

3.2. Genetic differentiation between populations

For each locus, 21 pair-wise comparisons of allele frequency distributions were made. Across six loci, significant heterogeneity of allele distributions was detected in 73 of 126 possible tests after sequential Bonferroni adjustment. Local stocks (Hat 1 and Hat 2) differed in allele frequencies at *Mbr-1* while Nat 1 and Hat 5 differed at *Mbr-5*. The samples from Nat 1, Hat 1, Hat 2 and Hat 5 were among the similar groups in which only 25% of the tests were significant (9 out of 36 tests). Large differentiations were detected between the Myanmar (Hat 3) and the India (Hat 4) from the others in which more than 75% of the tests were significant (42 out of 54 tests). Hat 3 and Hat 4 differed from the rest of the populations at four to six loci. When probabilities were combined across loci, all comparisons were statistically significant, suggesting strong differentiation among populations.

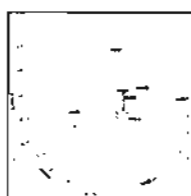
Overall and single locus estimates of F_{ST} indicated high levels of differentiation among populations. The overall F_{ST} value of 0.0621 (95% CI: 0.0415–0.0917) indicated that 6% of the detected variation arises from between population differences and 94% of within population differentiation. The values were significant ($P < 0.05$) over all loci. All population pair-wise comparisons exhibited significant F_{ST} values ($P < 0.05$, Table 3). The highest genetic differentiation using both D_A and F_{ST} distances was observed between the Myanmar (Hat 3) and the India (Hat 4) populations ($D_A = 0.0948$, $F_{ST} = 0.1222$). The ChaoPhaya population was very similar to the hatchery population (Hat 5) from Indonesia ($D_A = 0.0081$, $F_{ST} = 0.0113$). Among hatchery stocks, the local populations (Hat 1 and Hat 2) were very similar ($D_A = 0.0102$, $F_{ST} = 0.0143$). The MDS plot revealed genetic relationships of three different groups (Fig. 2). Group 1 consisted of five populations (Nat 1, Nat 2, Hat 1, Hat 2, and Hat 5). Groups 2 and 3 were Hat 3 (Myanmar) and Hat 4 (India) populations. The UPGMA dendrogram constructed from the D_A matrix corresponded to the MDS plot and origins of hatchery stocks (Fig. 3). The ChaoPhaya River, Hat 5, Hat 1, and Hat 2 populations were grouped together in a single major branch, which also contained the Kraburi River (Nat 2) population. The Hat 3 and Hat 4 populations were clearly separated from each other and from the major group of the ChaoPhaya River origin. The dendrogram constructed by the neighbor joining method displayed the same genetic relationships as the UPGMA clustering.

Table 3.

Matrix of pair-wise F_{ST} values (above diagonal) and genetic distances (D_A) between seven populations

Population	Nat 1	Nat 2	Hat 1	Hat 2	Hat 3	Hat 4	Hat 5
Nat 1		0.0441	0.0312	0.0261	0.0603	0.0635	0.0113
Nat 2	0.0318		0.0572	0.0480	0.0821	0.1110	0.0434
Hat 1	0.0224	0.0412		0.0143	0.0619	0.1092	0.0176
Hat 2	0.0191	0.0346	0.0102		0.0595	0.1019	0.0129
Hat 3	0.0441	0.0583	0.0458	0.0439		0.1222	0.0599
Hat 4	0.0486	0.0869	0.0865	0.0810	0.0948		0.0952
Hat 5	0.0081	0.0316	0.0125	0.0098	0.0450	0.0765	

All pair-wise F_{ST} values were significant at $P < 0.05$.



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Fig. 2. A multidimensional (MDS) plot based on D_A distances showing genetic relationships among seven populations of freshwater prawn.



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Fig. 3. UPGMA dendrogram clustering Nei's genetic distances (D_A) among seven populations of freshwater prawn.

4. Discussion

Freshwater prawn culture has been practiced in Thailand for more than three decades. Since the success of post-larval production in the 1970s, broodstocks have been kept in captivity without proper management of breeding programs. Breeding records including the size of founding population and the number of broodstock for each generation were not known. The issue of inbreeding and genetic deterioration of local stocks was raised by both prawn farmers and scientists as the probable cause of depressing farm production. As a result, several hatchery managers started to introduce non-native stocks from the neighboring country, i.e., Myanmar and from India to replace their own stocks. In this study, we found evidence of departures from HWE in all hatchery populations, in which heterozygote deficiencies were exhibited, except the Myanmar (Hat 3) population. The departures from HWE could have resulted from both small sample size and mixing of populations within samples. The practice of acquiring broodstocks of different genetic background is common among local hatcheries. It was difficult to determine the genetic relationship among broodstocks because the pedigree and sale records of broodstock were mostly non-existent. Inbreeding also affects allele frequencies by increasing the number of homozygotes, thus changing genotype distribution within populations relative to HWE. However, we found that all hatchery and wild populations exhibited similar and relatively high levels of genetic variation, including number of alleles per locus, allele richness and heterozygosities. It was, therefore, unlikely that inbreeding depression would be the cause of their departures from HWE or poorer performance of the stocks. Among hatchery stocks, the local population (Hat 2) showed the highest number of allele per locus and allele richness (10.67, 9.37), while the Myanmar population (Hat 3) had the smallest (7.5, 6.34). In this case, the Hat 3 population may have established from a small number of broodstocks. Founder effect and genetic drift may have eliminated rare alleles present in the original population resulting in the low allelic diversity and the heterozygosity excess of the Hat 3 samples. Among populations of the ChaoPhya River origin, the numbers of alleles existing in two local hatchery stocks (Hat 1 and Hat 2) for loci *Mbr-5* and *Mbr-7* were larger than the Nat 1. The absence of low frequency alleles at these loci from the Nat 1 population was likely due to sampling error.

Population pair-wise F_{ST} values suggested various levels of gene frequency differences among the studied populations. We have found evidence of significant population sub-structuring among all samples from hatcheries and natural waters. The MDS analysis using Nei's (1978) unbiased minimum distance clearly supported the above population differentiation analysis based on pair-wise F_{ST} values. The hatchery samples from Indonesia (Hat 5) appeared to be closely related to the ChaoPhya River (Nat 1) and the local hatcheries

(Hat 1 and Hat 2) samples. It might be possible that the hatchery stock of Indonesian (Hat 5) was introduced from Thailand. However, there was no documented record to confirm the assumption. Among local hatchery populations, the weak differences between Hat 1 and Hat 2 samples implied that they were of similar origins. According to our survey, most of the small or medium size hatcheries including Hat 1 and Hat 2 did not practice selective breeding program nor maintain their own broodstock. Alternatively, they sell post-larvae to grow-out farms in the area and purchase gravid females for producing post-larvae. Because of the similarity of Hat 1 and Hat 2 to the ChaoPhaya River samples, one might think that they were originated from the wild population of the ChaoPhaya River. However, the history of restocking program and our results suggested otherwise. The *Macrobrachium* population in the ChaoPhaya River had gone to nearly extinction after the closing of the ChaoPhaya dam in the 1970s interrupting migration of the species. The population reappeared after the Department of Fisheries (DOF) has made a decision to embark on the Intense restocking program to re-establish local population almost 30 years ago. It was, therefore, likely that the hatchery populations rather than the wild contributed to most of the genetic diversity detected in the ChaoPhaya River samples. For the Kraburi River samples, the situation differed because no restocking program has been done in the Kraburi River. The amount of genetic diversity detected in the samples was contributed by the wild population. Further, the slight but significant differentiation between these two populations was clearly due to geographical separation.

This study demonstrated that local hatchery stocks had more genetic variation than was expected based on the comparison to other wild populations. Reduced genetic diversity has been observed in several hatchery stocks of fish and crustacean, e.g., Japanese flounder (Sekino et al., 2002), Nile tilapia (Brummett et al., 2004), channel catfish (Simmons et al., 2006), Kuruma prawn (Luan et al., 2006) and tiger prawn (Xu et al., 2001). In most cases, selection and hatchery practices were the prime factors causing reduction of genetic variability in aquaculture populations. For freshwater prawn, common practices of using gravid females to produce larvae may also lead to a reduction of the effective population size and subsequent inbreeding. However, the negative effects of hatchery practices were undetectable in this study. The presence of high genetic variation in the local hatchery populations was due to the fact that the large numbers of broodstocks were used in each generation. To prevent the chance of inbreeding that might occur in later generations, hatchery managers should start keeping breeding records and pedigree information of breeders.

In conclusion, this study provided baseline for genetic investigations of freshwater prawn aquaculture and practical guidelines for hatchery managers. The high levels of genetic variation in local hatchery stocks suggested that genetic factors were unlikely to cause low production of this species. A proper management program of local hatchery stocks is needed to preserve genetic diversity for further development of prawn aquaculture. Introduction of non-native stocks to increase production may have adverse effects on the genetic integrity of local populations. Moreover, prawn producers should start investigating other potential problems that might have negative effects on productivity.

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References

- Archie, 1985 J.W. Archie, Statistical analysis of heterozygosity data: independent sample comparisons, *Evolution* **39** (3) (1985), pp. 623–637. Full Text via CrossRef
- Brummett et al., 2004 R.E. Brummett, D.E. Angoni and V. Poumogne, On-farm and on-station comparisons of wild and domesticated Cameroonian populations of *Oreochromis niloticus*, *Aquaculture* **242** (2004), pp. 157–164.

SummaryPlus | Full Text + Links | PDF (154 K) | View Record in Scopus | Cited By in Scopus (1)

Charoentawee et al., 2006 K. Charoentawee, S. Poompuang and U. Na-Nakorn, Isolation and characterization of microsatellites in giant freshwater prawn *Macrobrachium rosenbergii*, *Mol. Ecol. Notes* **6** (2006), pp. 823–825. Full Text via CrossRef | View Record in Scopus | Cited By in Scopus (0)

Excoffier et al., 2006 L. Excoffier, G. Laval and S. Schneider, Arlequin ver 3.11: An Integrated Software Package for Population Genetics Data Analysis, Computational and Molecular Population Genetics Lab (CMPG), Institute of Zoology University of Berne, Switzerland (2006).

FAO, 2006 FAO 2006. Fishstat Plus: Universal software for fishery statistical. Time series 1950–2004. Version 2.30. FAO Fisheries Department, Fishery Information, Data and Statistics Unit. www.fao.org/fi/statist/fisoft/FISHPLUS.asp.

Goudet, 2001 J. Goudet, FSTAT, A program to Estimate and Test Gene Diversities and Fixation Indices (version 2.9.3.2), Institute of Ecology, University of Lausanne, Switzerland (2001) <http://www.unil.ch/izea/software/fstat.html>.

Guo and Thompson, 1992 S.W. Guo and E.A. Thompson, Performing exact test of Hardy–Weinberg proportion for multiple alleles, *Biometrics* **48** (1992), pp. 361–372. Full Text via CrossRef

Kamonrat, 1996 Kamonrat, W., 1996. Spatial genetic structure of Thai silver barb *Puntius gonionotus* (Bleeker) populations in Thailand. PhD Thesis. Dalhousie University, Canada. 193 pp.

Liu and Cordes, 2004 Z.J. Liu and J.F. Cordes, DNA marker technologies and their applications in aquaculture genetics, *Aquaculture* **238** (2004), pp. 1–37. SummaryPlus | Full Text + Links | PDF (801 K) | View Record in Scopus | Cited By in Scopus (48)

Luan et al., 2006 S. Luan, J. Kong and Q.Y. Wang, Genetic variation of wild and cultured populations of the Kuruma prawn *Marsupenaeus japonicus* (Bate 1888) using microsatellites, *Aquacult. Res.* **37** (2006), pp. 785–792. Full Text via CrossRef | View Record in Scopus | Cited By in Scopus (0)

Miller, 1997 M.P. Miller, Tools for population genetic analysis (TFPGA) 1.3. A window program for the analysis of allozyme and population genetic data. Computer software distributed by the author (1997).

Nei, 1978 M. Nei, Estimation of average heterozygosity and genetic distance from a small number of individuals, *Genetics* **23** (1978), pp. 341–369. Full Text via CrossRef | View Record in Scopus | Cited By in Scopus (53)

Nei et al., 1983 M. Nei, F. Tajima and Y. Tateno, Accuracy of estimated phylogenetic trees for molecular data, *J. Mol. Evol.* **19** (1983), pp. 153–170. Full Text via CrossRef | View Record in Scopus | Cited By in Scopus (439)

Raymond and Rousset, 1995 M. Raymond and F. Rousset, GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism, *J. Heredity* **87** (1995), pp. 248–249.

Rice, 1989 W.R. Rice, Analyzing tables of statistical tests, *Evolution* **43** (1989), pp. 223–225. Full Text via CrossRef

Sekino et al., 2002 M. Sekino, M. Hara and N. Taniguchi, Loss of microsatellite and mitochondrial DNA variation in hatchery strains of Japanese flounder *Paralichthys olivaceus*, *Aquaculture* **213** (2002), pp. 101–122. SummaryPlus | Full Text + Links | PDF (326 K) | View Record in Scopus | Cited By in Scopus (28)

Simmons et al., 2006 M. Simmons, K. Mickett, H. Kucuktas, P. Li, R. Dunham and Z. Liu, Comparison of domestic and wild channel catfish (*Ictalurus punctatus*) populations provides no evidence for genetic impact, *Aquaculture* **252** (2006), pp. 133–146. [SummaryPlus](#) | [Full Text + Links](#) | PDF (483 K) | [View Record in Scopus](#) | [Cited By in Scopus](#) (3)

Sodsuk and Sodsuk, 1998 S. Sodsuk and P.K. Sodsuk, Genetic diversity of giant freshwater prawn from three locations in Thailand. Technical Paper No. 18/1998, National Aquaculture Genetics Research Institute, Pathumthani, Thailand (1998) 40 pp..

Taggart et al., 1992 J.B. Taggart, R.A. Hynes, P.A. Prodohl and A. Ferguson, A simplified protocol of routine total DNA isolation from salmonid fishes, *J. Fish Biol.* **40** (1992), pp. 963–965. [Full Text via CrossRef](#)

Wilkinson et al., 1992 L. Wilkinson, M. Hill, J.P. Welna and G.K. Birkenbeuel, SYSTAT for Windows: Statistics, Version 5 Edition, SYSTAT, Inc., Evanston, IL (1992).

Xu et al., 2001 Z. Xu, J.H. Primavera, L.D. de la Pena, P. Pettit, J. Belak and A. Alcivar-Warren, Genetic diversity of wild and cultured black tiger shrimp (*Penaeus monodon*) in the Philippines using microsatellites, *Aquaculture* **199** (2001), pp. 13–40. [SummaryPlus](#) | [Full Text + Links](#) | PDF (611 K) | [View Record in Scopus](#) | [Cited By in Scopus](#) (28)

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เอกสารแนบที่ 7

Pumitinsee, P, Senanan, W, Na-Nakom, U, Kamonrat, W, Koedprang, W.
Temporal genetic variation of orange-spotted grouper (*Epinephelus coioides*, Pisces:
Serranidae) juveniles collected from an important fishing ground in Thailand

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Abstract: *Epinephelus coioides* is a major species targeted for fisheries worldwide and for aquaculture in Asia. *E. coioides* fisheries target both adults and juveniles. It is, therefore, important to gather some basic genetic data for aggregations of *E. coioides* juveniles. The year-round natural production of *E. coioides* juveniles in a nursery area in the tropics raises an interesting biological question about possible distinct spawning times of spawners. If genetically isolated spawner groups exist, temporal genetic structure of *E. coioides* populations can raise a fisheries management concern about differential demographics of isolated stocks. To test whether temporal genetic isolation exists in this *E. coioides* population, we examined genetic heterogeneity of juveniles collected at different times of the year at a nursery area in coastal waters of Trang province, Thailand, with an outgroup coming from a distant geographic location, Chantaburi. Genetic variation at six polymorphic microsatellite genetic markers within each sample was moderate with observed heterozygosities across all loci ranging from 0.51 - 0.63 and number of alleles per locus ranged from 6.33 - 7.83. Genetic differentiation measures (Exact tests, F_{st} and Cavalli Sforza's genetic distance) indicated substantial differentiation between the two geographic distant samples, Trang and Chantaburi. Within Trang, July samples were genetically distinct from other samples ($F_{st} = 0.028 - 0.0643$, p -values < 0.05). The

Rannala and Moutain's (1997) method individual-based assignment also suggested a similar pattern with large proportions of individuals (more than 0.83) collected in July being correctly assigned to July. Temporal samples within Trang other than July were genetically similar. Our results suggested possible genetic isolation between spawning time of *E. coioides* at this nursery area. To manage this *E. coioides* fisheries in a sustainable manner, further investigation on optimal exploitation strategies for each genetic stock may be needed.

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- 4 1 Temporal genetic heterogeneity of juvenile orange-spotted grouper (*Epinephelus coioides*,
- 5 2 Pisces: Serranidae) collected from an important nursery ground in Thailand
- 6 3 Panuwat Pumltinsee¹, Wansuk Senanan¹, Uthairat Na-Nakorn², Wongpathom Kamonrat³,
- 7 4 and Worawut Keadprang⁴
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Abstract

Epinephelus coioides is a major species targeted for fisheries worldwide and for aquaculture in Asia. *E. coioides* fisheries target both adults and juveniles. It is, therefore, important to gather some basic genetic data for aggregations of *E. coioides* juveniles. The year-round natural production of *E. coioides* juveniles in a nursery area in the tropics raises an interesting biological question about possible distinct spawning times of spawners. If genetically isolated spawner groups exist, temporal genetic structure of *E. coioides* populations can raise a fisheries management concern about differential demographics of isolated stocks. To test whether temporal genetic isolation exists in this *E. coioides* population, we examined genetic heterogeneity of juveniles collected at different times of the year at a nursery area in coastal waters of Trang province, Thailand, with an outgroup coming from a distant geographic location, Chantaburi. Genetic variation at six polymorphic microsatellite genetic markers within each sample was moderate with observed heterozygosities across all loci ranging from 0.51 – 0.63 and number of alleles per locus ranged from 6.33 – 7.83. Genetic differentiation measures (Exact tests, F_{st} and Cavalli Sforza's genetic distance) indicated substantial differentiation between the two geographic distant samples, Trang and Chantaburi. Within Trang, July samples were genetically distinct from other samples ($F_{st} = 0.028 - 0.0643$, p -values < 0.05). The Rannala and Moutain's (1997) method individual-based assignment also suggested a similar pattern with large proportions of individuals (more than 0.83) collected in July being correctly assigned to July. Temporal samples within Trang other than July were genetically similar. Our results suggested possible genetic isolation between spawning time of *E. coioides* at this nursery area. To manage this *E. coioides* fisheries in a sustainable manner, further investigation on optimal exploitation strategies for each genetic stock may be needed.

1 Introduction

Orange-spotted grouper, *Epinephelus coioides* (Pisces: Serranidae) is economically and ecologically significance species. This species is widely distributed throughout the Indo-West Pacific from the Red Sea to South Africa, eastward to Palau and Fiji, north to the Ryukyu Island and south to the Arafura Sea and Australia (Heemstra and Randall, 1993). It is one of the major species targeted for fisheries worldwide (e.g., Grandcourt et al., 2005) and for aquaculture in Asia (e.g., Sadovy, 2000). In Southeast Asia, juveniles of serranid species are valuable commodity because of high demand for seeds for aquaculture (Sadovy, 2000; Mous et al., 2006).

Populations of *E. coioides* worldwide are under immense fishing pressure for both adults (e.g., Grandcourt et al., 2005) and juveniles (e.g., Sadovy, 2000; Mous et al., 2006). Current aquaculture operations rely heavily on juveniles harvested from the wild (more than 90% in some areas, Sadovy, 2000). Current fisheries management often overlooks the impacts of early life-phase capture on the adult populations (Mous et al., 2006). The impetus for our study, therefore, is to generate basic population genetic information of juvenile *E. coioides* collected at a major harvest site within SE Asia (Sadovy, 2000) and to gain insights about reproduction cycles of adult population(s) via the inference of juvenile data. Our study site in Trang province supports year-round natural production of juveniles (at least 400,000 – 500,000 individuals purchased from this site in 1999, Sadovy, 2000; Sheriff, 2004). Because of the heavy fishing pressure for all life stages and their life history characters that make them vulnerable to overexploitation (e.g., long life span, slow growing, spawn in aggregation, highly skewed sex ratio favoring females), this species has been listed as vulnerable to extinction under the Red List of Threaten Species (IUCN) (e.g., IUCN, <http://www.iucnredlist.org/>; Morris et al., 2000).

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6 2 There is still large knowledge gap about reproductive biology of tropical serranid species
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8 3 and the linkages between the dynamics of spawning adults in the oceanic environments and
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10 4 the abundance of juveniles present at a nursery area. Existing literature for Serranid fishes
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12 5 suggests that the spawning/adult habitats and nursery sites for *E. coioides* can be separated
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14 6 in a great distance (Gillanders et al., 2003) with juveniles inhabiting the mangrove and
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16 7 rocky reef estuarine environments and adults inhabiting oceanic environments (Sheaves,
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18 8 1995). Most knowledge about reproductive and population ecology of Serranid fishes was
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20 9 derived from temperate species (e.g., dusky grouper, *E. marginatus*, Marino et al., 2001;
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22 10 Nassua grouper, *Epinephelus striatus*, Sadovy and Collin, 1995) and our current
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24 11 understanding of tropical grouper species is quite limited. For example, the fish stock
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26 12 assessment data have only been reported for *Epinephelus coioides* populations within the
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28 13 Arabian Gulf (e.g., Grandcourt et al., 2005).
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36 15 Spawning time for *E. coioides* adults in tropical areas is another mysterious aspect of
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38 16 reproductive biology for this species. Observation of gonadal-somatic index (GSI) and
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40 17 level of vitellogenin of a closely related species, *E. malabaricus*, suggests that the peak GSI
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42 18 and the concentration of vitellogenin are expressed during November and March
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44 19 (Kongkumnerd, 1997). Yet, the small-size juveniles (2 – 10 cm.) of *E. coioides* can be
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46 20 captured at a nursery are year round (personal observation). The year-round presence of fry
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48 21 raises an important biological and management question: do aggregations of juveniles
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50 22 present at different times within a year in a nursing area derive from one or more
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52 23 genetically distinct spawner groups? In some species, variation in spawning time is
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54 24 heritable (e.g., Pacific Salmonids, Ford et al., 2006) and can lead to genetic isolation among
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56 25 groups. The stability of temporal genetic structure, however, will depend on the levels of
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1 gene flow between the spawner groups, if the spawner groups are reproductively isolated.

2 Compared to freshwater or anadromous species, observing spawning events of marine

3 species may be more challenging. Using molecular techniques (e.g., Hoarau et al., 2002,

4 Plaice, *Pleuronectes platessa* L.; Jørgensen et al., 2004, herring, *Clupea herengus*; Maes et

5 al., 2006, European eel, *Anguilla anguilla* L.; Shaw et al., 2004, Patagonian squid, *Loligo*

6 *gahi*), some studies detected temporal genetic isolation for marine species with pelagic

7 larvae (e.g. *Clupea herengus*, Jørgensen et al., 2004; *Anguilla anguilla*, Maes et al., 2006).

8 In these species, the authors speculated that there are at least two genetically isolated

9 groups of spawners utilizing the same spawning ground.

10

11 Despite the economic significance of Serranid species, there are only handful genetic

12 studies (e.g., Stevenson et al., 1998; Rhodes et al., 2003; Zantcoff et al., 2004) and only a

13 few focused on tropical species (e.g., Koedprang, 2002; Antoro et al., 2006). Very few

14 designed the sampling schemes to assess possible temporal genetic isolation. Genetic data

15 generated from previous studies indicated strong spatial differentiation among populations

16 (e.g., Antoro et al., 2006; Rhodes et al., 2003; Zantcoff et al., 2004). Within the Southeast

17 Asia region, Antoro et al. (2006) discovered strong spatial population genetic structure in *E.*

18 *coioides*, at least at a regional scale (Thailand vs. Indonesia, and the east and west coasts of

19 Thailand) although allozyme analysis could not distinguish populations located in close

20 proximity (with Trang province, Thailand, Koedprang, 2002).

21

22 Availability of high-resolution genetic markers, such as microsatellite DNA markers and

23 powerful statistical models (such as Bayesian individual-based assignment methods;

24 reviewed in Manel et al., 2005) allows for the detection of genetic variation at a fine

25 geographic scale in both freshwater and marine species (e.g., *Gadus morhua*, Knutsen et

al., 2003; *Pangasianodon hypophthalmus*, So et al., 2006). We, therefore, utilized microsatellite genetic markers and a variety of statistical models to evaluate potential temporal genetic isolation. The objectives of this study were to describe genetic variation within and among groups of juveniles collected at different times in a year and to determine potential genetic isolation among spawner stocks from which the juveniles were produced. Genetic insights about the temporal genetic variation of *E. coioides* juveniles would serve as a starting point to address the biological and fisheries management question about possible genetic distinct stocks that produce juveniles within a nursery area.

Methods

Study area and sampling scheme

We collected orange-spotted grouper (*E. coioides*) juveniles from Trang (UTM 47N055556/0805863; N = 313) and Chantaburi (UTM47N1385415/0821685; N = 45) Provinces, Thailand (Figure 1). The Trang site is one of the largest nursery grounds for grouper juveniles in Thailand as this area has relatively healthy mangrove coverage. In 1997, the mangrove areas in the three coastal districts of Trang accounted for roughly 12 % (33,036 hectares) of Thailand's total mangrove coverage (275,805 hectares; National Park, Wildlife and Plant Conservation Department, <http://ims.dnp.go.th/index.htm>). We analyzed nine samples, including eight samples from Trang and an outgroup from Chantaburi. We collected the Trang samples in January (JA), April (AP), July (JU) and November (NO) 2004 and the Chantaburi sample (CH) in January 2004 (Fork length, FL = 6.6 - 12.8 cm). For each sampling month, Trang samples consisted of two size classes, (1) either small (S, FL = 6.5-10.3 cm) or moderate (M, FL = 10-12.5 cm), and (2) large (L, FL = 17.5-29 cm). Each sample consisted of 33-40 individuals.

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6 2 Resolving microsatellite polymorphisms
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10 4 We analyzed six microsatellite genetic markers (CA2, CA6, CA7, Rivera et al., 2003;
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12 5 EM07, EM08 and EM10, Nugroho et al., 1998). The markers CA2, CA6 and CA7 were
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14 6 originally developed for *Epinephelus quernus* and consisted of all perfect CA repeats.
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16 7 EM07, EM08 and EM10 were developed for *Epinephelus merra* and consisted of imperfect
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18 8 GT repeats. The DNA analyses consist of three steps, (1) DNA extraction, (2) DNA
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20 9 amplification using the polymerase chain reaction and (3) gel electrophoresis. We
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22 10 extracted DNA from ethanol-preserved fin clips using a salting out protocol (adapted from
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24 11 Aljanabi and Martinez, 1997). Briefly, we incubated small pieces of fin clip tissue in lysis
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26 12 buffer (10mM Tris HCl, 2mM EDTA, 0.4M NaCl, 10%SDS, and 20 ng of Proteinase K) at
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28 13 55 °C overnight. We precipitated the lyzed protein using 300 µl of 7.5M Ammonium
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30 14 Acetate. The DNA was precipitated and washed using 100% and 70% ethanol,
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32 15 respectively.
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36 17 DNA was then amplified using polymerase chain reaction (PCR). PCR was performed in a
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38 18 thermocycler (Hybaid Touchdown or Biometra Tgradient). Each reaction mixture (10 µL)
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40 19 contained 1 µL template DNA solution or 50 ng purified template DNA, 2.5-5 µM of each
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42 20 primer, 1.0 mM MgCl₂, 0.01 mM of each dNTP, 1 X reaction buffer (Invitrogen, USA),
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44 21 and 0.2-0.3 unit of *Taq* polymerase (Invitrogen, USA). Temperature profiles for the PCR
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46 22 consisted of two steps: a seven-cycle profile consisting of denaturing at 94° C for 1 minute,
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48 23 annealing at primer specific temperatures (50 – 54 °C) for 30 seconds, and elongating at 72°
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50 24 C for 30 seconds, and a 33-cycle profile consisting of denaturing at 90° C for 30 seconds,
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52 25 annealing at primer specific temperatures for 30 seconds, and elongating at 72° C for 30
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seconds (adapted from Nugroho et al., 1998). We performed electrophoresis of PCR products on 6% polyacrylamide gel at 900 volts for 3 hours. We then visualized PCR products on the gel stained with Silver Staining Technique (Promega, USA). To score alleles, we compared the size of DNA fragments with a DNA sequence of PGEM plasmid (Promega, USA).

Genetic data analysis

We analyzed genetic variation within samples using following indexes: allele frequencies, number of allele/locus (A), the effective number of allele per locus (A_E), and heterozygosities. We tested for the deviation of observed genotypes proportions from those expected under Hardy-Weinberg equilibrium (HWE) using the Markov chain exact tests (Raymond and Rousset, 1995) implemented within the software GENEPOP (Raymond and Rousset, 1995; p-value estimated from 10000 dememorization number, in 100 batches with 1000 iterations per batch). To investigate the likelihood of the presence of null allele, we used the Brookfield estimator 1 in the software Microchecker (Van Oosterhout et al., 2004).

To assess among-sample genetic variation, we performed exact tests of allele frequency differences (Guo and Thompson, 1992), estimated subdivision index, F_{st} (Weir and Cockerham, 1984), and estimated genetic distance values (Cavalli-Sforza and Edwards' genetic distance, and Neighbor-Joining cluster analysis with 1,000 bootstrap replications). We performed exact tests using software GENEPOP (Raymond and Rousset, 1995) and calculated F_{st} (1,000 permutations) and Cavalli-Sforza and Edwards' genetic distance values (1,000 bootstrap replications) using and Microsatellite Analyzer software (MSA; Dieringer and Schlötterer, 2003). We constructed a genetic distance tree using the Neighbor Joining clustering method, included in NEIGHBOR and CONSENSE within the

1 PHYLIP software package (Felsenstein, 1993). The consensus tree was viewed in the
2 TREEVIEW software (Page, 1996).
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4 To determine whether samples collected from Trang derive from one or more genetically
5 distinct groups, we took two approaches: (1) conventional population-based and (2)
6 individual-based assignment methods. For the conventional approach, we assessed the
7 genetic homogeneity both for pooled data for all Trang samples and for each predefined
8 temporal sample. For the individual-based approach, we used the approach developed by
9 Rannala and Mountain (1997) and Pritchard et al. (2000). The former method estimates the
10 probabilities that an array of multi locus genotypes belongs to putative baseline populations
11 using Bayesian approach (10,000 simulated individuals and threshold value = 0.05).
12 Pritchard et al.'s (2000) approach simulates the number of 'true' populations (K), each of
13 which is characterized by a set of allele frequencies at a locus, and estimates the admixture
14 proportions (Q) of each individual belonging to one of the K populations (length of Burnin
15 Period = 30,000, number of Markov chain Monte Carlo Replications after Burnin: 100,000,
16 admixture model). The individual-based assignment methods were included in the
17 GeneClass2 (Piry et al., 2004) and Structure software (Pritchard et al., 2000).

18 19 **Results**

20 21 **Microsatellite DNA polymorphisms and within-sample genetic variation**

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23 All microsatellite markers examined were polymorphic in at least one sample. For each
24 locus, the alleles ranged from five (CA06) to 23 (EM10), average effective number of
25 alleles ranged from 2.59 (CA06) – 10.56 (EM10), and observed heterozygosities average

across samples ranged from 0.11 (CA07) to 0.82 (EM10) (Table 1). Within each sample, the number of allele per locus average across loci ranged from 6.33 (JUL) to 7.83 (APS), the average effective number of allele (A_E) ranged from 3.71 (JUL, Large size class collected in July) to 4.52 (JUM, Medium size class collected in July), and the average observed heterozygosities ranged from 0.51 (APL, Large size class collected in April; NOL, Large size class collected in November) to 0.63 (CH, Chantaburi sample) (Table 1). We detected private alleles in APS (EM08, freq = 0.018), JUM (EM10, freq = 0.018), JUL (CA02, freq = 0.015), NOS (CA06, freq = 0.013) and CH (CA7, freq = 0.047 and EM10 = 0.013).

Genotypic frequencies of most samples at all loci were consistent with those expected under the Hardy-Weinberg equilibrium (Exact tests, $p < 0.00093$ after the Bonferroni correction for multiple comparisons of 54 tests, Rice 1989). Of 54 tests, nine tests deviated from the Hardy-Weinberg proportions (EM07 in NOL sample, EM 08 in JAS samples, and EM10 in all samples, except NOS and CH). Most deviations occurred at EM10 (seven out of nine tests at this locus). Micochecker analysis (Van Oosterhout et al. 2004) for the presence of null allele suggested that the frequencies of null allele (r) at EM10 ranged from 0.053 (CH) to 0.206 (JUL). The average r -value for the Trang temporal samples was 0.131. We, therefore, removed genotypes at EM10 from further analyses that require Hardy-Weinberg equilibrium (HWE). After removing EM10, only two of 45 tests deviated from the expectations under HWE ($p < 0.0011$, Bonferroni correction for 45 tests). In addition to the tests for predefined temporal samples in Trang, we tested the genetic heterogeneity within the pooled sample using exact tests for heterozygote deficiency at six microsatellite loci. The test results indicated that genotypic frequencies at four out of six loci of the

1 pooled sample deviated from Hardy – Weinberg proportions (overall p value < 0.001).

2 This was the first indication of heterogeneity in the pooled sample.

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5 Genetic variation among samples

6 Exact tests for allele frequency differentiation at five microsatellite loci and pair-wise F_{st}

7 values revealed genetic differentiation between Chantaburi (CH) and Trang samples

8 ($p < 0.0014$ with the Bonferroni correction for 36 tests). For the Trang temporal samples,

9 global F_{st} indicated genetic differentiation among samples ($F_{st} = 0.026$, $p < 0.001$). July

10 samples (both sizes) were genetically distinct from most samples (Table 2, Table 3). Other

11 Trang samples are genetically similar although some exact tests revealed the p values at the

12 border of the significance level ($0.0014 < p < 0.0064$). The Cavalli-Sforza and Edwards'

13 genetic distance values suggested a similar pattern of population differentiation (Figure 2),

14 with CH samples being distinct from Trang samples and JUM-JUL being genetically

15 distinct from other the sampling months in the Trang samples. The remaining Trang

16 samples were clustered into two groups: (1) JAL, NOS, NOL (12.0 % supporting bootstrap

17 value) and (2) JAS, APS, APL (42.7 % bootstrap supporting value).

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19 Data generated from individual-based assignment methods were inconclusive. For Rannala

20 and Mountain's (1997) method, most individuals were assigned to more than one sampling

21 months (Table 4). Significant proportions of individuals (more than 0.70) sampled within

22 JAS, JAL, APS, APL, NOS, and NOL were assigned to at least one of these sampling

23 months. Similarly, large proportions (more than 0.83) of individuals sampled within JUM

24 and JUL were assigned to July samples. Even though these genetic relationships were

25 consistent across most genetic baselines, the proportions of assigned individuals varied in

26 the following reciprocal sample-baseline pairs: JAS-NOL, JAS-JUL, JAL-APL, JAL-NOL,

1 APS-APL, APS-NOL, APL-NOS, and JUM-JUL, and NOS-NOL. For the Pritchard et al.'s
 2 (2000) clustering method, we could not group individuals based on the likelihood of an
 3 individual belonging to one of the K clusters ($Q > 0.15$, data not shown).

4 **Discussion and conclusions**

5 **Genetic variation within samples**

6 The levels of microsatellite polymorphisms observed in temporal samples of *E. coioides* in
 7 this study were comparable to those observed in *E. coioides* samples previously collected
 8 for the spatial genetic analyses in the Southeast Asia region (mean $H_o = 0.36 - 0.55$, mean
 9 allele/locus = 7.25 – 8, Antoro et al., 2006), but were relatively lower than those observed
 10 in populations of other Serranid species (e.g., mean $H_o = 0.6805$, mean allele/locus = 18.75
 11 for *E. Mario*; mean $H_o = 0.664 - 0.689$, average allele/locus = 12.75 – 16 for *Mycteroperca*
 12 *phenax*, Zantcoff et al., 2004; mean $H_o = 0.735 - 0.885$, average allele/locus = 19 – 22.66
 13 for *E. polyphekadion*, Rhodes et al., 2003; mean $H_o = 0.51 - 0.69$, mean allele/locus = 7.71
 14 – 13.86 for *E. marginatus*, de Innocentiis et al., 2001). Low effective population size (N_e ;
 15 Table 5) due to several life history traits, such as highly skewed sex ratio (Grandcourt et al.,
 16 2005) and variance in family sizes, of *E. coioides* as well as limited gene flow among
 17 populations (Antoro et al., 2006) may have contributed to the lower-than-average genetic
 18 diversity.

