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Using micropylar ultrastructure for species identification and phylogenetic inference among four species of Sparidae

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Among *Sparus sarba*, *Acanthopagrus latus*, *A. schlegeli* and *Pagrus major*, *P. major* had the largest egg size, with the biggest micropyle funnel and the most numerous accessory openings. The reinforcement in the micropyle canals was species specific with eight spiral clockwise, five two-spiral clockwise, seven two-spiral clockwise, and 10 triangular ridges in *S. sarba*, *A. latus*, *A. schlegeli*, and *P. major*, respectively. A key to identify these four species based on micropyle characters is proposed for future applications. Cladistic analysis by using different parsimonious methods on the morphological character of the micropyle suggested that the generic interrelationship between *Sparus* and *Acanthopagrus* was closer than to the genus *Pagrus*. Furthermore, the congeneric species of *A. latus* and *A. schlegeli* were the most closely related, with *S. sarba* the second, and *P. major* the last. This result agreed with conclusions obtained from other character suites including morphological, biochemical and molecular data.

Key words: eggs; Sparidae; micropylar; ultrastructure; identification; phylogenetic relationship.

INTRODUCTION

In teleostean fishes, the comparison of the ultrastructures of egg membrane surfaces and micropyles using electronic microscopy for species identification was attempted quite early (Riehl & Schulte, 1978). Nevertheless, because of some practical reasons, most fish egg identification still uses traditional light microscopy. Characters that can be examined by light microscope include egg size (Ahlstrom & Moser, 1980), shape (Ahlstrom, 1984), oil droplet (Ahlstrom & Moser, 1980), pigment (Mikulin, 1981), hatching opening, chorion (Matarese & Sandknop, 1984), and egg surface ornamentations (Ahlstrom, 1984), etc. However, the number of these characters is still insufficient for further species or genus identification so that phylogenetic inferences have been very limited. Therefore, additional features obtained from scanning electron microscopy (SEM) would be very helpful in this regard. In fact, some previous papers have already reported that the microstructure of the micropyle contains a group of very useful taxonomic characters. These papers include studies of *Oncorhynchus mykiss* (Walbaum) and *Salmo trutta lacustris* L. (Riehl, 1980), *Engraulis japonica* (Schlegel) (Hirai & Yamamoto, 1986), *Saurida elongata* Temminck and Schlegel (Hirai, 1988), *Anarhichas lupus marisalbi* (Linnaeus) (Dzerzhinskiy *et al.*, 1992), *Oncorhynchus keta* (Walbaum) (Yamamoto & Kobayashi, 1992), Pleuronectinae

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(Hirai, 1993), and Luciocephalidae (Riehl & Koleoscha, 1993). Another paper examined the ultrastructure of the egg membrane in addition to using the micropyle for phylogenetic comparisons in studying the four genera of Anabantoidae (Britz *et al*., 1995). Although there are not many papers using ultrastructure of fish eggs for systematic studies, the potential of using this character suite for phylogenetic inferences as well as congruences with phylogenies reconstructed from other types of characters is still very promising and needs to be evaluated further.

For taxonomic purposes, the ultrastructure of the egg membrane usually can be applied to distantly related species, but not to closely related species, because they look too similar to each other. For example, flatfish have polygonal walls on the chlorion membrane surface (Sumida *et al*., 1979); the egg surface of *Oryzias latipes* (Temminck & Schlegel) has microvilli and attaching filaments (Yamamoto & Yamagami, 1975); and that of eels (Anguillidae) has central depressed knobs on the surface (Ohta & Iwamatsu, 1983), etc. As to the thickness of the membrane, although there could be differences among different kinds of fish, these might be affected by ecological environments and developmental stages (Ivankov & Kurdyayeva, 1973; Riehl, 1978). On the other hand, the micropyle plays an important role in gamete recognition during fertilization, so its morphology may be species specific (Ginzburg, 1968; Kobayashi & Yamamoto, 1981). As to the number of micropyles, most fish species have only one, but a few species may have more. For example, the sea wolf fish can have one to five micropyles (Dzerzhinskiy *et al*., 1992). In ultrastructural features, there are three kinds of micropylar canals: a cylindrical canal as in *Gadus morhua marisalbi* L.; conical canal as in *Mugil cephalus* L.; and funnels as in *Eleginus navaga* Pallas (Mikodina, 1987). During fertilization, although many sperm swim to the vicinity of the micropylar opening, only one sperm is allowed to enter the micropyle (Kobayashi & Yamamoto, 1981). When the sperm head goes through the micropyle and reaches the egg inner membrane, then fertilization is complete (Brummett & Dumont, 1979). During this time, the inner part of the micropylar canal becomes smaller and narrower, and the microvilli and plug-like deposit quickly form on the miropyle to block possible polyspermy (Kudo, 1980; Iwamatsu & Ohta, 1981; Kobayashi & Yamamoto, 1981; Ohta & Iwamatsu, 1983; Hart, 1990). The closure time of the micropyle is very short, usually less than half an hour, and its morphological changes are difficult to categorize and measure for taxonomic use. Thus, only unfertilized eggs were used in this study.

