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MOLECULAR PHYLOGENY OF GRAPSOID AND OCYPODOID CRABS WITH SPECIAL REFERENCE TO THE GENERA *METAPLAX* AND *MACROPHTHALMUS*

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A B S T R A C T

Some species of the genus *Metaplax* belonging to the family Grapsidae have occasionally been reported to perform waving display that is a characteristic behavior in the family Ocypodidae but uncommon in grapsids. The morphology and life styles of species of *Metaplax* are also quite similar to those of the ocypodid genus *Macrophthalmus*. To examine whether ecological and morphological similarities between *Metaplax* and *Macrophthalmus* are based on convergent evolution or their common evolutionary history, 841-bp nucleotide sequences from the 16S mitochondrial ribosomal RNA gene of 19 grapsids, 10 ocypodids, and 3 camptandriids, including four species of *Metaplax* and four species of *Macrophthalmus*, were analyzed. The resultant phylogenetic tree revealed that both families Grapsidae and Ocypodidae are polyphyletic. *Macrophthalmus* was distinct from any other ocypodid genera studied, forming a sister group relationship with grapsid species of the subfamily Varuninae, and *Metaplax*, *Cyclograpsus*, and *Helice* of the subfamily Sesarminae. *Metaplax*, *Cyclograpsus*, and *Helice* were found to be more closely related to Varuninae than to other Sesarminae species, indicating that the subfamily Sesarminae is polyphyletic. These relationships were in agreement with the distribution pattern of a $tRNA^{Val}$ gene rearrangement on the inferred tree. This molecular phylogenetic analysis suggests that the behavioral and morphological similarities observed between *Metaplax* and *Macrophthalmus* species are probably due to convergent evolution, despite a close phylogenetic relationship. The waving display in intertidal crabs of the families Grapsidae and Ocypodidae may have evolved several times in their lineages, associated with exposed semi-terrestrial habitat of the intertidal environment.

Many brachyuran species of the family Ocypodidae (consisting of Dotillinae, Heloeciinae, Macrophthalminae, and Ocypodinae) are known to perform waving displays, during which they move their chelipeds rhythmically. Waving in the ocypodid crabs has been considered to have both courtship and agonistic functions (e.g., Salmon, 1965, 1987; Crane, 1975; Wada, 1981, 1984, 1993; Zucker, 1988; Moriito and Wada, 1997, 2000). The family Grapsidae (consisting of Grapsinae, Plagusiinae, Sesarminae, and Varuninae) contains species living at intertidal to supratidal zones like the ocypodids and also includes some species that have been more or less known to have waving displays (e.g., Schöne and Schöne, 1963; Salmon and Atsaides, 1968; Schöne, 1968; Wright, 1968; Lindberg, 1980). For example, some species of the genus *Metaplax* are known to show waving display (e.g., Pretzmann, 1971; Beinlich and Polivka, 1989; Wang and Liu, 1996). Like many ocypodids, species of *Metaplax* are usually found on intertidal mud flats, burrowing there and deposit feeding by scooping the top of the substratum. In addition, their external morphology of broad and depressed carapace, elongate eyestalks, and elongate palm of chela is quite similar to those of species of the ocypodid genus *Macrophthalmus*.

A phylogenetic approach is one of the more important methods to study the evolution of behavioral and ecological characters (Brooks and McLennan, 1991; Harvey and Pagel, 1991). To understand morphological and ecological similarities between *Metaplax* and *Macrophthalmus* species, a reliable phylogenetic framework is required. Schubart *et al*. (2000a) reported *Macrophthalmus* species to be more closely

related to grapsid species, especially the subfamily Varuninae, than to any other ocypodids, based on partial mitochondrial 16S rRNA gene sequences. Schubart *et al.* (2000b) also suggested that *Metaplax* and other genera do not belong to the Sesarminae but to the Varuninae based on preliminary molecular data. However, the phylogenetic relationship between species of *Macrophthalmus* and *Metaplax* has not been examined directly and thus is still unknown. The aim of the present study is to provide a preliminary molecular phylogenetic relationship between species of *Metaplax* and *Macrophthalmus* based on partial nucleotide sequence from the 16S mitochondrial ribosomal RNA gene, in order to assess whether their behavioral similarities, such as occurrence of waving display, are due to convergent evolution or their common evolutionary history. The $tRNA^{Val}$ gene region between the 12S rRNA and 16S rRNA genes was also sequenced, because $tRNA^{Val}$ gene rearrangement had been reported in *Macrophthalmus banzai* (see Kitaura *et al.*, 1998) and there is a possibility that this rearrangement helps to resolve the relationships among the species of *Metaplax* and *Macrophthalmus*.

Materials and Methods

Samples, DNA Extraction, Amplification, and Sequencing

The 23 species of the family Grapsidae, Ocypodidae, and Camptandriidae (formerly placed in Ocypodidae) used in the DNA-analysis are listed with collection data in Table 1. *Eriphia sebana* (family Eriphiidae) and *Ovalipes iridescens* (family Portunidae) were used as outgroups. Collected specimens were preserved in 100% ethanol immediately after collection. In addition to the 25 species listed in Table 1, the sequence data of species studied in a previous study (Kitaura *et al.*, 1998) were also used for the phylogenetic analysis. A list of all 34 species examined in the phylogenetic analysis, along with GenBank accession numbers, source of reference, and the occurrence of the $tRNA^{Val}$ gene rearrangement, is provided in Table 2. All the specimens used in this study are deposited at Osaka Museum of Natural History, Japan (the collection number in Table 1).

