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MEIOTIC CHROMOSOME COMPLEMENTS AND NUCLEAR DNA CONTENTS OF FOUR SPECIES OF SHRIMPS OF THE GENUS *PENAEUS*

Seinen Chow, William J. Dougherty, and Paul A. Sandifer

ABSTRACT

Meiotic chromosome spreads were prepared from testicular lobes of 4 species of penaeid shrimps to determine numbers of chromosomes and meiotic phases. DNA content of nuclei isolated from shrimp hemocytes of these species was estimated by flow cytometry. Meiotic chromosome spreads indicated that the haploid chromosome number of *Penaeus aztecus, P. duorarum,* and *P. vannamei* was 44, while that of *P. setiferus* was 45. These counts are consistent with diploid chromosome numbers reported previously for each of these species. No trivalents or quadrivalents were found in meiotic prophase. These 4 species had approximately the same genome size (approximately 70% of the human genome) as measured by flow cytometry. It is suggested that Robertsonian chromosome rearrangement has accompanied the speciation process in the genus *Penaeus* without polyploidization.

Chromosome research in the decapod Crustacea has advanced slowly (Farmer, 1974; Milligan, 1976; Hughes, 1982; Hayashi and Fujiwara, 1988). Milligan (1976) and Hayashi and Fujiwara (1988) provided new techniques to obtain mitotic chromosome metaphase preparations in penaeid shrimps, but these works are still concerned only with chromosome counts and comparison of chromosome numbers between species. Thus, no karyotype has ever been described in this animal group. Although the small size and large numbers of chromosomes present some difficulty, determination of the genome size and examination of meiotic chromosome phase may provide additional information.

In this paper, we report observations on meiotic chromosome phases in 4 species of shrimps of the genus *Penaeus,* with determinations of their genome size.

MATERIALS AND METHODS

Penaeus aztecus, P. duorarum, and *P. setiferus* were collected from Charleston Harbor, South Carolina, and off (< 1 mile or 1.61 km) Cape Lookout and Cape Fear, North Carolina, during May through November 1988. The Pacific species *P. vannamei* was derived from laboratory stock.

Chromosome Observation.—The procedure for chromosome preparation was primarily based on the methods of Farmer (1974) and Hughes (1982), without colchicine. Our preliminary observations on shrimp organs indicated that cell division is rarely encountered in any tissues except for the testis. Therefore, we concentrated on the testis. Dissected testicular lobes were exposed to distilled water for 15–20 min. The swollen testicular lobes were then transferred to fixative (methanol : acetic acid at 3:1) and refrigerated (at approximately 4°C). The fixative was changed twice within 6 h. Since dividing cells were sometimes found clustering in the proximal or medial regions of a testicular lobe (unpublished), these portions were used. One small piece (1–2 mm) was cut from each region, placed on a well-cleaned glass slide with 2 or 3 drops of fixative, and mashed and spread using forceps. The wet slide was then placed in the oven at 60°C for a minimum of 30 min. The dried glass slide was washed in 70% ethanol, followed by distilled water, and dried prior to staining with 1% toluidine blue (1 g toluidine blue and 1 g sodium borate in 100 ml H_2O, pH 8.4–8.6). The slide was placed on a slide warmer (80°C) and the staining solution was dropped on it. After 1–2 min of staining, the slide was washed with running tap water followed by distilled water.

Measurements of Nuclear DNA Contents.—The method of Hülgenhof *et al.* (1988) was modified to prepare samples of nuclei. Shrimp blood was used to collect hemocyte nuclei. Hemolymph was collected from the heart using a syringe containing 1 ml of phosphate buffered saline-ribonuclease (PBS-RNase) solution. PBS (pH 7.4–7.6) was prepared by dissolving the following chemicals (g) in 1 liter of distilled water: NaCl (10), KCl (0.6), Na_2HPO_4 (0.146), KH_2PO_4 (0.04), sodium citrate (10), and sucrose (20). Two mg of RNase were dissolved in 1 ml of distilled water, and this stock solution was kept in boiling water for 5 min. PBS-RNase solution was prepared by mixing 1 ml of RNase stock solution with 49 ml of PBS. The collected blood samples (approximately 1.1–1.5 ml) were transferred to plastic microcentrifuge tubes, held for 30 min at room temperature, and centrifuged at 600 *g* for 5 min. To remove blood serum, the supernatant was discarded and the pellet was resuspended in 1 ml of PBS-RNase solution and centrifuged. After removing the supernatant, the pellet was resuspended in 0.3 ml of PBS, and 0.7 ml of refrigerated absolute ethanol were gradually added to the cell suspension with gentle shaking. The fixed sample was kept in the refrigerator (at 4°C) for a minimum of one day. To remove cytoplasmic membrane, 0.05 ml of 1% (v/v) NP–40 solution was