All previous taxonomic reports using the egg as a character seemed to ignore this problem since they did not mention whether eggs had been fertilized or not. Thus, the present study compares the morphology of the micropyle only before fertilization, not after fertilization, to ensure that the results are more accurate.

Sea breams (family: Sparidae) contain five sub-families (Sparinae, Denticinae, Pagellinae, Boopsinae, and Pagrinae), 29 genera, and *c.* 100 species in the world (Smith & Heemstra, 1986; Hayashi, 1993; Nelson, 1994). In Taiwan, three sub-families, six genera, and 10 species have been recorded so far. They are *Dentex tumifrons* Temminck & Schlegel of Denticinae; *Argyrops bleekeri* Swainson, *A. spinfer* (Forsskål), *Evynnis cardinalis* Lacépède, and *Pagrus major*

Temminck & Schlegel of Pagrinae; and *Acanthopagrus australis* Günther, *A. berda* (Forsskål), *A. latus* (Houttuyn), *A. schlegeli* (Bleeker), and *Sparus sarba* Forsskål of Sparinae (Jean *et al.*, 1992; Shen *et al.*, 1993). Among the above 10 Taiwanese species, *P. major* of Pagrinae and the last three species of Sparinae have been artificially bred successfully in Taiwan, but only the four species of *P. major*, *A. latus*, *A. schlegeli*, and *S. sarba,* representing three genera and two sub-families, are actually cultured in brackish-water ponds or net cages along the shore. Therefore, fresh eggs of all different development stages before or after fertilization can be collected for systematic studies. Phylogenetic relationships at the level of species, genus, or even sub-family can also be tested to determine whether they are congruent or not by using the characters of egg ultrastructure against the phylogenies recently obtained from morphological characters, 35 isozymes, and mtDNA sequences in the D-loop (phe-transfer RNA and partial 12 sRNA region) of five species of Sparinae (Jean *et al.*, 1992, 1995*a*,*b*). The two species of Sparinae, *A. australis* and *A. berda*, used in Jean *et al.*'s studies were not included in this study because egg material could not be obtained as they are not often reared in Taiwan due to the rarity of parent individuals caught from the wild, as well as a smaller body size, slower growth rate, and higher mortality compared to the other four species used in this study.

MATERIALS AND METHODS

Eggs of *S. sarba* and *P. major* were collected from the Penghu Branch of the Taiwan Fisheries Research Institute (TFRI), and of *A. latus* and *A. schlegeli* from the Tainan Branch and Taitung Branch of TFRI. These eggs were spawned either artificially or naturally. Sampling times were chosen to correspond to their spawning seasons. Spawning seasons of these four sparids are: *P. major* and *A. latus* from January to April, *A. schlegeli* from November to April, and *S. sarba* from November to March. Usually, healthy and mature female fishes for artificial spawning are approximately 4 years old. The maturity of these fishes could be checked first by determining whether the egg diameters were all >400 μm. These adult female fishes weighed *c.* 350 g and were injected with 350 units of HCG (human chorionic gonadotropin) into their abdomen. After 6 h postinjection, the eggs would be spawned artificially into a plastic bowl while hand-pressing their abdomen. Unfertilized eggs were collected immediately and fixed for SEM examination. Approximately 100–200 eggs for each species and each stage were sampled randomly in this step.