Total genomic DNA was extracted from the abdomen or ambulatory leg musculature of each crab using QIAamp Tissue Kit (QIAGEN). The target DNA segments were amplified by the polymerase chain reaction (PCR), with primers L2510C (5′-CGCCTGTTTAACAAAGACAT-3′) (Kitaura *et al.*, 1998) and H3062 (5′-CCGGTCTGAACTCAGATCA-3′) (modified from Palumbi *et al.*, 1991), in addition to L1496i (5′-GTACATATCGCCCGTCGCTT-3′) (Kitaura *et al.*, 1998), H2492i (5′-CAGACATGTTTTTAATAAACAGGC-3′) (modified from Palumbi *et al.*, 1991) and H2716i (5′-AAGTTTTATAGGGTCTTATCGTC-3′) (Kitaura *et al.*, 1998) for the $tRNA^{Val}$ gene. Double-stranded PCR products were obtained in a total reaction volume of 25 μl, containing 2.5 μl of 10 × PCR buffer II (Perkin-Elmer), 0.2 mM each dNTP, 0.5 μM of each primer, 3.5 mM $MgCl_2$, 0.125 μl of 5 units/μl *Taq* polymerase (Perkin-Elmer) and 1 μl template. The temperature regime for 35 cycles was 40 sec at 94°C, 1 min at 45–50°C, depending on the primer specificity for the different samples, and 1 min at 72°C.

Amplification products were checked for size by loading 5 μl on a 2% NuSieve (FMC) agarose gel with 0.5 μg/ml ethidium bromide. The remaining product was purified using QIAquick purification kit (QIAGEN), prior to direct sequencing using the dye-terminator cycle sequencing reaction (Perkin-Elmer) with ABI DNA Sequencer 373A. Sequencing reactions followed the protocol suggested by the manufacturer. All final sequences were obtained from both strands for verification. For each species studied, the sequence was determined using one specimen.

Sequence Alignment and Phylogenetic Analysis

The DNA sequences obtained were edited with the multiple sequence editor DNASIS (Hitachi Co. Ltd.). These were initially aligned using CLUSTAL W (gap penalty, 5–10; gap length penalty, 1–5; Thompson *et al.*, 1994). Then, sequences were slightly modified by eye using the sequence analysis software SeqPup 0.6 (Gilbert, 1996). The positions that could not be aligned with certainty were excluded from the data sets. The aligned sequence data used for phylogenetic analyses can be obtained directly from K. W.

Phylogenetic relationships were analyzed by three major phylogenetic procedures, i.e., maximum parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) methods, using PAUP* ver. 4.0b4a (Swofford, 2000).

The MP analyses were performed with a heuristic search with 10 replications of random stepwise additions, using the transition (TS)/transversion (TV) weightings TS1TV1, TS1TV2, and TS1TV3 (numerals indicate weights for TS and TV). The other options in PAUP were set at the default settings in the program. All phylogenetically uninformative sites were neglected from the analyses. To evaluate the consistency of nodes derived by parsimony analysis, 1,000 heuristic bootstrap replications (Felsenstein, 1985) were performed.

The NJ analyses (Saitou and Nei, 1987) were performed with genetic distance estimated based on the HKY85 (Hasegawa *et al.*, 1985) model of evolution. To evaluate the confidence in the nodes of the NJ trees, 1,000 bootstrap replications were executed.

The ML analyses were performed using the following method. First, the NJ tree was used to estimate base frequencies and substitution rate-matrix parameters via maximum likelihood, using the general time reversible (Yang, 1994) model of evolution, allowing some sites to be invariable and the variable sites to follow a gamma distribution. These ML estimates were then used in a heuristic search to find the ML tree topology.

Waving Display of *Metaplax* Species

The waving display behavior of species of *Metaplax* has so far been reported in *M. indica occidentalis* Pretzmann, 1971 (e.g., Pretzmann, 1971), *M. crenulata* (Gerstaecker, 1856) (see Beinlich and Polivka, 1989), and roughly in *M. elegans* (see Tweedie, 1954; Wang and Liu, 1996). In addition to these reports, we have recorded the waving display of *M. distincta* and *M. elegans* in a mangrove

Table 1. List of species studied, with collecting dates and locations, and collection numbers in Osaka Museum of Natural History (OMNH).

Family Grapsidae
- Subfamily Grapsinae
  - Genus *Grapsus*
    - *G. albolineatus* (Herbst, 1783) (7/12/1998; Minatogawa, Okinawa Is., Japan; OMNH Ar 4998)
  - Genus *Metopograpsus*
    - *M. thukuhar* (Owen, 1839) (8/4/1998; Nakama River, Iriomote Is., Japan; OMNH Ar 4999)
  - Genus *Pachygrapsus*
    - *P. minutus* A. Milne-Edwards, 1873 (6/28/1995; Hoshizuna, Iriomote Is., Japan; OMNH Ar 5000)
- Subfamily Varuninae
  - Genus *Gaetice*
    - *G. depressus* (De Haan, 1835) (6/11/1998; Shirahama, Wakayama Pref., Japan; OMNH Ar 5001)
  - Genus *Ptychognathus*
    - *P. ishii* Sakai, 1939 (8/24/1998; Sashiki, Okinawa Is., Japan; OMNH Ar 5002)
  - Genus *Parapyxidognathus*
    - *P. deianira* (De Man, 1888) (8/24/1998; Sashiki, Okinawa Is., Japan; OMNH Ar 5003)
  - Genus *Pseudograpsus*
    - *P. albus* Stimpson, 1858 (8/25/1998; Nakadomari, Okinawa Is., Japan; OMNH Ar 5004)
  - Genus *Hemigrapsus*
    - *H. sanguineus* (De Haan, 1835) (6/11/1998; Shirahama, Wakayama Pref., Japan; OMNH Ar 5005)
    - *H. penicillatus* (De Haan, 1835) (7/28/1996; Isahaya Bay, Nagasaki Pref., Japan; OMNH Ar 5006)
  - Genus *Varuna*
    - *V. litterata* (Fabricius, 1798) (8/23/1998; Sashiki, Okinawa Is., Japan; OMNH Ar 5007)
- Subfamily Sesarminae
  - Genus *Chiromantes*
    - *C. haematocheir* (De Haan, 1835) (6/11/1998; Shirahama, Wakayama Pref., Japan; OMNH Ar 5008)
  - Genus *Cyclograpsus*
    - *C. intermedius* (De Haan, 1835) (5/22/2000; Awaji Is., Hyogo Pref., Japan; OMNH Ar 5009)
  - Genus *Helice*
    - *H. tridens* (De Haan, 1835) (5/13/1998; Chikusa River, Hyogo Pref., Japan; OMNH Ar 5010)
  - Genus *Metaplax*
    - *M. distincta* H. Milne Edwards, 1852 (12/9/1999; Ranong, Thailand; OMNH Ar 5011)
    - *M. elegans* De Man, 1888 (12/11/1995; Haiphong, Vietnam; OMNH Ar 5012)
    - *M. shenii* Gordon, 1931 (12/7–8/1995; Haiphong, Vietnam; OMNH Ar 5013)
    - *M. takahashii* Sakai, 1939 (12/7–8/1995; Haiphong, Vietnam; OMNH Ar 5014)
  - Genus *Perisesarma*
    - *P. bidens* (De Haan, 1835) (8/23/1998; Sashiki, Okinawa Is., Japan; OMNH Ar 5015)
  - Genus *Sesarmops*
    - *S. intermedium* (De Haan, 1835) (6/11/1998; Shirahama, Wakayama Pref., Japan; OMNH Ar 5016)

