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Artificial interspecific hybridization between *Macrobrachium* species

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Abstract

Viable F_1 hybrids were obtained from crosses of female *Macrobrachium nipponense* and male *Macrobrachium hainanense* involving spermatophore transfer and artificial insemination. This represents the first successful known case of hybridization of two *Macrobrachium* species by means of artificial insemination. The hatching rate was over 90%. About 20–60% of newly hatched larvae metamorphosed to postlarvae. The morphological characteristics of the hybrids resembled a combination of features of both parents. Malate dehydrogenase (MDH) and esterase (EST) isozyme electrophoresis indicated parents and F_1 hybrids showed co-dominant expression of the paternal and maternal alleles controlling the isozymes and confirmed the hybridization.

Keywords: Freshwater prawn; *Macrobrachium*; Hybridization; Artificial insemination; Spermatophore transfer; Isozyme; Genetic breeding

1. Introduction

Macrobrachium nipponense is one of the most important freshwater prawns for aquaculture in China, especially in the southern regions of the country. Recently however, *M. nipponense* farming has seriously declined due to diseases and lower sizes available for

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markets. Genetic improvement may help this situation for *M. nipponense*. Cross breeding has potential as a classical method of genetic improvement and has been very successful in agriculture. Sankolli et al. (1982) reported a successful natural mating between *M. rosenbergii* and *M. malacolmsonii*. Shokita (1978) obtained hybrids from natural mating between *M. asperulum* and *M. shokitai*. Uno and Fujita (1972) reported experimental hybridization between *M. formosense* and *M. nipponense*. American (*H. americanus*) and European (*H. gammarus*) lobsters were found to be inter-fertile and hybrids between them had potential for genetic improvement (Carlberg et al., 1978). Sandifer and Smith (1979) developed methods for artificial insemination based on spermatophore transfer, and reported successful hybridization between two closely related *Palaemonetes* species. Interspecific hybridization between penaeid species by artificial insemination has been successful in several combinations (Lawrence, 1984; Lin and Ting, 1988; Bray et al., 1990). However, most *Macrobrachium* species do not hybridize interspecifically by natural mating. In addition, interspecific hybridization through artificial insemination between *Macrobrachium* species has also been unsuccessful, although several attempts have been made (Sandifer and Smith, 1979; Sandifer and Lynn, 1980; Graziani et al., 2002). Our research focuses on hybridization between *Macrobrachium* species using artificial insemination.

2. Materials and methods

M. nipponense was collected from the Taihu Lake of Jiangsu province, China and *M. hainanense* from the Oujiang River of Zhejiang province, China. Artificial insemination was performed according to the methods of Sandifer and Smith (1979) with some modifications.

2.1. Selection and rearing of parent prawns

Healthy female *M. nipponense* and male *M. hainanense* of body lengths between 6 and 7 cm with well-developed gonads were selected as parents. Female *M. nipponense* and male *M. hainanense* were reared separately in two $100 \times 50 \times 60$-cm tanks with water temperature maintained at 22–29 °C and constant aeration provided. Tree branches were placed in the tanks in order to provide habitat. The prawns were fed ad libitum with snail meat, potato and compound feedstuff.

2.2. Spermatophore transfer, artificial insemination and spawning

After each female underwent a pre-nuptial molt, a mature male was selected. Firstly, spermatophores were collected by a forceps, then the molted female was handled. The spermatophores were placed on the abdomen of the molted female between her fourth pereiopods in such a manner that it will insure its adherence to the female. This transfer process was completed usually within 5 h after the female's pre-nuptial molt. The female containing the transferred spermatophore was maintained individually in a spawning tank. The females generally spawned within 12–72 h after

the artificial insemination. Fertilization occurred automatically at the time of spawning and the fertilized females carrying their incubating eggs ("berried females") were maintained in a nursery/hatching tank that contained tree branches to provide habitat for the larvae after they hatch. The water temperature was maintained between 27 and 29 °C and constant aeration was provided.

2.3. Hatching, nursery and grow out

Larvae hatched from the brooding females egg mass after 10–20 days of incubation. The larvae were fed *Artemia* three times per day ad libitum. The larvae rearing tanks were cleaned twice daily to remove excess feed and wastes. Ideal water quality was maintained. After all, 20–60% the larvae metamorphosed into postlarvae. They were cultured in the nursery/hatching tank for an additional 10–15 days until the hybrids had grown into small juveniles of approximately 2.5 cm, which were then stocked into outdoor concrete ponds and raised into 5–8-cm-long adults.