In the fixing process, fresh eggs were first collected and cleaned using fish saline buffer, then they were pre-fixed in 5% glutaraldehyde and 4% paraformaldehyde in 0·1 M phosphate buffer at pH 7·4, and finally post-fixed in 1% osmium tetroxide. After buffer washing, the samples were dehydrated in a graded series of alcohol, and then the samples in absolute ethanol were placed into amylacetate and dried in a critical point dryer with liquid CO_2. The dried samples were coated with gold in a sputter coater. The samples were then studied with a scanning electron microscope (S-2400 Hitachi, Japan) under an accelerating voltage of 15 kV.

Egg morphological characters, especially the micropyle ultrastructure of these four sea bream species, were described and then compared with one another. These characters were then coded as binary (0, 1) or multistate (0–3) characters depending on whether the data type was qualitative or quantitative. For continuous or rank-ordered quantitative data, the number of states were determined using the results of Fishers' least-squares test of difference (LST). The parsimonious cladograms of these four seabream species were then reconstructed from a seven-character data matrix using software by Hennig 86 (version 1.5) (Farris, 1988) and PAUP version 3.1.1 (Swofford, 1991). When using PAUP, different Fitch, Wagner, Dollo, or Camin–Sokal parsimony methods were used.

For the phenetic clustering method, Bray–Curtis or Manhattan distance coefficients together with the UPGMA method in NTSYS-pc (version 1.50) (Rohlf, 1988) were tried for the same seven micropyle characters. Finally, the phylogenies obtained from the above cladograms and phenograms using the micropyle character suite were compared to the results obtained from other character suites including morphological biochemical, and molecular data (Jean *et al.*, 1992, 1995*a*,*b*).

RESULTS

ULTRASTRUCTURE OF FISH EGGS

Among all eggs collected, only those in the best fixation condition were chosen for further observation. Measurements of each character are given as average values (Table I). Individual or intraspecific variation against interspecific difference was examined first. The results showed that all qualitative characters were invariable within species. Quantitative characters showed overlapping values, and species differences were only significant between *P. major* and three other species.

Sparus sarba *[Fig. 1(a)]*

Before fertilization, eggs of *S. sarba* had a diameter of 748 μm with a variation of 73 μm. The micropylar canal opened outward like a funnel. The average maximum diameter of the funnel was *c.* 5·58 μm. The inside of the canal was reinforced by eight thickened annuli on the sides. Lots of little knobs were found on the thickened annuli of the canal. The outer opening of the canal was nearly always in the centre of the funnel. The annular thickenings showed a clockwise spiral arrangement from the bottom to the outer opening in the channel. Many accessory openings were found in the surroundings of the micropyle. The number of accessory openings was *c.* 120. The average size of the openings was 0·38 μm and varied within a range of 0·1–0·7 μm.

Acanthopagrus latus *[Fig. 1(b)]*

Unfertilized eggs of *A. latus* were spherical in shape and *c.* 824 μm in diameter. The micropyle consisted of a micropylar canal and many accessory openings. The micropylar canal was in an open stage before fertilization. The average diameter of the accessory openings was 0·45 μm with a great variation from 0·1 to 0·5 μm. Each egg had 127 accessory openings around the micropyle. Annular reinforcements were developed on the sides of the canal in two clockwise-spiral ridges. The reinforcements had no knobs and were smooth. From the centre of the canal to the outer opening were five thickened annuli as reinforcement. The outer opening had a diameter of 5 μm. No microvilli were found in the micropylar canal or accessory opening region.

Acanthopagrus schlegeli *[Fig. 1(c)]*

Unfertilized eggs of *A. schlegeli* were smaller than those of the other three species with a diameter of 667 μm. The micropyle consisted of a micropylar canal and many accessory openings. The accessory openings numbered *c.* 155, arranged randomly around the micropyle. Some microvilli were seen in the region of the accessory openings. The average diameter of the openings was 0·38 μm and ranged from 0·2 to 0·8 μm. The diameter of the outer canal of an

TABLE I. Comparison of microstructural characters of the eggs of four species in Sparidae