Family Ocypodidae
- Subfamily Macrophthalminae
  - Genus *Macrophthalmus*
    - *M. brevis* (Herbst, 1804) (8/19/1998; Sashiki, Okinawa Is., Japan; OMNH Ar 5017)
    - *M. quadratus* A. Milne-Edwards, 1873 (7/15/1998; Sashiki, Okinawa Is., Japan; OMNH Ar 5018)
    - *M. latreillei* (Desmarest, 1822) (8/31/1995; Ludmilla Creek, Darwin, Australia; OMNH Ar 5019)

Family Camptandriidae
- Genus *Cleistostoma*
  - *C. dilatatum* (De Haan, 1835) (7/28/1996; Isahaya Bay, Nagasaki Pref., Japan; OMNH Ar 5020)

Family Eriphiidae
- Genus *Eriphia*
  - *E. sebana* (Shaw and Nodder, 1803) (7/31/1998; Minatogawa, Okinawa Is., Japan; OMNH Ar 5021)

Family Portunidae
- Genus *Ovalipes*
  - *O. iridescens* (Miers, 1886) (5/6/1998; Maizuru Bay, Kyoto, Japan; OMNH Ar 5022)

swamp at Ranong, western Thailand. All recordings were made during daytime/low tide of 7 and 8 Dec. 1999, with bare eyes and an 8-mm video camera. Total recording periods were 2.5 h for *M. elegans* and 2.1 h for *M. distincta*.

Temporal components of the display were determined by frame analysis. Because the display behavior was recorded at 30 frames per second, estimates of temporal characteristics were determined by multiplying the number of frames involved by 0.033 seconds.

Table 2. All taxa examined in this analysis, along with occurrence of tRNA gene rearrangement.

Family	Subfamily	Species	$tRMA^{Val}$ gene rearrangement	DDBJ/EMBL/GenBank Accession No.	Source
Grapsidae	Grapsinae	*Grapus albolineatus*	−	AB057806	present study
	Grapsinae	*Metopograpsus thukuhar*	−	AB057807	present study
	Grapsinae	*Pachygrapsus minutus*	−	AB057808	present study
	Varuninae	*Gaetice depressus*	+	AB058626	present study
	Varuninae	*Hemigrapsus sanguineus*	+	AB058630	present study
	Varuninae	*Hemigrapsus penicillatus*	+	AB058628	present study
	Varuninae	*Ptychognathus ishii*	+	AB058621	present study
	Varuninae	*Parapyxidognathus deianira*	+	AB058619	present study
	Varuninae	*Pseudograpsus albus*	+	AB058618	present study
	Varuninae	*Varuna litterata*	+	AB058620	present study
	Sesarminae	*Chiromantes haematocheir*	−	AB057809	present study
	Sesarminae	*Cyclograpsus intermedius*	+	AB058627	present study
	Sesarminae	*Helice tridens*	+	AB058629	present study
	Sesarminae	*Metaplax distincta*	+	AB058622	present study
	Sesarminae	*Metaplax elegans*	+	AB058623	present study
	Sesarminae	*Metaplax shenii*	+	AB058624	present study
	Sesarminae	*Metaplax takahashii*	+	AB058625	present study
	Sesarminae	*Perisesarma bidens*	−	AB057810	present study
	Sesarminae	*Sesarmops intermedium*	−	AB057811	present study
Ocypodidae	Ocypodinae	*Ocypode stimpsoni*	−	AB002131	Kitaura *et al.* (1998)
	Ocypodinae	*Uca lactea*	−	AB002130	Kitaura *et al.* (1998)
	Dotillinae	*Dotilla wichmanni*	−	AB002126	Kitaura *et al.* (1998)
	Dotillinae	*Ilyoplax deschampsi*	−	AB002117	Kitaura *et al.* (1998)
	Dotillinae	*Scopimera globosa*	−	AB002124	Kitaura *et al.* (1998)
	Dotillinae	*Tmethypocoelis ceratophora*	−	AB002127	Kitaura *et al.* (1998)
	Macrophthalminae	*Macrophthalmus banzai*	+	AB002132	Kitaura *et al.* (1998)
	Macrophthalminae	*Macrophthalmus brevis*	+	AB058631	present study
	Macrophthalminae	*Macrophthalmus quadratus*	+	AB058633	present study
	Macrophthalminae	*Macrophthalmus latreillei*	+	AB058632	present study
Camptandriidae		*Cleistostoma dilatatum*	−	AB057812	present study
		Paracleistostoma depressum	−	AB002128	Kitaura *et al.* (1998)
		Baruna trigranulum	−	AB002129	Kitaura *et al.* (1998)
Eriphiidae		*Eriphia sebana*	−	AB057813	present study
Portunidae	Macropipinae	*Ovalipes iridescens*	−	AB057814	present study