19 We observed heterozygous deficiency at EM10 almost in all Trang samples (seven out of
 20 eight) and at two additional loci in some samples (EM08 and EM07 in JAS and APS,
 21 respectively). The heterozygote deficiency at EM10 may be due to admixture of
 22 individuals belonging to different sib groups or populations (Wahlund effect), the presence
 23 of null alleles or sampling error. We may have included full-sib families in our samples

1 instead of representatives from the entire population. The juveniles collected may have
 2 been schooling juveniles (size 6.5 cm – 29 cm). The presence of null alleles could also
 3 result in the deficiency of heterozygotes. Microchecker analysis (Van Oosterhout et al.
 4 2004) suggested the presence of null alleles at noticeable frequencies (more than 0.1) in the
 5 Trang samples. It is interesting to note that Antoro et al. (2006), whose samples also
 6 included a Trang sample, did not observe the deviation for HWE in their Trang sample (10
 7 – 15 cm juveniles collected from May to July 2003). Inadequacy of sample size for each
 8 sample may also play a significant role in the departure from HWE at this locus. This
 9 marker is highly variable (23 alleles); our sample size in each sampling month may not be
 10 adequate to include all possible genotypes (i.e., 105 – 190 genotypes for 14 to 19
 11 alleles/sample respectively). In our case, it may require at least 53 – 80 individuals per
 12 sample. Despite potential problems associated with sampling and null alleles, we still
 13 believe that the departures to HWE within the Trang sample might have some biological
 14 significance. The genetic analysis of pooled data (all sampling months, $n = 358$) revealed
 15 that the Trang samples were heterogeneous.

16 Genetic variation among samples

17 Examined genetic markers were adequately powerful to differentiate Trang and Chantaburi
 18 samples and to reveal subtle genetic differentiation within the Trang samples. The spatial
 19 differentiation (Chantaburi vs. Trang) might represent the differentiation between the
 20 Andaman Sea and Gulf of Thailand populations; it was consistent with the genetic
 21 differentiation patterns observed in *E. coicoides* (Antoro et al., 2006) and some other
 22 marine species in Thailand, such as black tiger prawn (*Peneaus monodon*; Supungul et al.,
 23 2001) and tropical abalone (*Haliotis asinina*; Tang et al., 2004) and Asian moon scallop
 24 (*Amusium pleuronectes*, Mahidol et al., in press). The genetic differentiation could be

1 attributed to the geographic barrier (the Malay peninsular) between the two coasts of
2 Thailand.

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4 An interesting aspect of our study is the temporal genetic variation. Within Trang samples,
5 July samples (both size classes) were genetically distinct from most remaining samples (F_{st}
6 values = 0.029 – 0.064; Cavalli-Sforza and Edwards' genetic distance = 0.19 - 0.26).
7 Neighbor-Joining cluster analysis based on genetic distance values also suggested some
8 subtle differences among sampling months other than July: (1) NOS, NOL, JAL and (2)
9 JAS, APS, APL. The Rannala and Mountain's (1997) method indicated somewhat unique
10 genetic identify for the July samples. The differentiation between July samples and other
11 sampling months was comparable to the differentiation between Chantaburi and Trang
12 samples (F_{st} values = 0.015 – 0.037). We hypothesize that the factors contributing to this
13 temporal genetic differentiation may include (1) the presence of discrete spawning
14 episodes, and (2) artifacts of sampling.

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16 Juveniles collected in different sampling months might have derived from discrete groups
17 of spawners. In some marine species, genetic evidence supports the hypothesis of
18 'spawning waves,' where different groups of spawners use the same spawning ground at
19 different time (e.g., herring, *Clupea herengus*; Jørgensen et al., 2004; European eel,
20 *Anguilla anguilla* L.; Maes et al., 2006). In herring (*Clupea herengus* L.) in the Baltic Sea,
21 Jørgensen et al. (2004) found that, spawners present earlier (March, April) and later (May)
22 in the spawning season were genetically distinct ($0.0019 < F_{st} < 0.0136$) at one of the two
23 study sites. Our results may suggest at least two spawning episodes that produce, (1) JAS,
24 JAL, APS, APL, NOS, NOL (during October to March, assuming a growth rate of fry was
25 2-3 cm per month, Rimmer, 2000), and (2) JUM, JUL (during June to August). Spawners

1 producing JAL might be genetically similar to the ones producing NOS and NOL in the
2 following year. Results from individual assignment indicated that there could be overlap
3 among spawning episodes (individuals were assigned to more than one sampling months).
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5 Based on understanding of reproductive biology of other grouper species, aggregations of
6 individuals within a population may spawn asynchronously (e.g. Levin and Grimes, 2002).
7 Peak spawning activities of each aggregation are often associated with lunar cycles (e.g.
8 Levin and Grimes, 2002). Based on growth data inferred from otoliths and behavior data,
9 the actual spawning time for each individual can be very brief ranging from days to weeks
10 (reviewed in Levin and Grimes, 2002) even though indexes for gonad maturation (e.g.,
11 gonado-somatic index) indicated that mature gonads may be retained for the period of 1-5
12 months in a year. The patterns of spawning activities of *E. coioides* in the Andaman Sea
13 are currently unknown. Information on reproductive season and behavior of closely related
14 species or the same species under semi-natural conditions is inconclusive. Kongkumnerd
15 (1997) suggested that a peak reproductive season for *E. malabaricus* is from November to
16 March, based on the levels of vitellogenin and gonado-somatic index. On the other hand,
17 Sudaryanto et al. (2004) observed spawning patterns of *E. coioides* in cages located in a
18 bay (Komodo, Flores, Indonesia) and found that *E. coioides* spawn in pairs (as opposed to
19 aggregations), with the males initiate the spawning activities. The authors also observed
20 spawning activities in September through March in some cages and April through July in
21 others (2-5 times per year per cage). It was not clear of how many times one individual can
22 spawn within a year and whether spawning activities are induced by biological (e.g., age,
23 social hierarchy, genetics) or environmental factors (e.g., temperature or salinity).

24

Demography of each spawner group of *E. coioides* could also facilitate genetic changes within and among discrete spawner groups. Genetic drift within each group could be substantial if the effective population size (N_e) of a group is small and could lead to genetic differentiation among groups. Antoro et al. (2006) speculated that genetically distinct populations of *E. coioides* examined in their study were quite isolated and each population was quite small. In our samples, N_e estimated from levels of linkage disequilibrium (Peel et al., 2004), indicated very small N_e for most sampling months, except for NOS and JAL. Even though our experimental design did not lend itself for proper evaluation of N_e (we could not verify the assumptions of closed population and our small sample sizes were quite small), the N_e estimates using our data may serve as an approximation. Estimates of small N_e suspected for this species is not surprising because many marine species tend to have small $N_e:N$ ratios (e.g., $N_e/N = 0.0001$ in New Zealand snapper, *Pagrus auratus*, Hausers et al., 2002). Factors that lead to small N_e in *E. coioides* samples may include highly skewed sex ratio, differential reproductive success among families and fluctuation in population sizes. Most species of the Order Serranidae are protogynous hermaphrodites (i.e., larger individuals within a group become a functional male while smaller individuals remain functional females) and thus, populations tend to have fewer males than females. In some instances, the sex ratios can be highly skew towards females (e.g., ratio of male to female = 1:48 in the southern Arabian Gulf; Grandcourt et al., 2005). Many marine species have high differential reproductive success among families (e.g., Hedgecock 1994). The successes of recruitment are usually variable from year to year depending on the oceanic conditions, which turn out to be highly variable.

Although the presence of discrete spawning episodes is a plausible explanation of the observed genetic structure within the Trang sample, the observed genetic differences may

also be an artifact of sampling either by collecting few full-sib families or a mixture of
 individuals from several spawning episodes, particularly when growth rates are unknown.
 Our rationale for including two distinct size classes for each sampling month in Trang was
 to avoid problems of sampling the same cohort in different sampling month. It is difficult
 to draw a boundary for genetically isolated groups when undertaking a study of temporal
 genetic variation, where a sampling location the same throughout a study. Even though we
 used a size class as a proxy for age, there may still be substantial overlap in size among age
 classes. A technique for determining ages of juveniles will be helpful.

Even though individual assignment methods did not reveal distinctive clustering of
 individuals belonging to distinct genetic groups, this approach may still be valuable for
 studies along the line of our study. A few factors may have reduced the power of the
 individual-based approach in our study: (1) low level of differentiation between the Trang
 samples ($0.0001 < F_{st} < 0.0643$), (2) relatively few highly polymorphic loci, and (3) lack of
 appropriate genetic baseline. Cornuet et al. (1999) simulated the relationships between F_{st}
 and percent correct assignment and showed that the percent correct assignment decreased
 rapidly at F_{st} below 0.05. Even though some studies indicated that relatively few loci were
 adequately powerful in discriminating individuals with distinct genetic background (e.g., 7
 loci used in So et al. 2006), the genetic diversity of the loci under investigation were
 generally high (total of 114 allele and $H_o = 0.734$ in So et al., 2006) and true population
 differentiation were generally high ($F_{st} > 0.05$; Castric and Bernatchez, 2004). In addition
 to the power of microsatellite markers and the true genetic difference of populations,
 baseline information is also important and in some cases, necessary (e.g., Rannala and
 Mountain, 1997). Hauser et al. (2006) illustrated that availability of baselines substantially
 improve the accuracy of all individual-based assignment methods.

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2 Implications for fisheries management and conservation

3 Our study is among a few to address temporal genetic variation in tropical marine species.

4 It is clear from our study and Antoro et al. (2006) that the population genetic structure
5 exists among populations inhabiting the Andaman Sea and the Gulf of Thailand. Our study
6 also provided some evidence for temporal genetic heterogeneity, possibly reflecting
7 discrete spawning waves. This finding raises several biological and management questions
8 for this species: (1) is this temporal genetic structure stable? (2) Do different spawning
9 groups (if any) share the spawning grounds? (3) If different genetic stocks exist, how
10 different are their demographic characteristics? (4) How different is the fishing pressure on
11 genetically distinct stocks? Both genetic and demographic information will be necessary
12 for sustainable fisheries management of this species.

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21 References

22 Aljanabi, SM, Martinez, I (1997) Universal and rapid salt-extraction of high quality
23 genomic DNA for PCR-based techniques. Nucleic Acid Research 25: 4692-4693.

1
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- 1 Antoro, S, Na-Nakorn, U, Koedprang, W (2006) Genetic Diversity of Orange-spotted
2 Grouper, *Epinephelus coioides* from Thailand and Indonesia using microsatellite
3 markers. Mar Biotechnol 8, 17-26.
- 4 Brookfield JFY, 1996. A simple new method for estimating null allele frequency from
5 heterozygote deficiency. Mol Ecol 5: 453-455.
- 6 Castric, V, and Bernatchez, L (2004) Individual assignment test reveals differential
7 restriction to dispersal between two salmonids despite no increase of genetic
8 differences with distance. Mol Ecol 13, 1299 – 1312.
- 9 Cornuet, J, Piry, S, Luikart, G, Estoup, A, Solignac, M (1999) New methods employing
10 multilocus genotypes to select or exclude populations as origins of individuals.
11 Genetics 153: 1989-2000.
- 12 De Innocentiis, S, Sola, L., Cataudella, S, Bentzen, P (2001) Allozyme and microsatellite
13 loci provide discordant estimates of population differentiation in the endangered
14 species dusky grouper (*Epinephelus marginatus*) within the Mediterranean Sea. Mol
15 Ecol 10, 2163 – 2175.
- 16 Dieringer, D, Schlötterer, C (2003) microsatellite analyser (MSA): a platform independent
17 analysis tool for large microsatellite data sets. Molecular Ecology Notes 3, 167 –
18 169.
- 19 Felsenstein, J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by
20 the author. Department of Genetics, University of Washington, Seattle.
- 21 Ford, MJ, Fuss, H, Boelts, B, LaHood, E, Hard, J, Miller, J (2006) Changes in run timing
22 and natural smolt production in a natural spawning coho salmon (*Oncorhynchus*
23 *kisutch*) population after 60 years of intensive hatchery supplementation. Can J Fish
24 Aquat Sci 63, 2343 – 2355.

- 1 Gillanders, BM, Able, KW, Brown, JA, Eggleston, D, Sheridan, P (2003) Evidence of
- 2 connectivity between juvenile and adult habitats for mobile marine fauna: an
- 3 important component of nurseries. *Mar Ecol Prog Ser* 247, 281 – 295.
- 4 Goudet, J (2000) FSTAT, a program to estimate and test gene diversities and fixation
- 5 indices (version 2.9.1). Available from
- 6 <http://www.unil.ch/izea/software/fstat.html>.
- 7 Grandcourt, EM, Al Abdessalaam, TZ, Francis, F, Al Shamsi, AT (2005) Population
- 8 biology and assessment of the orange-spotted grouper, *Epinephelus coioides*
- 9 (Hamilton, 1822), in the southern Arabian Gulf. *Fisheries Research* 74, 55-68.
- 10 Guo, SW, Thompson, EA (1992) Performing the exact test of Hardy-Weinberg proportion
- 11 for multiple alleles. *Biometrics* 48: 361-372.
- 12 Hauser, L, Andock, GJ, Smith, PJ, Bernal Ramirez, JH, Carvalho, GR (2002) Loss of
- 13 microsatellite diversity and low effective population size in an overexploited
- 14 population of New Zealand snapper (*Pagrus auratus*). *Proc. Natl. Acad. Sci. USA*
- 15 99: 11742 – 11747.
- 16 Hauser, L., Seamons, TR, Dauer, M, Naish, KA, Quinn, TP (2006) An empirical
- 17 verification of population assignment methods by marking and parentage data:
- 18 hatchery and wild steelhead (*Oncorhynchus mykiss*) in Forks Creek, Washington,
- 19 USA. *Mol Ecol* 15, 3157 – 3173.
- 20 Hedgecock, D (1994) Temporal and spatial genetic-structure of marine animal populations
- 21 in the California current. *California Cooperative Oceanic Fisheries Investigations*
- 22 *Reports* 35, 73 – 81.
- 23 Heemstra, P.C., Randall, J.E. (1993) *FAO species catalogue. Vol. 16. Groupers of the*
- 24 *world (Family Serranidae, Subfamily Epinephelinae). An annotated and illustrated*

- 1 catalogue of the grouper, rockcod, hind, coral grouper and lyretail species known to
- 2 date. FAO Fisheries Synopsis. No. 125, Vol. 16. Rome, FAO.
- 3 Jørgensen, HBH, Hansen, MM, Loeschcke, V (2004) Spring-spawning herring (*Clupea*
- 4 harengus L.) in the southwestern Baltic Sea: do they form genetically distinct
- 5 spawning waves? ICES J Mar Sci 62: 1065-1075.
- 6 Koedprang, W, Ngamsiri, T (2002). Study on Genetic diversity of Orange spotted
- 7 grouper(*Epinephelus coioides*) in Trang province using Isozyme analysis. Faculty of
- 8 Science and Fisheries. Rajamangala University of Technology Srivijaya. Trang
- 9 campus. Sikao, Trang. 21 p.
- 10 Knutsen, H, Jorde, P.E., Andre, C, Stenseth, NCHR (2003) Fine-scaled geographic
- 11 population structuring in a highly mobile marine species: the Atlantic Cod. Mol
- 12 Ecol 12, 385 – 394.
- 13 Kongkumnerd, J (1997) Vitellogenin levels during the annual reproductive cycle of grouper
- 14 (*Epinephelus malabaricus*). Technical Paper No. 2/1997. National Institute of
- 15 Coastal Aquaculture, Department of Fisheries, Ministry of Agriculture and
- 16 Cooperative. 16 pp. (in Thai).
- 17 Levin, PS, Grimes, CB (2002) Reef fish ecology and grouper conservation and
- 18 management. In: Sale, PF (ed) Coral Reef Fishes: Dynamics and Diversity in a
- 19 Complex Ecosystem. Academic Press, San Diego, USA.
- 20 Maes, G., Pujolar, JM, Hellemans, B, Volckaert, FAM (2006) Evidence for isolation by
- 21 time in the European eel (*Anguilla anguilla*, L.). Mol Ecol 15, 2095-2107.
- 22 Mahidol, C, Na-Nakorn, U, Sukmanomon, S, Taniguchi, N, Nguyen, TTT (*In press*)
- 23 Mitochondrial DNA diversity of the Asian moon scallop, *Amusium pleuronectes*
- 24 (Pectinidae) in Thailand. Mar Biotechnol.

- 1 Manel, S, Gaggiotti, OE, Waples, RS (2005) Assignment methods: matching biological
- 2 questions with appropriate technique. Trends in Ecology and Evolution 20: 136 –
- 3 142.
- 4 Marino, G, Azzurro, E, Massari, A, Finola, MG, Mandich, A (2001) Reproduction in the
- 5 dusky grouper from the southern Mediterranean. J of Fish Biol 58, 909 – 927.
- 6 Morris, AV, Roberts, CM, Hawkins, JP (2000) The threaten status of groupers
- 7 (Ephinephelinae). Biodiversity and Conservation 9: 919 – 942.
- 8 Mous, PJ, Sadovy, Y, Halim, A, Pet, JS (2006) Capture for culture: artificial shelters for
- 9 grouper collection in SE Asia. Fish and Fisheries 7, 58-72.
- 10 Paetkau, D, Slade, R, Burdens, M, Estoup, A (2004) Genetic assignment methods for the
- 11 direct, real-time estimation of migration rate: a simulation-based exploration of
- 12 accuracy and power. Mol Ecol 13: 55-65.
- 13 Page, RDM. (1996) TREEVIEW: An application to display phylogenetic trees on personal
- 14 computers. Computer Applications in the Biosciences 12, 357-358.
- 15 Peakall, R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic
- 16 software for teaching and research. Mol Ecol Notes 6: 288-295. Available from
- 17 <http://www.anu.edu.au/BoZo/GenAlEx/>.
- 18 Peel, D, Ovenden, JR and Peel, SL (2004). NeEstimator: software for estimating effective
- 19 population size, Version 1.3. Queensland Government, Department of Primary
- 20 Industries and Fisheries. Available from
- 21 <http://www.dpi.qld.gov.au/fishweb/11629.html>.
- 22 Piry S, Alapetite, A, Cornuet, JM, Paetkau D, Baudouin, L., Estoup, A. (2004) GeneClass2:
- 23 A Software for Genetic Assignment and First-Generation Migrant Detection.
- 24 Journal of Heredity 95, 536-539. Available from
- 25 <http://www.montpellier.inra.fr/CBGP>.

- 1 Rannala, B, Mountain, JL (1997) Detecting immigration by using multilocus genotypes.
2
3
4
5
6
7
8
9
10
11
12
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57
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59
60
61
62
63
64
65
- 1 Proc. Natl. Acad. Sci. USA 94, 9197-9221.
- 2
3 Rhodes, KL, Lewis, RI, Chapman, RW, Sadovy, Y (2003) Genetic structure of camouflage
4 grouper, *Epinephelus polyphekadion* (Pisces: Serranidae), in the western central
5 Pacific. Mar Biol 142: 771 – 776.
- 6
7 Rice, WR (1989) Analyzing table of statistical tests. Evolution 43, 223 -225.
- 8
9 Rimmer, M (2000) Review of grouper hatchery technology. SPG Live Reef Fish
10 Information Bulletin 1, 14 – 19.
- 11
12 Sadovy, Y, Colin, PL (1995) Sexual development and sexuality in the Nassau
13 grouper. J Fish Biol 46, 961-976.
- 14
15 Sadovy, Y (2000) Regional survey for fry/fingerling supply and current practices for
16 grouper mariculture: evaluating current status and long-term prospects for grouper
17 mariculture in South East Asia. Final report to the Collaboration APEC grouper
18 research and development network (FWG01/99).
- 19
20 Shaw, PW, Arkhipkin AI, Adcock, GJ, Burnett, WJ, Carvalho, GR., Scherbich, JN,
21 Villegas, PA (2004) DNA markers indicate that distinct spawning cohorts and
22 aggregations of Patagonian squid, *Loligo gahi*, do not represent genetically discrete
23 subpopulations. Mar Biol 144, 961–970
- 24
25 Sheaves, M (1995) Large lujanid and serranid fishes in tropical estuaries: are they adults or
26 juveniles. Mar Ecol Prog Ser 129, 31 – 40.
- 27
28 Sheriff, N (2004) Fisher livelihoods in southern Thailand: sustainability and the role of
29 grouper culture. Ph.D. dissertation Stirling University, UK.
- 30
31 So, N, Maes, GE and Volckaert, FAM (2006) High genetic diversity in cryptic population
32 of the migratory sutchi catfish *Pangasianodon hypophthalmus* in the Mekong River.
33
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53
54
55
56
57
58
59
60
61
62
63
64
65
- 1 Heredity 96, 166 – 174.

- 1 Sudaryanto, Meyer, T, Mous, PJ (2004) Natural Spawning of three species of grouper in
2 floating cages at a pilot broodstock facility at Komodo, Flores, Indonesia. SPC Live
3 Reef Fish Information Bulletin 12, 21-23.
- 4 Supungul, P, Sootanan, , Klinbunga, S, Kamonrat, W, Jarayabhand, P, Tassanakajon, A
5 (2000) Microsatellite Polymorphism and the Population Structure of the Black Tiger
6 Shrimp (*Penaeus monodon*) in Thailand Mar. Biotechnol. 2, 339–347.
- 7 Tang, S, Tassanakajon, A, Klinbunga, S, Jarayabhand and P, Menasveta, P (2004)
8 Population structure of tropical abalone (*Haliotis asinina*) in coastal waters of
9 Thailand determined using microsatellite markers. Mar. Biotechnol 6: 604-611.
- 10 Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) microchecker: software
11 for identifying and correcting genotyping errors in microsatellite data. Molecular
12 Ecological Notes, 4, 535–538.
- 13 Zancoff, MS, Ball, AO, Sedberry, GR (2004) Population genetic analysis of red grouper,
14 *Epinephelus morio*, and scamp, *Mycteroperca phenax*, from the Southeastern U.S.
15 Atlantic and Gulf of Mexico. Mar Biol 144: 769 – 777.

Table 1. Microsatellite genetic variation at six loci of grouper samples collected in January (JA), April (AP), July (JU) and November (NO) 2004 from Trang and Chantaburi (CH) provinces. Designated codes of samples represent time of collection, size classes (7.6-10.3 cm, S; 10-12.5 cm, M; 17.5-29 cm, L) and location. Within population genetic variation indexes include the number of allele per locus (A), effective number of allele per locus (A_E), observed heterozygosity (H_o), expected heterozygosity (H_e), and F_{is} . Values underlined indicate the deviation of heterozygotes from Hardy-Weinberg proportions (at $0.01 < p < 0.05$ and $p < 0.00093$ with Bonferroni correction for multiple comparisons, $0.05/54$, highlighted in bold type). EM10 was removed from further analyses that require HWE assumptions.

Loci		Sampling month /size classes (Trang samples)								CH	Across all samples
		JAS	JAL	APS	APL	JUM	JUL	NOS	NOL		
CA02	N	40	40	36	40	40	33	38	38	45	350
	A	6	6	5	5	5	7	6	5	6	8
	A_E	1.7	2	2.6	1.7	2.8	3.7	2.5	2.3	4.1	2.59
	H_o	0.43	0.55	0.56	0.48	0.60	0.85	0.71	0.53	0.64	0.59
	H_e	0.41	0.50	0.62	0.40	0.64	0.73	0.59	0.56	0.76	0.58
	F_{is}	-0.04	-0.10	0.11	-0.18	0.06	-0.17	-0.20	0.06	0.15	-0.02
CA06	N	40	40	40	40	40	33	40	38	41	352
	A	11	8	12	9	8	8	9	8	9	13
	A_E	4.3	4.8	5.1	4.98	4.7	4.75	5.1	4.6	5.04	4.83
	H_o	0.84	0.68	0.90	0.70	0.93	0.97	0.75	0.55	0.83	0.79
	H_e	0.77	0.79	0.81	0.80	0.79	0.79	0.80	0.78	0.80	0.79
	F_{is}	-0.10	0.15	-0.12	0.12	<u>-0.17</u>	<u>-0.23</u>	0.07	<u>0.30</u>	-0.03	0.00
CA07	N	40	40	39	39	36	31	39	39	43	346
	A	5	3	3	3	4	1	3	2	3	6
	A_E	1.2	1.1	1.1	1.05	1.2	1	1.2	1.03	1.2	1.12
	H_o	0.18	0.13	0.10	0.05	0.14	0.00	0.18	0.03	0.16	0.11
	H_e	0.17	0.12	0.10	0.05	0.16	0.00	0.17	0.03	0.15	0.10
	F_{is}	-0.06	-0.05	-0.04	-0.02	0.12	N/A	-0.09	-0.01	-0.07	-0.03
EM07	N	38	39	37	36	38	33	38	39	41	339
	A	6	6	6	5	6	5	6	6	6	6
	A_E	4.3	4.1	4.5	4.2	4.15	3.55	4.6	4.55	4.3	4.25
	H_o	0.84	0.77	0.97	0.75	0.84	0.70	0.84	0.79	0.88	0.82

		He	0.77	0.76	0.78	0.76	0.76	0.72	0.78	0.78	0.77	0.76
	Fis	-0.08	-0.02	<u>-0.25</u>	0.01	-0.11	0.03	-0.08	-0.02	-0.14	-0.08	
EM08	N	33	39	28	37	33	28	36	35	41	310	
	A	3	3	5	2	2	2	3	4	3	5	
	A _E	1.66	2.26	1.88	1.84	1.96	1.60	2.18	2.11	1.76	1.91	
	Ho	0.21	0.62	0.36	0.49	0.33	0.29	0.50	0.54	0.46	0.42	
	He	0.40	0.56	0.47	0.46	0.49	0.38	0.54	0.53	0.43	0.47	
	Fis	<u>0.46</u>	-0.10	0.23	-0.07	<u>0.32</u>	0.24	0.08	-0.03	-0.08	<u>0.10</u>	
EM10	N	38	36	36	36	36	31	36	39	39	327	
	A	15	16	16	19	18	15	17	14	15	23	
	A _E	10.6	10.4	11.7	11.6	12.3	7.7	10.8	8.6	9.6	10.36	
	Ho	0.63	0.72	0.67	0.61	0.75	0.48	0.75	0.62	0.79	0.67	
	He	0.91	0.90	0.91	0.91	0.92	0.87	0.91	0.88	0.90	0.90	
	Fis	<u>0.30</u>	<u>0.20</u>	<u>0.27</u>	<u>0.33</u>	<u>0.18</u>	<u>0.44</u>	<u>0.17</u>	<u>0.30</u>	<u>0.11</u>	<u>0.26</u>	
Across												
all loci	A	7.67	7.00	7.83	7.17	7.17	6.33	7.33	6.50	7.00		
	A _E	3.96	4.12	4.49	4.22	4.52	3.71	4.38	3.86	4.34		
	Ho	0.52	0.58	0.59	0.51	0.60	0.55	0.62	0.51	0.63		

Table 2. P-values for the exact tests (Fisher's method) of allele frequency differences for each population pair across five loci. Values underlined indicate statistical significance ($p < 0.0014$, after Bonferroni correction = $0.05/36$). Values in italics fall between 0.0014 and 0.01.

	JAS	JAL	APS	APL	JUM	JUL	NOS	NOL	CH
JAS	****								
JAL	<u>0.0028</u>	****							
APS	0.0356	<u>0.0020</u>	****						
APL	0.2054	0.0202	0.0734	****					
JUM	<u>0.0012</u>	<u>0.0064</u>	<u>0.0001</u>	<u>0.0005</u>	****				
JUL	<u>0.0007</u>	<u>0.0000</u>	<u>0.0020</u>	<u>0.0045</u>	0.0567	****			
NOS	0.0109	0.3329	0.1957	0.0120	<u>0.0025</u>	<u>0.0000</u>	****		
NOL	<u>0.0030</u>	0.4861	0.0477	0.0215	<u>0.0003</u>	<u>0.0001</u>	0.1268	****	
CH	<u>0.0000</u>	<u>0.0000</u>	<u>0.0000</u>	<u>0.0003</u>	<u>0.0000</u>	<u>0.0006</u>	<u>0.0000</u>	<u>0.0000</u>	****

Table 3. Pair-wise F_{st} values among samples calculated as Weir and Cockerham's (1984) theta using genetic information at five loci (excluding EM10). Underlined values are significantly higher than zero ($p < 0.05$).

	JAS	JAL	APS	APL	JUM	JUL	NOS	NOL	CH
JAS	0.0000		*						
JAL	0.0120	0.0000							
APS	0.0125	<u>0.0194</u>	0.0000						
APL	0.0045	0.0117	0.0047	0.0000					
JUM	<u>0.0605</u>	<u>0.0482</u>	<u>0.0474</u>	<u>0.0643</u>	0.0000				

JUL	<u>0.0440</u>	<u>0.0520</u>	<u>0.0288</u>	<u>0.0500</u>	0.0114	0.0000			
NOS	0.0148	0.0001	0.0034	0.0099	<u>0.0454</u>	<u>0.0404</u>	0.0000		
NOL	<u>0.0227</u>	0.0036	0.0078	0.0148	<u>0.0566</u>	<u>0.0479</u>	0.0013	0.0000	
CH	<u>0.0284</u>	<u>0.0286</u>	0.0161	<u>0.0259</u>	<u>0.0214</u>	<u>0.0235</u>	0.0157	<u>0.0349</u>	0.0000

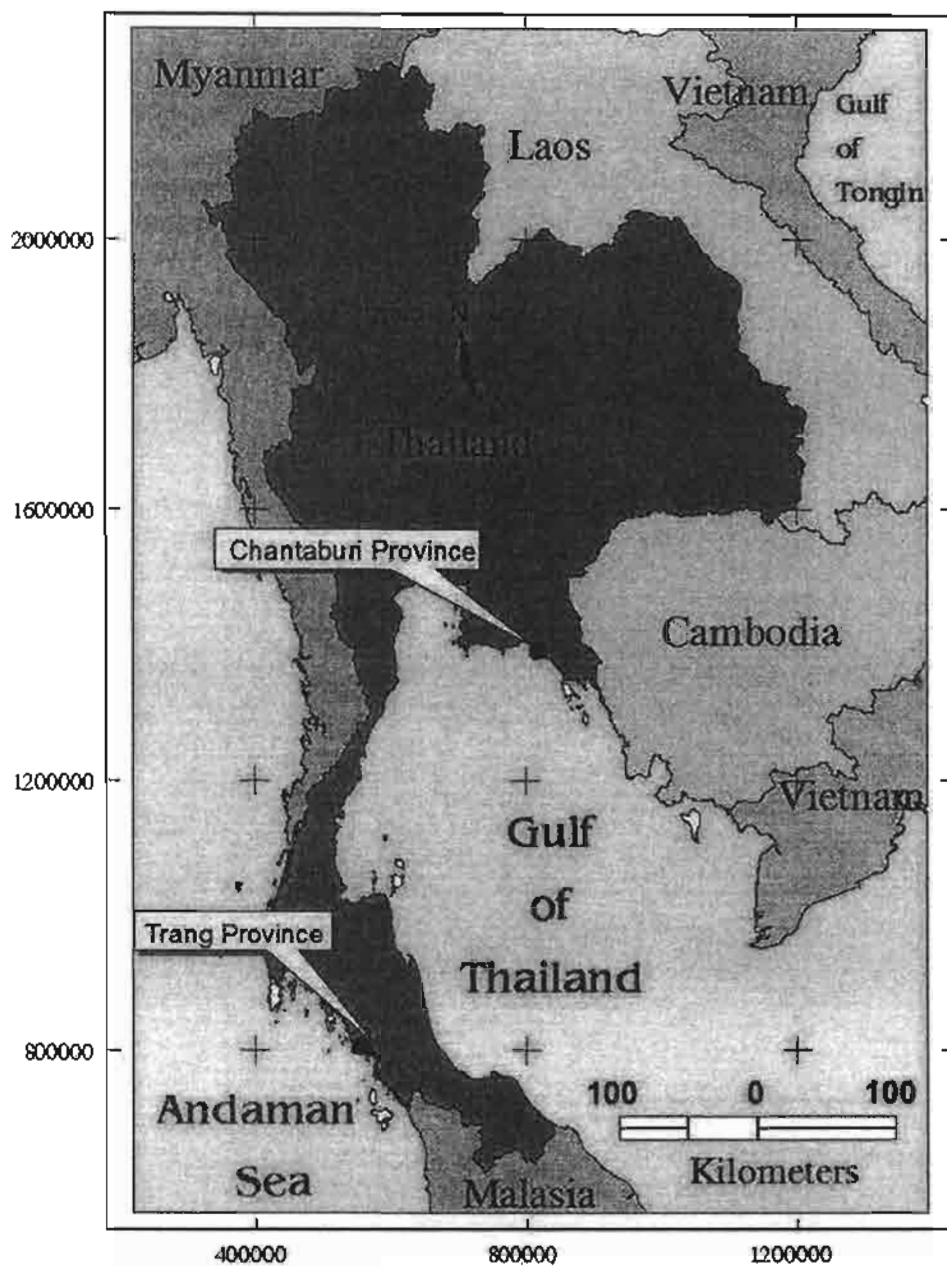
Table 4. Proportion of grouper juveniles within Trang samples assigned to each sampling month based on information from six microsatellite loci (using the Rannala and Mountain's method). The numbers in bold type are the proportion of individuals assigned to their respective sampling month.

	Excluded from all months	Assigned to							
		JAS	JAL	APS	APL	JUM	JUL	NOS	NOL
JAS	0.08	0.93	0.78	0.88	0.75	0.73	0.50	0.85	0.63
JAL	0.03	0.70	0.98	0.83	0.65	0.68	0.45	0.83	0.55
APS	0.03	0.85	0.65	0.98	0.63	0.48	0.43	0.80	0.53
APL	0.03	0.85	0.80	0.88	0.96	0.73	0.43	0.83	0.63
JUM	0.05	0.50	0.45	0.48	0.28	0.96	0.48	0.48	0.13
JUL	0	0.70	0.52	0.61	0.30	0.82	0.94	0.67	0.27
NOS	0.03	0.78	0.73	0.78	0.55	0.53	0.25	0.95	0.50
NOL	0.03	0.85	0.80	0.90	0.65	0.60	0.43	0.83	0.96

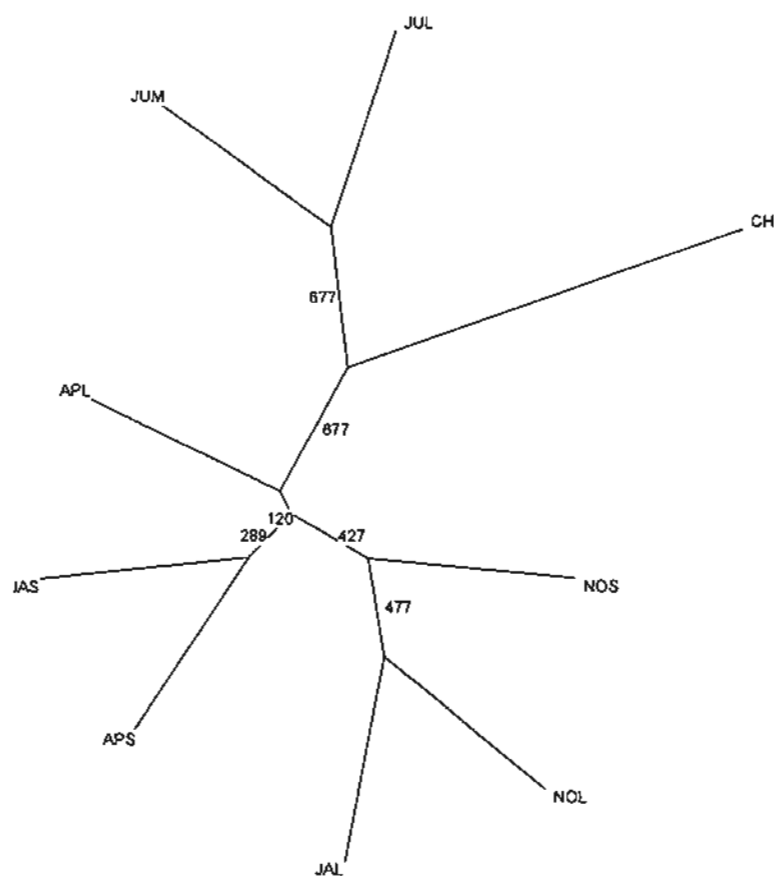
Table 5. Estimation of effective population size (N_e), based on the linkage disequilibrium method, of the *E. coioides* samples from Trang (Peel et al., 2004)

Samples	N_e estimates	95% CI
JAS	74.3	[43.1 200.3]
JAL	Infinity	[153.4 Infinity]
APS	38.8	[26.5 65.3]
APL	22.1	[16.6 30.8]
JUM	76.3	[41 288.6]
JUL	62.6	[31.8 315.4]
NOS	191.7	[66.0 Infinity]
NOL	29.9	[20.9 46.9]

1 Figure 1. Map of Thailand showing sampling locations, coastal waters of Trang and
2 Chantaburi provinces.
3



1 **Figure 2** Neighbor Joining clustering of samples based on Cavalli-Sforza's genetic distance
 2 (1 = JAS, 2 = JAL, 3 = APS, 4 = APL, 5 = JUM, 6 = JUL, 7 = NOS, 8 = NOL, 9 = CH). A
 3 value on each node indicates bootstrap replications (out of 1,000) for the node.



เอกสารแนบที่ 8

Koedprang, W, Na-Nakorn, U, Nakajima, M, Taniguchi, N. 2007. Evaluation of genetic diversity of eight grouper species *Epinephelus* spp. based on microsatellite variations. Fisheries Science 73(2), 227-236.