Characters	*S. sarba*	*A. latus*	*A. schlegeli*	*P. major*
Diameter of micropyle funnel	5·58 ± 0·69[a] (n=4)	5·36 ± 0·37[a] (n=4)	4·67 ± 0·8[a] (n=4)	6·59 ± 0·36[b] (n=4)
Number of accessory opening	120 ± 25[a] (n=15)	127 ± 28[a] (n=7)	155 ± 75[a] (n=8)	299 ± 104[b] (n=3)
Diameter of accessory opening	0·38 ± 0·07[a] (n=20)	0·45 ± 0·30[a] (n=20)	0·38 ± 0·19[a] (n=20)	0·19 ± 0·03[b] (n=20)
Reinforcement direction in micropyle canal	Clockwise	Clockwise	Clockwise	Triangular
Reinforcement type in micropyle canal	Spiral	Two-spiral	Two-spiral	No spiral
Reinforcement number in micropyle canal	8	5	7	10
Arrangement of acessory opening	Random	Random	Random	Slightly radial

Length unit: μm.
[a]Table values are the means calculated from various sample sizes. Means in a row with the same letter are not significantly different (P=0·05) in t-test (mean ± S.D.).
[b]Micropyle funnels were the outer micropyle apparatus, micropyle canals were the inner micropyle apparatus.