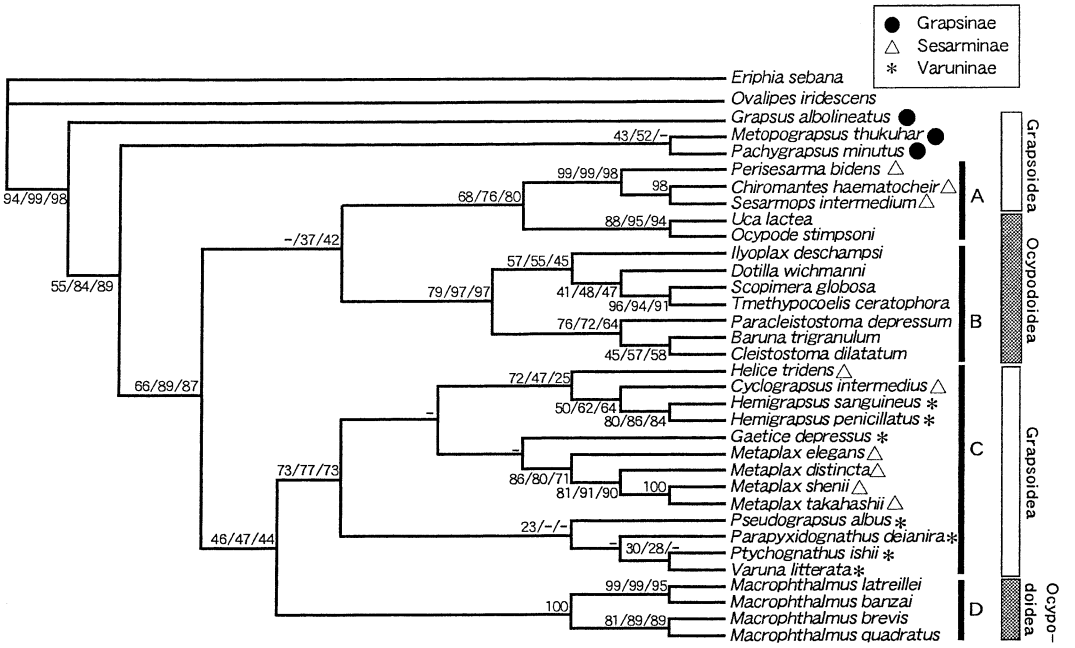

Fig. 1. The single most-parsimonious tree of 19 grapsid and 13 ocypodoid species and two outgroup taxa. A–D denotes four clusters commonly inferred from all of the tree reconstruction methods employed. Numbers at each branch indicate bootstrap values obtained after 1,000 replicates for each weighting scheme (TS1TV1/TS1TV2/TS1TV3). A single number indicates that the values were the same for all weightings. A dash indicates that a node was not recovered in bootstrap analysis.

Results

Phylogenetic Analysis

In the phylogenetic analysis, multiple-sequence alignment of the sequence-determined region of 19 grapsids, 10 ocypodids, 3 camptandriids, and 2 outgroup taxa resulted in sequences of 1,360 basepairs (= bp) with gaps. Of the aligned sequences, the regions which could not be aligned unambiguously and the $tRNA^{Val}$ gene region were discarded from the analyses, resulting in a total of 841 bp (including gaps) used for the analysis. *Metaplax elegans* and *M. distincta* were clearly genetically distinct from other *Metaplax* species, but *M. shenii* and *M. takahashii* turned out to be identical in their sequence. Three additional sequences of *M. takahashii* from the same locality gave identical results.

The three major phylogenetic procedures, MP, NJ, and ML methods, produced similar but slightly different topologies (Figs. 1–3). The MP method with the weighting of TS1TV1 provided one single most-parsimonious tree (Fig. 1), showing the subfamily Grapsinae branching off first, followed by several clusters named A–D (Fig. 1). The family Grapsidae and Ocypodidae were shown to be polyphyletic. Species of *Metaplax* formed a cluster with the subfamily Varuninae, together with *Cyclograpsus intermedius* and *Helice tridens* of the subfamily Sesarminae (cluster C in Fig. 1), and this cluster was most closely related with the clade of *Macrophthalmus* (cluster D in Fig. 1). A single MP tree with TV1TV2 weighting was different from the above tree in the relationship among the cluster C species. A strict consensus tree from six MP trees with TV1TV3 weighting was the same as that with TS1TV1 weighting, except for six polytomies within the cluster C.

The NJ tree (Fig. 2) also showed the four clusters A–D, but their branching order among A, B, C + D, the relationships among the cluster C species, the placement of *Dotilla wichmanni* in the cluster B, and the relationship between *Pachygrapsus minutus* and *Metopograpsus thukuhar* differed from that in the MP tree in Fig. 1.

The ML tree topology (Fig. 3) differed from that of the MP tree in Fig. 1 in the branching order of the major clusters A, B, C + D, the relationships among species of the cluster C, and the placement of *Dotilla wichmanni*.