added to the sample, and the sample was strongly shaken 3 times using vortex for 2 s each time. Since long exposure to NP-40 and hard treatment by vortex may damage the nuclear membrane, this procedure should be as short as possible. After confirming release of nuclei under the microscope, the sample was centrifuged at 600 *g* for 5 min, resuspended in 1 ml of refrigerated 70% ethanol, and filtered through 25-μm Nytex® mesh. Staining was carried out by adding 0.01 ml of 0.1% (w/v) propidium iodide solution per 1 ml of sample. Then, using flow cytometry (Spectrum 3, Ortho Diagnostic Systems, Inc., Irvine, California 92714), the fluorescence of hemocyte nuclei was determined in parallel with that of human lymphocytes prepared by the same procedures used for shrimp but using biological saline pH 7.4 as PBS.

Results

Chromosome Observation.—Five slides were made from each individual. Dividing cells were observed on at least one slide in 3 of 8 *P. aztecus*, 4 of 16 *P. duorarum*, 3 of 9 *P. setiferus*, and 4 of 11 *P. vannamei*. Some slides from an individual contained many dividing cells (Fig. 1), while others exhibited few or none. This finding indicates that the testicular pieces used for the preparation contained different stages of maturation of spermatogenic cells, supporting our preliminary observation in which the spermatogenic cell maturation is synchronized within a region of a testicular lobe.

All dividing cells observed were of meiosis I and no mitotic divisions were observed. Diakinesis was most frequently observed in all species (Figs. 2–7). In diakinesis, the bivalent nature of tetrads was clearly visible with some chiasmata (Figs. 2, 3, 5). No trivalent or quadrivalent forms were found in any species. The size of the chromosomes in meiosis I ranged from 1–4 μm.

Chromosome number varied within the range of 38–47 in all species, with a mode of 44 (Fig. 8). Count of a well-scattered diakinesis stage of *P. setiferus* (Fig. 7) gave $N = 45$, whereas a condensed figure (Fig. 6) showed $N = 44$. Such irregularities were often observed in these species, and apparently due to artifact.

Nuclear DNA Contents.—A representative histogram of fluorescence intensity (channel number) and number of nuclei for male *P. vannamei* is illustrated in Fig. 9. Here, 2 gaps are observed. Gap I shows a narrow distribution and has a distinct high peak, while gap II shows broad distribution and a low indistinct peak. Similar patterns were found in all species, but gap II was usually invisible. The channel number at the peak of gap I was adopted as a fluorescence intensity of nuclei of the individual and converted to a percentage of that of human lymphocytes. Thus, estimated DNA contents of the 4 shrimp species are expressed as percent of human DNA content (Table 1). Within each species, large variation of DNA content was usually observed among individuals, but comparable with the results of recent flow cytometric analysis in mammalian nuclear DNA content (Burton *et al.*, 1989). There was no significant difference (2-way ANOVA) in genome size between sexes of all species ($P > 0.25$), but the difference among species was significant ($P < 0.005$) (Table 1). Genome size for *P. duorarum* was significantly smaller than that for *P. setiferus* and *P. vannamei*, but not *P. aztecus*, as indicated by Duncan's multiple range test. The large variation observed makes precise determination of shrimp DNA content difficult and suggests that there is considerable room for technical improvement in the methods. Nevertheless, the averages suggest that the genome size of these 4 penaeid shrimp species is approximately 70% of the human genome.