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Evaluation of genetic diversity of eight grouper species *Epinephelus* spp. based on microsatellite variations

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ABSTRACT: Genetic diversity among eight species of grouper, *Epinephelus bleekeri*, *E. coioides*, *E. malabaricus*, *E. ongus*, *E. akaara*, *E. maculatus*, *E. merra* and *E. fuscoguttatus*, was studied using six microsatellite loci, *Em-01**, *Em-03**, *Em-07**, *Em-08**, *Em-10**, and *CA-07**, with the aim of exploring the feasibility of using microsatellite data for species identification. The results showed high levels of genetic differentiation among species ($F_{ST} = 0.4403$ and $R_{ST} = 0.4954$). Species identification based on fixed allelic differences was possible between *E. coioides*, *E. ongus*, and *E. fuscoguttatus* at *Em-01** and between *E. fuscoguttatus* and *E. ongus* at *Em-08**. Private alleles were found in all species, except for *E. ongus*. Pairwise F_{ST} ranged 0.238–0.578 ($P < 0.008$ Bonferroni correction), and Nei's genetic distance ranged 0.433–2.710. Size homoplasy was observed at *Em-03** 157 allele, which was characterized by a T–C transition at the 119th nucleotide site of PCR products. The genetic assignment test unambiguously assigned each individual to the correct species. Thus, this test can be used for species identification of unknown individuals when the multilocus genotypes of the six microsatellite loci are available. The phylogenetic (neighbor-joining) tree, which was constructed based on the genetic distance matrix, separated the eight grouper species into two main groups.

KEY WORDS: *Epinephelus*, genetic diversity, grouper species, homoplasy, microsatellite.

INTRODUCTION

The grouper genus *Epinephelus* is one of the largest genera among bony fish, with 129 congeneric species inhabiting marine habitats around the world.¹ Many of these species are economically important for both capture fisheries and aquaculture.

Species identification of grouper is problematic, since morphological traits overlap among species, and variations of body coloration during different life stages can make it difficult to correctly classify species. Although identification guides based on morphological characteristics are available for the identification of almost all grouper species in the world,² misidentification between species is still

common. For example, one grouper species, *E. coioides*, which is cultured in South-East Asia, is often misreported as *E. tauvina*,³ and the cases of misidentification between *E. coioides* and *E. malabaricus* are also not uncommon.² Other instances of misclassification include: (i) *E. guaza* inhabiting the Mediterranean and the Atlantic Ocean was often misidentified as *E. tauvina* (of the Indo-Pacific region); and (ii) confusion of *E. fuscoguttatus* with *E. polyphakadion*.²

Molecular genetic markers have been used to resolve taxonomic ambiguity in many taxa⁴ including fishes.⁵ For example, two morphologically different forms of stream-dwelling fishes of the genus *Ilyodon* in certain rivers in Mexico had been considered as different species, but later allozyme analysis suggested that they should not be genetically be delineated.^{6,7} The Bullhead *Cottus gobio*, previously thought to consist of one species, was divided into two subspecies based on fixed allelic differences of allozyme loci (10% of the examined

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loci).⁸ Further, species-specific genetic markers serve as an efficient tool for precise species identification and detection of introgression between species.^{9,10} Several types of molecular markers have been used to quantify genetic diversity of groupers, including allozymes,^{11,12} microsatellite DNA,^{12–14} mitochondrial cytochrome *b*,^{15,16} the 590-bp region of the 16S rRNA gene,¹⁷ and RAPDs.¹⁸

In this study we investigated genetic diversity among members of the genus *Epinephelus* in order to document the potential of microsatellite DNA loci for species identification in this genus. Microsatellites have been used to survey the extent of genetic differentiation between species in several fishes, such as sparids (*Pagrus major* and congeners),¹⁹ salmonids,²⁰ and species in genus *Seriola*,²¹ largely because of the (hypervariable) nature and transferability of microsatellite alleles, which has recently received much attention in the context of conservation and evolutionary population genetics. Size homoplasy, which explains the state that alleles with the same size have different nucleotide sequences, has frequently been found in compound and interrupted microsatellites^{22,23} in a wide range of taxa such as primates,^{24,25} invertebrates,^{26,27} and fishes.²² We report here the first evidence of size homoplasy among grouper species.

MATERIALS AND METHODS

DNA samples

We drew samples from eight species of groupers of the genus *Epinephelus*. Four species, *E. bleekeri* ($n = 34$), *E. coioides* ($n = 50$), *E. malabaricus* ($n = 50$), and *E. ongus* ($n = 50$), were sampled from natural habitats along the coast of the Andaman Sea in Trang Province, Thailand. Three other species, *E. akaara* ($n = 29$), *E. maculatus* ($n = 11$), and *E. merra* ($n = 10$), were collected from natural habitats around Okinawa, Japan. *Epinephelus fuscoguttatus* ($n = 49$) was collected from a hatchery in Taiwan. Sampling sites are shown in Figure 1.

Before collecting tissue samples for DNA extraction, we identified the species of these fish samples based on their morphological characteristics following Heemstra and Randall.² Then, we cut small pieces of caudal fins and preserved them individually in 90% ethanol until DNA extraction was performed. Genomic DNA was extracted by the phenol-chloroform method, and the concentration was checked.

Microsatellite DNA analysis

A total of six microsatellite primer sets were used, with five developed from *E. malabaricus*: three

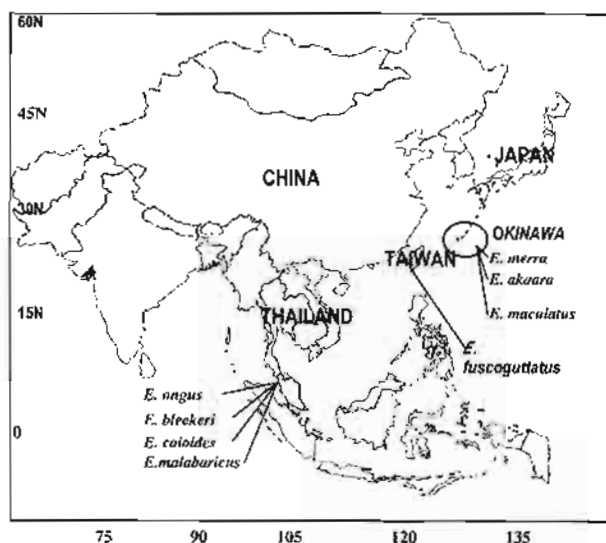


Fig. 1 Sampling sites of eight species of groupers of the genus *Epinephelus* included in this study.

sets²⁸ (*Em-01*, *Em-03*, and *Em-08*) and two sets²⁹ (*Em-07* and *Em-10*). One primer set (*CA-07*, GENBANK accession number AF539609) was derived from *E. quernus*.³⁰ The forward primer of each primer set was biotin-labeled at its 5' end to visualize PCR products. The PCR mixture was prepared according to Nugroho *et al.*²⁸

The conditions for the PCR cycles were as follows: seven cycles of 1 min at 94°C, 30 s at the optimal annealing temperature (T_{ann}), and 30 s at 72°C followed by 33 cycles of 30 s at 90°C, 30 s at optimal T_{ann} , and 30 s at 72°C. After PCR cycling, deionized formamide stop dye was added to each reaction mixture.

Electrophoresis was performed on 6% polyacrylamide urea gel after denaturation of PCR products (at 95°C for 15 min), and the alleles were sized relative to the sequence ladder generated from *M13mp18*.

Chemiluminescence detection was performed according to the method described by Perez-Enriquez *et al.*³¹ After electrophoresis, DNA was transferred to a nylon membrane by blotting, and then the transferred membrane was dried and UV-cross linked. DNA on the membrane was detected using a Phototope Star Detection Kit (New England Biolabs, Beverly, MA, USA).

Data analysis

Genotype data were used to calculate the number of alleles at each locus, and observed (H_o) and

expected (H_e) heterozygosity. Subsequent calculation of the effective number of alleles (A_e) was made using the formula $A_e = 1/\sum x_i^2$, where x_i is the frequency of the allele for each locus.³²

Genetic differentiation was assessed using Wright's F_{ST} -statistic.^{33,34} R_{ST} over all samples was also estimated following Rousset.⁴⁵ Pairwise F_{ST} was calculated to examine genetic differentiation between species. All calculations were performed using GENEPOP software.³⁶ Nei's unbiased genetic distances⁴⁷ were calculated and subsequently used to construct a phylogenetic dendrogram by a neighbor-joining method³⁸ using PHYLIP software.³⁹ The tree was evaluated by 1000 resamplings of loci. Visualization of the tree was done on Tree-Explorer software.⁴⁰

Species assignment test

Species assignment of individuals was verified to explore the feasibility of using microsatellite data for species identification. The test is on the basis of the multilocus genotypes of microsatellite loci by a maximum likelihood method using WHICHRUN⁴¹ to allocate individuals to their most likely source population.

Assessment of microsatellite DNA homoplasy

We addressed size homoplasy of microsatellite alleles at one locus (*Em-03*), where seven of the eight species shared a common allele (*Em-03**157, Appendix Table A1). Three species (*E. bleekeri*, *E. coioides*, and *E. malabaricus*) showed no allelic variation at this locus. The *Em-03**157 allele was sampled from 10 homozygous individuals each from *E. akaara*, *E. bleekeri*, *E. coioides*, *E. fuscoguttatus*, and *E. malabaricus*, and four homozygotes from *E. maculatus*. Alleles from *E. merra* were not analyzed because no homozygote *Em-03**157/157 was found in this species and we were unable to elute the *157 allele from the heterozygotes. DNA from each individual was amplified using the *Em-03* primer set, and PCR products were resolved on 2% agarose gel. The *157 bands were extracted and purified from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Both strands of purified 157-bp PCR products were sequenced using a DNA sequencer (Prism 377, ABI, Foster City, CA, USA). The DNA sequences were manually aligned and polymorphism was detected.

RESULTS

Private alleles and diagnostic loci

Allele frequencies are shown in Appendix Table A1. Private alleles were found in seven species (all species studied except for *E. ongus*), and across seven loci examined six alleles were specific to *E. akaara*, five alleles in *E. bleekeri*, two in *E. coioides*, thirteen in *E. fuscoguttatus*, four in *E. maculatus*, six in *E. malabaricus*, and eight alleles in *E. merra*. For example, at locus *Em-01*, two alleles (*270 and *274) appeared in *E. akaara* but not in the other species. Likewise, *235 and *239 were unique for *E. maculatus*; *220, *222, *224, and *226 for *E. merra*; *225 for *E. fuscoguttatus*; and *255 for *E. malabaricus*.

Two diagnostic loci that refer to loci that are monomorphic at different alleles in different species were observed between *E. coioides*, *E. ongus* (fixed at *Em-01**257), and *E. fuscoguttatus* (fixed at *Em-01**224), and between *E. fuscoguttatus* (fixed at *Em-08**204) and *E. ongus* (fixed at *Em-08**202).

Genetic variability within species

The levels of polymorphism varied considerably among loci. The mean number of alleles over loci across eight species ranged from 2.8 (*E. ongus*) to 6.8 (*E. fuscoguttatus*), and the mean effective numbers of alleles ranged from 1.6 (*E. ongus*) to 3.6 (*E. merra*) (Table 1). The average H_e of eight species ranged from 0.32 (*E. ongus*) and 0.58 (*E. merra*), while H_e was between 0.36 (*E. coioides*) and 0.62 (*E. merra*).

Genetic differentiation

Genetic differentiation among species in terms of the overall F_{ST} ($F_{ST} = 0.4403$, $P < 0.008$ after Bonferroni correction) was statistically significant (Table 2). The loci that contributed much to the genetic differentiation were *Em-01**, *Em-08**, and *Em-03** (F_{ST} of each locus across species = 0.7414, 0.7015, and 0.5735, respectively).

R_{ST} over all samples was 0.4954. We could not test the significance of the R_{ST} because the core repeat sequences of the microsatellite markers developed by Nugroho *et al.*²⁸ were not available. Three loci (*Em-01**, *CA-07**, and *Em-08**) generated high R_{ST} values (*Em-01** [$R_{ST} = 0.981$], *CA-07** [$R_{ST} = 0.605$], and *Em-08** [$R_{ST} = 0.585$]).

Pairwise F_{ST} values between species ranged 0.238–0.578 and were statistically different from

Table 1 Within-species genetic variability of eight grouper species estimated based on six microsatellite loci

Locus	EA	EB	EC	EF	EMac	EMal	EMer	EO
<i>Em-01</i> *								
<i>n</i>	29	34	50	49	11	41	10	50
<i>A</i> (<i>A_e</i>)	2 (1.9)	2 (1.4)	1 (-)	1 (-)	2 (1.8)	2 (1.9)	4 (2.6)	1 (-)
<i>H_o</i>	0.86	0.35	0	0	0.54	0.46	0.40	0
<i>H_e</i>	0.49	0.33	0	0	0.48	0.50	0.65	0
<i>F_{IS}</i>	-0.750	-0.065	-	-	-0.132	0.077	0.405	-
<i>Em-03</i> *								
<i>n</i>	29	34	50	44	11	41	10	50
<i>A</i> (<i>A_e</i>)	2 (1.1)	1 (-)	1 (-)	2 (1.5)	2 (1.7)	1 (-)	3 (2.8)	2 (1.6)
<i>H_o</i>	0.17	0	0	0.45	0.63	0	0.90	0.46
<i>H_e</i>	0.16	0	0	0.35	0.45	0	0.67	0.39
<i>F_{IS}</i>	-0.077	-	-	-0.284	-0.429	-	-0.350	-0.157
<i>Em-07</i> *								
<i>n</i>	29	34	50	35	11	41	10	50
<i>A</i> (<i>A_e</i>)	5 (2.6)	8 (2.8)	3 (2.2)	10 (3.2)	4 (2.4)	6 (2.7)	4 (3.3)	5 (2.3)
<i>H_o</i>	0.82	0.47	0.58	0.60	0.45	0.53	0.90	0.16
<i>H_e</i>	0.62	0.65	0.55	0.69	0.61	0.64	0.74	0.57
<i>F_{IS}</i>	-0.322*	0.282*	-0.052*	0.141	0.275	0.167	-0.227	0.722*
<i>Em-08</i> *								
<i>n</i>	29	34	50	49	11	41	10	50
<i>A</i> (<i>A_e</i>)	3 (1.2)	2 (1.0)	4 (2.1)	1 (-)	2 (1.2)	2 (3.7)	2 (1.1)	1 (-)
<i>H_o</i>	0.13	0.02	0.44	0	0	0.75	0.10	0
<i>H_e</i>	0.19	0.02	0.51	0	0.17	0.74	0.10	0
<i>F_{IS}</i>	0.293	-	0.155	-	1.00	-0.016	-	-
<i>Em-10</i> *								
<i>n</i>	29	34	50	49	11	41	10	50
<i>A</i> (<i>A_e</i>)	8 (2.64)	16 (8.5)	21 (11.2)	22 (10.8)	5 (4.3)	12 (7.4)	10 (8.3)	5 (3.8)
<i>H_o</i>	0.96	0.97	1.00	0.97	1.00	0.90	1.00	0.92
<i>H_e</i>	0.63	0.89	0.92	0.91	0.80	0.87	0.92	0.74
<i>F_{IS}</i>	-0.540*	-0.085	-0.087	-0.070	-0.257*	-0.030*	-0.084	-0.266*
<i>CA-07</i> *								
<i>n</i>	29	34	50	49	11	41	10	50
<i>A</i> (<i>A_e</i>)	6 (2.5)	8 (3.5)	4 (1.2)	5 (2.4)	9 (5.3)	3 (2.4)	3 (2.4)	3 (2.0)
<i>H_o</i>	0.51	0.82	0.18	0.38	0.81	0.48	0.20	0.42
<i>H_e</i>	0.61	0.73	0.16	0.59	0.85	0.59	0.62	0.50
<i>F_{IS}</i>	0.167	-0.128	-0.069	0.354*	0.043	0.302	0.692*	0.169
Mean								
<i>n</i>	29	34	50	45	11	41	10	50
<i>A</i> (<i>A_e</i>)	4.3 (2.0)	6.1 (2.9)	5.6 (2.7)	6.8 (3.0)	4.0 (2.8)	4.4 (3.0)	4.3 (3.6)	2.8 (1.6)
<i>H_o</i>	0.58	0.44	0.36	0.40	0.57	0.52	0.58	0.32
<i>H_e</i>	0.45	0.44	0.36	0.42	0.56	0.55	0.62	0.37
Overall								
<i>F_{IS}</i>	-0.275*	-0.002*	-0.017*	0.056*	-0.020	0.063	0.061*	0.119*

EA, *E. akaara*; EB, *E. bleekeri*; EC, *E. coioides*; EF, *E. fuscoguttatus*; EMac, *E. maculatus*; EMal, *E. malabaricus*; EMer, *E. merra*; EO, *E. ongus*.

n, sample size; *A*, number of observed alleles; *A_e*, effective number of alleles; *H_o* and *H_e*, observed and expected heterozygosity, respectively; *F_{IS}*, inbreeding coefficient, where statistically significant *F_{IS}* value is indicated by adding an asterisk (**P* < 0.001, simultaneous tests).

zero (*P* < 0.008 after Bonferroni correction). Nei's unbiased genetic distances suggested high levels of inter-specific genetic differentiation, as the distances ranged 0.433–2.710 (Table 2).

Homoplasy at *Em-03**157

Sequencing of the 157-bp products obtained from multiplication reaction primed by the primer

Em-03 revealed that the reverse strands of all six species comprised 154 nucleotide sequences (accession numbers AY736036–41) plus an A stretch (3 nt), which was probably added to the 3' end of the PCR product because of terminal transferase activity of *Taq* polymerase. The 157-bp products obtained from the six species comprised the same sequences except for a T–C transition at the 119th nucleotide site observed in the PCR products

Table 2 Pairwise F_{ST} between eight grouper species (upper matrix) and Nei's unbiased genetic distance (lower matrix)

Species	EA	EB	EC	EF	EMac	EMal	EMer	EO
EA	–	0.466	0.513	0.385	0.396	0.391	0.437	0.578
EB	1.219	–	0.447	0.462	0.292	0.323	0.305	0.460
EC	1.199	0.748	–	0.494	0.487	0.304	0.504	0.518
EF	0.666	1.064	0.976	–	0.431	0.391	0.456	0.554
EMac	1.010	0.478	1.160	1.118	–	0.327	0.236	0.424
EMal	1.112	0.654	0.433	0.945	0.975	–	0.349	0.433
EMer	1.713	0.566	1.538	1.614	0.598	1.385	–	0.396
EO	2.710	0.842	0.960	1.721	0.788	1.033	0.715	–

EA, *E. akaara*; EB, *E. bleekeri*; EC, *E. coioides*; EF, *E. fuscoguttatus*; EMac, *E. maculatus*; EMal, *E. malabaricus*; EMer, *E. merra*; EO, *E. ongus*.

All pairwise F_{ST} were statistically significant (Bonferroni corrected $P < 0.05/28 = 0.0018$, where the critical value 0.05 is adjusted because of multiple tests by dividing by number of tests = 28.)

Table 3 Percent of species assignment test of individuals of eight grouper species

Sample	Species of origin							
	EA	EB	EC	EF	EMac	EMal	EMer	EO
EA	100	0	0	0	0	0	0	0
EB	0	100	0	0	0	0	0	0
EC	0	0	100	0	0	0	0	0
EF	0	0	0	100	0	0	0	0
EMac	0	0	0	0	100	0	0	0
EMal	0	0	0	0	0	100	0	0
EMer	0	0	0	0	0	0	100	0
EO	0	0	0	0	0	0	0	100

EA, *E. akaara*; EB, *E. bleekeri*; EC, *E. coioides*; EF, *E. fuscoguttatus*; EMac, *E. maculatus*; EMal, *E. malabaricus*; EMer, *E. merra*; EO, *E. ongus*.

Individuals were assigned to each species at a level of LOD > 2.

	5'	3'
<i>E. akaara</i>	T.....	
<i>E. bleekeri</i>	C.....	
<i>E. coioides</i>	T.....	
<i>E. fuscoguttatus</i>	T.....	
<i>E. maculatus</i>	C.....	
<i>E. malabaricus</i>	T.....	
Consensus	T TCTGCTGTAGCTATGTGTGTGCGTGTGTCG	

Fig. 2 Reverse strand sequence of microsatellite locus *Em-03* determined for six grouper species. The nucleotide site where a point mutation (T–C transition) appeared (119th nucleotide site) and adjacent sequences are shown. Repeat domain of this locus is underlined in the consensus sequence.

from *E. bleekeri* and *E. maculatus*. The transitional site was at the first GT repeats of (GT)₃ (Fig. 2). There was no sequence polymorphism among conspecific individuals.

Species assignment test

The species assignment was accomplished using the data of six microsatellite loci. The individuals

from eight grouper species were successfully assigned to their origin. The percent of collected species is shown in Table 3.

Phylogenetic dendrogram

The neighbor-joining tree generated from the distance matrix (Fig. 3) showed that the eight grouper species are clustered into two main groups. The first group comprised the two temperate species *E. fuscoguttatus* and *E. akaara* with a bootstrapping value of 63%. The second group included all the remaining species in which all the internal nodes except for a node that grouped *E. coioides* and *E. malabaricus* were not supported by bootstrap values.

The phylogenetic dendrogram regenerated taking into account the size homoplasy (without *E. merra*) resulted in a slight change of the original tree topology (figure not shown). However, both the original and new trees were statistically not well supported at most of the internal nodes.

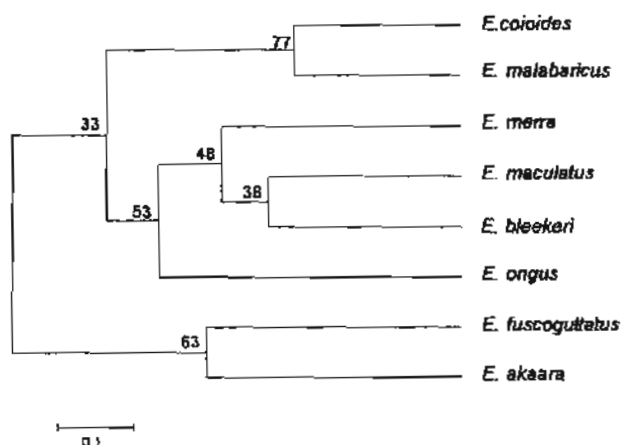


Fig. 3 Neighbor-joining tree based on Nei's unbiased genetic distance.³⁷ Bootstrap value is shown at each node, scale bar = genetic distance.

DISCUSSION

Genetic differentiation between species

The eight grouper species were readily differentiated using microsatellite loci, as the F_{ST} values between species were much higher than intraspecific F_{ST} values for *E. marginatus* ($F_{ST} = 0.018$)¹² and *E. coioides* ($F_{ST} = 0.074$).⁴² Genetic distance obtained in this study ($D = 0.433$ – 2.710) was also higher than that reported for intraspecific difference of the three species of *Epinephelus* ($D = 0.051$ – 0.470 for *E. marginatus*,¹² $D = 0.023$ – 0.123 for *E. polyphekadion*,¹³ $D = 0.016$ – 0.086 for *E. coioides*⁴²).

The diagnostic alleles observed at *Em-01** (between *E. coioides*, *E. ongus*, and *E. fuscoguttatus*) and *Em-08** (between *E. ongus* and *E. fuscoguttatus*) are useful for species identification. Although polymorphism at *Em-01** was observed in four different populations of *E. coioides*,⁴² the allele sizes did not overlap with the alleles from *E. fuscoguttatus*, with approximately 30-bp difference, suggesting that this locus can be used for species differentiation between *E. coioides* and *E. fuscoguttatus*. In addition to the diagnostic alleles, private alleles, which were found in several species, will provide an objective means to discriminate the grouper species, for example, between *E. akaara* and *E. bleekeri*–*E. coioides*–*E. maculatus*–*E. ongus* and between *E. merra*–*E. fuscoguttatus* and *E. maculatus*. Although in the case of species identification for adult fish little taxonomic confusion occurs between these species based on external characteristics alone, the private alleles can be useful for taxonomic classification of larvae. Because the sample size is small,

it is very unlikely that we were able to sample all alleles for every locus and species; therefore, it is necessary to extensively survey the allelic diversities of other populations in each species.

Homoplasy at *Em-03*157*

Size homoplasy was observed between species of grouper but not within species, and this finding complements those of previous studies that size homoplasy always occurred at compound and interrupted microsatellites.^{22,23,27,43} Most of the previously reported homoplasy was caused by deletion and additions of repeat units, and in some instances, especially between species, by nucleotide variations in microsatellite flanking regions (deletion, addition, and point mutation).²⁵

The presence of size homoplasy is expected to result in reduction of the branch length of a phylogenetic tree⁴³ or alteration of tree topology.²⁶ When we scored the homoplasy alleles as different alleles, the tree topology was altered (figure not shown), but the confidence level of the reconstructed tree was low as the bootstrap values ranged 52–79. Hence, our data sets do not suffice to evaluate how much the presence of size homoplasy influences the inference of phylogenetic relationships among species; this topic should be investigated further.

Species assignment test

The eight grouper species were unambiguously separated by the assignment test, when the individuals were completely assigned to their species at the level of logarithm of the odds ratio (LOD) > 2. The microsatellite DNA markers provide more statistical power than was previously afforded by allozymes, both for detecting linkage disequilibrium, a signal of sample admixture, and for assigning individuals to populations of origin.⁴¹ An example is the non-winter contaminants of chinook salmon *Oncorhynchus tshawytscha* in winter-run broodstock, which were assigned by microsatellite loci data using the WHICHRUN⁴⁴ program. Removing 17 individuals determined to be likely of non-winter origin from these stock restored equilibrium conditions.⁴⁴ Further identification of any unknown grouper can be facilitated by the assignment test (WHICHRUN program) using our data as a baseline, providing that the same microsatellite loci are scored.

Phylogenetic dendrogram

The dendrogram obtained in this study did not show a robust relationship between species

because most of the internal nodes were not supported by high bootstrap values. Nevertheless, at least the close relationship between *E. coioides* and *E. malabaricus* was supported (bootstrap value = 77%) and is in agreement with the study based on RAPDs.¹⁸

CONCLUSION

This study demonstrated that: (i) the eight species of the genus *Epinephelus* could be readily differentiated using microsatellite markers; (ii) the fixed allelic different and private alleles provide an additional tool for species identification; (iii) the eight species included in this study were divided into two distinct groups; and (iv) size homoplasy was present.

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REFERENCES

1. Froese R, Pauly D. *FishBase*. 2005 September. [Cited 10 September 2005]. Available from URL: <http://www.fishbase.org>, version 09/2005.
2. Heemstra PC, Randall JE. *FAO Species Catalogue: Groupers of the World (Family Serranidae, Subfamily Epinephelinae)*. FAO Fisheries Synopsis no. 125. FAO, Rome. 1993.
3. Leong TS. Grouper culture. In: De Silva SS (ed.). *Tropical Mariculture*. Academic Press, Toronto. 1998; 381–448.
4. Frankham R, Ballou JD, Briscoe DA. *Introduction to Population Genetics*. Cambridge University Press, Cambridge, UK. 2000.
5. Avise JC. *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York, NY. 1994.
6. Grudzien TA, Turner BJ. Genic identity and geographic differentiation of trophically dichotomous *Ilyodon* (Teleostei: Goodeidae). *Copeia* 1984; 1984: 102–107.
7. Turner BJ, Grosse DJ. Trophic differentiation in *Ilyodon*, a genus of stream-dwelling goodeid fishes: speciation versus ecological polymorphism. *Evolution* 1980; 34: 259–270.
8. Hanfling B, Brandl R. Genetic differentiation of the bullhead *Cottus gobio* L. across watersheds in Central Europe: evidence for two taxa. *Heredity* 1998; 80: 110–117.
9. Senanan W, Kapuscinski AR, Na-Nakorn U, Miller LM. Genetic impacts of hybrid catfish farming (*Clarias macrocephalus* x *C. gariepinus*) on native catfish populations in central Thailand. *Aquaculture* 2004; 235: 167–184.
10. Na-Nakorn U, Kamonrat W, Ngamsiri T. Genetic diversity of walking catfish, *Clarias macrocephalus*, in Thailand and evidence of genetic introgression from introduced farmed *C. gariepinus*. *Aquaculture* 2004; 240: 145–163.
11. Sugama K, Tridjoko, Haryanti, Budi S, Cholik F. Genetic variation and population structure in the humpback grouper, *Cromileptes altivelis*, throughout its range in Indonesian waters. *Indo Fish. Res. J.* 1999; 1: 32–38.
12. De Innocentis S, Sola L, Cataudela S, Bentzen P. Allozyme and microsatellite loci provide discordant estimates of population differentiation in the endangered dusky grouper (*Epinephelus marginatus*) within the Mediterranean Sea. *Mol. Ecol.* 2001; 10: 2163–2175.
13. Rhodes KL, Lewis RI, Chapman RW, Sadovy Y. Genetic structure of camouflage grouper, *Epinephelus polyphekadion* (Pisces: Serranidae), in the western central Pacific. *Mar. Biol.* 2003; 142: 771–776.
14. Zlatoff MS, Ball AO, Sedberry GR. Population genetic analysis of red grouper, *Epinephelus morio*, and scamp, *Mycteroperca phenax*, from the southeastern U.S. Atlantic and Gulf of Mexico. *Mar. Biol.* 2004; 144: 769–777.
15. Gilles A, Miquelès A, Quignard JP, Faure E. Molecular phylogeography of western Mediterranean dusky grouper *Epinephelus marginatus*. *Life Sci.* 2000; 323: 195–205.
16. Carlin JL, Robertson DR, Bowen BW. Ancient divergences and recent connections in two tropical Atlantic reef fishes *Epinephelus adscensionis* and *Rypticus saponaceus* (Percoidae: Serranidae). *Mar. Biol.* 2003; 143: 1057–1069.
17. Craig MT, Pondella DJ, Franck JPC, Hafner JC. On the status of the serranid fish genus *Epinephelus*: evidence for paraphyly based upon 16S rDNA sequence. *Mol. Phylogenet. Evol.* 2001; 19: 121–130.
18. Govindaraju GS, Jayasankar P. Taxonomic relationship among seven species of Grouper (genus *Epinephelus*; Family Serranidae) as revealed by RAPD fingerprinting. *Mar. Biotechnol.* 2004; 6: 229–237.
19. Takagi M, Taniguchi N, Cook D, Doyle RW. Isolation and characterization of microsatellite loci from red sea bream *Pagrus major* and detection in closely related species. *Fish. Sci.* 1997; 63: 199–204.
20. Morris DB, Richard KR, Wright JM. Microsatellites from rainbow trout (*Oncorhynchus mykiss*) and their use for genetic study of salmonids. *Can. J. Fish. Aquat. Sci.* 1996; 53: 120–126.
21. Nugroho E, Taniguchi N. Isolation of greater amberjack microsatellite DNA and their application as genetic marker to species of genus *Seriola* from Japan. *Fish. Sci.* 1999; 65: 353–357.
22. Taylor JS, Justina SP, Breden F. Microsatellite allele size homoplasy in the guppy (*Poecilia reticulata*). *J. Mol. Evol.* 1999; 48: 245–247.
23. Adams RJ, Brown KM, Hamilton MB. The impact of microsatellite homoplasy on multilocus population structure estimates in a tropical tree (*Corythophora alba*) and an anadromous fish (*Morone saxatilis*). *Mol. Ecol.* 2004; 13: 2579–2588.

24. Blanquer-Maumont A, Crouau-Roy B. Polymorphism, monomorphism and sequence in conserved microsatellites in Primates species. *J. Mol. Evol.* 1995; 41: 492-497.
25. Grimaldi M, Crouau-Roy B. Microsatellite allelic homoplasy due to variable flanking sequences. *J. Mol. Evol.* 1997; 44: 336-340.
26. Viard F, Franck P, Dubois M, Estoup A, Jarne P. Variation of microsatellite size homoplasy across electromorphs, loci, and populations in three invertebrate species. *J. Mol. Evol.* 1998; 47: 42-51.
27. Angers B, Estoup A, Jarne P. Microsatellite size homoplasy, SSCP, and population structure: a case study in the freshwater snail *Bulinus truncatus*. *Mol. Biol. Evol.* 2000; 17: 1926-1932.
28. Nugroho E, Takagi M, Sugama K, Taniguchi N. Detection of GT repeats microsatellite loci and their polymorphism for grouper of the genus *Epinephelus*. *Fish. Sci.* 1998; 64: 836-837.
29. Taniguchi N, Nugroho E. Genetic characteristics of introduced fishes and study of genetic evaluation of fish genetics and breeding. In: Japan Seawater Fisheries Cultivation Association (ed.). *A Report of Project for Counter Measure on the Problems Caused in Fish Culture*. Japan Seawater Fisheries Cultivation Association, Kobe, Japan. 2000; 141-188 (in Japanese).
30. Rivera MAJ, Graham GC, Roderick GK. Isolation and characterization of nine microsatellite loci from the Hawaiian grouper *Epinephelus quernus* (Serranidae) for population genetic analyses. *Mar. Biotechnol.* 2003; 5: 126-129.
31. Perez-Enriquez R, Takamura M, Taniguchi N. Microsatellite DNA detection by chemiluminescence in red sea bream: a practical manual. *Fish. Genet. Breed. Sci.* 1998; 26: 73-79 (in Japanese). ◆
32. Nei M. *Molecular Evolutionary Genetics*. Columbia University Press, Columbia, NY. 1987.
33. Wright S. The genetic structure of populations. *Ann. Eugenics* 1951; 15: 323-354.
34. Wright S. *Evolution and the Genetics of Populations, Vol. 4, Variability within and among Natural Populations*. University of Chicago Press, Chicago, IL. 1978.
35. Rousset F. Equilibrium values of measures of population subdivision for stepwise mutational processes. *Genetics* 1996; 142: 1357-1362.
36. Raymond M, Rousset F. GENEPOP (ver. 1.2): a population genetics Software for exact test and ecumenicism. *J. Hered.* 1995; 86, 248-249.
37. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 1978; 89: 583-590.
38. Saitoh N, Nei M. The neighbor-joining method: a new method for reconstruction phylogenetic trees. *Mol. Biol. Evol.* 1987; 4: 406-425.
39. Felsenstein J. *PHYLIP (Phylogeny Inference Package), Version 3.6*. Department of Genetics, University of Washington, Seattle, WA. 2004.
40. Koichiro T. *Tree-Explorer software, Version 2.12*. Evolutionary Genetics Laboratory, Tokyo Metropolitan University, Tokyo. 1999.
41. Banks MA, Eichert W. WHICHRUN (ver. 4.1): a computer program for population assignment of individuals based on multilocus genotype data. *J. Hered.* 2000; 91: 87-89.
42. Antoro S, Na-Nakorn U, Koedprang W. Study of genetic diversity of orange-spotted grouper, *Epinephelus coioides* from Thailand and Indonesia using microsatellite markers. *Mar. Biotechnol.* 2006; 8: 17-26.
43. Banks MA, Rashbrook VK, Calavetta MJ, Dean CA, Hedgcock D. Analysis of microsatellite DNA resolves genetic structure and diversity of chinook salmon (*Oncorhynchus tshawytscha*) in California's Central Valley. *Can. J. Fish. Aquat. Sci.* 2000; 57: 915-927.
44. Estoup A, Tailliez C, Cornuet JM, Solignac M. Size homoplasy and mutational processes of interrupted microsatellites in two bee species, *Apis mellifera* and *Bombus terrestris* (Apidae). *Mol. Biol. Evol.* 1995; 12: 1074-1084.