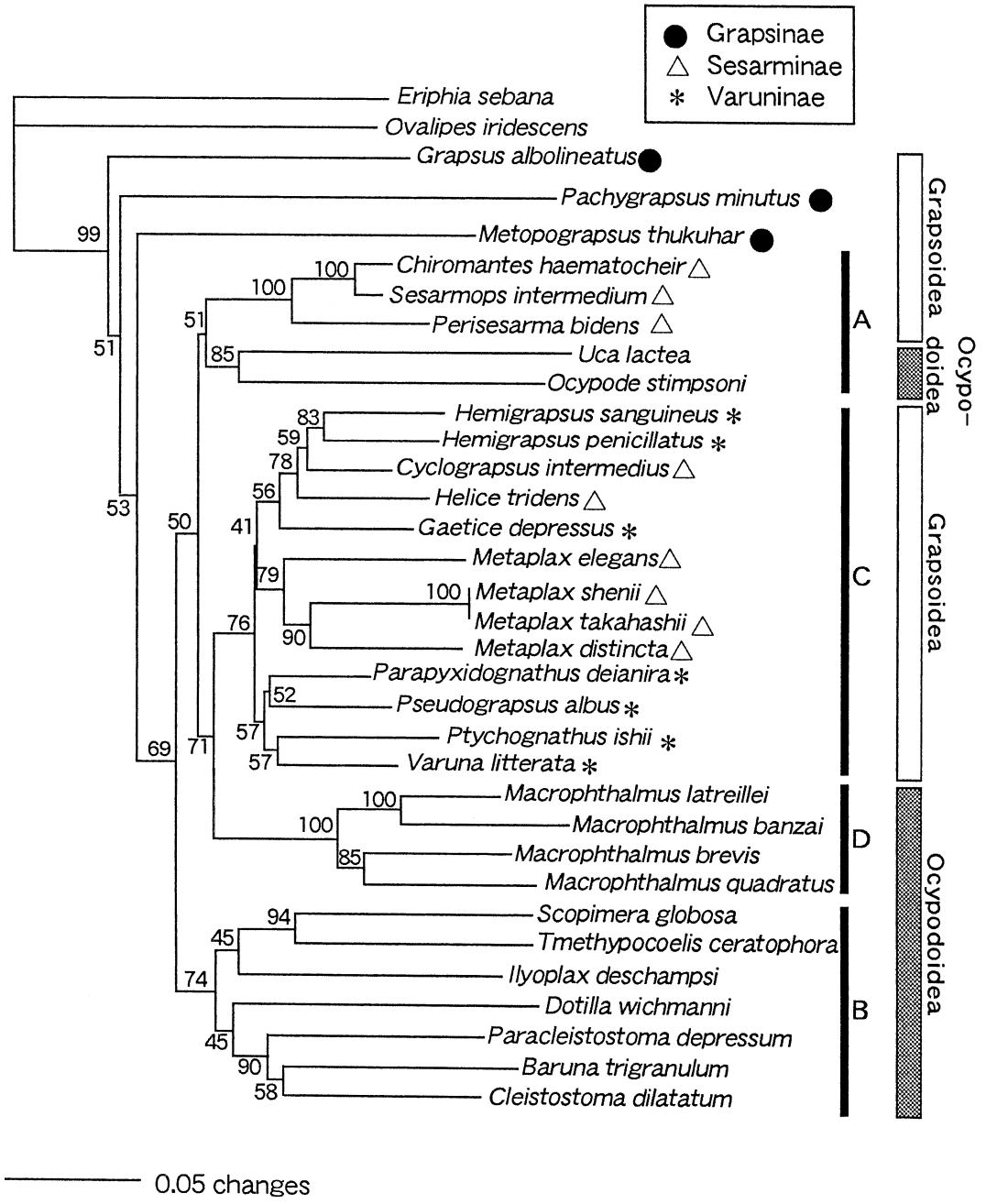

Fig. 2. Neighbor-joining tree of 19 grapsid and 13 ocypodoid species and two outgroup taxa, using the HKY85 model of evolution. Numbers are bootstrap values obtained after 1,000 replicates. A–D denotes four clusters commonly inferred from all of the tree reconstruction methods employed.

The discrepancies in the phylogenetic resolution among these tree reconstruction methods were due to ambiguity in the relationship among three major clusters A, B, C + D, the relationship among species of the cluster C, the placement of *Dotilla wichmanni*, and the relationship between *Pachygrapsus minutus* and *Metopograpsus thukuhar*. The other parts of

