig. 2) together with Littlewood's (1994) morphological matrix. All three gryphaeid taxa (*Hyotissa hyotis*, *Parahyotissa numisma*, and *Neopycnodonte cochlear*) were designated as outgroups. The respective number of steps are indicated above each branch and the decay index and bootstrap values (if >50) supporting each node are presented below the branches.

shell microstructure characters and placed the Mesozoic "*Lopha*-like" oysters in a separate family (the Paleolophidae), considering them to be phylogenetically distinct from the modern Lophinae. According to Malchus (1990, 1998), the first appearance of the Lophinae in the fossil record may have occurred as late as the Miocene. Littlewood's (1994) cladistic analysis of oysters, based on 13 morphological characters, yielded a topology congruent with Stenzel's (1971) interpretation, except that it did not recover a monophyletic Ostreidae. In contrast, our molecular trees (Figs. 2–4), which employ nonostreoidean outgroups, find a ro-

bustly monophyletic Ostreidae and are broadly congruent with Malchus (1990, 1995, 1998) in consistently placing the Crassostreinae basally and the brooding taxa (including the Lophinae) in a derived position. A number of other molecular and anatomical features of extant ostreids may also be consistent with a later origin for brooding lineages within the ostreid clade. For a homologous 450-nt fragment of the mt 16S RNA gene, Asian and American species of *Crassostrea* differ by 15–16% (Ó Foighil *et al.*, 1995), whereas pairwise distances among the entire clade of brooding ostreids are less than 10% (Jozefowicz and Ó Foighil, 1998). In addition, Stenzel (1971) identified as plesiomorphic a number of characters shared exclusively among extant ostreoideans by crassostreinids and gryphaeids: presence of a promyal passage (indicated by the top exhalent stream in Fig. 1a) and a nonincubatory mode of reproduction.

Our phylogenetic trees are very pertinent to systematic studies of extant taxa. Harry (1985) employed both shell and soft part characters in the most recent comprehensive reclassification of living oysters. His radical taxonomic rearrangement of the two brooding ostreid subfamilies proved to be largely incongruent with subsequent mitochondrial 16S gene trees based on 14 ostreinid and lophinid species (Jozefowicz and Ó Foighil, 1998). Although fewer brooding taxa were included in this present study, and 28S evolves at a much slower rate than 16S (Hillis and Dixon, 1991), many topological elements are common to both 28S and 16S trees and are at odds with Harry's (1985) conclusions. These include the phylogenetic distinctiveness of regional southern hemisphere flat oyster lineages, the clade ((*Ostrea chilensis*) (*O. edulis, O. angasi*)), the paraphyletic position of the ostreinid *O. algoensis*, and the paraphyly of the lophinid genus *Dendostrea*.

The nonbrooding oysters yielded tree topologies that were more consistent with Harry's (1985) taxonomic conclusions. We obtained comprehensive support for the Pycndonteinae and its constituent taxa: the Hyotissini (*Hyotissa hyotis, Parahyotissa numisma*) and the Neopycnodontini (*Neopycnodonte cochlear*). Combined data analysis (Fig. 5) yielded monophyletic Crassostreinae, Crassostreini (Asian and Atlantic *Crassostrea* species), and Striosteini (*Saccostrea commercialis, Saccostrea cucullata* and *Striostrea margaritacea*). This topology was obtained from molecular characters alone only in distance analyses employing pycnodonteinid outgroups (not shown), although a monophyletic Crassostreini was also recovered by maximum-likelihood analysis (Fig. 4). We could not replicate Littlewood's (1994) finding of a robust sister relationship among the *Saccostrea* taxa and northwestern Atlantic *Crassostrea* lineages.

Phylogenetic studies of reproductively heterogeneous marine invertebrate radiations are providing numerous empirical case histories detailing evolutionary transitions in life history attributes (Strathmann and Eernisse, 1994; McHugh and Rouse, 1988). Our ostreid results, demonstrating that broadcast spawning and an obligate feeding pelagic larva are plesiomorphic conditions, are consistent with the overall evolutionary trends revealed by most (but not all) such studies on diverse marine invertebrate taxa. Clear distinctions in the tempo of such evolutionary transitions are apparent among taxa, even when the trends are parallel, e.g., oysters and asterinid seastars (Hart *et al.*, 1997). Such diversity reflects the unique evolutionary histories experienced by diverse marine invertebrate lineages and is likely to be a prominent feature of this particular field of study.

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