Table A1 Microsatellite allele frequencies in eight grouper species[illegible]

Table A1 Continued

Allele n	EA 29	EB 34	EC 50	EF 49	EMac 11	EMal 50	EMer 10	EO 50
177	0	0	0	0.227	0	0	0	0
<i>Em-07*</i>								
155	0	0.412	0.570	0.327	0	0.309	0.150	0.620
157	0.414	0	0	0	0.136	0.012	0	0.170
159	0.448	0.015	0.080	0	0.182	0	0	0.040
161	0	0	0	0.011	0.091	0	0	0
163	0	0.015	0	0.041	0	0	0	0
165	0	0.029	0	0	0	0	0.300	0
167	0	0	0	0.029	0	0	0	0
169	0.034	0	0	0.029	0	0	0.400	0
171	0	0.426	0.350	0.443	0.591	0.451	0.150	0
173	0.086	0.059	0	0	0	0.024	0	0
175	0	0.015	0	0	0	0.037	0	0.130
177	0.017	0	0	0.041	0	0.085	0	0
179	0	0.029	0	0	0	0	0	0
181	0	0	0	0.011	0	0	0	0.040
183	0	0	0	0.057	0	0	0	0
185	0	0	0	0.011	0	0	0	0
<i>Em-08*</i>								
196	0	0	0.020	0	0	0	0	0
200	0	0	0	0	0.091	0	0	0
201	0	0	0	0	0	0.244	0	0
202	0	0.985	0	0	0.909	0	0.950	1.000
204	0.897	0.015	0	1.000	0	0	0.050	0
206	0.052	0	0.570	0	0	0	0	0
207	0	0	0	0	0	0.134	0	0
208	0.052	0	0.400	0	0	0	0	0
210	0	0	0.010	0	0	0	0	0
211	0	0	0	0	0	0.329	0	0
213	0	0	0	0	0	0.281	0	0
215	0	0	0	0	0	0.012	0	0
<i>Em-10*</i>								
94	0	0	0.030	0	0	0	0.050	0
96	0	0.074	0.010	0	0	0	0.150	0
98	0	0	0	0	0	0	0.100	0
100	0	0.015	0	0	0	0	0.100	0
101	0.397	0	0	0	0	0	0	0
102	0	0	0	0	0	0.037	0	0
103	0.466	0	0	0	0	0	0	0
104	0	0	0.010	0	0	0	0.100	0
105	0.017	0	0	0	0.227	0	0	0
106	0	0.015	0.050	0	0	0.073	0	0.170
107	0.052	0	0	0	0.227	0	0	0
108	0	0.015	0.040	0	0	0.061	0	0.170
109	0	0	0	0	0.273	0	0	0
110	0	0.103	0.080	0.122	0	0.085	0.100	0.130
111	0.017	0	0	0	0.227	0	0	0
112	0	0	0.030	0.010	0	0.073	0.200	0.420
113	0.017	0	0	0	0.045	0	0	0
114	0	0.103	0.160	0.122	0	0	0.100	0.110
115	0.017	0	0	0	0	0	0	0
116	0	0.221	0.100	0.010	0	0.024	0	0
118	0	0.088	0.160	0.031	0	0.085	0.050	0
120	0	0.162	0.080	0.051	0	0.182	0.050	0
121	0.017	0	0	0	0	0	0	0
122	0	0	0.030	0.010	0	0.244	0	0

Table A1 Continued

Allele <i>n</i>	EA 29	EB 34	EC 50	EF 49	EMac 11	EMal 50	EMer 10	EO 50
124	0	0.059	0.030	0.020	0	0.098	0	0
126	0	0.029	0.030	0.031	0	0.024	0	0
128	0	0.015	0.010	0.112	0	0.012	0	0
130	0	0.044	0.010	0.184	0	0	0	0
132	0	0.015	0.010	0.041	0	0	0	0
134	0	0.015	0.010	0.031	0	0	0	0
136	0	0	0.060	0.051	0	0	0	0
138	0	0	0.020	0.031	0	0	0	0
140	0	0.029	0.040	0.061	0	0	0	0
142	0	0	0	0.020	0	0	0	0
148	0	0	0	0.020	0	0	0	0
150	0	0	0	0.031	0	0	0	0
156	0	0	0	0.010	0	0	0	0
CA-07*								
117	0	0	0	0	0	0	0.250	0
119	0	0	0	0	0	0	0.550	0
201	0	0	0	0	0	0	0.200	0
203	0	0.412	0	0	0	0	0	0
205	0	0.294	0	0	0	0	0	0
207	0.017	0.044	0	0	0	0	0	0
209	0.224	0.029	0	0	0.045	0	0	0
211	0.121	0	0	0.591	0.273	0	0	0.590
213	0	0	0	0	0.045	0.524	0	0.390
215	0	0.132	0	0	0	0.122	0	0.020
217	0.034	0	0.010	0	0.045	0	0	0
219	0.569	0	0.010	0	0.273	0	0	0
221	0.034	0.029	0.910	0.216	0.091	0	0	0
223	0	0.015	0.070	0.102	0.045	0.354	0	0
225	0	0	0	0.068	0.045	0	0	0
229	0	0	0	0.023	0	0	0	0
231	0	0	0	0	0.136	0	0	0
237	0	0.044	0	0	0	0	0	0

EA, *E. akaara*; EB, *E. bleekeri*; EC, *E. coioides*; EF, *E. fuscoguttatus*; EMac, *E. maculatus*; EMal, *E. malabaricus*; EMer, *E. merra*; EO, *E. ongus*. *n*, sample size.

เอกสารแนบที่ 9

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Study of Genetic Diversity of Orange-Spotted Grouper, *Epinephelus coioides*, from Thailand and Indonesia Using Microsatellite Markers

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Abstract

Genetic diversity of *Epinephelus coioides* (Hamilton, 1822), which inhabits coastal reefs from the western Indian Ocean to the western Pacific Ocean, was studied based on four polymorphic microsatellite loci. Two hundred and fifty individuals were collected from two locations in Thailand (Nakorn-rithammarat-N and Trang-T) and four in Indonesia (Sibolga-S, Lampung-L, Jepara-J, and Flores-F). The genetic variation of *E. coioides* was relatively low; the observed heterozygosities (H_o) ranged between 0.36 (F) and 0.55 (N). The average number of alleles/locus was between 3.57 (L) and 5.09 (J). Genotypic distribution for most population pairs was significantly different after Bonferroni correction ($P < 0.0024$) except for J and F. Population structuring was significant ($F_{ST} = 0.074$). The genetic distances between populations ranged between 0.016 (L and N) to 0.086 (F and S). Mantel's test showed no correlation between genetic distance and geographical distance. The NJ tree clearly separated N from the others which comprised two subgroups, T-S and L-J-F.

Keywords: Genetic variation — grouper — population structure — southeast Asia

Introduction

Epinephelus coioides, the orange-spotted grouper, belongs to the subfamily Epinephelinae (family Serranidae) and inhabits areas from the Red Sea, south to at least Durban, east to the western Pacific from the Ryukyu Islands to Australia, and eastward

to Palau and Fiji (Heemstra and Randall, 1993). *E. coioides* and other members of the genus *Epinephelus* and the two sister genera *Plectropomus* and *Cromileptes* significantly contributed to capture fisheries production, with more than 233,000 metric tonnes landed in 2002 (FAO online at <http://www.fao.org/fi/statist/statist.asp>). Moreover, *E. coioides* is among a few species of *Epinephelus* that are accepted for aquaculture and showed an increasing trend for the world's annual production (from 9,577 metric tonnes in 2000 to 22,808 metric tonnes in 2002) (FAO online at <http://www.fao.org/fi/statist/statist.asp>).

Despite the economic importance of *E. coioides*, little is known about its genetic diversity. Genetic variation within populations of groupers may be low for one or more of the following reasons: decreased abundance as a result of fishing activities during their spawning aggregation (Collin, 1996; Sala et al., 2001) and catching large quantities of immature groupers for either consumption (Al Janhi et al., 2005) or aquaculture (Cesar et al., 2000), reduction of effective population size owing to skewed sex ratio, and inbreeding.

Groupers are believed to mate with a skewed sex ratio because most are consecutive hermaphrodites (Collin, 1996); hence large fish comprise only one sex and are caught more frequently than the smaller ones (Shapiro, 1987). Empirical data showed that the sex ratio of *E. coioides* caught around the United Arab Emirates was one male to 23.6 females (Al Janhi et al., 2005). This unequal sex ratio leads to a reduction of effective population size which determines levels of inbreeding and eventually results in loss of genetic diversity (Falconer, 1985).

Genetic diversity among populations is an invaluable resource for the sustainability of the

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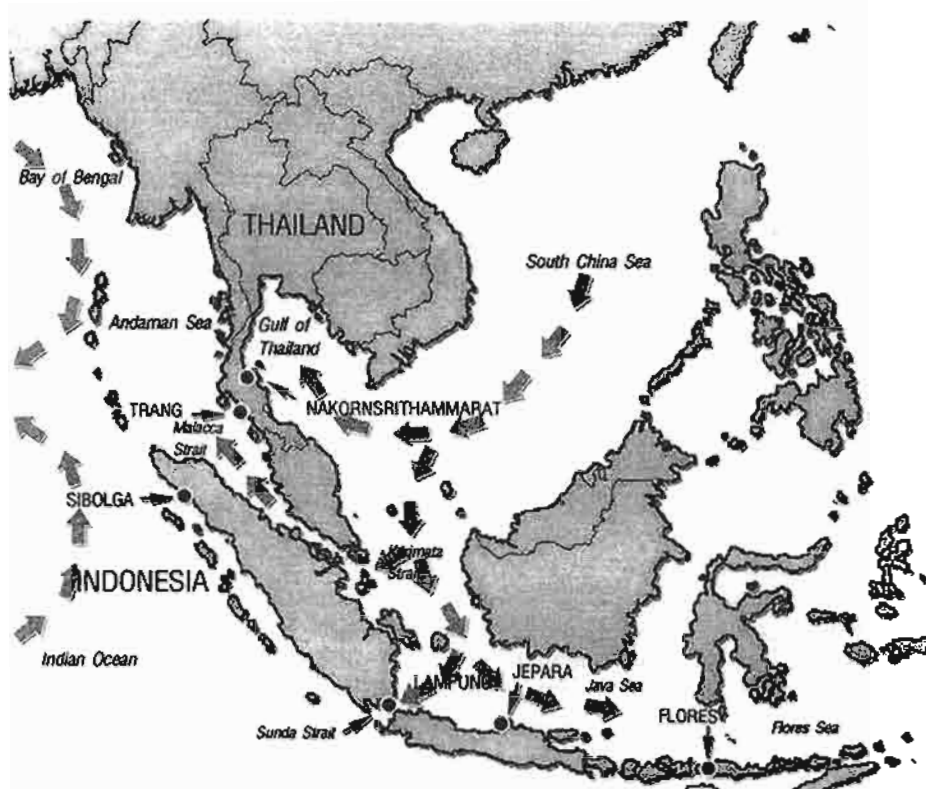


Fig. 1. Sampling locations of *E. coioides* and sea surface current (0 to 10 m) in the Gulf of Thailand during northeast monsoon (December to February). Source: Snidvongs and Sojisuoporn (1999).

species. It is likely that Serranid species as well as other demersal marine fishes possess a distinct population structure because of their relatively sedentary nature at adult stages. However, gene flow is facilitated by dispersal of planktonic eggs and larvae by oceanic current (Thompson and Munro, 1978; Shaklee, 1984; Garcia de Leon et al., 1997; Bagley et al., 1999; Medioni et al., 2001). Genetic differentiation depends on duration of the larval period, degree of larval retention, survival of the dispersed larvae (Rhodes et al., 2003), and other factors precluding larval dispersal (e.g., river outflow and oceanic expanses and historical differentiation) (Carlin et al., 2003).

Information on genetic diversity of groupers is still limited, especially among the species of south-east Asia. Most studies revealed significant population structuring of grouper populations inhabiting a

wide range of geographic areas (Sugama et al., 1999; De Innocentiis et al., 2001; Carlin et al., 2003).

The objectives of this study were to quantify genetic diversity of *E. coioides* from six populations throughout their natural range in Thai and Indonesian waters, and assess their genetic relationship. The information obtained will facilitate better understanding of the genetic diversity of sedentary marine species such as *E. coioides*, especially the species of southeast Asian waters. It will also provide information on genetically distinct populations useful for fishery management and aquaculture.

Materials and Methods

Sample Collection. Two hundred and fifty samples of *E. coioides* (31 to 50 fish/site) were collected from May to July, 2003, covering six sampling sites

Table 1. Details for Collection Sites, Size, and Number of Samples of *Epinephelus coioides* Collected from Six Locations in Thailand and Indonesia

Collection sites	Country	Lat./Long.	Sizes of samples (cm)	No. of samples
Nakornsrihammarat	Thailand	100°15' E/8°45' N	20–25	50
Trang	Thailand	99°30' E/7°30' N	10–15	46
Sibolga	Indonesia	98°25' E/1°45' N	10–15	42
Lampung	Indonesia	105°20' E/5°45' S	20–30	43
Jepara	Indonesia	110°45' E/6°25' S	20–30	38
Flores	Indonesia	119°45' E/8°45' S	40–60	31

(Figure 1 and Table 1): two in Thailand, Trang (Andaman Sea) and Nakornsrithammarat (Southern Gulf of Thailand), and four in Indonesia, Sibolga (Western Coast of North Sumatra Island), Lampung (Southern tip of Sumatra Island), Jepara (Northern Coast of Central Java Island), and Flores (Flores Sea).

A small piece of a caudal fin was cut and preserved in 90% ethanol and delivered to Fish Genetics Laboratory, Department of Aquaculture, Faculty of Fishery, Kasetsart University. Before fin tissue was taken, the fish were identified according to Heemstra and Randall (1993).

DNA extraction was performed using a standard phenol/chloroform method (Taggart et al., 1992) with slight modifications. The individual DNA was resuspended in TE buffer (10 mM Tris-HCl, pH 7.2; 1 mM EDTA, pH 8.0) and stored at 4°C.

The DNA of each individual was amplified via polymerase chain reaction (PCR) primed by five microsatellite primers developed from *E. merra* DNA libraries, *Em-01*, *Em-03*, and *Em-08* (Nugroho et al., 1998), *Em-07*, and *Em-10* (Taniguchi and Nugroho, 2000). PTC (Programmable Thermal Controller)-100 was used to perform amplification. Each 10 µl of PCR reaction contained 5 to 15 ng of template DNA, 0.5 µM of reverse and forward primers, reaction buffer (8.8 mM Tris-HCl, pH 8.3 and 43.9 mM KCl), 1.2 to 1.5 mM MgCl₂, 100 µM of dNTP mix, and 0.2 U of *Taq* polymerase (Pharmacia Biotech.). The PCR cycles consisted of one cycle at 94°C for 1 minute, followed by 35 cycles of 94°C (30 sec), appropriate annealing temperature (30 second), and 72°C (1 minute), and then terminated by one cycle of 72°C for 1 minute.

Following amplification, each PCR product was mixed with 5 µl of sequencing dye and denatured at 95°C for 5 min. Three microliters of each PCR product were electrophoresed on polyacrylamide gel 6% acrylamide, 700 µl of 10% ammonium persulfate, and 14 µl of N,N,N',N'-tetramethylethylenediamine (TEMED). Gel fixation and silver staining were performed following the method described by Sambrook et al. (1989). Sizes of alleles were determined according to a M13 sequence ladder.

Data Analyses. Because of the monomorphism of the *Em-03*, only data from the remaining four microsatellites were used for the following analyses. The number of alleles per locus, number of effective alleles per locus, and observed and expected heterozygosities were calculated. Conformity to Hardy-Weinberg equilibrium was tested using the Markov chain method (dememorization: 1000, batches: 500 and iterations per batches: 1000). All of these calculations were performed using

the GENEPOP computer package (Raymond and Rousset, 1995). The independent *t*-test comparison was employed to check differentiation of observed heterozygosity among populations (Archie, 1985).

A test for the existence of a bottleneck was performed according to Cornuet and Luikart (1996) using the program BOTTLENECK version 1.2.02 (Cornuet and Luikart, 1996). The program performed tests that aimed to detect allele deficiency by comparing observed heterozygosity and the heterozygosity expected from the observed number of alleles. Populations showing heterozygosity excess were considered as having experienced a recent genetic bottleneck.

The GENEPOP package was used to calculate *F*-statistics (F_{IS} , F_{IT} , and F_{ST}), test of genetic disequilibrium for each pair of loci, and genetic differentiation between populations (dememorization: 1000, batches: 100 and iterations per batches: 1000). Because of the small number of loci used, the package did not allow for a significant test of the overall F_{ST} . Therefore, a heterogeneity chi-square for each locus was employed to test whether F_{ST} was significantly different than from (Halliburton, 2004). The locus-wise F_{IS} for each population was also calculated to detect effects of inbreeding and Wahlund effects.

Genetic distance between populations was based on the Cavalli-Sforza and Edwards (1967) chord distance because it is among the best measures to recover the true tree topology (Takezaki and Nei, 1996). A neighbor joining tree including bootstrap values was constructed using the PHYLIP computer package (Felsenstein, 1995). Mantel's test was performed to evaluate correlation between genetic distances and geographical distances (Manly, 1993) using TFGPA version 1.3 (Miller, 1997).

Results

Private Alleles and Allele Fixation. Six private alleles at three loci were observed in Flores, *Em-07**170, *172, *Em-08**216, *Em-10**70, *72, while one allele, *Em-10** 98, was private for Sibolga. Alleles fixation (gene frequency = 1) was observed in Trang (at *Em-01**256). Gene frequencies are shown in Table 2.

Hardy-Weinberg Equilibrium (HWE) and Linkage Disequilibrium. The F_{IS} value for each population across all loci (Table 2) indicated heterozygote deficiency ($P < 0.05$) in all but one population (Nakornsrithammarat). However, when the Bonferroni correction was applied, three

Table 2. Allele Frequencies at Four Microsatellite Loci and F_{IS} Across Loci for Each Population of *E. coioides* Collected from Two Locations in Thailand and Four Locations in Indonesia

Alleles	Trang	Nakornsrithammarat	Sibolga	Lampung	Jepara	Flores
Em-01	45	50	42	43	38	31
*254	0.00	0.00	0.04	0.01	0.07	0.03
*256	1.00	0.80	0.89	0.95	0.80	0.95
*258	0.00	0.20	0.07	0.04	0.13	0.02
F_{IS}	NA	-0.24	-0.08	0.23	0.15	0.66*
Em-07	44	50	42	43	38	31
*162	0.11	0.03	0.09	0.05	0.09	0.06
*164	0.50	0.36	0.32	0.46	0.39	0.24
*168	0.08	0.12	0.05	0.11	0.17	0.22
*170	0.00	0.00	0.00	0.00	0.00	0.14
*172	0.00	0.00	0.00	0.00	0.00	0.02
F_{IS}	0.40*	0.26*	0.14	0.34*	0.44*	0.63*
Em-08	46	50	42	43	38	31
*204	0.00	0.01	0.00	0.02	0.00	0.00
*206	0.65	0.64	0.62	0.55	0.01	0.03
*208	0.35	0.34	0.38	0.39	0.23	0.31
*210	0.00	0.01	0.00	0.04	0.43	0.48
*212	0.00	0.00	0.00	0.00	0.32	0.14
*214	0.00	0.00	0.00	0.00	0.01	0.02
*216	0.00	0.00	0.00	0.00	0.00	0.02
F_{IS}	0.15	-0.04	0.10	-0.06	0.33*	0.51*
Em-10	45	50	42	43	38	31
*70	0.00	0.00	0.00	0.00	0.00	0.02
*72	0.00	0.00	0.00	0.00	0.00	0.03
*94	0.02	0.01	0.01	0.01	0.01	0.00
*98	0.00	0.00	0.01	0.00	0.00	0.00
*104	0.01	0.00	0.00	0.02	0.00	0.02
*106	0.06	0.05	0.03	0.00	0.04	0.05
*108	0.04	0.17	0.07	0.11	0.04	0.05
*110	0.08	0.03	0.05	0.04	0.05	0.00
*112	0.04	0.04	0.05	0.09	0.07	0.06
*114	0.17	0.09	0.06	0.26	0.14	0.08
*116	0.08	0.07	0.09	0.06	0.08	0.06
*118	0.17	0.15	0.11	0.12	0.12	0.31
*120	0.10	0.10	0.13	0.07	0.03	0.19
*122	0.03	0.07	0.05	0.08	0.12	0.02
*124	0.03	0.02	0.09	0.05	0.03	0.06
*126	0.02	0.02	0.05	0.01	0.03	0.02
*128	0.01	0.02	0.04	0.00	0.00	0.00
*130	0.01	0.02	0.05	0.01	0.03	0.01
*132	0.00	0.06	0.01	0.03	0.03	0.02
*134	0.03	0.01	0.01	0.01	0.08	0.00
*136	0.02	0.01	0.00	0.01	0.04	0.00
*138	0.03	0.02	0.00	0.01	0.01	0.00
*140	0.00	0.00	0.04	0.00	0.03	0.00
*142	0.02	0.01	0.00	0.00	0.01	0.00
*144	0.01	0.02	0.04	0.00	0.00	0.00
*146	0.01	0.00	0.00	0.00	0.00	0.00
*148	0.01	0.00	0.01	0.01	0.01	0.00
*150	0.00	0.01	0.00	0.00	0.00	0.00
F_{IS}	-0.02	0.06	0.01	0.03	0.18*	0.06

NA = not available.

*Statistically significant after Bonferroni correction ($P < 0.05/24$).

populations, Nakornsrithammarat, Sibolga, and Lampung, conformed to HWE ($P > 0.05/24$). There was no significant genotypic linkage disequilibrium between each pair of loci within each of the seven populations.

Genetic Variation Within Populations and Existence of Bottlenecks. Genetic variability within populations is shown in Table 3. Mean numbers of alleles per locus ranged from 7.25 in populations from Trang, Sibolga, and Lampung to

Table 3. Genetic Variability of Six *E. coioides* Populations Collected from Thailand and Indonesia Based on Four Microsatellite Loci

Population	Mean no. alleles/locus	Effective allele no. across loci	Mean heterozygosity	
			Observed	Expected
Trang	7.25	4.13	0.43 ± 0.10	0.50 ± 0.13
Nakornsrihammarat	7.75	4.24	0.55 ± 0.19	0.59 ± 0.19
Sibolga	7.25	4.81	0.52 ± 0.15	0.55 ± 0.15
Lampung	7.25	3.57	0.48 ± 0.16	0.54 ± 0.17
Jepara	8.00	5.09	0.47 ± 0.10	0.66 ± 0.12
Flores	7.50	3.65	0.36 ± 0.16	0.60 ± 0.17

8.0 in a population from Jepara. However, when allelic evenness was considered, the effective allele numbers across loci were reduced to between 3.57 in Lampung and 5.09 in Jepara. Observed heterozygosities ranged between 0.36 in the Flores population and 0.55 in the Nakornsrihammarat population. Expected heterozygosities were between 0.50 and 0.66.

An independent *t*-test (Archie, 1985) showed that neither observed nor expected heterozygosity was significantly different between populations.

The detection of bottlenecks gave different results between sign test, standard difference test (SDT), Wilcoxon sign-rank test, and the mode-shift test. The sign test failed to reject the hypothesis of mutation-drift equilibrium, thus showing no evidence of a recent bottleneck. The SDT rejected the hypothesis under SMM (stepwise mutation model) in two populations, Lampung ($T_2 = -2.385$; $P = 0.008$) and Flores ($T_2 = -3.271$; $P = 0.001$), and under IAM (infinite allele model) in Jepara ($T_2 = 1.734$; $P = 0.041$) while Wilcoxon test showed a presence of a bottleneck for Nakornsrihammarat under IAM ($P = 0.031$) (Table 4). Nevertheless, the allele frequency distribution was approximately L-shaped for all populations, which implied no recent bottlenecks occurred in these populations.

Population Structuring and Population Differentiation. F_{ST} was 0.074 and significantly different from 0 (Halliburton, 2004), thus clearly showing that populations of *E. coioides* divided into subpopulations. Four of 15 population pairs were not significantly different ($P < 0.003$, Bonferroni corrected), Sibolga and Nakornsrihammarat, Lampung and Nakornsrihammarat, Lampung and Trang, and Lampung and Sibolga.

The locus-wise F_{IS} for each population were shown in Table 2. It was significant after Bonferroni correction ($F < 0.0021$) at one locus in Nakornsrihammarat, Trang, and Lampung and three loci in Flores ($Em-07^*$, -08^* and -10^* and Jepara ($Em-01^*$, -07^* , -08^*).

Genetic Distance and a Phylogenetic Dendrogram. Cavalli-Sforza and Edwards chord distance between populations fall within a wide range, from 0.183 (Lampung and Nakornsrihammarat) to 0.416 (Flores and Sibolga) (Table 5).

Mantel's test showed nonsignificant correlation between genetic and geographical distance ($r = 0.3292$; $Z = 1539.0326$; upper tail $P = 0.1588$; lower tail $P = 0.8452$).

The NJ tree (Figure 2) clearly separated Nakornsrihammarat from the others which com-

Table 4. Results of Bottleneck Test

Populations	Sign test			SDT			Wilcoxon sign-rank test			Mode-shift
	IAM	TPM	SMM	IAM	TPM	SMM	IAM	TPM	SMM	
Trang	0.077	0.613	0.592	0.065	0.287	0.332	0.031	0.156	0.906	Normal
Nakorn.	0.309	0.661	0.656	0.133	0.401	0.254	0.094	0.437	0.843	Normal
Sibolga	0.341	0.378	0.413	0.056	0.148	0.465	0.062	0.156	0.437	Normal
Lampung	0.394	0.552	0.201	0.359	0.267	0.008	0.437	0.844	0.969	Normal
Jepara	0.107	0.451	0.185	0.041	0.169	0.477	0.031	0.062	0.844	Normal
Flores	0.573	0.197	0.180	0.492	0.103	0.001	0.562	0.937	0.968	Normal

The values show probabilities (P) to reject the hypothesis of mutation-drift equilibrium. ($P < 0.05$) indicates evidence of a recent bottleneck. The normal mode-shift indicates the approximately L-shaped allele frequency distribution; hence no recent bottlenecks occur. SDT = standardized different test.

Table 5. Matrix of Genetic Distance and Geographical Distance

Populations/species	N	T	S	L	J	F
Nakornsrihammarat (N)	...	2117	2959	1914	2088	3103
Trang (T)	0.024	...	1160	1856	1972	2987
Sibolga (S)	0.017	0.020	...	1218	1856	2871
Lampung (L)	0.016	0.018	0.021	...	638	1827
Jepara (J)	0.074	0.085	0.080	0.064	...	1044
Flores (F)	0.081	0.084	0.086	0.068	0.042	...

Above diagonal is geographical distance (km); below diagonal is genetic distance according to Cavalli-Sforza and Edwards (1967) chord distance.

prising two clusters (bootstrap value = 86.9%). Trang and Sibolga (1160 km apart) were clustered but the node was not supported by bootstrap value (56.6%). The populations along the Java Island formed a distinct cluster wherein Jepara and Flores were the most similar despite the geographic distance of 1044 km. Lampung clustered to the previous pair with a deep branch supported by a 68.4% bootstrap value although it is only 638 km away from Jepara.

Discussion

Departures from Hardy-Weinberg equilibrium (HWE). Three populations (Trang, Jepara, and Flores) did not conform to HWE toward homozy-

gote excess. In this case, because of significant F_{IS} at three of four loci in Jepara and Flores, we surmised that inbreeding, resulting from small effective population sizes and/or unequal number of male and female parents, might have occurred and caused departure from HWE in these populations. An evidence for the occurrence of the Wahlund effect, undetectable breeding subunits are not found inside the population samples and therefore are unlikely to be the reason for the homozygote excess (Halliburton, 2004) observed here. The presence of null alleles at *Em-07** is possible and may be responsible for the departure from HWE (Garcia de Leon et al., 1997; Valles-Jimenez et al., 2005) because this locus showed significant F_{IS} in all but one population. In addition, homozygote excess may be a result of inclusion of a few fullsib families in the sample rather than representative of the population (Nakorn et al., 2004). This situation was also supported in Jepara and Flores by significant F_{IS} at three of four loci. Sampling of a few fullsibs was possible because all of our samples (except Nakornsrihammarat) were obtained from cages initially stocked with schooling fingerling.

Genetic Variation Within Populations. The genetic variation of *E. coioides* (H_o = 0.3630–0.555; A = 7.25–8.00) revealed in the present study was comparable with that reported for other epinephelin species, *E. bonthoides*, *E. coromandelicus*, *E. ongus*, *E. fuscoguttatus*, and *Cromilepis altivelis* (H_o ranged between 0.41 and 0.57; A = 4–11) (Nugroho et al., 1998), and *E. quernus* (H_o = 0.54; A = 2–18) (Rivera et al., 2003), except for the endangered *E. marginatus* (H_o = 0.62; A = 7.71–13.86) (De Innocentiis et al., 2001). But despite the dispersal ability of their pelagic larvae, which enhanced substantial gene flow, level of heterozygosity of epinephelin fishes, which are rather sedentary, was relatively low compared to the migratory fishes, such as cod (H_e = 0.898; A = 40.8) (Bentzen et al., 1996), red sea bream (H_o = 0.808; A = 13.5) (Takagi et al., 1997), and king fish (H_o = 0.729; A = 21.5) (Nugroho et al., 2001).

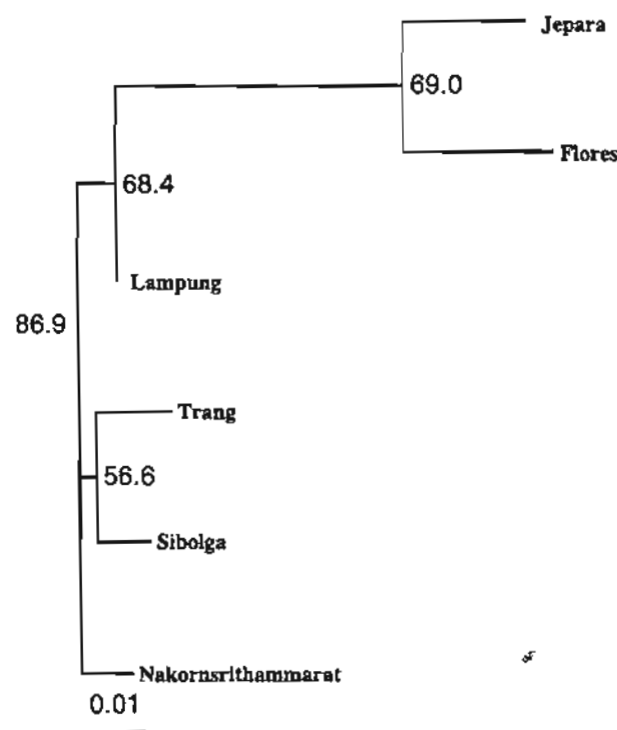


Fig. 2. Neighbourjoining tree based on Cavalli-Sforza chord distance. [Note: the numbers on the branches indicate bootstrap values.]

Relatively low genetic variation could have been a result of population isolation, small population size, or historical population bottleneck (Hartl and Brandl, 1998). The evidence for occurrence of bottleneck in our samples was not conclusive because the results varied with the tests. The SDT revealed recent bottlenecks in Lampung, Jepara, and Flores while the Wilcoxon test demonstrated the same for Nakornsrithammarat. The Wilcoxon test provided relatively high power whereas the SDT may have suffered from a low number of loci used in our study (this test requires at least 20 loci).

Small genetic variation can result in decline of adaptability to changing environments (Hedrick, 1999; Hoelzel, 1999); loss of alleles might decrease the degree of disease resistance (Allendorf and Phelps, 1980). Low genetic variation has been correlated with low variation of major histocompatibility complex (MHC) and acceptance of intrapopulation skin grafts in pocket gophers, *Thomomys bottae* (Sanjayan et al., 1996), while it resulted in adverse effects on physiological traits such as salinity tolerance among populations of wild guppy (Shikano et al., 2000; Shikano and Taniguchi, 2002).

Population Structuring. The population structure of *E. coioides* was strong relative to pelagic species such as Atlantic cod ($F_{ST} = 0.015$; Bentzen et al., 1996), yellowtail kingfish ($F_{ST} = 0.046$; Nugroho et al., 2001) or other demersal fishes such as European sea bass ($F_{ST} = 0.007$; Garcia de Leon et al., 1997), vermillion snapper ($F_{ST} = 0.004$; Bagley et al., 1999), dusky grouper ($F_{ST} = 0.0179$; De Innocentiis et al., 2001), and Hawaiian grouper ($F_{ST} = 0.0649$; Rivera et al., 2004). The level of population differentiation was slightly higher than average population differentiation of marine fishes revealed by isozyme marker ($F_{ST} = 0.062$) (Ward et al., 1994). Strong population structuring and low genetic variation suggest that populations of *E. coioides* are small and isolated.

Despite limited information on dispersal ability of adults and larval stages of *E. coioides*, its strong population structuring suggests that it tends to have strong site fidelity such as highly specific habitat preferences, male territorial behavior, and stable spawning aggregations like other epinephelin species (Heemstra and Randall, 1993; Sadovy et al., 1994). Such characteristics enhance population differentiation.

Effects of Sea Current Patterns and Geographical Events on Genetic Diversity Among Populations. The genetic relationship revealed by the phylogenetic dendrogram did not agree with geo-

graphic distances. Jepara and Flores were the most similar despite the distance of 1044 km, whereas Lampung, only 638 km away from Jepara, was significantly separated from the first pair (bootstrapped value = 68.4%). Such a relationship suggested that sea current may play a role in the shaping genetic relationships of these populations.

Although information for *E. coioides* is lacking, serranid fishes are generally high fecundity fish (ranging from 2.4×10^4 to more than 2.4×10^6 eggs depending on body weight) (Collins et al., 2002; Whiteman et al., 2005). They release large amounts of pelagic larvae dispersing through oceanic current (Thompson and Munro, 1978). In the case of *E. coioides*, anecdotal information indicated spawning occurs during the months of the northeast monsoon (November to March with peak season from December to January).

The northeast monsoon forces sea surface current from the South China Sea southward to Karimata Strait toward Sunda Strait and eastward passing Java Sea to Flores Sea. In Malacca strait, surface current always flows from the South China Sea toward Andaman Sea during either the northeast or southeast monsoon (Sharp, 1996) (Figure 1). As such the current facilitates dispersal of *E. coioides* larvae from Jepara to Flores, resulting in a close relationship between them. The current flowing through the Sunda strait may enhance dispersal of larvae from Lampung toward the Indian Ocean rather than eastward to Jepara, thus enhancing genetic differentiation between Lampung and Jepara and supporting the similarity between Lampung and Trang. Similar results supporting the effect of sea current on pattern of genetic relationship among conspecific populations were reported in the six bar wrasse (*Thallasoma hardwicki*) in the northern South China Sea (Chen et al., 2004).

The distinct separation between the two Thai populations, Nakornsrithammarat and Trang, may be partly explained by a geographic barrier caused by the Malay peninsula. Our result was concordant with the report in the tropical abalone (*Haliotis assinina*) in which marked differentiation existed between populations of the Andaman sea and the Gulf of Thailand (Tang et al., 2005).

Conclusion and Recommendations

Our study revealed strong population structuring of *E. coioides* in Thailand and Indonesia. The six populations included in this study had relatively low genetic variation, which may impair adaptability to the changing environment. Specifically, recent bottlenecks were observed in Nakorn-

srithammarat, Jepara, Lampung, and Flores. Therefore, measures should be established to avoid or minimize the additional threats caused by anthropogenic activities on these populations, for example, establishment of reserved area, restriction of fishing sizes, sales bans during spawning aggregation period, and so forth (Rhodes and Sadovy, 2002).

Distinct genetic differentiation suggested that these populations should be managed separately. Moreover, genetically differentiated populations may show variation of traits important for aquaculture (Kumagai et al., 2004). Therefore, economically important traits, such as growth, disease resistance, and so forth, of these populations should be evaluated. Incorporating information on genetic variation and performances would enable efficient exploitation of these resources for aquaculture and genetic improvement programs.

Furthermore, the baseline information established here would enable efficient monitoring of the impact of natural and/or anthropogenic activities on natural populations of *E. coioides*. For example, unique alleles occurring in Flores (Em-07*170, *172; Em-08*216; Em-10*70, *72) and in Sibolga (Em-10*98) may be useful for further genetic monitoring of stock translocation including these populations.