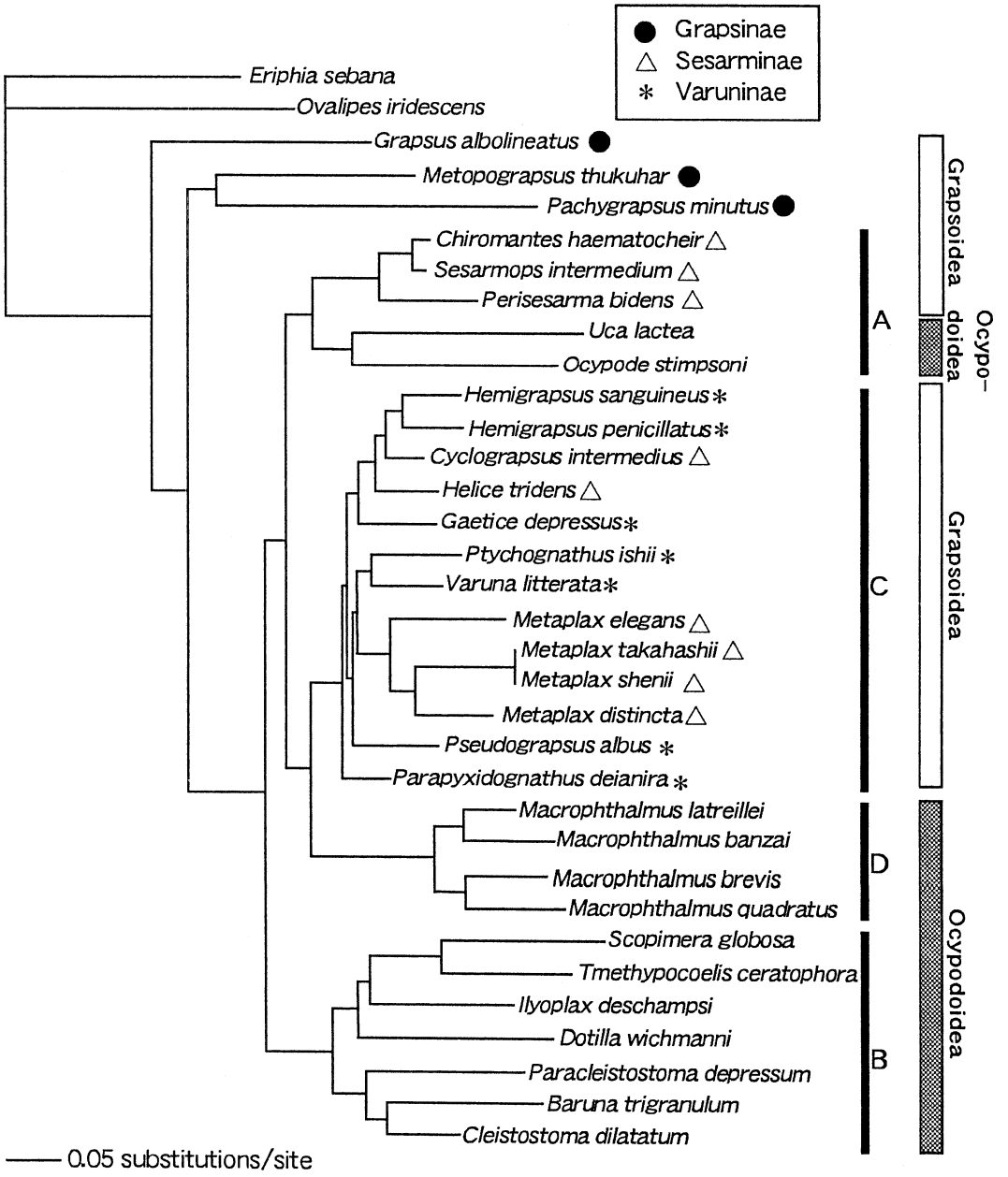

Fig. 3. Maximum likelihood tree with GTR + I + Γ model of evolution for 19 grapsid and 13 ocypodoid species and two outgroup taxa. –Ln likelihood = 9303.46034, percentage invariant sites = 0.302, gamma shape parameter = 0.597 estimated with four rate categories.

the phylogenetic relationship did not change under different analysis conditions.

Rearrangement of the $tRNA^{Val}$ Gene Region

The sequence between the 12S rRNA and 16S rRNA genes for all four species of *Macrophthalmus*, and all seven species of the subfamily Varuninae and six species of the subfamily Sesarminae other than *Chiromantes haematocheir*, *Perisesarma bidens*, and *Sesarmops intermedium* were very variable and deviated from those of the remaining species. The anticodon triplet (UAC) region could not be found in the first group of species, and the

Fig. 4. The sequence between the 12S rRNA and 16S rRNA gene of 19 grapsid and 13 ocypodoid species and two outgroup taxa compared with homologous sequences from *Penaeus monodon* (see Wilson *et al*., 2000) and *Daphnia pulex* (see Crease, 1999). The $tRNA^{val}$ gene regions of *Penaeus monodon* and *Daphnia pulex* are boxed, and the anticodon is shaded. Identification of both the 5′ and 3′ ends of $tRNA^{Val}$ gene, 3′ end of the 12S rRNA gene and 5′ end of the 16S rRNA gene of *Penaeus monodon* and *Daphnia pulex* are from Wilson *et al*. (2000) and Crease (1999), respectively.

DNA sequences are shorter than the one in the other crabs (Fig. 4). These evidences suggest that the $tRNA^{Val}$ gene region in the above 17 species is not functional and that a functional $tRNA^{Val}$ gene exists somewhere in the mitochondrial genome, as previously pointed out for *Macrophthalmus banzai* Wada and Sakai, 1989 (see Kitaura *et al*., 1998). The result of the presence or absence of the $tRNA^{Val}$ gene rearrangement is summarized in Table 2. Species having this rearrangement exclusively corresponded to the species clusters C or D (Fig. 5).

Waving Display of *Metaplax*

The waving gesture of *M. elegans* was performed with one cheliped (right or left) as reported in *M. crenulata* (see Beinlich and Polivka, 1989). Both chelipeds at the beginning remained flexed in front of the buccal region (gesture 1 in Fig. 6), and one cheliped (right or left) was moved as the basis of the chela described a circle. During this motion (gestures 2 and 3 in Fig. 6), the suborbital crenellation was rubbed by the base of the chela. Waving display behavior was observed in both sexes (eight males, 13 waves; one female, one wave), and the waving by females was performed in the same way as by males. Waving by a male crab was often performed when the male repelled a neighbor, when the male approached a female, or sometimes without directing the wave to a particular individual. In addition to the waving display, the male sometimes drummed with one chela against the substrate, producing several strokes per series.

In contrast to *M. elegans*, waving in *M. distincta* was performed with both chelipeds (Fig. 7). One cheliped (right or left) was moved as the basis of the chela described a circle in the same way as in *M. elegans*, but the other chela was also moved accompanying this motion. During the gestures from 3 to 6 in Fig. 7, the suborbital crenellation was also rubbed by the base of chela as in *M. elegans* and *M. crenulata* (see Beinlich and Polivka, 1989). The waving was observed in males only (six males, 15 waves). Waving of a male crab was often performed when the male repelled a neighbor or sometimes without directing the wave to a particular individual.