Discussion

Previous studies of chromosomes in penaeid shrimp are few in number. Milligan (1976) reported diploid chromosome numbers to be 90 for *P. setiferus* and 88 for *P. aztecus* and *P. duorarum*. More recently, Hayashi and Fujiwara (1988) reported diploid and haploid chromosome number in *P. japonicus* to be 86 and 43, respectively. Our results are similar and agree with Milligan (1976). We observed the mode of haploid chromosome numbers at 44 in 4 species of

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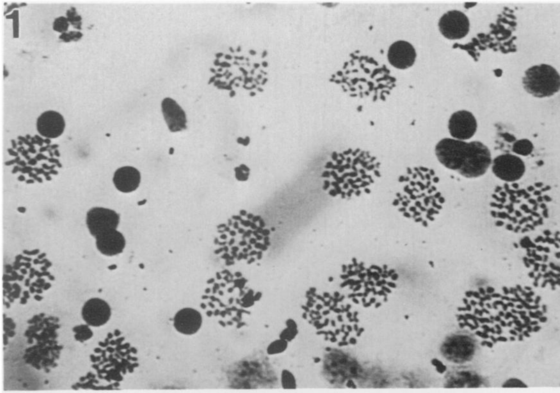
Figs. 1–7. Dividing cells in testis of *Penaeus*. Fig. 1. Clustered dividing cells on a slide prepared from a piece of the testicular lobe of *P. vannamei*. Figs. 2–7. Diakinesis stage of *P. aztecus* (2), *P. duorarum* (3), *P. vannamei* (4, 5), and *P. setiferus* (6, 7); bars are 5 μm, and arrows indicate chiasmata.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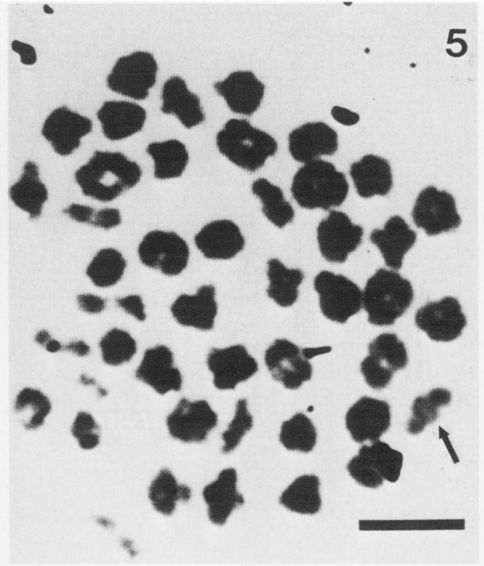
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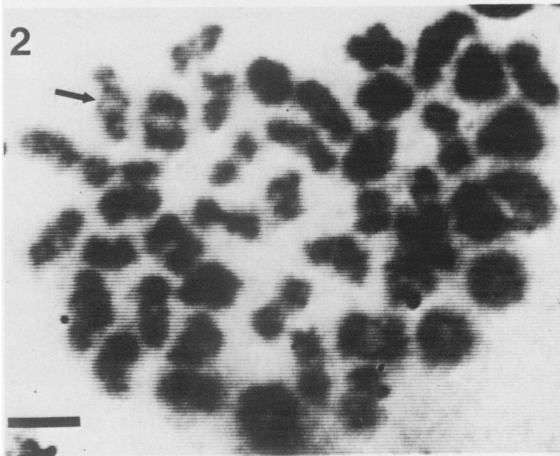