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References

- Al Janhi A, Samuel M, Al Zabi A, Al Yasi A, Anwahi A (2005). Age, growth, reproductive biology and spawning season of *Epinephelus coioides* in UAE. Available at http://www.uae.gov.ae/uaeagricent/FISHERIES/hamoor_en.stm [accessed May 1, 2005]
- Allendorf FW, Phelps SR (1980) Loss of genetic variation in a hatchery stock of cutthroat trout. *Trans Am Fish Soc* 109, 537-543
- Archie JW (1985) Statistical analysis of heterozygosity data independent sample comparison. *Evolution* 39, 623-637
- Bagley MJ, Linquist DG, Geller JB (1999) Microsatellite variation, effective population size, and population genetic structure of vermillion snapper, *Rhomboplites aurorbens*, off the southeastern USA. *Mar Biol* 134, 609-620
- Bentzen P, Taggart CT, Ruzzante DE, Cook D (1996) Microsatellite polymorphism and the population structure of Atlantic cod (*Gadus morhua*) in the northwest Atlantic. *Can J Fish Aquat Sci* 53, 2706-2721
- Carlin JL, Robertson DR, Bowen BW (2003) Ancient divergences and recent connections in two tropical Atlantic reef fishes *Epinephelus adscensionis* and *Ryp-ticus saponaceous* (Percoidae: Serranidae). *Mar Biol* 143, 1057-1069
- Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis: Models and estimation procedure. *Evolution* 21, 550-570
- Cesar HSI, Warren KA, Sadovy Y, Lau P, Meijer S, van Ierland E (2000) "Marine market transformation of the live reef fish food trade in southeast Asia." In: *Collected Essays on the Economics of Coral Reefs*, Cesar HSI, ed. (Kalmar, Sweden: Department of Biology and Environmental Sciences, Kalmar University) pp 137-157
- Chen CA, Ablan MCA, McManus JW, Bell JD, Tuan VS, Cabanban AS, Shao K (2004) Population structure and genetic variability of Six Bar Wrasse (*Thalassoma hardwicki*) in northern South China Sea revealed by mitochondrial control region sequences. *Mar Biotechnol* 6, 312-326
- Collin PL (1996) Longevity of some coral reef fish spawning aggregations. *Copeia* 1996, 189-192
- Collins LA, Fitzhugh GR, Lombardi-Carlson LA, Lyon HM, Walling WT, Oliver DW (2002) Panama City Laboratory, Contribution Series 2002-07. (Panama City: National Marine Fisheries Service)
- Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144, 2001-2014
- De Innocentis S, Sola L, Cataudela S, Bentzen P (2001) Allozyme and microsatellite loci provide discordant estimates of population differentiation in the endangered dusky grouper (*Epinephelus marginatus*) within the Mediterranean Sea. *Mol Ecol* 10, 2163-2175
- FAO online at <http://www.fao.org/fi/statist/statist.asp> [accessed May 1, 2005]
- Falconer DS (1985) Introduction to Quantitative Genetics, 2nd ed. (London: Longman) 340 pp.
- Felsenstein J. (1995) PHYLIP (Phylogeny Inference Package) Version 3.57c. Available Source: <http://evolution.genetics.washington.edu/phyliip.html> [accessed January 1, 2003]
- Garcia de Leon FJ, Chiki L, Bonhomme P (1997) Microsatellite polymorphism and population subdivision in natural populations of European sea bass *Dicentrarchus labrax* (Linnaeus, 1758). *Mol Ecol* 6, 51-62
- Halliburton R (2004) Introduction to Population Genetics. (Upper Saddle River, NJ: Pearson Education International)

- Hanfling B, Brandl R (1998) Genetic variability, population size and isolation of distinct populations in the freshwater fish *Cottus gobio* L. *Mol Ecol* 7, 1625–1632
- Hedrick PW (1999) *Genetics of Population*, 2nd ed. (Sunderbury, MA: Jones and Bartlett)
- Heemstra PC, Randall JE (1993) "FAO species catalogue. Groupers of the world (Family Serranidae, Subfamily Epinephelinae)". (Rome: FAO)
- Hoelzel AR (1999) Impact of population bottlenecks on genetic variation and the importance of life-history, a case study of the northern elephant seal. *Biol J Linn Soc* 68, 23–39
- Kumagai K, Barinova AA, Nakajima M, Taniguchi N (2004) Genetic diversity between Japanese and Chinese threeline grunt (*Parapristipoma trilineatum*) examined by microsatellite DNA markers. *Mar Biotechnol* 6, 221–228
- Manly KF (1993) A Macintosh program for storage and analysis of experimental genetic mapping data. *Mamm Genome* 4, 303–313
- Medioni E, Finiger RL, Loureiro N, Planes S (2001) Genetic and demographic variation among color morphs of cabrilla sea bass. *J Fish Biol* 58, 1113–1124
- Miller MP (1997) Tools for Population Genetic Analyses (TFPGA) 1.3: A Windows Program for the Analysis of Allozyme and Molecular Population Genetic Data. (Arizona: Northern Arizona University)
- Na-Nakorn U, Kamonrat W, Ngamsiri T (2004) Genetic diversity of walking catfish, *Clarias macrocephalus*, in Thailand and evidence of genetic introgression from introduced farmed *C. gariepinus*. *Aquaculture* 240, 145–163
- Nugroho E, Takagi M, Sugama K, Taniguchi N (1998) Detection of GT repeats microsatellite loci and their polymorphism for grouper of the genus *Epinephelus*. *Fish Sci* 64, 836–837
- Nugroho E, Ferrel DJ, Smith P, Taniguchi N (2001) Genetic divergence of kingfish from Japan, Australia and New Zealand inferred by microsatellite DNA and mitochondrial DNA control region markers. *Fish Sci* 67, 843–850
- Raymond M, Rousset F (1995) GENEPOP (ver. 1.2): a population genetics software for exact test and ecumenicism. *J Heredity* 86, 248–249
- Rhodes KL, Sadovy Y (2002) Temporal and spatial trends in spawning aggregations of camouflage grouper, *Epinephelus polyphekadion* in Pohnpei, Micronesia. *Environ. Biol. Fishes* 63, 27–39
- Rhodes KL, Lewis RI, Chapman RW, Sadovy Y (2003) Genetic structure of camouflage grouper, *Epinephelus polyphekadion* (Pisces: Serranidae), in the western central Pacific. *Mar Biol* 142, 771–776
- Rivera MAJ, Graham GC, Roderick GK (2003) Isolation and characterization of nine microsatellite loci from the Hawaiian grouper *Epinephelus quernus* (Serranidae) for population genetic analyses. *Mar Biotechnol* 5, 126–129
- Rivera MAJ, Kelly CD, Roderick GK (2004) Subtle population genetic structure in the Hawaiian grouper *Epinephelus quernus* (Serranidae) as revealed by mitochondrial analyses. *Biol J Linn Soc* 81, 449–468
- Sadovy Y, Colin PL, Domeier ML (1994) Aggregation and spawning in the tiger grouper, *Mycteroperca tigris* (Pisces: Serranidae). *Copeia* 2, 511–516
- Sala E, Ballesteros E, Starr RM (2001) Rapid decline of Nassau Grouper spawning aggregations in Belize: Fishery management and conservation needs. *Fisheries* 26, 23–30 (<http://www.fisheries.org>)
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory)
- Sanjayan MA, Crooks K, Zegers G, Foran D (1996) Genetic variation and the immune response in natural populations of Pocket Gophers. *Conserv Biol* 10, 1519–1527
- Shaklee JB (1984) Genetic variation and population structure in the damselfish, *Stegastes fasciatus*, throughout the Hawaiian Archipelago. *Copeia* 3, 629–640
- Shapiro DY (1987) "Reproduction in grouper." In: *Tropical Snappers and Groupers: Biology and Fisheries, Management*, Polovina JJ, Rabton S, eds. (Boulder: Westview Press) pp 295–327
- Sharp G.D. (1996) "Oceanography of the Indonesian Archipelago and adjacent areas" In: *Baseline Studies of Biodiversity: The Fish Resources of Western Indonesia ICLARM Stud Rev*, Pauly D, Martosubroto P, eds. 23 pp. 321.
- Shikano T, Taniguchi N (2002) Relationships between genetic variation measured by microsatellite DNA markers and a fitness-related trait in the guppy (*Poecilia reticulata*). *Aquaculture* 209, 77–90
- Shikano T, Chiyokubo T, Nakadate M, Fujio Y (2000) The relationship between allozyme heterozygosity and salinity tolerance in wild and domestic populations of the guppy (*Poecilia reticulata*). *Aquaculture* 184, 233–245
- Snidvongs A, Sojisuorn P (1999). Numerical simulations of the current in the Gulf of Thailand under different monsoon regimes. In: *Proceedings of The First Technical Seminar on Marine Fishery Resources Survey in the South China Sea* (Samutprakan, Thailand) pp. 54–72
- Sugama K, Tridjoko, Haryanti, Budi S, Cholik F (1999) Genetic variation and population structure in the humpback grouper, *Cromileptes altivelis*, throughout its range in Indonesian waters. *Indo Fish Res J V*(1), 32–38
- Taggart JB, Hynes RA, Prodohl PA, Ferguson A (1992) A simplified protocol for routine total DNA isolation from salmonid fishes. *J Fish Biol* 40, 963–965
- Takagi M, Taniguchi N, Cook D, Doyle RW (1997) Isolation and characterization of microsatellite loci from red sea bream *Pagrus major* and detection in closely related species. *Fish Sci* 63, 199–204
- Takezaki N, Nei M (1996) Genetics distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* 144, 389–399
- Tang S, Tassanakajorn A, Klinbunga S, Jarayaphand P, Menasveta P (2005) Population structure of tropical abalone (*Haliotis assinina*) in coastal waters of Thailand determined using microsatellite markers. *Mar Biotechnol* (in press)
- Taniguchi N, Nugroho E (2000) "Genetic characteristics of introduced fishes and study of genetic evaluation of fish genetics and breeding". (Japan: Japan Seawater Fisheries Cultivation Association) pp 141–188 (In Japanese)

- Thompson R, Munro JL (1978) Aspects of the biology and ecology of Caribbean reef fishes: Serranidae (hinds and groupers). *J Fish Biol* 12, 115–146
- Valles-Jimenez R, Cruz P, Perez-Enriquez R (2005) Population genetic structure of Pacific White Shrimp (*Litopenaeus vannamei*) from Mexico to Panama: microsatellite DNA variation. *Mar Biotechnol* 6, 475–484
- Ward RD, Woodward M, Skibinski DO (1994) A comparison of genetic diversity levels in marine, freshwater and anadromous fishes. *J Fish Biol* 44, 213–232
- Whiteman EA, Jennings CA, Nemeth RS (2005) Sex structure and potential female fecundity in a *Epinephelus guttatus* spawning aggregation: applying ultrasonic imaging. *J Fish Biol* 66, 983–995

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Mitochondrial DNA Diversity of the Asian Moon Scallop, *Amusium pleuronectes* (Pectinidae), in Thailand

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Abstract

Sequence variation of the mitochondrial DNA 16S rRNA region of the Asian moon scallop, *Amusium pleuronectes*, was surveyed in seven populations along the coast of Thailand. A total of 16 unique haplotypes were detected among 174 individuals with a total 27 variable sites out of 534 bp sequenced. The mitochondrial haplotypes grouped into two distinct arrays (estimated to differ by about 2.62% to 2.99% nucleotide divergence) that characterized samples collected from the Gulf of Thailand versus the Andaman Sea. Low levels of intrapopulation variation were observed, while in contrast, significant divergence was observed between populations from the Gulf of Thailand and Andaman Sea. Results of AMOVA reveal a high F_{ST} value (0.765) and showed that the majority of the total genetic variance (76.03%) occurred among groups (i.e., Andaman Sea and the Gulf of Thailand) and little among populations within the group (0.52%) and within populations (23.45%). The genetic differentiation between the populations recorded in the present study is similar to that observed in a variety of marine species in the Indo-Pacific. The implications of the findings for management of *A. pleuronectes* genetic resources in Thailand are discussed.

Keywords: 16S rRNA—genetic diversity—mtDNA—*Amusium pleuronectes*—the Gulf of Thailand—Andaman Sea

Introduction

Amusium pleuronectes, commonly referred to as Asian moon scallop, is present over much of Indo-

Pacific coastal areas. The species has been recorded from the Indian Ocean, South China Sea, Indo-China, Japan, the Philippines, New Guinea, Indonesia, Java, and Australia (Morton, 1980) and at a depth of 18 to 40 m (Minchin, 2003). In Thailand, the species is of significant commercial value, with about 500 to 1100 metric tonnes/year harvested during 1994–1999. However, the recent catch statistics showed that production declined to 100 to 300 metric tonnes/year between 2000 and 2003 (FAO, 2006). An effort has been made to reduce pressure on wild-caught fisheries, through aquaculture development of this species although this is at an early stage.

An understanding of genetic variability and population structure are critical for management and conservation of exploited aquatic species. It is now widely recognized that this information could be achieved through the recently developed molecular genetic techniques. Molecular genetic data provide fishery managers with essential information regarding the existence and distribution of discrete populations and/or management units (Ward and Grewe, 1995). In addition, for newly emerging aquaculture species such as the Asian moon scallop, genetic information would provide useful insight into the establishment of baseline stocks for selective breeding and development of broodstock management strategies (Carvalho and Pitcher, 1995).

Molecular genetic studies using allozyme and DNA markers have provided useful insights into a number of areas of biology of many scallop species, including evolution, taxonomy, and fishery management. For example, the identification of genetically distinct populations of *Pecten maximus* (Wilding et al., 1997; Heipel et al., 1999) and *Patinopecten yessoensis* (Dolganov and Pudovkin, 1998) are of

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significant value to the development of management strategies of these genetic resources.

Mitochondrial DNA (mtDNA) is haploid and generally believed to be maternally inherited (Birky et al., 1983). Therefore, the effective population sizes estimated from mtDNA is smaller than those from nuclear DNA, increasing its sensitivity to genetic drift and bottleneck effects. MtDNA has been a successful marker for addressing questions relating to evolutionary relationships among scallop species (Frischer et al., 1998; Capana et al., 1999; Matsumoto and Hayami, 2000; Capana et al., 2000a,b) or within bivalve in general (Giribet and Wheeler, 2002). At the population level, mtDNA has proven to be useful in determining population genetic structure of some scallop species such as *Pecten maximus* (Wilding et al., 1997) and Japanese scallop, *Mizuhopecten yessoensis* (Nagashima et al., 2005).

Along the Gulf of Thailand, the moon scallop is found from Chonburi Province to Rayong, Chantaburi, Trat and down to Narathiw in the south (Figure 1). Different spawning seasons were observed at different locations—July to January in Rayong Bay (Roongratri, 1996) and January to March in Trat (Nugranad, 1990), which are less than 100 km apart. Reproductive isolation among these populations is likely and hence significant population subdivision is expected.

The overall objective of the current study was to conduct a baseline survey on the genetic resources of *A. pleuronectes* and determine population genetic

structure of this species along the coast of Thailand using nucleotide sequences of partial 16S rRNA gene of the mtDNA. The information obtained will provide useful insight into management practices of scallop resources in Thailand.

Materials and Methods

Sample Collection. A total of 174 individuals of *A. pleuronectes* from six locations in the Gulf of Thailand and one location from the Andaman Sea were collected from April to December 2005 by trawling. Details on sample sizes and localities are presented in Table 1 and Figure 1.

Fresh specimens of *A. pleuronectes* were identified following Okutani (2000). Approximately 150 mg of adductor muscle was taken from each individual and preserved in 90% ethanol. All samples were transferred and kept at 4°C at the Fish Genetics Laboratory, Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Thailand until required.

DNA Extraction, Polymerase Chain Reaction (PCR), and Sequencing. DNA extraction was performed following the method of Taggart et al. (1992). Approximately 50 mg tissue sample was taken and macerated in 500 µl of lysis buffer (0.1 M Tris-OH, pH 8.0, 0.05 M EDTA, 0.2 M NaCl, and 1% sodium dodecyl sulfate [SDS] with 0.5 mg/ml of proteinase K) followed by incubation at 65°C for 2 h.

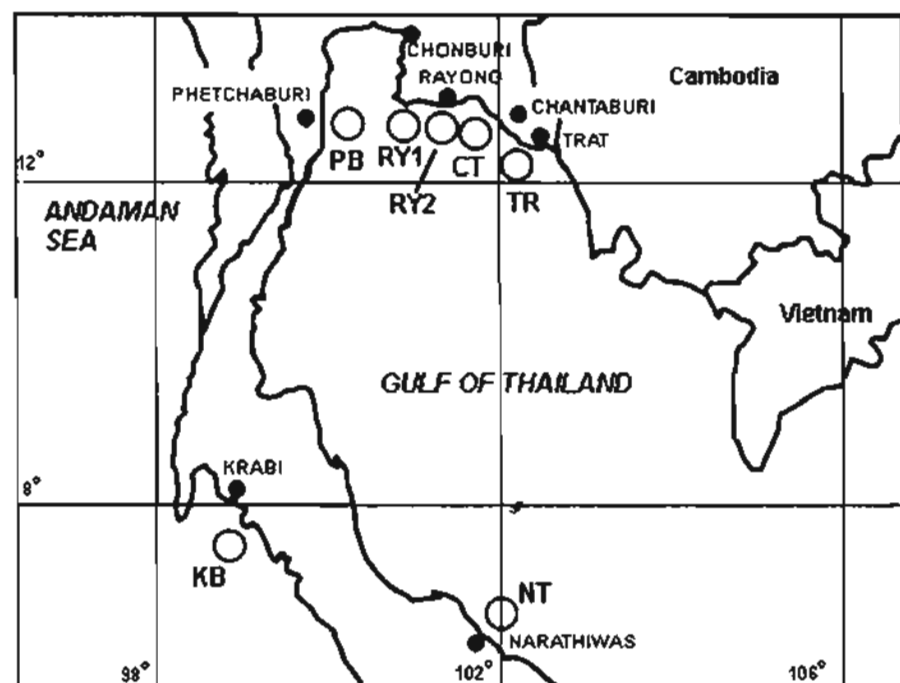


Figure 1. Sampling localities of seven *A. pleuronectes* populations analyzed in the present study. Closed circles represent the adjacent town; open circles are sampling sites.

Table 1. Sample code, sample size and collecting localities of seven populations of *A. pleuronectes* analyzed in the present study

Sample code	Locality	Coordinates	Sample size
CT	Chantaburi Province, Gulf of Thailand	12° 22' 21" N 101° 52' 48" E	10
KB	Krabi Province, Andaman Sea	07° 33' 24" N 98° 52' 38" E	34
NT	Narathiwat Province, Gulf of Thailand	06° 32' 3" N 102° 04' 07" E	5
PB	Phetchaburi Province, Gulf of Thailand	13° 05' 14" N 100° 04' 10" E	26
RY1	Rayong Province, Gulf of Thailand	12° 35' 42" N 101° 15' 12" E	39
RY2	Rayong Province, Gulf of Thailand	12° 32' 38" N 101° 33' 40" E	11
TR	Trat Province, Gulf of Thailand	12° 04' 29" N 102° 07' 14" E	49

The digested products were then extracted twice with phenol (saturated with 10 mM Tris, 1 mM EDTA, pH 8.0) and once with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with ethanol, dried, and dissolved in sterile deionized water.

PCR reactions were performed using the universal primer for 16S rRNA developed by Palumbi et al. (1991); 16S_{FL} 5'-CGC CTG TTT AAC AAA AAC AT-3' and 16S_{R1} 5'-CCG GTC TGA ACT CAG ATC ATG T-3'. PCR was performed in a total volume of 35 µl containing 50 ng/µl of template DNA, 1 × PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM of each primer, 1 U of *Taq* polymerase (Promega). The PCR profile comprised an initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

Sequencing was performed at the Laboratory of Population Genetic Informatics, Tohoku University, Japan where the PCR products were purified using ExoSAP-IT[®] (usb) before sequencing via the BigDye[™] Terminator Cycle Sequencing Ready Reaction Kit. The fragments were separated on a ABI Prism[®] 377 DNA Sequencer (Applied Biosystems).

Data Analysis. Sequences were viewed, edited, and aligned using MEGA software (Kumar et al., 2004). Levels of mtDNA 16S rRNA variability within samples of *A. pleuronectes* were examined by computing the nucleotide and haplotype diversity indices via Arlequin version 3.1 (Schneider et al., 2000). Statistical testing for population differentiation involved an exact test (Raymond and Rousset, 1995) of a contingency table based on haplotype frequencies and pairwise comparisons of the F_{ST} using analysis of molecular variance (Excoffier et al., 1992) based on 20,000 permutations of the data matrix. Minimum spanning network showing relationships among haplotypes and average pairwise differences among populations (Nei and Li, 1979) were estimated via the same software. Multiple tests of the

same null hypothesis were subjected to table-wide sequential Bonferroni correction to avoid elevated type I error rates.

Results

A total of 534 base pairs of the 16S rRNA gene fragment were successfully sequenced for 174 individuals from seven populations of *A. pleuronectes*. Sixteen unique haplotypes with 27 variable sites (5.06%) were identified, of which 21 were transitions, 5 transversions, and 1 position was an insertion/deletion. Sequences representing each haplotype were submitted to GenBank (GenBank accession nos. DQ640830 to DQ640845). The mean total nucleotide composition was A = 24.9%, T = 31.5%, C = 16.4%, and G = 27.2%. Mean nucleotide divergence among haplotypes observed within the Gulf of Thailand was 0.004, and that of the Andaman Sea sample was 0.002. The average difference between haplotypes from the Gulf of Thailand samples and that of the Andaman Sea

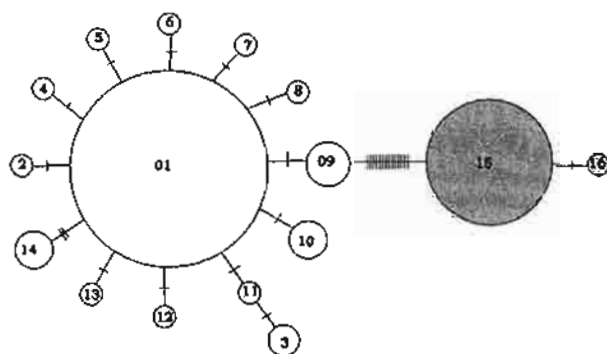


Figure 2. Minimum spanning network showing relationships among 16 haplotypes of mtDNA 16S rRNA of *A. pleuronectes* observed in the present study. Each bar with a crossed connection between haplotypes indicates one mutation. Shaded circles are haplotypes that occur only in the KB population, and open circles are those observed only in populations from the Gulf of Thailand.

Table 2. Distribution of 16 observed mtDNA 16S rRNA haplotypes, nucleotide diversity, number of haplotypes, haplotype diversity, and number of polymorphic sites of populations of *A. pleuronectes*

Haplotype frequency																									
Nucleotide site																									
1 2																									

The vertical numbers indicate the position of variable nucleotides within the 534-bp sequence. Dots indicate that the same nucleotide is present as in Hap01 and a dash (-) indicates a deletion. Numbers under each population indicate the frequencies of individuals with that haplotype in each population.

was 0.029. Relationships among haplotypes are shown in Figure 2.

An alignment of variable sites of the 16 haplotypes and the distribution of these haplotypes among populations are presented in Table 2. Haplotype 01 was dominant (70% to 100%) in all populations from the west coast (CT, PB, RY1, RY2, TR, and NT). Almost all populations, except for NT, had at least two haplotypes that were not shared with other populations, although with low frequencies (0.020 to 0.115). None of the haplotypes from the west coast population (KB) were recorded elsewhere.

Intrapopulation diversity indices are shown in Table 2. Overall, low levels of intrapopulation diversity were observed. Of all the populations examined, the highest nucleotide diversity (0.0017) and haplotype diversity (0.511) occurred in the population from Chantaburi Province (CT), and the lowest diversity in the population from Narathiwat Province (NT), probably associated with its small sample size (five individuals).

The average number of pairwise differences between populations and pairwise F_{ST} values are shown in Table 3. Pairwise genetic differences ranged from 0.091 (between NT and RY2) to 1.000 (between KB and others). Significant pairwise F_{ST} values (0.767 to 0.948, $P < 0.05$) and significant probabilities ($P < 0.05$) based on 20,000 permutations of haplotype frequencies after sequential Bonferroni correction were observed between KB and all other populations. AMOVA with two groups (i.e., Gulf of Thailand versus Andaman Sea) revealed a high F_{ST} value (0.765), and showed that the majority of the total genetic variance (76.03%) occurred among groups, and to a lesser extent within populations (23.45%) and little among populations within the group (0.52%).

Discussion

Levels of Population Divergence. The levels of intrapopulation variation of *A. pleuronectes* revealed

by sequences of the mtDNA 16S rRNA gene region in the present study are relatively low. Mean nucleotide diversity of 0.0006 and mean haplotype diversity of 0.237 observed in each population are considerably lower than that reported for other scallop species. Saavedra and Peña (2004) used sequences of the same gene region, with limited sample sizes (six to eight individuals), and observed higher haplotype diversities and nucleotide diversities ($h = 0.857, 0.667$, and 0.800 ; and $\pi = 0.039, 0.015$, and 0.002 for *Pecten maximus*, *P. japonicus*, and *P. novaezealandiae*, respectively). In another study, Wilding et al. (1997) examined genetic variation of *P. maximus* populations from the United Kingdom and Atlantic coasts using PCR-restriction length polymorphism (RFLP) of about 6000 bp of the mtDNA and revealed much higher within-population diversity ($h = 0.7056$ to 0.9011 , $\pi = 0.0145$ to 0.0265). However, levels of within-population diversity reported herein seem similar to that observed in pearl oyster, *Pinctada margaritifera* ($h = 0.087$ to 0.600 , $\pi = 0.004$ to 0.0044) (Arnaud-Haond et al., 2003). No diagnostic haplotypes or high-frequency haplotypes confined to any particular population were observed, except for two haplotypes (haplotypes 15 and 16) representing a population from the Andaman Sea (KB).

Klinbunga et al. (2001), using RFLP analysis of two mitochondrial gene regions (16S rRNA and COI-COII), demonstrated that geographic samples of *Penaeus monodon* in a similar geographical region showed much higher levels of intrapopulation variation ($h = 0.831$ to 0.887 , $\pi = 0.032$ to 0.038) compared to *A. pleuronectes* samples in the present study. Similarly, three abalone species (*Haliotis asinina*, *H. ovina*, and *H. varia*) studied by Klinbunga et al. (2003) also showed much higher levels of intrapopulation variation in general.

No significant genetic structure was observed within the Gulf of Thailand, and even though populations from Rayong Bay and Trat had differential

Table 3. Average number of pairwise differences (below diagonal) and pairwise F_{ST} values (above diagonal) between populations of *A. pleuronectes* examined in the present study

	CT	NT	PB	RY1	RY2	TR	KB
CT		0.046	0.030	0.103	0.053	0.028	0.824*
NT	0.300		-0.031	-0.082	-0.089	-0.034	0.948*
PB	0.435	0.192		0.020	-0.006	0.007	0.815*
RY1	0.372	0.103	0.275		-0.031	0.007	0.868*
RY2	0.364	0.091	0.266	0.184		-0.009	0.910*
TR	0.443	0.204	0.357	0.283	0.276		0.767*
KB	1.000	1.000	1.000	1.000	1.000	1.000	

Significant F_{ST} ($P < 0.05$) and significant probabilities ($P < 0.05$) based on 20,000 permutations of haplotype frequencies after Bonferroni correction among samples are indicated with an asterisk (*).

spawning seasons (Nugranad, 1990; Roongratri, 1996), they did not show genetic differentiation, as evident from low genetic distances (0.276 to 0.283) and nonsignificant pairwise F_{ST} (-0.009 to 0.007, $P > 0.05$). This is not surprising, as gene flow between these populations (less than 100 km apart) is possible, as a result of migratory behaviour during spawning and larval dispersal in *A. pleuronectes* (Morton, 1980). The differences in spawning season are therefore most likely the result of annual fluctuation, reflecting environmental variation, as the two studies of Nugranad (1990) and Roongratri (1996) were conducted in two different years.

In contrast to the low levels of genetic variation observed within populations and within the Gulf of Thailand, substantial inter-population variation of *A. pleuronectes* was observed between these populations and that from the Andaman Sea. This deep genetic division between these populations observed in the present study is similar to that described for a variety of marine species in the Indo-Pacific, including fish (Keenan, 1994; Lacson and Clark, 1995; McMillan and Palumbi, 1995; Chenoweth et al., 1998), crustaceans (Lavery et al., 1996; Duda and Palumbi, 1999; Williams et al., 1999; Barber et al., 2000), starfish (Williams and Benzie, 1998; Benzie, 1999a), and molluscs such as abalone, *Haliotis ovina* (Klinbunga et al., 2003). We admit that our interpretation relies to a major degree on inference from the biogeographic patterns in other species, as we sampled only one population from the Andaman Sea. Accordingly, the observed association between genetic divergence and the separation of the Pacific and Indian Oceans is thought related to past low sea level events associated with the ice age (Palumbi, 1997; Benzie, 1999b; Alfaro et al., 2004).

The existence of two distinct lineages is clear evidence of a relatively old geographically based pattern of divergence in *A. pleuronectes*. Two different hypotheses could be proposed for such contemporary divergence: (1) colonization of the two different isolated ancestral populations or (2) reproductive/physical isolation of two subpopulations between an ancestral panmictic population. These hypotheses could be tested with more intensive sampling coupled with a nested analysis approach.

Estimated time of divergence (based on 2% nucleotide differences per million years) between haplotypes of populations from the Gulf of Thailand and the Andaman Sea since a shared ancestor haplotype was from 1.3 to 1.5 million years ago, indicating that limited gene flow could occur since the Pleistocene during the connection of the Asian landmass to the Greater Sunda Islands due to lowered sea levels and emergence of the Sunda

Shelf. Further, even under the contemporary sea level, gene flow between the Gulf of Thailand and the Andaman Sea is likely inhibited due to the constant north-flowing current in the Strait of Malacca (Great Britain Hydrographic Office, 1958).

Management Implications. The sequence data of the 16S rRNA gene region identified significant differences between populations from the Gulf of Thailand and Andaman Sea of *A. pleuronectes*. Accordingly, it is desirable to manage these distinct populations separately. Transfer of individuals of *A. pleuronectes* between the Gulf of Thailand and the Andaman Sea is discouraged, as it may threaten the genetic component of species diversity because of interbreeding and competition (Johnson, 2000). Moreover, the long-term isolation of these populations could lead to the possibility of local adaptation to specific environment conditions, and hence translocation of individuals between distinct populations could also bring about indirect genetic risks such as disease transmission.

The 16S rRNA sequence data did not reveal significant genetic structure among populations within the east coast of Thailand. However, the absence of divergence among samples does not necessarily indicate that such populations are panmictic (Johnson, 2000). As Klinbunga et al., (2003) noted, no genetic differentiation was observed in *M. asinina* using mitochondrial DNA markers, but substantial population structure was revealed when nuclear markers were employed (i.e., 18S rRNA, RAPD and microsatellites). This suggests the need for further investigations using more variable genetic markers such as microsatellites, which have stronger resolving power compared to allozymes (Arnaud-Haond et al., 2003) and mitochondrial DNA (Hoarau et al., 2004).

Finally, if aquaculture of *A. pleuronectes* is to expand, care must be taken when selecting broodstock. It is advisable to select from residing populations and develop clear broodstock management strategies so that genetic contamination is avoided, especially if the progeny are used for restocking purposes. These strategies will minimize the risk associated with hybridization and dilution of the gene pool, which have been well documented in many aquaculture species (Hindar et al., 1991; Crozier, 1993).

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References

- Alfaro ME, Karns DR, Voris HK, Abernathy E, Sellins SL (2004) Phylogeny of *Cerberus* (Serpentes: Homalopsinae) and phylogeography of *Cerberus rynchops*: diversification of a coastal marine snake in Southeast Asia. *J Biogeogr* 31, 1277–1292
- Arnaud-Haond S, Bonhomme F, Blanc F (2003) Large discrepancies in differentiation of allozymes, nuclear and mitochondrial DNA loci on recently founded Pacific populations of pearl oyster *Pinctada margaritifera*. *J Evol Biol* 16, 388–398
- Barber PH, Palumbi SR, Erdmann MV, Kasim Moosa M (2000) A marine Wallace's line. *Nature* 406, 692–693
- Benzie JAH (1999a) Major genetic differences between crown-of-thorns starfish (*Acanthaster planci*) populations from the Indian and Pacific Oceans. *Evolution* 53, 1782–1795
- Benzie JAH (1999b) Genetic structure of coral reef organisms: ghosts of dispersal past. *Am Zool* 39, 131–145
- Birky CW, Maruyama T, Fuerst P (1983) An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplast and some results. *Genetics* 103, 513–527
- Capana A, Barucca M, Marinelli A, Olmo E (1999) A molecular approach to the systematics of Antarctic scallop *Adamussium colbecki*. *Ital J Zool* 66, 379–382
- Capana A, Barucca M, Caputo V, Marinelli A, Nisi Cerioni P, Olmo E (2000a) A molecular analysis of the systematics of three Antarctic bivalves. *Ital J Zool Suppl* 1, 127–132
- Capana A, Barucca M, Marinelli A, Olmo E (2000b) Molecular data from the 16S rRNA gene for the phylogeny of Pectinidae (Mollusca: Bivalvia). *J Mol Evol* 50, 93–97
- Carvalho GR, Pitcher TJ, eds. (1995) *Molecular Genetics in Fisheries* (London: Chapman & Hall)
- Chenoweth SE, Hughes JM, Keenan CP, Lavery S (1998) When oceans meet: a teleost shows secondary intergradation at an Indian-Pacific interface. *Proc R Soc Lond B Biol Sci* 265, 415–420
- Crozier W (1993) Evidence of genetic interaction between escaped farmed salmon and wild Atlantic salmon (*Salmo salar* L.) in a northern Irish river. *Aquaculture* 113, 19–29
- Dolganov SM, Pudovkin AI (1998) Population genetic structure of the Japanese scallop *Mizuhopecten (Patinopecten) yessoensis* from Sakhalin Island and the southern Kuril Islands. *Russ J Genet (Genetika)* 34, 1196–1204
- Duda TF, Palumbi SR (1999) Population structure of the black tiger prawn, *Penaeus monodon*, among western Indian Ocean and western Pacific populations. *Mar Biol* 134, 705–710
- Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 147, 499–513
- FAO (2006) Fishstat Plus: universal software for fishery statistical. Time series 1950–2004. Version 2.30. FAO Fisheries Department, Fishery Information, Data and Statistics Unit. www.fao.org/fi/statist/fissoft/FISHPLU-S.asp
- Frischer ME, Williams J, Kenchington E (1998) "A molecular phylogeny of some major groups of Pectinidae inferred from 18S rRNA gene sequences". In: Johnston PA and Haggart JW (Eds) *Bivalves: An Eon of Evolution. Paleobiological Studies Honoring Norman D. Newell* (Calgary: University of Calgary Press) pp 213–221
- Giribet G, Wheeler W (2002) On bivalve phylogeny: a high-level analysis of the Bivalvia (Mollusca) based on combined morphology and DNA sequence data. *Invertebr Biol* 121, 271–324
- Great Britain Hydrographic Office (1958) *Malacca Strait pilot*. 4th ed. Admiralty. UK: Hydrographic Department
- Heipel DA, Bishop JDD, Brand AR (1999) Mitochondrial DNA variation among open-sea and enclosed populations of the scallop *Pecten maximus* in western Britain. *J Mar Biol Assoc UK* 79, 687–695
- Hindar K, Ryman N, Utter F (1991) Genetic effects of cultured fish on natural fish populations. *Can J Fish Aquat Sci* 48, 945–957
- Hoarau G, Piquet AM-T, van der Veer HW, Rijnsdorp AD, Stam WT, Olsen JL (2004) Population structure of plaice (*Pleuronectes platessa* L.) in northern Europe: a comparison of resolving power between microsatellites and mitochondrial DNA data. *J Sea Res* 51, 183–190
- Johnson MS (2000) Measuring and interpreting genetic structure to minimise the genetic risk of translocation. *Aquac Res* 31, 133–143
- Keenan CP (1994) Recent evolution of population structure in Australian Barramundi, *Lates calcarifer* (Bloch): an example of isolation by distance in one dimension. *Aust J Mar Freshw Res* 45, 1123–1148
- Klinbunga S, Siludjai D, Wudthijinda W, Tassanakajon A, Jarayaphand P, Menasveta P (2001) Genetic heterogeneity of the giant tiger prawn (*Penaeus monodon*) in Thailand revealed by RAPD and mitochondrial DNA RFLP analyses. *Mar Biotechnol* 3, 428–438
- Klinbunga S, Pripue P, Khamnamtong N, Puanglarp N, Tassanakajon A, Jarayaphand P, Hirano I, Aoki T, Menasveta P (2003) Genetic diversity and molecular markers of the tropical abalone (*Haliotis asinina*) in Thailand. *Mar Biotechnol* 5, 505–517
- Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5, 2
- Lacson JM, Clark S (1995) Genetic divergence of Maldivian and Micronesian demes of the damselfishes *Stegastes nigricans*, *Chrysiptera biocellata*, *C. glauca* and *C. leucopoma* (Pomacentridae). *Mar Biol* 121, 585–590

- Lavery S, Moritz C, Fielder DR (1996) Indo-Pacific population structure and evolutionary history of the coconut crab *Birgus latro*. *Mol Ecol* 5, 557-570
- Matsumoto M, Hayami I (2000) Phylogenetic analysis of the family Pectinidae (Bivalvia) based on mitochondrial cytochrome C oxidase subunit I. *J Mollusc Stud* 66, 477-488
- McMillan WO, Palumbi SR (1995) Concordant evolutionary patterns among Indo-West Pacific butterflyfishes. *Proc R Soc Lond B Biol Sci* 260, 229-236
- Minchin D (2003) Introductions: some biological and ecological characteristics of scallops. *Aquat Living Resour* 16, 521-532
- Morton N (1980) Swimming in *Amusium pleuronectes* (Bivalvia: Pectinidae). *J Zool* 190, 375-404
- Nagashima K, Sato M, Kawamata K, Nakamura A, Ohta T (2005) Genetic structure of Japanese scallop population in Hokkaido, analysed by mitochondrial haplotype distribution. *Mar Biotechnol* 7, 1-10
- Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* 76, 5269-5273
- Nugranad J (1990) Population Dynamics of the Asian Moon Scallop (*Amusium pleuronectes*, Linn) Around the Chang Islands, Trat Province. Master's Thesis, Chulalongkorn University, 128 pp
- Okutani T (2000) *Marine Mollusks in Japan*. (Tokyo: Tokai University Press)
- Palumbi SR (1997) Molecular biogeography of the Pacific. *Coral Reefs* 16, S47-S52
- Palumbi SR, Martin AP, Romano S, McMillan WO, Stice L, Grabowski G (1991) *The Simple Fool's Guide to PCR*. (Honolulu: Department of Zoology, University of Hawaii), pp 1-45
- Raymond M, Rousset F (1995) An exact test for population differentiation. *Evolution* 49, 1280-1283
- Roongratri M (1996) *Biology of the Asian Moon Scallop [Amusium pleuronectes, Linn] in Rayong Bay, Rayong Province*. Technical Paper No. 61: Eastern Marine Fisheries Development Center, Department of Fisheries, Ministry of Agriculture and Cooperatives
- Saavedra C, Peña JB (2004) Phylogenetic relationships of commercial European and Australasian king scallops (*Pecten* spp.) based on partial 16S ribosomal RNA gene sequences. *Aquaculture* 235, 153-166
- Schneider S, Roessli D, Excoffier L (2000) Arlequin: a software for population genetics data analysis. Genetics and Biometry Lab, Department of Anthropology, University of Geneva
- Taggart JB, Hynes RA, Prodohl PA, Ferguson A (1992) A simplified protocol for routine total DNA isolation from salmonid fishes. *J Fish Biol* 40, 963-965
- Ward RD, Grewe PM (1995) "Appraisal of molecular genetic techniques in fisheries". In: *Molecular Genetics in Fisheries*, Carvalho GR, Pitcher TJ, eds. (London: Chapman & Hall), pp 29-54
- Wilding CM, Beaumont AR, Latchford JW (1997) Mitochondrial DNA variation in the scallop *Pecten maximus* (L.) assessed by a PCR-RFLP method. *Heredity* 79, 178-189
- Williams ST, Benzie JAH (1998) Evidence of a biogeographic break between populations of a high dispersal starfish: congruent regions within the Indo-West Pacific defined by colour morphs, mtDNA and allozyme data. *Evolution* 52, 87-99
- Williams ST, Knowlton N, Weight LA (1999) "Indo-Pacific molecular biogeography of the coral-dwelling snapping shrimp *Alpheus lottini* (Decapoda: Caridea: Alpheidae)". In: *Ecology of the Chagos Archipelago* Linnean Society Occasional Publications, Sheppard, CRC, Seaward MRD, eds. (London: Linnean Society) pp, 195-206

เอกสารแนบที่ 11

Mahidol, C, Na-Nakorn, U, Sukmanomon, S, Yoosuk, W, Taniguchi, N, Nguyen, TTT.
2007. Phylogenetic relationships among nine scallop species (Bivalvia: Pectinidae)
inferred from nucleotide sequences of one mitochondrial and three nuclear gene regions.
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PHYLOGENETIC RELATIONSHIPS AMONG NINE SCALLOP SPECIES (BIVALVIA: PECTINIDAE) INFERRED FROM NUCLEOTIDE SEQUENCES OF ONE MITOCHONDRIAL AND THREE NUCLEAR GENE REGIONS

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ABSTRACT Current knowledge of the evolutionary relationships among scallop species (Mollusca: Bivalvia: Pectinidae) in the Indo-Pacific region is rather scanty. To enhance the understanding of the relationships within this group, phylogenies of nine species of scallops with the majority from coastal regions of Thailand, were reconstructed by maximum parsimony, maximum likelihood, and Bayesian methods using sequences of the 16S rRNA of the mitochondrial genome, and a fragment containing the ITS1, 5.8S and ITS2 genes of the nuclear DNA. The trees that resulted from the three methods of analysis were topologically identical, however, gained different levels of support at some nodes. Nine species were clustered into two major clades, corresponding to two subfamilies (Pectininae and Chlamydominae) of the three currently recognized subfamilies within Pectinidae. Overall, the relationships reported herein are mostly in accordance with the previous molecular studies that used sequences of the mtDNA cytochrome oxidase subunit I, and the classification system based on microsculpture of shell features and morphological characteristics of juveniles. Levels of divergences were different among genes (i.e., the 5.8S gene showed the lowest levels of nucleotide divergence at all levels, whereas the 16S rRNA showed the highest level of variation within species, and ITS2 gene revealed the highest level of divergence at higher levels).