2.4. Isozyme analysis

2.4.1. Sample preparation

Muscle was sampled directly from the abdomen of the prawns after sacrifice. The fresh muscles were washed out from contaminating fluids with distilled water, homogenized in distilled water at a ratio of 1 g/4 ml water, and centrifuged at 14,000 rpm for 20 min. The supernatants were stored at −20 °C for later use.

2.4.2. Electrophoresis and staining

Esterase (EST) and Malate dehydrogenase (MDH) were analyzed by vertical polyacrylamide gel electrophoresis. Electrophoresis for esterase (EST) was performed 1.5 h at 240 V with a gel concentration of 7.5% in a buffer system made up of 0.155 M Tris–0.043 M Citric acid and maintained at pH 7.4. Malate dehydrogenase (MDH) analysis was performed for 2 h at 270 V in a 8.5% gel concentration made with a buffer system of 0.25 M Tris–0.057 M citric acid, and maintained at pH8.0. Gels were stained according to the method of Shaw and Prasad (1970).

3. Results

3.1. Hybridization between M. nipponense (female) and M. hainanense (male)

In total, five single pair crosses were attempted, four resulted in successful spawning and berried females. Three of these clutches hatched a family of hybrids with a hatching rate over 90%. The total survival rate from early zoeae to postlarvae was 20%, 27% and 60%, respectively, and from postlarvae to subadults was 23%, 26% and 52% for the above three clutches of hybrids (Table 1). The duration from fertilization to hatching was about 10 days at 28 °C, and from hatching to postlarvae

Table 1
Results of hybridization between *M. nipponense* (female) × *M. hainanense*

Cross number	Spawning	Zoeae	Postlarvae (survival rate)	4–6-cm Subadult (survival rate)
1#	+	0	0	0
2#	+	500	100 (20%)	23 (23%)
3#	+	2300	630 (27%)	165 (26%)
4#	–	0	0	0
5#	+	1200	720 (60%)	372 (52%)

was 17–25 days at 28–30 °C. About 3.2% surviving late zoeae were unable to metamorphose into postlarvae and finally died after an additional 5–10 days of surviving. A total of 236 hybrids developed into adults.

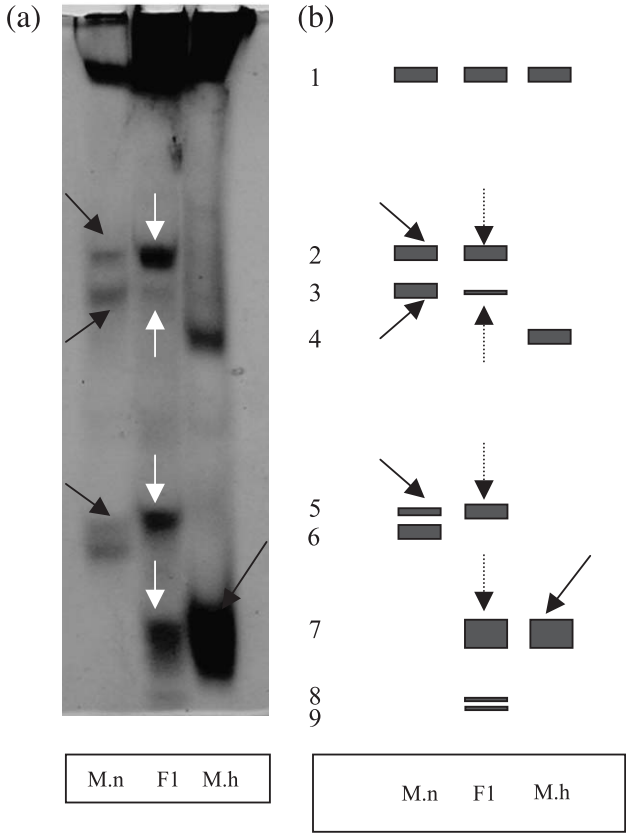

Fig. 1. (a) Stained gel for Esterase (EST) isozymes. Dark single arrows show Bands 2, 3, and 5 from M.n and Band 7 of M.h; similar counterpart bands in the hybrid are shown with white arrows. (b) Drawing interpretation of stained gel in frame (a). Arrows indicate gel bands of interest as in frame (a). Lane pattern from left to right is M.n: *M. nipponense*, "F1": hybrid, M.h: *M. hainanense*. Arabic numbers 1–9 in frame (b) represent the serial numbers of electrophoretic bands.