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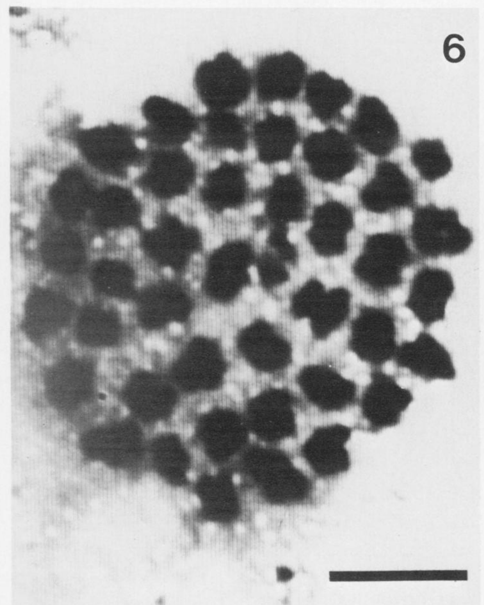
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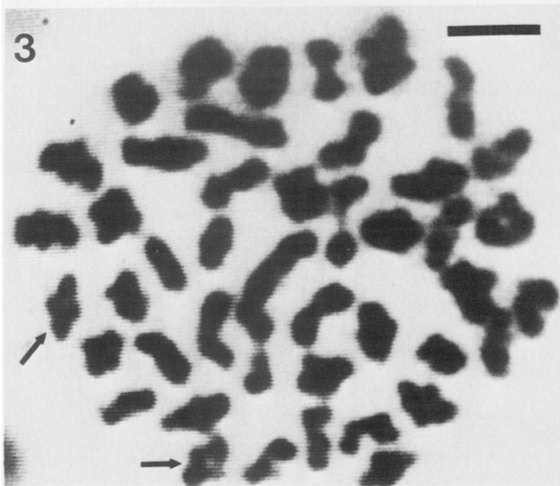
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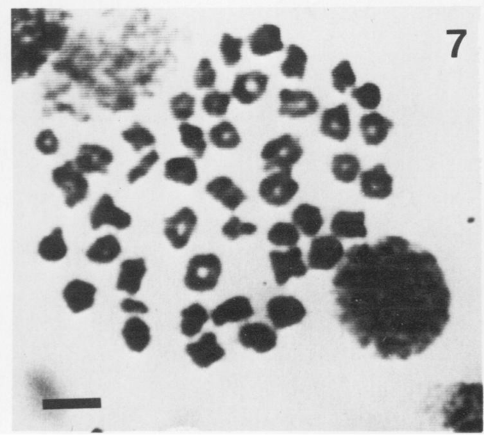
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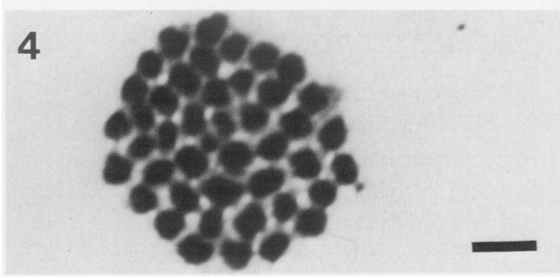
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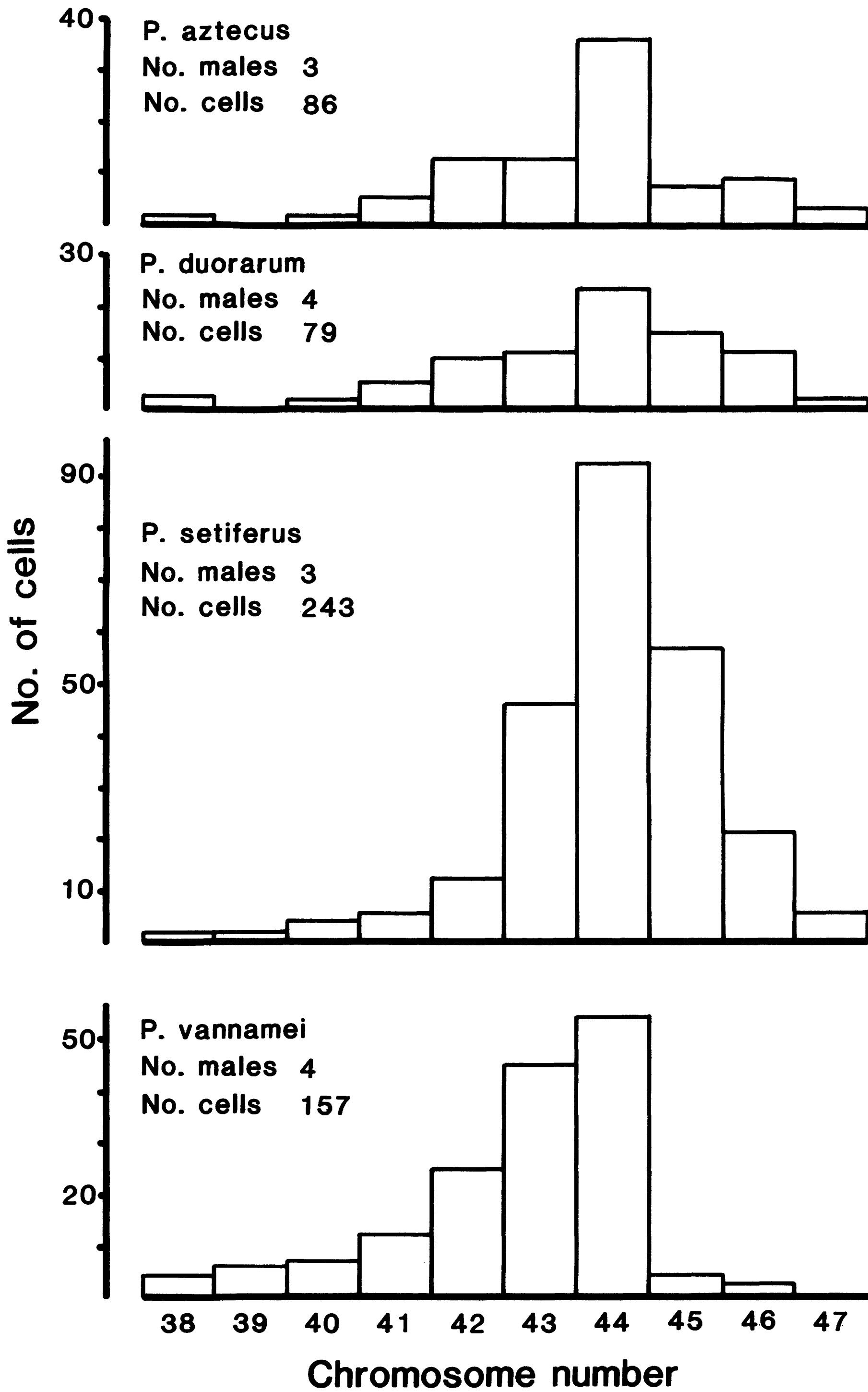