KEY WORDS: Pectinidae, scallops, phylogeny, nucleotide sequences

INTRODUCTION

Bivalves of the family Pectinidae, often referred to as scallops, are among the better-known shellfishes. Scallops are distributed worldwide and inhabit a wide variety of environments in all seas from polar regions to the tropics (Brand 2006). Scallops are also well known because of their commercial importance and contribute significantly to commercial fisheries as well as aquaculture production. Many species are currently cultured, and the average annual aquaculture production of scallops in the period 2000–2004 was about 1.17 million t, valued at \$63.6 million USD, accounting for about 61.2% global scallop production (FAO 2006). Given their importance, scallops have been the subject of much research (Shumway & Parsons 2006).

Currently about 400 living scallop species are recognized and are reputed to have a very complex taxonomy. A number of classification systems have been proposed based on morphological characters (e.g., Hertlein 1969, Korobkov 1960, Waller 1991, 1993, 2006). The current consensus and well-accepted system is that of Waller, who classified Pectinidae, based on microsculpture of shell features and morphological characteristics of juveniles. Waller (1991, 1993) suggested the division of Pectinidae into three subfamilies, comprising of Camptonectinae, Chlamydominae, and Pectininae. The subfamily Chlamydominae was further divided into four tribes: Chlamydini, Crassadomini, Mimachlamydini and Acquiptectini, and Pectininae into three tribes: Palliolini, Decatoplectini, and Pectinini. In a recent revision, Waller (2006) suggested the additional tribe Amusiini in Pectininae.

With the recent advances in the field of molecular phylogenetics, several attempts have been made to reconstruct the phylogeny of Pectinidae using molecular data. Sequences from a number of gene regions have been used to infer scallop phylogenies, such as the 16S rRNA and 12S rRNA (Capana et al. 1999, 2000b, Capana et al. 2000a) and the cytochrome oxidase subunit I (COI) (Giribet & Wheeler 2002, Matsumoto & Hayami 2000) of the mitochondrial genome, and 18S rRNA (Capana et al. 1999, Frischer et al. 1998, Giribet & Carranza 1999, Giribet & Wheeler 2002, Winnepenninckx et al. 1996) and the internal transcribed spacer I (ITS1) to internal transcribed spacer II (ITS2) (Insua et al. 2003) of the nuclear DNA. In general, phylogenies recovered from molecular DNA sequences support the classification system proposed by Waller (1991, 1993) (Barucca et al. 2004, Matsumoto & Hayami 2000). However, most studies used scallop samples from the north Atlantic and the north Pacific regions, and little attention has been paid to species from the Indo-Pacific. The only study to date on scallop systematics in this region was that of Matsumoto and Hayami (2000), but was confined to Japan.

The Indo-Pacific coastal region harbors a rich scallop fauna, with about 185 known species belonging to 38 genera currently listed in the Ocean Biogeographic Information System (OBIS) Indo-Pacific Molluscan Database (<http://data.acnatsci.org/obis/>). Many scallop species found in the Indo-Pacific region are of commercial importance and some are being cultured, such as for example *Chlamys farreri* and *Mimachlamys nobilis* in China (Guo & Luo 2006), *Patinopecten yessoensis* in Japan (Kosaka & Ito 2006), and *Pecten* spp. in Australia (Saavedra & Peña 2004). The Asian region has been leading cultured scallop production over the last many decades, contributing about

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97.9% to that of the world (FAO 2006). In Thailand, as in most of SE Asia, many wild populations of scallops have been overexploited and efforts have been made, although still at a very early stage, in filling the gap between demand and supply through aquaculture. In this regard for example, hatchery production techniques have been developed for species such as *Minachlamys senatoria* (Nugranad & Promjinda 1997).

The objective of this study is to undertake a phylogenetic analysis of sequences from four gene regions, including partial 16S rRNA gene region of the mitochondrial DNA, and the complete sequences of three nuclear genes (i.e., ITS1, 5.8S, and ITS2) to investigate phylogenetic relationships among nine scallop species in the Indo-West Pacific region with samples mainly obtained from Thailand coastal areas.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

A total of 29 individuals of scallops, believed to be from nine species were collected from 2004–2006 along the coast of Thailand. Upon capture, a small portion (approximately 150 mg) of adductor muscle was preserved in 95% ethanol. Voucher specimens comprising of preserved tissues and shells were held at the Kasetsart University Museum of Fisheries, Bangkok, Thailand. For comparative purposes, three individuals of *Decatopecten radula* from Lampung, Indonesia; three and two individuals of *Mimachlamys nobilis* were also obtained from Hainan, China and Kochi Prefecture, Japan, respectively. Details on localities and sample sizes are given in Table 1 and Figure 1.

Total genomic DNA was extracted using phenol/chloroform standard method as described by Taggart et al. (1992) with a slight modification. The individual DNA was resuspended in TE buffer (10 mM Tris-HCl pH 7.2; 1 mM EDTA pH 8.0) and stored at -20°C until required.

PCR Amplification and Sequencing

A fragment of the large ribosomal mitochondrial gene (16S rRNA) was amplified using primers 16Sar (5'-CGC CTG TTT AAC AAA AAC AT-3') and 16Sbr (5'-CCG GTC TGA ACT CAG ATC ATG T-3') (Palumbi et al. 1991). The transcribed spacer (ITS) region, comprising of ITS1, the 5.8 rRNA gene, and ITS2, was amplified using primers designed by Heath et al. (1995), which anneal to the 3'-end of the 18S gene and to the 5'-end of the 28S gene (forward - 5' GTT TCC GTA GGT GAA CCT G 3', reverse - 5' CTC GTC TGA TCT GAG GTC G 3'). PCR was performed in a total volume of 30 µl containing approximately 50 ng template DNA, 1X PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM of each primer, and 1 unit Taq Polymerase (Promega). PCR conditions were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

The majority of samples were analyzed at the Laboratory of Population Genetic Informatics, Tohoku University, Japan where PCR products were purified with the ExoSAP-IT (usb) and sequenced using an ABI Prism 377 DNA Sequencer

TABLE 1.

Details of specimens of Pectinid species used in the present study. Most of the samples were from Thailand otherwise indicated.

Species	Sample Code	Sample Size	Locality
<i>Amusium</i>	Ap-T	2	Trat
<i>pleuronectes</i>	Ap-NT	1	Naratiwas Province
<i>Annachlamys</i>			
<i>macassarensis</i>	Am-NT	4	Naratiwas Province
<i>Decatopecten</i>			
<i>radula</i>	Dr-IN	3	Lampung, INDONESIA
<i>Decatopecten</i>	Dp-PJ	4	Bangsapan, Prachuabkhirakhan
<i>plica</i>			
<i>Mimachlamys</i>	Mn-BC	3	Bangsare, Chonburi
<i>nobilis</i>	Mn-SC	3	Samasarn, Chonburi
	Mn-CN	3	Hainan, CHINA
	Mn-JP	2	Kochi Prefecture, JAPAN
	Mn-GB		GenBank Accession Number AJ571620 ¹ and AY690599 ²
<i>Mimachlamys</i>	Ms-BC	1	Bangsare, Chonburi Province
<i>senatoria</i>	Ms-SC	2	Samasarn, Chonburi Province
	Ms-PB	1	Cha-am, Phetchaburi Province
	Ms-PJ	1	Bangsapan, Prachuabkhirakhan
	Ms-PK	2	Phuket
<i>Minachlamys</i> spp.	M?-KBI	2	Lanta Noi Island, Krabi Province
<i>Mimivola</i>	Mp-KB	3	Lanta Noi Island, Krabi Province
<i>pyridata</i>			
<i>Mimachlamys</i>	Mv-GB		GenBank Accession Number AJ243575 ³ and AJ534978 ⁴
<i>varia</i>			
<i>Semipallium</i>	Sf-CP	2	Cha-am, Phetchaburi Province
<i>fulvicostatum</i>			

¹ Barucca et al. (2004)

² Bao et al. (unpublished)

³ Capana et al. (2000b)

⁴ Insua et al. (2003)

(Applied Biosystems) by using the BigDye Terminator (Version 3) Cycle Sequencing Ready Reaction Kit. Remaining samples were sent to Macrogen Inc., Korea for purification and sequencing.

Data Analysis

The data set included a total of 39 sequences of nine scallop species. In addition, sequences from the GenBank for *M. nobilis* (16S rRNA: AJ571620, Barucca et al. [2004]; ITS1, 5.8S and ITS2: AY690599; Bao et al. [unpublished]), and *M. varia* (16S rRNA: AJ243575, Capana et al. [2000b]; ITS1, 5.8S and ITS2: AJ534978, Insua et al. [2003]) were also included for comparative purposes. Sequences of *Ostrea edulis* (Genbank Accession No. DQ280032 [Giribet et al. 2006] for 16S rRNA, and U88709 [Carnegie, unpublished] for other gene regions) were used as an outgroup.

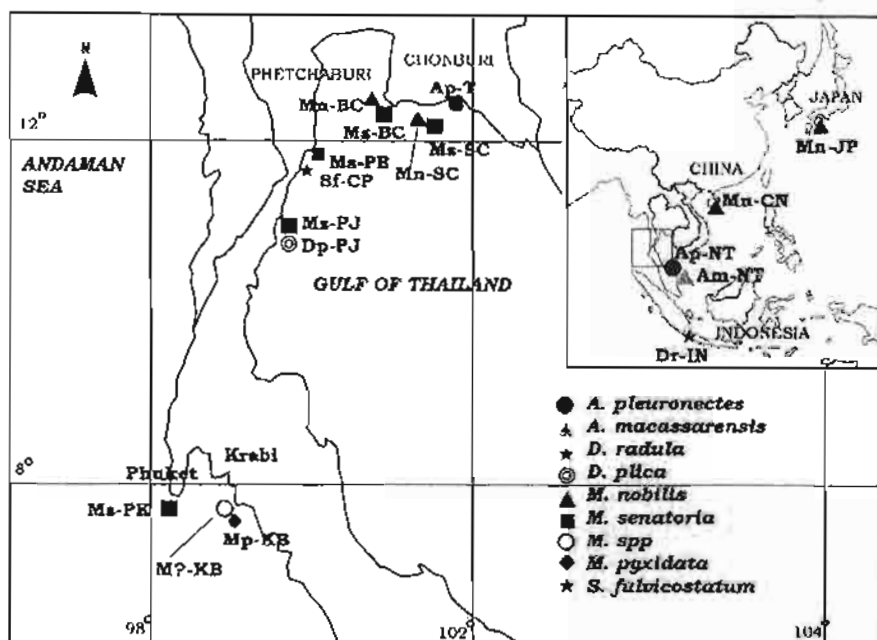


Figure 1. Sampling localities of nine scallop species in the present study.

The 5.8S gene fragment was aligned by eyes and the amino acid sequences were translated with reference to *Pecten maximus* (GenBank Accession No. AJ428410) as a test of the presence of nuclear paralogues. Alignment of the 16S rRNA, ITS1 and ITS2 gene regions was undertaken using the program SOAP (Löytynoja & Milinkovitch 2001). This program generates and compares alignments corresponding to 30 different sets of alignment parameters (gap extension penalty from 12–17 in steps of 1; gap opening penalty from 6–8 in steps of 0.5). A strict consensus of all of these alignment combinations was then used for further analysis.

Significant differences in base composition were tested for each data partition using homogeneity χ^2 analysis as implemented in PAUP* 4.0b10 (Swofford 2001). The presence of significant heterogeneity between partitions was assessed using partition homogeneity test (Farris et al. 1995) as implemented in PAUP* 4.0b10 (Swofford 2001).

Maximum parsimony (MP), maximum likelihood (ML) and Bayesian approaches were used to estimate phylogenetic relationships among the subject taxa. MP and ML phylogenetic analyses were executed in PAUP* 4.0b10 (Swofford 2001). For the MP analysis, trees were generated using the heuristic search option with TBR branch swapping using 1,000 random taxon additions and gaps were treated as missing data. The best fit substitution model was estimated using MODELTEST version 3.07 (Posada & Crandall 1998). The best-fit maximum likelihood score was chosen using Akaike's information criterion (AIC), because this reduced the amount of unnecessary parameters that contribute little to describing the data by penalizing more complex models (Burnham & Anderson, 2002; Nylander et al., 2004). For the ML analyses heuristic searches with TBR branch swapping and 100 random additions of taxa were also performed.

Uncorrected ("p") sequence divergence values were calculated between samples. Phylogenetic confidence in the nodes recovered from parsimony was estimated by nonparametric bootstrapping (Felsenstein 1985), analyzing 1,000 pseudoreplicates of data sets, whereas because of computational constraints only 200 pseudo-replicates were performed for ML. Bayesian inferences were used to investigate optimal tree space using the program MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001). For each analysis, four Markov chains were run, with each chain starting from a random tree and three million generations generated. Sampling from the chain occurred every 500th tree for each of the four partitions (16S rRNA, ITS1, 5.8S, ITS2), followed by the total evidence. In these combined analyses, genes were partitioned according to substitution models selected using MODELTEST, using unlinked parameters. A fifty percent majority rule consensus tree was generated from the trees retained, with posterior probabilities for each node estimated by the percentage of time the node was recovered. For the Bayesian analyses, data sets were run a minimum of four times to test whether they converge on the same topology.

We used Bayesian analysis (MrBayes 3.0b4) to estimate base frequencies, transition matrices, proportion of invariant sites and Γ -shapes for each partition by unlinking estimates of these for each partition. For all runs, stationarity was reached after about 2.2×10^6 generations and parameter estimates were based on the last 1,000 trees (i.e., last half million generations).

RESULTS

A total of 39 sequences for each gene (16S rRNA, ITS1, 5.8S, and ITS2) were obtained from the nine scallop species used in the present study. All sequences were deposited in GenBank (GenBank Accession Numbers DQ873890–DQ873916 for the

regions from ITS1 to ITS2, DR873917-DQ873942 for 16S rRNA). Alignment of 16S rRNA resulted in 606 base pairs (bp), of which four regions consisting of 158 bp (between bases 220–239, 389–399, 550–565, and 597–603 in the original alignment) were deemed unstable and removed from further analysis. Similarly, of 320 bp obtained from the ITS1 region, six regions with a total of 77 bp (between bases 22–29, 95–108, 124–135, 198–203, 248–255, and 297–330 in the original alignment) were inconsistent between alignments and therefore excluded from further analysis. Four regions of the ITS2 gene, consisting of 78 bp (between bases 123–139, 224–242, 247–283, and 248–352) were found unstable and removed. Only 14 bp at the 3'-end of the 18S rRNA gene was obtained, and these were invariable among samples examined and as such also excluded from further analysis.

Chi-square tests in base composition among taxa indicated no significant differences for any of the genes ($df = 111$, $P = 0.99 - 1.00$, Table 2). No significant heterogeneity between data partitions was detected under the partition homogeneity test ($P = 0.09$), and as such all data partitions were combined for further analysis.

In a total of 1142 bp of the combined data set, 771 sites were variable, of which 447 sites were parsimony informative. Bayesian estimates for GTR substitution matrices, proportions of invariant sites, and Γ -shapes are given in Table 2. These data indicated very different evolutionary dynamics for each of the partitions, in particular Γ -shape values varied among the partitions and extremely high in the ITS1 and ITS2 gene regions. Levels of divergence between species estimated as uncorrected "p" distances are presented in Table 3. Overall, low levels of intraspecific variation were observed, ranging from 0.000 (*D. radula*) to 0.003 (*M. senatoria*). The lowest level of between species differentiation (0.073) was observed between *M. nobilis* and *M. senatoria*. All species showed high levels of variation to the outgroup, *O. edulis* with an average genetic distance of 0.528.

A comparison among gene partitions on level of divergence is shown in Figure 2. Among the partitions, the 5.8S gene showed the lowest variation at all levels (0.000 within species, 0.011 between species, 0.018 between genera and 0.021 between subfamilies), whereas the ITS2 gene fragment showed the highest level of divergence at species level or higher (0.150 between species, 0.317 between genera, and 0.471 between subfamilies). The 16S rRNA gene region showed the highest level of intraspecific variation (0.005), however, less nucleotide divergence was found at higher levels compared with the two ITS genes.

The maximum parsimony analysis for the combined data set with all sites weighted equally gave three most parsimonious trees at the length of 1547. A strict consensus of these trees recovered identical topology to the tree obtained from Bayesian analysis (Fig. 3). The MP tree was supported by high bootstrap values (79% to 100%) at major nodes, but support from ML analysis was lower than 50% at some nodes, and Bayesian posterior probabilities ranged from moderate to high (0.54–1.00).

The tree was bifurcated into two major clades. The first clade (Pectininae) represented tribes Amusiini, Decatopectini, and Pectinini, in which Decatopectinini was found to be more closely related to Pectinini with high level of support (60% to 80% bootstrap, 0.82 posterior probability). Except for the three samples of *D. radula*, which were monomorphic, all others in this clade were polymorphic at least in one gene region. Three haplotypes were observed among Thai samples of *A. pleuronectes*, which showed an average of 0.003 divergence to the sequence of the same species obtained from GenBank.

The remaining samples were clustered into the second group, comprising of Chlamydiae (Chlamidini and Mimachlamyidini) with low to moderate support (posterior probability of 0.54, bootstrap 79% for MP and <50 for ML) and a sample of Pectininae (*Mimivola pyxidata*). Within Mimachlamyidini, a strong correlation between patterns of genetic variation with

TABLE 2.

Base frequencies of each gene partition and chi-square tests of bias among taxa, and substitution rate matrices, proportion of invariant sites and Γ -shapes (α), estimated using Bayesian analyses of 16S rRNA, ITS1, 5.8S and ITS2 partitions, and the combined data set (estimates are based on means of 1,000 saved trees representing generations $2.5-3 \times 10^6$ in the MCMC analysis).

Gene Partitions	Base Frequencies				χ^2	P	Substitution Rate Matrices					α
	A	C	G	T			C	G	T	$p(\text{inv})$		
16S rRNA	0.23	0.19	0.29	0.29	37.16	1.00	A	0.033	0.383	0.120	0.155	0.36
							C		0.012	0.398		
							G			0.054		
ITS1	0.33	0.27	0.20	0.20	65.97	0.99	A	0.190	0.150	0.126	0.294	105.24
							C		0.134	0.204		
							G			0.195		
5.8S	0.24	0.26	0.28	0.21	5.36	1.00	A	0.122	0.223	0.229	0.107	2.63
							C		0.056	0.260		
							G			0.110		
ITS2	0.27	0.24	0.25	0.24	71.54	0.99	A	0.130	0.169	0.166	0.0147	119.37
							C		0.132	0.255		
							G			0.148		
Combined data	0.26	0.23	0.26	0.25	36.49	1.00	A	0.149	0.158	0.209	0.122	68.56
							C		0.087	0.238		
							G			0.159		

TABLE 3.

Mean uncorrected "p" genetic distances between species examined in the present study based on the combined data set. The numbers on the diagonal are within species variation, dash (—) indicates only one sample was sequenced.

Species	1	2	3	4	5	6	7	7	9	10	11
1. <i>Amusium pleuronectes</i>	0.001										
2. <i>Amusium</i> <i>macassarensis</i>	0.226	0.007									
3. <i>Decatopecten radula</i>	0.117	0.145	0.000								
4. <i>Decatopecten plica</i>	0.186	0.150	0.100	0.001							
5. <i>Mimachlamys nobilis</i>	0.232	0.243	0.242	0.235	0.002						
6. <i>Mimachlamys senatoria</i>	0.242	0.236	0.242	0.237	0.073	0.003					
7. <i>Mimachlamys spp.</i> ¹	0.191	0.232	0.236	0.238	0.121	0.115	0.001				
8. <i>Mimachlamys pyxidata</i>	0.213	0.230	0.235	0.231	0.134	0.123	0.104				
9. <i>Mimachlamys varia</i> ¹	0.255	0.246	0.250	0.261	0.184	0.172	0.169	0.166			
10. <i>Semipallium</i> <i>fulvostatum</i>	0.226	0.225	0.246	0.241	0.181	0.168	0.178	0.176	0.204		
11. <i>Ostrea edulis</i> ²	0.516	0.215	0.520	0.535	0.536	0.538	0.530	0.528	0.539	0.527	

¹ Capana et al. (2000b) and Insua et al. (2003) for 16S rRNA and ITS1, 5.8S and ITS2, respectively

² Giribet et al. (2006) and Carnegie (unpublished) for 16S rRNA and ITS1, 5.8S and ITS2, respectively

geographical distribution was observed. *Mimachlamys varia*, a species that is distributed through out Mediterranean and extending to the North Sea, and was genetically differentiated (0.178 nucleotide divergence) from its congeners occurring in the Indo-West Pacific region.

DISCUSSION

Phylogenetic Relationships

The phylogenetic analysis of a total of 1,142 bp comprising of mitochondrial and nuclear genes indicates that scallop species included in the present study constitute clearly distinct lineages. Nine scallop species from Thailand were well grouped into two subfamilies of the three currently recognized subfamilies of Pectinidae, corresponding to Pectininae and Chlamydeinae of Waller (1991, 2006). The subfamily Pectininae appears to be paraphyletic considering the position of *M. pyxidata*, a species that has not been examined by Waller (1991, 2006) and or

subjected to other molecular studies (Capana et al. 2000b, Giribet et al. 2006, Matsumoto & Hayami 2000).

Overall, the phylogenetic relationships presented herein are complimentary to those suggested by Waller (2006), as slightly modified from Waller (1991, 1993). With the addition of nine species from the Indo-Pacific region used in the present analysis, the findings conform to Waller's system not only in the constituent genera but also in the ranking of subfamilies and tribes. This observation is also supported by the results of Matsumoto and Hayami (2000), who inferred the relationships among seven Japanese scallop species using sequences of the COI mtDNA gene region. As suggested by Matsumoto and Hayami (2000), molecular data seem to support Waller's classification system probably because Waller used a cladistic approach based on microscopic characters appearing in the early dissoconchs, such as shell microstructure, patterns of radial ribs, and dentition. These characters are believed to be less influenced by changes in life history, unlike in the case of adult shells (Hertlein 1969, Korobkov 1960, Thiele 1935).

In a recent revision, Waller (2006) suggested that genus *Amusium* be removed from the tribe Pectinini, and included in a new tribe (Amusiini) together with the other two genera. Our data support this view considering the position of *A. pleuronectes* in association with Decatopectinini and Pectinini species. In addition, the mean genetic distance between *A. pleuronectes* and Decatopectinini (0.170) is higher than that between Decatopectinini and Pectinini (0.148), justifying the recognition of Amusiini as a distinct tribe. It is also noted that this observation is similar to that of Matsumoto and Hayami (2000), but these authors, however, suggested that a subfamilial status for *Amusium* was unwarranted, and did not discuss about its tribal status.

The phylogenetic tree recovered from our data is not in conformity to that of Waller (2006) at one occasion. Although limited number of taxa were examined in the present study, our findings and that of Matsumoto and Hayami (2000) indicate the close relationship between Decatopectinini and Pectinini species (posterior probability of 0.82, and bootstrap supports resulted from MP and ML analysis are 82 and 60, respectively). Waller (2006) using morphological data and fossil records,

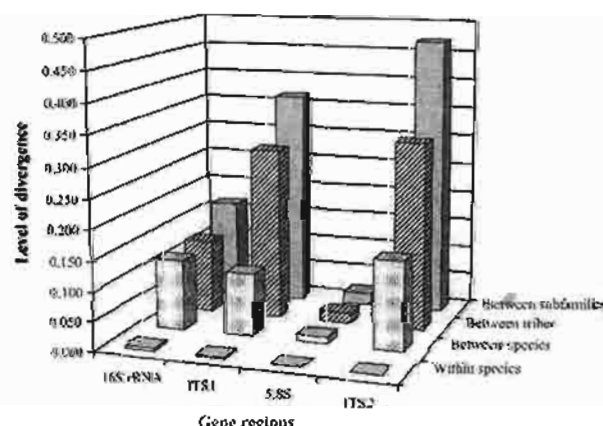


Figure 2. Comparison of levels of nucleotide divergences between four different gene fragments used in the present study.

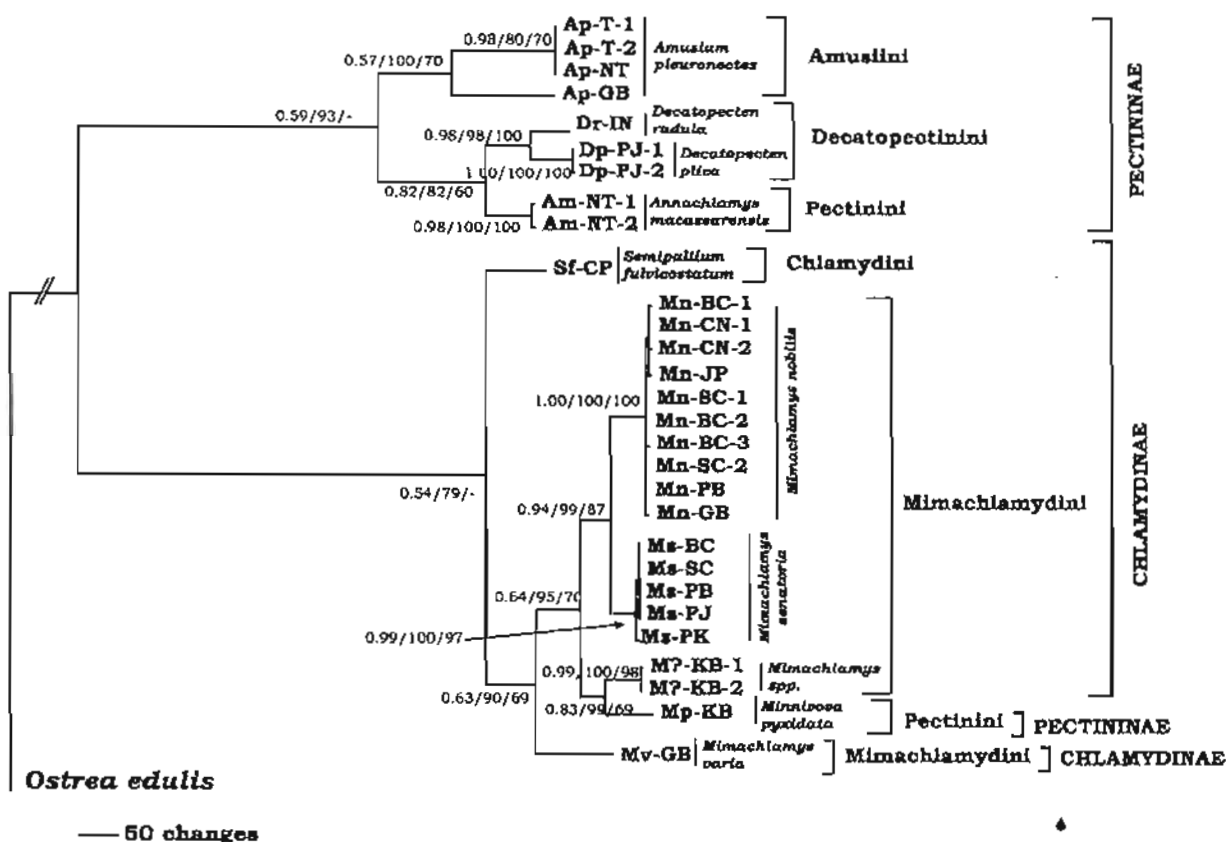


Figure 3. Bayesian estimation of the phylogenetic relationships among 10 scallop species, and the outgroup using combined data of four gene fragments (16S rRNA of the mitochondrial genome, and ITS1, 5.8S and ITS2 of the nuclear DNA). Tree produced from 3×10^6 generations using the GTR + I+J model of sequence evolution unlinked across all partitions. Numbers at each node represent posterior probabilities, bootstrap support for MP and bootstrap support for ML, respectively.

however, suggested that Decatopectinini is a sister group of the (Pectinini + Amusini). Further investigations with broader taxon sampling (i.e., more species representing each tribe) would be useful in resolving this uncertainty.

It is difficult to compare the phylogenetic relationships recovered from our data to that of Winnepekinckx et al. (1996) and Frischer et al. (1998) because of differences in taxon samplings. However, it is noted that the tree recovered by Frischer et al. (1998) using 18S rRNA gene sequences of seven scallop species from the north Atlantic and northeast Pacific were interpreted as partially inconsistent with Waller's system, although bootstrap supports were low (<50%) at some nodes. For example, species that represents the subfamily Pectininae (*Placopecten magellanicus*) appeared to constitute Chlamydiinae. Similar branching patterns were also observed by Winnepekinckx et al. (1996). The different observations among molecular studies indicate the need for an evaluation of the utility of different gene regions for Pectinidae systematic studies with additional species.

The placement of *M. minivola* (Pectinini) (Dijkstra 1998) within the Chlamydiinae group raises an intriguing question relating to paraphyletic status of Pectinini. This finding indicates that there are problems with shell morphological based taxonomy. It is noted that members of genus *Mimivola* were

not examined by Waller (1991, 1993, 1996) or any other molecular studies and as such its position is based solely on morphological features of adult shells. There could be a possibility that the gene trees generated in this study do not reflect the true phylogenetic relationship among taxa or there is possible presence of molecular convergence. However, the use of combination of one mitochondrial gene region and other three nuclear genes would be sufficient to eliminate these doubts.

Levels of Divergence

Although sequences of the two gene fragments used in the present study for phylogenetic inference have been examined previously (Barucca et al. 2004, Capana et al. 2000b, Insua et al. 2003), this is the first study that used a combination of these genes. In addition to COI sequences obtained by Matsumoto and Hayami (2000), data presented herein provide useful insight into the phylogeny of Pectinidae and the utility of a particular gene in systematic studies of this group. Overall, the fragment from 5.8S ribosomal coding gene showed the least variation, and the fragments of ITS genes showed considerably greater levels of divergence between major groups. The 16S rRNA gene of the mitochondrial genome although showed the highest level of intraspecific variation, it reveals lower a level

of variation at higher levels compared with the two nuclear ITS genes.

The levels of divergence at intraspecific level observed in our study are lower than those previously reported. For example, the highest level of divergence was observed within *M. nobilis* in the 16S rRNA gene (0.006), is much lower than that found in European species (i.e., *Pecten jacobaeus* [0.200] and *Pecten maximus* [0.420], and Australian king scallops, *Pecten novaezelandiae* [0.300] (Saavedra & Peña 2004). These differences could be because of a number of factors, including differences in taxon sampling, sample size, the models used for estimating genetic distances, and probably the exclusion of a number of sites in our data set after alignment in the data set.

Levels of interspecific variation of the 16S rRNA gene region observed in our study are, however, very similar to that reported by Saavedra and Peña (2004). The latter study reported average values for interspecific comparisons among two European species and two Australasian species and ranged from 0.042–0.160, whereas our results ranged from 0.072–0.166. Frischer et al. (1998) reported only 0.095 at the maximum divergence observed among seven pectinids for the 18S rRNA gene, and according to Matsumoto and Hayami (2000) this level of variation indicates 18S rRNA gene is too conservative compared with COI gene, which has 30% amino acid variable sites among 17 pectinids. However, the level of divergence observed herein for the 5.8S gene sequences are even lower than

that of the 18S rRNA gene reported by Frischer et al. (1998). As for the ITS genes, level of variation observed in the present study are commensurate with that determined by Insua et al. (2003).

In conclusion, scallops species analyzed in this study belong to two subfamilies, Pectininae and Chlamydinac of Pectinidae. Analysis of both mitochondrial and nuclear genes of these samples has resulted in a phylogeny that is largely consistent with those previously described based on nonadaptive morphological characters of scallop species. Our sampling was mainly confined to a relatively small geographic region, and an extension to this study with additional number of species in the Indo-Pacific region is warranted to understand better the evolutionary relationships within this group.

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LITERATURE CITED

- Barucca, M., E. Olmo, S. Schiaparelli & A. Capana. 2004. Molecular phylogeny of the family Pectinidae (Mollusca: Bivalvia) based on mitochondrial 16S and 12S rRNA genes. *Mol. Phylog. Evol.* 1:89–95.
- Burnham, K. P. & D. R. Anderson. 2002. Model selection and multi model inferences, a practical information-theoretic approach. New York: Springer.
- Brand, A. R. 2006. Scallop ecology: distributions and behaviour. In: S. E. Shumway & G. J. Parsons, editors. *Scallops: biology, ecology and aquaculture*. The Netherlands: Elsevier B. V. pp. 561–744.
- Capana, A., M. Barucca, A. Marinelli & E. Olmo. 1999. A molecular approach to the systematics of Antarctic scallop *Adamussium colbecki*. *Ital. J. Zool. (Modena)* 66:379–382.
- Capana, A., M. Barucca, A. Marinelli & E. Olmo. 2000b. Molecular data from the 16S rRNA gene for the phylogeny of Pectinidae (Mollusca: Bivalvia). *J. Mol. Evol.* 50:93–97.
- Capana, A., M. Barucca, V. Caputo, A. Marinelli, P. Nisi Cerioni & E. Olmo. 2000a. A molecular analysis of the systematics of three Antarctic bivalves. *Ital. J. Zool. (Modena)* (Suppl. 1):127–132.
- Dijkstra, H. H. 1998. Pectinoidea (Mollusca: Bivalvia: Pectinidae: Propeamussiidae) from Hansa Bay, Papua New Guinea. *Mollusc. Res.* 19:11–52.
- FAO. 2006. Fishstat plus: universal software for fishery statistical. Time series 1950–2004. Version 2.30. FAO Fisheries Department, Fishery Information, Data and Statistics Unit.
- Farris, S. J., M. Källersjö, A. G. Kluge & C. Bult. 1995. Constructing a significant test for incongruence. *Syst. Biol.* 44:570–572.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution Int. J. Org. Evolution* 39:783–791.
- Frischer, M. E., J. Williams & E. Kenchington. 1998. A molecular phylogeny of some major groups of Pectinidae inferred from 18S rRNA gene sequences, bivalves: an Eon of evolution. *Paleobiological studies honoring Norman D. Newell*. University of Calgary Press, Calgary. pp. 213–221.
- Giribet, G. & S. Carranza. 1999. What can 18S rRNA do for bivalve phylogeny? *J. Mol. Evol.* 48:256–258.
- Giribet, G. & W. Wheeler. 2002. On bivalve phylogeny: a high-level analysis of the bivalvia (Mollusca) based on combined morphology and DNA sequence data. *Invert. Biol.* 121:271–324.
- Giribet, G., A. Okusu, A. R. Lindgren, S. W. Huff, M. Schödl & M. K. Nishiguchi. 2006. Evidence for a clade composed of molluscs with serially repeated structures: Monoplacophorans are related to chitons. *Proc. Nat. Acad. Sci. USA* 103:7723–7728.
- Guo, X. & Y. Luo. 2006. Scallop culture in China. In: S. E. Shumway & G. J. Parsons, editors. *Scallops: biology, ecology and aquaculture*. 2nd ed. The Netherlands: Elsevier, Amsterdam. pp. 1143–1161.
- Heath, D. D., P. D. Rawson & T. J. Hilbish. 1995. PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. *Can. J. Fish. Aquat. Sci.* 52:2621–2627.
- Hertlein, L. G. 1969. Pectinidae. In: R. C. Moore, editor. *Treatise on invertebrate paleontology*. Lawrence: Geological Society of America and University of Kansas Press. pp. N349–N373.
- Huelsenbeck, J. P. & F. Ronquist. 2001. MrBayes: bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–755.
- Insua, A., M. J. López-Piñón & R. J. M. Freire. 2003. Sequence analysis of the ribosomal DNA internal transcribed spacer region in some scallop species (Mollusca: Bivalvia: Pectinidae). *Genome* 46:595–604.
- Korobkov, I. A. 1960. Family Pectinidae Lamarck. 1801. In: U. A. Orlov, editor. *Osnovy Paleontologii, Mollusca-Loricata, Bivalvia and Scaphopoda*. Moscow: Academy Nauk USSR. pp. 82–85.
- Kosaka, Y. & H. Ito. 2006. Japan. In: S. E. Shumway & G. J. Parsons, editors. *Scallops: biology, ecology and aquaculture*, 2nd ed. The Netherlands: Elsevier Amsterdam. pp. 1093–1136.
- Löytynoja, A. & M. C. Milinkovitch. 2001. SOAP, cleaning multiple alignments from unstable blocks. *Bioinformatics* 17:573–574.
- Matsumoto, M. & I. Hayami. 2000. Phylogenetic analysis of the family Pectinidae (Bivalvia) based on mitochondrial cytochrome C oxidase subunit I. *J. Mollusc. Stud.* 66:477–488.
- Nugranad, J. & K. Promjinda. 1997. An experiment on hatchery seed production of the scallop *Chlamys senatoria* Gmelin.