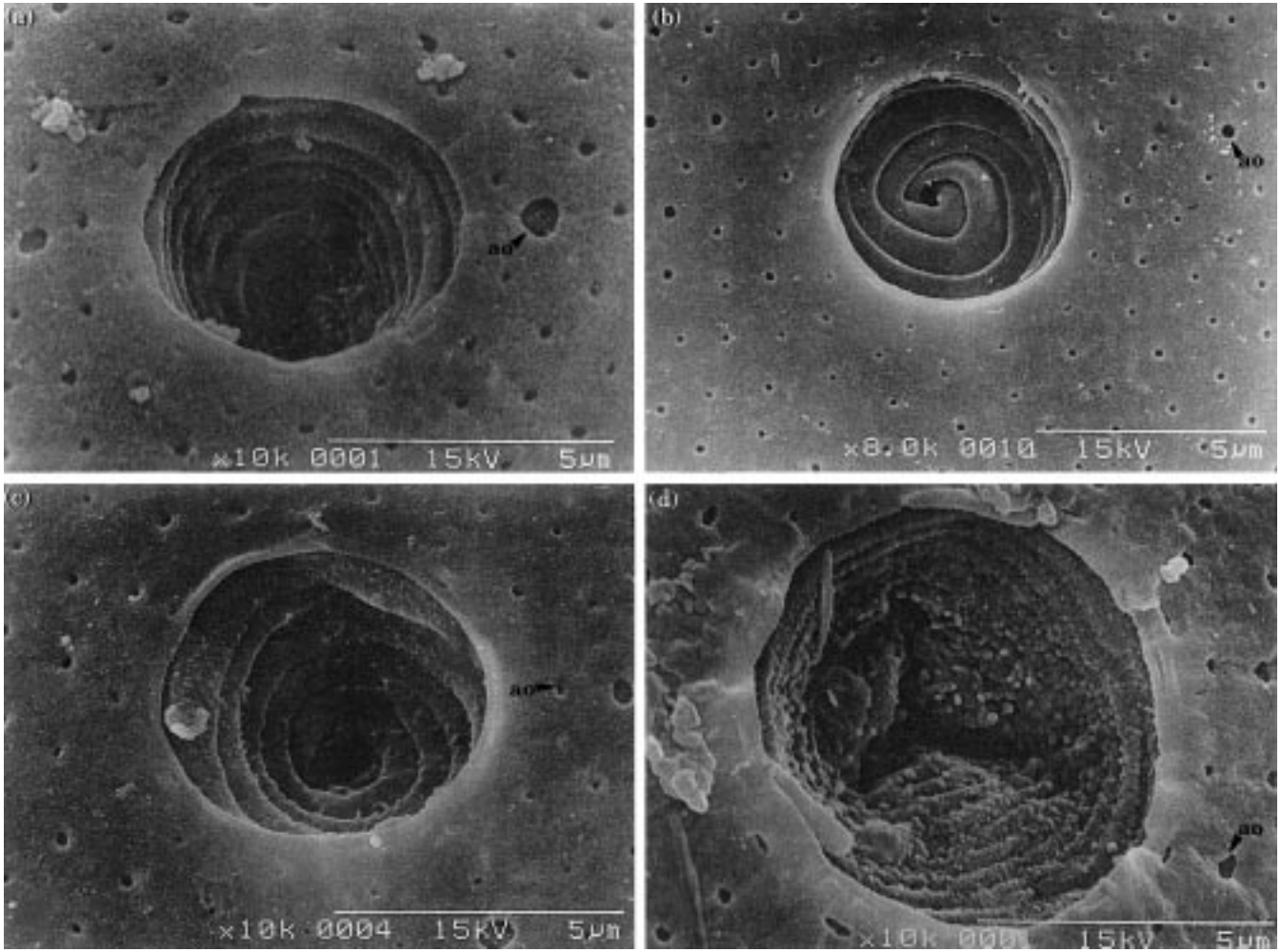

FIG. 1. Unfertilized micropyle of four species of Sparidae. (a) *Sparus sarba*; (b) *Acanthopagrus latus*; (c) *Acanthopagrus schlegeli*; (d) *Pagrus major*. ao: Accessory openings.

unfertilized mature egg micropyle was *c.* 5 μm. The micropylar canal was also reinforced by two clockwise-spiral thickened annuli in the canal wall. The annular reinforcements had many knobs but no microvilli before fertilization. Seven thickened annuli were found from the centre of the canal to the outer opening.

Pagrus major *[Fig. 1(d)]*

Before fertilization, eggs of *P. major* were the largest among the four species studied. Their average diameter was 1019 μm with a variation of 95 μm. The micropyle also consisted of a micropylar canal and many accessory openings. The accessory openings were arranged around the canal with a somewhat regular radial pattern. The average diameter of the accessory openings was 0·19 μm with a range of 0·3–0·5 μm. The micropylar canal was surrounded by 299 accessory openings on average with a variation of 73. Some long microvilli, with lengths of 0·90 μm, were found in the accessory opening region. The most distinctive and noteworthy feature in the eggs was the microstructure of the micropylar canal. The outer opening of the micropylar canal had a diameter of 7·2 μm. The micropyle canals were reinforced by 10 thickened annuli in the canal sides. Many knobs were found on the thickened annuli. Since the reinforcements were strongly developed from three directions in the canal sides, the centre of the canal became Y-shaped, and the reinforcements surrounding the canal wall had triangular thickened annuli.

Taxonomic key for identifying the four species of Sparidae based on micropyle characters (ao, accessory openings):

1a. Reinforcements 10, shape of canal triangular with no spirals; arrangement of ao slightly radial ..... *P. major*
1b. Reinforcements 5–8, direction in canal clockwise and spiral; arrangement of ao random 2
2a. Reinforcements 8, and arrangement in canal spiral ..... *S. sarba*
2b. Reinforcements 5–7, and arrangements in canal two—spiral 3
3a. Reinforcements 5 ..... *A. latus*
3b. Reinforcements 7 ..... *A. schlegeli*

PHYLOGENETIC INFERENCE

A total of seven characters were used in the analysis of the four sparids (Tables II and III). Based on this character data matrix, several different cladograms were reconstructed using different parsimonious methods including those by Wagner (both from Hennig86 and PAUP), and by Dollo, Fitch and Camin–Sokal (PAUP). Three cladograms obtained from the Camin–Sokal method were neglected here since all three trees had longer length ($L=13$) and worse consistency and retention indices (CI=0·769, RI=0·625). The four trees all had the same shortest tree lengths ($L=10$) with no homoplasy (HI=0) (Fig. 2). The first tree with higher resolution was constructed from both Wagner and Dollo methods. The remaining three trees with lower resolution of multifurcation were obtained from the Fitch method. The reason for obtaining the latter three topologies was simply due to the assumption of Fitch that characters are unordered, i.e. any state is capable of transforming directly to any other state, with equal cost. The character state changes mapped on to these three

TABLE II. Character coding table used for cladistic analysis

	Characters	States
1	Diameter of micropyle funnel	(0) 5·58[a], 5·36[a], 4·67[a]; (1) 6·59[b]
2	Number of accessory opening	(0) 120[a], 127[a], 155[a]; (1) 299[b]
3	Diameter of accessory opening	(0) 0·19[b]; (1) 0·38[a], 0·45[a], 0·38[a]
4	Reinforcement direction in micropyle canal	(0) clockwise; (1) triangular
5	Reinforcement type in micropyle canal	(0) no spiral; (1) spiral; (2) two-spiral
6	Reinforcement number in micropyle canal	(0) 5; (1) 7; (2) 8; (3) 10
7	Arrangement of accessory opening	(0) random; (1) slightly radial

Superscripts a and b indicate the mean separation by Fishers' least-square test of difference (LST).