Fig. 8. The number of cells for each chromosome number in 4 species of *Penaeus*.

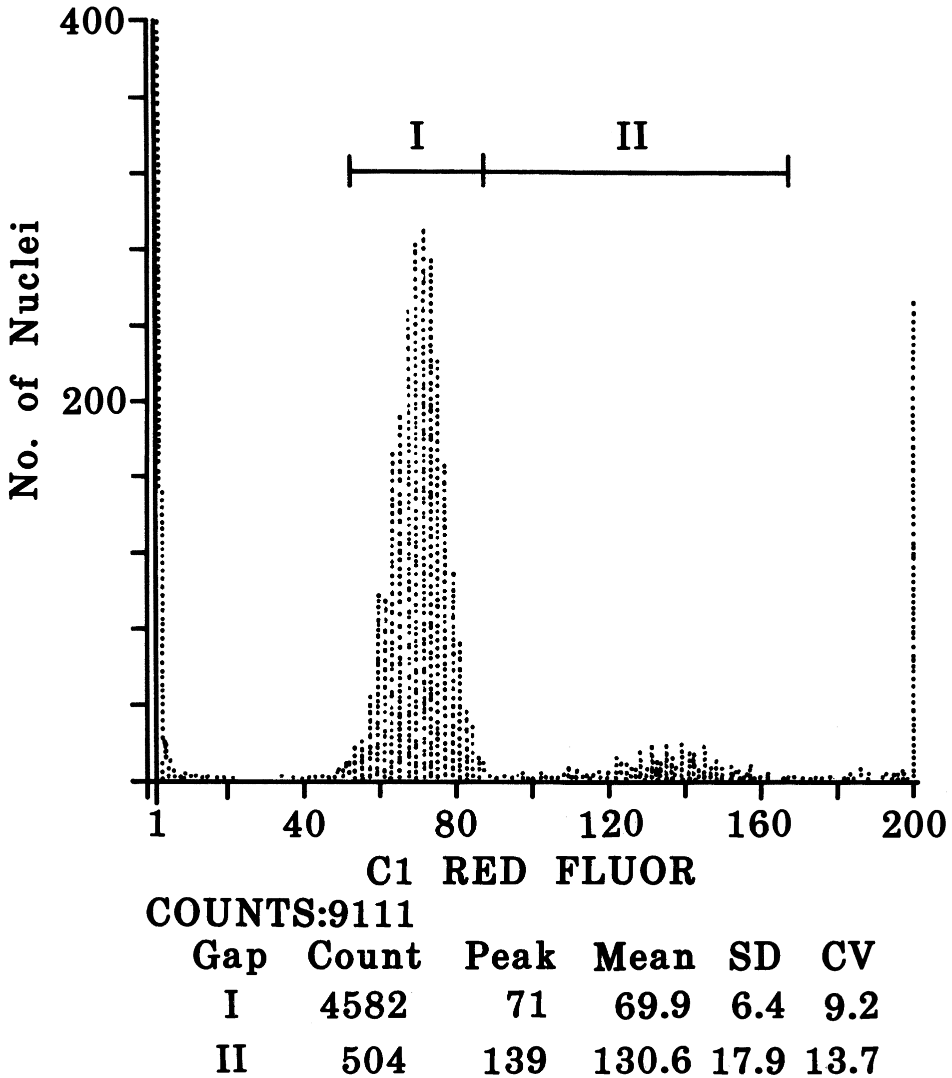

Fig. 9. DNA histogram of nuclei of shrimp hemocytes. Peak channel number of human lymphocytes was 100 at this time.

Penaeus. We also recorded extensive variation in apparent chromosome numbers of all 4 species examined. It is probable that this extensive variation was due to chromosome overlapping and loss, and possibly to contamination from other chromosome spreads because of the closely clustered nature and small size of the chromosomes. It is highly probable that the mode of our haploid chromosome number in *P. setiferus* (44) is caused by chromosome overlapping.

Karyotyping for decapod crustaceans has been difficult because of their small chromosomes. Though Milligan (1976) did not mention chromosome morphology, mitotic figures presented by him show that *P. setiferus* has 39 pairs of meta- and submetacentric and 6 pairs of acrocentric chromosomes, while *P. aztecus* and *P. duorarum* possess 40 pairs of meta- and submetacentric and 4 pairs of acrocentric chromosomes. Based on morphological features such as an open thelycum, the absence of a gastrofrontal carina, and the presence of a hepatic carina, Perez Farfante (1969) suggested that the subgenus *Litopenaeus* (represented by *P. setiferus* and *P. vannamei* in this study) is the most primitive group in

Table 1. DNA content in *Penaeus* (expressed as a percentage of that of human DNA). Number of individuals is in parentheses. Means with same superscripts in a given column are not significantly different (2-way ANOVA, Duncan's Multiple Range Test, $\alpha = 0.05$).

Species	Percentage DNA content ± SD		
	Male	Female	Total
Penaeus aztecus	68.5 ± 5.9 (7)	70.4 ± 3.6 (16)	69.8 ± 4.5^{ab}
Penaeus duorarum	68.2 ± 3.6 (9)	67.3 ± 4.2 (18)	67.6 ± 4.1^{b}
Penaeus setiferus	70.2 ± 6.1 (32)	72.8 ± 4.8 (30)	71.6 ± 5.7^{a}
Penaeus vannamei	71.7 ± 4.8 (21)	71.4 ± 5.6 (34)	71.5 ± 5.2^{a}

the genus *Penaeus.* Robertsonian fusion (Robertson, 1916) may have occurred between two pairs of acrocentric chromosomes without drastic changes in nuclear DNA content. This has been noted in some fish and mammalian species (Ohno, 1970). Although Hayashi and Fujiwara (1988) did not mention chromosome types in *P. japonicus,* the diploid chromosome number of 86 suggests that an additional Robertsonian fusion may have occurred in this species. We suggest that chromosome rearrangements have accompanied the speciation process in the genus *Penaeus,* possibly with minor tandem gene duplication or chromosomal deletion but without polyploidization.