3.2. Isozyme analysis indicated hybrids expressed both paternal and maternal genes

3.2.1. Esterase (EST)

A total of five bands were seen in female *M. nipponense*. Four of these were expressed in the hybrids (Bands 1, 2, 3, 5) but one (Band 6) failed to be expressed. Three bands appeared in male *M. hainanense*, of which two (Bands 1 and 7) were exhibited in the hybrids and one (Band 4) did not appear. Bands 2, 3 and 5 of hybrids matched those of *M. nipponense*, and Band 7 of hybrids matched that of *M. hainanense*. The most anodal two pale bands in the hybrids did not match any band of *M. nipponense* and *M. hainanense*; these were probably a recombination of isozyme subunits from the parents. The slowest band expressed in the hybrids, with intensive staining, was probably an overlapping expression of the parental alleles (Fig. 1).

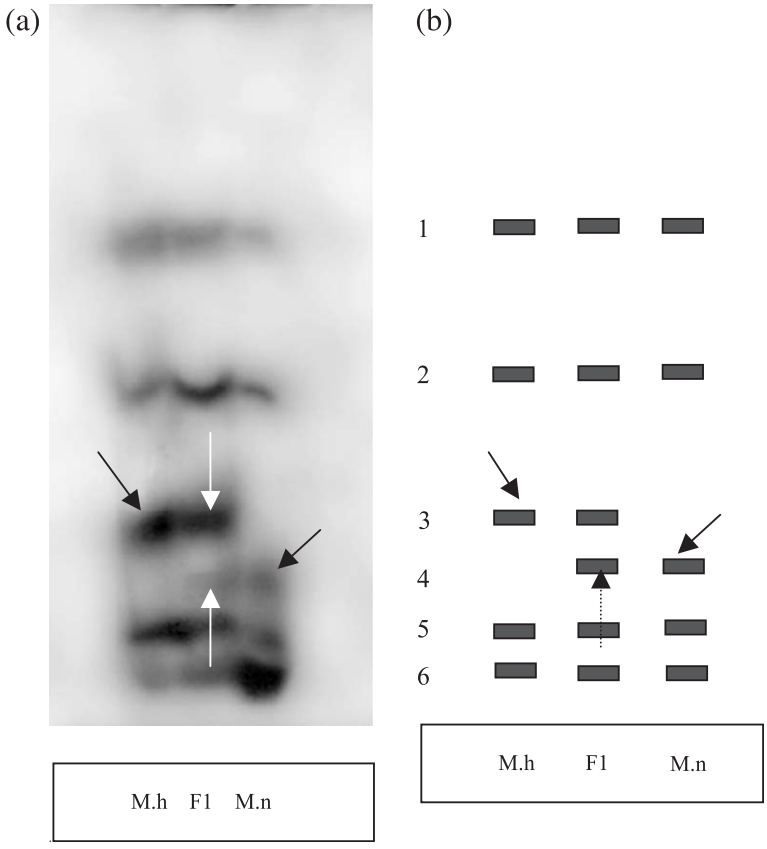

Fig. 2. (a) Stained gel for Malate dehydrogenase (MDH) isozymes. Dark single arrows show Bands 3 from M.h. and Band 4 of M.n; similar counterpart bands in the hybrid are shown with white arrows. (b) Drawing interpretation of stained gel in frame (a). Arrows indicate gel bands of interest as in frame (a). Lane pattern from left to right are M.h: *M. hainanense*, "F1": hybrid, M.n: *M. nipponense*. Arabic numbers 1–6 in frame (b) represent the serial numbers of electrophoretic bands.