Discussion

Phylogenetic Relationships

The phylogenetic relationships among ocypodid and grapsid species derived from the present molecular data greatly differ from the traditional classification. The analyses strongly suggest that the grapsid subfamilies Sesarminae and Varuninae are not monophyletic. *Metaplax*, *Cyclograpsus*, and *Helice* (subfamily

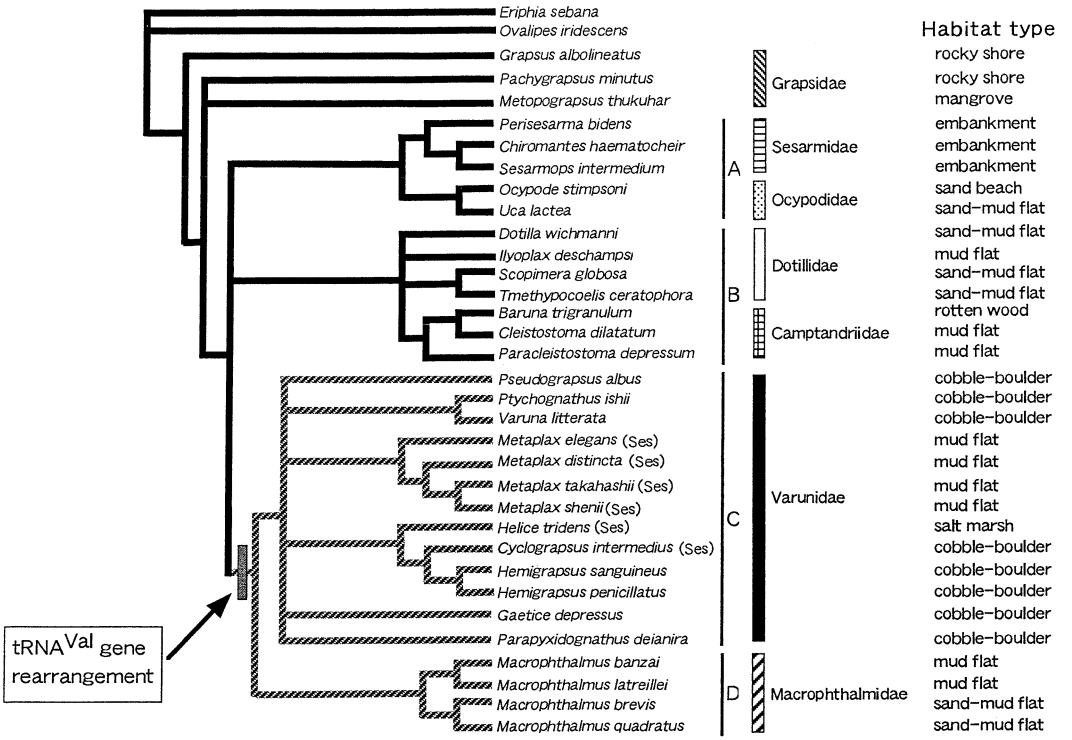

Fig. 5. The $tRNA^{Val}$ gene rearrangement information mapped on the strict consensus of the three trees, the MP, NJ, and ML trees shown in Figs. 1–3. Habitat type (rocky shore, cobble-boulder, salt marsh, mangrove, embankment, sand-mud flat) is also shown next to each species. A–D denotes four clusters commonly inferred from all of the tree reconstruction methods employed. Taxonomy after Schubart *et al.* (2000b).

Sesarminae) formed a cluster with species from the subfamily Varuninae. This relationship was also evident with respect to the $tRNA^{Val}$ arrangement data. The $tRNA^{Val}$ rearrangement occurred in all the species of Varuninae as well as in *Metaplax*, *Cyclograpsus*, and *Helice*, but it does not occur in other species of Sesarminae, such as in *Chiromantes haematocheir*, *Perisesarma bidens*, or *Sesarmops intermedium*. These results indicate that the taxonomy of these subfamilies needs to be revised, and *Metaplax*, *Cyclograpsus*, and *Helice* should be transferred from the Sesarminae to the Varuninae. Schubart *et al.* (2000b) also reached the same conclusion for these three as well as other genera based on mtDNA sequence and morphological data.

The present analyses also suggest that the family Grapsidae and Ocypodidae are polyphyletic. Some species of Sesarminae (family Grapsidae), namely *Chiromantes haematocheir*, *Perisesarma bidens*, and *Sesarmops intermedium*, formed a cluster with species of Ocypodinae (family Ocypodidae) in all tree reconstruction methods (51–80% bootstrap values). Furthermore, the species of the genus *Macrophthalmus* (cluster D in Figs. 1–3) were more closely related to varunine species including *Metaplax*, *Cyclograpsus*, and *Helice* (cluster C in Figs. 1–3) than to any other ocypodid species (44–71% bootstrap values), and this result is compatible with the distribution pattern of the $tRNA^{Val}$ gene rearrangement. Figure 5 summarizes the result of phylogenetic analysis of the $tRNA^{Val}$ rearrangement on the ML tree, indicating that this rearrangement occurred only once in the common ancestor of cluster C + D. This is strong genetic evidence for a closer relationship of the genus *Macrophthalmus* (cluster D) to the Varuninae, including *Metaplax*, *Cyclograpsus*, and *Helice* species (cluster C), than to other ocypodids. A similar conclusion has been reached by Schubart *et al.* (2000a).