The nuclear DNA content of decapod Crustacea was first reported by Mirsky and Ris (1951), followed by Vaughn and Locy (1969), Musich and Skinner (1972), Bachmann and Rheinsmith (1973), and Rheinsmith *et al.* (1974). These investigators used the Feulgen reaction photocytometry methods. Bachmann and Rheinsmith (1973) and Rheinsmith *et al.* (1974) reported that the nuclear DNA content of *P. duorarum* is 82 and 74% (of human content), respectively, while we recorded a value of 68% here. Similarly, Vaughn (1976) reported the DNA content of *P. setiferus* as 101% of human content, while we found it to be 72%. Theoretically, there should be little difference between DNA content determination with Feulgen reaction photocytometry and flow cytometry, but the sample size used for the former was usually quite small. For example, most reports of nuclear DNA content for decapods referenced above are based on only 1 or 2 individual animals. Furthermore, shrimp DNA content values determined by us using flow cytometry were slightly lower than those reported based on Feulgen reaction photocytometry. A similar tendency has also been observed among plant species (Hülgenhof *et al.,* 1988). However, large variations within species observed in the present study generally overshadowed such differences related to methods, with the exception of the high value for *P. setiferus* reported by Vaughn (1976). Intraspecific variations in DNA content would be expected to be insufficient to account for the wide range of values observed.

We reviewed the relative nuclear DNA content of 58 decapod species (Table 2). Since some of the references did not express DNA amount relative to that for human DNA, we converted the DNA quantity (in picograms) to percentage of human DNA content.

Although the brachyurans were the most extensively studied group of decapod crustaceans, with reports on 26 species of 19 genera in 9 families, the range of DNA values observed (38–130% of that for human DNA) was much narrower than that seen in carideans (90–1,000% of human DNA) and anomurans (35–230% of human DNA). The average nuclear DNA content of the five species of *Penaeus* studied to date is 74% of that of human DNA, or approximately twice the minimum level observed in other decapods. Evolution by polyploidization could account for the great variation in the genome size of decapod crustaceans (Bachmann and Rheinsmith, 1973; Rheinsmith *et al.,* 1974). However, Vaughn (1975, 1976), using reassociation kinetics of heat-denatured DNA, observed that there is insufficient DNA in the haploid decapod genome to support the polymeric chromatid model. He concluded that the chromatid structure of decapod crustaceans is uninemic, and that there was no detectable occurrence of polyploidization in the recent evolutionary history of decapods. Vaughn also suggested that addition of repeated sequences to linear uninemic chromatids may account for the genome size increases. A

Table 2. Comparison of nuclear DNA content in 58 decapod crustacean species (DNA content is expressed as a percentage of that of human DNA).