TABLE III. Multistate character data matrix for the micropyle ultrastructure of Sparidae fish egg before fertilization

Taxon species	Characters						
	1	2	3	4	5	6	7
Pagrus major	1	1	0	1	0	3	1
Acanthopagrus schlegeli	0	0	1	0	2	1	0
Acanthopagrus latus	0	0	1	0	2	0	0
Sparus sarba	0	0	1	0	1	2	0

trees [Fig. 2(b)–(d)], could be self-explanatory. However, it is quite obvious that the three species of *A. latus*, *A. schlegeli*, and *P. major* are more closely related to each other than to *P. major*, because there are only one to three steps among each of the former three species but there are seven or more steps separating them from *P. major*. Thus, the cladogram of Fig. 2(a) should be our final chosen phylogeny. Additionally, only this cladogram contained two synapomorphic characters, i.e. characters 5 and 6, which supports the assumption that the two congeneric species of *Acanthopagrus* are a sister group. The other three cladograms have only autapomorphic characters with no synapomorphy.

A UPGMA phenogram (Fig. 3) was obtained from the same data matrix of micropyle characters (Table III). The topology is the same as in Fig. 2(a). Thus, there is no doubt that the congeneric species *A. latus* and *A. schlegeli* are a sister group, with *S. sarba* second, and *P. major* last. The generic interrelationship between *Sparus* and *Acanthopagrus* is closer than that with the genus *Pagrus* (Fig. 2).

DISCUSSION

ULTRASTRUCTURAL COMPARISONS AMONG SPECIES

The surface microstructure of external egg membranes has been used as a taxonomic character for species identification of fish eggs (Riehl & Schulte, 1977). The outer surface of the chorion and the microstructure of the micropyle