Although the phylogenetic relationships derived from the present molecular data indicated that the family Grapsidae and Ocypodidae are polyphyletic, the subfamilies of these groups

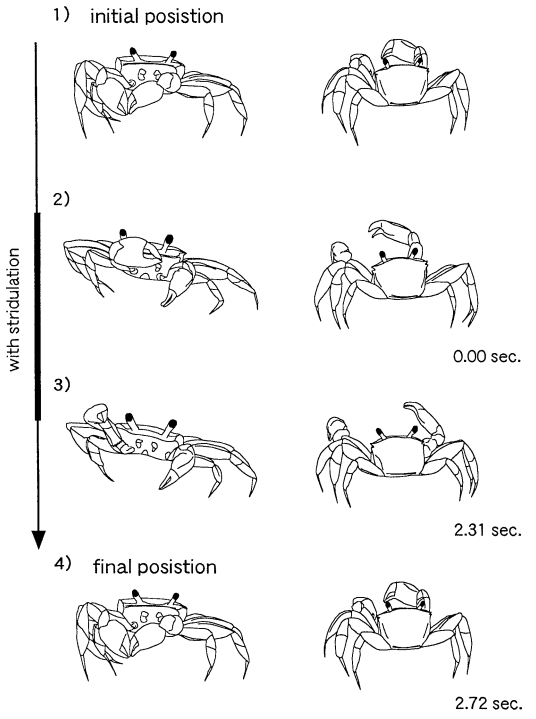

Fig. 6. Successive postures of waving display by *Metaplax elegans*. Drawings are traced from videotapes (left: front view; right: dorsal view). Values below crabs indicate time in sec from the gesture 2 of the wave (the mean value of six waves).

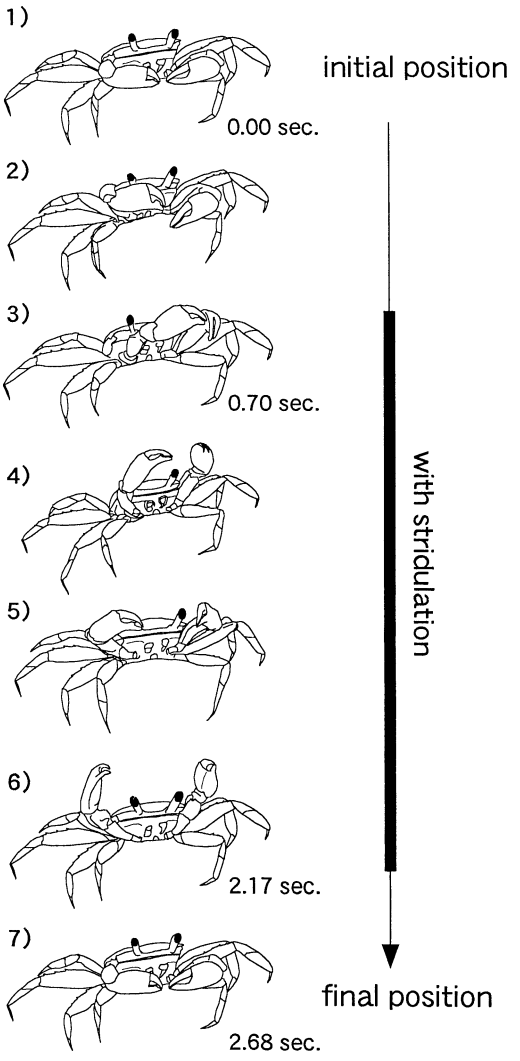

Fig. 7. Successive postures of waving display by *Metaplax distincta*. Drawings are traced from videotapes. Values below crabs indicate time in sec from the beginning of the wave (the mean value of seven waves).

by themselves were mostly monophyletic. Schubart *et al*. (2000b) proposed elevation of all grapsid subfamilies to family level, e.g., Grapsidae, Sesarmidae, Varunidae. If we adopt the family status of Grapsidae, Sesarmidae (with the exclusion of a few genera: *Metaplax*, *Cyclograpsus*, and *Helice*) and Varunidae (including these genera), the ocypodid subfamilies should be placed at the same taxonomic level (i.e., Ocypodidae, Dotillidae, Macrophthalmidae) to make taxonomy and phylogeny congruent (Fig. 5).

Evolution of Waving Display

The present phylogenetic hypothesis suggests that the behavioral similarities between *Metaplax* and *Macrophthalmus* come from convergent evolution if we consider the gain and loss of the waving behavior occurred with equal probabilities.

This leads to the question of what factors are responsible for the evolution of behavioral similarities such as occurrence of waving display. In general, a visual signal is considered to play an important role in the social communication of semi-terrestrial grapsids and ocypodids (Tweedie, 1954; Schöne, 1968). Salmon and Atsaides (1968) hypothesized that the following factors are advantageous for the development of visual signaling in the semi-terrestrial crabs: the substrate, which is flat and relatively free from the vegetational obstruction and other discontinuities; the diurnal activity of crabs; and the feeding proximity to their shelters, which leads crabs to live in colonies so that social contacts are frequent. These habitat and behavioral conditions are characteristic of many

ocypodids, such as the Ocypodinae, the Dotillinae, and *Macrophthalmus*, as well as of *Metaplax*. The habitat type of each species is shown in Fig. 5. All species studied here live at the intertidal to supratidal zone, but most species that have been classified within the family Grapsidae occur in vertical habitat, such as underneath/gap of stones or in a vegetation, where visual communication is difficult. In contrast to these species, *Metaplax* and most ocypodid species, including *Macrophthalmus,* occur in an exposed habitat, such as sand beach and mud/sand flat. Species of *Metaplax* burrow into the mud bottoms like many ocypodids. However, other closely related species of Varuninae occur in sheltered habitats (cobble-boulder or salt marsh), where visual communication is difficult, and actually these species have not been known to perform waving display. Thus, the common ecological conditions observed in *Metaplax* and many waving ocypodids—that is, horizontal and exposed semi-terrestrial habitat—are considered to play an important role in the evolution of the waving display signals. The other ecological factors suggested by Salmon and Atsaides (1968) may also have contributed to the evolution of waving display, but the available data on the diurnal activity and the feeding proximity to shelters of the species studied are not sufficient to be discussed. Future comparative studies of these ecological conditions between the species performing waving displays and species without this behavior from more taxonomic groups living in various habitats should clarify the situation for evolution of the waving display in these crabs.

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