Suborder	Species	Percentages in references						
		(1)	(2)	(3)	(4)	(5)	(6)	(7)
Dendrobranchiata	*Penaeus aztecus*	—	—	—	—	—	—	70
	Penaeus duorarum	—	—	—	82	74	—	68
	Penaeus marginatus	—	—	—	66	—	—	—
	Penaeus setiferus	—	—	—	—	—	101	72
	Penaeus vannamei	—	—	—	—	—	—	72
Pleocyemata Caridea (Infraorder)	*Atya bisulcata*	—	—	—	197	—	—	—
	Antecaridina sp.	—	—	—	90	—	—	—
	Halocaridina rubra	—	—	—	129	—	—	—
	Palaemon debilis	—	—	—	225	—	—	—
	Palaemonetes kadiakensis	—	—	—	—	226	—	—
	Macrobrachium acanthurus	—	—	—	—	185	—	—
	Macrobrachium grandimanus	—	—	—	219	—	—	—
	Macrobrachium lar	—	—	—	200	—	—	—
	Macrobrachium ohione	—	—	—	—	633	—	—
	Alpheus crassimanus	—	—	—	225	—	—	—
	Alpheus heterochaeli	—	—	—	—	466	—	—
	Metabetaeus lohena	—	—	—	112	—	—	—
	Pandalus platyceros	—	—	—	—	—	1,000	—
Astacidea	*Procambarus clarkii*	—	—	—	170	—	—	—
	Cambarus sp.	—	—	—	—	161	—	—
Palinura	*Panulirus interruptus*	—	—	—	—	—	126	—
Anomura	*Clibanarius vittatus*	—	—	—	—	35	—	—
	Clibanarius zebra	—	—	—	79	—	—	—
	Dardanus gemmatus	—	—	—	230	—	—	—
	Calcinus laevimanus	—	—	—	156	—	—	—
	Calcinus latens	—	—	—	121	—	—	—
	Pagurus longicarpus	—	—	—	—	143	—	—
	Pagurus pollicaris	—	—	—	—	124	—	—
	Pagurus samuelis	—	—	—	208	—	208	—
	Petrochirus diogenes	—	—	—	121	—	—	—
	Petrolisthes galathinus	—	—	—	—	60	—	—
	Emerita analoga	—	79	—	—	—	—	—
Brachyura	*Randalia ornata*	—	—	—	—	47	—	—
	Hepatus epheliticus	—	—	—	49	48	—	—
	Libinia emarginata	—	—	—	63	60	—	—
	Libinia sp.	—	—	—	107	—	—	—
	Libinia sp.	—	—	—	—	130	—	—
	Cancer anthoai	—	—	—	—	49	—	—
	Cancer borealis	—	—	—	—	—	60	—
	Callinectes sapidus	—	—	—	52	61	58	—
	Scylla serrata	—	—	—	58	—	—	—
	Thalamita edwardsi	—	—	—	55	—	—	—
	Etisus laevimanus	—	—	—	68	—	—	—
	Eurypanopeus depressus	—	—	—	77	86	—	—
	Menippe mercenaria	—	—	—	—	76	—	—
	Neopanope texana	—	—	—	—	82	—	—
	Neopanope sp.	—	—	—	—	76	—	—
	Ocypode ceratophthalmus	—	—	—	66	—	—	—
	Uca crenulata	—	—	—	—	56	—	—
	Uca pugilator	—	—	—	60	65	—	—
	Uca sp.	—	—	—	—	52	—	—
	Pachygrapsus crassipes	—	—	—	77	80	—	—
	Metopograpsus messor	—	—	—	66	—	—	—
	Sesarma cinereum	—	—	—	121	114	—	—
	Plagusia depressa	50	—	—	49	—	—	—
	Grapsus grapsus	—	—	—	—	38	—	—
	grapsid crab	—	—	—	—	87	—	—
	Gecarcinus lateralis	—	—	44	—	—	—	—

References: (1) Mirsky and Ris (1951), (2) Vaughn and Locy (1969), (3) Musich and Skinner (1972), (4) Bachmann and Rheinsmith (1973), (5) Rheinsmith *et al.* (1974), (6) Vaughn (1976), (7) present study.
In reference (1) the percentage of genome size was calculated by the human diploid genome size (6.0 pg).
In references (2)–(6) 7.3 pg diploid human genome size was used to calculate the relative DNA content.

relatively simple electrophoretically detectable gene-enzyme system in decapods (Nelson and Hedgecock, 1980; Chow and Fujio, 1987) was also negative for polyploidization (see also Hedgecock *et al.*, 1982: 285–289). Our meiotic chromosome figures do not indicate any traces of polyploidization. Yet, chromosomal diploidization may be complete in this animal group, in contrast to some salmonid fishes and amphibians which are still in the process of diploidization (Ohno, 1970). Furthermore, recent research on ontogenic changes in isozyme gene expression in penaeid shrimps (Lester and Cook, 1987) leaves room for consideration of polyploidization.

Whether polyploidization has occurred or not, obvious differences in genome size occur among major groups of decapod crustaceans, and there may be a tendency toward greater variation in phylogenetically older groups. Thus, it would be very interesting to determine DNA content for species of the suborder Dendrobranchiata.

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Addresses: (SC) Rosenstiel School of Marine and Atmospheric Science, 4600 Rickenbacker Causeway, Miami, Florida 33149; and (PAS) Marine Resources Research Institute, South Carolina Wildlife and Marine Resources Department, P.O. Box 12559, Charleston, South Carolina 29412; (WJD) Marine Biomedical Research Program, Medical University of South Carolina, P.O. Box 12559, Charleston, South Carolina 29412.