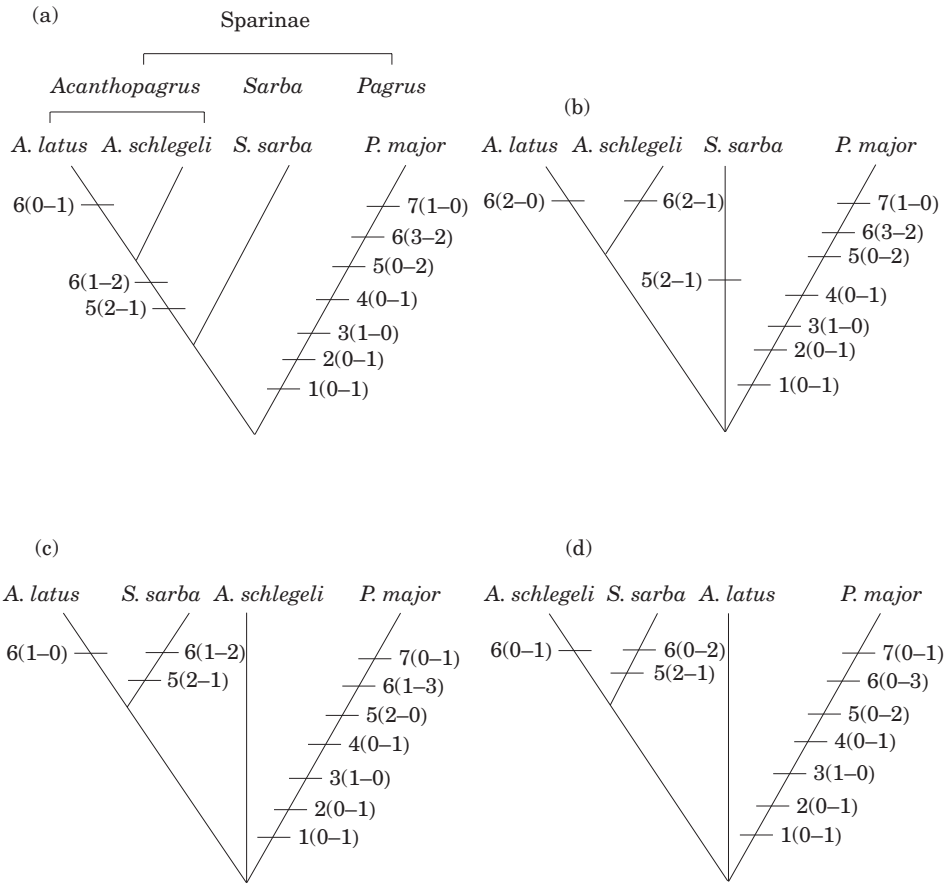

FIG. 2. Four proposed phylogenies obtained from different parsimonious methods. (a) The shortest tree with $L=10$, CI=1·0 and RI=% obtained from the Wagner method of Hennig 86 and PAUP, and the Dollo method of PAUP. (b) (c) and (d) are three equally short trees obtained from the Fitch method of PAUP with $L=10$, CI=1·0 and RI=% but showing three different typologies.

are the noteworthy features for egg identification and phylogenetic study. However, the outer surface of the chorion generally does not show remarkable differences in microstructure among species in a genus or family. Because the micropyle is the initial isolating mechanism for preventing interspecific hybridization, micropylar microstructure has been described (Gary *et al.*, 1982) and is considered to be species specific (Riehl, 1980). The usefulness of micropylar microstructure for identification of fish eggs was reported even earlier (Riehl & Schulte, 1978). Three types of micropyles were described by Riehl & Schulte (1977): type I, micropyles with a deep micropylar pit and short micropylar canal; type II, micropyles with a flat pit and a correspondingly longer canal; type III, micropyles without a pit, only with a canal. The micropyles of the four species of Sparidae from this study should be classified as type III because no micropylar pits were found under SEM but only a canal was found (Fig. 1). Micropylar canals of Sparidae might be even broader at the upper end on the external egg membrane than at the bottom of the internal egg membrane.

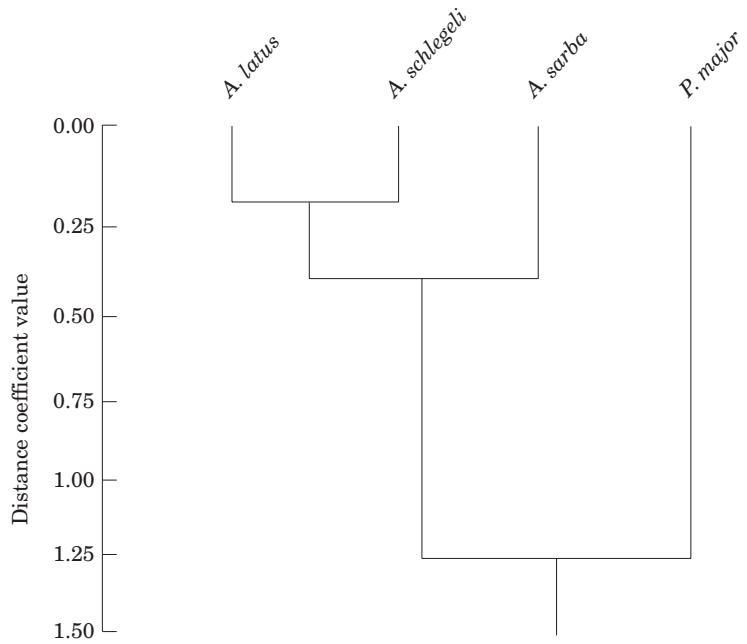

FIG. 3. UPGMA phenogram constructed from the multistate data matrix of Table III and using the Manhattan distance coefficient.