- Phuket Marine Biological Center Special Publication* 17: 241–249.
- Nylander, J. A. A., F. Ronquist, J. P. Huelsenbeck & J. L. Nieves-Aldrey. 2004. Bayesian phylogenetic analysis of combined data. *Syst. Biol.* 53:47–67.
- Palumbi, S. R., A. P. Martin, S. Romano, W. O. McMillan, L. Stice & G. Grabowski. 1991. The simple fool's guide to PCR. Department of zoology, University of Hawaii, Honolulu. pp. 1–45.
- Posada, D. & K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–818.
- Saavedra, C. & J. B. Peña. 2004. Phylogenetic relationships of commercial European and Australasian king scallops (*Pecten* spp.) based on partial 16S ribosomal RNA gene sequences. *Aquaculture* 235:153–166.
- Shumway, S. E. & G. J. Parsons. 2006. *Scallops: biology, ecology and aquaculture*. The Netherlands: Elsevier, Amsterdam.
- Swofford, D. L. 2001. *PAUP*: phylogenetic analysis using parsimony (*and other methods)*, ver. 4b10. Sunderland: Sinauer.
- Taggart, J. B., R. A. Hynes, P. A. Prodohl & A. Ferguson. 1992. A simplified protocol for routine total DNA isolation from salmonid fishes. *J. Fish Biol.* 40:963–965.
- Thiele, J. 1935. *Handbuch der systematischen Weichtierkunde*. Gustav Fischer, Jena, Zweiter Band. pp. 79–154.
- Waller, T. R. 1991. Evolutionary relationship among commercial scallops (Mollusca: Bivalvia: Pectinidae). In: S. E. Shumway, editor. *Scallops: biology, ecology and aquaculture*, 1st ed. The Netherlands: Elsevier, Amsterdam. pp. 1–73.
- Waller, T. R. 1993. The evolution of '*Chlamys*' (Mollusca: Bivalvia: Pectinidae) in the tropical western Atlantic and eastern Pacific. *Am. Mala. Bull.* 10:195–249.
- Waller, T. R. 1996. Bridging the gap between the eastern Atlantic and eastern Pacific: a new species of *Crassadoma* (Bivalvia: Pectinidae) in Pliocene of Florida. *J. Paleon.* 70:941–946.
- Waller, T. R. 2006. New phylogenies of the Pectinidae (Mollusca: Bivalvia): Conciling morphological and molecular approaches. In: S. E. Shumway & G. J. Parsons, editors. *Scallops: biology, ecology and aquaculture*, 2nd ed. The Netherlands: Elsevier, Amsterdam. pp. 1–73.
- Winnepenunckx, B., T. Backeljau & R. De Wachter. 1996. Investigation of molluscan phylogeny on the basis of 18S rRNA sequences. *Mol. Biol. Evol.* 13:1306–1317.

เอกสารแนบที่ 12

Mahidol, C., U. Na-Nakorn, S. Sukmanomon and T.T. Nguyen. Genetic diversity of *Mimachlamys senatoria* in Thailand based on variation of microsatellite DNA

Genetic diversity of *Mimachlamys senatoria* in Thailand based on variation of microsatellite DNA

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ABSTRACT

Four microsatellite primers were developed from a partial DNA library of *Mimachlamys* (*Chlamys*) *senatoria*. They were subsequently used to study genetic diversity of four populations of *M. senatoria*, three from the Gulf of Thailand and one from the Andaman Sea. The results revealed low genetic variation within populations in the Gulf of Thailand (A_o ranged 6.14-6.51; A_r ranged 5.71-8.75; H_o ranged 0.55-0.61) while it was relatively high for a population from Andaman Sea (A_o =10.6; A_r =7.76; H_o =0.72). Population structuring exists with F_{ST} =0.022 ($P<0.01$). All population pairs were genetically different. The deep genetic divergence between the populations from the Gulf of Thailand and the Andaman Sea was observed.

INTRODUCTION

The bivalves of the family Pectinidae significantly contribute to the annual world fishery production (e.g. 1,166,756 metric tonnes in 2004 with 69% contribution from aquaculture) (FAO, 2006). Recently aquaculture of the Pectinids has been expanding worldwide, for example 12% expansion of annual production of *Patinopecten yessoensis* in Japan during 1990-2004 (FAO, 2006); culture of *Chlamys nobilis* in China increased by 5 folds during the same period (annual production was 910,352 in 2004; FAO, 2006).

As in other countries, scallops also gains popularity among consumers in Thailand. The attempts to develop aquaculture of scallops was encouraged by the successful breeding of *Chlamys* or *Mimachlamys senatoria* (Nugranad and Promjinda, 1997) and *Amusium pleuronectes* (Chaithanavisuti, 1987) in the late 1980s. Due to possible adverse impacts of aquaculture on genetic diversity of the local stocks (Hindar *et al.*, 1991; Crozier, 1993; Bentzen and Thodesen, 2005) the present study was initiated to study genetic diversity of the conspecific natural stocks. The information obtained will be considered as a baseline for genetic monitoring. Moreover the conservation and fishery management authorities can utilize our results to develop management strategies for these stocks.

The study based on sequence of the 16S rRNA genes revealed no genetic differentiation among samples of *Amusium pleuronectes* within the Gulf of Thailand while the difference between the Gulf's population and the Andaman's population was distinct (Mahidol *et al.*, 2007a). Similarly clear genetic divergence between the two coasts was shown in *Mimachlamys senatoria* based on 16S rRNA sequences despite a low sample size (Mahidol *et al.*, 2007b). However, owing to the conservative nature of the 16S rRNA more polymorphic markers should be used to study the fine scale differentiation of the stocks especially the Gulf's populations.

Microsatellite loci, tandem repeats of subunits of di, tri or tetra nucleotides, are widely used for studies on genetic diversities especially among the closely related populations due to the hypervariable, codominant nature. They were used to detect genetic differences between natural and aquaculture stocks in Turbot, *Scophthalmus maximus* (Coughlan *et al.* 1998), Kuruma prawn, *Marsupeneus japonicus* (Luan *et al.*, 2006) and Pacific oyster, *Crassostrea gigas* (Appleyard and Ward, 2006), assessed the impacts of aquaculture stocks on natural stocks (Kamonrat, 1996; Nannakorn *et al.*, 2004).

None of microsatellite primers are developed from *M. senatoria* to date. Therefore the first objective of this study was to develop microsatellite primers from DNA of *M. senatoria*. Then the primers were used for studying the genetic diversity of this species in Thailand.

MATERIALS AND METHODS

Isolation and characterization of microsatellite loci

Total DNA was extracted from adductor muscle tissue obtained from an individual *M. senatoria* following the standard phenol-chloroform protocol (Taggart *et al.*, 1995). Then a partial microsatellite library was constructed using the modified enrichment procedure (Billotte *et al.*, 1999) wherein the genomic DNA was digested with a restriction enzyme, *MseI*, and ligated to the *MseI*-Adaptor. Then the ligated DNA was amplified using *MseI* adaptor as a primer. The PCR product was hybridized to 6 streptavidin-biotinylated oligo SSR complexes, (AG)₁₀, (TG)₁₀, (TAC)₁₀, (CAA)₁₀, (CAG)₁₀ and (GAT)₁₀. The bound enriched DNA was eluted and re-amplified. The amplified fragments were cloned using pGEM-T Easy vector and transformed into *E. coli* DH10 B. Six DIG-labelled oligo SSRs were used to screen the colonies which represented the repeat inserts. Eighty-two positive clones were identified and subsequently sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem) and ABI PRISM 377 DNA Sequencer (PE Applied Biosystem). The cloning and design of primers was done by DNA Technology Laboratory, Kampaengsaen, Thailand.

The study on genetic diversity

Sample collection: A total of 128 samples of *M. senatoria* were collected from four localities in Thailand as detailed in Table 1 and Figure 1. They were found attached to rocks and corals at the depth between 3-10 m. The live specimens were delivered to shore and 150 g of adductor muscle was collected from each individual, preserved in 99% alcohol and delivered to Laboratory of Fish Genetics, Faculty of Fisheries, Kasetsart University, Bangkok for further DNA analyses. The identification was performed on shell morphology following the description of Poutiers (1998).

Microsatellite DNA analyses: The PCR conditions were as follow: each of 10 µl reactions contained 5 ng DNA template, 10X PCR buffer, 0.25 µM each of forward and reversed primers, 1.5 mM MgCl₂ and 0.2 U Taq DNA polymerase (Promega).

PCR reactions were performed using PTC-100 Peltier Thermal Cycler (MJ Research) with 3 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 30 s at annealing temperature (see Table 2) and 60 s at 72 °C and a final extension step of 5 min at 72 °C. The PCR products were subsequently separated on 4.5% denaturing acrylamide gel and visualized by silver staining. Alleles were sized based on M13 ladder.

Data analyses: First the test for conformation to Hardy-Weinberg expectations (HWE) and linkage disequilibrium were performed using GENEPOP ver.3.4 (Raymond & Rousset, 2004). Then parameters for genetic variation within populations, average number of alleles/locus (A), effective number of alleles/locus (A_e), observed and expected heterozygosity per individual (H_o and H_e respectively) were calculated using POPGENE ver 1.31 (Yeh *et al.*, 1999). Allelic richness (A_r), allele diversity independent of sample sizes, was calculated using the FSTAT V2.9.3 computer program (Goudet, 2002).

To analyze the genetic diversity between populations, population structuring was studied using F-statistics calculated using the program FSTAT V2.9.3 and the overall F_{ST} was tested against 0 using the TFGA program (Miller, 1997). The difference between pairwise populations was tested using the program Arlequin (Schneider *et al.*, 2000).

The genetic distance (Cavalli-Sforza and Edwards, 1967) was calculated using the program PHYLIP (Felsenstein, 1995) and a subsequent reconstruction of the neighbor joining tree was facilitated by the same program.

RESULTS

Microsatellite loci: Among a total of one hundred twenty sequenced inserts, forty-three contained microsatellite and only six primers could be designed. Four primer pairs were able to amplify DNA of *M. senatoria*. The PCR conditions were as follow: each of 5 μ l reactions contained 5 ng DNA template, 100 mM $MgCl_2$, 0.25 μ M each of forward and reversed primers, 100 μ M dNTPs and 0.2 U Taq DNA polymerase (Promega). PCR reactions were performed using PTC-100 Peltier Thermal Cycler (MJ Research) with 3 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 30 s at annealing temperature (see Table 2) and 60 s at 72 °C and a final extension step of 5 min at 72 °C. The PCR products were subsequently separated on 5% denaturing acrylamide gel and visualized by silver staining. Alleles were sized based on M13 ladder. The pictures of microsatellite profiles are shown in Fig 2.

Allele frequency: Scoring of microsatellite loci across 128 *M. senatoria* individuals resulted in the allele size range between 220-346 for Cse 1, 189-297 for Cse 2, 156-180 for Cse 3 and 172-190 for Cse 4. Number of alleles across populations was 35, 31, 5 and 6 for Cse 1, Cse 2, Cse 3 and Cse 4 respectively. Allele frequencies for each population are in Table 3.

Tests for conformation to Hardy-Weinberg equilibrium and linkage disequilibrium: The Fisher's exact test showed the departure from HWE towards homozygote excess in two populations (PJ and PK) (Table 4). However the locus-wise F_{IS} showed significant departure from HWE at only one out of four loci in either PJ or PK (Table 4). No linkage disequilibrium was observed between loci in each population.

Genetic variation within populations: Genetic variation of *M. senatoria* in Thailand (Table 5) was quite low regarding allele diversity ($A_e = 9.39 \pm 9.17$); $A_r = 7.03 \pm 1.47$) and varied considerably among populations (e.g. A_e ranged from 6.14 in CB-1 to 10.60 in PK; A_r ranged from 5.71 in CB-1 to 8.75 in CB-2). Likewise H_o of the

populations from the Gulf of Thailand was low (0.552-0.612) while it was quite high in the population from the Andaman Sea (0.716).

Genetic variation between populations

Population structuring: The overall F_{ST} was 0.022 and was statistically greater than 0 (95% CI = 0.0346 and 0.0070) and hence suggested the subdivision of the populations.

The genotypic differentiation test showed that all population pairs were statistically different (Table 6). The pairwise F_{ST} (Table 6) revealed significant differences of all population pairs except for CB-1 and CB-2. The Bayesian assignment test assigned most individuals to the correct population (Table 7)

Genetic distances: The Carvalli-Sforza chord distance (D) ranged between 0.0311 (PJ and CB-1) and 0.0474 (PK and CB-2). The D between the populations from Andaman Sea (PK) and the Gulf of Thailand (ranged 0.0378 – 0.0474) was slightly higher than D within the Gulf of Thailand (ranged 0.0311 – 0.0359) (Table 8).

Phylogenetic dendrogram: The UPGMA dendrogram (Figure 3) clearly separated the populations in the Gulf (CB-1, CB-2, PJ) from the Andaman's (PK). Wherein within the Gulf, CB-1 and PJ formed the closest cluster but was not supported by the bootstrap (bootstrap = 46.9%) while CB-2 was separated from the first group with a bootstrap of 100.

DISCUSSION

The microsatellite primers developed from DNA of *Mimachlamys senatoria* showed similar level of polymorphism to those developed from other species (e.g. 16 primers from *Patinopecten caurinus* by Elfstrom *et al.*, 2005; 10 primers from *Mizuhopecten yessoensis* by An *et al.*, 2005; 15 primers from *Patinopecten yessoensis* by Zhao *et al.*, 2006; 35 primers from *Nodipecten subnodosus* by Ibarra *et al.*, 2006; 10 primers from *Chlamys nobilis*, by Hui *et al.*, 2006).

Despite large number of microsatellite developed only limited studies on genetic diversity within species were available to date. The genetic variation reported for *M. senatoria* in this study was lower than those of two species of scallops, *Placopecten magellanicus* ($H_o=0.802$; Gjetvaj *et al.*, 1997) and *Mizuhopecten yessoensis* (H_o ranged 0.65 to 0.76; Sato *et al.*, 2005), the European flat oyster, *Ostrea edulis* (H_o ranged 0.801-0.845) (Sobolewska and Beaumont, 2005) but similar with Asian abalone, *Haliotis asinina* collected from the same area ($A_e=6.04-9.37$; $H_o=0.58-0.78$) (Tang *et al.*, 2004). The low genetic variation of *M. senatoria* agrees well with the low abundance and patchy occurrence of this species in Thai waters.

Among the four populations included in this study CB-2 and PK showed relatively high allele diversity hence indicating that they did not experience serious genetic drift (Allendorf, 1986). Nevertheless low heterozygosity of CB-2 suggested that it may have limit distribution (Hedrick, 1985; Allendorf and Luikart, 2007) while high heterozygosity of PK may indicate better dispersal ability. Although the adult *M. senatoria* attaches to substrate most of the time its distribution can be varied between localities due to different dispersal ability of the planktonic larvae (reviewed by Brand [1991]). In the reviews Brand (1991) stated that long pelagic larval period of scallops enhances dispersal of larvae which significantly determine local distribution. However, larvae may be retained in some particular places with certain geographic and environmental features such as persistent gyres, two layer circulation and fronts.

CB-1 and PJ showed relatively low A_r and H_o hence suggested that they may have passed bottleneck resulted in contemporary small population sizes. It is of

concern that the environment around the Gulf of Thailand is rapidly deteriorated (Chongprasith and Praekulvanich, 2003). As such the populations with low genetic variation would be more vulnerable to extinction due to the compromised adaptability to changing environment (Allendorf and Luikart, 2007).

Nevertheless, we also concerned that the estimates of genetic variation in this study based on small number of populations and loci with remarkable variation in level of polymorphism among loci. Hence, further study including more loci and covering wide distribution of populations is recommended.

Genetic divergence between populations from the Gulf of Thailand and Andaman Sea

Microsatellite revealed distinct genetic divergence between populations of *M. senatoria* in the Gulf of Thailand and Andaman Sea which has been reported in wide range of taxa, e.g. mollusks (*Heliotis asinina*: Klinbunga *et al.*, 2003; *Amusium pleuronectes*: Mahidol *et al.*, 2007a), crustacean (*Penaeus monodon*: Klinbunga *et al.*, 2001), teleost (*Epinephelus coioides*: Antoro *et al.*, 2006). Thus it is obvious that such divergence related to historical low sea level events that separated the Pacific and Indian Oceans (reviewed by Mahidol *et al.*, 2007a).

The genetic divergence among populations of *M. senatoria* within the Gulf was significant for all pairs as revealed by pairwise F_{ST} . This is quite contrast with lack of genetic divergence observed in *A. pleuronectes* collected from areas with longer geographic distance (e.g. between Narathiwat and Rayong) (Mahidol *et al.*, 2007a). The difference may associate with different dispersal ability of adults wherein *A. pleuronectes* is a mobile species showing a certain degree of spawning migration (Morton, 1980) hence resulted in genetic homogeneity of populations through out the Gulf. *M. senatoria* is relatively sessile hence gene flow depended largely on larval dispersal. Thus, although it is rather a speculation, our results indicated that adult dispersal was more efficient in enhancing gene flow than the dispersal of larvae.

Application for conservation and aquaculture

Genetic differentiation among populations of *M. senatoria* suggested that stock translocation should be avoided in order to conserve the genetic integrity. Stock translocation which is always practiced in scallop aquaculture may have adverse impacts, e.g. diluting or destroying the unique genetic stocks while hybridization may break the useful adaptive linkage group (Beaumont, 2000).

Low genetic variation within populations of CB-1 and PJ should receive special attention. Genetic monitoring is recommended and a restoration plan should be applied if the decline of genetic variation is observed.

Although genetic diversity based on neutral loci does not always relate with performance of stocks, e.g. no correlation between growth rate and heterozygosity at single locus nuclear restriction fragment length polymorphisms of the scallop *Placopecten magellanicus* (Pogson and Zouros, 1994), nor between mortality and microsatellite diversity of Gila topminnow (*Poeciliopsis occidentalis*) (Giese and Hedrick, 2003), it is possible that the genetic divergent stocks may show differential phenotypic performances of some economic traits. Thus further studies to evaluate economic traits of the stocks are encouraged in order for efficient utilization of these gene pools for aquaculture.

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REFERENCES

- Allendorf, F.W. 1986. Genetic drift and the loss of alleles versus heterozygosity. *Zoo Biol.* 5: 181-190.
- Allendorf, F.W., Luikart, G. (2007) Conservation and the Genetics of Populations. Blackwell Publishing, Oxford, UK. 642 pp.
- Appleyard, S.A. and R.D. Ward. 2006. Genetic diversity and effective population size in mass selection lines of Pacific oyster. *Aquaculture* 254: 148-159.
- An, H.S., J.Y. Park, Y.G. Lee and C. Lee. 2005. Ten polymorphic microsatellite loci in the giant scallop (*Mizuhopecten yessoensis*). *Mol. Ecol. Notes* 5, 806-808. doi: 10.1111/j.1471-8286.2005.01069.x
- Antoro, S., U. Na-Nakorn and W. Koedprang. 2006. Genetic Diversity of Orange-spotted Grouper, *Epinephelus coioides* from Thailand and Indonesia using microsatellite markers. *Mar. Biotechnol.* 8: 17-26.
- Beaumont, A. 2000. Genetic considerations in transfers and introductions of scallops. *Aquaculture International* 8: 493-512.
- Bentzen, H.B., Thodesen, J. 2005. Genetic interactions between farmed and wild fish, with examples from the Atlantic salmon case in Norway. Pp. 319-334, *In* T. Gjerdem (ed.) Selection and Breeding Programs in Aquaculture. Springer, Netherlands.
- Billotte, N., P.J.L. Lagoda, A.M. Risterucci and F.C. Baurens. 1999. Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. *Fruits* 54: 277-288.
- Brand, A.R. 1991. Scallop ecology: Distribution and behavior. Pp. 517-584, *In* S.E. Shumway (ed.) Scallops: Biology, Ecology and Aquaculture, Elsevier, Amsterdam.
- Chaithanavisuti, N. 1987. Experimental breeding and larval of the common scallop, *Amusium pleuronectes* Linnaeus (Bivalvia: Pectinidae). Technical Report No.4/1987. Aquatic Resources Research Institute, Chulalongkorn University.
- Chongprasith, P. and E. Praekulvanich. 2003. Coastal pollution management in Thailand. Poster presented at Diffuse Pollution Conference, Dublin 2003. Available at http://www.ucd.ie/dipcon/docs/theme14/theme_21PDF. Downloaded on 23 February 2007.
- Coughlan, J.P., Imsland, A.K., Galvin, P.T., Fitzgerald, R.D., Naevdal, G., Cross, T.F. 1998. Microsatellite DNA variation in wild populations and farmed strains of turbot from Ireland and Norway: a preliminary study. *J. Fish Biol.* 52, 916-922.
- Elfstrom, C.M., C.T. Smith, K.C. Jones and J.E. Seeb. 2005. Characterization of 16 polymorphic microsatellite loci in weathervane scallop *Patinopecten caurinus*. *Mol. Ecol. Notes* 5, 514-516. doi:10.1111/j.1471-8286-2005.00974.x.
- Giese, A.R. and P.W. Hedrick. 2003. Genetic variation and resistance to a bacterial infection in the endangered Gila minnow. *Anim. Conserv.* 6: 369-377.

- Gjetvaj, B., R.M. Ball, S. Burbridge, C.J. Bird, E. Kenchington and E. Zouros. 1997. Amounts of polymorphism at microsatellite loci in the sea scallop *Placopecten magellanicus*. J. Shellfish Res. 16: 547-553.
- Goudet, J. 2002. FSTAT, A program to Estimate and Test Gene Diversities and Fixation Indices (version 2.9.3.2). Institute of Ecology, University of Lausanne, Switzerland. <http://www.unil.ch/izea/software/fstat.html>.
- Hedrick, P.W. 1985. Genetics of Populations. Jones and Bartlett, Boston. 629 pp.
- Hui, M., Z. Bao, A. Zhan, X. Hu, W. Lu, D. Chang and J. Hu. 2006. Ten polymorphic dinucleotide microsatellite markers of the noble scallop *Chlamys nobilis*. Mol. Ecol. Notes 6, 1033-1035.
- Ibarra, A.M., J.L. Peterson, T.R. Famula and B. May. 2006. Characterization of 35 microsatellite loci in the Pacific lion-paw scallop (*Nodipecten subnodosus*) and there cross-species amplification in four other scallops of the Pectinidae family. Mol. Ecol. Notes 6: 153-156.
- Kamonrat, W. 1996. Spatial Genetic Structure of Thai Silver Barb *Puntius gonionotus* (Bleeker) Populations in Thailand. Ph.D. Thesis, Dalhousie University, Dalhousie, Canada.
- Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120.
- Klinbunga, S., P. Pripue, N. Khamnamtong, N. Puanglarp, A. Tassanakajon, P. Jarayaphand, I. Hirono, T. Aoki and P. Menasveta. 2003. Genetic diversity and molecular markers of the tropical abalone (*Haliotis asinina*) in Thailand. Marine Biotechnology 5: 505-517.
- Klinbunga, S., D. Siludjai, W. Wudthijinda, A. Tassanakajon, P. Jarayaphand and P. Menasveta. 2001. Genetic heterogeneity of the giant tiger prawn (*Penaeus monodon*) in Thailand revealed by RAPD and mitochondrial DNA RFLP analyses. Marine Biotechnology 3: 428-438.
- Luan, S., Kong, J., Wang, Q.Y. 2006. Genetic variation of wild and cultured populations of the Kuruma prawn *Marsupenaeus japonicus* (Bate 1888) using microsatellites. Aquaculture Research 37, 785-792.
- Mahidol, C., U. Na-Nakorn, S. Sukmanomon, N. Taniguchi and T.T.T. Nguyen. 2007a. Mitochondrial DNA diversity of the Asian moon scallop, *Amusium pleuronectes* (Pectinidae) in Thailand. Marine Biotechnology, DOI: 10.1007/s10126-006-6137-y
- Mahidol, C., U. Na-Nakorn, S. Sukmanomon, W. Yoosuk, N. Taniguchi and T.T.T. Nguyen. 2007b. Phylogenetic relationships among nine scallop species (Bivalvia: Pectinidae) inferred from nucleotide sequences of one mitochondrial and three nuclear gene regions Journal of Shellfish Research 26(1): 25-32.
- Miller, M.P. 1997. Tool for population genetic analyses (TFPGA) 1.3: A window program for the analysis of allozyme and molecular population genetic data. Computer software distributed by the author. Available at <http://www.marksgeneticsoftware.net/tfpga.htm>. Download date: February 5, 2007.
- Morton, N. 1980. Swimming in *Amusium pleuronectes* (Bivalvia: Pectinidae). Journal of Zoology 190: 375-404.
- Na-Nakorn, U., W. Kamonrat and T. Ngamsiri. 2004. Genetic diversity of walking catfish, *Clarias macrocephalus*, in Thailand and evidence of genetic introgression from Introduced farmed *C. gariepinus*. Aquaculture 240: 145-163.

- Nugranad, J. and K. Promjinda. 1997. An experiment on hatchery seed production of the scallop *Chlamys senatoria* Gmelin. *Phuket Marine Biological Center Special Publication* 17: 241-249.
- Piry, S., A. Alapetite, J.-M. Cornuet, D. Paetkau, L. Baudouin and A. Estoup. 2004. GeneClass 2: A software for genetic assignment and first-generation migrant detection. *J. Heredity* 95: 536-539.
- Pogson, G.H. and E. Zouros. 1994. Allozyme and RFLP heterozygosities as correlates of growth rate in the scallop *Placopecten magellanicus*: a test of the associative overdominance hypothesis. *Genetics* 137: 221-231.
- Poutiers, J.M. 1998. Bivalves (Acephala Lamellibranchia Pelecypoda). Pp.198-210. In K.E. Carpenter and V.H. Niem eds. *The Living Marine Resources of the Western Central Pacific Vol. 1*. FAO, Rome.
- Raymond M. and F. Rousset. 2004. *GENEPOP (version 3.4): population genetics software for exact tests and ecumenicism*. Available at: <http://wbiomed.curtin.edu.au/genepop/>.html.
- Saavedra, C. and J.B. Peña. 2004. Phylogenetic relationships of commercial European and Australasian king scallops (*Pecten* spp.) based on partial 16S ribosomal RNA gene sequences. *Aquaculture* 235: 153-166.
- Sato, M., K. Kawamata, N. Zaslavskaya, A. Nakamura, T. Ohta, T. Nishikiori, V. Brykov and K. Nagashima. 2005. Development of microsatellite markers for Japanese scallop (*Mizuhopecten yessoensis*) and their application to a population genetic study. *Mar. Biotechnol.* 7: 713-728.
- Singh, S.M. and R.H. Green. 1984. Excess of allozyme homozygosity in marine mollusk and its possible biological significance. *Malacologia* 25: 569-581.
- Smith, P.J. 1990. Protein electrophoresis for identification of Australian fish stocks. *Aust. J. Mar. Freshwater Res.* 41: 823-833.
- Sobolewska, H. and A.R. Beaumont. 2005. Genetic variation at microsatellite loci in northern populations of European flat oyster (*Ostrea edulis*). *J. Mar. Biol. Assoc. UK* 85: 955-960. (Abstract)
- Taggart, J.B., Hynes, R.A., Prodohl, P.A. and A. Ferguson. 1995. A simplified protocol for routine total DNA isolation from salmonid fishes. *J. Fish Biol.* 49: 963-965.
- Tang, S., Tassanakajorn, A., Klinbunga, S., Jarayaphand, P., and Menasveta, P. (2005). Population structure of tropical abalone (*Haliotis assinina*) in coastal waters of Thailand determined using microsatellite markers. *Mar Biotechnol* 6: 604-611.
- Yeh, F.C., R.C. Yang and T. Boyle. 1999. *POPGENE VERSION 1.31 Microsoft windows-based freeware for population genetic analysis*. University of Alberta and Centre for International Forestry Research, Alberta, Canada.
- Zhao, Y.-Y., X.-C. Zhu, X.-W. Sun and L.-Q. Liang. 2006. Polymorphic microsatellite loci for population studies of the Japanese scallop, *Patinopecten yessoensis*. *Mol. Ecol. Notes* doi:10.1111/j.1471-8286.2006.01626.x.

Table 1 Details for sampling locations, sample sizes and population abbreviation of *Chlamys senatoria*.

Populations	Pop. Abbrev.	Sampling locations	Lat/Long	No.
Bangsarae	CB-1	Amphur Bangsarae, Chonburi Province, Gulf of Thailand	Lat.12° 36' 40"N Long. 100° 58' 22"E	27
Sattahip	CB-2	Amphur Sattahib, Chonburi Province, Gulf of Thailand	Lat.12° 38' 10"N Long. 100° 53' 23"E	24
Prajuabkirikhan	PJ	Amphur Bangsaphan, Prajuabkirikhan Province, Gulf of Thailand	Lat.11° 08' 45"N Long. 99° 31' 47"E	40
Phuket	PK	Amphur Muang, Phuket Province, Andaman Sea	Lat.7° 53' 32"N Long. 98° 15' 22"E	37
Total				128

Table 2 Details of microsatellite loci developed for *Mimachlamys senatoria*.

Loci	Primer sequences (5' → 3')	Repeat units	Sequence accession no.	Ann. T.	Product size range (bp)
Cse 1	F CAT GTT TTG CTT CGA GTT TAG TTG R TGG AAA CAA CCA CTG ATG TTC TAT G	(GA) ₂₁	DQ641501	55	220-356
Cse 2	F ACA ACA ACA ACA ACA TCA TCC ACA R TCC AAA ACC GTT CAC ACT AAA GC	(CAA) ₈ N ₂₄ (CAA) ₅	DQ641502	60	189-297
Cse 3	F CAA CAA CAA CTT CAC CAA CAA CG R CAA CAT TTG CTG TCG TTC TGT CT	(CAA) ₅ N ₂₇ (CAA) ₆ N ₂₄ (CAA) ₁₂ N ₁₂ (CAA) ₄	DQ641503	55	156-180
Cse 4	F ATA GGA CAC ACA CCG TGT CA R ATT TTG ATC GGA CAC ACT GC	(TA) ₄ N ₁₀ (CA) ₃	DQ641504	55	172-190

Ann. T. = annealing temperature

Table 3 Allele frequency at four microsatellite loci in four populations of *Mimachlamys senatoria* in Thailand.

Loci	Allele size	CB-1	CB-2	PJ	PK	Private allele
Cse 1		27	24	40	37	
	220	0.0000	0.0000	0.0000	0.0135	PK
	222	0.0000	0.0000	0.0000	0.0135	PK
	224	0.0000	0.0000	0.0000	0.0270	PK
	226	0.0000	0.0208	0.0000	0.0000	CB-2
	228	0.0000	0.0625	0.0000	0.0135	
	232	0.0000	0.0625	0.0125	0.0270	
	236	0.0000	0.0208	0.0000	0.0135	
	238	0.0000	0.0417	0.0375	0.0000	
	240	0.0185	0.0417	0.1000	0.0135	
	242	0.0370	0.0208	0.0625	0.0405	
	244	0.0556	0.0833	0.0625	0.0946	
	246	0.0741	0.0417	0.0625	0.0270	
	248	0.0556	0.0417	0.1250	0.0405	
	250	0.1111	0.0000	0.0500	0.0270	
	252	0.0741	0.0000	0.0625	0.0811	
	254	0.0370	0.0417	0.0000	0.0000	
	256	0.0741	0.1042	0.0625	0.0405	
	258	0.0370	0.0417	0.1375	0.0811	
	260	0.1111	0.0625	0.0125	0.0676	
	262	0.0185	0.0417	0.0875	0.0811	
	264	0.1111	0.0208	0.0125	0.0541	
	266	0.0000	0.0833	0.0375	0.0541	
	268	0.0000	0.0000	0.0375	0.0135	
	270	0.0000	0.0000	0.0000	0.0405	PK
	272	0.0000	0.0625	0.0000	0.0541	
	274	0.0185	0.0625	0.0000	0.0541	
	286	0.0000	0.0000	0.0000	0.0270	PK
	308	0.0185	0.0000	0.0000	0.0000	CB-1
	310	0.0185	0.0000	0.0000	0.0000	CB-1
	316	0.0185	0.0000	0.0000	0.0000	CB-1
	318	0.0185	0.0000	0.0125	0.0000	
	322	0.0000	0.0417	0.0250	0.0000	
	326	0.0556	0.0000	0.0000	0.0000	CB-1
	330	0.0185	0.0000	0.0000	0.0000	CB-1
	346	0.0185	0.0000	0.0000	0.0000	CB-1
Cse 2		26	24	24	37	
	189	0.0000	0.0000	0.0000	0.0135	PK
	195	0.0000	0.0000	0.0000	0.0135	PK
	201	0.0000	0.0000	0.0000	0.0135	PK
	204	0.0000	0.0000	0.0000	0.0135	PK
	216	0.0000	0.0417	0.0000	0.0135	
	219	0.0185	0.0000	0.0000	0.0135	
	222	0.1111	0.0000	0.0000	0.0541	
	225	0.0000	0.0000	0.0125	0.0270	

228	0.0556	0.0625	0.0375	0.0135	
231	0.0370	0.0625	0.0125	0.0676	
234	0.0926	0.1250	0.0750	0.1081	
237	0.1111	0.1042	0.0500	0.0541	
240	0.1667	0.0625	0.0625	0.0135	
243	0.2593	0.3333	0.1000	0.0405	
246	0.0370	0.1250	0.1375	0.0135	
249	0.0556	0.0000	0.1250	0.0811	
252	0.0185	0.0000	0.1625	0.0405	
255	0.0370	0.0000	0.0625	0.0270	
258	0.0000	0.0625	0.0625	0.0541	
261	0.0000	0.0000	0.0500	0.0405	
264	0.0000	0.0000	0.0375	0.0405	
267	0.0000	0.0208	0.0000	0.0000	CB-2
270	0.0000	0.0000	0.0000	0.0270	PK
273	0.0000	0.0000	0.0125	0.0405	
279	0.0000	0.0000	0.0000	0.0135	PK
282	0.0000	0.0000	0.0000	0.0541	PK
285	0.0000	0.0000	0.0000	0.0135	PK
288	0.0000	0.0000	0.0000	0.0135	PK
291	0.0000	0.0000	0.0000	0.0405	PK
294	0.0000	0.0000	0.0000	0.0135	PK
297	0.0000	0.0000	0.0000	0.0270	PK
Cse 3	27	24	40	37	
156	0.0000	0.0208	0.0125	0.0000	
168	0.0000	0.0000	0.0000	0.0541	PK
174	0.0556	0.0833	0.1250	0.1081	
177	0.9444	0.8958	0.8500	0.8378	
180	0.0000	0.0000	0.0125	0.0000	PJ
Cse 4	26	24	40	37	
172	0.0000	0.0000	0.0000	0.0135	PK
180	0.0000	0.0000	0.0000	0.0405	PK
182	0.4444	0.6250	0.5625	0.4865	
184	0.5556	0.3750	0.4375	0.2432	
188	0.0000	0.0000	0.0000	0.0676	PK
190	0.0000	0.0000	0.0000	0.1486	PK

Table 4 Genetic variation at four microsatellite loci of four populations of *Mimachlamys senatoria* in Thailand and results of Hardy-Weinberg test. (n=sample size; A= average no. alleles/locus; A_e =effective no. alleles/locus; H_o , H_e =observed and expected heterozygosity respectively; numbers in parentheses were standard deviation)

Populations	n	A	A_e	A_r	H_o	H_e
Bangsarae (CB-1)	27	9.25 (9.14)	6.14 (6.06)	5.71 (4.66)	0.565 (0.390)	0.609 (0.387)
Sattahip (CB-2)	24	8.75 (8.30)	6.41 (7.15)	8.75 (8.30)	0.552 (0.277)	0.620 (0.350)
Prajuabkirikhan (PJ)	40	9.75 (7.93)	6.56 (5.73)	5.92 (4.36)	0.612 (0.302)	0.652 (0.327)
Phuket (PK)	37	15.75 (13.27)	10.60 (9.72)	7.76 (5.05)	0.716 (0.274)	0.723 (0.318)
Overall	128	19.25 (15.97)	9.39 (9.17)	7.03 (1.47)	0.621 (0.302)	0.669 (0.345)

Note: α after Bonferroni correction = 0.05/20

Table 5 The locus-wise F_{IS} and the exact P-values by the Markov chain method (in parentheses) for four populations of *Mimachlamys senatoria* in Thailand. (a critical value for $P=0.0025$ after Bonferroni correction for 4 multiple tests). The P-values for the overall F_{IS} were estimated following the Fisher's method.