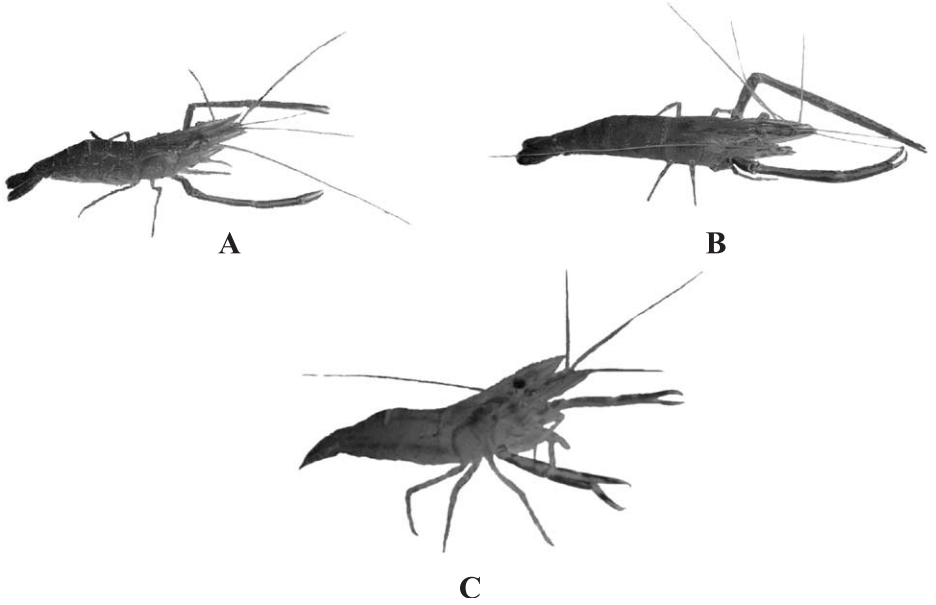

Fig. 3. The morphological characteristics of the hybrids appeared to be a combination of the traits of their parents. Body shape of the hybrid resembled the female parent (*M. nipponense*). The second pereiopod of hybrids had clearly circular flecks and curved fingers, which were similar to that of *M. hainanense* but different from *M. nipponense*. The flecks of the second pereiopod of *M. nipponense* are irregular and unclear, its fingers are straight. (A) *M. nipponense*; (B) *M. hainanense*; (C) F_1 Hybrid.

3.2.2. Malate dehydrogenase (MDH)

A total of five bands appeared in both female *M. nipponense* and male *M. hainanense* (Fig. 2). The mobility of Bands 1, 2, 5 and 6 of *M. nipponense* was similar to that of Bands 1, 2, 5 and 6 of *M. hainanense*, respectively. Band 4 of *M. nipponense* was not found in *M. hainanense*, but shared with the hybrid. Similarly, Band 3 was found in *M. hainanense* but not in *M. nipponense* and this band was shared with the hybrid. This provides direct electrophoretic evidence of the parental origin of the hybrids. Fig. 2 shows the stained gel (frame a) and an interpretive drawing of the gel banding pattern, frame (b).

3.2.3. General morphology

The morphological characteristics of the hybrids appeared to be a combination of the traits of their parents. Body shape of the hybrid resembled the female parent (*M. nipponense*). The second pereiopod of hybrids had clearly circular flecks and curved fingers, which were similar to that of *M. hainanense* but different from *M. nipponense*. The flecks of the second pereiopod of *M. nipponense* are irregular and unclear, its fingers are straight (Fig. 3).

4. Discussion

M. nipponense and *M. hainanense* coexisted in several rivers of China but no hybrids were found in natural waters. Our control experiments also showed that no any sex attraction and mating behavior existed between them. Species are generally isolated from one another by temporal, spatial, behavioral, physiological or developmental barriers. Any one or a combination of factors related to these could play a role in the fact that most *Macrobrachium* species cannot hybridize naturally and that the attempts to hybridize them using artificial insemination have had limited success. Our work apparently circumvented any spatial, behavioral and temporal barriers between *M. nipponense* and *M. hainanense*. It showed that the isolation between the two species is chiefly because of physiological barriers and no developmental barriers to interspecific hybribization existed.

Artificial interspecific hybridization between freshwater prawns species including *Macrobrachium* species seems to be more difficult than between species of *Penaeus*. This is most probably due to the fact that female penaeid shrimp have a thelycum, a ventral external structure that can store the male spermatophores for weeks or months in nature. The thelycum is not present in freshwater prawns. This allows artificial insemination to be performed in penaeid shrimp within a broad time span well before spawning. In contrast, for the analogous procedure to be successful in freshwater prawns, it must be completed within a narrow window of time; only a few hours after the female undergoes her pre-nuptial molt.

Our success in this work was probably because we made some modifications according to Sandifer and Smith's method (Sandifer and Smith, 1979). In their method, firstly, the females were blotted with paper toweling and wrapped in damp toweling, then collected the spermatophores from the males. In our work, firstly, spermatophores were collected by a forceps, then the molted females were handled so as to lessen disturbances to the females and their duration out of water. We also maintained the females containing transferred

spermatophores individually in a spawning tank contained tree branches, which provided a peaceful and quiet environment and lowered the possibility of dislodging spermatophores. In addition, we paid careful attention to the females' exact molting condition and applied the spermatophore at the right time. All those improvements were important and certainly raised the probability of success. On the other hand, *M. nipponense* and *M. hainanense* are most related species. No genetic barrier existed between them for the whole development, which is the basis of our success.

5. Conclusion

Our work has shown that the progeny of crosses of *M. nipponense* and *M. hainanense* was undoubtedly the F_1 hybrids of these parental groups. This is the first successful known case of the artificial interspecific hybridization of two *Macrobrachium* species.

This result holds significance in theory and value for the genetic improvement of freshwater prawns through possibilities to utilize hybrid vigor (heterosis) and genetic selection to produce new varieties for commercial freshwater prawn farming.

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