Among the four species *P. major* eggs were the largest with a diameter of 1019 μm, followed by those of *A. latus*, which were significantly larger than eggs of both *S. sarba* and *A. schlegeli* (Table I). The sizes of micropyles of *S. sarba*, *A. latus*, and *A. schlegeli*, at 5·58, 5·36 and 4·67 μm, respectively, were similar and hard to distinguish. Only *P. major* possessed much larger micropyles than any of the other three species. On the other hand, the diameter of accessory openings of the four species showed great variation ranging from 0·1 to 0·8 μm. It is difficult to distinguish eggs of these species by using the diameter of accessory openings although their size in *P. major* was significantly smaller than that of the other three species. The number of accessory openings around the micropyle showed differences among the four species. The significant feature of the accessory openings of *P. major* was the largest number (299) of openings, but that of *S. sarba* was low (120) and showed no significant differences with *A. latus* or *A. schlegeli* [Fig. 1(a), (b) and (d); Table I].

The microstructure of the micropylar canals was the most significant character identifying the four species of Sparidae. Although there was no significant difference in the diameter of the micropyle funnels among *S. sarba*, *A. latus* and *A. schlegeli*, the size of the funnel of *P. major* was remarkably bigger and could be used as a diagnostic character. Another important feature for egg identification was the reinforcement in the sides of the micropylar canal. In *S. sarba*, *A. latus*, and *A. schlegeli* the canal was reinforced by helical ridges [Fig. 1(a)–(d)]. In *P. major* the canal was reinforced by 10 thickened annuli in the canal sides, but without a helical pattern. A Y-shaped canal was formed by the reinforcements in three directions from the canal sides. The spiral ridges from the centre

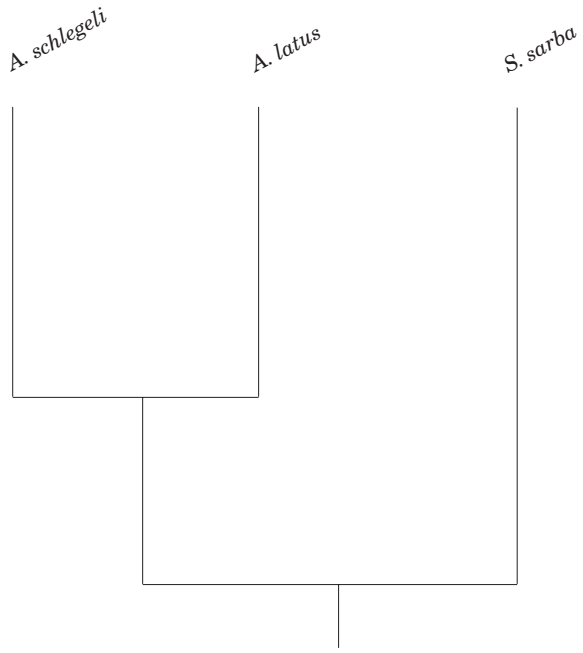

FIG. 4. Phylogenies proposed by using the UPGMA clustering method based on morphological, allozyme, and mtDNA data in Jean *et al.* (1992, 1995*a*,*b*).

of the canal to the outer opening had five and seven annuli in *A. latus* and *A. schlegeli*, respectively [Fig. 1(b) and (c)]. However, the annular reinforcements had slight knobs in *A. schlegeli*, but were without knobs in *A. latus*. Therefore, spiral ridges in the reinforcements in *A. latus* were smooth and sharp, and those in *A. schlegeli* were coarse. Micropylar canals in *S. sarba* were reinforced by eight annuli with slight knobs (Fig. 1).

PHYLOGENETIC RELATIONSHIPS

Phylogenies proposed from the data matrix were recoded from morphological, allozyme, and mtDNA data of Jean *et al.* (1992, 1995*a*,*b*) (Fig. 4). Because they did not include *P. major* in their studies, only three species could be used to compare the results here. In general, the UPGMA phenogram was the same for three different character suites. Again this demonstrates that the general appearance or body shape of the three species of *A. latus*, *A. schlegeli*, and *S. sarba* are quite similar to each other, but the two congeneric species of *Acanthopagrus* are the sister group.

The result of character analysis from Fig. 2(a) also demonstrates that the reinforcement type and number in the micropylar canal are the two most useful characters among all seven characters to distinguish generic differences among the three genera of this study. As to the common ancestor of these four sparids, the authors suspect that it should be *P. major* but would not jump to any conclusion because no appropriate outgroup could be chosen for this study. Probably, further molecular work on additional sea bream species including *P. major* might give more evidence to clarify the most primitive species.

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