Pop.	loci				overall
	Cse01	Cse02	Cse03	Cse04	
Bangsarae (CB-1)	0.023 (0.6553)	0.029 (0.0390)	-0.040 (1)	0.268 (0.2440)	0.073 (0.2543)
Sattahip (CB-2)	0.090 (0.0919)	0.266 (0.0007)	-0.075 (1)	-0.045 (1)	0.112 (0.0127)
Prajuabkirikhan (PJ)	0.089 (0.0041)	0.099 (0.0000)	0.247 (0.0071)	-0.156 (0.3548)	0.062 (High. Sign.)
Phuket (PK)	0.069 (0.0002)	0.047 (0.0060)	-0.131 (1)	-0.066 (0.1312)	0.010 (0.0001)

Table 6 Genotypic differentiation (P- value) (above diagonal) and pairwise F_{ST} (below diagonal) of *Mimachlamys senatoria* in Thailand.

Pop.	CB-1	CB-2	PJ	PK
Bangsarae (CB-1)	-	0.00272	0.00002	0.00000
Sattahip (CB-2)	0.0169 ^{ns}	-	0.00033	0.00001
Prajuabkirikhan (PJ)	0.0237*	0.0187*	-	Infinity
Phuket (PK)	0.0355*	0.0253*	0.0179*	-

Note: $P=0.005$ (Bonferroni correction)

Table 7 Genetic distance (Cavalli-Sforza chord distance) between populations of *Mimachlamys senatoria* in Thailand.

Populations	CB-1	CB-2	PJ	PK
Bangsarae (CB-1)	***			
Sattahip (CB-2)	0.0359	***		
Prajuabkirikhan (PJ)	0.0311	0.0337	***	
Phuket (PK)	0.0470	0.0474	0.0378	***

Table 8 Summary of population assignment for four populations of *Mimachlamys senatoria* in Thailand based on microsatellite loci.

Sample	Correctly assigned	Assigned to other sample		
		Assigned to	Score %	-log(L)
CB-1	92.6%	CB-2	59.83	4.62
		PJ	57.87	4.62
CB-2	87.5%	CB-1	67.99	4.75
		PJ	62.17	4.44
PJ	95.0%	CB-1	62.65	4.81
		CB-2	64.27	4.09
PK	(86.5%)	CB-1	74.07	4.35
		PJ	61.69	5.73

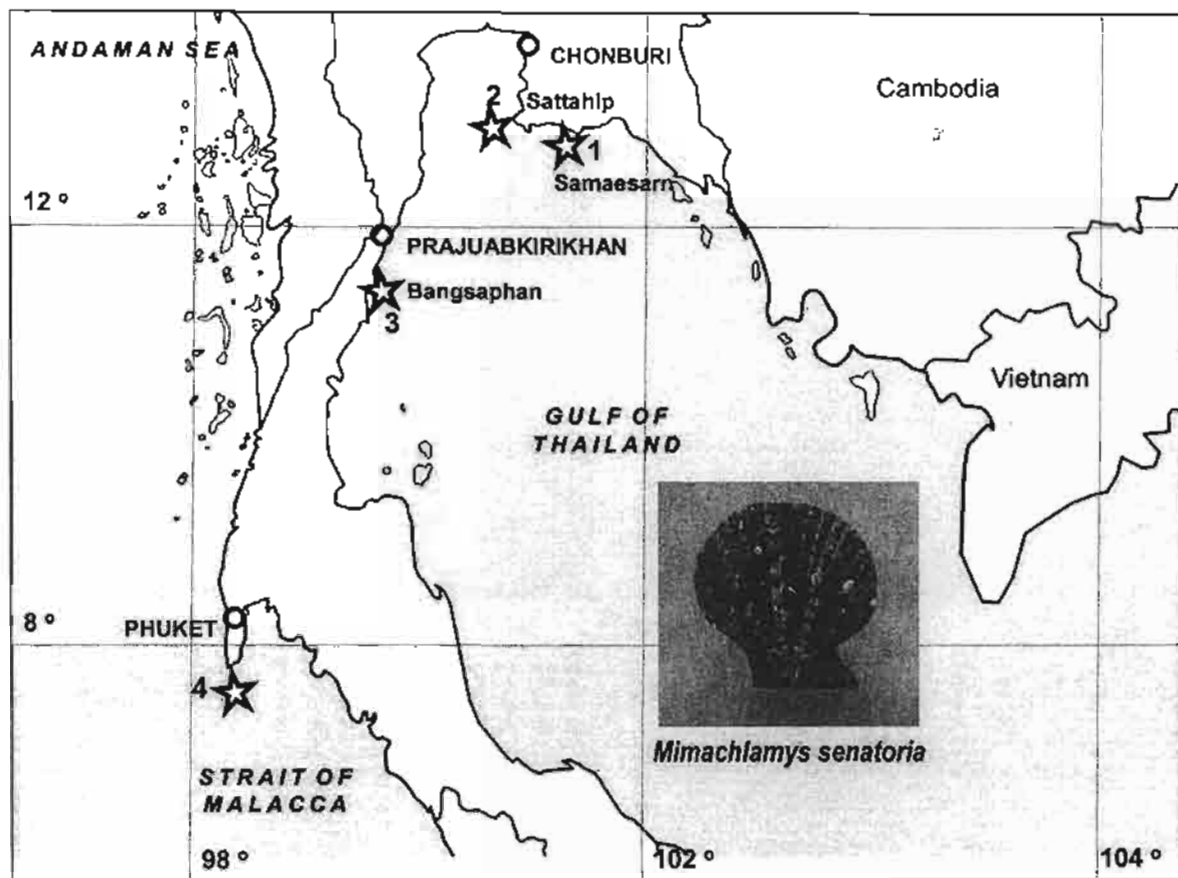


Figure 1 Sampling sites of *Mimachlamys senatoria* used for this study.

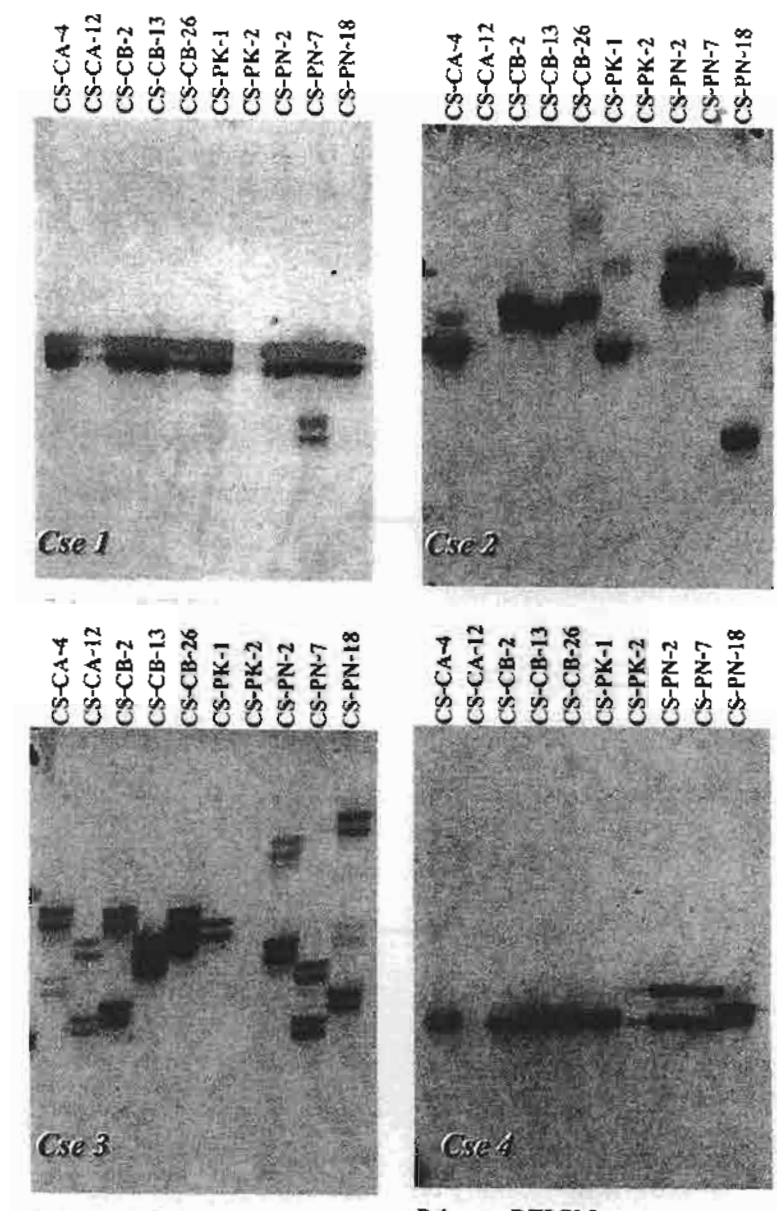


Figure 2 Profile of microsatellite loci, Cse 1, Cse 2, Cse 3 and Cse 4 developed from *Mimachlamys senatoria*.

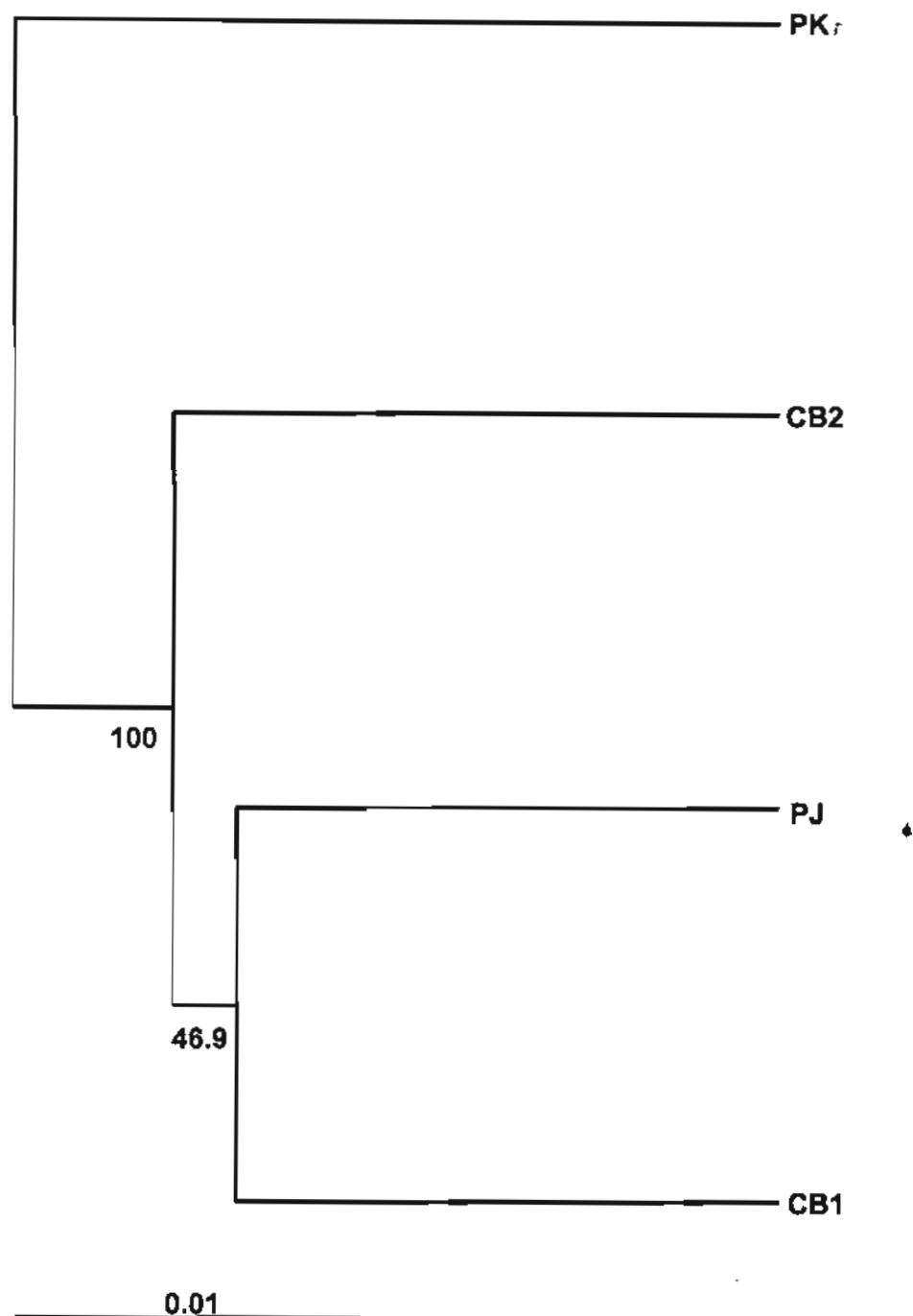


Figure 3 A neighbor joining tree based on microsatellite variation of four populations of *Mimachlamys senatoria* in Thailand.

เอกสารแนบที่ 12

หนังสือเรื่องพันธุศาสตร์ประชากรเพื่อการเพาะเลี้ยงสัตว์น้ำ

เอกสารแนบที่ 13

เอกสารเผยแพร่ “กายวิภาคของกึ่งกำมกรามและการผลิตลูกกึ่งเพศผู้ล้วนโดยการทำลายต่อม
แอนโดรเจนิก”

เอกสารแนบที่ 14

เอกสารแสดงเรื่องการยอมรับเทคโนโลยีของภาคเอกชน

60 AQUATIC LIFE MAGAZINE

ฉบับที่ 205 ประจำปี ๒๕๔๙

คลังสมองของผู้ประกอบการสัตว์น้ำ

No. 2 **ปรับวิธีเลี้ยงกุ้ง**
ด้วยระบบสมดุลย์
เพื่อผลิตกุ้งที่ยั่งยืน

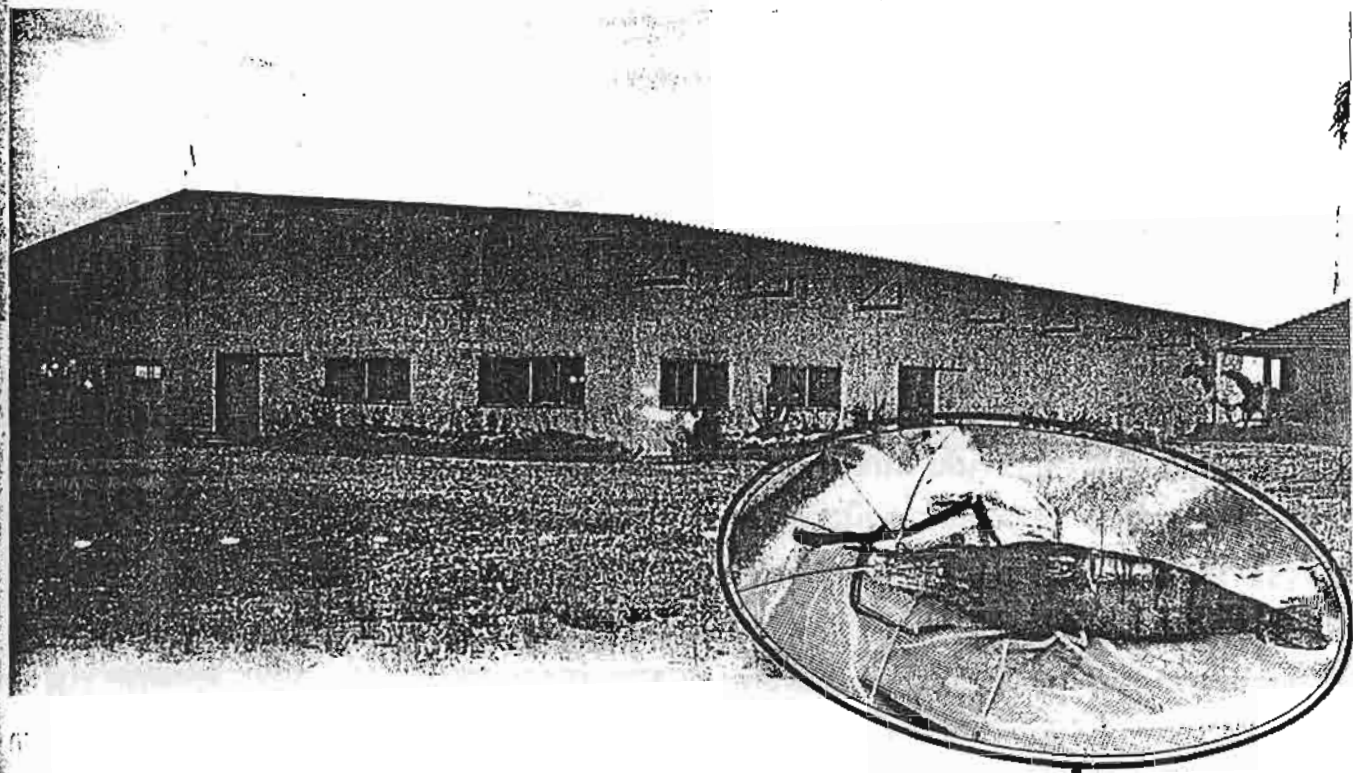
**ชาวบางกะปิ ย้ายนกมาดำ
อันดับ 1 ของประเทศ**

สามชุกกล้วยปลานิลในกระชัง
ประกันราคา 38 บาท/กก.

ប្រាក់កម្រៃ ៧ លាន ៤ ពាន់/កក.

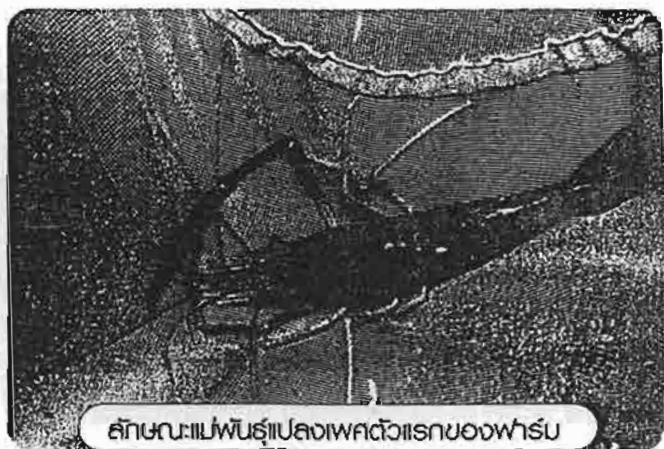
โศคาฟาร์มได้แล้ว!

“แม่กุ้งกำมกรามกะทาย”...พร้อมให้ลูกผู้ด้อย



จากเมื่อประมาณเดือนกุมภาพันธ์ที่ผ่านมา ทางทีมงานนิตยสารสัตว์น้ำได้นำเสนอในเรื่อง การสร้างแม่พันธุ์กุ้งกำมกรามที่ให้ลูกเป็นตัวผู้ล้วน ด้วยวิธีการทำลายต่อม androgenic gland โดยใช้เทคนิคการดิงออกมาซึ่งเป็นผลงานของ อ.วิกรม รังสินธุ์ ที่ไปดิงต่อม androgenic gland ให้กับทางโศคาฟาร์ม เป็นครั้งแรกที่ทำในรูปแบบออนฟาร์ม ในครั้งนั้น ทางทีมงานได้เดินทางไปดูวิธีการดิงต่อมกุ้งกำมกรามเพื่อแปลงเพศกุ้งตัวผู้ให้เป็นตัวเมีย ทำการดิงโดย อ.วิกรม และได้นำมาลงตีพิมพ์เผยแพร่ลงในนิตยสารสัตว์น้ำ ฉบับที่ 199 ซึ่งเมื่อได้นำเสนอออกไปแล้วนั้น มีผู้สนใจในเรื่องนี้เป็นจำนวนมาก ทางทีมงานจึงได้ไปติดตามผลงานที่มีการดิงต่อม androgenic gland ในครั้งนั้นมานำเสนอในฉบับนี้

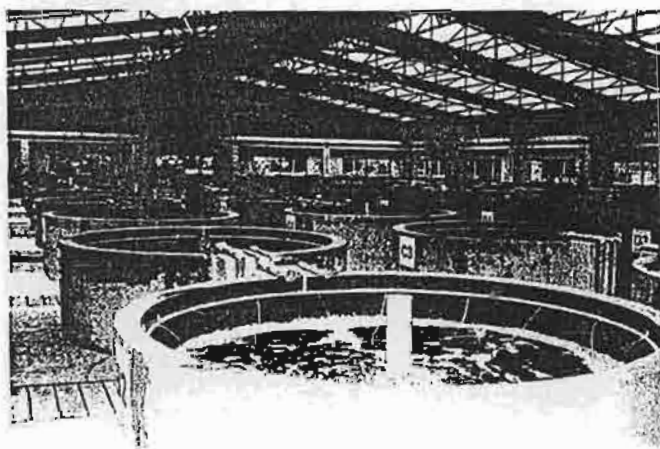
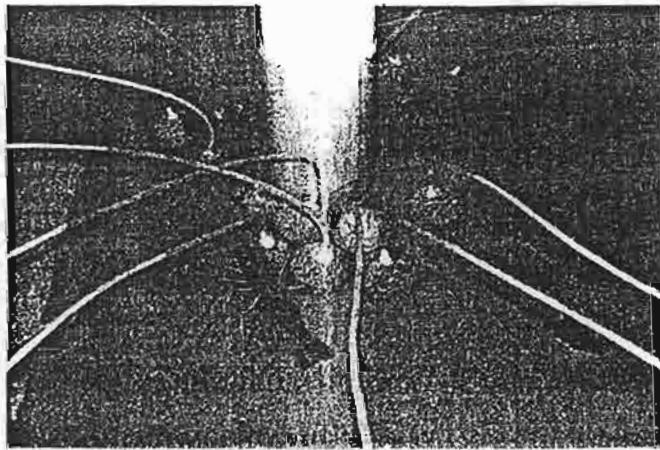
จะขอเริ่มอย่างนี้ก่อนว่า ทางทีมงานได้มีการติดต่อพูดคุยกับทางโศคาฟาร์มที่มี คุณธนกร กะถิ่นเทศ อยู่เป็นประจำถึงความคืบหน้าของลูกกุ้งตัวผู้ที่ถูกดิงต่อมและเลี้ยงจนมาเป็นแม่พันธุ์ที่จะสามารถให้ลูกตัวผู้ล้วนได้ โดยเมื่อประมาณต้นเดือนสิงหาคมที่ผ่านมาทางทีมงานได้รับโทรศัพท์จากคุณธนกร แจ้ง



ลักษณะแม่พันธุ์แปลงเพศตัวแรกของฟาร์ม

ว่าจะมีการลากอวนแม่พันธุ์ที่ได้รับการดัดต่อม androgenic gland ในบ่อที่เลี้ยงไว้จนมีอายุ 6 เดือน และสามารถนำขึ้นมาเพื่อให้สลัดไข่ได้แล้ว (จะนำแม่พันธุ์ที่มีไข่แก่ขึ้นมาให้สลัดไข่ในบ่อซีเมนต์ที่มีความเค็มของน้ำ 15 พีพีที) ซึ่งพอทางทีมงานได้รับการแจ้งข่าวแล้วจึงได้เดินทางไปโกคาฟาร์ม อ.ท่าม่วง จ.กาญจนบุรี ในอีก 2 วันต่อมา เนื่องจากว่าจะมีการลากอวนแม่พันธุ์ชุดนี้ขึ้นมาอีกครั้งหนึ่ง ซึ่งครั้งแรกได้ทำการลากแม่พันธุ์ขึ้นมาแล้ว 1 ครั้ง ได้แม่พันธุ์กึ่งก้ามกรามที่ได้มีการแปลงเพศที่มีไข่แก่ขึ้นมา 1 ตัว แล้วได้นำไปสลัดไข่และเป็นลูกกึ่งแล้วอายุประมาณ 13 วัน

วันที่ทางทีมงานเดินทางไปยังโกคาฟาร์มนั้น เป็นการลากแม่พันธุ์กึ่งก้ามกรามขึ้นมาเป็นครั้งที่ 2 โดยในครั้งนี้ได้แม่พันธุ์ที่มีไข่แก่ขึ้น 3 ตัว ซึ่งถือได้ว่ามีปริมาณที่เพิ่มขึ้นจากครั้งแรก จากการสอบถามคุณธนกร ผู้จัดการฟาร์มได้รับการเปิดเผยว่า



ลักษณะของคราบน้ำพันธุ์แปลงเพศ

การดัดต่อม androgenic gland ในครั้งแรกที่ทาง อ.วิกรม ได้มาช่วยดัดให้ ครั้งนั้นทำได้จำนวนไม่มากนักเนื่องจากมีลูกกึ่งตัวผู้ที่มีอายุที่สามารถทำได้มีจำนวนไม่มาก และด้วยเงื่อนไขของระยะเวลาที่จะต้องทำจึงได้กึ่งที่ผ่านการแปลงเพศได้ไม่มาก แต่พอหลังจากนั้นมีลูกกึ่งตัวผู้ที่มีอายุที่สามารถดัดต่อมได้มากขึ้น ทางฟาร์มจึงให้นักวิชาการที่ผ่านการฝึกอบรมการดัดต่อม androgenic gland ทำการดัดต่อมเพื่อแปลงเพศกึ่งมาเรื่อยๆ กว่าจะได้แม่พันธุ์กึ่งก้ามกรามที่ได้มาจากการแปลงเพศที่มีอายุตั้งแต่ 6 เดือนลงมาที่มีอยู่ในปัจจุบันนี้ประมาณ 4,000 ตัว ใช้ระยะเวลาในการดัดประมาณเกือบ 1 เดือน แต่สำหรับกึ่งที่ถูกแปลงเพศชุดแรกที่มีอายุมากกว่า 6 เดือนที่สามารถมีไข่และนำมาเพาะลูกได้มีอยู่ประมาณ 30 ตัว ตรงนี้จะต้องใช้ระยะเวลาและจะมีจำนวนที่มากขึ้นเรื่อยๆ เพราะลูกกึ่งตัวผู้ก็นำมาแปลงเพศนั้นอายุไม่เท่ากัน โดยการแยกเลี้ยงไว้ 2 บ่อ คือกึ่งที่มีอายุมากกว่า 3 เดือนขึ้นไปแยกได้บ่อหนึ่งจำนวน 2,500 ตัว และอีกบ่อหนึ่งเป็นกึ่งที่มีอายุน้อยกว่า 3 เดือน จำนวน 1,500 ตัว ซึ่งในบ่อแรกนั้นได้มีการปล่อยกึ่งตัวผู้ลงไปเลี้ยงด้วย จะใช้จากแหล่งที่มาจากเพชรบุรี เพื่อให้มีการผสมพันธุ์กันตามธรรมชาติภายในบ่อ หลังจากนั้น

ก็จะทยอยลากแม่พันธุ์ที่มีไข่แก่ขึ้นมาเพาะลูกกุ้งตัวผู้ส่วนต่อไป

ส่วนลักษณะของแม่พันธุ์กุ้งก้ามกรามที่ได้รับการแปลงเพศมาแล้ว โดยการดึง ต่อม androgenic gland จากการสังเกตลักษณะทางกายภาพภายนอก ซึ่งจะเห็นว่ากุ้งจะมีลักษณะที่คล้ายคลึงกับกุ้งตัวผู้มาก คือมีลักษณะของก้ามคู่หน้าที่ยาวกว่ากุ้งตัวเมียธรรมดา และมีขนาดตัวที่ใหญ่กว่าตัวเมียธรรมดา แต่มีลักษณะการเคลื่อนไหวช้ากว่ากุ้งธรรมดา เล็กน้อย สำหรับเรื่องที่มีขนาดตัวใหญ่กว่าตัวเมียธรรมดานั้น น่าจะเกิดมาจากกุ้งแปลงเพศยังมีพันธุกรรมที่ยังคงเป็นตัวผู้อยู่ก็เป็นได้

สำหรับการฟอรั่มไข่ของกุ้งก้ามกรามที่ถูกแปลงเพศนั้น เท่าที่ได้สังเกตจากแม่พันธุ์ที่ได้ขึ้นมาทั้ง 4 ตัว ปริมาณของไข่ไม่เต็มท้องจะมีอยู่ 3 ใน 4 ของปริมาณไข่เมื่อเปรียบเทียบกับแม่กุ้งธรรมชาติใน



ลูกกุ้งที่ได้ตัวใส่แก้วขึ้นมาดู

ขนาดตัวเท่ากัน ยกตัวอย่างเช่น โดยปกติจะได้ไข่แก่ที่กุ้งสลัดออกมาประมาณ 20,000 ฟอง/ตัว แต่ในแม่กุ้งแปลงเพศทั้ง 4 ตัวได้ไข่ประมาณ 15,000 ฟอง/ตัว ซึ่งตรงนี้อาจจะหลุดไปในระหว่างขั้นตอนของการลากอวนขึ้นมาเพื่อคัดแม่พันธุ์ที่มีไข่แก่ก็เป็นไปได้ ต้องทดลองจากการลากอวนแม่พันธุ์ไข่แก่ขึ้นมาเรื่อยๆ หลายครั้งจึงจะสามารถระบุได้ว่าเกิดมาจากสาเหตุอะไร ตรงนี้ทางฟาร์มยังไม่ได้สอบถามไปยัง อ.วิกรม ว่าเกิดมาจากสาเหตุใด

ส่วนอัตราการรอดของลูกกุ้งที่ได้มาจากแม่พันธุ์ที่ถูกแปลงเพศมานั้น ยังเป็นปกติเหมือนกับลูกกุ้งที่ได้มาจากแม่พันธุ์

ธรรมชาติ โดยในตอนนี้ลูกกุ้งเพาะเลี้ยงไว้มีอายุได้ 13 วัน ซึ่งถือว่าในระยะนี้จะเป็นช่วงหนึ่งที่ลูกกุ้งก้ามกรามมักที่จะมีปัญหาเกิดขึ้นมาอยู่บ่อยๆ และอีกช่วงหนึ่งคือในระยะที่ลูกกุ้งคว่ำประมาณ 20-25 วัน แต่ถ้าผ่าน 2 ช่วงนี้ไปได้ถือว่าปลอดภัยแล้ว เพราะลูกกุ้งที่ติดเชื้อจะแสดงอาการใน 2 ช่วง ระยะนี้โดยเฉพาะเชื้อ MrNV และ XSV จะแสดงอาการออกมาในระยะที่ลูกกุ้งคว่ำและจะทำให้มีอัตราการดต่ำ เพราะจะเกิดความเสียหายจากลูกกุ้งไม่ว่าตัว ซึ่งลูกกุ้งชุดนี้ยังอยู่ในช่วงแรกอยู่ก็จะต้องรอดต่อไปจนกว่าลูกกุ้งจะคว่ำถึงจะรู้อัตราการที่แน่นอน เท่าที่ผ่านถือว่าลูกกุ้งชุดนี้ค่อนข้างจะมีอัตราการรอดที่ดี

เป้าหมายในการผลิตลูกกุ้งก้ามกรามนั้น สำหรับลูกกุ้งก้ามกรามเพศผู้ส่วนที่ได้จากแม่พันธุ์ที่ผ่านการแปลงเพศมาคุณธนกร กล่าวว่าการที่ได้พูดคุยกับคุณวิทยา ศุภศิริ โภคาผู้บริหารฟาร์ม ลูกกุ้งก้ามกรามเพศผู้ส่วนจะทำออกมาจำหน่ายได้คงน่าจะเป็นประมาณต้นปี 50 ในเรื่องของจำนวนที่จะเพาะผลิตออกมาเท่าไรนั้น ตรงนี้ยังระบุไม่ได้ โดยในปัจจุบันจะเป็นการฝึกประสบการณ์ให้มีทักษะการดึง ต่อม androgenic gland ของ



บุคลากรที่มีอยู่ในตอนนี้ของทางฟาร์มให้มากขึ้นไปก่อน และกำลังที่จะหาบุคลากรเพิ่มเพื่อให้เข้ามาช่วยงานตรงนี้ เพราะจะต้องทำแม่พันธุ์กึ่งก้ามกรามแปลงเพศผสมให้ได้จำนวนที่มากขึ้นกว่านี้

สำหรับสัดส่วนในการที่จะผลิตลูกกึ่งก้ามกรามทั้ง 2 แบบ คือลูกกึ่งแบบปกติธรรมดาและลูกกึ่งแบบเพศผู้ล้วนที่จะผลิตเพื่อจำหน่ายในต้นปีหน้านั้น ในระยะแรกจะเน้นไปทางลูกกึ่งแบบปกติธรรมดา มากกว่า สัดส่วนประมาณ 60:40 ซึ่งตรงนี้ยังคาดเดาไม่ได้จะต้องดูตามยอดออเดอร์การสั่งจองเข้ามาประกอบไปด้วยว่าจะเพาะผลิตลูกกึ่งตัวไหนมากหรือน้อย โดยเฉพาะลูกกึ่งเพศผู้ล้วนนั้นเท่าที่ผ่านมาได้มีการสั่งจองจากลูกค้าเข้ามาบ้างแล้ว แต่ทางฟาร์มบอกกับไปว่าขอเป็นประมาณต้นปีหน้า

โดยรวมแล้วยอดการผลิตลูกกึ่งทั้งหมดของ



โกดาฟาร์มในปัจจุบันอยู่ที่ประมาณ 5 ล้านตัว/เดือน ซึ่งจะเป็นลูกกึ่งจากแม่พันธุ์ธรรมชาติทั้งหมด ส่วนเป้าหมายการผลิตของฟาร์มที่สามารถทำได้เต็มกำลังนั้นทั้ง 2 โรงเรือนอยู่ประมาณ 20-25 ล้านตัว/เดือน ที่ผลิตลูกกึ่งได้จำนวนไม่มาก เนื่องจากยังติดปัญหาตรงที่ว่ายังมีพ่อแม่พันธุ์ในปริมาณที่น้อยโดยเฉพาะแม่พันธุ์กึ่ง จึงทำให้ได้ไข่แก่น้อยในแต่ละรอบการผลิต ซึ่งตรงนี้ทางฟาร์มพยายามจะหาพ่อ-แม่พันธุ์ผสมให้มากขึ้น

อยู่เรื่อยๆ

สำหรับพ่อ-แม่พันธุ์ที่ไม่ได้แปลงเพศเพาะเลี้ยงไว้ในบ่อดินของทางฟาร์มที่มีการทำอยู่ยังมีอายุน้อยประมาณ 3 เดือน ซึ่งยังนำมาใช้เป็นพ่อ-แม่พันธุ์ไม่ได้ โดยมีจำนวนที่ปล่อยเลี้ยงไว้ในบ่อมีอยู่ประมาณ 250,000 ตัว จะมีการแยกในแต่ละแหล่งน้ำและเลี้ยงจนได้อายุครบวัยเจริญพันธุ์ จากนั้นจึงนำมาไขว้สายพันธุ์กันเพื่อลดปัญหาการเกิดเลือดชิด วัตถุประสงค์ในการผลิตพ่อ-แม่พันธุ์ของทางฟาร์มขึ้นมา ตรงนี้เพื่อเป็นการสร้างพ่อ-แม่พันธุ์ที่ปลอดจากเชื้อโรคต่างๆ โดยเฉพาะเชื้อ MrNV และ XSV ที่สร้างความเสียหายให้กับวงการกึ่งก้ามกรามในปัจจุบัน ซึ่งพอทำตรงนี้ขึ้นมาแล้วจะเป็นการลดความเสี่ยงที่จะต้องไปนำพ่อ-แม่พันธุ์ที่ได้มาจากธรรมชาติ

แต่ทางฟาร์มก็ยังคงมีระบบการป้องกันโดยการสุ่มตรวจพ่อ-แม่พันธุ์ที่ได้มาจากธรรมชาติในแต่ละแหล่ง ถ้ามีการตรวจพบจะทำการขายทิ้งเป็นกึ่งเนื้อทั้งหมดจะไม่มาเพาะพันธุ์ สำหรับพ่อ-แม่พันธุ์ที่ผ่านการสุ่มตรวจจะนำมาเพาะพันธุ์เมื่อได้ลูกกึ่งออกมาแล้ว จะมีการสุ่มตรวจอีกครั้งหนึ่ง เพื่อให้แน่ใจว่าได้ลูกกึ่งที่มีคุณภาพจริงๆ สำหรับลูกกึ่งที่ได้ส่วนหนึ่งทางฟาร์มจะเก็บไว้แปลงเพศ เพื่อจะทำเป็นแม่พันธุ์ให้ลูกเพศผู้ล้วนต่อไป อีกส่วนหนึ่งจะจำหน่ายให้แก่ลูกค้าที่มีออเดอร์สั่งจองเข้ามาซึ่งส่วนใหญ่จะอยู่ในเขตภาคกลาง

ในเรื่องการพิสูจน์ลูกกึ่งชุดนี้ที่ได้มาจากแม่พันธุ์แปลงเพศนั้น ตรงนี้จะต้องรอการพิสูจน์อีกประมาณ 45-50 วัน หลังจากที่ได้ลูกกึ่งแล้ว จะต้องรอดูว่าลูกกึ่งชุดนี้จะเป็นเพศผู้ประมาณกี่เปอร์เซ็นต์ ซึ่งทางทีมงานนิตยสารสัตว์น้ำจะติดตามและเข้าร่วมการพิสูจน์ต่อไปเพื่อจะนำมาเสนอให้แก่ผู้อ่านในโอกาสต่อไป

